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In vivo analysis using variants of zebrafish BMPR-IA: range of action and involvement of BMP in ectoderm patterning

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SUMMARY

It has been an intriguing problem whether the polypeptide growth factors belonging to the transforming growth factor-β (TGF-β) superfamily function as direct and long-range signaling molecules in pattern formation of the early embryo. In this study, we examined the mechanism of signal propagation of bone morphogenetic protein (BMP) in the ectodermal patterning of zebrafish embryos, in which BMP functions as an epidermal inducer and a neural inhibitor. To estimate the effective range of *zbmp-2*, we first performed whole-mount in situ hybridization analysis. The *zbmp-2*-expressing domain and the neuroectoderm, marked by *otx-2* expression, were complementary, suggesting that BMP has a short-range effect in vivo. Moreover, mosaic experiments using a constitutively active form of a zebrafish BMP type I receptor (CA-BRIA) demonstrated that the cell-fate conversion, revealed by ectopic expression of *gata-3* and repression of *otx-2*, occurred in a cell-autonomous manner, denying the involvement of the relay mechanism. We also found that *zbmp-2* was induced cell autonomously within the transplanted cells in the host ectoderm, suggesting that BMP cannot influence even the neighboring cells. This result is consistent with the observation that there is no gap between the expression domains of *zbmp-2* and *otx-2*. Taken together, we propose that, in ectodermal patterning, BMP exerts a direct and cell-autonomous effect to fate uncommitted ectodermal cells to become epidermis.

Key words: Zebrafish, BMP, BMP receptor, Ectoderm patterning

INTRODUCTION

In vertebrates, embryonic ectoderm is fated to become either neuroectoderm or non-neural epidermal ectoderm, and this cell-fate decision is believed to occur during gastrulation. It has long been thought that neural fate is induced by a positive regulator emanating from Spemann's organizer, with epidermal fate as the default state. However, recent studies demonstrate that epidermal fate is induced by positive regulators derived from the ventral ectoderm (for review, Hemmati-Brivanlou and Melton, 1997; Graff, 1997).

BMP-4 and BMP-7 are secreted factors from the TGF-β superfamily. There are several lines of evidence from experiments performed in *Xenopus* to suggest that these factors function as endogenous epidermal inducers and neural inhibitors. Firstly, when expressed in prospective ectodermal explants (animal caps), a dominant negative form of the BMP-2/4 receptor and a processing mutant of BMP ligand, both of which interfere with BMP signaling, cause neuralization (Graff et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Xu et al., 1995). Secondly, all known organizer-derived neural inducers (Chordin, Noggin and Follistatin) act as BMP antagonists through direct binding to the BMP receptor, thereby inhibiting its activation (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). In keeping with this, *bmp-4* transcripts are excluded from the presumptive neural plate and restricted to the epidermal cells (Fainsod et al., 1994: Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). Finally, BMP can induce epidermis and repress neuralization in dispersed ectodermal cells, which otherwise differentiate into neuralized tissue (Wilson and Hemmati-Brivanlou, 1995).

Although it is generally agreed that the antagonistic interaction between BMP and its binding proteins determines ectodermal cell fate, the mechanism of BMP signal propagation in the ectoderm remains obscure. Above all, the effective range of BMP is presently the most controversial question not only in the determination of ectodermal fate, but in other situations, such as mesoderm specification in vertebrate and disc development of fly. Jones et al. (1996) demonstrated by conjugation analysis that *Xenopus Brachyury* (*Xbra*), a mesodermal marker gene induced by BMP-4, is expressed only in tissue producing BMP-4. This suggested that BMP-4 acts as a short-range, or almost cell-autonomous signaling molecule in mesoderm induction. In other

experimental systems, however, BMP-4 (Dosch et al., 1997) and Dpp (Lecuit et al., 1996; Nellen et al., 1996), a *Drosophila* counterpart of vertebrate BMP-2/4, have been shown to act as long-range signaling molecules, as revealed by their non-cell-autonomous effects.

In addition to the effective range of BMP, another interesting question is whether BMP directly induces epidermis by a 'direct diffusion mechanism' or indirectly by a 'relay mechanism' with the aid of secondary diffusible factor(s) triggered by BMP. A useful approach to this problem would be an ectopic expression experiment using a constitutively active BMP receptor that can transmit BMP signals in a ligand-independent manner. If a secondary secreted molecule(s) is involved in epidermal induction, such constitutively active receptors should induce epidermal properties, not only in the cells expressing the receptors, but also in surrounding cells in a non-cell-autonomous manner. On the contrary, if BMP directly induces epidermal fate, the induction should be cell-autonomous and restricted to the cells expressing the activated receptors. Similar experiments have been performed in other animal systems. In *Drosophila*, use of a constitutively active form of Tkv, a receptor for Dpp, demonstrated that Dpp directly induced some marker genes during wing disc development (Lecuit et al., 1996; Nellen et al., 1996).

In an effort to investigate the mechanism of BMP signal propagation, we have isolated a *BMPR-IA* from zebrafish. Zebrafish embryos are fairly translucent and lend themselves well to making genetic chimeras by cell transplantation. By exploiting the experimental advantages of zebrafish, we show here that BMP acts as a direct and cell autonomously effective signaling molecule in ectodermal patterning.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Nikaido et al., 1997) with both digoxigenin- and fluorescein-labeled antisense RNA probes. For two-color in situ hybridization, hybridization with each probe was visualized successively. The fluorescein-labeled probe was first detected by alkaline phosphatase-conjugated anti-fluorescein antibody using Fast Red (Boehringer Mannheim) as a substrate. After inactivating the first alkaline phosphatase, the second digoxigenin-labeled probe was detected using BM purple (Boehringer Mannheim) as a substrate for alkaline phosphatase-conjugated anti-digoxigenin antibody. In single-color in situ hybridization, a combination of 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt (BCIP) or BM purple was used for detection.

BMP bead transplantation

Xenopus recombinant BMP-4 protein was kindly provided by S. Iemura and T. Natsume (Nippon Meat Packers Inc.). For preparation of BMP- or BSA-soaked beads, agarose beads (Affigel-Blue, 100-200 mesh, Bio-Rad) were washed three times with PBS(−) (PBS lacking Mg^{2+} and Ca^{2+}). Recombinant BMP protein or crystallized BSA was dissolved into PBS(−) to concentrations of 10 ng/μl and incubated with washed beads at 37°C for 30 minutes. Zebrafish embryos obtained by natural spawning were cultured in one-third Ringer's solution (39 mM NaCl, 0.97 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM Hepes, pH 7.2) at 28.5°C. At the shield stage, protein-soaked beads were transferred by glass pipette into the medium surrounding the embryos, 2% methyl cellulose in one-third Ringer's solution, and transplanted into the dorsoanimal region of embryos using a tungsten needle. Before this operation, embryos were dechorionated with pronase (Sigma). After transplantation, embryos were cultured at 26°C in one-third Ringer's solution until the 80% epiboly stage and fixed with 4% paraformaldehyde in PBS(−) for in situ hybridization.

Construction of constitutively active BMPR-IA

Replacement of the glutamine positioned at amino acid 228 of BMPR-IA with aspartate was carried out using the Transformer™ Site-Directed Mutagenesis Kit (Clontech). The experimental procedure was performed according to the manufacturer's instructions. For the selection primer, a commercially supplied *Sca*I-*Stu*I oligomer was used, and the mutagenic primer was as follows: 5′-CGCACTATCCGAAAGGACATCCAGACAGTG-3′ (The replaced nucleotides are underlined).

RNA injection

For the ectopic expression experiment, we synthesized capped mRNA encoding CA-BRIA by using SP6 and T7 RNA polymerase. However, in both cases, synthesized RNAs contained shorter transcripts in addition to full-length ones. Because similar short transcripts were also observed when we performed the same experiment using the cDNA encoding *Xenopus* ALK-3 as a template, it seemed likely that these results were probably caused by sequences conserved in between the zebrafish and *Xenopus* receptors. We performed the overexpression experiment using this mixture of full-length and short RNAs. The mixture of mRNAs (approximately 0.2 μg/μl) was injected at the 1- or 2-cell stage at 100 pg per embryo. For the dominant negative form of BMPR-IA (DN-BRIA), capped mRNA (approximately 0.1 μg/μl) was injected at the same stage at 50 pg per embryo. Injected embryos were allowed to develop at 28.5˚C in one-third Ringer's solution and were then subjected to whole-mount in situ hybridization at the appropriate stage. Synthetic mRNA encoding β-galactosidase was used as a negative control.

Cell transplantation

Donor embryos at the 1- or 2-cell stage were injected with the synthetic mRNA mixture used in the overexpression experiment of CA-BRIA described above. Next, a mixture of rhodamine B isothiocyanate-dextran (Sigma) and lysine fixable biotin-dextran (Molecular Probes) dissolved in 0.2 M KCl was injected into the yolk cell of the same embryo as a lineage tracer. Injected donor and uninjected host embryos obtained at the same time were synchronously cultured at 28.5°C. At the sphere stage, enzymatically dechorionated donor and host embryos were oriented in 2% methyl cellulose. Donor cells were drawn up into a glass pipette and transplanted into the animal pole region of host embryos of the same stage. Chimeras that had received mRNA and biotin-dextran-injected cells were fixed at the 80% epiboly stage and subjected to whole-mount in situ hybridization, followed by detection of biotin-dextran using the VECTASTAIN ABC-kit (Vector Laboratory). For sectioning, stained embryos were embedded in resin, Technovit 8100 (Kulzer) and 6 μm sections were cut using a standard microtome.

RESULTS

BMP is expressed in non-neural ectoderm and regulates dorsoventral patterning of the ectoderm

As reported previously, *bmp* is expressed exclusively in the ventroanimal ectoderm of *Xenopus* and zebrafish embryos, and it is thought that the *bmp*-free domain corresponds to neuroectoderm (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995; Nikaido et al., 1997). Moreover, dispersion-reaggregation experiments using *Xenopus* animal caps have shown that BMP protein can induce

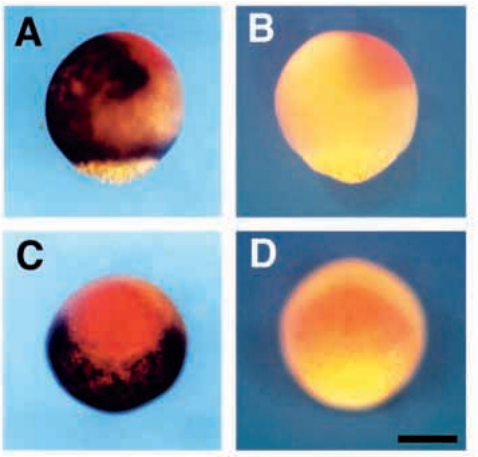

Fig. 1. Comparison of the expression domain of *zbmp-2* with that of *otx-2*. (A,C) At 80% epiboly, *zbmp-2* (brown) and *otx-2* (red) are expressed in contact with each other in the ectodermal region. (B,D) Embryos hybridized with an antisense probe for *otx-2* (red) and a sense probe for *zbmp-2*. (A,B) Lateral views are oriented with the dorsal side to the right. (C,D) Animal pole views show the dorsal side up. Scale bar, 200 μm.

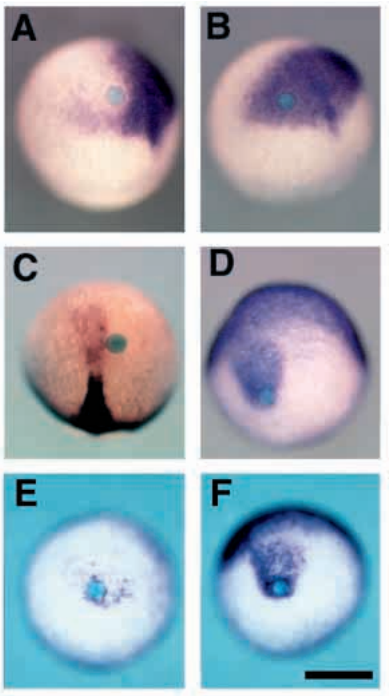

Fig. 2. BMP-soaked beads can repress neural differentiation and induce epidermal ectoderm without perturbing normal gastrulation. (A) *otx-2* expression was down regulated around the BMP-soaked bead. The effect of the BMP-soaked bead tended to be stronger in the region anterior to the bead than posterior to it. (B) Control BSA-soaked beads did not inhibit *otx-2* expression at all. (C) In the same experiment, BMP-soaked beads did not perturb axial mesoderm formation at the morphological and molecular level. Neither *gsc* nor *ntl* in chordamesoderm was repressed. The non-neural (epidermal) ectoderm marker, *gata-3* (D), was induced by BMP-soaked beads. As shown in Fig. 2A, a stronger effect was observed anterior to the bead. (E) Overexpression of DN-BRIA can repress the induction of *gata-3* caused by BMP-soaked beads. In control embryo injected with β-galactosidase mRNA (F), *gata-3* is expressed normally. Scale bar, 200 μm.

epidermis and inhibit neuralization (Wilson and Hemmati-Brivanlou, 1995). These observations suggest that BMP acts to determine neural and non-neural cell fate and establishes the boundary between the two cell populations.

To examine whether the *bmp*-expressing and neuroectoderm regions are complementary to each other, we performed two-color whole-mount in situ hybridization using antisense probes for *zbmp-2* (in zebrafish, BMP-2 is likely to be the functional homolog of *Xenopus* BMP-4 (Kishimoto et al., 1997; Nikaido et al., 1997)) and *otx-2*, an anterior neuroectoderm marker (Mori et al., 1994). Fig. 1 shows that, at the 80% epiboly stage, the *zbmp-2* -expression domain (brown) contacts that of *otx-2* (red) in the lateral (Fig. 1A) and animal (Fig. 1C) regions. This suggests that *zbmp-2* signaling is only effective within its own expressing domain.

Since it is known that BMP is a neural inhibitor and epidermal inducer, we next tested the ability of BMP to induce epidermal markers and repress neuroectodermal markers in the zebrafish ectoderm. So as to prevent perturbation of dorsoventral patterning of the mesoderm, the method of exogenous BMP application that we chose was transplantation of BMP-soaked beads. A similar approach has been used successfully to analyze the action of BMP on the dental mesenchyme in tooth development (Vainio et al., 1993). It is possible that the operation of transplanting beads interferes with gastrulation. Fortunately, however, due to the translucency of the zebrafish embryo, the axial mesoderm can be identified under the stereomicroscope. Therefore, only those embryos with normal axial mesoderm were selected for further analysis.

BMP beads were prepared as previously described (Vainio et al., 1993; also see Materials and Methods). The BMP-soaked beads were implanted into the dorsal blastoderm of embryos at the shield stage, because at earlier stages, BMP beads induce mesodermal tissue. After fixation of the host embryos, we examined the expression of several marker genes using whole-mount in situ hybridization. To confirm that the axial mesoderm was intact we analyzed the expression of *goosecoid* (*gsc)*, which marks prechordal mesoderm (Stachel et al., 1993), and *no tail* (*ntl)*, which marks chordamesoderm (Schulte-Merker et al., 1992). Expression of these markers was normal, despite the presence of the BMP beads (in 29 of 29 embryos) (Fig. 2C). Expression of the anterior neuroectoderm marker, *otx-2*, was repressed around the beads (in 40 of 40 embryos) (Fig. 2A). By contrast, the BMP beads induced *gata-3*, an epidermal ectoderm marker (Neave et al., 1995), in the epiblast (Fig. 2D) (in 30 of 30 embryos). Interestingly, for *otx-2* and *gata-3*, the range of BMP's effect was always broader in the region anterior to the bead than posterior to it. These results suggest that, in zebrafish, BMP expressed in the ventral ectoderm functions to suppress the neuroectoderm by inducing the epidermis.

Molecular cloning of the BMP type I serine/threonine kinase receptor

The next question that we wanted to address was whether BMP directly regulates cell fate in the ectoderm or whether other secreted factors, induced in response to BMP stimulation, are

involved. To answer this, we isolated a BMP type I receptor from zebrafish. Previous studies using various model animals have demonstrated that constitutively active forms of receptors are useful tools for solving such problems (Jones et al., 1996; Lecuit et al., 1996; Nellen et al., 1996). Briefly, if epidermal induction by BMP requires a secondary factor (relay mechanism), then the epidermal marker gene *gata-3* would be induced in a non-cell-autonomous manner when a constitutively active BMP receptor was ectopicaly expressed in the ectoderm. In contrast, if BMP can directly convert neural fate to epidermal without the aid of a secondary factor (direct diffusion mechanism), *gata-3* would be induced in a cell-autonomous manner. Using a previously reported BMP type I receptor (Suzuki et al., 1994) as a probe, we screened a zebrafish gastrula cDNA library and isolated a cDNA encoding a 527-amino acid receptor-like protein. The predicted amino acid sequence of this clone is shown in Fig. 3A. The presence of conserved cysteine residues in the extracellular domain (lightly shaded, Fig. 3A) and a typical GS domain (SGSGSGLP, boxed area, Fig. 3A) in the juxtamembrane

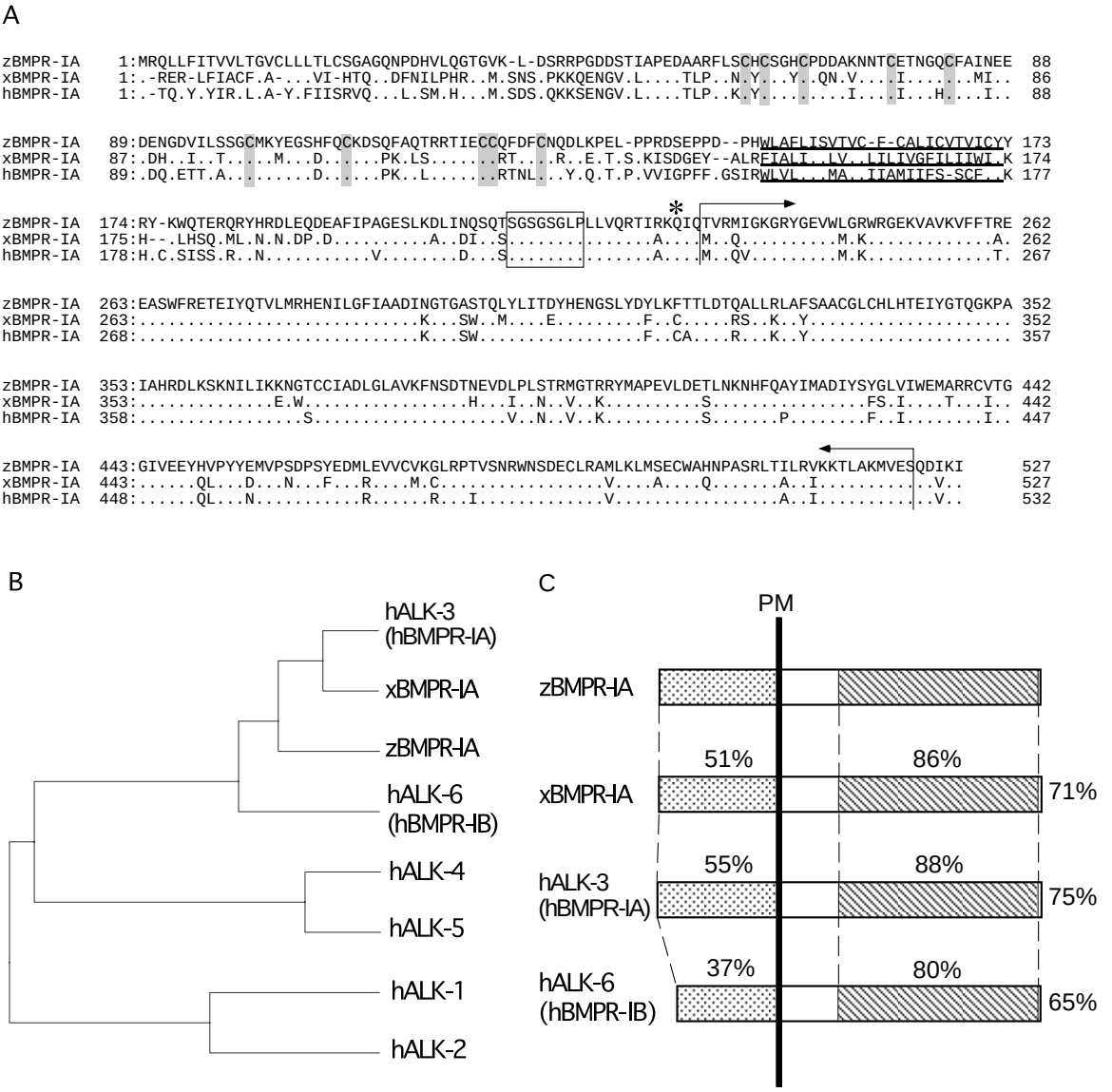

Fig. 3. Zebrafish BMPR-IA. (A) The alignment compares the amino acid sequences of zebrafish (GenBank Accession number: AB011826), *Xenopus* (GenBank Accession number: U16654) and human (GenBank Accession number: Z22535) BMPR-IA (ALK-3). To obtain maximum matching, gaps are introduced in these sequences and identical amino acids are indicated by dots. Ten conserved cysteine residues in the extracellular domain are lightly shaded. The deduced transmembrane region is underlined and the GS domain (SGSGSGLP), which exists in all type I receptors is boxed. The asterisk and bent arrows indicate the glutamine (Q) that was replaced to generate a constitutively active variant and a serine/threonine kinase domain, respectively. (B) An evolutionary tree generated using the UPGMA algorithm, illustrating the phylogenetic relationships among kinase domains of various type I receptors. The length of the two parallel lines connecting the two sequences is proportional to the estimated genetic distance between them. (C) The amino acid sequence identity of other BMP receptors with zebrafish BMPR-IA. The percentage of similarity in the extracellular and the kinase domains are indicated above the corresponding regions in the drawings. The identity in the full-length receptor are shown alongside each drawing. Abbreviation: PM, plasma membrane.

region confirmed that this receptor was a type I receptor of the TGF-β superfamily. Furthermore, comparison of the amino acid sequence of the serine/threonine kinase domain (marked with arrows, Fig. 3A) with that of other type I receptors showed that this receptor is most closely related to the type IA or ALK-3 (Fig. 3B) class of BMP receptors (ten Dijke et al., 1994). Based on this structural similarity, we tentatively designated this receptor as zebrafish BMPR-IA. Subsequently, we examined the expression pattern of this gene during gastrulation, when ectodermal and mesodermal patterning takes place. Whole-mount in situ hybridization revealed that the transcripts encoding BMPR-IA are distributed ubiquitously in all stages examined, from the blastula (sphere stage, Fig. 4A) to the late gastrula (80% epiboly, Fig. 4D). At the 80% epiboly stage, sagittal sections showed that BMPR-IA is equally expressed in the hypoblast and epiblast (data not shown) in the embryo. In addition, northern blot analysis reveals that transcripts are present maternally and persist until at least 24-hour postfertilization (Fig. 4E). Thus, BMPR-IA is a good candidate for the endogenous BMP type I receptor participating in the dorsoventral patterning of the ectoderm and mesoderm during gastrulation.

CA-BRIA can transduce a BMP-like ventralizing signal in mesoderm

To confirm that the cloned receptor is the functional homolog of BMPR-IA of other species, we carried out functional analyses. In *Xenopus* and zebrafish, BMP-2/4 ventralizes both the ectoderm and mesoderm during early embryogenesis. Thus, for zebrafish BMPR-IA to be the homologue of the BMPR-IA, a constitutively active form of zebrafish BMPR-IA should also ventralize ectoderm and mesoderm in a ligand-independent manner.

For the TGF-β type I receptor (TβRI), it has been shown that substitution of a threonine residue adjacent to the GS domain with an aspartate residue results in a mutant receptor that mimics the activated phosphorylation state of wild-type TβRI, in a ligand-independent manner (Wieser et al., 1995). This threonine is not conserved in zebrafish BMPR-IA. However, like Tkv (Lecuit et al., 1996; Nellen et al., 1996) and mammalian ALK-3 (Hoodless et al., 1996), it has a glutamine residue located in the position corresponding to the threonine of TβRI. Substitution of this glutamine residue in Tkv and ALK-3 with aspartate residue is known to make constitutively active forms of these receptors. So, we replaced the corresponding glutamine residue in zebrafish BMPR-IA (position 228, marked with an asterisk in Fig. 3A) with an aspartate residue. was replaced with aspartate (Fig. 5a). By analogy with TβRI, Tkv and ALK-3, we predicted that this mutant zebrafish type I BMP receptor would show ligand-independent activity. By injecting synthetic mRNA encoding this constitutively active variant (CA-BRIA), we next investigate the in vivo role of BMPR-IA in mesoderm patterning.

Injection was carried out at the 1- or 2-cell stage. Injected embryos were allowed to develop to appropriate stages, after which marker gene expression along the dorsoventral axis was analyzed by whole-mount in situ hybridization. Fig. 5bA and B show that expression of *ntl* in the chordamesoderm region was repressed at the tail bud stage in injected embryos, although it was not repressed in the tail bud domain. Similarly, expression of *gsc*, a specific marker for prechordal mesoderm, was strongly repressed in injected embryos of the same stage (Fig. 5bC,D). By contrast, *wnt-8*, a marker for ventrolateral mesoderm (Kelly et al., 1995), was induced in the whole area of the blastoderm margin (Fig. 5bE,F). Strikingly, *wnt-8* was not induced in the animal hemisphere although CA-BRIA protein translated from injected mRNA is thought to be distributed ubiquitously throughout the embryo. This result suggests that CA-BRIA can ventralize the mesoderm, but cannot induce the ventral mesoderm ectopically in the presumptive ectodermal region.

Like embryos injected with *zbmp-2* (Nikaido et al., 1997), CA-BRIA is sufficient to ventralize the mesoderm, suggesting that endogenous BMPR-IA can transduce BMP signals for mesodermal patterning in vivo. The lack of ectopic *wnt-8* expression in the animal pole region of injected embryos suggests that the ability of the blastoderm to differentiate into ventral mesoderm in response to CA-BRIA may be restricted to the blastoderm margin where mesoderm was previously induced.

A truncated BMPR-IA can promote ventral expansion of neuroectoderm

The experiments described above show that the constitutively active CA-BRIA, causes the same phenotype as gain of function of BMP-2 or BMP-4, indicating that BMPR-IA is an endogenous receptor for BMP-2/4. In this section, we provide evidence that a dominant negative form of this receptor (DN-BRIA) causes phenotypes similar to those of loss of function of BMPs. The dominant negative receptor is truncated at the C-terminal end of the putative transmembrane region and lacks the GS domain and the entire ser/thr kinase domain. According to studies, mainly in *Xenopus*, depletion of active BMP in ectoderm by overexpression of either a dominant negative BMP receptor or its ligand (Graff et al., 1994; Suzuki et al., 1994; Hawley et al., 1995; Xu et al., 1995), or BMP antagonists, Chordin (Sasai et al., 1995) or Noggin (Lamb et al., 1993; Zimmerman et al., 1996), respecifies ectoderm to neural fate. Thus, if DN-BRIA neuralizes the ventral part of the ectoderm, this would suggest that the phenotype results from the depletion of BMP ligand by the dominant negative receptor. This in turn would suggest that wild-type BMPR-IA can interact with BMP ligand in vivo.

To determine whether DN-BRIA neuralizes ventral ectoderm, synthetic mRNA encoding DN-BRIA was injected into 1- or 2-cell-stage embryos. These embryos were harvested at the 80% epiboly stage and analyzed for marker gene expression by in situ hybridization. Both the anterior and the posterior neuroectoderm expanded to the ventral region, to the same extent, in embryos injected with DN-BRIA, as revealed by the ectopic expression of *otx-2* and *hoxa-1* (Alexandre et al., 1996), respectively (Fig. 6A,C). In both cases, the ectopic expression induced in the ventral region was restricted in epiblast (data not shown), while the anterior and posterior boundaries of expression of these genes were not perturbed (arrowheads in Fig. 6A-D). By contrast, the expression domain of *gata-3*, which marks non-neural ectoderm in the anteroventral region of the embryo was reduced (Fig. 6E,F). Similarly, *eve 1*, which is expressed mainly in the posteroventral epiblast at the 80% epiboly

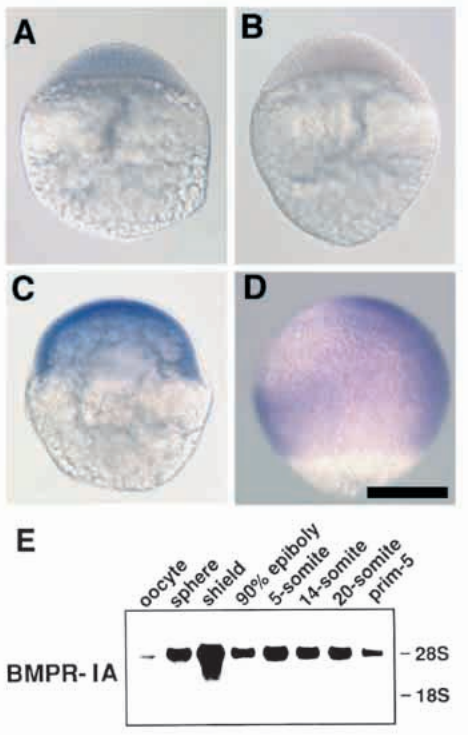

Fig. 4. Spatiotemporal expression pattern of BMPR-IA. Whole-mount in situ hybridization analysis at different stages; sphere stage (A,B, blastula), shield stage (C, early gastrula) and 80% epiboly (D, late gastrula). As shown in B, in the sphere stage, detectable signals were not obtained using sense probes as a negative control. Note that BMPR-IA was expressed ubiquitously during early embryogenesis. At 80% epiboly stage (D), signals were rarely detected in the ventroanimal region in whole embryos, but in sagittal sections faint signals were observed in this region (data not shown). Lateral views are oriented with their dorsal side to the right except for (A). Scale bar; 200 μm. (E) Northern blot analysis demonstrated that BMPR-IA transcripts were present at low levels maternally. Zygotically, strong signals persist during early embryogenesis with a peak at the shield stage. The positions of 28S and 18S ribosomal RNA are indicated.

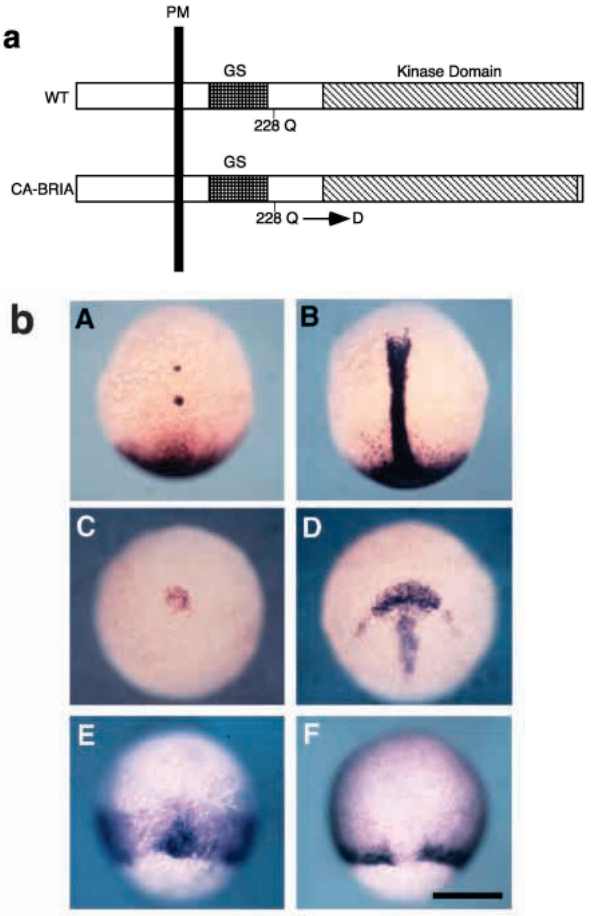

Fig. 5. CA-BRIA ventralizes mesoderm like BMP ligand. (a) A schematic drawing of wild-type and constitutively active form of BMPR-IA. The glutamine (Q) residue located at position 228 was replaced with aspartate (D) in the constitutively active variant. (b) Dorsal views (A,B,E,F) and dorsoanimal views (C,D) of experimental (left column) and β-galactosidase mRNA-injected (right column) embryos. All embryos are oriented with their animal pole up. Suppression of *ntl* (A) and *gsc* (C) expression in the embryos injected with mRNA encoding CA-BRIA revealed the absence of axial mesoderm. By contrast, expansion of *wnt-8* to the dorsal part demonstrated the mesoderm ventralization instead of dorsal mesoderm. Scale bar, 200 μm.

stage (Joly et al., 1993), was significantly reduced (Fig. 6G,H).

As BMP is thought to act as an epidermal inducer and neural inhibitor in dorsoventral patterning of the ectoderm, *zbmp-2* should be downregulated in DN-BRIA mRNA-injected embryos. As expected, *zbmp-2* expression was reduced in these embryos (Fig. 6I,J). This is considered to represent the inhibition of autoactivation pathway of *zbmp-2* by DN-BRIA. The disruption of BMP signaling by the DN-BRIA was also observed in embryos with transplanted BMP-soaked beads. As shown in Fig. 2E, induction of the non-neural epidermal marker, *gata-3*, around the BMP-soaked beads was reduced when the host embryo was injected with DN-BRIA mRNA. In the same embryo, endogenous expression of *gata-3* was reduced. In the control embryo, however, *gata-3* was induced normally.

It is known that organizer-derived structures, such as the notochord, are not formed in the secondary axis induced by the dominant negative BMP receptors (Suzuki et al., 1994; Frisch and Wright, 1998). Consistent with this, axial *ntl* expression, revealing the existence of notochord, was not observed in the embryo in which *otx-2* expression domain was expanded most ventrally (Fig. 6K).

From these results, we concluded that the DN-BRIA can interfere with BMP signaling, presumably by trapping BMP ligands or forming an inactive complex with the wild-type II BMP receptor.

CA-BRIA directly converts neural fate to epidermal in a cell-autonomous manner

The experiments that we have described using CA-BRIA and DN-BRIA strongly suggest that the BMPR-IA cloned in this study is an endogenous receptor for zebrafish BMP-2/4. Using this clone, we performed a cell-transplantation experiment to investigate whether BMP induced epidermis directly or indirectly. Embryos injected with a mixture of rhodamine-dextran and biotin-dextran were injected with synthetic mRNA

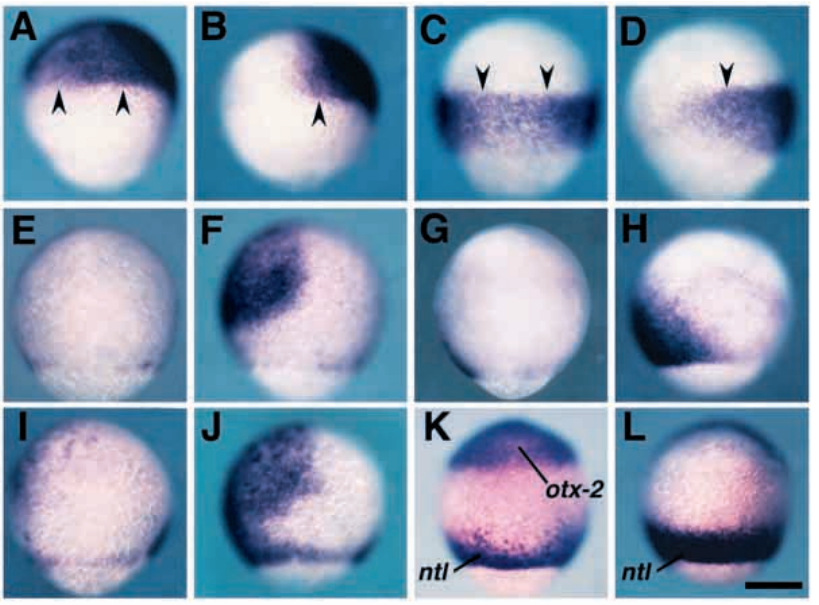

Fig. 6. DN-BRIA causes the expansion of the neuroectoderm to the ventral part without any perturbation of the anterior-posterior pattern. At the 80% epiboly stage, embryos injected with mRNA encoding DN-BRIA (A,C,E,G,I,K) and β-galactosidase (B,D,F,H,J,L) were stained with the specific probes for *otx-2* (A,B), *hoxa-1* (C,D), *gata-3* (E,F), *eve-1* (G,H) and *zbmp-2* (I,J). For the embryos shown in K and L, a mixture of probes for *otx-2* and *ntl* was used. (A-J) Embryos are oriented with their dorsal side to the right and animal pole to the top; (K,L) ventral views with the animal pole at the top. In embryos injected with DN-BRIA mRNA, expression domains of anterior (*otx-2*, A) and posterior (*hoxa-1*, C) neuroectoderm marker genes expanded to the ventral part of the embryo, compared with the negative control embryos (B,D, stained with probes for *otx-2* and *hoxa-1*, respectively). The posterior boundary of *otx-2* and anterior boundary of *hoxa-1* were maintained (arrowheads) in the DN-BRIA-injected embryos. By contrast, ventral non-neural ectodermal marker genes, *gata-3* and *eve-1*, are reduced by the overexpression of DN-BRIA (E and G, respectively). Similarly, *zbmp-2* is also reduced (I). This suggests that the neuralization of the ectoderm (A,C) and reduction of non-neural ectoderm (E,G) presented here results from the down-regulation of *zbmp-2*, caused by interference with the autoactivation process by DN-BRIA. (K,L) DN-BRIA cannot induce involuting dorsal mesoderm as revealed by the axis-like expression pattern of *ntl* even in the embryo in which neuroectoderm is expanded to the most ventral part. Scale bar, 200 μm.

encoding CA-BRIA at the 1- or 2-cell stage and used as donors in a transplantation experiment. To avoid a contribution by involuting mesoderm, labeled donor cells were transplanted close to the animal pole of the host embryo. By such a targeted transplantation, placing donor cells in the ectoderm, we could exclude the possibility that mesoderm locally ventralized by BMP would indirectly change the gene expression in the overlying ectoderm. These chimeras were allowed to develop until the 80% epiboly stage and those embryos that went through normal gastrulation were fixed to determine their phenotypes by whole-mount in situ hybridization, followed by staining with HRP-conjugated avidin-biotin complex to visualize the location of transplanted cells.

As shown in Fig. 7A,B, *gata-3* was induced cell autonomously in chimeras with a successful dorsal transplantation of cells expressing CA-BRIA. In addition, sagittal sections of these chimeric embryos demonstrated that *gata-3* expression was induced exclusively in the epiblast far

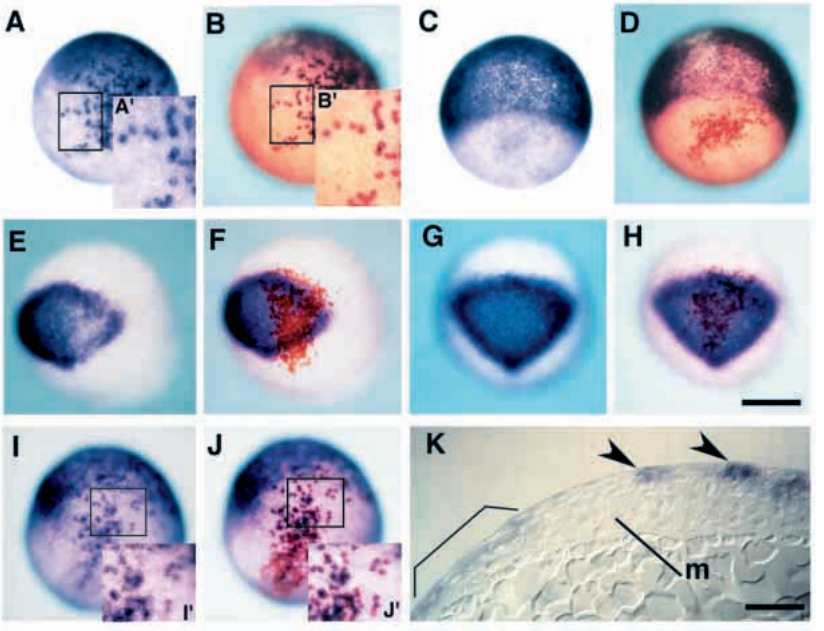

Fig. 7. Constitutively active BMPR-IA can simulate BMP signaling in a direct and cell-autonomous manner in ectoderm patterning. (A-J) Chimeras containing cells derived from injected embryos with biotinylated dextran as a lineage tracer and mRNA encoding either constitutively active receptor (A,B, E,F, I,J) or β-galactosidase (C,D, G,H). Embryos after operation are processed for whole-mount in situ hybridization at 80% epiboly with *gata-3*, epidermal marker gene (A,C), *otx-2*, neural marker gene (E,G) and *zbmp-2* (I). The same embryos are stained with avidin-biotin HRP complex for the purpose of detection of donor cells (B,D), (F,H), (J), respectively). The insets (A′,B′, I′,J′) show a magnification of the boxed area in the same figures. Note that BMP signaling can activate the expression of *gata-3* and *zbmp-2* only in cells derived from injected embryo (compare Fig. 7A′ with 7B′ and 7I′ with 7J′, respectively). By contrast, *otx-2* is downregulated only in donor cells expressing CA-BRIA (E,F). In chimeric embryos transplanted cells containing β-galactosidase (C,D, G,H), neither ectopic induction nor repression of marker genes were detected. (K) Sagittal section of chimeric embryos containing CA-BRIA in ectoderm. Ectopic *gata-3* signals are observed in ectoderm (arrowheads), suggesting that ectoderm ventralization is independent of mesoderm ventralization. Endogenous *gata-3* expression is marked by bracket. Abbreviation: m, axial mesoderm. Scale bar, 200 μm (H), 50 μm (K). A-J are the same magnification.

from the mesoderm (indicated by arrowheads in Fig. 7K), showing that the induction of *gata-3* occurred independently of the mesoderm. In contrast, the neuroectodermal marker, *otx-2*, was downregulated in the donor cells (Fig. 7E,F). These results demonstrate that a secondary secreted molecule induced by BMP is not involved in ectodermal patterning. *bmp* is, however, known to be induced by BMP through the autoactivation pathway. Indeed, we analysed the expression pattern of *zbmp-2* in the chimeric embryos and found a cell-autonomous induction of *zbmp-2* (Fig. 7I,J). *zbmp-2* is induced by CA-BRIA, but the effect of zBMP-2 does not appear to reach even the adjacent cells, even though it has a potential to act as a diffusible factor. From these observations, we concluded that, in vivo, BMP directly converts neural to epidermal fate without the aid of other secreted molecules and that its effects are restricted to the cells in which it is expressed.

DISCUSSION

BMP acts as a direct and short-range signaling molecule

Based on previous reports, *bmp* is thought to be expressed in non-neural ectoderm. In this study, we have shown that the relative positions of the zebrafish *bmp*-2-expressing domain and the neuroectoderm at the molecular level. As shown in Fig. 1, there is no gap between the expression domains of *bmp-2* and *otx-2*, suggesting that BMP activity is tightly confined within its expressing region. However, when we implanted BMP-conjugated beads, marker genes were repressed or activated from a distance of several cell diameters (Fig. 2A,D). This suggests that BMP-4 does act as a long-range signaling molecule, as a result of either its diffusibility or through its induction of a secondary factor. The mosaic analysis shown in Fig. 7 provides an interpretation of this paradox. This experiment demonstrated that *zbmp-2* was induced only in donor cells expressing a constitutively active BMPR-IA protein (CA-BRIA) (Fig. 7I,J). If BMP protein had affected the adjacent cells, *zbmp-2* should have been induced in them, since *zbmp-2* is known to be induced through the autoactivation pathway. Therefore, cell-autonomous expression of *zbmp-2* suggests that the effect of BMP is restricted to its expression domain in vivo. This restriction of BMP activity may be caused by the interaction of BMP with the extracellular matrix and extracellular inhibitors, such as Chordin and Noggin (see below). Therefore, we concluded that this apparent long-range effect shown in Fig. 2 was mainly due to direct diffusion caused by using a high concentration (10 ng/μl) of BMP solution to treat the beads. The long-range effect observed in the bead implantation experiment could also be explained by mechanical disruption of tight cell-cell contact in the blastoderm during implantation. Jones et al. (1996) suggested that the extracellular matrix (ECM) might trap BMP-4 by showing that dissociation of the animal cap that removes the ECM made BMP act as a long-range signal.

The next question raised is why BMP preferentially affected cells located anterior to the beads; *otx-2* repression and *gata-3* induction are not radially symmetrical to the implanted bead (Fig. 2A,D). There are several possible explanations for the asymmetric marker gene induction and repression. First, Chordino, the zebrafish homolog of Chordin, which is one of the two major BMP antagonists identified in *Xenopus*, is expressed in the shield, the most dorsal part of the zebrafish embryo and the region corresponding to Spemann's organizer (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). Given that Chordino acts to prevent excess BMP protein activity, the anterior part of the neuroectoderm most distant from the shield, the source of Chordino, may be more sensitive to BMP. Alternatively, the greater anterior extent of BMP influence may be explained by cell migration and proliferation. BMP protein, as discussed above, may associate with extracellular components so efficiently that BMP stays on the cells that produce and secrete it. Similarly, in the bead transplantation experiment, it is thought that BMP proteins diffusing from the bead may stay on the cells near it. However, cells surrounding the bead proliferate and move toward the animal pole region by conversion-extension as gastrulation proceeds. As a result, BMP proteins associated with these moving cells might also be conveyed towards the region anterior to the beads and could induce specific responses in these cells. Even the cells previously exposed to BMP could be affected by BMP after migration, assuming that the concentration of BMP solution used in our experiment is high enough. From this point of view, our data presented in Fig. 2A,D may represent a trait of this sort of cell migration. The hypothesis that BMP is conveyed by migration and/or proliferation of cells has been proposed for *Drosophila* Dpp in wing disc patterning, in which Dpp is thought to be carried from its source in part by the proliferation of nonsecreting cells during disc development (Lecuit et al., 1996; Nellen et al., 1996). In addition to this, there is evidence from work in *Xenopus* showing that BMP requires the aid of cell migration to demonstrate its long-range effect. In this experiment, animal cap explants expressing BMP-4 were conjugated to recipient caps labeled with a fluorescent lineage tracer and the effect of BMP was monitored by assaying the expression of *Xbra* in the fluorescent explant. Using this assay, it was demonstrated that *Xbra* was not induced far (no more than a few cell diameters) in the recipient explant, suggesting that BMP-4 cannot travel across the explant-explant boundary that prevents intermigration of cells (Jones et al., 1996). In whole embryos, however, ectopically expressed *Xenopus* BMP-4 has been shown to act over a long range, when mRNA encoding BMP-4 is injected into only a few blastomeres of 32-cell-stage embryos (Dosch et al., 1997). In this experiment, it is thought that such cell migration is not perturbed.

As discussed above, the spatial regulation of secreted factors, such as BMP, may involve many factors including the cellular environment, attachment to the ECM and cell migration. Because of this tight regulation, BMP may not be effective outside the region where it is expressed, thereby establishing the sharp boundary between neural and non-neural tissue.

Anteroposterior prepattern is not affected by depletion of BMP

In amphibia, the anteroposterior pattern of the neural tissue has been thought to be regulated through the influences of the organizer in a two-step process (two-signal model, Nieuwkoop, 1952). Initially, anterior axial mesoderm neuralizes the overlying ectoderm with the anterior property (activation step). The anterior neural tissue (e.g., forebrain) induced by this

process is then posteriorized by the involuting chordamesoderm to form more posterior structures such as hindbrain and spinal cord (transformation step). Recent work in zebrafish, however, suggests that axial mesoderm is not the only player in the anteroposterior patterning of the ectoderm. Using transplantation experiments, Koshida et al. (1998) proposed the existence of a circumferentially established prepattern independent of the organizer. Briefly, they demonstrated that aggregates of COS-7 cells transfected with *Xenopus noggin* and *chordin*, which are the potent neural inducers (Lamb et al., 1993; Sasai et al., 1995), caused the ectopic expression of some region-specific neuroectodermal markers, depending on the location they were transplanted. Namely, expression of the anterior neural marker, *otx-2*, is induced when the aggregate is located near the animal pole region, while the posterior neural marker, *hoxa-1*, is induced when it is located near the blastoderm margin. Since the neural inducing activity of Noggin and Chordin is thought to be attributed to their ability to bind to and inactivate BMP protein, the most plausible scenario for neural patterning is as follows. By early gastrula stages, BMP expressed in the ectoderm masks the circumferentially established prepattern along the anteroposterior axis. Afterwards, the organizer-derived molecules, Noggin, Chordin, and/or Follistatin, sequester active BMP protein only in the dorsal ectoderm, resulting in the emergence of neural tissue with correct anteroposterior polarity according to the prepatterned positional information. In our present work, this hypothesis is further confirmed. As shown in Fig. 6A,C, the neuroectodermal marker genes, *otx-2* and *hoxa-1*, are ectopically induced by DN-BRIA keeping the normal anteroposterior boundary. Consistent with the observation in *Xenopus*, this epidermal-to-neural fate conversion occurs without induction of the axial mesoderm (Fig. 6K). The most likely explanation for our data is that DN-BRIA acts to remove BMP in the ectoderm, like Noggin or Chordin, and as a result the circumferential anteroposterior pattern is unveiled regardless of the dorsoventral axis.

BMP plays a major role in the determination of the ectodermal fates, neural or non-neural (epidermal). Neural tissue emerges as a result of depletion of BMP; however, the anteroposterior pattern is presumably regulated by another cue, as discussed above. According to recent work, one of the signal sources that may establish such a prepattern is the nonaxial mesoderm (Woo and Fraser, 1997; Koshida et al., 1998). More recently, it has been reported that the small group of ectodermal cells located close to the animal pole region in the embryo have the ability to anteriorize surrounding neural tissue (Houart et al., 1998). This suggests that these cells are involved in the formation of the anteroposterior prepattern. To understand the exact mechanism for anteroposterior patterning in neural tissue regulated by these organizing tissues, identification of the substances emanating from them will certainly be necessary.

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