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A DOMINANT NEGATIVE BONE MORPHOGENETIC PROTEIN 4 RECEPTOR CAUSES NEURALIZATION IN *XENOPUS* ECTODERM*

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SUMMARY: Injection of DN-BR mRNA encoding a dominant negative type I receptor for bone morphogenetic protein 4 (BMP4) converted prospective ectoderm into neural tissue in *Xenopus* animal cap explants, in the absence of expression of mesodermal marker genes. The injected caps expressed a general neural marker NCAM and the forebrain marker opsin. Coinjection of wild-type BMP4 receptor mRNA completely reversed the neuralization by DN-BR. No expression of known neuralizing factors, i.e., noggin and follistatin, was detected in the DN-BR-injected animal caps. Furthermore, neuralization elicited by noggin or 3m, a LIM domain mutant of *Xlim*-1, was substantially inhibited by co-injection of BMP4 mRNA. Since BMP4 is expressed in the prospective ectoderm during gastrulation, our results suggest that the ventralizing factor BMP4 acts also as a physiological inhibitor of neuralization in the development of *Xenopus* ectoderm.

Xenopus animal cap (the prospective ectoderm) explant culture is a unique system to study neural and mesodermal induction, since it has the potential to give rise to either neural or epidermal tissue as well as mesoderm in the presence of suitable inducers. During gastrulation, the neural plate is induced in the dorsal ectoderm by the underlying dorsal mesoderm (1-3). If animal cap is dissected at blastula stage, such

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caps are seperated from embryonic neural-inducing activity and commit an epidermic differentiation. However, it has been shown that even in the absence of mesoderm, the ectodermal cells can differentiate to neural tissue by cell dissociation and dilution (4-6), suggesting that the ectodermal cells undergo cell-autonomous neuralization in a default neural state when the intercellular signaling is inhibited. Therefore, some factor(s) in animal cap is thought to play a suppressing effect on the default neural state. One of such factors is suggested to be activin, a member of the transforming growth factor-β superfamily. It was recently reported that inhibition of activin signaling by a dominant negative activin type II receptor or follistatin (a natural inhibitory binding protein for activin) initiates neuralization of the ectoderm (7, 8).

Another member of the transforming growth factor-β superfamily, bone morphogenetic protein 4 (BMP4), has been identified as a ventralizing factor for mesoderm patterning in the early development of *Xenopus* embryo, since BMP4 overrides the dorsal mesoderm formation by activin in animal caps (9, 10). Furthermore, misexpression of BMP4 in the dorsal mesoderm ventralizes the embryo; BMP4 rescues embryo dorsalized by LiCl treatment (11); and a dominant negative BMP4 type I receptor (DN-BR) converts the ventral mesoderm to dorsal mesoderm (12-14). Although BMP4 is suggested to play a role in the mesoderm patterning, BMP4 transcripts are present in the entire ectodermal region at the early gastrula stage (11), implying another role of BMP4 in the ectoderm. This possibility has been suggested by data reported by Maéno M. et al. (13), in which the animal cap injected with DN-BR dorsalizes the ventral mesoderm to initiate formation of muscle and neural tissues including eyes.

To analyze a role of BMP4 in the ectoderm, we first examined the effect of DN-BR on cell differentiation of animal caps. The DN-BR-injected animal caps, which would otherwise differentiate to ectoderm, were found to form neural tissue. The neuralization was indicated by the appearance of cement gland and the expression of a general neural marker, neural cell adhesion molecule (NCAM) (3). There was neither induction of mesodermal markers nor expression of known neuralizing factors, i.e., noggin or follistatin in the DN-BR-injected animal caps. Furthermore, neuralization initiated by 3m, a LIM domain mutant of the homeobox gene *Xlim*-1 (15), or noggin (16, 17) was dramatically suppressed by BMP4. Therefore, we propose that the ventralizing factor BMP4 may also act as a general neural inhibitor in the development of *Xenopus* ectoderm.

MATERIALS AND METHODS

mRNA Preparation and Embryo Injections. Plasmids used for mRNA synthesis are DN-BR, wild type BMP4 receptor (WT-BR) (12), BMP4 (18), 3m (15) and noggin (16). All these plasmids were linearized and subjected to synthesis of capped mRNA using an in vitro transcription kit (Ambion, Austin, TX) as described by Moon and Christian (19).

Xenopus laevis embryos were obtained by artificial insemination after induction of females with 500 IU human chorionic gonadotropin. Developmental stages were designated according to Nieuwkoop and Faber (20). At the 2-cell stage each blastomere was injected with the synthetic mRNA. Animal caps were dissected at stages 8.5 to 9, and cultured at 22°C in 67% Leibovitz's L-15 medium (GIBCO-BRL) and 7 mM Tris-HCl (pH 7.5) and gentamicin 50 μg/ml. The explants were harvested at equivalent of gastrula or tailbud stages for assay of cell differentiation using molecular markers.

Detection of Molecular Markers. RNA extraction from the animal cap explants, and design of oligonucleotides for the reverse transcription-polymerase chain reaction (RT-PCR) analysis were as described by Hemmati-Brivanlou A. et al. (7, 8). RT-PCR was used for detection of molecular markers including muscle actin, NCAM, opsin, elongation factor-1α (EF-1α), *Xbra*, *goosecoid* (*gsc*), noggin and follistatin (7, 8). Primers for *Xlim*-1 (21) were designed as follows: upstream, 5'-TGGTGGACAGATTAGAGCCG-3' and downstream, 5'-AGAGTGCATGGAACCTGGTA-3'. In some experiments, NCAM was also determined by Western blot. Briefly, a mouse anti-NCAM cytoplasmic domain antibody 4d (from Development Studies Hybridoma Bank, Iowa University) was used at dilution of 1:20. In each experiment, five to six animal caps per group were combined and the lysate equivalent to one animal cap was loaded on SDS-PAGE. A blotted nitrocellulose filter was incubated with 4d, and followed by the peroxidase-conjugated anti-mouse IgG antibody. The presence of NCAM on the resulting blot was visualized by ECL system (Amersham).

RESULTS

DN-BR Causes Neuralization in Animal Cap in the Absence of Expression of Mesodermal Markers. One ng of DN-BR mRNA was injected into the two blastomeres of *Xenopus* embryos at the two-cell stage. Animal caps were dissected at stages 8.5-9 followed by culture. At the equivalent of the tailbud stage, all DN-BR-injected animal caps began to elongate and develop cement gland, whereas, control β-galactosidase (β-gal) mRNA-injected animal caps remained roughly round without the morphological change (Fig. 1). Cement gland is the anteriomost ectodermal structure (22, 23), which has never been observed in the control caps. Co-injection of 2 ng of WT-BR mRNA completely reversed the morphological change caused by DN-BR (data not shown). We next examined neural differentiation in the DN-BR-injected caps using RT-PCR analysis. As shown in Fig. 2A, NCAM as well as opsin, a marker of photoreceptor of the eye (24), were expressed in the DN-BR-injected animal caps. The expression of these neural markers caused by DN-BR was totally

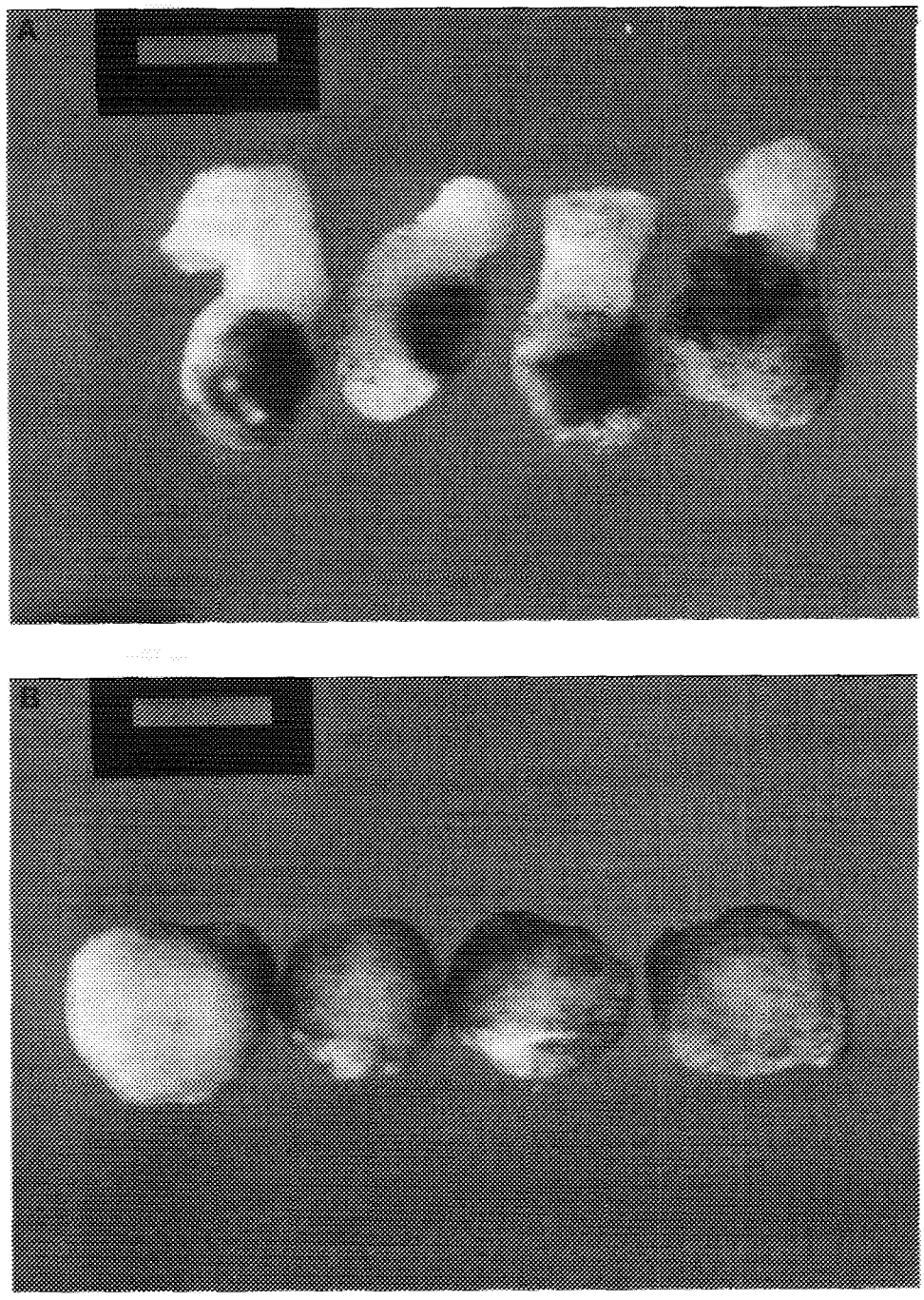

Fig. 1. Morphological Change of Animal Cap Injected with DN-BR. One ng of DN-BR or β-gal RNA was injected into the two blastomeres of *Xenopus* embryos at the two-cell stage. Animal caps were dissected at stages 8.5-9 followed by culture. At equivalent of tailbud stage, the DN-BR-injected animal caps elongated and developed cement gland (A), whereas, control β-gal RNA-injected animal caps remained roughly round without the morphological change (B).

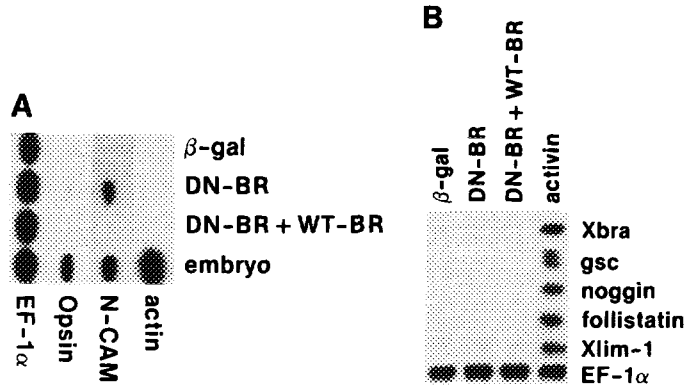

Fig. 2. Effect of DN-BR on Gene Expression in the Animal Cap. Embryos were injected with RNAs encoding β-gal, DN-BR or DN-BR plus WT-BR, at 1 ng/embryo for each RNA. The blastula stage animal caps were dissected and cultured in buffer until sibling controls reached tailbud (A) or gastrula (B) stages. RNAs were extracted, gene expression was determined by RT-PCR (see Materials and Methods). As a control, EF-1α gene expression was determined and a comparable amount of RNA was used in each set.

eliminated by co-injection of WT-BR mRNA (Fig. 2A), suggesting that the neuralization by DN-BR is through the specific inhibition of the BMP4 signaling. However, expression of muscle actin, a mesodermal marker (25) (Fig. 2A), *Xbra*, an early general mesodermal marker (26), *gsc* (27) and *Xlim*-1 (21), two early dorsal mesodermal markers (Fig. 2B), were not initiated by DN-BR injection, suggesting that the mesoderm induction is not involved in the neuralization by DN-BR. However, at present, we do not know how the DN-BR-injected animal caps elongated in the absence of mesoderm induction. It may imply that, in addition to neural tissue, some unidentified tissue(s) other than mesoderm was generated by DN-BR injection.

DN-BR Does Not Affect Gene Expression of Known Neural Inducers. So far, a few secreted molecules have been identified to have neural inducing activity, such as noggin (16, 17) and follistatin (8). To determine whether these neural inducers are involved in the neuralization by DN-BR, we analyzed the expression of these genes in the animal caps injected with DN-BR. The RT-PCR analysis showed that DN-BR injection did not cause the expression of noggin or follistatin in the gastrulating animal caps (Fig. 2B), excluding the possibility that DN-BR causes neuralization by activating the expression of these neural inducers.

BMP4 Inhibits Neuralization in the Animal Cap. According to the above results, we postulate that the DN-BR-injected animal caps commit a neural fate, probably resulting from the expression of the default neural state after removing the

BMP4 supression, instead of the induction of the neuralizing factors. Then we asked whether BMP4 inhibits the animal cap neuralization. To test this possiblity, we used the neural inducing agents 3m and noggin. 3m has been shown to elicit neural differentiation of the prospective ectoderm, probably by activating a gene encoding a neural inducer other than noggin (15). Injection of mRNA encoding noggin (0.25 ng/embryo) or 3m (1 ng/embryo) into animal caps gave rise to NCAM expression as shown by Western blot, however, this expression was substantially supressed by co-injection of BMP4 mRNA (0.25 ng/embryo) (Fig. 3). These results provide direct evidence for BMP4 as a natural inhibitor of neuralization in the animal cap.

DISCUSSION

In this study, we have demonstrated that elimination of the BMP4 signal by DN-BR directly converts the proectodermal cells to neural tissue (Fig. 1, 2A); this neuralization is neither consequent to the mesodermal induction nor through the induction of the known neural inducers (i.e., noggin and follistatin) (Fig. 2B). On the other hand, exogenous BMP4 inhibits the neuralization in the animal cap induced by noggin and 3m (Fig. 3). Based on these results, we propose that the ventralizing factor BMP4 is a physiological inhibitor of neuralization in the animal cap. A critical support for our proposal is the fact that BMP4 is first detected in the animal cap in early gastrula (11),

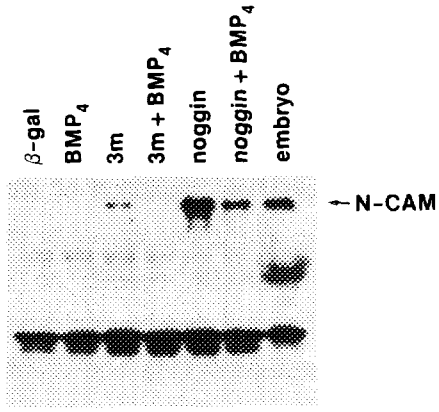

Fig. 3. BMP4 Inhibits NCAM Expression Elicited by 3m or Noggin in Animal Cap. Embryos were injected with β-gal, BMP4, 3m, 3m plus BMP4, noggin or noggin plus BMP4 RNAs. The amounts of RNA (ng/embryo) were β-gal (1.0), BMP4 (0.25), 3m (1.0) and noggin (0.25). The blastula stage animal caps were dissected and cultured until the equivalent of early tailbud stage. The lysates were prepared and tested by Western blot analysis (see Materials and Methods) for the expression of the general neural marker, NCAM. The embryo lane represents proteins extracted from the stage 28 embryos and provides a positive control.

providing spatiotemporal evidence for BMP4 to play an inhibitory role in the ectodermal neuralization.

We also suggest that disruption of the BMP4 signal in the animal cap is the essential step for the formation of the neural tissue. Other investigators have induced neuralization by dispersing the animal cap cells and diluting the extracellular signals. One of the possible extracellular signals is BMP4. Results from this type of experiment are consistent with our model, because neuralization occurs in a cell-autonomous fashion in the dissociated animal cap cells (4-6).

Both noggin and 3m cause neuralization in the animal cap (15, 17). However, 3m, thought to be an active form of LIM homeobox gene *Xlim*-1, does not induce expression of the neuralizing factor noggin (15), implicating that they function through different mechanisms. This is supported by the fact that en-2, a midbrain marker, is expressed in 3m-injected caps (15) but not in noggin-treated caps (17). Nevertheless, BMP4 inhibits the neuralization elicited by either 3m or noggin (Fig. 3), suggesting that BMP4 is a general inhibitor of neuralization. Therefore, we conclude that BMP4 plays a role in the control of dorsoventral patterning of ectoderm as well as mesoderm.

According to the reports by Hemmati-Brivanlou A. et al. (7, 8) and our data in this paper, depletion of either activin or BMP4 signal seems to cause neuralization in animal caps. At present, it is not yet known which pathway is more physiologically relevant, although these two pathways are not mutually exclusive (12, 21, 28). If the activin type II receptor and the BMP4 type I receptor interact with each other as shown by the Drosophila homologous genes *tkv* and *punt* (29, 30), there may be a common pathway for inhibiting neuralization. Alternatively, if both activin and BMP4 pathways are required for inhibiting neuralization, depletion of either the activin or BMP4 pathway could lead to neuralization. We prefer the latter possibility for the following reasons: (i) since follistatin is reportedly specific to antagonize activin (31), neuralization by follistatin can not be mediated by depletion of BMP4 signal, and (ii) BMP4 is strongly expressed in the ectoderm (11), and inhibits neuralization by noggin and 3m (Fig. 3). We speculate that quantitative balance among neural inducer(s), activin and BMP4 may determine the fate of the ectodermal cells. Thus, our data on the inhibitory role of BMP4 in neuralization shed a new insight into the mechanism of neural induction.

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