PV.1 induced by FGF-Xbra functions as a repressor of neurogenesis in *Xenopus* embryos

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During *Xenopus* early development, FGF signaling is involved in mesoderm formation and neurogenesis by modulating various signaling cascades. FGF-MAPK signaling induces Xbra expression, which maintains mesodermal fate through an autocatalytic-loop. Interestingly, previous reports have demonstrated that basic FGF (bFGF) treatment alone does not induce neurogenesis in ectodermal explants, even though FGF signaling inhibits BMP signaling via phosphorylation in Smad1 linker region. In addition, the overexpression of dominant-negative Xbra induces neurogenesis in ectodermal explants. However, the detailed mechanism underlying these phenomena has not yet been clarified. In this work, we showed that bFGF-Xbra signaling increased the PV.1 expression. DN-Xbra was found to decrease PV.1 expression, and the co-injection of PV.1 with DN-Xbra reduced neurogenesis in ectodermal explants. Furthermore, the knockdown of PV.1 induced neurogenesis in bFGF-treated ectodermal explants. Taken together, our results demonstrate that FGF-Xbra signaling induces PV.1 expression and that PV.1 functions as a neural repressor in the FGF-treated ectoderm. [BMB Reports 2014; 47(12): 673-678]

INTRODUCTION

The basic body plan of a vertebrate embryo is initiated with the germ-layer specification (1). A fertilized embryo forms three germ-layers at the early embryonic stage: ectoderm, mesoderm, and endoderm. The ectoderm differentiates into epidermis and neural cells, the mesoderm differentiates into muscle and blood cells, and the endoderm differentiates into the internal organs (1). These developing processes are modulated by various molecular signaling pathways, including the TGF-β (nodal and BMP), Wnt, and FGF pathways (2).

FGF (fibroblast growth factor) signaling is involved in cell differentiation, apoptosis, migration, and development via complex signaling cascades (3). In particular, FGF induces and maintains the mesodermal fate during the early development of vertebrate embryos (4). The pan-mesodermal marker Xbra is induced by FGF signaling through the auto-catalytic loop (FGF-ras-AP-1-Xbra) and maintains the mesodermal fate (5). Xbra is a transcription factor with a T-box DNA-binding domain and acts as a transcriptional activator through its C-terminal domain (6). Interestingly, previous studies have demonstrated that the ectopic expression of Xbra with a truncated C-terminal domain induces neurogenesis in ectodermal explants of *Xenopus* embryos (7). Our data shows that dominant-negative Xbra (DN-Xbra) also induces neurogenesis. Because the suppression of BMP signaling is required to lead the ectodermal explants to the neural ectoderm, the data suggest that DN-Xbra may reduce BMP signaling. However, the mechanism through which truncated or dominant-negative Xbra induces neurogenesis is not fully understood.

The basic and embryonic FGF (FGF2 and FGF4) signaling has been reported to be involved in neurogenesis (8). For the neurogenesis of ectodermal explants of *Xenopus* embryos, both FGF signaling and the inhibition of BMP signaling are required (9). Interestingly, the FGF stimulus triggers the phosphorylation of Smad1 linker by activating Erk. The phosphorylation on the linker region inhibits Smad1 activation and translocation into the nucleus (10). Therefore, if the inhibitory function of FGF in BMP signaling is sufficient for neurogenesis, treatment with high concentration of FGF may induce neurogenesis in ectodermal explants of *Xenopus* embryos. However, various studies indicated that the activation of FGF signaling alone does not induce neurogenesis in ectodermal explants of *Xenopus* embryos (8, 10, 11).

To investigate why FGF treatment alone does not induce neurogenesis in ectodermal explants, even though FGF signaling has a BMP antagonistic function via Smad1 inactivation, we studied the target genes downstream of FGF in the present investigation. The results showed that treatment with bFGF or the ectopic expression of wild-type Xbra (WT-Xbra) induces the expression of PV.1 (Vent1b), which is well known as a downstream target of BMP signaling (12). In contrast, the dominant-negative Xbra (DN-Xbra) decreases PV.1 expression and increased neurogenesis in ectodermal explants of *Xenopus*

embryos. A cyclohexamide treatment assay demonstrated that Xbra directly induces PV.1 expression. Furthermore, bFGF treatment was found to induce neurogenesis in ectodermal explants injected with PV.1 morpholino oligos.

In summary, we revealed that FGF-Xbra signaling induces the expression of PV.1, which functions as a neural repressor.

RESULTS

bFGF treatment induces PV.1 expression in ectodermal explants of *Xenopus* embryos

Various studies have demonstrated that FGF signaling inhibits the BMP signal cascade via the phosphorylation of the Smad1 linker region (10). Although the suppression of BMP signaling is usually sufficient to induce neurogenesis in the ectoderm, basic FGF (bFGF) or embryonic FGF (eFGF) treatment does not commonly lead to neurogenesis in ectodermal explants of *Xenopus* embryos (9). To re-examine the role of FGF in the neurogenesis of the *Xenopus* ectoderm, we analyzed which genes are regulated by FGF stimulation. As shown in Fig. 1A,
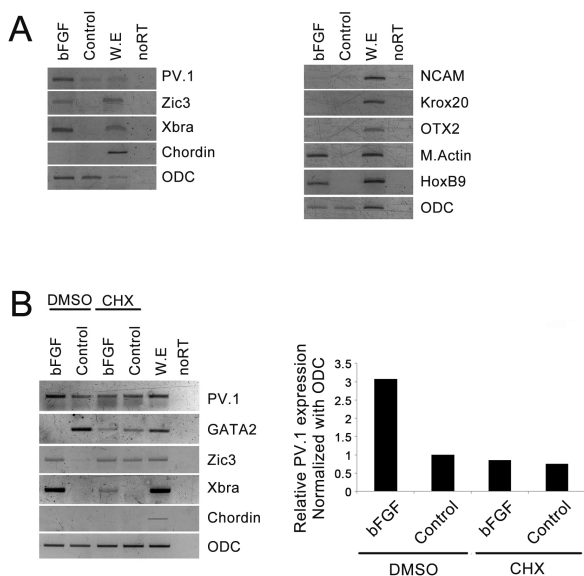

Fig. 1. bFGF induces PV.1 expression. (A) Ectodermal explants were dissected at stage 8 and incubated in animal cap media containing bFGF (50 ng/ml) until stage 11 or 24. The relative gene expression was analyzed by RT-PCR. (B) Ectodermal explants were dissected at stage 8 and incubated in animal cap media containing either bFGF alone or bFGF and CHX (5 ng/ml) until stage 11. The relative gene expression was analyzed by RT-PCR. The graph indicates the band intensity of PV.1 (right panel). ODC, loading control; no RT, control reaction without reverse transcriptase; WE, whole-embryo positive control; Xbra, pan-mesoderm marker; chordin, dorsal mesoderm marker; PV.1, GATA2, and XVent-1a, ventral-specific markers; Zic3, early neural marker; NCAM, pan-neural marker; OTX2, anterior neural marker; HoxB9, posterior neural marker; Krox20, mid-brain marker; muscle actin (M. Actin), late dorsal mesoderm marker.

basic FGF treatment significantly induced the expression of mesodermal genes, including Xbra and actin. However, bFGF treatment did not induce neural markers including NCAM, Krox20, and OTX2, at the tadpole stage, although Zic3 was slightly induced at stage 12. Interestingly, bFGF treatment induced PV.1 expression, which was not expected. The PV.1 is known as a transcriptional repressor and a downstream target of BMP signaling, where it acts as a neural suppressor. Based on these data, we established a hypothesis that FGF signaling does not induce neurogenesis, because FGF increases PV.1 expression even though the FGF-Erk signaling pathway inhibits BMP signaling. We then performed a cycloheximide (CHX) assay to investigate whether bFGF signaling directly induces PV.1 expression. The CHX is an inhibitor of protein synthesis and is generally used to discriminate direct and indirect effects of a given signaling pathway (13). The treatment with bFGF increased PV.1 expression, but CHX treatment abolished this increase (Fig. 1B). In addition, Zic3 expression was induced by bFGF but was not altered in CHX-treated ectodermal explants, compared to the control. These data suggest that bFGF indirectly increases PV.1 expression.

Taken together, we showed that bFGF treatment does not cause neurogenesis in ectodermal explants and that bFGF unexpectedly increases PV.1 expression in an indirect manner.

Xbra directly induces PV.1 expression

Xbra is a well-known target gene of FGF signaling and acts as a transcriptional activator via its C-terminal domain (14, 15). We examined whether Xbra increases PV.1 expression. The results showed that the ectopic expression of Xbra increases PV.1 expression in ectodermal explants of *Xenopus* embryos (Fig. 2A). A previous research has demonstrated that the expression of PV.1 is mainly regulated by BMP-Smad1 signaling (16). Therefore, we investigated the relevance of BMP signaling in PV.1 induction through the overexpression of Xbra. As shown in Fig. 2B, the ectopic expression of the dominant-negative BMP receptor (DNBR) abolished the PV.1 expression induced by Xbra. These data suggest that PV.1 expression induced by Xbra requires BMP signaling. We then performed a CHX assay to investigate whether Xbra directly regulates PV.1 expression. As shown in Fig. 2C, Xbra induces PV.1 expression in the presence of CHX. However, the expressions of Vent1a and GATA2 were not directly regulated by the ectopic expression of Xbra.

Taken together, our data indicate that Xbra directly induces PV.1 expression.

DN-Xbra causes neurogenesis via suppression of PV.1

It was previously reported that the ectopic expression of the C-terminus-truncated Xbra mutant B304 induces neurogenesis in ectodermal explants of *Xenopus* embryos (7). In this study, we used a dominant-negative Xbra that contains an engrailed repressor domain instead of a transcriptional activation domain in the C-terminal region (Fig. 3A) (5). If PV.1 is a target of Xbra, DN-Xbra may reduce PV.1 expression and thereby in-
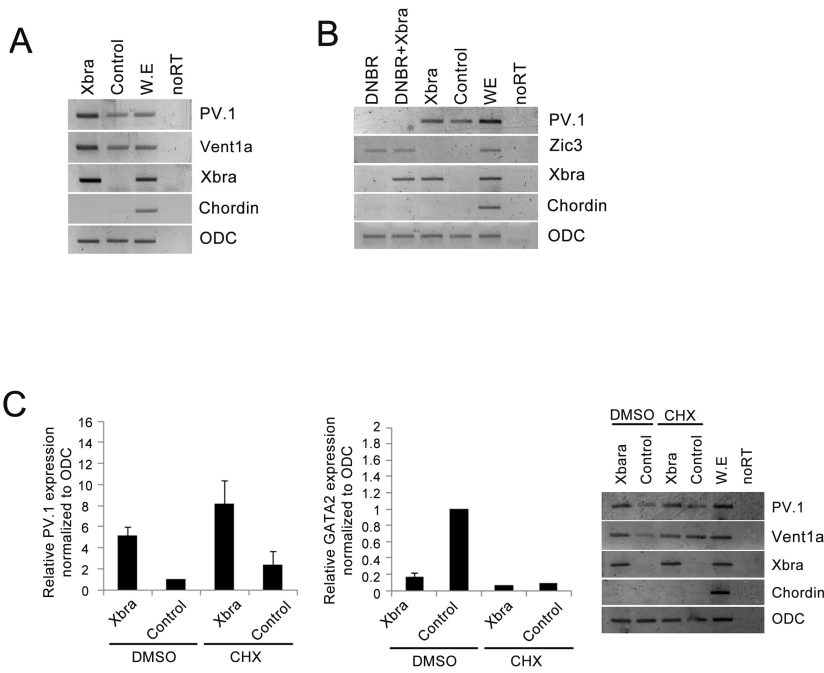
Fig. 2. DN-Xbra reduces the expression of target genes downstream of BMP. (A) Xbra RNA (1 ng) was injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated until stage 11. The relative gene expression was analyzed by RT-PCR. (B) DNBR RNA (1 ng) and Xbra RNA (1 ng) were co-injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated until stage 11. The relative gene expression was analyzed by RT-PCR. (C) Xbra RNA (1 ng) was injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated in animal cap media containing CHX (5 ng/ml) until stage 11 or 24. The relative gene expression was analyzed by RT-PCR. The data shown represent the means ± the S.D.s from at least three independent experiments. The differences were considered significant at P < 0.05.
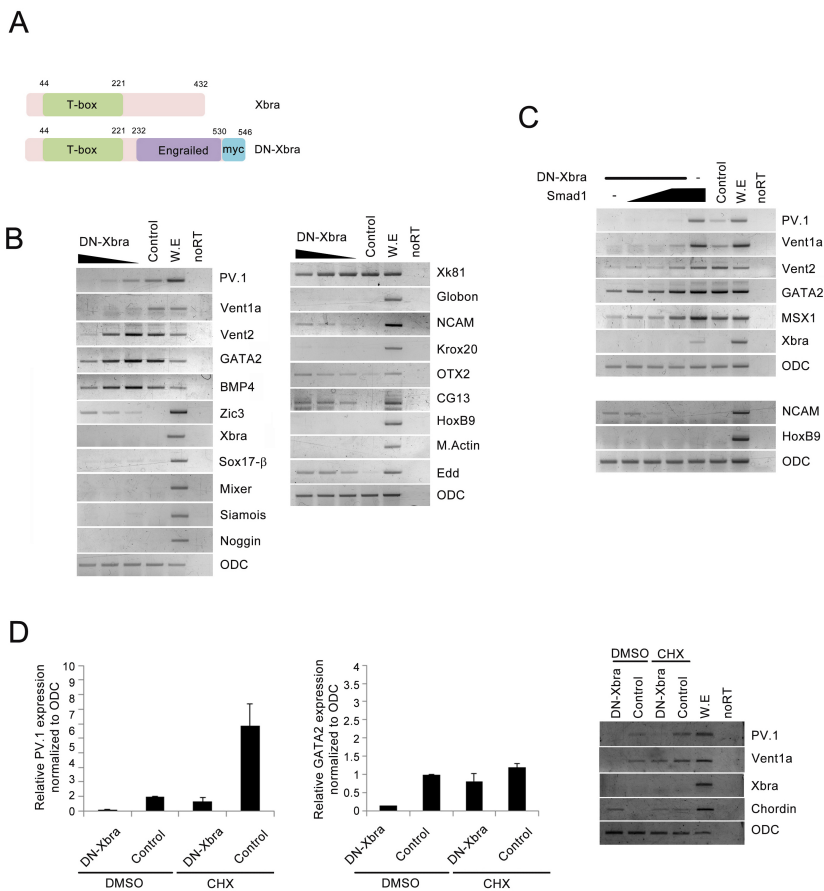

Fig. 3. Xbra directly induces PV.1 expression. (A) Schematic representation of Xbra and DN-Xbra. Xbra and DN-Xbra have a DNA-binding domain (T-box), but DN-Xbra has an engrailed repressor domain instead of a transcriptional activation domain in the C-terminal region. (B) DN-Xbra RNA (2, 1, or 0.5 ng) was injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated until stage 11 or 24. The relative gene expression was analyzed by RT-PCR. (C) Smad1 RNA (2, 1, or 0.5 ng) and DN-Xbra RNA (2 ng) were co-injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated until stage 11 or 24. The relative gene expression was analyzed by RT-PCR. (D) DN-Xbra RNA (2 ng) was injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated in animal cap media containing CHX (5 ng/ml) until stage 11 or 24. The relative gene expression was analyzed by RT-PCT. Noggin, dorsal mesoderm marker; Mixer, Sox17b, and Edd, endoderm markers; Xk81, epidermis marker; globin, blood marker; CG13, cement glad marker. The data are shown as the means ± the S.D.s of the values of at least three independent experiments. Differences were considered significant at P < 0.05.

duce neurogenesis. To verify this hypothesis, we analyzed the relative gene expression regulated by DN-Xbra. As shown in Fig. 3B, the ectopic expression of DN-Xbra abolished PV.1 expression. In addition, the expression levels of BMP4 and its downstream genes, including GATA2, Vent1a, Vent2, and the epidermis marker Xk81, were significantly reduced by DN-Xbra in a dose-dependent manner. Furthermore, it is consistent with the results of a previous study that neural markers
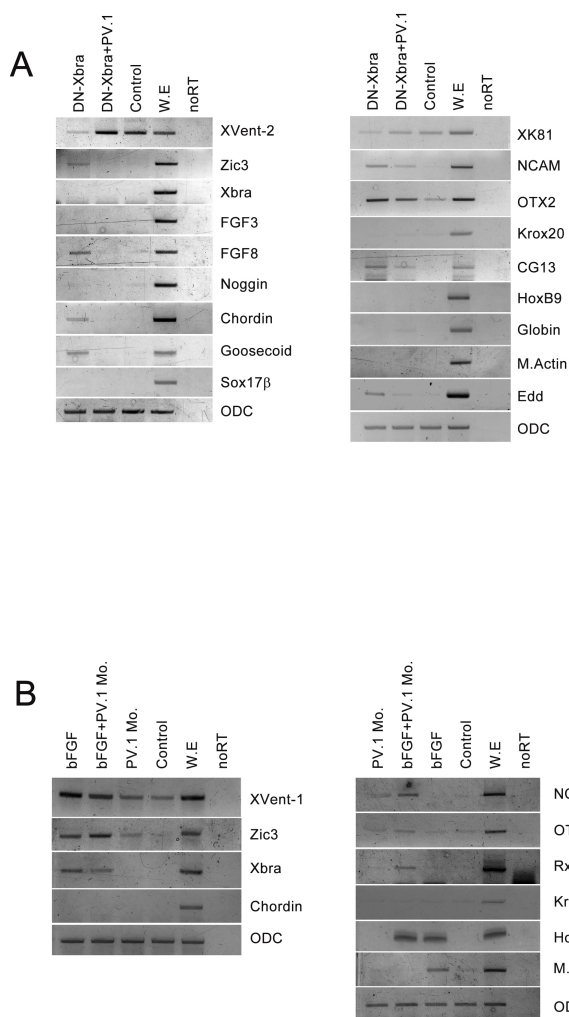
including Zic3, NCAM, OTX2, and CG13 were found to be induced by DN-Xbra. These data suggest that DN-Xbra induces neurogenesis via the suppression of BMP signaling. We then examined whether an increase in BMP signaling abolishes neurogenesis induced by DN-Xbra. The overexpression of Smad1 rescued the expression of BMP downstream genes, such as GATA2, MSX1, Vent1a and Vent2, in a dose-dependent manner, but the expression of the pan neural marker NCAM was reduced (Fig. 3C). Interestingly, PV.1 expression was not rescued by the overexpression of Smad1, even at a high dose. This result suggests that DN-Xbra tightly represses PV.1 expression. We also investigated whether DN-Xbra directly regulates PV.1 expression. As shown in Fig. 3D, Xbra induces PV.1 expression in the presence of CHX. The ectopic expression of DN-Xbra decreases PV.1 expression in the presence of CHX, which is consistent with the results obtained with the overexpression of Xbra (Fig. 3B). In addition, we showed that the expression of Zic3 is not directly regulated by DN-Xbra.

Taken together, our data suggest that DN-Xbra directly suppresses the expression of PV.1, resulting in neurogenesis.

FGF signaling induces neurogenesis in the absence of PV.1

Our previous data showed that PV.1 is a direct target of Xbra and DN-Xbra. To investigate the relevance of PV.1 in FGF signaling and neurogenesis during the early embryo development, we performed functional studies of PV.1. The overexpression of PV.1 decreased the levels of neural markers including NCAM, CG13, and OTX2 which were induced by DN-Xbra, and rescued the expressions of XVent-2 and the epidermis marker Xk81 (Fig. 4A). We then postulated that FGF treatment may lead to neurogenesis in the absence of PV.1. To verify this hypothesis, we used morpholino oligos of PV.1. The treatment with bFGF alone was not sufficient to induce neurogenesis. However, the treatment with bFGF in conjunction with the microinjection of PV.1 morpholino oligos (PV.1 Mo) strongly induced neural markers including the early neural marker Zic3, the anterior neural marker OTX2, the pan-neural marker NCAM, and the eye-specific marker Rx1.

The results suggest that FGF signaling alone does not induce neurogenesis in ectodermal explants, because FGF-Xbra signaling induces the expression of PV.1 which acts as a neural repressor. However, the FGF treatment combined with the knockdown of PV.1 can induce neurogenesis.

DISCUSSION

In this study, we attempted to obtain answers to two unsolved questions. The first question is why the FGF signaling alone is not sufficient for neurogenesis, even in the presence of the inhibitory function of Smad1 (17). The second question is how the truncated Xbra or DN-Xbra causes neurogenesis, even though Xbra is not expressed in ectodermal explants (7). Our data showed that FGF-Xbra induces PV.1, a potent repressor of

Fig. 4. The knockdown of PV.1 induces neurogenesis in bFGF-treated ectodermal explants. (A) PV.1 RNA (0.5 ng) and DN-Xbra RNA (2 ng) were co-injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated until stage 11 or 24. The relative gene expression was analyzed by PCR. (B) Anti-sense morpholino oligos of PV.1 (20 ng) were injected at the one-cell stage. Ectodermal explants were dissected at stage 8 and incubated in animal cap media containing bFGF (50 ng/ml) until stage 11 or 24. The relative gene expression was analyzed by RT-PCR.

neurogenesis (Figs. 1 and 2). In the absence of PV.1, the treatment with bFGF increases neurogenesis in ectodermal explants of *Xenopus* embryos (Fig. 4B). In the present study, we found that Xbra directly induces PV.1 (Fig. 2C) and that DN-Xbra inhibits its expression (Fig. 3D). We also showed that PV.1 is a potent neural repressor (Fig. 4). Taken together, we provided the first demonstration that FGF treatment leads to neurogenesis in the absence of PV.1 and that DN-Xbra induces neurogenesis via PV.1 repression.

FGF-Xbra signaling induces the expression of PV.1

Various studies have demonstrated that BMP target genes inhibit neurogenesis (18). For example, the overexpression of GATA1b, Msx1, Vent1a and Vent2 suppresses neural gene expression in ectodermal explants of *Xenopus* embryos (19-21). The ectopic expression of dominant-negative BMP receptor and dissociation conditions reduce BMP signaling and the expression of the BMP target genes, resulting in neural induction (22). In general, the neuroectoderm arises from the dorsal ectoderm through Spemann's organizer (23-27). The BMP signaling induces neural repressors in the ventral mesoderm and ectoderm (24, 28). We postulate that FGF/Xbra may induce PV.1, which at least partially contributes to the inhibition of neurogenesis in the lateral mesoderm. Although FGF-treated ectodermal explants do not transform into the neuroectoderm, activin-treated ectodermal explants generally develop into the neuroectoderm and the dorsal mesoderm containing organizer genes. In Spemann's organizer at the dorsal mesoderm region, PV.1 expression is completely absent even though FGF/Xbra signaling is present. We found that goosecoid represses PV.1 expression by binding directly to the PV.1 promoter (unpublished data). Activin-treated ectodermal explants and dorsal mesoderm include both a direct repressor of PV.1 expression, namely goosecoid, and the BMP antagonizers chordin and noggin, which also contribute negatively to PV.1 expression (18). Therefore, we suggest that PV.1 expression contributes to the discrimination of activin-treated ectodermal explants from those treated with FGF, during neuroectoderm specification. In summary, both activin and FGF signaling induce the mesodermal fate during the early development of *Xenopus* embryos (23). The activin signaling can elicit neuroectoderm and organizer genes and thereby suppress BMP signaling and the expression of target genes downstream of BMP. However, FGF signaling alone does not induce neuroectoderm development. This phenomenon may be due to the induction of PV.1 by FGF-Xbra signaling. It remains unclear whether PV.1 is a unique repressor of neurogenesis in FGF-treated ectodermal explants and how the promoter of PV.1 is regulated by both BMP-Smad1 and FGF/Xbra signaling (29).

FGF signaling induces neurogenesis in the absence of PV.1

Although the inhibition of BMP signaling is usually sufficient to elicit neurogenesis in the ectoderm, the treatment with bFGF signaling is not sufficient to induce neurogenesis, even though

it inhibits Smad1, a key intracellular signaling molecule of BMP. Previously, Rao (1994) demonstrated that the conversion of the mesodermalizing molecule Xbra induces neurogenesis in ectodermal explants of *Xenopus* embryos. However, the details of these mechanisms are elusive. Our results showed that DN-Xbra directly inhibits PV.1 expression, but the knockdown of PV.1 is not sufficient for neurogenesis (Fig. 2A and Fig. 4B). Thus, we expected that DN-Xbra inhibits PV.1 expression and that the suppression of PV.1 reduces overall BMP signaling by inducing chordin and goosecoid expression, because PV.1 can also inhibits the expression of organizer genes (30). In contrast, the overexpression of PV.1 reduced not only the neural gene expression, but also the expression of organizer genes induced by DN-Xbra (Fig. 4A). The neuralizing activity of DN-Xbra suggests the possibility that Xbra prevents neural differentiation in ectodermal cells. In addition, Hemmati-Brivanlou and Melton (1994) reported that low levels of Xbra may suppress neuralization and maintain a balance between mesodermal fate and neural fate (31). In our study, we showed that Xbra directly induces PV.1 expression. Although bFGF alone does not induce neurogenesis, the knockdown of PV.1 using anti-sense morpholino oligos induced neurogenesis in bFGF-treated animal cap explants. Taken together, our results demonstrated that bFGF does not induce neurogenesis, because FGF-Xbra induces the expression of PV.1 which acts as a neural repressor. However, the detailed mechanism by which FGF signaling modulates the mesoderm and neuroectoderm by the tight regulation of Xbra and PV.1 in the presence and absence of BMP signaling remains to be elucidated.

MATERIALS AND METHODS

Methods of Embryo injection and animal cap assay, Reverse transcription polymerase chain reaction (RT-PCR), and Real-time PCR analysis are available as supplementary materials on BMB Reports online.

Morpholino oligos

The antisense morpholino-oligos (MOs) were obtained from Gene Tools (Philomath, OR, USA). The PV.1 MO sequence 5'-GTCAATAGAGAATCCCTGTTGAACC-3' was designed to bind to complementary sequences found in *Xenopus* PV.1 mRNA (Accession No. BC170526) and to prevent the translation of the PV.1 mRNA transcripts. The oligos were resuspended in sterile water, and 20 ng of the oligos were injected into each embryo.

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