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BMP signal attenuates FGF pathway in anteroposterior neural patterning

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ABSTRACT

In vertebrate early development, the neural tissue is specified along the antero-posterior (A-P) axis by the activity of graded patterning signals such as Wnt, Nodal and FGF. Attenuation of these signals has been shown to play critical roles in the determination of anterior neural region, but it remains poorly understood how FGF action is counteracted in the neural plate. Here, we show that BMP signal acts as an antagonist of FGF signaling for AP neural patterning in *Xenopus* embryo. During the neurula stages, BMP signal was up-regulated in the anterior neural plate, displaying a graded pattern along the AP axis. Inhibition of the late BMP signaling after mid-gastrulation abrogated the expression of anterior neural markers. We found that BMP signaling interfered with FGFs-induced ERK phosphorylation and neural caudalization. This inhibitory action of BMP signal involved repression of the expression of *Flrt3*, a positive regulator of FGF signaling. Furthermore, the gain- and loss-of-function of *Flrt3* inhibited and expanded the expression of forebrain marker genes, respectively. Together, these results demonstrate that BMP signal can down-regulate FGF pathway via inhibition of *Flrt3* expression for anterior neural formation, revealing stage-specific roles of BMP signaling and its novel crosstalk with FGF pathway in neural development.

1. Introduction

Formation of the vertebrate nervous system is initiated by neural induction that demarcates the neural plate from the non-neural (epidermal) ectoderm, which is followed by neural patterning that induces position-specific gene expressions in the neural ectoderm along the anterior–posterior (A-P) axis. In the two-signal model proposed by Nieuwkoop, the neural induction generates initially anterior neural tissue (forebrain), which is subsequently modified into more posterior neural tissues (midbrain, hindbrain and spinal cord) by caudalizing signals [1]. Several works in amphibians revealed that inhibition of BMP signaling is sufficient to induce neural tissue in naïve ectodermal tissue [2]. Thus, neural induction requires secreted signals, in particular bone morphogenetic protein (BMP) antagonists such as Noggin and Chordin, emanating from the dorsal mesoderm or Spemann's organizer region of the embryo [3]. In addition, studies in the chick and frog embryo demonstrated that fibroblast growth factor (FGF) signal is implicated in the neural induction process [4,5].

The caudalizing factors involved in the A-P patterning of induced neural tissue include Wnt, FGF, Nodal and retinoic acid [6]. The Spemann organizer also regulates the A-P neural patterning by secreting inhibitors of caudalization factors such as *Dickkopf-1*, *Lefty*, *Frzb-1* and *Cerberus* [7–9]. These inhibitory factors play critical roles in anterior neuroectoderm formation by restricting the actions of Wnt, Nodal and BMP signals in a non-cell autonomous manner. FGF ligands such as FGF4 and FGF8 are expressed in the posterior mesoderm and posterior neuroectoderm, which is also considered a critical factor in the A-P patterning of neural tissue. Although attenuation of FGF signals by Shisa in the anterior neuroectoderm, which is an endoplasmic reticulum (ER) protein inhibiting the trafficking of FGF receptor to the cell surface, has been reported [10], the mechanism by which the low level of FGF signaling is maintained in the anterior neuroectoderm has yet to be fully elucidated.

Several developmental processes appear to be regulated by crosstalk between BMP and FGF signaling pathways. It seems likely that FGFs promote neural induction through the MAPK-mediated phosphorylation of the linker region of BMP-specific Smad1, which results in cytoplasmic retention of Smad1 and suppression of BMP signaling [11,12]. Inhibition of BMP signal has been shown to initiate neural induction by activating FGF4 expression. Furthermore, BMP inhibition of FGF signaling plays critical roles in limb patterning [13] and heart differentiation [14]. What has emerged from these studies is that both FGF- and BMP-mediated pathways should be maintained at the proper levels for normal development. Therefore, maintaining the balance between FGF and BMP signaling appears to require reciprocal regulatory mechanisms.

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In this study, we show that BMP signal also antagonizes FGF signaling for neural patterning. After gastrulation, BMP signaling is activated in the anterior neuroectoderm in *Xenopus* early embryo. This late activation of BMP signal is essential for maintenance of anterior neural fate. This activity of BMP signal involves down-regulation of FGF signaling, which is achieved by inhibition of the expression of *Flrt3*, a positive regulator of the latter pathway [15,16]. Thus, we suggest a novel role of BMP signal as an inhibitor of FGF caudalizing pathway in the A-P patterning of induced neural tissue.

2. Materials and methods

2.1. Embryo manipulation and chemical treatments

In vitro fertilization, microinjection and embryo culture were performed as described previously [17]. Developmental stages of embryos were determined according to the Nieuwkoop and Faber's normal table of development [18]. For the induction of human glucocorticoid receptor ligand binding domain (hGR)-fused constructs, dexamethasone (DEX; 10 μM, Sigma) was added at the indicated time. SU5402 (20 μM, Calbiochem) was treated at the indicated stages to block FGF signaling.

2.2. Plasmid constructs and morpholino oligonucleotides

A full-length *Xenopus laevis Flrt3* (accession No. AJ605776) was amplified by PCR and inserted into the *BamHI*/*XhoI* sites of pCS2+ vector. The anti-sense morpholino oligos (MOs) were purchased from Gene Tools. The sequences of MOs are as follows; *Flrt3* MO-a, 5′-AATATCCAGTAGGCAGAAAGCAGCC-3′; *Flrt3* MO-b, 5′-GTTTCTGTAGACATGGTCACTGATG-3′. For complete depletion of *Flrt3*, equal amounts of *Flrt3* MO-a and *Flrt3* MO-b were mixed and microinjected [15]. Control MO was a standard morpholino oligo from Gene Tools whose sequence is 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

2.3. Immunostaining and Western blotting

Embryos were collected and fixed for 1 h at room temperature (RT) in MEMFA and then incubated for 10 min in 100 mM lysine in PBS + 0.1% Tween20 at RT. Embryos were washed several times

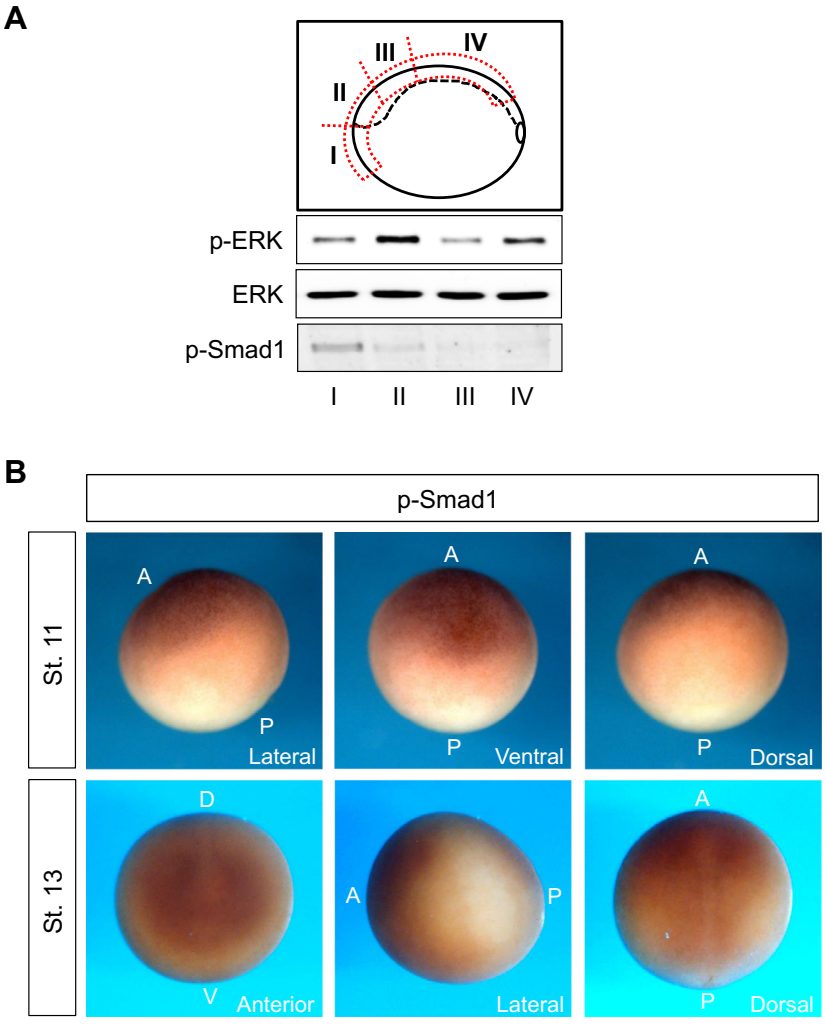

Fig. 1. BMP signal forms a graded pattern along the antero-posterior axis of a neurulae. (A) Western blotting analysis showing the activities of FGF and BMP signaling in the dorsal neural ectoderm, which is subdivided into four parts (I, II, III and IV), along the A-P axis of a neurula stage *Xenopus* embryo. An embryo in the cartoon is viewed laterally with anterior to the left. (B) Whole embryos were subjected to immunohistochemistry against phospho-Smad1 at stage 11 and 13. Embryos are viewed as indicated. A, anterior; P, posterior; D, dorsal; V, ventral.

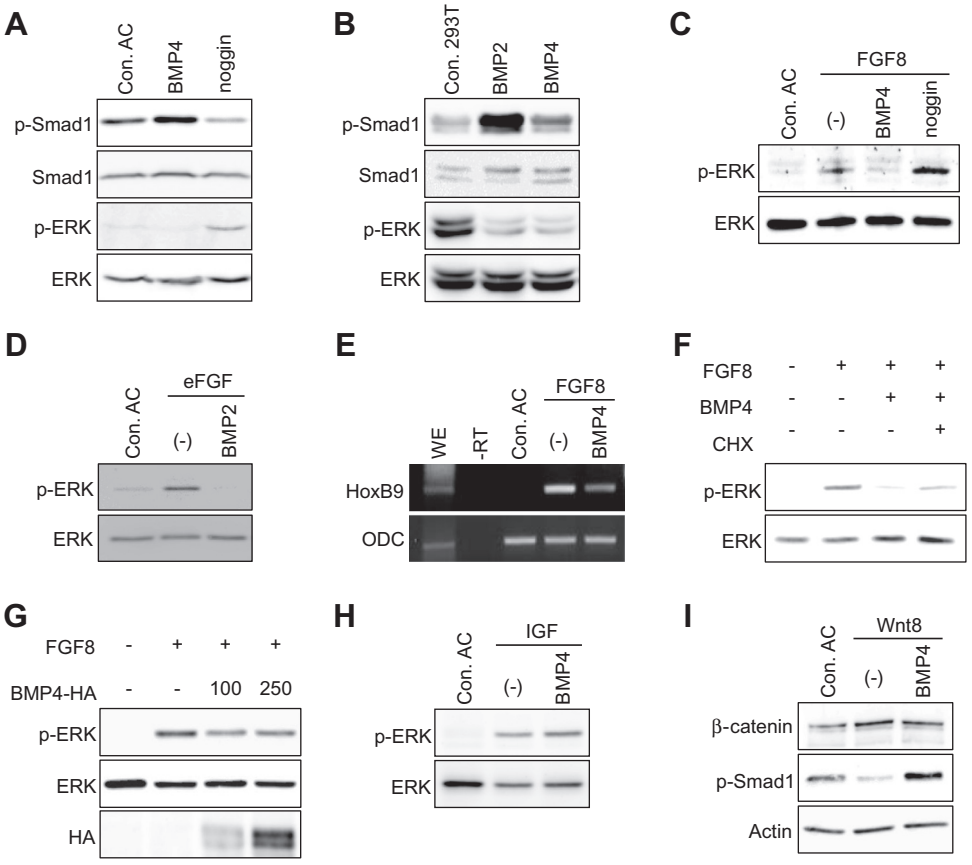

Fig. 2. BMP signals down-regulate FGF signaling. (A, C–I) Four-cell stage embryos were injected in the animal pole region with the indicated combination of *BMP4* (200 pg), *BMP4-HA* (100, 250 pg), *BMP2* (200 pg), *noggin* (10 pg), *FGF8* (1 ng), *eFGF* (1 ng), *IGF1* (1 ng) and *Wnt8* (200 pg) mRNA, and then the animal cap explants were dissected at stage 8 and cultured until stage 12 for Western blotting (A, C, D, F–I) or until stage 15 (E) for RT-PCR analyses. WE, whole embryo; -RT, a control in the absence of reverse transcriptase; Con. AC, uninjected control animal caps. *ODC* and ERK serve as loading controls. (F) After excision, the animal caps were cultured to stage 12 in the presence or absence of cycloheximide (CHX; 10 μg/ml). (B) HEK 293T cells were transfected with *BMP2* (0.5 μg) or *BMP4* (0.5 μg) DNA and harvested for Western blotting analysis 24 h later.

in PBT (PBS, 2 mg/ml BSA, 0.1% Triton X-100) and stored in PBT at 4 °C for no longer than 24 h prior to immunostaining. For immunostaining, embryos were blocked in 10% normal goat serum (NGS) (Jackson Immunoresearch) in PBT, incubated in 1:20 dilution of anti-pSmad1/5/8 antibody in 10% NGS overnight at 4 °C, washed six times for 15 min each in PBT and then incubated with HRP conjugated secondary antibody (1:250). Staining was developed using an ImmunoPure Metal-enhanced DAB Kit (Pierce) as per manufacturer's instructions for 15–20 min. Western blotting was performed according to the standard protocol with p44/42 MAPK (Erk1/2) (1:1000, Cell signaling), phospho-p44/42 MAPK (Erk1/2) (1:1000, Cell signaling), phospho-Smad1/5/8 (1:1000, Cell signaling), Smad1 (1:1000, Santa Cruz) and anti-Actin (1:1000, Santa Cruz) antibodies.

2.4. Lineage tracing, in situ hybridization and RT-PCR

For lineage tracing, β-galactosidase mRNA (β-gal, 250 pg) was co-injected and its activity was visualized with Red-Gal substrate (Sigma). Whole-mount *in situ* hybridization was performed as described in [19]. Anti-sense *in situ* RNA probes were *in vitro* synthesized using digoxigenin-labeled nucleotides. For RT-PCR analysis, total RNA was extracted from whole embryos and tissue explants using TRI Reagent (Molecular Research Center), and RNA was transcribed using M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. PCR products were analyzed on 2% agarose gels. The numbers of PCR cycles for each primer set were determined empirically to maintain amplification in the linear range.

3. Results

3.1. BMP and FGF signals form a graded pattern of activity

Since FGF signal regulates the A-P patterning of induced neural tissue [20], we wanted to examine whether the levels of FGF signaling are directly relevant to its function in neural patterning. Thus, we measured the levels of phosphorylated extracellular signal-related kinase (p-ERK), readout of FGF signaling activity, in the dorsal ectoderm along the A-P axis of a neurula stage embryo. Consistent with its role as a posteriorizing factor in neural patterning [20], FGF signaling activity was generally higher in the caudal than in the rostral region of embryo (Fig. 1A). We also examined the pattern of BMP signaling activity in the same tissues. In contrast to the levels of p-ERK, the phosphorylated Smad1 (p-Smad1), which is produced by BMP activation, was more prominent in the anterior region that appears to include cement gland and forebrain than in the posterior region including mid-hindbrain and spinal cord (Fig. 1A). This graded pattern of p-Smad1 was also observed in neurula stage whole embryos (Fig. 1B). These patterns of BMP and FGF signaling activities prompted us to hypothesize that BMP signal could down-regulate FGF signaling in antero-posterior neural patterning.

3.2. BMPs attenuate FGF signaling

To test this possibility, we first investigated whether BMP signal could antagonize FGF signaling. In *Xenopus* animal cap cells, the

basal level of p-ERK was too low to be detected effectively (Fig. 2A), possibly due to the low activity of FGF signaling as noted previously [20]. Expression of a BMP inhibitor, *noggin* increased its level (Fig. 2A), suggesting that the low basal level of p-ERK also results from the high level of BMP signaling in animal cap cells. HEK 293T cells had relatively high level of p-ERK, which was down-regulated by coexpression of *BMP2* or *BMP4* (Fig. 2B). In addition, *FGF8* or *eFGF*-enhanced levels of p-ERK were decreased and further increased by co-expression of *BMPs* or *noggin*, respectively (Fig. 2C,D). Consistently, *FGF8*-induced ectopic expression of a posterior neural marker, *HoxB9* was inhibited by co-injection of *BMP4* (Fig. 2E). However, *BMP4* did not efficiently interfere with *FGF8*-induced phosphorylation of ERK in the presence of a protein synthesis inhibitor, cycloheximide (Fig. 2F), indicating that the mechanism of BMP inhibition of FGF signaling involves transcriptional induction of a negative regulator of FGF pathway. Intriguingly, *BMP4* inhibition of FGF8-induced ERK phosphorylation reached a plateau, with a certain level of FGF activity still remaining even in the presence of increased levels of *BMP4* (Fig. 1G). As ERK phosphorylation can be triggered by factors other than FGFs, we examined whether *BMP4* could also inhibit ERK phosphorylation in response to insulin-like growth factor-1 (*IGF-1*), with the result that *BMP4* had no effect on it (Fig. 1H). Furthermore, *BMP4* signal could not affect beta-catenin stabilization induced by Wnt signaling, another caudalization pathway (Fig. 2I). Together, these results suggest that BMP signal down-regulates FGF signaling through induction of a specific inhibitor of this pathway.

3.3. Late activation of BMP signal is essential for anterior neural development

Although simple BMP inhibition by its antagonists such as noggin and chordin induces a neural fate of anterior character in animal cap explants [21], several lines of evidence suggest that low level of FGF signal should be combined with BMP inhibition for this neural induction [5,22]. Since BMP inhibition up-regulates FGF signaling (Fig. 2A and C), we next determined whether this FGF signal enhanced by BMP inhibition might be involved in anterior neural induction. As expected, suppression of FGF signaling by treatment of a pharmacological inhibitor, SU5402 could impair *noggin* or *chordin*-induced expression of an anterior neural marker, *Otx2* and a pan-neural marker, *NCAM* (Fig. 3A). These results indicate that inhibition of BMP signaling might function to derepress FGF signal which is sufficient for anterior neural induction in animal cap cells. Given that FGF signal induces directly several neural markers in a dose-dependent manner with higher doses eliciting more caudal neural genes (Fig. 3B), the FGF signal in animal cap cells, which induces anterior neural tissue when derepressed by BMP inhibition, might be at low levels. As BMP signaling is activated in the anterior neural plate after gastrulation (Fig. 1B) and functions as a specific inhibitor of FGF pathway as shown above, we assumed that BMP activation, but not its inhibition, could induce anterior neural tissue in animal cap cells with high levels of FGF signal. In support of this, a high dose of *FGF8* induces the expression of posterior neural markers, *HoxB9* and *Krox20* only, but not an anterior neural marker, *Otx2*, and both early and late

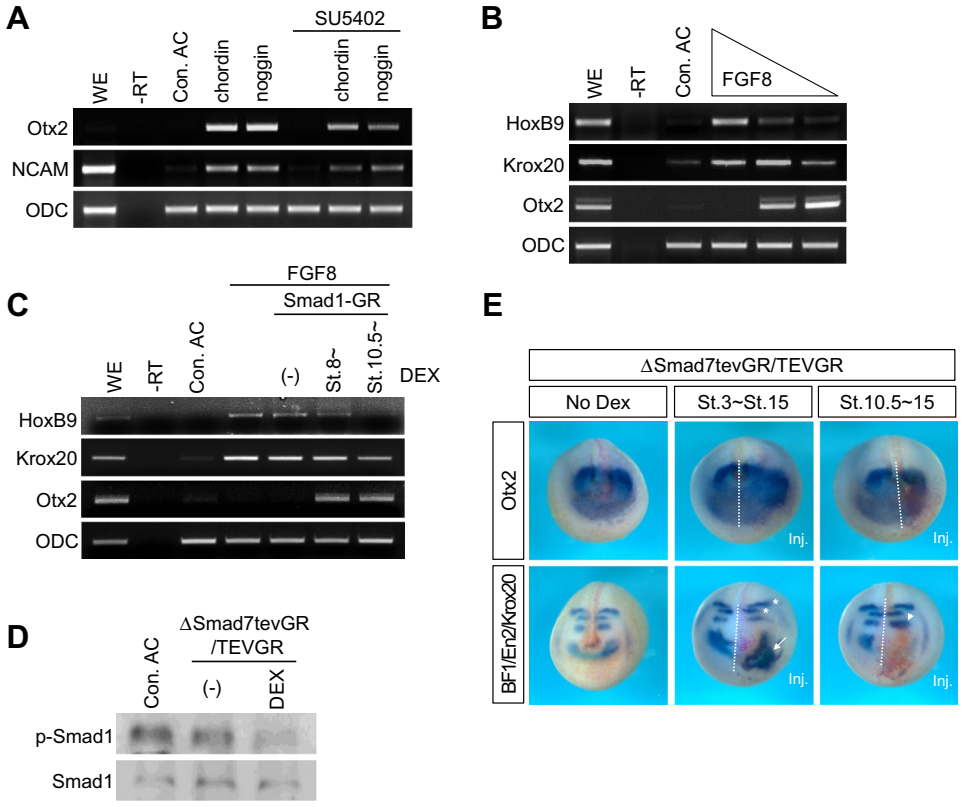

Fig. 3. BMP activation is necessary for maintenance of anterior neural fate. (A–D) Four-cell stage embryos were injected in the animal pole region as indicated with *chordin* (200 pg), *noggin* (10 pg), *FGF8* (B, 100–500–1000 pg; C, 1000 pg), *Smad1-GR* (200 pg), Δ*Smad7tevGR* (250 pg) and *TEVGR* (10 pg) mRNA, and then the animal cap explants were dissected at stage 8 and cultured until stage 12 for Western blotting (D) or stage 15 for RT-PCR (A–C). (A, C, D) Animal caps were treated with SU5402 (20 μM) from stage 8 to 15 (A) or dexamethasone (DEX; 10 μM) from stage 8 or 10.5 to 15 (C) or from stage 8 to 12 (D) as indicated. (E) *Smad7tevGR* (250 pg) and *TEVGR* (10 pg) mRNAs were injected into the dorso-animal region of one blastomere at the 4-cell stage, and the injected whole embryos were cultured in the presence of DEX for the indicated periods and harvested for whole-mount *in situ* hybridization against *Otx2, Bf1, En2 and Krox20*. An arrow and arrowhead indicate the expansion of *Bf1* and *En2* expression, respectively. Asterisks denote *Krox20* expression. Embryos are shown in anterior view with dorsal to the top. Inj., injected side.

activation of BMP signaling, which were mediated by *Smad1-GR* activated before and after mid-gastrulation, could reverse this pattern of gene expression (Fig. 3C). Therefore, these data suggest that anterior neural tissue could be induced by either inhibition or activation of BMP signaling, depending on the levels of FGF signal in cells.

We further investigated whether BMP activation is indispensable for anterior neural development *in vivo*. To this end, we employed a previously described hormone-inducible system in which both Δ*Smad7tevGR* and *TEV2GR* are co-expressed and activated upon treatment of dexamethasone (DEX) [23] in order to impair BMP signaling at specific stages of development. Expression of Δ*Smad7tevGR* and *TEV2GR* in animal cap cells inhibited efficiently Smad1 phosphorylation in a DEX-dependent manner (Fig. 3D). Since neural tissue is induced at the blastula stage by the blastula chordin- and noggin-expressing (BCNE) center [3], inhibition of BMP signaling at an early stage by injection of *noggin* induces a dramatic increase in anterior neural tissue. Similarly, embryos that were co-injected with Δ*Smad7tevGR*/*TEV2GR* RNAs and treated with DEX from stage 3 to 15 displayed expanded expression of a cement gland and forebrain marker, *Otx2* and a forebrain marker, *Bf1* (94.8%, $n = 39$ for *Otx2*; 87.5%, $n = 40$ for *Bf1*; Fig. 3E). In contrast, the expression of these anterior neural markers was markedly impeded in embryos injected with the same RNAs and treated with DEX from stage 10.5 to 15 (73.6%, $n = 38$ for *Otx2*; 79.4%, $n = 39$ for *Bf1*; Fig. 3E). Inhibition of late BMP signaling increased the expression of a mid-hindbrain marker, *En2* with no effect on that of a hindbrain marker, *Krox20* (Fig. 3E). Taking together, we conclude that BMP signaling should be inhibited and activated before and after mid-gastrula stages, respectively, in order to achieve the optimal levels of FGF signaling for normal anterior neural formation.

3.4. BMP antagonizes FGF signaling by repressing Flrt3 expression

In an attempt to identify the mechanism by which BMP inhibition up-regulates FGF signaling, we focused our attention on *Flrt3* gene, because it is a positive regulator of the FGF-MAP kinase signaling and is expressed in the anterior neural tissue of *Xenopus* embryo [15,16]. Interestingly, *Flrt3* expression could be strongly induced by *noggin* or a dominant negative BMP receptor (*tBR*)-mediated BMP inhibition in ectodermal explants (Fig. 4A). *BMP4* could also repress *FGF8*-induced *Flrt3* expression, whereas expression of *Flrt2*, a close isoform of *Flrt3* was not affected by BMP inhibition or activation (Fig. 4A and B), indicating the specific effect of BMP signal on *Flrt3* expression. Knockdown of *Flrt3*, which is mediated by anti-sense morpholino oligos (MOs), inhibited *FGF8*-induced ERK phosphorylation (Fig. 4C). Moreover, depletion of *Flrt3* impeded ERK phosphorylation induced by *noggin* (Fig. 4D) and its co-injection rescued *BMP4*-induced inhibition of ERK phosphorylation (Fig. 4E), thereby confirming that BMP inhibits FGF signaling by suppressing *Flrt3* expression. We next examined whether the level of *Flrt3* expression might be relevant to the AP neural patterning. As shown in Fig. 4F–I, injection of *Flrt3* MO in the anterior neural tissue expanded the expression of anterior neural markers, *Otx2* and *Bf1* (95%, $n = 40$ for *Otx2*; 72%, $n = 39$ for *Bf1*), whereas *Flrt3* RNA suppressed their expression (84%, $n = 38$ for *Otx2*; 78%, $n = 41$ for *Bf1*) when injected in the same tissue. While knockdown of *Flrt3* decreased the expression of *En2* and *Krox20* markers, its overexpression had reverse effect, extending *Krox20* laterally and

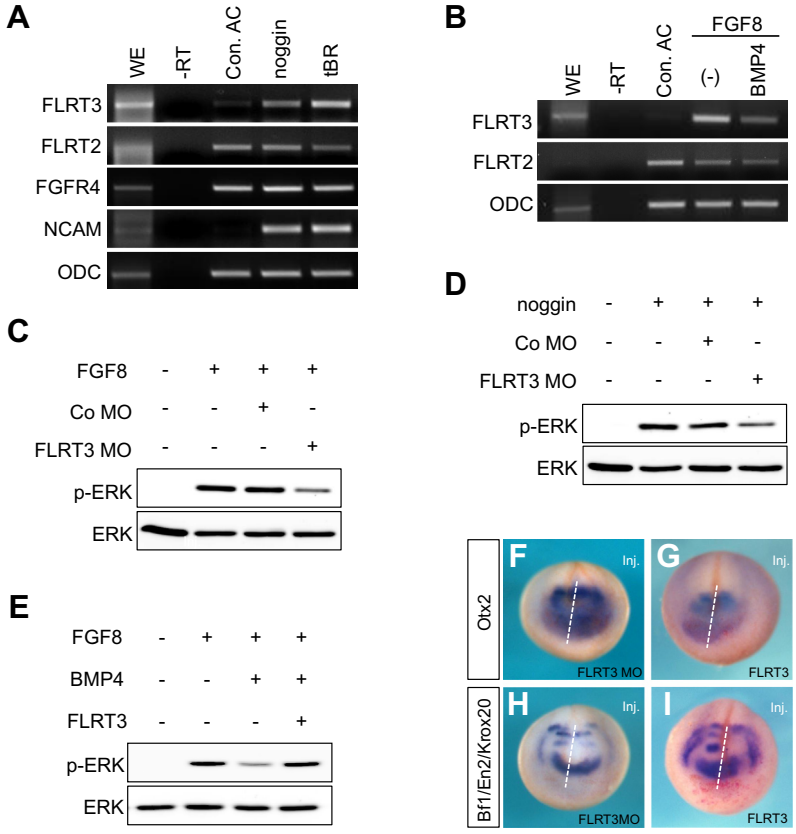

Fig. 4. BMP signal inhibits *Flrt3* expression to down-regulate FGF pathway. (A–E) Four-cell stage embryos were injected in the animal pole region as indicated with *noggin* (10 pg), *tBR* (100 pg), *FGF8* (1 ng), *BMP4* (200 pg), *Flrt3* (200 pg), *Flrt3* MO (12.5 ng) and Co MO (12.5 ng), and then the animal cap explants were excised at stage 8 and cultured to stage 12 for RT-PCR (A, B) or Western blotting (C–E) analyses. (F–I) Four-cell stage embryos were injected dorso-animally with *Flrt3* (200 pg) or *Flrt3* MO (10 ng), cultured until stage 15 and then subjected to *in situ* hybridization against *Otx2, Bf1, En2* and *Krox20*. Anterior view with dorsal to the top.

forward (Fig. 4H and I), indicative of a critical role of *Flrt3* expression level in posterior neural formation.

4. Discussion

In this study, we show that BMP signaling is necessary for anterior neural development in *Xenopus*. Anterior neural induction has been shown to occur at the pre-gastrula stages [3]. Inhibition of BMP signaling only after mid-gastrulation reduces the expression of anterior neural markers, suggesting that this late BMP signal is critical for maintenance of anterior neural character. The mechanism underlying this role of BMP signaling involves its attenuation of posteriorizing FGF signal through repression of *Flrt3* expression. In support of this, knockdown of *Flrt3* suppresses *noggin* up-regulation of ERK phosphorylation and its coexpression recovers BMP inhibition of *FGF8*-induced ERK phosphorylation. Furthermore, *Flrt3*-depleted embryos show expanded expression of anterior neural markers but reduced expression of relatively posterior neural markers, *En2* and *Krox20*, thereby confirming that the level of *Flrt3* expression is relevant to the A-P neural patterning. FGF signal has been shown to inhibit BMP signaling through the MAPK-mediated phosphorylation of Smad1 [12]. FGF signaling can also induce *Flrt3* expression, creating a positive feedback loop. Together, these results suggest that the antagonism between BMP and FGF signaling plays a pivotal role in precise control of A-P neural patterning.

It has been reported that BMP signal inhibits MAPK activity without protein synthesis in *Xenopus* early gastrula ectoderm via a TAK1/p38 pathway [24]. By contrast, our data shows that BMP4 does not efficiently impede *FGF8*-induced ERK phosphorylation in the presence of cycloheximide. This discrepancy could be explained by two possibilities. First, the embryonic stages at which the MAPK activity is measured might be critical. While Goswami et al., examined the MAPK activity within 1 h after animal cap isolation, presumably at the very early gastrula stage, we cultured the dissected animal caps to late gastrula stages for detection of ERK phosphorylation. Possibly, protein synthesis may be necessary for the continued inhibition by BMP signal of the MAPK activity in gastrula ectoderm. Second, the BMP-4/TAK1 signal could down-regulate the MAPK activity induced by several kinds of FGFs including FGF1, FGF4 and FGF8. However, BMP inhibition of *Flrt3* expression would be specific for FGF8 signaling, since *Flrt3* appears to have a preference for FGF8 in control of gene expression [15]. Thus, our results reveal a novel crosstalk between the BMP and FGF pathways that is a key mechanism underlying neural development.

Given that FGF signal which could induce anterior neural tissue is derepressed by BMP inhibition, BMP signaling appears to promote epidermal differentiation by inhibiting FGF signaling prior to gastrulation. After mid-gastrulation, the BMP signal goes on suppressing FGF signaling for maintenance of the anterior neural fate but not of the epidermal character. It seems likely that these stage-specific effects that BMP inhibition of FGF signaling has on ectodermal differentiation result from the different cellular levels of FGF signals in the ectoderm during early developmental stages. The level of MAPK activity has been shown to be relatively low at the cleavage and early blastula stages and to increase dramatically from the mid-blastula to mid-gastrula stages [20]. During the blastula stages, BMP signaling pathway seems to repress completely the low level of FGF signal, thereby resulting in epidermal differentiation in the ectoderm. Thus, BMP antagonists such as chordin and noggin from the organizer of early embryo function to derepress the low FGF signal for anterior neural induction. Since the high level of FGF signal at the gastrula stages can also antagonize BMP signaling [11,12], the late BMP signal activated in the anterior neural plate is likely to inhibit the FGF signal to a certain level which is optimal for the normal anterior development. Our data reveals a stage-specific role of BMP signal as a novel anti-caudalization factor which down-regulates FGF signaling for anterior neural development.

Mutually antagonistic interactions between BMP and FGF pathways could ensure their balances which are critical for demarcating spatial boundaries or thresholds of developmental commitment. Inhibition of FGF signaling by BMP pathway is required for the commitment of ectodermal cells to epidermal fate and the maintenance of anterior neural character. BMP inhibition of FGF signaling was shown to reach a plateau even in the presence of increasing levels of BMP signal (Fig. 2G), suggesting a possibility that FGF signal could prevent BMP pathway, for example, via inhibitory phosphorylation of Smad1, from suppressing the activity of the former below a threshold. This counteraction of FGF signal might be necessary for anterior neural induction by BMP antagonists and posterior neural patterning after gastrulation. Future experiments are warranted to investigate whether the inhibition of the *Flrt3*/FGF pathway by BMP signal might occur in developmental events other than neural patterning and to identify other target genes of BMP signal which would mediate the crosstalks between BMP and other signaling pathways.

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