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A BMP-*Shh* negative-feedback loop restricts *Shh* expression during limb development

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Normal patterning of tissues and organs requires the tight restriction of signaling molecules to well-defined organizing centers. In the limb bud, one of the main signaling centers is the zone of polarizing activity (ZPA) that controls growth and patterning through the production of sonic hedgehog (SHH). The appropriate temporal and spatial expression of *Shh* is crucial for normal limb bud patterning, because modifications, even if subtle, have important phenotypic consequences. However, although there is a lot of information about the factors that activate and maintain *Shh* expression, much less is known about the mechanisms that restrict its expression to the ZPA. In this study, we show that BMP activity negatively regulates *Shh* transcription and that a BMP-*Shh* negative-feedback loop serves to confine *Shh* expression. BMP-dependent downregulation of *Shh* is achieved by interfering with the FGF and Wnt signaling activities that maintain *Shh* expression. We also show that FGF induction of *Shh* requires protein synthesis and is mediated by the ERK1/2 MAPK transduction pathway. BMP gene expression in the posterior limb bud mesoderm is positively regulated by FGF signaling and finely regulated by an auto-regulatory loop. Our study emphasizes the intricacy of the crosstalk between the major signaling pathways in the posterior limb bud.

KEY WORDS: SHH, BMP, ZPA, Noggin, FGF signaling, Wnt signaling, Limb development, Pattern formation

INTRODUCTION

The anatomy of the amniote limb is characterized by clear asymmetries in the antero-posterior axis and is particularly well displayed by the different shapes of the digits. A multitude of observations and experiments in the past years indicates that the signaling molecule sonic hedgehog (SHH) has a crucial role in specifying the number and identity of the digits by controlling both proliferation and patterning in the developing limb (Bastida and Ros, 2008; McMahon et al., 2003; Towers and Tickle, 2009).

SHH is specifically secreted by the zone of polarizing Activity (ZPA), a group of cells located at the posterior margin of the bud (Riddle et al., 1993). The ZPA was originally identified by its capacity to direct antero-posterior patterning; it was later shown that all its properties are due to the production of SHH (Lopez-Martinez et al., 1995; Riddle et al., 1993; Yang et al., 1997). SHH has been considered to function as a morphogen diffusing from the ZPA and establishing a concentration gradient across the digit-forming field (Tickle, 2003).

SHH is a very potent molecule, and its expression thus needs to be tightly regulated because modifications in the strength, duration or spreading of the signal have profound phenotypic consequences (Harfe et al., 2004; Lewis et al., 2001; Li et al., 2006; Scherz et al., 2007; Towers et al., 2008; Zhu et al., 2008). For example, misregulation of *Shh* expression leading to its ectopic activation at the anterior border of the bud is considered to be at the root of many preaxial polydactylies (Hill, 2007; Talamillo et al., 2005). Also, naturally occurring variations in the intensity or duration of the SHH signal, even if subtle, have been shown to correlate with changes in limb morphology across species (Hockman et al., 2008; Shapiro et al., 2003; Stopper and Wagner, 2007; Thewissen et al., 2006). Accordingly, SHH signaling is a highly regulated process that includes a series of transcriptional activators and repressors, multiple intra- and extracellular modulators of secretion, and spreading of the protein (Jiang and Hui, 2008).

Proper activation of *Shh* in the ZPA requires the previous establishment of an early anterior-posterior limb polarity, called prepattern, which depends at least on the collinear activation of Hox genes and the mutual antagonism between *Gli3* and *Hand2* (Hill, 2007; Kmita et al., 2005; Tarchini and Duboule, 2006; te Welscher et al., 2002). Very little is presently known about the factors that restrict *Shh* to the ZPA within the ample permissive area. Once activated, the domain of *Shh* expression remains of a relatively similar size throughout the period of *Shh* expression in the limb bud, although it becomes progressively distally displaced. Particularly in the mouse (Harfe et al., 2004), but also in the chick (Sanz-Ezquerro and Tickle, 2000; Towers et al., 2008), cell lineage analysis has demonstrated that *Shh* descendants extend far beyond the expression domain of *Shh*, leading to the notion that the number of *Shh*-expressing cells is tightly controlled to prevent an excess of SHH signaling.

In the established limb bud, a positive-feedback loop between SHH from the ZPA and fibroblast growth factors (FGFs) from the apical ectodermal ridge (AER) coordinates limb patterning and growth (Laufer et al., 1994; Niswander et al., 1994). SHH, through BMP (Nissim et al., 2006) and GREM1 (Benazet et al., 2009; Khokha et al., 2003; Michos et al., 2004) maintains FGF gene expression in the AER. In turn, FGFs from the AER maintain *Shh* expression in the ZPA (Laufer et al., 1994; Niswander et al., 1994). WNT7A secreted by the dorsal ectoderm is another factor required for *Shh* expression (Parr and McMahon, 1995; Yang et al., 1997). Within the area of FGF and Wnt signaling, the tight restriction of *Shh* expression to the posterior margin relies on the transcription factors TBX2 and TBX3 (Nissim et al., 2007). Finally, it is known that SHH can sense and regulate its own domain of transcription. In the chick wing bud this auto-regulation is achieved by controlling the number of *Shh*-expressing cells through apoptosis (Sanz-Ezquerro and Tickle, 2000).

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Bone morphogenetic proteins (BMP) are signaling molecules that are prominently expressed during limb development and are known to have a range of functions (Bandyopadhyay et al., 2006; Robert, 2007). Several BMP gene members are proven targets of SHH signalling, although in the early limb bud they are expressed before *Shh* (Bandyopadhyay et al., 2006; Duprez et al., 1996; Geetha-Loganathan et al., 2006; Nissim et al., 2006; Robert, 2007). In the chick limb bud, BMPs have been shown to exert a weak polarizing activity and to enhance the polarizing effect of a brief SHH application (Drossopoulou et al., 2000). In the digital plate, the identity of each digit relies on a particular level of BMP signaling (Dahn and Fallon, 2000; Suzuki et al., 2008).

Interestingly, in a number of different developmental contexts, including Hensen's node and the dental epithelium, BMP signaling negatively regulates *Shh* expression (Patten and Placzek, 2002; Piedra and Ros, 2002; Zhang et al., 2000). There is evidence indicating that this could also be the case for the limb bud (Zhang et al., 2000), but the control of *Shh* expression by BMPs has not been explored in depth, neither has it been integrated into current models for limb development. Therefore, the aim of the present work was to analyze the role of BMP signaling in the control of *Shh* expression in the ZPA and how the BMP pathway integrates with the other pathways already operating in the complex area of the posterior limb bud mesoderm.

We show that BMP activity negatively regulates *Shh* transcription and that this function is achieved indirectly, at least in part, by interfering with the FGF- and Wnt-signaling pathways. Since BMP gene are known targets of SHH, we describe a negative-feedback loop operating between BMPs and SHH to control *Shh* expression. Our study also shows that BMP gene expression is positively regulated by FGF signaling and subject to an autoregulatory loop, therefore placing BMP gene expression as an important point of crosstalk between the SHH, Wnt and FGF pathways in the posterior limb bud mesoderm.

MATERIALS AND METHODS

Embryos and in situ hybridization

Chick and mouse embryos of the desired embryonic stage were obtained following standard protocols (Hamburger and Hamilton, 1951; Ros et al., 2000). The *Bmp7*$^{-/-}$ mutant line (Godin et al., 1998) was kindly provided by Elisabeth Robertson (University of Oxford, Oxford, UK). Digoxigenin-labeled antisense riboprobes were prepared, and whole-mount in situ hybridization analysis performed according to standard procedures (Nieto et al., 1996). The probes used were *Shh, Fgf8, Fgf4, Gremlin1, Bmp2, Bmp4, Bmp7, Msx2, Dusp6*, *Fgfr1*, *Wnt7a, Dkk1* [kindly provided by A. Joyner (Sloan-Kettering Institute, New York, USA), G. Martin (University of California at San Francisco, San Francisco, USA), B. Robert (Institut Pasteur, Paris, France), U. Ruther (Heinrich-Heine-University, Düsseldorf, Germany) and C. Tabin (Harvard Medical School, Boston, USA)].

Application of proteins using beads as carriers

Heparin acrylic beads (Sigma, H5263) were soaked for at least 1 hour at room temperature in recombinant human BMP2 (0.1 μg/μl; R&D Systems), NOG (1 μg/μl; R&D Systems), FGF8 (0.5 μg/μl; R&D Systems) or SHH (9 μg/μl) protein solutions and AG1-X2 (Bio-Rad Laboratories) formate beads were incubated for at least 1 hour at room temperature in the specific MAPK kinase inhibitor PD184352 (20 mM in DMSO, University of Dundee, UK) or in the specific PI3K inhibitor LY294002 (25 mM in DMSO; Promega). The beads were implanted into the mesoderm of chick wing buds in ovo or mouse forelimbs in vitro, in the position, and for the time, desired. Mouse embryo trunk fragments with forelimbs were cultured either in a medium-air interface system (Zuniga et al., 1999) or ventral side down on semisolid medium (Unda et al., 2000). For all experiments, beads soaked in the corresponding vehicle were used as control; their application never resulted in modification of gene expression.

Cycloheximide and lithium chloride treatment

Cycloheximide dissolved in DMSO was diluted to a concentration of 1 mg/ml in PBS and 100 μl was added on top of the embryo in ovo at the time of bead implantation (Nissim et al., 2006). LiCl (Sigma) was dissolved in PBS to a concentration of 28 mM and 500 μl added on top of the embryo in ovo 1 hour before the insertion of the bead (Knobloch et al., 2007).

Cell death and immunoblot analysis

In situ detection of DNA fragmentation and immunoblot (western blot) analysis were performed as described (Bastida et al., 2003; Andrews and Faller, 1991). Detailed protocols can be provided upon request.

RESULTS

BMP signaling downregulates *Shh* expression in the chick ZPA

To examine whether *Shh* expression was under the control of BMP signaling in the ZPA, we experimentally manipulated the level of BMP signaling in the posterior limb mesoderm. For this purpose, we applied exogenous human recombinant BMP2 protein, loaded on heparin acrylic beads, into the posterior mesoderm of stage 20 wing buds (Fig. 1). In our experiments, we used a low concentration of BMP2 (0.1 μg/μl) to minimize its apoptotic effect (Bastida et al.,

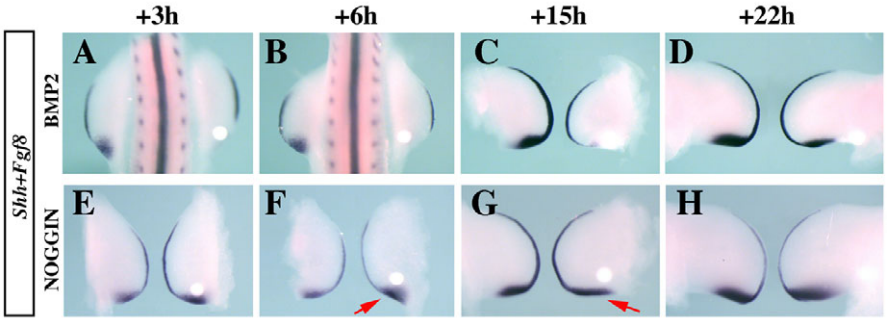

Fig. 1. BMP signaling negatively regulates *Shh* expression in the chick ZPA. (**A-D**) The implantation of a BMP2-soaked bead into the posterior mesoderm results in the drastic downregulation of *Shh* expression 3 (A) and 6 (B) hours after the application. The expression of *Shh* remains undetectable at 15 hours (C) and returns to normal by 22 hours (D). (**E-H**) Reciprocally, the application of a NOG-soaked bead results in an expansion of the *Shh* domain of expression that is barely discernible at 3 hours (E) but evident after 6 and 15 hours of exposure (arrows in F and G). The expression of *Shh* returned to normal at 22 hours (H). In this and following figures the experimental limb is shown on the right accompanied by the contralateral limb, used as control, on the left. Anterior is always to the top.

2004; Piedra and Ros, 2002). The embryos were collected 3, 6, 15 and 22 hours after bead implantation to examine *Shh* expression. Interestingly, we observed that *Shh* expression was drastically downregulated after 3 hours of BMP2 treatment and it became undetectable within 6 hours of exposure (25 out of 25; Fig. 1A-B). *Shh* expression remained undetectable at 15 hours (Fig. 1C) but appeared notably recovered 22 hours after the BMP application, probably because the BMP protein from the bead became exhausted (Fig. 1D). We note that *Shh* downregulation occurred in the presence of normal *Fgf8* transcription (Fig. 1A-D) as discussed below.

To assess whether the endogenous level of BMP signaling was involved in controlling *Shh* expression, we implanted beads soaked in the BMP antagonist noggin (NOG) within the endogenous *Shh* domain. NOG caused a moderate expansion of *Shh* expression domain clearly seen at 6 (Fig. 1F) and 15 (Fig. 1G) hours after the application. In the experimental limb, the *Shh* transcripts extended up to the body wall (arrows in Fig. 1F-G), whereas in the contralateral control limb they were more distally restricted (Fig. 1E-H).

In all these experiments, we used the expression of *Msx2* as reporter of BMP2 activity (Hogan, 1996a; Maas et al., 1996; Vainio et al., 1993). *Msx2* expression was always induced around the BMP2 bead and abolished around the NOG bead (100%, n=10; see Fig. S1 in the supplementary material) (Bastida et al., 2004). These observations support the notion that during normal chick limb development BMPs negatively regulate *Shh* in the ZPA. Since it is well documented that several BMP genes are targets of *Shh* in a variety of developmental contexts, including the ZPA (McMahon et al., 2003), it is possible that SHH induces the BMP genes as a negative-feedback loop to control its own domain of expression.

To assess which BMP family member could fulfil this function endogenously, we re-evaluated the pattern of expression of different BMP genes in relation to that of *Shh* during chick limb development. We found that *Bmp2* and *Bmp7* were excellent candidates because their expression pattern always overlapped with that of *Shh* (see Fig. S2 in the supplementary material), whereas the pattern of expression of *Bmp4* notably diverged, particularly at later stages.

BMP-dependent downregulation of *Shh* is not mediated by loss of FGF gene expression in the AER or cell death

BMPs have been shown to exert a detrimental effect on the expression of FGF genes in the AER, and it is known that AER maintenance requires the antagonism of BMP function by GREM1 (Fernandez-Teran and Ros, 2008; Michos et al., 2004). Since FGF signaling is required for *Shh* expression (Laufer et al., 1994; Niswander et al., 1994), it is possible that the BMP-dependent repression of *Shh* is secondary to the downregulation of FGF gene expression in the AER. However, expression of *Fgf8* and *Fgf4* in the AER was not appreciably modified 3 and 6 hours after the application of the BMP2 bead (Fig. 2A,B and data not shown), demonstrating that the effect of BMP on *Shh* expression was not mediated by loss of FGF gene transcription in the AER. It should be noted that bead placements more distal than those we have performed might interfere with AER-FGF gene expression. *Msx2* was also used as reporter of BMP activity in these experiments (Fig. 2C).

Also, since BMPs have been shown to induce cell death in a variety of embryonic organs including the limb (Graham et al., 1994; Guha et al., 2002), it is possible that the BMP-dependent *Shh* downregulation is caused by the death of *Shh*-expressing cells. Therefore, we examined the treated limbs for apoptosis using the TUNEL assay, and found that the distribution of apoptotic cells in the limb bud was normal after 3 and 6 hours of exposure to exogenous BMP2 (Fig. 2D-E). It was only after 9 hours of treatment that an abnormal cluster of apoptotic cells was observed around the bead (Fig. 2F). Thus, we concluded that cell death did not contribute to the ability of BMP2 to block *Shh*.

In the chick limb bud, SHH controls its own domain of expression through cell death (Sanz-Ezquerro and Tickle, 2000). Since our data indicate that the *Shh* domain of expression is regulated by BMP signaling levels, we analyzed whether the proposed SHH-dependent control of apoptosis could be mediated by BMP, as already considered by Sanz-Ezquerro and Tickle (2000). To examine this possibility, we implanted a SHH bead and then a NOG bead into the ZPA (Fig. 2G). The increase in cell death that normally follows excess of SHH signaling in the posterior mesoderm (Sanz-Ezquerro and Tickle, 2000) was completely abolished by NOG (Fig. 2G), demonstrating that SHH-induced cell death is mediated by BMP signaling.

In the chick wing bud, a normal area of programmed cell death called the posterior necrotic zone (PNZ) evolves at the posterior margin of the bud overlapping the ZPA from stage 23 to 25

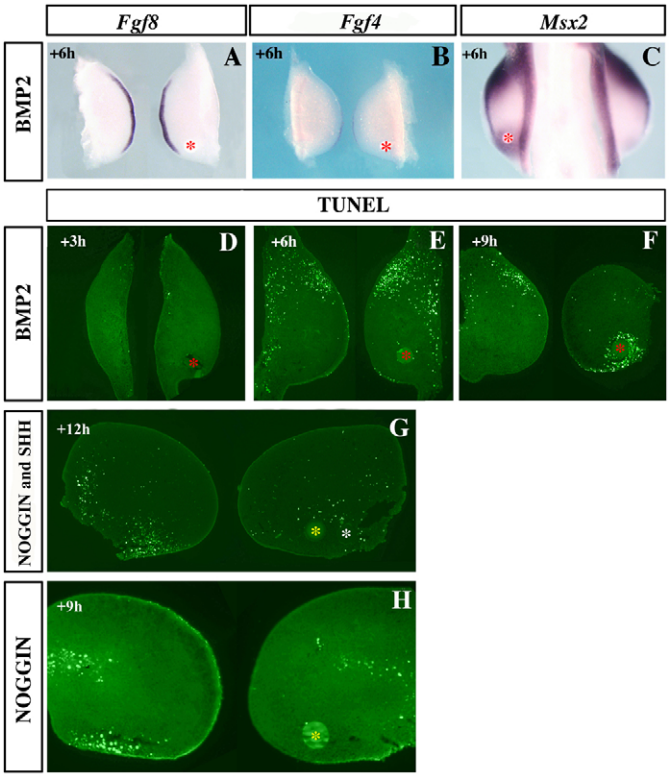

Fig. 2. BMP-dependent downregulation of *Shh* expression is not mediated by downregulation of FGF gene expression in the AER or by cell death. (**A-C**) The transcription of *Fgf8* (A) and *Fgf4* (B) in the AER is not affected 6 hours after the application of a BMP2-soaked bead into the posterior mesoderm, whereas *Msx2* expression, a target of BMP signaling, is clearly induced (C). (**D-F**) The TUNEL assay shows a normal pattern of cell death 3 (D) and 6 (E) hours after BMP2 application with abnormal cell death observed around the bead only after 9 hours of exposure (F). (**G**) A NOG bead prevents the increase in cell death normally caused by a SHH bead. (**H**) The application of a NOG-soaked bead into the posterior mesoderm of stage 22-23 wing buds prevents the establishment of the posterior necrotic zone. The red asterisk labels the BMP2 bead; the yellow asterisk labels the NOG bead and the white asterisk labels the SHH bead.

(Fernandez-Teran et al., 2006). To determine whether this area was dependent on BMP signaling, we applied NOG beads at later stages (stages 22-23), just before the onset of the PNZ. Interestingly, the blockade of BMP signaling by NOG was sufficient to prevent the establishment of the PNZ, demonstrating that the PNZ is mediated by BMP signaling (Fig. 2H).

BMP signaling downregulates *Shh* expression in the mouse ZPA

We next asked whether BMP signaling also participates in the control of *Shh* expression in the mouse ZPA. For this, we replicated the experiments of gain and loss of BMP signaling function in cultured mouse limb buds. Trunks with forelimb buds attached were dissected from E10.5 wild-type embryos and cultured (see the Materials and methods). Before culturing, a bead was implanted into the posterior mesoderm of the right forelimb bud, leaving the left forelimb as control. As in the chick, BMP2 beads (0.1 μg/μl) produced a rapid loss of *Shh* expression (analyzed at 5 and 10 hours, Fig. 3A-C) that occurred before modifications in *Fgf8* transcription in the AER or induced cell death could be observed (not shown). Conversely, application of NOG resulted in a variable expansion of the *Shh* domain of expression that varied from moderate (shown after 5 hours Fig. 3E) to prominent (shown after 10 hours Fig. 3F). According to the pattern of expression of different BMP gene family members in the mouse limb bud (Lewis et al., 2001), *Bmp4* and *Bmp7* are the best candidates to endogenously fulfil this function because of the overlap in their pattern of expression with that of *Shh*.

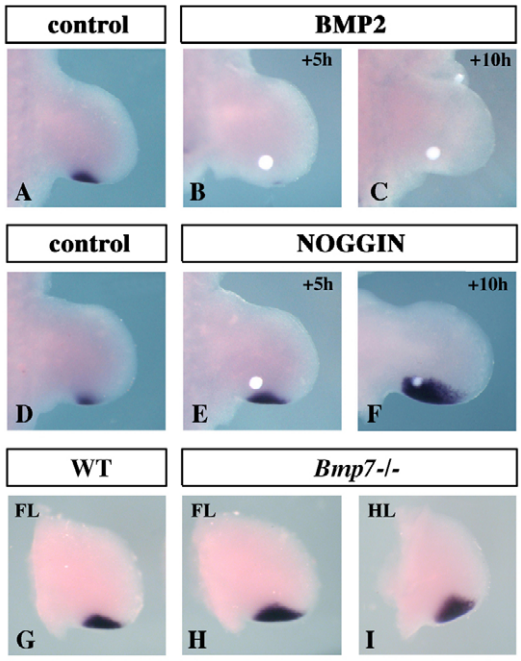

Fig. 3. BMP signaling regulates *Shh* expression in the mouse ZPA. (**A-C**) The implantation of a BMP2-soaked bead into the posterior limb bud mesoderm results in an extreme downregulation of *Shh* expression at 5 (B) and 10 (C) hours after the experiment, compared with the control (A). (**D-F**) Reciprocally, the application of a NOG-soaked bead results in an evident expansion of the *Shh* domain of expression at 5 (E) and 10 (F) hours after the experiment, compared with the control (D). (**G-I**) The *Shh* expression domain in the *Bmp7* mutant limb is expanded moderately in the forelimb (H) and very notably in the hindlimb (I), compared with the control (G).

It is worth noting that the domain of *Shh* expression has already been reported to be expanded in situations of reduced BMP signaling, such as overexpression of the BMP antagonists *Grem1* or *Nog* (Capdevila et al., 1999; Merino et al., 1999; Zuniga et al., 1999), and in different situations of conditional removal of one or several BMP genes in the limb mesoderm (Bandyopadhyay et al., 2006; Selever et al., 2004). This prompted us to carefully analyze *Shh* expression in the *Bmp7* mutant that, unlike *Bmp2* and *Bmp4* conventional knockouts, is not lethal at early embryogenesis (Dudley et al., 1995; Hofmann et al., 1996; Luo et al., 1995). *Bmp7* mutant embryos are characterized by anterior polydactyly with variable penetrance and expressivity (Hofmann et al., 1996). In accordance with our hypothesis, we found an expansion of the *Shh* domain in *Bmp7*-null limb buds that was more prominent in the hindlimb than in the forelimb (Fig. 3G-I). This expansion was very similar to that reported in the conditional removal of *Bmp4* in the limb mesoderm [compare Fig. 3I with Fig. 5B in results published by Selever and colleagues (Selever et al., 2004)].

Therefore, these results indicate that the negative effect of BMP signaling on *Shh* expression in the ZPA is conserved between birds and mammals.

Downregulation of *Shh* by BMP requires de novo protein synthesis

To test whether BMP activity downregulates *Shh* directly or indirectly, we implanted BMP2-soaked beads into the ZPA in the presence of cycloheximide, which is an inhibitor of de novo protein synthesis. Whereas BMP2 rapidly and dramatically downregulates *Shh* expression (100%; Fig. 1), in the presence of cycloheximide, the BMP bead had no effect on *Shh* expression for up to 6 hours (Fig. 4A-B; 6 out of 6), suggesting that BMP acts indirectly by a secondary signal either secreted or intracellular. The BMP2 bead was able to upregulate *Msx2* expression in the presence of cycloheximide, indicating that *Msx2* is a direct target of BMP signaling (Fig. 4C). We note that cycloheximide did not modify the pattern of expression of *Shh* (Fig. 4D), and that it caused the death of the embryo 6-9 hours after its administration.

BMP antagonizes FGF signaling in the posterior limb mesoderm

Since there is evidence that FGF from the AER has a major role in the induction and maintenance of *Shh* expression in the ZPA (Laufer et al., 1994; Niswander et al., 1994; Ros et al., 1996), we asked whether BMP could interfere with FGF signaling even in the presence of normal FGF gene transcription in the AER. For this, we implanted FGF8 (0.5 μg/μl)-soaked beads in the proximal posterior mesoderm, a procedure that is known to result in the induction of *Shh* expression (Yang and Niswander, 1995). At 6 hours after the application, when 100% of the embryos analyzed showed an extension of the endogenous domain of *Shh* towards the FGF bead (n=8; Fig. 5A), we applied a second bead, which was this time soaked in BMP2 (0.1 μg/μl). As a consequence, both the endogenous and the induced domains of *Shh* expression became progressively downregulated (Fig. 5B-C), whereas *Shh* upregulation continued in the specimens that only received the FGF8 bead (inset in Fig. 5C). This result showed that BMP can antagonize the induction of *Shh* by FGF8, even in the presence of excess FGF protein, and suggested that BMP signaling interferes with the reception or transduction of the FGF signal.

To further analyze this interference, we examined the state of *Fgfr1* expression, the main FGF receptor expressed in the limb bud mesoderm. We found that *Fgfr1* expression was not appreciably

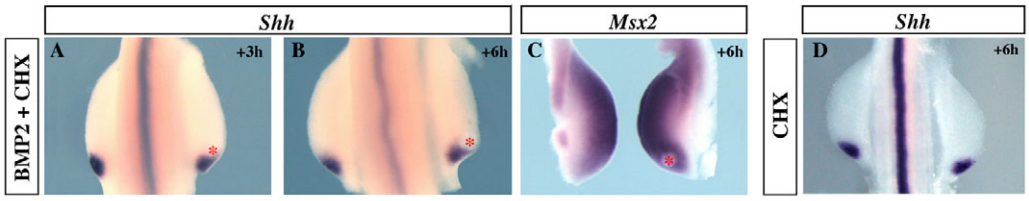

Fig. 4. BMP-dependent downregulation of *Shh* requires de novo protein synthesis. (**A-D**) In the presence of cycloheximide, the BMP2 bead does not downregulate *Shh* expression after 3 (A) and 6 (B) hours of exposure. By contrast, the presence of cycloheximide does not impair the ability of BMP to induce *Msx2* (C). Note that although *Msx2* expression is altered by cycloheximide, the additional upregulation by BMP2 can still be appreciated. Incubation with cycloheximide alone for 6 hours does not modify *Shh* pattern of expression (D). Red asterisks label the BMP2 beads.

modified by the BMP treatment (see Fig. S3 in the supplementary material), indicating that the observed BMP-dependent antagonism of the FGF pathway is probably downstream of the reception of the signal.

We also analyzed the expression of *Dusp6*, which is a good readout of FGF signaling (Mariani et al., 2008); it was slightly downregulated around the BMP2 bead (Fig. 5D-E). However, we noted that the endogenous expression of *Dusp6* was very low in the posterior mesoderm, a site in which FGF signaling is thought to occur at high levels (Laufer et al., 1994; Niswander et al., 1994) (read arrowhead in Fig. 5D and E). Therefore, to refine these results, we analyzed some components of the FGF intracellular pathway by immunoblotting. For this, we dissected the posterior half of limbs treated with BMP2 for 4 hours and used the posterior half of the contralateral non-treated limbs as a control (Fig. 5F). We used antibodies against phosphorylated and total ERK (Santa Cruz) because it has been shown that the ERK1/2 MAPK signaling pathway is one of the main transduction pathways of FGF signaling in the limb bud (Eblaghie et al., 2003; Kawakami et al., 2003; Smith et al., 2006). We analyzed separately the nuclear and cytoplasmic fractions, because ERK translocates to the nucleus upon activation. BMPs signal through BMP receptors (BMPRs) that are serine/threonine protein kinases that phosphorylate the transcription factors SMAD1/5/8 at their C-terminus, causing their activation and nuclear translocation (Shi and Massague, 2003). Therefore, we first used an antibody against phospho-SMAD1/5/8 (pSmad; Santa Cruz) to check the effect of the BMP treatment. As expected, the protein blot analysis showed that the exposure to exogenous BMP2 for 4 hours raised the cytoplasmic and nuclear pools of pSMAD1/5/8 (Fig. 5F). There was a 1.73-fold (±0.18) rise in the nuclear pool of pSMAD1/5/8 that was associated with a 0.58-fold (± 0.07) decrease in the amount and therefore activity of phosphorylated ERK (pERK), in the nucleus. The cytoplasmic pool of pERK, as well as both the nuclear and cytoplasmic pools of total ERK remained mostly unaffected by the BMP treatment (Fig. 5F). The immunoblot for RhoGDI (Fig. 5F) and PARP1/2 (not shown) showed the absence of cross-contamination between the cytoplasmic and nuclear fractions.

The results of the immunoblotting show that at least one important functional component of FGF intracellular signaling, pERK, is decreased in the nucleus upon increased BMP signaling. This indicates that BMP signaling antagonizes FGF signaling at least by negatively regulating the ERK1/2 MAPK pathway. To further confirm this result and also to analyze the possible involvement of the PI3K-Akt pathway, another important FGF transduction pathway in limb development, we conjointly applied FGF8 with the specific inhibitor of each of these two pathways. The co-implantation of an FGF8 bead with a bead soaked in PD184352, a specific MAPK kinase inhibitor, resulted in the local inhibition of *Shh* induction by FGF8 (Fig. 5G).

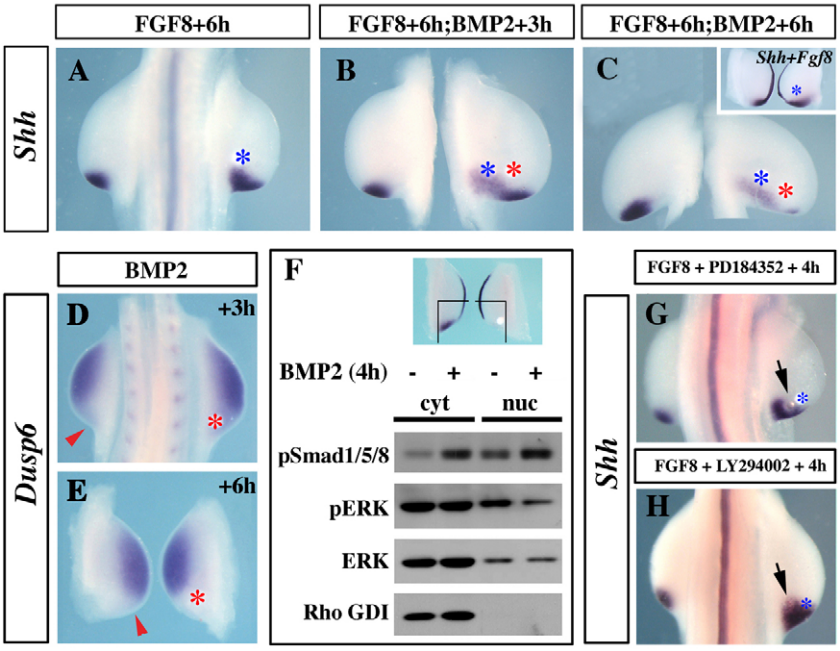

Fig. 5. BMP signaling interferes with FGF signaling in the presence of FGF8 protein. (**A**) The application of an FGF8 bead into the proximal-posterior mesoderm results in the induction of *Shh* shown 6 hours after the application. (**B**,**C**) The subsequent application of a BMP2 bead progressively overrides the FGF effect shown at 3 (B) and 6 (C) hours, whereas *Shh* upregulation continues in the samples that only received the FGF8 bead (inset in C). (**D**,**E**) The expression of *Dusp6*, a readout of FGF signaling, is slightly downregulated by the BMP2 bead. However, note that the endogenous expression of *Dusp6* is much lower in the posterior wing bud mesoderm (arrowhead in D and E). (**F**) Immunoblot showing that a rise in BMP signaling as indicated by the increase in the nuclear pool of pSmad1/5/8 is accompanied by a decrease by half in the nuclear pool of pERK. Only the posterior half of experimental and contralateral control limb were used for the inmunoblots as indicated in the drawing. (**G**,**H**) When coimplanted with an FGF8-bead, the PD184352 bead locally inhibits the FGF-dependent induction of *Shh* at 4 hours (arrow in G), whereas the LY294002 bead does not (arrow in H). The red asterisk labels the BMP2 bead and the blue asterisk labels the FGF8 bead.

However, the co-implantation of an FGF8 bead with a bead soaked in LY294002, a specific PI3K-inhibitor, had no effect (Fig. 5H). These results strongly indicate that FGF regulates *Shh* through the ERK1/2 MAPK pathway and together with the immunoblot results (Fig. 5F) indicate that BMPs negatively regulate *Shh* by interfering with the activation of the ERK1/2 MAPK pathway by FGFs.

The antagonism of Wnt/β-catenin signaling might contribute to BMP-dependent downregulation of *Shh*

Another essential factor for the maintenance of *Shh* expression in the ZPA is WNT7A, which is secreted by the dorsal ectoderm (Parr and McMahon, 1995; Yang and Niswander, 1995). Since the deficit in WNT7A, whether genetic or experimental, results in a drastic downregulation of *Shh* expression (Parr and McMahon, 1995; Yang and Niswander, 1995), it is possible that BMPs could repress *Shh* expression by repressing *Wnt7a*. To check this possibility, we examined *Wnt7a* expression after application of BMP, and found that it was not appreciably modified at 3 or 6 hours after the application (Fig. 6B-C). This result rules out transcriptional regulation of *Wnt7a* as a possible mediator of the effect of BMP on *Shh* expression. However, as previously noted for cell death (Macias et al., 1997), it is possible that BMP signaling does not affect the ectoderm, but it might nevertheless affect Wnt signaling in the subjacent mesoderm.

WNT7A signals, at least partially, through the canonical Wnt signaling and it is known that it interacts with the gene encoding Dickkopf1 (*Dkk1*), a negative regulator of Wnt signaling (Adamska et al., 2004; Hill et al., 2006). Since *Dkk1* is regulated by BMP signaling (Grotewold and Ruther, 2002), we asked whether DKK1 could mediate BMP-dependent repression of *Shh*. As expected, *Dkk1* was strongly and rapidly upregulated in our experiments of gain-of-function of BMP signaling (Fig. 6D-E) and, conversely, became rapidly undetectable upon application of NOG (Fig. 6F-G). This result indicates an inhibition of the Wnt/β-catenin signaling pathway in the posterior limb mesoderm upon increased BMP signaling. To further analyze this point, we performed immunoblots to examine the nuclear and cytoplasmic fractions of pSMAD1/5/8 and β-catenin. As previously shown, treatment with BMP increased the cytoplasmic and nuclear levels of pSMAD1/5/8, demonstrating activation of BMP signaling (Fig. 6H). This was accompanied by a 0.53-fold (±0.09) reduction of the nuclear pool of β-catenin (Fig. 6H) indicating that BMP signaling antagonizes the canonical Wnt/β-catenin signaling by inducing *Dkk1*.

To further test this result, we investigated whether BMP could block *Shh* in the presence of Wnt-signaling activators operating downstream of DKK1. For this, we repeated the experiments in the presence of LiCl, an inhibitor of GSK3β that prevents β-catenin phosphorylation and degradation therefore activating the Wnt/β-catenin pathway (Klein and Melton, 1996). Interestingly, the presence of LiCl abrogated the negative effect of the exogenous BMP on *Shh* expression in the majority of cases analyzed at 3 hours (Fig. 6I and 6K; 5 out of 6). At 6 hours after treatment, the majority of the embryos (71.4%; 5 out of 7) showed either no modification or only partial reduction of *Shh* expression (Fig. 6J) with only two out of seven embryos analyzed (28.6%) showing the expected reduction in *Shh* expression (Fig. 6L). The fact that LiCl can abrogate the effect of BMP on *Shh* indicates that BMP-dependent downregulation of *Shh* occurs upstream of GSK3β, supporting the involvement of DKK1. LiCl did not interfere with the induction of *Msx2* by BMP2, which was used as a control of BMP activity (Fig. 6M-N).

The induction of *Shh* by FGF requires protein synthesis

Our data indicate that BMP-dependent downregulation of *Shh* is achieved by counteracting the positive effects of the FGF and Wnt pathways, indicating a tight crosstalk among the BMP-, FGF- and Wnt-signaling pathways in the posterior limb bud mesoderm to control the domain of *Shh* expression.

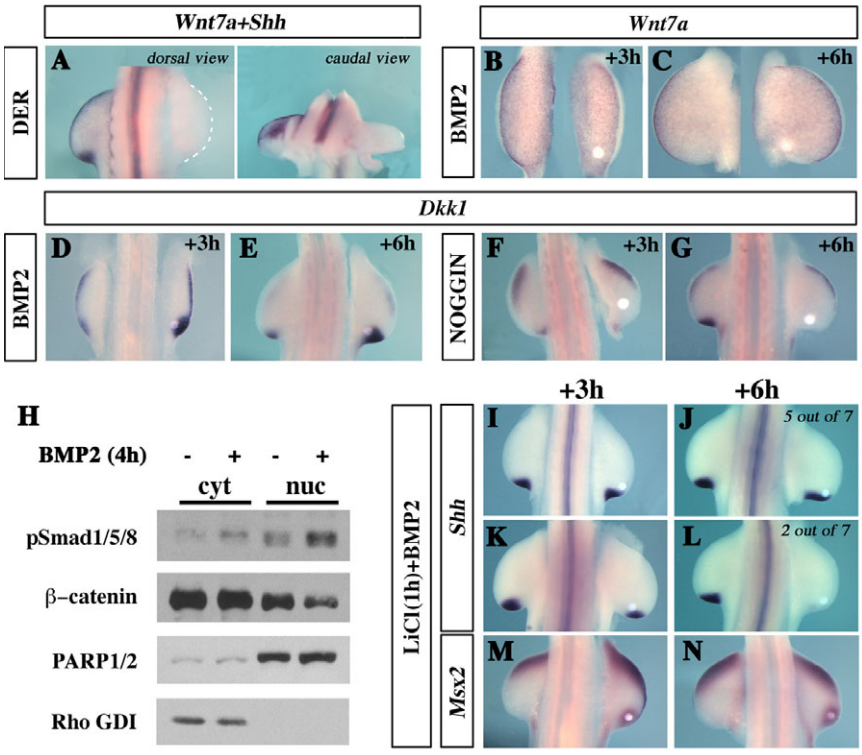

Fig. 6. BMP signaling interferes with Wnt signaling. (**A**) Dorsal ectoderm removal (DER) leads to a rapid and complete downregulation of *Shh* within 6 hours. The removal of the dorsal ectoderm is assessed by the absence of *Wnt7a* expression, shown in dorsal and caudal view as indicated. (**B**,**C**) Application of exogenous BMP2 into the posterior mesoderm does not affect *Wnt7a* expression in the dorsal ectoderm. (**D**,**E**) Application of a BMP2 bead into the posterior mesoderm strongly upregulates the expression of *Dkk1* around the bead shown 3 (D) and 6 (E) hours after application. (**F**,**G**) Reciprocally, a NOG-soaked bead results in the complete downregulation of *Dkk1* 3 (F) and 6 (G) hours after the application. (**H**) Immunoblotting showing that a rise in BMP signaling as indicated by the increase in the nuclear pool of pSmad1/5/8 is accompanied by a decrease in the nuclear pool of β-catenin. (**I-N**) In the presence of LiCl, the BMP2 bead is unable to downregulate *Shh* expression at 3 hours (I,K) and only in some embryos at 6 hours (J,L). LiCl does not interfere the ability of BMP2 to induce Msx2 (M,N).

Given these complex signaling interactions, we considered the possibility that the induction of *Shh* by FGFs could be mediated by a potential downregulation in BMP gene expression. As a first step to analyze this possibility, we examined whether FGF8 was able to induce *Shh* directly, in the absence of protein synthesis. Remarkably, FGF8 beads applied to the posterior limb bud mesoderm were unable to upregulate *Shh* in the presence of cycloheximide (n=4; analyzed at 4 and 6 hours and shown in Fig. 7A-B at 4 hours), indicating that this induction requires protein synthesis. Next, we analyzed the state of BMP gene transcription in the posterior mesoderm after FGF application. We found that the FGF8 beads strongly upregulated *Bmp2* (Fig. 7C) as well as *Bmp4* and *Bmp7* (not shown), and that this upregulation did not require protein synthesis as it was not modified by the presence of cycloheximide (Fig. 7D). As previously shown for *Shh* (Fig. 4D), cycloheximide alone did not alter *Bmp2* expression (inset in Fig. 7D). To determine which FGF transduction pathway, ERK1/2 MAPK or PI3K/Akt, was involved in the direct activation of BMP gene expression by FGF, we concomitantly applied FGF8 and the corresponding specific inhibitor. Although PD184352 locally blocked FGF8 induction of *Bmp2* (Fig. 7E), LY294002 had no effect (Fig. 7F), indicating that the ERK1/2-MAPK-signaling pathway mediates this induction.

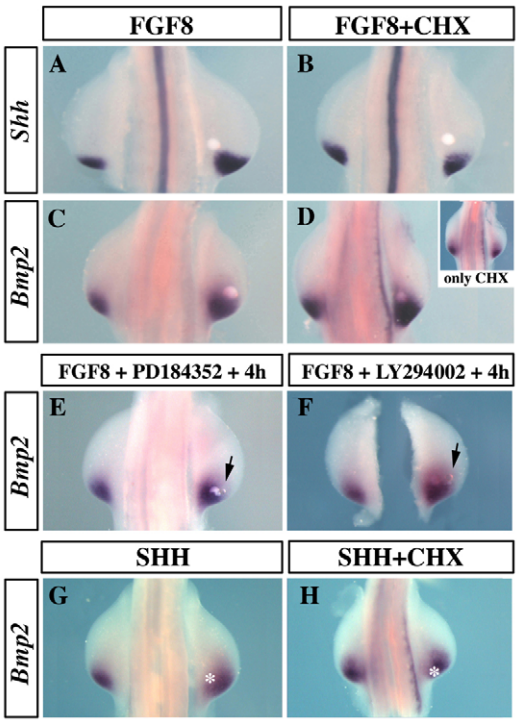

Fig. 7. FGF8-dependent induction of *Shh* requires protein synthesis. (**A**,**B**) The implantation of an FGF8 bead into the posterior mesoderm results in the expansion of the *Shh* domain of expression (A), an effect that is abrogated by cycloheximide (B). (**C**,**D**) By contrast, the FGF8 bead upregulates *Bmp2* (C) irrespective of the presence of cycloheximide (D). Incubation with cycloheximide alone does not modify *Bmp2* expression (inset in D). (**E**,**F**) When coimplanted with an FGF8 bead, a PD184352 bead locally inhibits the FGF-dependent induction of *Bmp2* at 4 hours (arrow in E), whereas an LY294002-bead does not (arrow in F). (**G**,**H**) The SHH-dependent upregulation of *Bmp2* (G) is abrogated by cycloheximide (D). The white asterisk labels the SHH bead.

Finally, we asked whether the well-known induction of BMP genes by SHH was cycloheximide sensitive. We found that SHH beads were unable to expand the *Bmp2* expression domain in the presence of cycloheximide (Fig. 7G-H), indicating that this induction required protein synthesis.

An auto-regulatory loop controls BMP gene expression in the posterior mesoderm

Our results indicate that, in the posterior limb bud mesoderm, both SHH (indirectly) and FGF (directly) signaling pathways positively regulate *Bmp2* expression. It is also reported that the Wnt/β-catenin signaling pathway upregulates BMP genes in the limb bud mesoderm (Hill et al., 2006). Therefore, all these three pathways converge in the positive regulation of BMP gene transcription raising the question of how BMP gene expression is controlled in the posterior mesoderm. In several systems such as the inner ear, it has been shown that the expression of several BMP genes is subject to an auto-regulatory loop (Pujades et al., 2006), and indeed, it has also been suggested for the limb (Capdevila and Johnson, 1998). Therefore, we analyzed the endogenous expression of *Bmp2*, *Bmp7* and *Bmp4* after the experimental manipulation in BMP signaling. We observed that the expression of *Bmp2* (Fig. 8A-C), *Bmp7* (Fig. 8D-F) and *Bmp4* (Fig. 8G-I) was rapidly and drastically downregulated by the experimental rise in BMP2 levels. This effect was clearly seen at 3 and 6 hours, and in the process of returning to normal by 15-22 hours after the application (Fig. 8A-I). Conversely, the expression of *Bmp2* (Fig. 8J-L), *Bmp7* (Fig. 8M-O) and *Bmp4* (Fig. 8P-R) was dramatically upregulated by NOG, strongly supporting the existence of an auto-regulatory loop that operates in the posterior mesoderm to control BMP gene expression. Interesting, this auto-regulatory loop requires de novo protein synthesis because the BMP2 beads were unable to downregulate *Bmp2* expression in the presence of cycloheximide (Fig. 8S-T).

DISCUSSION

We propose that BMP signaling levels have a specific role in controlling the *Shh* domain of expression by negatively regulating its transcription. During normal development, the appropriate level of BMP signaling in the posterior limb bud mesoderm is the result of a delicate balance between positive signals (SHH, FGF and Wnt pathways) and counteracting factors (auto-regulatory loop and BMP inhibitors).

A BMP-*Shh* negative-feedback loop controls *Shh* expression

BMPs are known to have important roles in AER induction, maintenance and regression (Ahn et al., 2001; Pizette et al., 2001), in the determination of digit identity (Dahn and Fallon, 2000; Suzuki et al., 2008) and in the regulation of apoptosis and skeletogenesis (Hogan, 1996a; Hogan, 1996b; Robert, 2007). Here, we describe a new BMP function in the control of the ZPA. We show that an increase in BMP signaling in the posterior limb mesoderm causes a rapid decline in *Shh* expression, whereas blocking BMP signaling conversely results in an expansion of the *Shh* domain of expression, both in chick and in mouse. These results indicate that the net level of endogenous BMP signaling is an important parameter in the control of *Shh* transcription in the posterior limb bud mesoderm. Since the expression of several BMP genes in the posterior mesoderm is regulated by SHH (Drossopoulou et al., 2000), our studies unravel a negative-feedback loop (BMP-*Shh*) that serves to control *Shh* expression within the ample area of positive regulators and that is interconnected with the well-established FGF-*Shh*

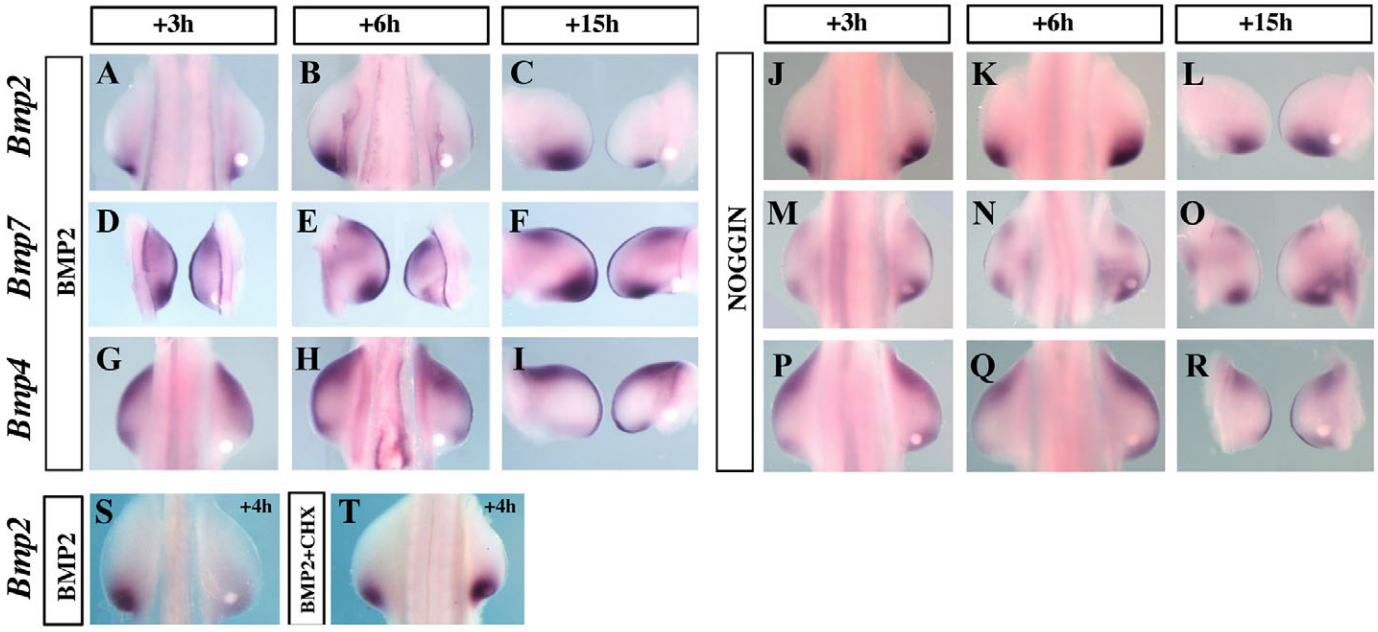

Fig. 8. Auto-regulation of *Bmp2, Bmp4 and Bmp7* expression in the posterior limb bud mesoderm. (**A-T**) The experimental rise of BMP2 levels in the posterior mesoderm by the placement of a BMP2-bead results in the marked downregulation in endogenous expression of *Bmp2* (A-C), *Bmp7* (D-F) and *Bmp4* (G-I). Reciprocally, the experimental blockade of BMP signaling by a noggin bead results in a marked upregulation of *Bmp2* (J-L), *Bmp7* (M-O) and *Bmp4* (P-R) expression. The presence of cycloheximide, abrogates the BMP2-dependent downregulation of *Bmp2* (S,T).

positive-feedback loop. Thus, we show that FGF signaling, in addition to promoting *Shh* expression, also induces BMP signaling, which in turn blocks *Shh* expression. Interestingly, *Etv4* and *Etv5* are other *Shh* inhibitors that are positively regulated by FGFs (Mao et al., 2009; Zhang et al., 2009).

This model can account for and is supported by previously reported evidence linking decreased BMP signaling to expanded *Shh* expression. This evidence includes the overexpression of BMP antagonists (Capdevila et al., 1999; Merino et al., 1999; Zuniga et al., 1999), the conditional removal of *Bmp4* (Selever et al., 2004) and the compound conditional removal of *Bmp4* and *Bmp2* (Bandyopadhyay et al., 2006). All these situations involve upregulation of *Shh*, which was interpreted to be due to an increase in FGF signaling in the AER. Our study supports an alternative interpretation whereby upregulation of *Shh* is the result of a decrease in BMP signaling. Consistent with this, in the current study, we show that the *Shh* domain of expression is expanded in the *Bmp7* mutant limb (Dunn et al., 1997; Guha et al., 2002; Katagiri et al., 1998).

An expansion of the *Shh* domain has not been reported after NOG overexpression in the chick, but this might depend on the observation time or on the establishment of compensatory mechanisms (Capdevila and Johnson, 1998; Pizette and Niswander, 1999).

SHH-dependent cell death in the posterior mesoderm is mediated by BMP signaling

Sanz-Ezquerro and Tickle (Sanz-Ezquerro and Tickle, 2000) showed that, in the developing wing bud, SHH regulates its own expression by controlling the number of *Shh*-expressing cells through apoptosis. They proposed that the domain of *Shh* would be maintained at the appropriate size and position by a balance between the distal positive influence of the AER, which will maintain expression in the distal cells within *Shh* domain, and the loss of *Shh*-expressing cells proximally by cell death. This mechanism nicely accounts for the distal displacement of the *Shh* domain that normally occurs during development, as well as provides an explanation for the predominantly proximal expansion of the *Shh* domain observed after NOG application in the chick.

Here, we show that the mechanism by which SHH induces apoptosis is indirect and mediated by BMPs, as was previously hypothesized (Sanz-Ezquerro and Tickle, 2000). Furthermore, we show that BMPs can downregulate *Shh* transcription without inducing cell death, at least in part, by counteracting the positive inputs of the FGF- and Wnt-signaling pathways. Our results suggest that *Shh* autoregulation is mediated by the induction of BMP genes, which in turn represses *Shh*, establishing a negative feedback mechanism that, when integrated into the network of signals operating in the posterior limb bud mesoderm, controls *Shh* transcription. This model does not require cell death and applies to all types of limbs independently of the existence of an area of cell death overlapping the ZPA, which is present in the chick wing bud but not in the mouse limb bud (Fernandez-Teran et al., 2006; Saunders and Fallon, 1967). We further show that the PNZ is dependent on BMP signaling because it can be abolished by NOG.

BMPs counteract FGF and Wnt/β-CATENIN signaling in the posterior limb bud

Several studies have provided evidence for the interaction between the BMP- and FGF-signaling pathways in different systems. In the limb, this interaction has not been completely elucidated. Genetic and experimental manipulations in mouse and chick have shown that BMP signaling blocks FGF gene transcription in the AER leading to the idea that GREM1 is a critical AER maintenance factor (Fernandez-Teran and Ros, 2008; Khokha et al., 2003; Michos et al., 2004; Pizette and Niswander, 1999). Conversely, the abolition of BMP signaling in the AER results in the temporal extension of FGF gene expression in the AER (Pajni-Underwood et al., 2007; Maatouk et al., 2009).

At the intracellular level, it is known that FGF-MAPK signals oppose BMP-SMAD1 in many developmental organs (De Robertis and Kuroda, 2004). Four MAPK phosphorylation sites are present in the middle region of SMAD1/5/8 and it is known that the phosphorylation of these sites promotes polyubiquitylation, thereby terminating SMAD activity (Eivers et al., 2008; Fuentealba et al., 2007). Here, we provide evidence of regulation in the opposite direction. The immunoblot analysis we performed showed that in the posterior limb mesoderm, excess BMP signaling notably reduces the pool of nuclear phosphorylated ERK in the presence of normal FGF gene transcription in the AER. Since there is no evidence that BMPs can interfere with FGF binding to its receptor, and we show that BMP signaling does not modify *Fgfr1* expression in the posterior chick mesoderm (Yoon et al., 2006), we conclude that the BMP interference with the FGF signaling pathway occurs downstream of the reception of the signal. Indeed, the increase in BMP signaling is accompanied by a decrease in the pull of nuclear pERK, indicating inhibition of this intracellular pathway. Accordingly, using specific inhibitors for the ERK1/2-MAPK and the PI3K-Akt pathways, we show that the FGF induction of *Shh* (and also BMP genes) is mediated by the classical ERK1/2 MAPK transduction pathway. Together, all these observations allow the conclusion that BMP regulates *Shh* by interfering with the ERK1/2MAPK pathway that is activated by FGFs.

We also present evidence indicating that BMP downregulation of *Shh* is mediated by a downregulation in the Wnt/β-catenin signaling pathway that is normally involved in the maintenance of *Shh* expression (Hill et al., 2006; Parr and McMahon, 1995; Yang and Niswander, 1995). Interference with this pathway appears to be mediated by the BMP-dependent upregulation of *Dkk1* because it is abrogated by LiCl. The observation that LiCl frequently rescues the negative effect of BMP on *Shh* expression might reveal a predominance of the Wnt pathway over the FGF pathway in *Shh* maintenance of expression. However, it is also possible that the activation of the Wnt/β-catenin pathway by LiCl is sufficient to compensate the concomitant blocking of the FGF pathway. Therefore, based on our data, we propose that BMPs downregulate *Shh* in part by interfering with the FGF- (intracellular) and Wnt (extracellular)-signaling pathways.

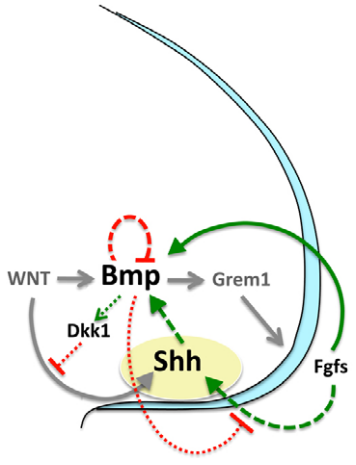

Fig. 9. The regulatory pathways operating in the posterior mesoderm. Arrows and T-bars indicate positive and negative regulation respectively. The interactions analyzed in this paper are shown in green and red: solid lines indicate direct interactions, dashed lines indicate interactions that require protein synthesis. Dotted lines represent undetermined interactions.

Crosstalk between major signaling pathways in the posterior limb bud

Our data stresses the importance of BMP signaling in *Shh* expression as well as the interconnection among signaling pathways in the posterior limb bud mesoderm (Fig. 9). Recently, Benazet and co-workers (Benazet et al., 2009) by combining mouse molecular genetics with mathematical modeling, have unraveled two feedback loops: a fast-operating BMP4-GREM1 module and a slower-operating SHH-GREM1-FGF module, which coordinates antero-posterior patterning by regulating BMP levels through GREM1. Our study reveals the involvement of additional feedback circuits that converge in the induction of BMP genes, which are in turn subject to an auto-regulatory loop (Fig. 9). Both studies coincide, in that the level of BMP signaling is an important point of crosstalk between the major signaling pathways operating in the posterior limb bud.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/22/3779/DC1

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