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Developmental Biology 299 (2006) 12–21
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Regulation of *Gremlin* expression in the posterior limb bud

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Received for publication 15 December 2005; revised 20 May 2006; accepted 23 May 2006
Available online 26 May 2006

Abstract

Proper outgrowth of the limb bud requires a positive feedback loop between *Sonic hedgehog* (*Shh*) in the zone of polarizing activity (ZPA) and *Fgfs* in the overlying apical ectodermal ridge. The Bmp antagonist *Gremlin* is expressed in a domain anterior to the ZPA and is thought to act as a signaling intermediate between *Shh* and *Fgf*. It is currently unclear whether *Shh* acts directly or indirectly to initiate and maintain *Gremlin*. In this study, we confirm that Bmp activity is necessary and sufficient for induction of *Gremlin*. Beads soaked in the Bmp antagonist Noggin downregulate *Gremlin*, while beads soaked in Bmp2 cause its upregulation. Furthermore, Bmp2 is also capable of upregulating Gremlin in *oligozeugodactyly* mutant limbs that lack Shh activity, demonstrating that Gremlin expression does not depend on the combined exposure to both these factors. In spite of the ability of Bmp2 to induce Gremlin, beads soaked in high concentrations of Bmp2 downregulate *Gremlin* around the bead without apparent induction of cell death, whereas another target gene *Msx2* is upregulated around the bead. Consistent with this concentration-dependent effect, we find that low concentrations of Bmp2 upregulate *Gremlin* while high concentrations of Bmp2 downregulate *Gremlin* in limb mesenchyme cultures. These data implicate Bmp activity as a required intermediate in the Shh–Fgf4 signaling loop. Though we show that Bmp activity is sufficient to upregulate *Gremlin*, *Gremlin* expression is excluded from a posterior domain of the limb, and expansion of this domain as limb outgrowth proceeds is important in terminating the Shh–Fgf4 signaling loop. We find that the posterior limb is refractory to *Gremlin* induction in response to Bmp2, suggesting that termination of the Shh–Fgf4 signaling loop results from inability of Bmp activity to induce *Gremlin* in the posterior. In contrast, in the *oligozeugodactyly* limb, we find that beads soaked in Bmp2 can induce *Gremlin* in the posterior, demonstrating that Shh activity is required for exclusion of *Gremlin* in the posterior. Finally, by blocking Shh activity with cyclopamine, we find evidence that continued Shh activity is also required to maintain refractoriness to *Gremlin* expression in response to Bmp activity.

Keywords: *Gremlin*; Bmp; Shh; Fgf; Bone morphogenetic protein; Sonic hedgehog; Fibroblast growth factor; Limb bud; *oligozeugodactyly*

Introduction

Proper outgrowth and patterning of the vertebrate limb require the coordination of several classical signaling centers. The Zone of Polarizing Activity (ZPA) in the posterior limb mesenchyme is a source of the secreted factor Sonic hedgehog (Shh) which is responsible for anterior–posterior patterning of the limb (Laufer et al., 1994; Riddle et al., 1993). Another signaling center, the apical ectoderm ridge (AER), is a specialized epithelial structure at the distal tip of the limb that is a source of several members of the fibroblast growth factor (FGF) family necessary to maintain proliferation of underlying mesenchyme and distal outgrowth of the limb (Fallon et al., 1994; Martin, 1998; Niswander et al., 1994). A positive feedback loop between Shh from the ZPA and Fgfs from the AER coordinates the activity of these two signaling centers. Misexpression of *Shh* in the anterior limb is sufficient to ectopically induce *Fgf4* in the anterior AER (Laufer et al., 1994; Niswander et al., 1994), and mice lacking *Shh* lose *Fgf4*, *Fgf9*, and *Fgf17* (Sun et al., 2000; Zuniga et al., 1999), demonstrating a direct requirement of *Shh* to maintain these Fgfs in the overlying AER. Likewise, Fgfs from the AER are required to maintain the expression of *Shh* (Laufer et al., 1994; Niswander et al., 1994). This interdependence of the ZPA and AER is critical in regulating limb outgrowth.

The bone morphogenetic protein (Bmp) antagonist *Gremlin* is an important intermediate in the signaling loop between Shh and Fgf. *Gremlin* is expressed in a domain anterior to the ZPA (Ganan et al., 1996) and is thought to block Bmps from

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doi:10.1016/j.ydbio.2006.05.026

downregulating Fgfs in the overlying AER (Capdevila et al., 1999; Pizette and Niswander, 1999; Zuniga et al., 1999). *Gremlin* activity is also required for the integrity of the AER itself and is thereby necessary for Fgf8 expression, although Fgf8 is not regulated by Gremlin at the transcriptional level (Khokha et al., 2003). *Shh* is both necessary and sufficient for *Gremlin* expression (Zuniga et al., 1999), and *Gremlin* in turn is both necessary and sufficient for expression of *Fgf4* in the AER (Khokha et al., 2003; Zuniga et al., 1999). While BMP antagonism by several disparate factors such as Noggin can mimic the ability of Gremlin to upregulate *Fgf4* in the AER, only Gremlin has been shown to have a physiologic role in early limb outgrowth; *Gremlin* mutant mice have distal skeletal defects resulting from a disrupted Shh–Fgf4 loop (Brunet et al., 1998; Khokha et al., 2003). Thus, the current model has Shh inducing the expression of *Gremlin* which, in turn, activates *Fgf* expression in the adjacent AER by antagonizing BMP activity.

While *Gremlin* expression is lost in *Shh* null mice and anterior misexpression of Shh induces ectopic *Gremlin* expression (Zuniga et al., 1999), it is currently not clear whether Shh acts directly or indirectly to initiate and maintain *Gremlin*. It has been previously hypothesized that members of the Bmp family regulate their own activity by induction of *Gremlin* (Capdevila et al., 1999). Evidence for this hypothesis comes from the observation that implantation of a bead soaked in Bmp7 is sufficient to upregulate *Gremlin* (Merino et al., 1999); moreover, retroviral infection of *Noggin*, another Bmp antagonist, in presumptive limb mesenchyme in an HH stage 10 embryo entirely abolishes *Gremlin* expression in the limb at HH stage 22–23 (Capdevila et al., 1999). However, as the retroviral infection is very broad and is applied very early, it is unclear whether the loss of *Gremlin* is a direct or indirect consequence. Moreover, if Bmp activity is indeed necessary for *Gremlin* expression, it is not clear if Shh and Bmps are both required in concert for *Gremlin* expression or if Bmps act as a secondary signal downstream of Shh to regulate *Gremlin*.

Another aspect of *Gremlin* regulation that remains unclear is the basis for the exclusion of *Gremlin* expression from the posterior-most limb. While Shh and Bmps have been implicated in the induction and/or maintenance of *Gremlin*, *Gremlin* is not expressed in the posterior limb where expression levels of *Shh*, *Bmp2*, and *Bmp7* are highest. Because the region of cells excluding *Gremlin* expands over time to distance the domain of *Shh* expression from the domain of *Gremlin* expression, this refractoriness plays an important role in the eventual breakdown of the Shh–Fgf4 signaling loop and therefore regulates limb outgrowth (Scherz et al., 2004). By tracing the descendants of cells expressing *Shh*, it has been demonstrated that former *Shh*-expressing cells cannot express *Gremlin* (Scherz et al., 2004). This refractoriness appears to be cell-autonomous. However, it is not clear if establishment of the block in *Gremlin* expression in ZPA cells depends upon Shh activity.

In this study, we find that Bmp activity is necessary and sufficient for induction of *Gremlin*. Moreover, in the context of the *oligozeugodactyly* (*ozd*) mutant limb in which Shh activity is absent, Bmp2 is sufficient to induce *Gremlin*. Using beads containing varying concentrations of Bmp2 in vivo or culturing limb mesenchyme with varying concentrations of Bmp2 in vitro, we find evidence supporting the idea that Bmp activity regulates *Gremlin* in a concentration-dependent fashion. These data implicate Bmp activity as a required intermediate in the Shh–Fgf4 signaling loop. We further demonstrate that the Shh–Fgf4 signaling loop breaks down when Bmp activity can no longer upregulate *Gremlin* in the posterior. In the posterior of *ozd* mutant limbs, *Gremlin* can be induced by Bmp2, suggesting that refractoriness to *Gremlin* induction is dependent on Shh activity. By blocking Shh activity using cyclopamine, we provide evidence that Shh activity is also required to maintain refractoriness to express *Gremlin* in response to BMP signaling.

Results

Previous studies have demonstrated that Shh signaling is both necessary and sufficient for *Gremlin* induction (Capdevila et al., 1999; Zuniga et al., 1999). To test if Shh acts directly or indirectly to upregulate *Gremlin*, we applied a bead soaked in Shh protein to the anterior of an HH stage 21 limb bud in the presence of cycloheximide, an inhibitor of de novo protein synthesis. Whereas Shh normally results in robust upregulation of *Gremlin* (n = 5/5) (Fig. 1A), Shh fails to upregulate *Gremlin* in the presence of cycloheximide (n = 10/10) (Fig. 1B), suggesting that Shh acts indirectly, either by a secondary secreted signal or secondary intracellular signal.

If Shh regulates *Gremlin* via a secondary secreted signal, good candidates for that signal include Bmp2 and Bmp7, established downstream targets of Shh (Laufer et al., 1994; Chiang et al., 2001). Expression of *Gremlin* appears adjacent to mesenchymal expression of *Bmp2* and *Bmp7* in the HH stage 22 limb (Figs. 1C, D, F). Though it is not believed to be regulated by *Shh*, *Bmp4* is also expressed in limb mesenchyme at this stage (Fig. 1E). To test if Bmp activity is necessary for *Gremlin* expression in a more controlled way than the previously published retroviral misexpression studies (Capdevila et al., 1999), we applied a bead soaked in Noggin protein in HH stage 22 chick limbs. Consistent with the retroviral results, we observed downregulation of *Gremlin* immediately around the bead within 6 h (n = 10/10) (Fig. 1G). These results reinforce the conclusion that *Gremlin* expression is directly dependent on Bmp activity.

In the Shh null mouse, *Gremlin* expression is initially present but disappears by E10.25, indicating that Shh activity is necessary to maintain *Gremlin* (Zuniga et al., 1999). Therefore, Shh and Bmp activity may both be independently required to maintain *Gremlin* in the posterior. Alternatively, the requirement of Shh activity in *Gremlin* maintenance may be solely to induce Bmp activity. To distinguish these possibilities, we tested whether Bmp activity is sufficient to induce *Gremlin* in the anterior limb mesenchyme far from Shh activity. Indeed, a Bmp2-soaked bead applied to the anterior is sufficient to induce *Gremlin* (Figs. 2A, B). At a lower concentration (0.1 mg/ml), Bmp2

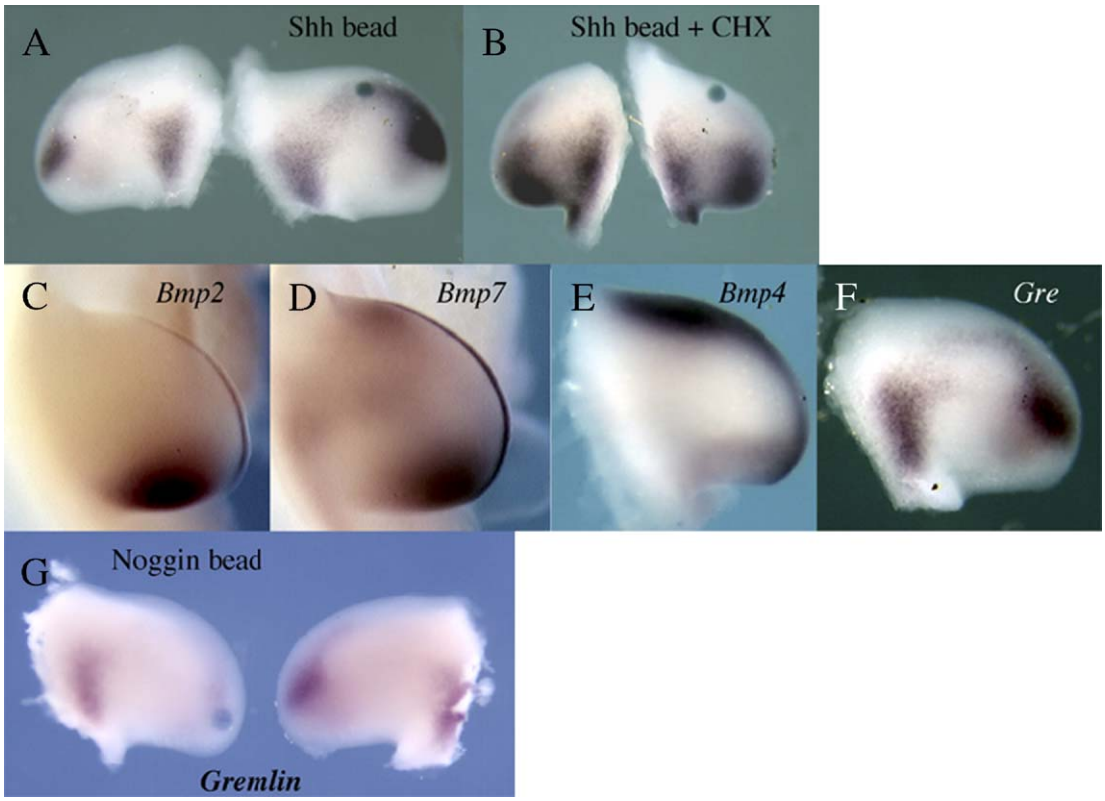

Fig. 1. *Gremlin* expression is not directly induced by Shh and requires Bmp activity. (A, B) Whole-mount *in situ* hybridization for *Gremlin* at HH 22–23 following implantation of a bead soaked in Shh. Compared to control (A), Shh cannot induce *Gremlin* in the presence of cycloheximide (B). (C–F) Relative expression of *Bmp2* (C), *Bmp7* (D), *Bmp4* (E), and *Gremlin* (F) at HH stage 22. (G) Downregulation of *Gremlin* after 6 h around a bead soaked in Noggin.

induces *Gremlin* immediately around the bead (n = 12/12) (Fig. 2A). However, in contrast to the effect of an Shh bead (Fig. 1A), high concentration of Bmp2 (1.0 mg/ml) induces a significant ectopic domain of *Gremlin* at a distance from the bead and appears to downregulate endogenous expression of *Gremlin* immediately around the bead (n = 12/12) (Fig. 2B).

To further corroborate that Bmp activity is sufficient to induce *Gremlin* independently of Shh, we examined the effect of Bmp in the context of the *ozd* chick mutant in which *Shh* is not expressed in the limbs (Maas and Fallon, 2004; Ros et al., 2003). A Bmp2-soaked bead applied to *ozd* limbs is sufficient to induce *Gremlin*, though at levels less than in wild-type limbs (n = 4/4) (Fig. 2C). This result rules out the possibility that Shh and Bmp activities are both required, together, to induce *Gremlin* and lends strong support for the hypothesis that *Bmp2* acts as a secondary signal downstream of *Shh* to induce *Gremlin*. Finally, we tested whether Bmp activity could upregulate *Gremlin* directly or indirectly. In contrast to an Shh-soaked bead, a Bmp2-soaked bead was sufficient to upregulate *Gremlin* in the presence of cycloheximide (n = 12/15) (Fig. 2D), while in control limbs cycloheximide by itself did not induce ectopic *Gremlin* expression (data not shown). These results indicate that Bmp activity does not require a secondary signal to regulate *Gremlin*. We noted that induction of *Gremlin* by Bmp2-soaked beads in cycloheximide-treated limbs appears to be less than in wild-type limbs, and this may indicate that there are, additionally, other factors that are also required for *Gremlin* induction.

Gremlin induction is sensitive to Bmp levels

In our experiments studying the ability of beads soaked in Bmp2 to induce *Gremlin*, we repeatedly observed that higher concentrations of Bmp2 appeared to downregulate endogenous expression of *Gremlin* immediately around the bead, in contrast to lower concentrations of Bmp2. This was true whether the Bmp2 bead was applied to the anterior (Figs. 2A, B) or to the posterior (Figs. 6A, B). The “halo” of cells not expressing *Gremlin* around the Bmp2 bead suggested that *Gremlin* may be downregulated by high levels of Bmp activity. Bmp activity is important in mediating cell death in the interdigital mesenchyme of the later limb (Ganan et al., 1996; Zou and Niswander, 1996). Therefore, to rule out the possibility that the “halo” of no *Gremlin* expression around a bead of Bmp2 ensues from apoptosis, we examined cell death by TUNEL analysis. We observed no increase in cell death around a bead soaked in 1.0 mg/ml Bmp2, though cell death was observed in the normal “posterior necrotic zone” and in the AER as expected (n = 5/5) (Fig. 3A). Moreover, the same high level of Bmp activity induced *Msx2*, a downstream target of Bmp, immediately around the bead (n = 5/5) (Fig. 3B). Put together, these observations suggest that, while Bmp

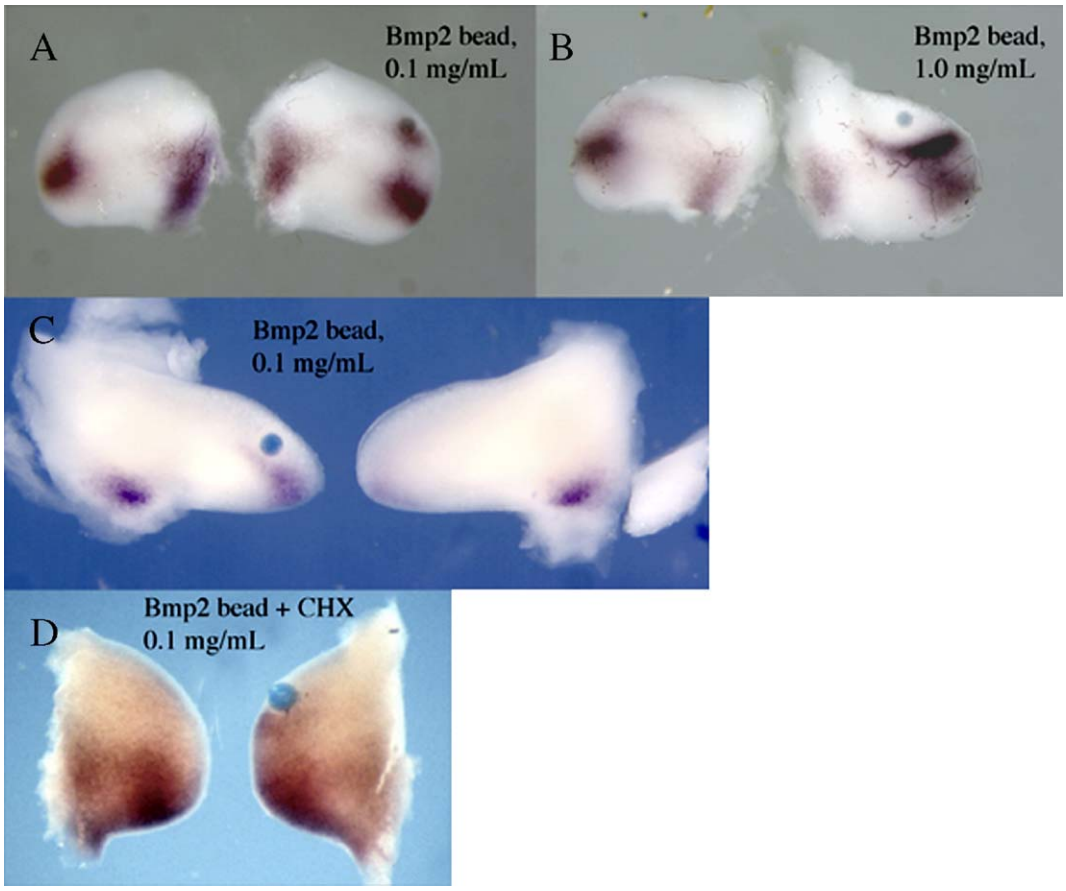

Fig. 2. Bmp2 is sufficient to activate *Gremlin* independently of Shh. (A, B) Upregulation of *Gremlin* in the anterior limb in response to a bead soaked in 0.1 mg/ml (A) or 1.0 mg/ml Bmp2. Note that *Gremlin* is induced at a greater distance from the bead with the higher concentration of Bmp2. (C) Upregulation of *Gremlin* in an *ozd* mutant limb. (D) Upregulation of *Gremlin* in response to a bead soaked in 0.1 mg/ml Bmp2 and applied to limbs treated with cycloheximide.

activity is necessary for *Gremlin* expression, high levels of Bmp activity downregulate *Gremlin*.

To further examine the possibility that *Gremlin* activation is sensitive to Bmp levels, we tested the ability of various concentrations of Bmp2 to induce *Gremlin* expression in limb mesenchyme cultures. Cultures were generated by dissociation of HH stage 22 anterior limb mesenchyme where *Gremlin* is absent or expressed at low levels (for example, see Fig. 1F). Cells were incubated with medium containing various concentrations of Bmp2, and *Gremlin* expression was assessed after 10 h. The intensity of *Gremlin* upregulation in response to Bmp2 varied with concentration of Bmp2 (Fig. 4A). Strongest upregulation of *Gremlin* occurred in response to Bmp2 concentrations of 100–150 ng/ml. In contrast, weak or no upregulation of *Gremlin* occurred in response to higher concentrations of Bmp2. The concentration of Bmp2 that resulted in strongest induction of *Gremlin* was much lower than concentrations of Bmp2 used to soak beads for implantation. To verify that this difference was not an artifact of the in vitro culture system, we added beads soaked in 0.1 mg/ml, the same concentration used in vivo, to the limb mesenchyme cultures and observed strong induction of *Gremlin* in cells around the bead (n = 4/4) (Fig. 4B); in contrast, beads soaked in 100 ng/ml did not upregulate *Gremlin* (n = 4/4) (Fig. 4C). This confirms that the effective concentration of Bmp2 released from the bead is orders of magnitude lower than the concentration of Bmp2 in which the bead was soaked. These in vitro data show that *Gremlin* induction is sensitive to levels of Bmp activity and support the in vivo observations that high levels of Bmp activity downregulate *Gremlin*.

We have argued that Bmp proteins are required secondary signals downstream of Shh in the induction of *Gremlin* expression. The activities of the ZPA and AER signaling centers in the limb are coordinated by a signaling loop between *Shh*, *Gremlin*, and AER *Fgfs*. In this loop, *Shh* is required to maintain *Gremlin*, which is required to maintain *Fgfs* in the AER, which in turn are required to maintain *Shh* (Laufer et al., 1994; Niswander et al., 1994; Zuniga et al., 1999). In demonstrating that Bmp activity is necessary and sufficient for *Gremlin* induction, these data therefore place *Bmp2* as an intermediate in this signaling loop, as previously hypothesized (Capdevila et al., 1999).

Breakdown of the Shh–Fgf4 feedback loop

Having implicated *Bmp2* as an intermediate in the *Shh–Fgf4* signaling loop, we sought to understand where and why this signaling loop breaks down. Termination of this loop by HH stage 28 is critical for proper patterning of the

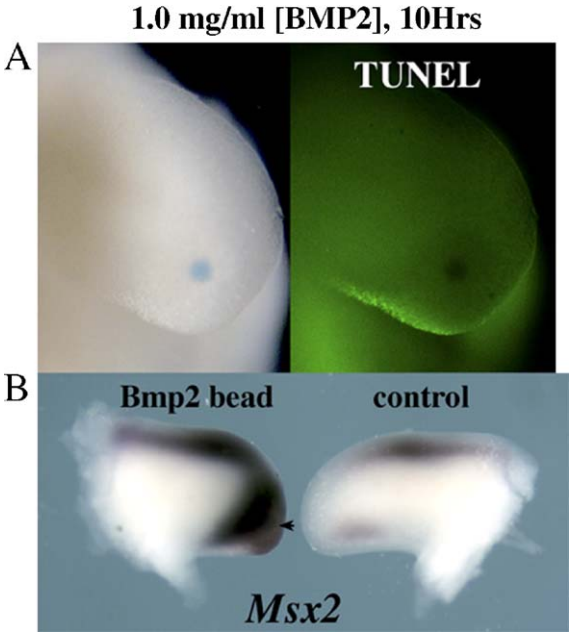

Fig. 3. Downregulation of *Gremlin* around a bead of high (1.0 mg/ml) Bmp2 is not due to cell death. (A) Bright-field and whole-mount TUNEL labeling shows cell death in a "posterior necrotic zone" and in the AER, but not around the Bmp2 bead where we have shown *Gremlin* is repressed (Fig. 2). (B) *Msx2* is induced immediately around a bead (arrow) of high Bmp2.

limb since prolonging it by ectopically introducing *Shh*, *Gremlin*, or *Fgf* results in longer growth than normal (Sanz-Ezquerro and Tickle, 2003; Scherz et al., 2004). Recently, it was demonstrated that the termination of this loop results from the failure of Shh to induce *Gremlin* in the posterior limb (Scherz et al., 2004). We therefore examined whether this termination results from an inability of Shh to induce *Bmp2* or an inability of Bmp2 to induce *Gremlin* in the posterior.

We applied beads soaked in Shh or Bmp2 protein at HH stage 25 and examined whether *Bmp2* or *Gremlin* was ectopically expressed at HH stage 28 when *Gremlin* disappears from the posterior. As previously shown, a bead soaked in Shh and applied to the posterior is unable to maintain *Gremlin*, but it can ectopically upregulate *Gremlin* if applied to the anterior (n = 8/8 anterior; n = 8/8 posterior) (Figs. 5A, B; Scherz et al., 2004). In contrast, an Shh bead is able to upregulate *Bmp2* in the posterior as well as in the anterior (n = 8/8 anterior; n = 8/8 posterior) (Figs. 5C, D). This indicates that inability to induce *Bmp2* downstream of *Shh* cannot explain the loss of *Gremlin* in the posterior. Indeed, *Bmp2* is itself endogenously expressed in the posterior at stage 28 when *Gremlin* expression disappears, though *Shh* is no longer present (Figs. 5C, D). To examine responsiveness to Bmp2, a bead soaked in Bmp2 was placed in the anterior and posterior HH stage 25 forelimb. Whereas the Bmp2 bead in the anterior induces robust upregulation of *Gremlin*, a Bmp2 bead in the posterior is unable to maintain *Gremlin* (n = 8/8 anterior; n = 10/10 posterior) (Figs. 5E, F). This indicates that cells in the posterior are refractory to *Gremlin* induction by Bmp2 and suggests that failure of Bmp activity to induce *Gremlin* in the posterior is the mechanism by which the termination of the Shh–Fgf4 signaling loop is initiated.

We noted that the pattern of *Gremlin* upregulation in the anterior differs in response to a bead soaked in Shh compared to a bead soaked in Bmp2 (Fig. 5A vs. E). The Shh bead is only able to induce *Gremlin* subapically, whereas the Bmp2 bead can induce *Gremlin* away from the AER. A subapical pattern of *Gremlin* induction was also noted when an Shh bead was applied to an earlier HH stage 21 limb, and this pattern differs dramatically from that in response to a Bmp2 bead (compare Fig. 1A vs. Figs. 2A, B). This observation is consistent with the previous demonstration that Shh requires cooperation with Fgfs from the AER to induce *Bmp2* (Marigo et al., 1996). Therefore, Shh can only induce *Bmp2* subapically. Because the pattern of *Gremlin* upregulation in response to Shh is defined by the pattern of *Bmp2* upregulation, *Gremlin* upregulation in response to Shh also occurs only subapically (compare Figs. 5A, C vs. E).

Refractoriness is dependent on activity of Shh

The refractoriness of the posterior limb to *Gremlin* expression is critical for terminating the Shh–Fgf4 signaling loop (Scherz et al., 2004). However, *Gremlin* expression is excluded from the posterior of earlier limbs as well (Fig. 1F), before this zone of refractoriness expands and thereby terminates the Shh–Fgf4 loop. To examine whether the posterior of earlier limbs is refractory to *Gremlin* upregulation in response to Bmp activity, we applied beads soaked in Bmp2 to a location slightly anterior to the ZPA in HH stage 23 limbs. Indeed, the Bmp2 beads upregulated *Gremlin* only on the anterior side of the bead but never in the most posterior region of the limb (n = 8/8 low [Bmp2], n = 8/8 high [Bmp2]) (Figs. 6A, B).

Former *Shh*-expressing cells exclude *Gremlin* expression in a cell-autonomous manner (Scherz et al., 2004). This cell-autonomous exclusion of *Gremlin* can be explained if refractoriness to *Gremlin* is regulated by the same upstream factors as *Shh* expression. Alternatively, highest levels of Shh activity, such as that only seen in cells which express *Shh*, may cause cells to become refractory to *Gremlin* induction. An excellent context in which to distinguish these models is the chick *ozd* mutant limb. In *ozd* mutants, factors acting upstream to regulate *Shh* expression in the ZPA are intact, but a defect mapping to a *cis*-regulatory element that controls limb-specific expression of *Shh* renders *ozd* limbs unable to express *Shh* (Maas and Fallon, 2004; Ros et al., 2003). Therefore, an observation that *Gremlin* can be induced in the posterior of *ozd* limbs would support the model that refractoriness to *Gremlin* requires Shh activity. In contrast, an observation that *Gremlin* cannot be induced in the posterior of *ozd* limbs would indicate that exclusion of *Gremlin* may be regulated in the same way as expression of *Shh* but is independent of Shh activity.

We applied beads soaked in Bmp2 to a location slightly anterior to the ZPA in HH stage 23 limbs, a stage when *ozd*

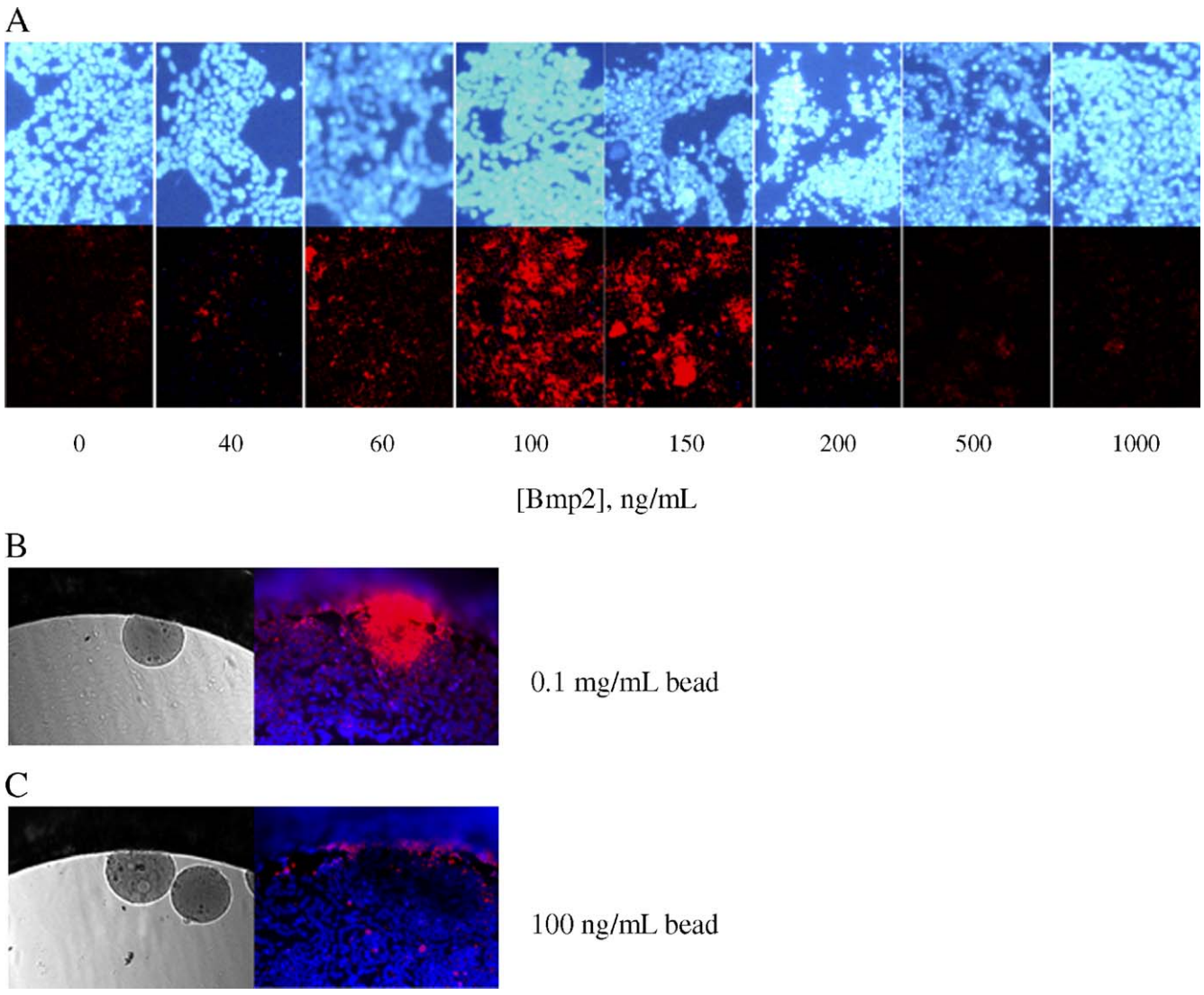

Fig. 4. *Gremlin* induction is sensitive to concentration of Bmp2. (A) DAPI and *Gremlin in situ* hybridization of cultures incubated with Bmp2 at 0–1000 ng/ml. (B, C) Bright-field and DAPI overlay with *Gremlin in situ* hybridization of cultures incubated with beads soaked in Bmp2 at 0.1 mg/ml (B) or 100 ng/ml (C).

mutant limbs cannot be distinguished from wild-type limbs. Whereas the Bmp2 beads failed to upregulate *Gremlin* in the most posterior region of wild-type limbs (Figs. 6A, B), Bmp2 beads induced *Gremlin* in the posterior of *ozd* mutant limbs (Fig. 2C). This result suggests that the refractoriness of the posterior to *Gremlin* induction by Bmp2 is dependent on Shh activity.

We next tested whether Shh activity is necessary not only for the initiation but also the maintenance of refractoriness to *Gremlin* expression. To this end, we examined the ability of Bmp2 to induce *Gremlin* in the posterior after blocking the activity of Shh with cyclopamine. Cyclopamine is a small steroidal alkaloid that blocks the cellular response to Shh, and genes dependent upon Shh are downregulated as quickly as 4 h after cyclopamine is added to the limb (Incardona, 1998). Whereas the *ozd* mutant limbs never see Shh activity, wild-type limbs treated with cyclopamine see normal Shh activity until the drug is provided. As in control limbs, when cyclopamine was added an hour before applying a Bmp2 bead to an HH stage 23 limb bud, the Bmp2 bead was unable to induce *Gremlin* in the posterior limb after 10 h (n = 8/8 low [Bmp2], n = 8/8 high [Bmp2]) (Figs. 6C, D). This indicates that the initial Shh activity prior to the addition of cyclopamine is sufficient to establish refractoriness to *Gremlin* induction. However, when cyclopamine was added 24 h earlier at HH stages 20–21 followed by a Bmp2 bead applied at HH stage 23 for 10 h, the Bmp2 bead induced *Gremlin* all around the bead, including in the posterior limb (n = 8/8 low [Bmp2], n = 8/8 high [Bmp2]) (Figs. 6E, F). Importantly, when cyclopamine was added 1 h before a Bmp2 bead was applied at HH stages 20–21, the Bmp2 bead was unable to induce *Gremlin* in the posterior limb (n = 8/8) (Fig. 6G), demonstrating that the posterior of the HH stages 20–21 limb is indeed refractory to *Gremlin* expression at the time cyclopamine is added and this refractoriness is lost if Shh activity is blocked for 34 h (Figs. 6E, F). These results suggest that Shh activity is required not only to establish refractoriness to *Gremlin* expression in *Shh*-expressing cells

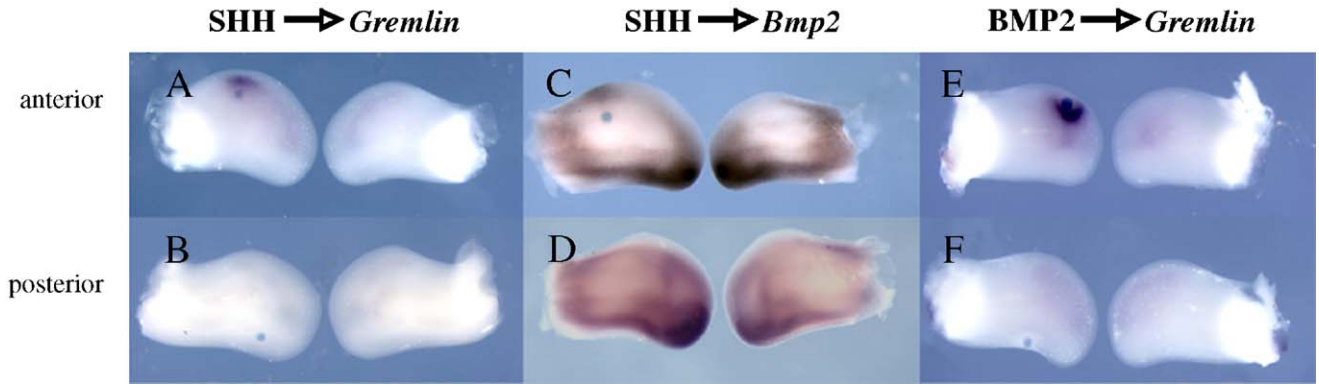

Fig. 5. The Shh–Fgf4 signaling loop terminates in failure of Bmp activity to induce *Gremlin*. The ability of Shh to induce *Gremlin* (A, B) of Shh to induce *Bmp2* (C, D) and of Bmp2 to induce *Gremlin* (E, F) was tested by applying beads of either Shh or Bmp2 to HH stage 25 limbs and assessing *Bmp2* or *Gremlin* expression at HH stage 28. In the anterior (A, C, E), Shh is able to induce *Gremlin* (A) and *Bmp2* (C), and Bmp2 is able to induce *Gremlin* (E). In the posterior (B, D, F), Shh is unable to induce *Gremlin* (B) but can upregulate *Bmp2* (D), and Bmp2 is unable to induce *Gremlin* (F).

but also to maintain refractoriness in *Shh*-expressing cells and their descendants.

Discussion

Bmp activity acts downstream of Shh to upregulate Gremlin

In this study, we show that Bmp activity is both necessary and sufficient for *Gremlin* upregulation. The ability of Bmp2 to induce *Gremlin* in the *ozd* mutant limb rules out the possibility that Shh activity is also independently required for *Gremlin* induction. Instead, the requirement of Shh activity in *Gremlin* expression appears to be solely to induce Bmp activity as a secondary signal that in turn regulates *Gremlin*. In the *Shh* null mouse, *Gremlin* expression is initiated but not maintained in the posterior limb (Zuniga et al., 1999). Consistent with our conclusion, *Bmp2* is also initiated in the posterior of *Shh* mutant limbs, though at reduced levels (Chiang et al., 2001). Similarly, the *ozd* chick mutant lacks Shh activity in the limbs but has a low level of *Gremlin* expression in the posterior (Fig. 2C) that corresponds with a reduced domain of *Bmp2* in the posterior (Ros et al., 2003).

Which Bmps comprise the Bmp activity that regulates *Gremlin* in the posterior limb? Good candidates for this secondary signal are *Bmp2* and *Bmp7*, both established downstream targets of Shh. *Bmp4*, which is expressed in the anterior and posterior limb, may also contribute to the regulation of *Gremlin*, though *Bmp4* does not appear to be regulated by Shh. All three Bmps present at this stage of limb development can be antagonized by Noggin, so our data do not identify which Bmps are necessary for *Gremlin* expression.

Loss-of-function studies in mouse have demonstrated the negative influence of Bmp activity on AER maintenance. Mice lacking *Bmp7* have hindlimb polydactyly associated with an expanded AER (Dudley et al., 1995; Hofmann et al., 1996; Luo et al., 1995). Similarly, conditional inactivation of *Bmp4* in the limb mesoderm results in hindlimb polydactyly associated with an enlarged AER (Selever et al., 2004). In the current study, we demonstrate that Bmp activity is required as an intermediate to induce *Gremlin* in the Shh–Fgf4 signaling loop. If this is true, why is the Shh–Fgf4 signaling loop intact and even prolonged in the Bmp mutants? These seemingly contradictory findings in fact support the model that the negative influence of Bmp activity on AER maintenance is self-regulated by induction of *Gremlin*. According to this model, any reduction in *Gremlin* activation in a Bmp mutant may be offset by a diminished negative influence of Bmp activity on AER maintenance. Hence, the Shh–Fgf4 signaling loop remains intact in Bmp mutants. In contrast, loss of *Gremlin* will have dire effects because the negative influence of Bmp activity on AER maintenance goes unmitigated. Indeed, in *Gremlin* mutants, the AER lacks *Fgf4* expression and is reduced or disorganized; as a result, the Shh–Fgf4 feedback loop is interrupted and severe skeletal deficiencies ensue (Khokha et al., 2003). Whether *Gremlin* is required to antagonize Bmp activity in the mesoderm, AER, or both remains to be elucidated.

Our data also provide evidence that Bmp activity acts in a concentration-dependent manner to regulate *Gremlin* in the limb. Both in vivo and in vitro data suggest that Bmp activity is necessary for *Gremlin* expression, but high levels of Bmp activity downregulate *Gremlin*. In other contexts, a gradient of Bmp activity has been implicated in the induction of different cell fates in a concentration-dependent manner. For example, progressively higher levels of Bmp activity are thought to specify the neural plate, neural crest cells, and ectodermal placodes at specific threshold concentrations during *Xenopus* neurulation (Marchant et al., 1998; Wilson et al., 1997). The notion that Bmps can behave as morphogens in vertebrate patterning accords well with our understanding of Dpp action in *Drosophila*. Dpp has been demonstrated to activate different genes in a concentration-dependent manner in the *Drosophila* wing disc (Nellen et al., 1996), and a Dpp gradient is also thought to induce distinct cell types during early dorsal–ventral patterning in the fly

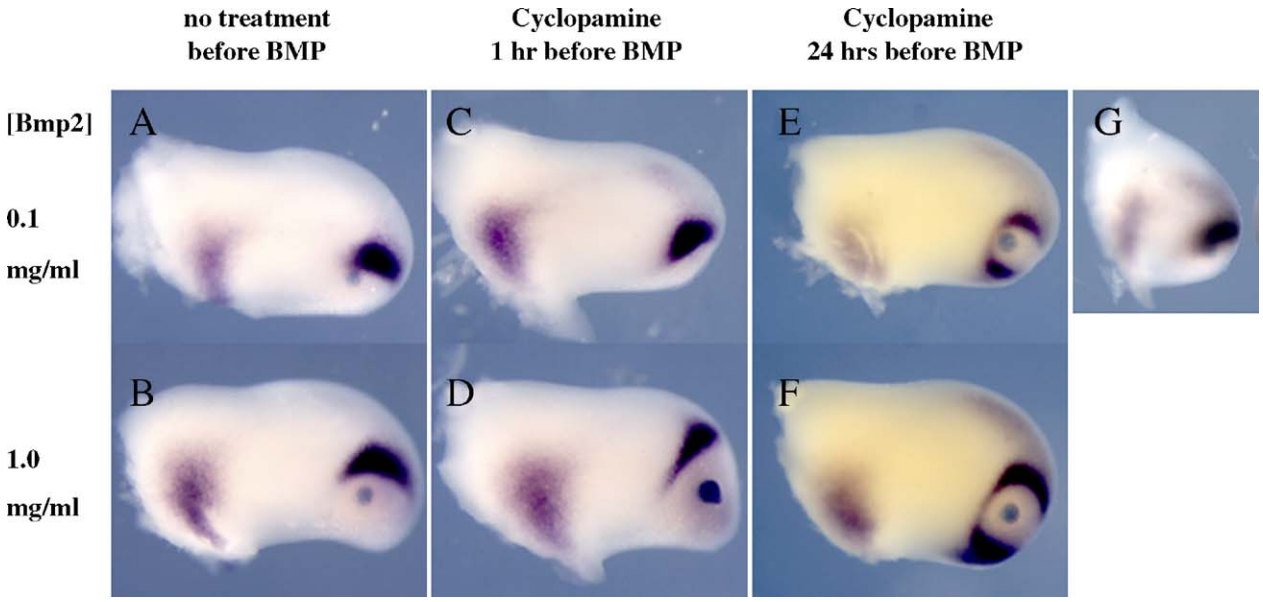

Fig. 6. Induction of *Gremlin* in the posterior limb in response to Bmp2 at 0.1 mg/ml (A, C, E, G) or 1.0 mg/ml (B, D, F). (A, B) Bmp2 fails to induce *Gremlin* in the posterior of an HH stage 24 limb. (C, D) The posterior remains refractory to *Gremlin* induction if Shh activity is blocked an hour before the Bmp2 bead is applied. (E, F) *Gremlin* can be induced in the posterior after cyclopamine is added at HH stages 20–21, 24 h before a Bmp2 bead is applied. (G) The posterior of an HH stage 21 limb is refractory to *Gremlin* induction in response to Bmp2.

(Ferguson and Anderson, 1992; Wharton et al., 1993). Our observation that high levels of Bmp2 downregulate *Gremlin* but strongly upregulate *Msx2* suggest that, in the developing limb as well, different genes may have different thresholds for activation and/or repression in response to a Bmp activity gradient.

The fact that, in particular, a BMP antagonist, *Gremlin*, is induced only within an intermediate concentration range of BMP protein may have a profound effect on the distribution of BMP signaling activity within the limb bud. For example, far from the source of BMP synthesis, BMP activity will be low. Scanning through the limb mesenchyme towards the source of BMPs, the level of signaling will start to gradually rise with increasing concentration, until the threshold for *Gremlin* induction is reached. At that point, moving closer to the source of BMPs will continue to result in higher BMP protein exposure, but this will be partially countermanded by BMP antagonist activity, flattening the rate of increase in BMP signaling. Higher concentrations of BMP protein will be met by higher levels of induced *Gremlin*, until the threshold of BMP signaling required for *Gremlin* repression is reached. At that point, BMP signaling will again begin to rise steeply in relation to BMP concentration. Thus, a curve tracking BMP signaling across the limb bud will be transformed from an exponential to a sigmoidal shape.

The concentration-dependent effect of BMP signaling may also, in part, explain why *Gremlin* expression does not completely correspond spatially with expression of *Bmp* genes in the limb. For example, *Gremlin* expression does not overlap expression of *Bmp4* and *Bmp7* in the anterior, and this may reflect downregulation by high levels of Bmp activity (Figs. 1D–F). However, as discussed below, it is likely that other mechanisms explain the exclusion of *Gremlin* from the posterior, where *Bmp2*, *Bmp4*, and *Bmp7* are all expressed.

Refractoriness to Gremlin expression requires Shh activity

It was previously shown that descendants of *Shh*-expressing cells in the mouse limb are refractory to *Gremlin* upregulation in response to Shh (Scherz et al., 2004). Having demonstrated that Shh acts via Bmps to induce *Gremlin*, we show that Bmp2 is unable to induce *Gremlin* in the posterior chick limb. This posterior refractory region may represent descendants of *Shh*-expressing cells in the chick limb. As in the mouse limb, this zone of *Gremlin* exclusion expands over time, and this likely reflects expansion of the *Shh* descendant population. We show that Shh is able to upregulate *Bmp2* in the posterior but that Bmp2 is unable to induce *Gremlin* in the posterior. These data suggest that termination of the Shh–Fgf4 signaling loop occurs as a result of failure of Bmp activity to upregulate *Gremlin* in the posterior, and as a result, the negative influence of Bmp activity on the AER goes unmitigated.

What is the mechanism by which *Shh* descendants become refractory to Bmp upregulation of *Gremlin*? In the *Drosophila* wing, it has been demonstrated that HH expression can non-autonomously attenuate responsiveness to Dpp by downregulating the Dpp receptor *thick veins* (*tkv*) (Tanimoto et al., 2000). However, our data suggest that the refractoriness in *Shh* descendants is not at the level of Bmp responsiveness since Bmp2 can induce *Msx2* in the posterior where *Gremlin* is excluded (Fig. 3B). Thus, the refractoriness in *Shh* descendants appears specific to *Gremlin* expression.

Our data also provide evidence that *Gremlin* upregulation is sensitive to levels of Bmp activity. Both in vivo and in vitro, we demonstrate that high levels of Bmp2 down-regulate *Gremlin* within 10 h, and this effect is not due to cell death (Fig. 3). This raises the possibility that exclusion of *Gremlin* in the posterior results from high levels of Bmp activity. However, refractoriness to *Gremlin* expression is a cell-autonomous property of *Shh* descendants, and *Gremlin* expression can be detected in cells immediately adjacent to *Shh* descendants (Scherz et al., 2004). If exclusion of *Gremlin* expression in the posterior was due to high levels of Bmp activity, it seems unlikely that it would be limited to *Shh* descendants but not adjacent cells. Moreover, in tissue grafting experiments, anterior grafts expressing *Gremlin* continue to express *Gremlin* when placed in the posterior, even though these grafts would presumably be exposed to high levels of Bmp in the posterior (Scherz et al., 2004). Thus, it seems unlikely that exclusion of *Gremlin* in the posterior results from high levels of Bmp activity.

The cell-autonomous manner in which *Gremlin* is excluded from *Shh* descendants can be explained if refractoriness is regulated in the same way as *Shh* expression or it is a consequence of high Shh activity in *Shh*-expressing cells. To distinguish these models, we examined *Gremlin* induction in the *ozd* mutant limb, in which a *cis*-acting mutation prevents *Shh* expression even though all upstream factors regulating *Shh* expression are intact. The ability of Bmp2 to induce *Gremlin* in the posterior *ozd* limb demonstrates that Shh activity is required to establish refractoriness. The *ozd* mutant limb closely resembles the limbs of *Shh* null mice, and *Shh* descendants (descendants of cells that attempt to express *Shh* and therefore have the factors that regulate *Shh* expression) make up approximately the posterior half of *Shh* null limbs at E12.5 (data not shown). Thus, while it is not currently possible to genetically label *Shh* descendants in the chick limb, it is likely that *Gremlin* induction in the posterior of *ozd* limbs is occurring in *Shh* descendants. Our data therefore suggest that Shh activity is required for *Gremlin* exclusion in *Shh* descendants. High levels of Shh activity in *Shh* descendants may be altering the state of chromatin or inducing transcription factors that prohibit the expression of *Gremlin*. Interestingly, *Gremlin* can be induced in the posterior after Shh activity is blocked with cyclopamine. This was observed when limbs were treated with cyclopamine for 24 h followed by a Bmp2 bead for 10 h. Thus, after 34 h in which Shh activity was blocked, the posterior was no longer refractory to *Gremlin* induction in response to Bmp2. These data lead us to speculate that Shh activity may be required not only to establish but also to maintain refractoriness to *Gremlin* expression. We propose that in the establishment phase the cells are rendered refractory to Gremlin expression only at very high levels of Shh, attained by autocrine signaling. Hence, only the former ZPA cells become refractory. In contrast, in the maintenance phase, lower levels of Shh suffice, such that the refracting property is maintained throughout the domain of former Shh-expressing cells, not just in the cells expressing Shh at later stages. The molecular mechanism for this refractoriness remains to be elucidated.

Materials and methods

Embryos

Experiments on wild-type chick embryos were performed on standard specific pathogen-free white Leghorn chick embryos provided by SPAFAS (Norwich, Connecticut). *Oligozeugodactyly* (*ozd*) mutant embryos were generously provided by the Fallon laboratory and were obtained from a heterozygous mating flock maintained at the University of Wisconsin (Madison, WI). Eggs were incubated, windowed, and staged as described previously (Hamburger and Hamilton, 1951).

Bead implants

Affi-Gel Blue beads (BioRad) were used to administer Shh, Bmp2, or Noggin protein to limbs. Beads were washed in PBS and incubated at room temperature for 1 h in human modified version of active N-terminal peptide of Shh (CURIS) at 1.0 mg/ml, Bmp2 (generous gift of Vicki Rosen) at 0.1 or 1.0 mg/ml, or recombinant human Noggin (PeproTech) at 1.0 mg/ml. Beads were implanted into a small slit made in the limb.

Cyclopamine and cycloheximide treatment

Cyclopamine (Toronto Research Chemicals) was added directly over the forelimb in ovo as previously described (Incardona et al., 1998). Briefly, 5 μl of 1.0 mg/ml cyclopamine in 45% HBC (Sigma) in PBS was added over the limb. Cycloheximide dissolved in DMSO was diluted to a concentration of 1.0 mg/ml in PBS and 100 μl was added directly over the forelimb in ovo.

In vitro studies

Limb mesenchyme from anterior third of HH stage 22 limbs was dissected and collected in PBS. Limb tissue was trypsinized for 15 min, 37°C, triturated, and suspended in DMEM + 1% fetal calf serum. Trituration was continued until suspension contained mostly single cells. Cells were plated in microwell minitrays (NUNC) at a density of 36,000 cells/well. After 2 h, cells were washed with PBS and Bmp2 in serum-free medium was added. Cell cultures were incubated for 6–10 h at 37°C and then fixed in 4% paraformaldehyde for 5 min. Cells were rinsed in PBT and then dehydrated and stored in methanol at −20°C.

Gremlin expression was visualized by in situ hybridization using fluorophore tyramide amplification (Perkin-Elmer). Briefly, cells stored in methanol were rehydrated into PBT. Acetylation was performed by incubating cells in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Cells were washed in PBT and then hybridized with DIG-labeled RNA probe for *Gremlin* overnight at 65°C. Cells were washed with 0.2× SSC twice for 20 min at 65°C and then rinsed in TNT (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20). Endogenous peroxidase activity was inactivated by incubating in 6% hydrogen peroxide in TNT for 30 min. Blocking was performed by incubating cells in TNB (1.0 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking buffer (Perkin-Elmer)) + 10% sheep serum for 30 min at room temperature. Cells were then incubated for 30 min at room temperature in secondary antibody (1:1000 anti-DIG-HRP, Roche) dissolved in TNB + 2% sheep serum. Cells were washed 3 times in TNT, and then fluorophore tyramide solution was added as described in kit (1:50 in amplification buffer + 0.0015% hydrogen peroxide). Fluorophore reaction was stopped after 15–20 min by rinsing cells in TNT and post-fixing in 4% paraformaldehyde in PBS. Cells were briefly incubated in DAPI nuclear stain before visualizing.

Whole-mount in situ hybridization and TUNEL

Whole-mount in situ hybridization was performed as previously described (Dietrich et al., 1997) with minor modifications. Briefly, embryos were fixed overnight in 4% paraformaldehyde, washed in PBS, dehydrated

into methanol, and bleached for 1 h in 6% hydrogen peroxide in methanol. Embryos were then rehydrated into PBT and permeabilized with 10 μg/ml proteinase K in PBT for 20–30 min depending on embryonic stage. Embryos were then washed in PBT, post-fixed in 4% paraformaldehyde/0.2% glutaraldehyde for 20 min, washed in PBT, and prehybridized for 1 h at 70°C in hybridization buffer (50% formamide, 5× SSC pH 4.5, 2% SDS, 2% blocking reagent (Roche), 250 μg/ml tRNA, 100 μg/ml heparin). The embryos were then hybridized overnight at 70°C in hybridization buffer with probes. After hybridization, the embryos were washed four times for 30 min each in 50% formamide/2× SSC pH 4.5/1% SDS and then washed in MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween 20). Embryos were blocked for 1 h in 2% blocking reagent/MABT followed by 1 h in 2% blocking reagent/20% heat inactivated goat serum/MABT and then incubated overnight at 4°C with secondary antibody (1:2500 anti-DIG AP, Roche). Embryos were then washed multiple times in MABT at room temperature and washed overnight at 4°C. Embryos were then equilibrated in NTM (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$), and color detection was performed with NBT/BCIP (Sigma). DIG-labeled probes were generated for *Gremlin* (Capdevila et al., 1999), *Shh* (Riddle et al., 1993), *Bmp2* (Francis et al., 1994), *Bmp4* (Francis et al., 1994), and *Bmp7* (received from L. Niswander).

To detect cell death in whole mount, an apoptosis detection kit for sectioned tissue (Roche) was modified for whole-mount embryos. In particular, embryos were permeabilized with proteinase K as for in situ hybridization and then equilibrated in TdT buffer (Roche). The fluorescein labeling TdT reaction was then performed in whole mount, and fluorescence was visualized to detect cell death.

Acknowledgments

We thank Paul Scherz for experimental assistance.

This work was supported by a grant, 2 R37 HD032443, from the NIH to CJT and NIH HD3255 to JFF.

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