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The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb

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SUMMARY

In this study, we have analyzed the expression and function of Gremlin in the developing avian limb. Gremlin is a member of the DAN family of BMP antagonists highly conserved through evolution able to bind and block BMP2, BMP4 and BMP7. At early stages of development, *gremlin* is expressed in the dorsal and ventral mesoderm in a pattern complementary to that of *bmp2*, *bmp4* and *bmp7*. The maintenance of *gremlin* expression at these stages is under the control of the AER, ZPA, and BMPs. Exogenous administration of recombinant Gremlin indicates that this protein is involved in the control of limb outgrowth. This function appears to be mediated by the neutralization of BMP function to maintain an active AER, to restrict the extension of the areas of programmed cell death and to confine chondrogenesis to the central core mesenchyme of the bud. At the stages of digit formation, *gremlin* is expressed in the proximal boundary of the interdigital mesoderm of the chick autopod. The anti-apoptotic influence of exogenous Gremlin, which results in the formation of soft tissue syndactyly in the chick, together with the expression of *gremlin* in the duck interdigital webs, indicates that Gremlin regulates the regression of the interdigital tissue. At later stages of limb development, *gremlin* is expressed in association with the differentiating skeletal pieces, muscles and the feather buds. The different expression of Gremlin in relation with other BMP antagonists present in the limb bud, such as Noggin, Chordin and Follistatin indicates that the functions of BMPs are regulated specifically by the different BMP antagonists, acting in a complementary fashion rather than being redundant signals.

Key words: Duck limb, Apoptosis, Syndactyly, Shh, FGFs

INTRODUCTION

Bone Morphogenetic Proteins (BMPs) constitute a large family of secreted growth factors belonging to the TGFβ superfamily. Although these proteins were first identified by their capacity to promote endochondral bone formation, they are now considered as components of an evolutionary conserved signalling pathway that is responsible for many developmental processes (Hogan, 1996). Like other members of the TGFβ superfamily, BMPs perform their biological function by interacting with cell surface receptors consisting of heterodimers of type I and type II receptors with intracellular serine/threonine kinase domains (Massagué, 1996). Ligand binding of BMPs to both of these receptors activates an intracellular pathway involving members of the Smad family.

Several members of the BMP family exhibit a regulated pattern of expression during embryonic limb development. Thus, *bmp2*, *bmp4* and *bmp7* are expressed in the undifferentiated limb mesoderm, apical ectodermal ridge (AER) and in the interdigital mesenchyme (Francis et al., 1994; Francis-West et al., 1995; Lyons et al., 1995; Laufer et al., 1997). Among the functions assigned to these BMPs are: control of mesodermal cell proliferation (Niswander and Martin, 1993), regulation of growth and regression of the AER (Gañan et al., 1998; Pizette and Niswander, 1999), chondrogenic differentiation (Duprez et al., 1996a; Macias et al., 1997; Merino et al., 1998; Enomoto-Iwamoto et al., 1998), control of muscle formation (Duprez et al., 1996b; Amthor et al., 1998), induction of apoptosis (Zou and Niswander, 1996; Gañan et al., 1996; Yokouchi et al., 1996; Kawakami et al., 1996; Macias et al., 1997; Zou et al., 1997b) and, possibly, regulation of the anteroposterior axis of the early limb bud (Duprez et al., 1996c, but see also Zou et al., 1997a). The molecular mechanisms accounting for this functional diversity are not totally understood. Some of the different functions of BMPs, e. g. the induction of cell death in the undifferentiated mesenchyme versus the promotion of growth and differentiation of prechondrogenic blastemas, might be

regulated by the type of BMP receptor expressed by the target cells (Kawakami et al., 1996; Merino et al., 1998). However, in other cases, the temporal and/or spatial distribution of BMP transcripts are not strictly correlated with their potential function. For example, while these BMPs are potent inducers of cell death, their domains of expression in the limb are considerably larger than the areas of apoptosis (Macias et al., 1997). Moreover, the interdigital expression of these *bmp* genes is similar in chick and duck leg buds despite differences between these species in their patterns of interdigital regression (Laufer et al., 1997). Similarly, BMPs are responsible for AER regression, but BMP are expressed by the AER cells throughout the whole period of limb morphogenesis (Pizette and Niswander, 1999). All these findings indicate that the activity of BMPs is fine-tuned by other factors.

Recently, a growing number of secreted proteins have been discovered that are antagonists of BMP function. These BMP antagonists share the functional property of binding specifically to BMPs, thus preventing their interaction with their receptors. The developmental function of these factors has been studied mainly during gastrulation (Smith and Harland, 1992; Piccolo et al., 1996, Zimmerman et al., 1996; Hsu et al., 1998). In the developing limb, a number of recent studies have analyzed the distribution and function of various BMP antagonists, such as Noggin (Brunet et al., 1998; Merino et al., 1998; Capdevila and Johnson 1998; Pizette and Niswander, 1999), DAN (Stanley et al., 1998; Pearce et al., 1999), Drm (Pearce et al., 1999), Chordin (Francis-West et al., 1999) and Follistatin (Merino et al., 1999). These studies suggest that the functions of BMPs are spatially and temporally regulated by different specific BMP antagonists. Thus, *noggin* is expressed in the chondrogenic condensations and appears to regulate the shape and size of the cartilaginous skeleton by controlling the effect of BMPs (Merino et al., 1998; Capdevila and Johnson, 1998; Brunet et al., 1998); *chordin* may regulate joint formation (Francis-West et al., 1999) while *follistatin* is involved in tendon and muscle differentiation (Merino et al., 1999). The possible participation of other BMP antagonists in limb development remains to be analyzed.

In this study, we have analyzed the expression and function of Gremlin in the developing avian limb. Gremlin is a member of the DAN family of BMP antagonists highly conserved through evolution (Hsu et al., 1998). The ability of Gremlin to bind and block BMP2, BMP4 and BMP7 activity has been demonstrated both in vivo and in vitro (Hsu et al., 1998 and A. N. E. unpublished data). Our findings show that *gremlin* is expressed in the developing limb under the control of the AER and ZPA in a pattern complementary to that of these BMPs. Local administra/tion of Gremlin protein provides evidence for a function of this BMP antagonist in the control of limb outgrowth, regulating the activity of the AER, delimiting the apoptotic areas and restricting chondrogenesis to the central core mesenchyme of the bud.

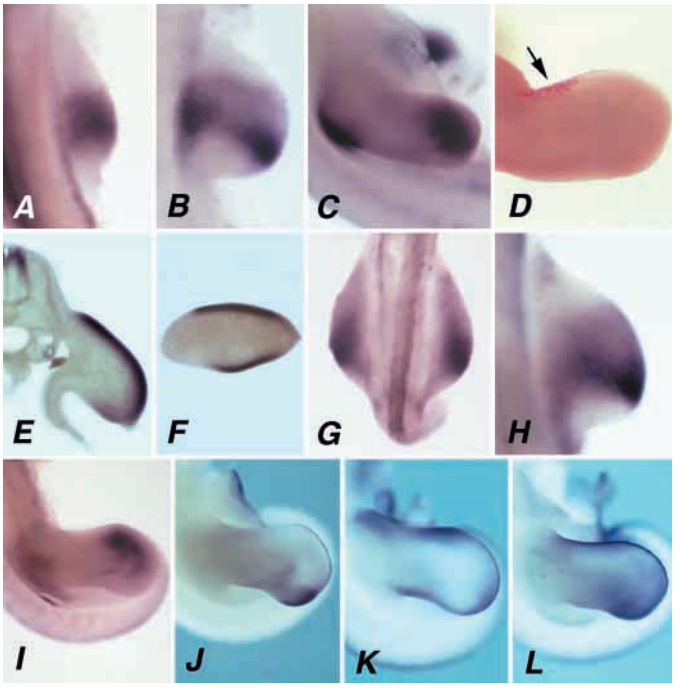

Fig. 1. Expression of *gremlin* in the limb bud at early stages of development. (A-C) *gremlin* expression in the wing bud at stages 20 (A), 23 (B) and 26 (C). (D) Wing bud at stage 26 showing the ANZ (arrow) after neutral red vital staining for cell death. (E,F) Transverse sections of stage 23 (E) and 26 (F) limb buds showing the distribution of *gremlin* transcripts in the dorsal and ventral mesenchyme of the bud. (G-I) Expression of *gremlin* in the leg bud at stages 20 (G), 22 (H) and 26 (I). (J-L) Expression of *bmp2* (J), *bmp4* (K) and *bmp7* (L) in the leg bud at stages 25-26 showing that *gremlin* and *bmp* genes are expressed in a complementary fashion.

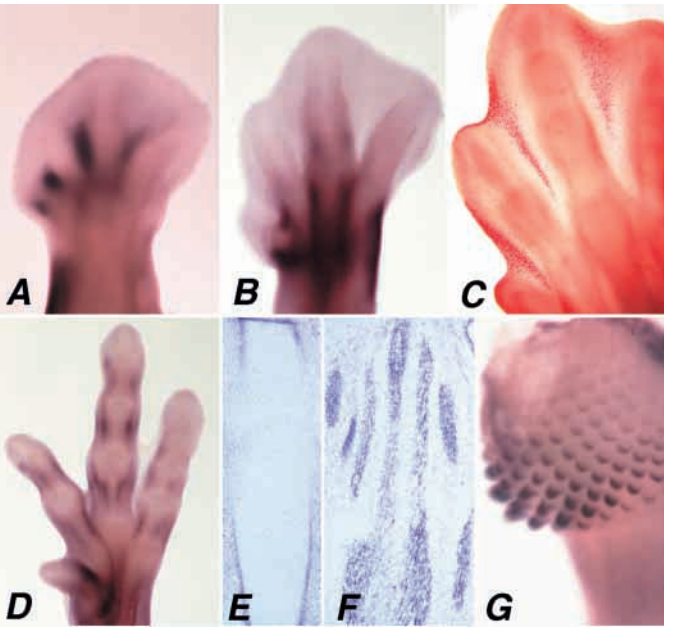

Fig. 2. Expression of *gremlin* during the formation of the digits in the chick leg bud. (A,B) Expression of *gremlin* in the chick limb at stages 28 (A) and 31 (B). Note that the interdigital domains observed at stage 28 disappear by stage 31 when interdigital cell death starts. (C) Illustration of the areas of interdigital cell death by vital staining with neutral red at stage 32. (D,E) Whole-mount (D) and tissue section (E) in situ hybridizations showing the expression of *gremlin* in the perichondrium of the phalanxes but not in the joint-forming regions of the digits at stage 35. (F,G) Expression of *gremlin* in the differentiating muscles (F) and in the developing feathers (G) of stage 33 and 35, respectively.

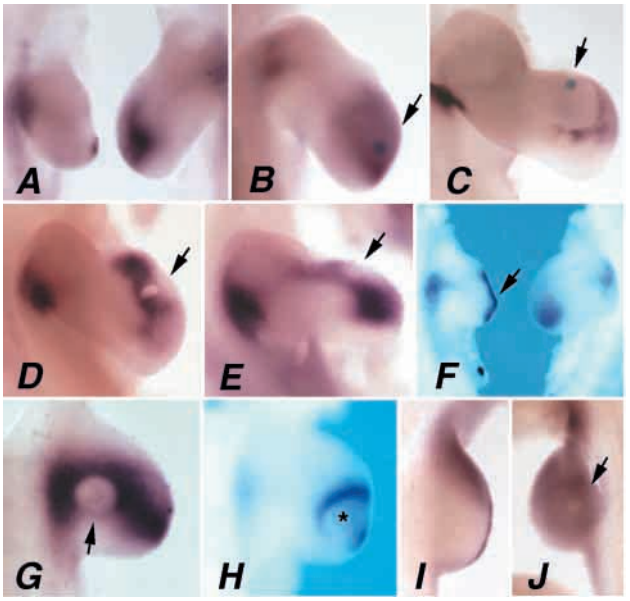

Fig. 3. Regulation of *gremlin* expression by AER, ZPA and BMPs. (A) Right experimental and left control wing buds of the same embryo 20 hours after AER removal, showing the intense downregulation of *gremlin* in the experimental limb. (B) Expression of *gremlin* in the wing bud is maintained when the removal of the AER is accompanied by the implantation of a FGF-bead (arrow). (C) Downregulation of *gremlin* 24 hours after the implantation at stage 21 of a FGF bead (arrow) in the anterior margin mesoderm. (D) Anterior expansion of *gremlin* expression 24 hours after grafting a ZPA (arrow) in the anterior margin of the bud. (E) Expansion of *gremlin* expression 24 hours after implantation of a Shh bead (arrow) in the anterior margin mesoderm. (F) Expression of *gremlin* 20 hours after removal the AER accompanied by the implantation of a Shh bead (arrow). Note that the distal mesenchyme of the operated right limb still exhibits a thin strip of *gremlin* expression. (G,H) Regulation of *gremlin* 7 hours after implantation of a BMP7 bead in the dorsal surface of a stage 20 (arrow; G) and in the distal mesoderm of a stage 23(*; H) limb buds (the apparent small size of the limb bud in G is due to the oblique angle at which the photograph was taken). (I,J) Expression of *msx-2* in (I) a control and (J) 7 hours after implantation of a BMP7 bead (arrow) in the dorsal surface of the limb bud. Note the upregulation of this gene in the treated bud in contrast with the downregulation of *gremlin* around the bead (G,H).

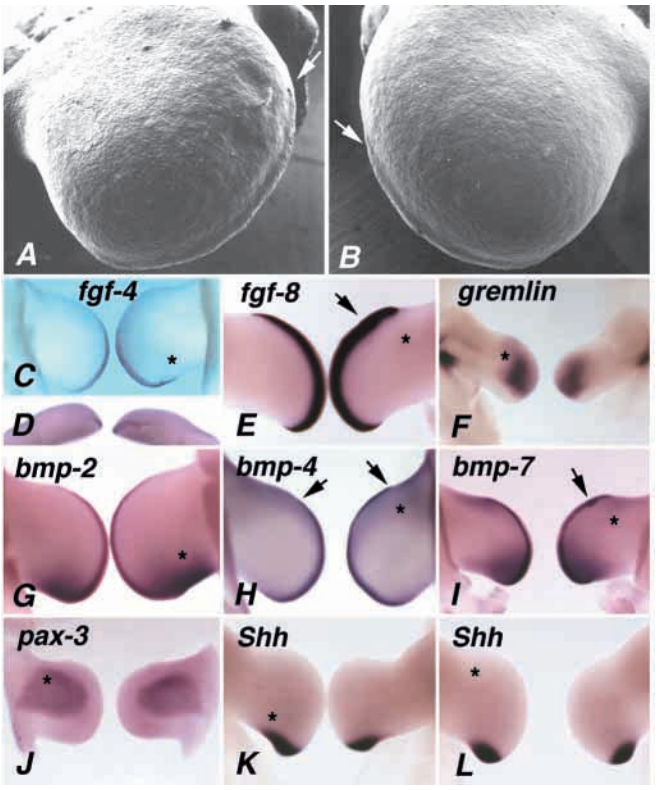

Fig. 4. Effect of implantation of Gremlin beads in the early limb bud. (A,B) Scanning electron images of the experimental (A) and control (B) wing buds of the same embryo 15 hours after the implantation of a Gremlin bead in the anterior margin mesoderm. Arrow indicates the anterior limit of the AER to show that this structure is extended anteriorly after Gremlin treatment (compare the position of arrow in A and B). (C,D) Expression of *fgf4* 15 hours after implantation of a Gremlin bead (*) in the posterior margin of the bud; (C) dorsal and (D) caudal views of the wing buds of the same embryo. (E) Expression of *fgf8* 20 hours after the implantation of the Gremlin bead (*) in the anterior bud margin. Note the thickening of the AER (arrow) in the zone of bead implantation. (F) Expression of *gremlin* 20 hours after implantation of a Gremlin bead (*) in the anterior margin of the bud showing a moderate increase in their domain of expression. (G-I) Expression of *bmp2* (G), *bmp4* (H) and *bmp7* (I) 20 hours after implantation of Gremlin beads (*). Note that the domain of expression of these genes increases in proportion to the increase of the size of the bud. Arrows show the enlargement of the AER in the zones close to the implantation of the bead. (J) Expression of *Pax3* 15 hours after the implantation of a Gremlin bead (*). Note that Pax3 increases in proportion to the increased size of the bud. (K,L) Expression of *Shh* 20 hours after the implantation of the Gremlin beads (*) in the posterior (K) and anterior (L) margins of the bud. Note the moderate increase of the domain of *shh* when the bead is implanted in the posterior mesenchyme, and the unchanged distribution following anterior implantation of the bead.

MATERIALS AND METHODS

We have employed Rhode Island chick embryos ranging from 3 to 9 days of incubation (stages 20-35, Hamburger and Hamilton, 1951) and Royal Pekin duck embryos ranging from day 4 to day 10 of incubation.

Experimental manipulations of the limb

Local application of Gremlin and BMPs was performed in chick embryos using heparin beads; FGF2 was applied in Affi-Gel blue beads; Shh protein was applied using either heparin or Affi-Gel blue beads. The beads were incubated in PBS or in the selected recombinant human protein solutions (see below) and implanted into the limb mesenchyme. Beads were implanted at different locations and stages as indicated in Results. Treatments prior to stage 24 were performed on the right wing bud and in later stages on the right leg bud. In all cases, the left limb was employed as the control.

Surgical removal of the AER was performed in chick wing buds at stages 20-22 using fine tungsten needles. In some cases, AER removal was followed by implantation of a bead incubated in FGF2 or Shh. After the operation, the eggs were returned to the incubator and used at different time intervals to study changes in *gremlin* expression by in situ hybridization.

For ZPA grafting experiments, the posterior margin mesoderm was excised from stage 22 chick wing buds and grafted into the anterior wing margin of host embryos at stage 20. After the operation, the

embryos were returned to the incubator and employed for gene expression studies.

Morphological analysis of the limb

The morphology of the limbs subjected to experimental manipulations was studied after cartilage staining with Alcian green or by scanning electron microscopy. The pattern of cell death was analyzed by vital staining with neutral red and by Tdt-mediated dUTP nick end labeling (TUNEL) in tissue sections as described previously (Macias et al., 1997).

Preparation of beads

Heparin acrylic (Sigma) or Affi-Gel blue (BioRad) were employed as carriers for administration of the selected proteins. Beads ranging between 100 and 150 μm in diameter were selected, washed in PBS and incubated for 1 hour at room temperature in the selected protein solution. Recombinant human Gremlin (Regeneron Pharm Inc. Tarrytown, NY) was employed at 1.4 mg/ml. Recombinant human BMP7 (Creative Biomolecules, Hopkinton) was employed at 0.5 and 0.1 mg/ml. FGF2 (R&D Systems) was employed at 1 mg/ml. Shh protein (obtained from J. C. Izpisua-Belmonte) was employed at 7.5 mg/ml. Control beads were incubated in PBS.

Probes and in situ hybridization

In the limbs treated with Gremlin, in situ hybridization was used to analyze the expression of *Pax3*, *MyoD*, *fgf4*, *fgf8*, *bmp7* and *shh* (obtained from J. C. Izpisua-Belmonte); *bmpR1b* (obtained from L. Niswander); *bmp2*, *bmp4* (obtained from P. Francis-West); *sox9* (obtained from P. T. Sharpe); and *msx2* (obtained from A. Kuroiwa).

Fragments of chicken and duck *gremlin* (507 bp) were obtained by RT-PCR. First-strand cDNA was synthesized with a mixture of random hexamers (Promega) and 1 μg of total RNA from chick or duck autopods at day 7.5 and 8 of incubation, respectively. The following primers (5′ to 3′) were used:

5′ primer, 5′-TCCTCCTGACAAGGATCAGC-3′

3′ primer, 5′-CTCACACTGGCAATGATTGC-3′.

PCR reactions were performed in a total volume of 100 μl using Taq DNA polymerase (Gibco BRL). The cycling conditions were 1 minute at 94°C for denaturation, 2 minutes at 60°C for annealing, 3 minutes at 72°C for elongation, and then 10 minutes at 72°C after the last cycle (35 cycles). The PCR products were subsequently cloned into pGEM-T (Promega) and the authenticity of the fragments was confirmed by dideoxy sequencing. A BLAST search revealed that the duck PCR product corresponded to a fragment of the duck homologue of *gremlin*.

In situ hybridization of control and treated limbs was performed in whole-mount specimens and in tissue sections. For whole-mount in situ hybridization, samples were treated according to their size and stage of development with 10 μg/ml of proteinase K for 25 to 40 minutes at 20°C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Reactions were developed with purple AP substrate (Boehringer-Mannheim). In situ hybridization in tissue sections was performed using digoxigenin-labeled antisense RNA probes as described by Zou et al. (1997b). Specificity of labeling was controlled using sense RNA probes.

RESULTS

Expression of *gremlin* in the developing chick limb correlates inversely with chondrogenesis and apoptosis

Gremlin exhibited a dynamic pattern of expression in the limb mesoderm throughout all the studied stages. Expression was similar in the wing and in the leg bud (Fig. 1). Prior to stage 23, *gremlin* transcripts were found in the superficial mesoderm of the ventral and dorsal surface of the bud, excluding the anterior and posterior margins (Fig. 1A,B,E,G,H). By stage 24-25, *gremlin* expression appeared progressively divided into a proximal domain located in the zone of limb implantation into the trunk and into a distal domain distributed through the superficial mesoderm of the autopod (Fig. 1C,F,I). This autopodial domain was partially displaced anteriorly (Fig. 1I). Throughout this period the distribution of *gremlin* showed an inverse relationship with the expression of *bmp* genes (Fig. 1J-L) and with the distribution of the areas of programmed cell death (ANZ, Fig. 1D; PNZ, not shown).

Between stages 27 and 30, *gremlin* transcripts were concentrated in the most proximal interdigital mesoderm (Fig. 2A). From stage 31, interdigital expression of *gremlin* was lost (Fig. 2B) preceding the establishment of the areas of interdigital cell death (Fig. 2C). At these advanced stages of limb development, *gremlin* was progressively expressed in the perichondrium of the developing digits except in the zones of joint formation (Fig. 2D,E) in the differentiating muscles (Fig. 2F) and in the the developing feather buds (Fig. 2G).

Regulation of gremlin expression by the AER, ZPA and BMPs

The possible influence of the AER on the distal displacement of *gremlin* expression observed in the course of limb outgrowth was analyzed by AER removal experiments. Surgical removal of the AER at stages 20-22 was followed 15 or 20 hours later by an intense downregulation of the distal domain of *gremlin* expression without affecting expression in the zone were the limb is implanted into the embryonic body (n=8; Fig. 3A). When the removal of the AER was accompanied by implantation of a FGF bead, expression of *gremlin* remained intense in the distal mesoderm (n=5; Fig. 3B). However, a direct effect of FGFs on *gremlin* expression could not be demonstrated since application of FGF beads at the anterior or posterior margin mesoderm of intact limb buds, was followed by downregulation of *gremlin* expression in the mesoderm close to the bead (n=6; Fig. 3C).

The influence of the ZPA was studied by grafting a ZPA into the anterior margin of stage 20-22 limb buds. Under these conditions, the mirror-limb duplications induced by the ZPA grafts were accompanied by the expansion of the distal autopodial domain of *gremlin* (n=5; Fig. 3D). Implantation of beads bearing Shh protein into the anterior margin mesoderm also expanded the domain of *gremlin* expression (n=5; Fig. 3E). Further evidence for an influence of Shh in the expression of *gremlin* was obtained in the experiments in which removal of the AER was accompanied by the implantation of a bead incubated in Shh. Under these conditions, in half of the experimental limbs (n=6), *gremlin* expression was maintained in the distal margin of the truncated limb (Fig. 3F), although at a level considerably lower to that obtained by FGF beads following AER removal.

The patterns of *bmp* genes and *gremlin* expression, which tended to occur in mutually exclusive domains in these stages, led us to analyze the possible influence of BMPs on *gremlin* expression. For this purpose, beads incubated in BMP7 at 0.5 or 0.1 mg/ml were implanted into dorsal surface of stage 20-21 wing buds (n=9; Fig. 3G) or in the progress zone mesoderm at stage 23 (n=4; Fig. 3H). This treatment induced an ectopic area of cell death detectable 10 hours after the implantation of

the bead (Macias et al., 1997). The appearance of the area of cell death was preceded by the induction of a large ectopic domain of *msx2* gene expression (Fig. 3I,J) and by downregulation of *gremlin* in the mesenchyme close to the bead (Fig. 3G,H), although the expression of this gene appeared upregulated at some distance from the bead.

Implantation of PBS beads at different positions of the limb bud, used as controls, failed to change the pattern of *gremlin* expression (data not shown).

Gremlin modulates early limb outgrowth

The potential role of Gremlin during early limb development was explored by implanting beads incubated in Gremlin into the anterior and/or posterior limb mesoderm of stage 20-21 limb buds (n=53). This treatment was followed by a mild but constant enlargement of the bud along the anteroposterior axis detectable from 12-15 hours after the treatment not observed in control experiments using beads incubated in PBS. The enlargement of the limb bud induced by Gremlin was transitory, and 30 or 40 hours after the treatment (presumably when the bead was no longer active) the experimental limb buds were indistinguishable from their contralateral control limbs. In accordance with previous studies of *noggin* misexpression (Pizette and Niswander, 1999), the anteroposterior enlargement of the limb bud appeared to be mediated by a transitory enlargement and thickening of the AER in the proximity of the bead as deduced by the morphological analysis of the bud (Fig. 4A,B), and by the pattern of expression of the *fgf4* (Fig. 4C,D) and *fgf8* genes (Fig. 4E). Expression of *bmp2*, *bmp4* and *bmp7* genes were not significantly modified by this treatment although there was a moderate expansion in their expression, which paralleled the increased in the size of the limb bud and that of the AER (Fig. 4G-I). Similarly, *gremlin* expression in this Gremlin-treated limb bud was expanded in correlation with the enlargement of the bud (Fig. 4F). *Pax3* and *MyoD* genes were employed here as markers for the proliferating and differentiating myogenic cells respectively (Amthor et al., 1998). *Pax3* exhibited a mild enlargement of its domain of expression in the proximity of the bead (Fig. 4J) and *MyoD* expression was not modified by the treatments (not shown). Gremlin did not caused ectopic expression of *shh* following implantation of the beads either in the anterior or posterior limb margins (Fig. 4K,L). In addition, the skeletal elements of the limbs treated in these early stages (prior to the appearance of the prechondrogenic aggregates) developed normally (n=12), ruling out a possible influence of gremlin in the establishment of the anteroposterior axis of the limb.

Gremlin inhibits chondrogenesis

The possible inhibitory role of Gremlin in chondrogenesis was analyzed in vivo by implanting Gremlin beads in the progress zone mesoderm in the stages of formation of the digits (stages 24-29; n=50). This treatment was followed by inhibition of chondrogenic differentiation. When the beads were implanted prior to stage 27, inhibition of chondrogenesis was restricted to the proximal elements of the digital rays in the course of formation at the time of treatment (metatarsal and first phalanx), but distal elements of the digits were formed (Fig. 5A). From stage 27, the treated digits appeared truncated at the level of the second or third phalanx (Fig. 5B). Early molecular markers of the differentiating cartilage such as *bmpR1b* (Fig. 5C,D) and *sox9* (Fig. 5E,F; Merino et al., 1998; Healy et al., 1999) were excluded from the mesenchyme surrounding the bead and remained confined proximally in the cartilage already differentiated at the time of bead implantation (Fig. 5C-F).

Gremlin modulates programmed cell death

Implantation of Gremlin beads in the interdigital regions between stages 28 and 30 caused an intense inhibition of interdigital cell death as assessed by neutral red staining (Fig. 6A,B) or TUNEL assay (Fig. 6C,D). When the beads were implanted prior to stage 29, the inhibition of cell death was transitory and, by stage 34, the limb exhibited, in most cases, a mild syndactyly or a normal phenotype. In contrast, severe soft tissue syndactyly was observed when two Gremlin beads were implanted sequentially at stages 28 and 28+20 hours (Fig. 6E,F) or when a single bead was implanted at stage 29. In accordance with the proposed role for BMPs in the expression of *msx* genes, the inhibition of interdigital cell death following local application of Gremlin beads was preceded by an intense downregulation of *msx-2* gene expression (Fig. 6G,H).

Since the formation of webbed digits in the duck is due to a reduced extension of the areas of interdigital cell death (compare Figs 2C and 7D) and is correlated with a reduced interdigital domain of *msx* gene expression (Fig. 6I; Gañan et al., 1998), we performed a comparative analysis of the expression of *gremlin* in the duck leg bud to further analyze its possible physiological role in the control of cell death. To this end, a fragment of the duck homologue of *gremlin* was cloned by PCR. The cloned fragment of duck *gremlin* showed 97% homology with the equivalent chick fragment.

In early stages of limb development, the expression of *gremlin* in the duck limb was essentially identical to that of the chick (not shown). Differences were detected in the autopod in the stages of digit formation. Thus, between days 8 and 10 of incubation, the interdigital spaces of the duck leg exhibited domains of *gremlin* expression not observed in the chick at equivalent stages of development (Fig. 7A-C). These interdigital domains correlated with the reduced extension of the areas of interdigital cell death observed in the duck (Fig. 7C,D)

DISCUSSION

Here we have shown that the BMP-antagonist Gremlin exhibits a precise and dynamic pattern of expression in the course of morphogenesis of the avian limb. This pattern of expression is rather coincident with that described for *Drm* in the developing limb of the mouse (Pearce et al., 1999). In addition, we have shown that exogenous Gremlin modulates limb outgrowth and inhibits chondrogenesis and cell death. These findings are in accordance with the ability of Gremlin to neutralize BMP2, BMP4 (Hsu et al., 1997) and BMP7 (A. N. E., unpublished data), which are signals involved in those processes during limb morphogenesis. Three main periods can be distinguished according to the distribution of Gremlin in the developing limb.

The first period (stages 20-25) precedes the formation of the digits, and *gremlin* is expressed in the mesoderm subjacent to the dorsal and ventral ectoderm excluding the central mesodermal core of the limb where chondrogenesis occurs.

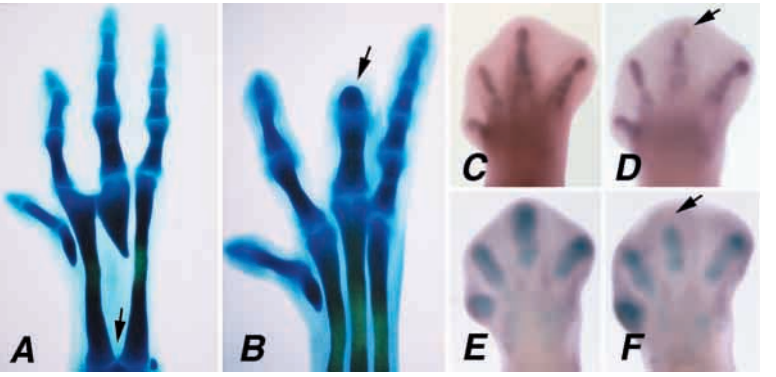

Fig. 5. Effects of Gremlin treatments in chondrogenesis. (A,B) Inhibition of digit chondrogenesis following the implantation of the Gremlin beads (arrow) at the tip of the digit forming region of stage 24 (A) and stage 28 (B) limbs. (C-F) Expression of *bmpR1b* (C,D) and *sox9* (E,F) genes in control (C,E) and in experimental limbs (D,F) 15 hours after the implantation of Gremlin beads (arrows) at the tip of digit 3. Note the intense downregulation of these genes in the treated digits.

Gremlin expression is also excluded from the anterior and posterior margins of the bud, which correspond to the anterior and posterior zones of programmed cell death (ANZ and PNZ). During this period, *gremlin* expression is influenced by the AER and ZPA. AER removal is followed by intense downregulation of *gremlin* and implantation of a FGF bead after AER removal restores *gremlin* expression. However, this effect of AER on *gremlin* expression appears to be indirect, as deduced by the observed downregulation of *gremlin* following the application of FGF beads in the intact limb bud. ZPA grafts into the anterior region of the limb lead to duplication of the distal domain of *gremlin* expression. Similarly, application of beads incubated in Shh protein upregulates *gremlin* expression. Taking into account that the function of the ZPA requires the integrity of the AER (Vogel and Tickle, 1993; Li et al., 1996), it can be presumed that the observed influence of the AER on the maintenance of *gremlin* expression is mediated by the ZPA.

It is also noteworthy that the expression of *gremlin* and the expression of *bmp2*, *bmp4* and *bmp7* are mutually exclusive at these stages. Furthermore, local treatment of the limb with beads incubated in BMPs downregulates *gremlin* expression close to the bead but its expression is upregulated at some distance from the bead in a fashion resembling the expression of these factors in the early limb bud. It has previously been observed that FGFs and BMPs play opposite roles in limb outgrowth, promoting and repressing mesodermal proliferation respectively (Niswander and Martin 1993; Macias et al., 1996). The role BMPs in limb outgrowth includes their ability to trigger apoptosis in the areas of programmed cell death which sculpt the limb morphology (ANZ, PNZ and INZ; see Macias et al., 1997). Hence, the presence of *gremlin* and its regulation by the AER/ZPA complex, may contribute to direct the polarized outgrowth of the limb modulating the anti-proliferative and apoptotic influence of BMPs (see Discussion below for the role of Gremlin in the control of apoptosis). The enlargement of the limb bud observed here after implantation of Gremlin beads in the anterior or posterior mesoderm supports this interpretation. In addition, the negative influence of BMPs on limb outgrowth also appears to involve a negative influence in the maintenance of the AER (Gañan et al., 1998; Pizette and Niswander, 1999). The presence of *gremlin* in the mesoderm close to the AER in these early stages of limb development and the enlargement and thickening of the AER observed after implantation of Gremlin beads suggest that this BMP antagonist is physiologically involved in the maintenance of an active AER.

In these early stages of limb development, outgrowth is accompanied by the differentiation of the mesodermal cells of the limb core to form the prechondrogenic aggregates of the skeleton. Our findings indicate that Gremlin may be involved in this process. BMPs appear to regulate the early events of chondrogenic differentiation as deduced from in vitro studies (Roark and Greer, 1994) and from the formation of limbs with

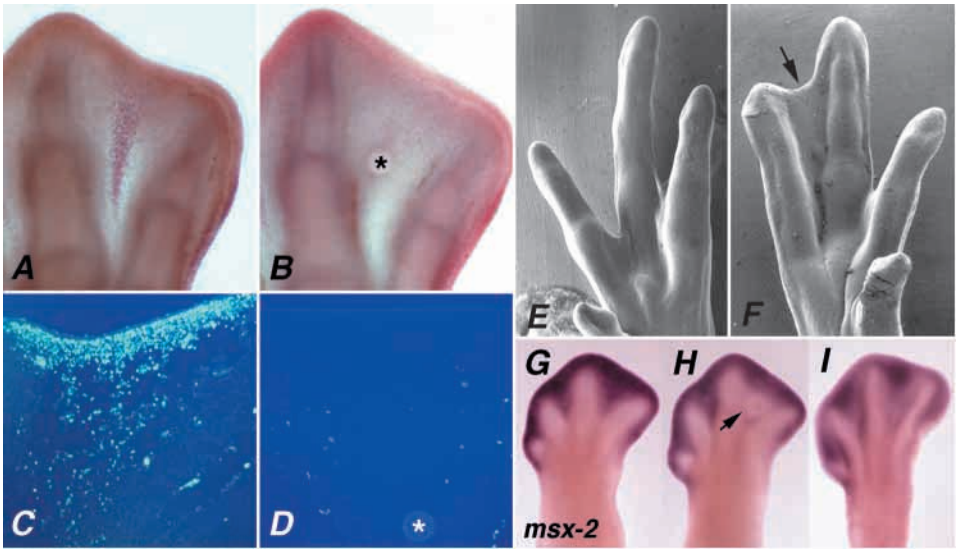

Fig. 6. Inhibition of interdigital cell death following Gremlin treatment. (A,B) Control (A) and experimental (B) interdigits vital stained with neutral red for cell death 20 hours after implantation of a Gremlin bead (*). Note the lack of interdigital cell death in the treated limb. (C,D) Distribution of TUNEL-positive apoptotic cells in the control interdigit (C) and its absence 20 hours after implantation of a Gremlin bead (D). (E,F) Scanning micrographs showing a control (E) and a duck-like syndactyly (F) in a chick limb 3 days after interdigital implantation of two Gremlin beads at stages 28 and 28+20 hours. (G-I) Expression of *msx-2* after Gremlin treatment. (G) Control chick leg bud. (H) Downregulation of *msx-2* in the chick limb 15 hours after interdigital implantation of a Gremlin bead (arrow). (I) Expression of *msx-2* in the duck leg bud at an equivalent stage to H and I. Note the similarity in the expression of *msx-2* between the duck and the chick treated interdigit.

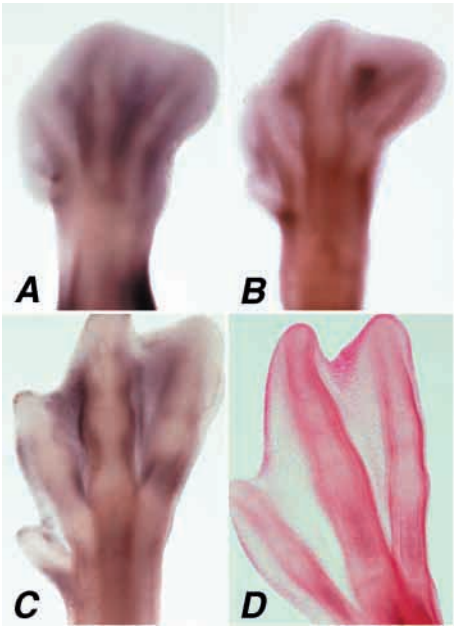

Fig. 7. Expression of *gremlin* during the formation of the digits in the duck. (A-C) Interdigital expression of *gremlin* in the duck limb at days 8.5 (A), 9 (B), and 10 (C) of incubation. (D) Duck leg bud at day 10 of incubation vital stained with neutral red showing the areas of interdigital cell death. Note that the distribution of cell death corresponds with the most distal mesenchyme of the interdigit lacking *gremlin* expression (compare C and D).

dramatically enlarged skeletal elements following retrovirus induced misexpression of *bmp2* or *bmp4* genes (Duprez et al., 1996a). Characteristically, in physiological conditions, prechondrogenic condensations exclude the superficial mesoderm subjacent to the ectodermal layer (Solursh, 1984). In this study, we have found that these non-chondrogenic regions correspond nicely to the zone of *gremlin* expression. Taking into account the high potential of exogenous Gremlin to block chondrogenesis in vivo, our results indicate that Gremlin has the function of confining chondrogenesis to the central core of the limb. In accordance with this possible role of gremlin in the control of chondrogenesis, its expression in the zone of implantation of the limb into the trunk may be related to the formation of the shoulder girdle skeleton which is also influenced by BMPs (Hofmann et al., 1998). It is interesting to note that, unlike Gremlin, the BMP antagonist Noggin is expressed in the chondrogenic condensations and appears to control the growth and shape of the cartilages (Merino et al., 1998) rather than the establishment of the prechondrogenic condensations. Thus, while both Noggin and Gremlin seem to be involved in the control of chondrogenesis, they may act in a complementary fashion rather than being redundant signals.

The presence of *gremlin* in the dorsal and ventral mesoderm might be related to the establishment of the developing muscle masses in these regions. In vivo and in vitro studies indicate that BMPs induce apoptosis of myogenic cells (Duprez et al., 1996b; Amthor et al., 1998) while at low concentration they may regulate muscle differentiation (Amthor et al., 1998). The distribution of *gremlin* transcripts is compatible with a role of this factor protecting the premyogenic cells from the apoptotic influence of BMPs. However, our findings indicate that Gremlin does not regulate early myogenic differentiation as deduced from the lack of changes in the expression of *MyoD* following Gremlin treatments.

The second period of *gremlin* expression in the limb corresponds to the stages of formation of the digits (stages 27-32). During these stages, in addition to being expressed in the zones of feather formation and in the differentiating muscles, *gremlin* is observed in the interdigital mesenchyme. Major differences are appreciable in this period between the chick and duck leg. In the chick, only the most proximal region of the interdigits exhibits a transitory expression of *gremlin*. From stage 30, coinciding with the onset of interdigital cell death, *gremlin* expression disappears from the chick interdigits. Unlike the chick, *gremlin* expression is maintained in the interdigital mesoderm of the duck leg. The difference between the webbed digits of the duck and the free digits of the chick is related to the extension of the areas of interdigital cell death (Saunders and Fallon, 1967). In addition, the role of BMPs in controlling interdigital regression in the chick has been demonstrated by a variety of experimental approaches (Zou and Niswander, 1996; Gañan et al., 1996; Yokouchi et al., 1996; Kawakami et al., 1996; Macias et al., 1997). However, surprisingly, the interdigital webs of the duck exhibit a pattern of *bmp* gene expression virtually identical to that of the chick. (Laufer et al., 1997). Thus, the continued expression of *gremlin* in the duck interdigit observed here may serve to neutralize interdigital BMPs. In accordance with this interpretation, we have observed that a duck-like syndactyly is induced in the chick by application of exogenous Gremlin in the interdigital mesoderm. In addition, the presence of *gremlin* in the duck interdigit may also explain the reduced expression of *msx* genes in this species (Gañan et al., 1998). This is a significant finding since *msx-2* gene appear to be required in the apoptotic pathway mediated by BMPs (Graham et al., 1994; Gañan et al., 1998; Rodriguez-Leon et al., 1999).

The third period of *gremlin* expression in the limb covers the stages of maturation of the limb tissues once the anatomical components of the limb have been established. In this late period of limb development, *gremlin* transcripts are found in the differentiating perichondrium except in the zones of joint formation. This expression is coincident with that of *bmp7* (Macias et al., 1997) and may be related to the control of cartilage growth and osteogenic differentiation by BMPs (Enomoto-Iwamoto et al., 1998). As mentioned above for the previous period, *gremlin* is also expressed at these late stages of development in the developing feathers, a process in which BMPs play a central role (Jung et al., 1998).

In conclusion, the present study provides evidence for a key role of Gremlin as a mediator of the early signalling centers responsible for limb outgrowth (AER and ZPA), which modulates the action of BMPs on growth, apoptosis and early skeletogenesis. In addition, Gremlin appears also involved in the control of interdigital tissue regression and in later stages in the regulation of the differentiation of the skeletal and muscular limb tissues.

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