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BMPs negatively regulate structure and function of the limb apical ectodermal ridge

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SUMMARY

The apical ectodermal ridge (AER), a transient specialized epithelium at the distal limb tip, is essential for vertebrate embryonic limb outgrowth along the proximodistal axis. Among all the molecules expressed in the AER, only the Fibroblast Growth Factors (FGFs) have been shown to substitute for its function in limb outgrowth. After specification of the skeletal progenitors is complete, the AER regresses, having fulfilled its function. However, the cellular processes underlying AER regression remain largely unclear, and the molecular ones, totally unknown.

Members of the Bone Morphogenetic Protein (BMP) family are expressed in the AER throughout its life and in the mesenchyme. Our studies using misexpression of Noggin, a BMP inhibitor, reveal an unsuspected role for BMPs in the negative regulation of *Fgf* expression and AER function. We find that BMPs limit limb outgrowth by promoting AER regression, as BMP inhibition results in persistence of the AER, prolonged *Fgf* expression and excess soft-tissue growth. In addition, the Noggin misexpression studies uncover an earlier role for BMPs in repression of AER function. Noggin overexpression results in extension of the AER anteriorly and loss of AER asymmetry. We show that overall the AER becomes taller, and its anterior half becomes more similar to a normal posterior AER. In addition, *Fgf4* transcripts, which are usually restricted to the posterior half of the AER, are now also expressed anteriorly. Moreover, ectopic*Fgf4* expression is induced independently of Sonic Hedgehog, contrary to current models of *Fgf4* regulation in the limb. Our studies also provide insight into the activity of the hypothesized apical ectodermal maintenance factor (AEMF), which is thought to maintain the tall shape of the posterior part of the AER. Our work shows that the AER is negatively regulated by BMP.

Key words: Apical ectodermal ridge, Bone Morphogenetic Protein, *Bmp*, Chick, *Fgf*, Limb development, Noggin

INTRODUCTION

The apical ectodermal ridge (AER) is a thickened epithelium located at the dorsoventral interface of the distal tip of the vertebrate limb. The AER sustains embryonic limb outgrowth, as its surgical removal results in limb truncation. The proximodistal level of truncation depends on the stage when the AER is removed (Saunders, 1948; Summerbell, 1974). The AER signals to the underlying mesenchyme cells in a region called the progress zone to maintain these cells in an undifferentiated and proliferative state. Previous studies indicate that Fibroblast Growth Factors (FGF) positively mediate AER function, as beads soaked in FGF protein rescue limb growth following AER removal (Martin, 1998). FGFs are good candidates for the endogenous signal as at least three (*Fgf2, Fgf4* and *Fgf8*) are expressed in the AER (Martin, 1998).

Despite the identification of positive mediators of AER signaling, a number of questions regarding regulation of AER function and morphology remain. For instance, very little is known about AER regression. The AER is a columnar pseudostratified epithelium in birds (Todt and Fallon, 1984) but near the end of its life it gradually flattens to a simple cuboidal epithelium (Pautou, 1978). AER regression takes place after the most distal skeletal progenitors are specified and is believed to limit limb outgrowth (Saunders, 1948; Rubin and Saunders, 1972; Summerbell, 1974; Rowe and Fallon, 1982). So far, the mechanism by which this occurs is controversial and no molecule has been implicated in AER regression.

Another question is how AER morphology is regulated. The chick AER is asymmetric along the anteroposterior axis, being taller posteriorly (Todt and Fallon, 1984). Classical experiments describe an activity called the apical ectodermal maintenance factor (AEMF), thought to be localized in the posterior part of the limb mesenchyme and involved in maintaining the tall shape of the AER posteriorly (Zwilling and Hansborough, 1956; Saunders and Gasseling, 1963). The zone of polarizing activity (ZPA) is also located in the posterior limb mesenchyme (Saunders and Gasseling, 1968). Transplantation of the ZPA into the anterior margin of the limb bud causes the

anterior AER to adopt a tall shape similar to that of the posterior AER, and ultimately results in mirror-image duplication of the limb skeleton (Saunders and Gasseling, 1968). As Sonic Hedgehog (SHH) is the key molecule of the ZPA (Riddle et al., 1993), it is a good candidate for the AEMF. FGF10 has also been proposed to be the AEMF. *Fgf10* is initially expressed in the posterior half of the progress zone and participates in maintaining *Fgf8* expression in the AER (Martin, 1998).

The only other known sign of AER asymmetry lies in the pattern of *Fgf4* expression. Other AER markers are expressed throughout the mature AER, whereas at early stages *Fgf4* transcripts are confined to the posterior half of the AER (Laufer et al., 1994; Niswander et al., 1994). Introduction of SHH under the anterior AER demonstrates that SHH can induce *Fgf4* expression (Laufer et al., 1994). Based on this, it has been hypothesized that the distribution of *Fgf4* mRNA is limited by the spatial restriction of its inducer. Thus, SHH may play a role in inducing and/or maintaining both the structural and molecular asymmetry of the AER.

BMPs, members of the TGFβ superfamily of extracellular signaling molecules, are involved in numerous developmental processes (Hogan, 1996). They signal through a heteromeric complex of transmembrane serine/threonine kinase receptors, the type I BMP receptors (BMPR-IA and BMPR-IB) and the type II BMP receptor (BMPRII) (Hogan, 1996). A number of BMPs are expressed in dynamic and partially overlapping patterns during limb development. *Bmp2*, *Bmp4* and *Bmp7* are expressed in the limb mesenchyme from early limb bud stages to later ones when their transcripts are detected in the maturing cartilage and interdigital mesenchyme (Francis et al., 1994; Francis-West et al., 1995). These three *Bmps* are also expressed in the AER throughout its existence. *BmpRIA* RNA is widely expressed in the chick limb at low levels, whereas *BmpRIB* transcripts are specific to areas of condensing mesenchyme (Kawakami et al., 1996; Zou et al., 1997b; Merino et al., 1998). *BmpRII* is detected in the AER and throughout the limb mesenchyme (Kawakami et al., 1996).

Gain-of-function studies performed in the chick embryonic limb using retroviruses encoding BMPs or constitutive active BMPRs or BMP-protein-soaked beads demonstrate roles for these factors in apoptosis and chondrogenesis (Duprez et al., 1996a; Gañan et al., 1996; Zou and Niswander, 1996; Macias et al., 1997; Zou et al., 1997b). These roles have been confirmed by loss-of-function studies using dominant negative BMPRs (Kawakami et al., 1996; Yokouchi et al., 1996; Zou and Niswander, 1996; Zou et al., 1997b). In addition, pellets of BMP2-expressing cells applied to the anterior margin of the limb bud show a weak polarizing activity (Duprez et al., 1996b). As chick *Bmp2* is expressed at early stages in an overlapping domain with *Shh*, it has been suggested that BMP2 is a downstream effector of SHH (Francis et al., 1994).

Finally, loss-of-function experiments in mice have not depicted the total spectrum of BMPs activities throughout limb development. Embryos homozygous for targeted mutations of *Bmp2*, *Bmp4* or *BmpRIA* die during early embryogenesis and are not informative in this respect. However, the limbs of *Bmp7* homozygous and *Bmp4* heterozygous mutants display preaxial polydactyly (references in Hogan, 1996 and Zhang and Bradley, 1996; Dunn et al., 1997). As BMPs are postulated to play many roles in limb development, the relatively mild limb phenotype may be explained by redundant functions of the many BMPs in the limb.

Recent studies have explored the regulation of BMP activity. In *Xenopus* embryos, BMPs ventralize mesoderm and direct the ectoderm towards an epidermal lineage whereas molecules secreted by the Spemann organizer induce dorsal and neural fates in the mesoderm and the ectoderm, respectively (reviewed in Hogan, 1996). Some of these dorsalizing factors are able to counteract BMP action by directly binding BMP and preventing their interaction with the BMPRs (Piccolo et al., 1996; Zimmerman et al., 1996). These inhibitors share specific, as well as overlapping, binding affinities for different members of the TGFβ superfamily (Piccolo et al., 1996; Zimmerman et al., 1996; Hsu et al., 1998).

Here we used one of these inhibitors, Noggin, that specifically binds BMP2 and BMP4 with high affinity and BMP7 with lower affinity (Zimmerman et al., 1996), to extend our analysis of BMP function in the developing limb. A replication-competent retrovirus was used to misexpress Noggin in the embryonic chick limb. The observed phenotypes confirm most of the functions ascribed to BMPs, however, our studies also uncover new roles. In addition to their participation in apoptosis and chondrogenesis, we find that BMPs repress AER function. BMPs promote AER regression, thereby limiting late limb outgrowth. They also negatively regulate earlier AER function by maintaining AER asymmetry with respect to restricted *Fgf4* expression and AER height. The mechanism for how the hypothesized AEMF may regulate AER structure is discussed.

MATERIALS AND METHODS

Noggin cloning and viral misexpression

The chicken *Noggin* homolog was isolated from a Hamburger-Hamilton (Hamburger and Hamilton, 1951) stage 12-15 chick embryo cDNA library (a kind gift from D. Wilkinson) by low-stringency hybridization using a *Xenopus Noggin* probe (Smith and Harland, 1992; provided by R. Harland). Its coding sequence was cloned into two forms of the RCAS replication-competent retroviral vectors (Hughes et al., 1987), which differ in their envelope subgroup to generate RCASBP(A)-*Noggin* and RCASBP(B)-*Noggin*. Viral stocks were prepared (Morgan and Fekete, 1996) and virus was injected into the presumptive limb field of stage 12-14 White Leghorn chick embryos (SPAFAS; Norwich, CT) to elicit *Noggin* misexpression throughout the limb. This protocol was used for studying the early limb phenotype. Early infection of the limb with RCAS(A)-*Noggin* invariably caused lethality around embryonic day 7 (E7). Therefore to study the effects of *Noggin* misexpression in the limb at subsequent stages, we used RCAS(B)-*Noggin*. This virus has a lower level of infectivity than RCAS(A) even in presence of polybrene, as assessed by whole-mount in situ hybridization with a viral probe (data not shown). RCAS(B)-*Noggin*-infected embryos displayed a weaker phenotype at early stages but many survived until E9 and some lived until E14. Alternatively, for analysis of late stage limb phenotypes, RCAS(A)-*Noggin* virus was introduced into the amniotic cavity at stage 20 to restrict expression to the ectoderm. This minimized the effects of *Noggin* misexpression on cartilage formation. These infection protocols produced similar effects on AER regression, the extent of the phenotype depending on the extent of infection. RCAS(A)-*dnBMPR-IB* was introduced under the vitelline membrane at stage 8-10 to restrict expression to the ectoderm to study early AER asymmetry or into stage 20 limb buds to study AER regression.

Experimental techniques

Whole-mount and section non-radioactive RNA in situ hybridizations were performed as in Zou et al. (1997b). Probes were as described in the following: Robert et al., 1991; Riddle et al., 1993; Francis et al., 1994; Niswander et al., 1994; Crossley et al., 1996; Marigo et al., 1996; Myat et al., 1996; Laufer et al., 1997. Bromodeoxyuridine (BrdU) incorporation, Nile blue staining and TUNEL assay as in Shen et al. (1997) and Zou et al. (1997b). For the BrdU experiments, all sections along the anteroposterior axis were examined in both control and *Noggin*-infected stage 27 limbs. Noggin-expressing cells were prepared from primary chick embryonic fibroblasts transfected with RCAS(A)-*Noggin* DNA and grown for 7 days. Stably transfected Shh-expressing cells, preparation of cell pellets and grafting procedure as in Yang and Niswander (1995). For cell death induction, heparin beads (Sigma H5263) were soaked in 500 μg/ml BMP2, which gives maximal cell death in uninfected limbs as determined in our laboratory and by Macias et al. (1997). The concentration of BMP4 used (50 μg/ml) gave extensive cell death in 100% of control cases. RCAS(A)-*Noggin* was introduced into the presumptive limb at stage 14, BMP beads were applied under the AER at stage 22 and the embryos stained with Nile blue 20 hours later. For plastic sections, limbs were embedded in Spurr's resin, sectioned at 3 μm and stained with methylene blue and Azure II. Number of nuclei was based on counting nuclei within the AER boundaries. All sections were counted in control and Noggin-infected limbs. Cell counts presented are from the anterior and posterior thirds of the AER.

RESULTS

Noggin misexpression leads to limb soft tissue overgrowth at late stages

A stage 12 to 15 chick embryonic cDNA library was screened with a Xenopus Noggin probe and three chicken Noggin clones identical in their coding sequence were isolated (GenBank accession number AF057364; see also Connolly et al., 1997). Chick Noggin was cloned into RCASBP(A) and RCASBP(B) retroviral vectors and the recombinant viruses injected into the presumptive limb field at stages 12 to 14 (see also Materials and Methods). Phenotypic analyses indicate that RCAS-Noggin-infected legs and wings at late stages (examined from E8 to 14) exhibit feather fusion (S. P. and L. N., unpublished observations; Noramly and Morgan, 1998) and interdigital webbing (Fig. 1A,E). Interestingly, an abnormal and persistent excess of distal soft tissue was also evident (data not shown). However, due to Noggin inhibition of BMP signaling, which is necessary for cartilage formation (Brunet et al., 1998; Capdevila and Johnson, 1998; Merino et al., 1998), Noggin misexpression was accompanied by digit truncation (all phenotypes observed in >95% of cases). To separate chondrogenic alterations from the presence of extra soft tissue, we performed RCAS-Noggin injection into the amnion at stage 20, which restricts viral infection to patches of ectoderm. Using this protocol, chondrogenesis was not affected or only slightly affected. Small regions of outgrowth were still observed and these correlated with the presence of viral transcripts (Fig. 1A, limb on right and data not shown). More widespread superficial Noggin infection resulted in excess soft tissue across much of the anteroposterior axis (Fig. 1A, limb on left). The fact that excess soft tissue could be found over both the digital and interdigital regions and regardless as to whether digit truncation occurred or not (Fig. 1A), strongly suggests that this phenotype arises independently of webbing or digit truncation.

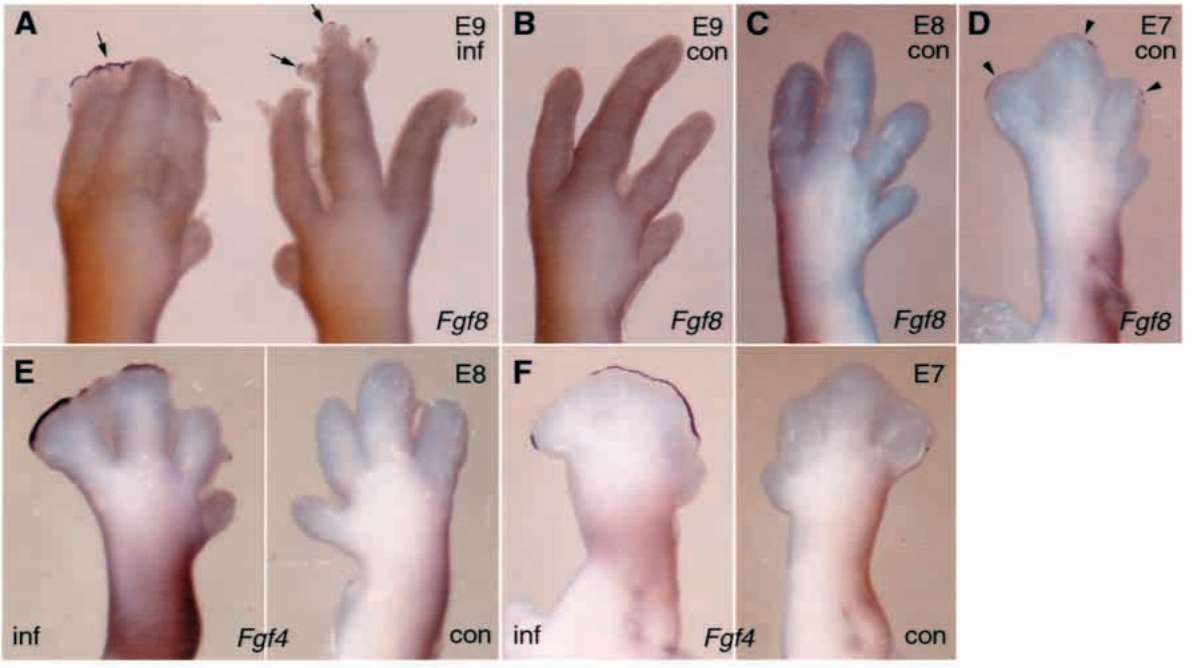

Fig. 1. *Noggin* misexpression leads to distal soft-tissue overgrowth that correlates with prolonged *Fgf* expression. In situ hybridization with digoxigenin-labeled probes of limbs following RCAS-*Noggin* infection (inf) into the amnion (A) or limb field (left limb in E,F) and of control limbs (con) (B-D and right limb in E,F). (A,B) E9, (C) E8 and (D) E7 legs hybridized with *Fgf8* probe (purple stain marks regions of gene expression; arrows in A point to distal soft-tissue outgrowth and arrowheads in D to regions of faint *Fgf8* expression). (E) E8 and (F) E7 legs hybridized with *Fgf4* probe. *Fgf4* and *Fgf8* expression are detected in infected limbs at least 3 and 2 days later than normal, respectively.

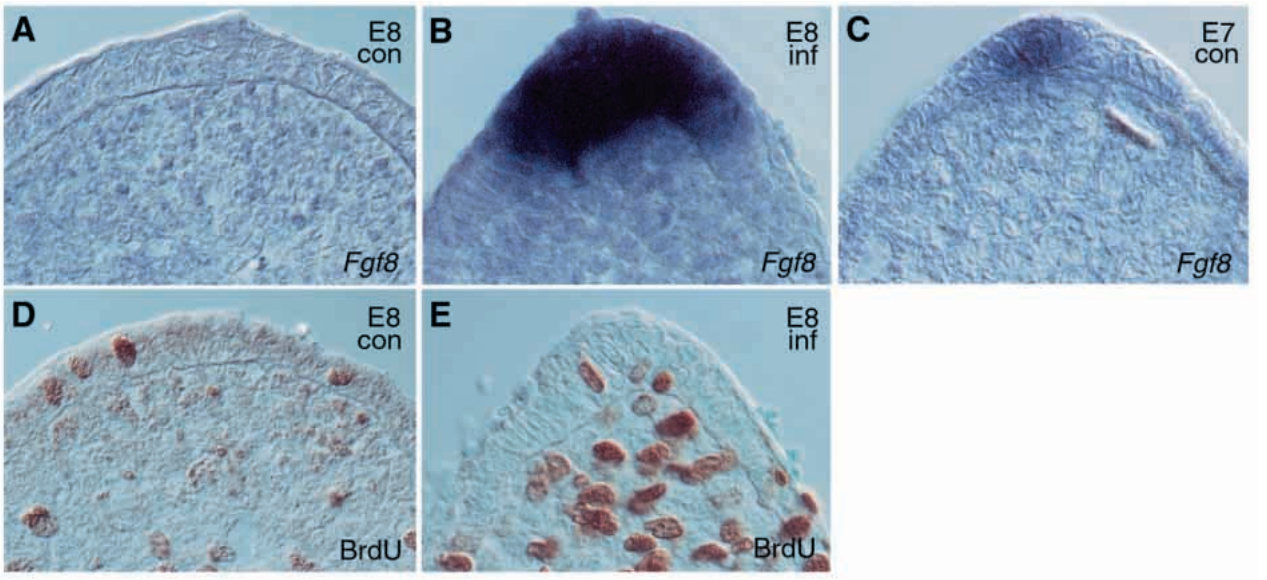

Fig. 2. AER regression is inhibited by *Noggin* misexpression. Representative transverse paraffin sections through the digit tip of E8 (A,B; stage 35) and E7 (C; stage 32) legs hybridized with *Fgf8* probe. (D,E) Alternate sections to A,B assayed for BrdU incorporation. In *Noggin*-infected limbs (B,E, amnion infection), a morphological AER is still present at E8 and mesenchyme proliferation is stimulated (compare to control in A,D). Also note that *Fgf8* expression is stronger, and the AER taller, in E8 infected limbs (B) compared to E7 control (C).

Persistence of the AER in *Noggin*-infected limbs

De-regulation of the extent of time that the AER continues to promote proliferation might be responsible for the presence of additional distal soft tissue. Therefore, we examined whether AER regression had taken place. Normally by stage 31-32 (E7), the AER overlying the interdigital regions has regressed, forming a flat simple cuboidal epithelium. By stage 35 (E8), AER regression is complete, encompassing the ectoderm at the tip of the digits (Fig. 2A). In *Noggin*-infected limbs, an epithelium with tall columnar cells retaining some radial organization characteristic of the AER was still present at stage 35 (Fig. 2E, compare to 2D). Moreover, it was much taller than the regressing AER of a stage 32 control (Fig. 2C). This AER-like epithelium was observed in accordance with the site of viral misexpression (data not shown). Thus, *Noggin* misexpression inhibits AER regression, at least at the morphological level.

To further link BMP function to AER regression, we studied the pattern of expression of *Bmp2*, *Bmp4* and *Bmp7* during this process. At stage 32 (E7; Fig. 3), all three genes were expressed in the interdigital apoptotic zones as previously described (Francis et al., 1994; Zou and Niswander, 1996). In addition, *Bmp4* and *Bmp7* continued to be expressed in the AER. *Bmp4* transcripts were present only in the remnants of the AER at the tip of the digits, whereas *Bmp7* transcripts were present along the entire distal aspect of the limb including over the interdigital mesenchyme where the AER is morphologically absent (Fig. 3B,C). *Bmp2* was no longer detected in the distal ectoderm (Fig. 3A). Therefore, any or all of these BMPs may mediate AER regression, either by a paracrine or an autocrine mechanism.

AER-specific gene expression is sustained in the persistent AER

Next, we investigated if the persistent AER continued to express AER-specific genes. We focused on members of the *Fgf* family, known for their role in stimulating distal limb mesenchyme proliferation. In contrast to other studied AER markers, *Fgf4* expression is normally downregulated quite early, long before final AER regression (not detected by whole-mount in situ hybridization after stage 27). Conversely, *Fgf8* transcripts remain visible in the AER until it has undergone complete regression (Fig. 1C,D). Following RCAS-*Noggin* infection, *Fgf8* transcripts were detected at least 2 days later than normal (stage 36, E9; Fig. 1A-D; *n*=7 at E8 and *n*=5 at E9). *Fgf4* mRNA was detected in the AER at stage 35 (E8), at

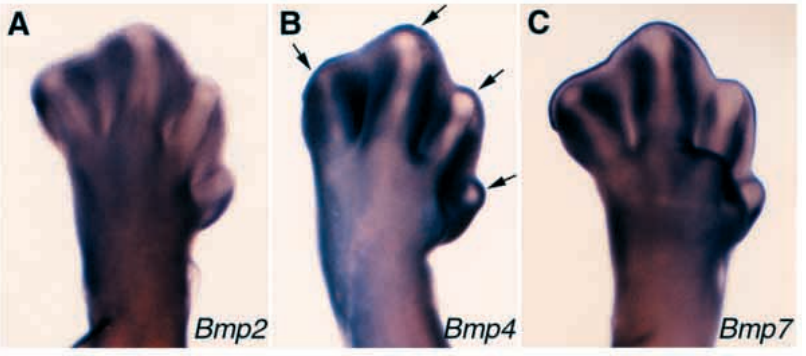

Fig. 3. Pattern of *Bmp* expression in the regressing AER. Dorsal views of uninfected limbs at stage 32 processed for whole-mount RNA in situ hybridization with digoxigenin-labeled probes for (A) *Bmp2*, (B) *Bmp4* and (C) *Bmp7*. *Bmp2* expression is undetectable in the AER by this stage. *Bmp4* mRNA is only detected in the remnants of the AER over the digits (arrows). In contrast, *Bmp7* is expressed along the entire anteroposterior extent of the distal ectoderm, including the flattened ectoderm overlying the interdigital region. All three *Bmp*s are expressed in the interdigital mesenchyme.

least 3 days later than normal (Fig. 1E,F; *n*=10 at E7 and *n*=7 at E8). Ectopic *Fgf* expression was equally found over digital and webbed interdigital regions, and could extend across the entire anteroposterior axis, as observed for the inhibition of AER regression (Fig. 1A,E,F).

In *Noggin*-infected limbs, other AER-specific genes such as *Notch1*, *R-Fringe* and *Bmp4* (Francis et al., 1994; Myat et al., 1996; Laufer et al., 1997) exhibited prolonged expression (data not shown; *n*>5 for each probe except *Notch1* *n*=2). Hence, inhibition of AER regression is accompanied by sustained gene expression.

Distal mesenchyme proliferation is maintained in *Noggin*-infected limbs

Finally, we sought to determine whether distal outgrowth was associated with ectopic *Fgf* expression. Therefore, we analyzed *Fgf8* mRNA distribution and bromodeoxyuridine (BrdU) incorporation in stage 35 *Noggin*-infected limbs. Alternate sections revealed that *Fgf8* expression always corresponded with a significantly higher number of BrdU-labeled mesenchyme cells underlying the AER. In contrast, uninfected limbs lacked both *Fgf8* expression and distal proliferation (Fig. 2A,B,D,E; *n*=4 for control and *n*=4 for infected limbs). Thus, there was a clear correlation between continued *Fgf8* expression and distal mesenchyme proliferation in *Noggin*-infected limbs.

Altogether these results (persistence of the AER, sustained *Fgf* expression and mesenchyme proliferation) indicate that BMPs are critical components in the control of AER function. Moreover, by promoting AER regression, these factors could serve to limit the pool of proliferating distal mesenchyme cells. In support of a specific role for Noggin in blocking BMPs, we found that infection (within the mesenchyme or restricted to the ectoderm) with RCAS virus expressing a dominant-negative version of BMP receptor-IB resulted in webbing and digit truncation (Zou and Niswander, 1996), as well as additional soft-tissue outgrowth and sustained *Fgf8* expression (examined at stage 35; *n*=14; 100%; data not shown). In conclusion, these data implicate BMPs in the negative control of limb growth.

Earlier role for BMPs in regulation of the AER

As BMPs control late AER function we tested if BMP signaling may regulate the AER earlier in development. 2-3 days after infection of the presumptive wing or leg bud at stage 12 to 14 with RCAS-*Noggin*, the majority of the developing limbs were wider than normal along the anteroposterior axis (Figs 4A, 5B, 8B,D). However, in ~15% of cases following injection at stage 12, small limbs formed that were narrower along the anteroposterior axis. This latter phenotype will be described elsewhere (S. P. and L. N., unpublished data). All the data presented below derive from embryos injected at stage 14 and from limbs that display the wider shape.

Asymmetry of AER morphology is altered in *Noggin*-infected limbs

The posterior half of the AER is normally taller than its anterior half. In RCAS-*Noggin*-infected wings or legs, asymmetry in AER height was lost. As early as stage 24 the AER anteriorly became significantly taller than normal, as seen in whole-mount preparations and in sections through the AER (Fig. 4A-D; *n*=16; at E4-E5 100% of limbs). The AER also appeared taller than normal on the posterior side (Fig. 4A) and was relatively symmetrical in height along the anteroposterior axis.

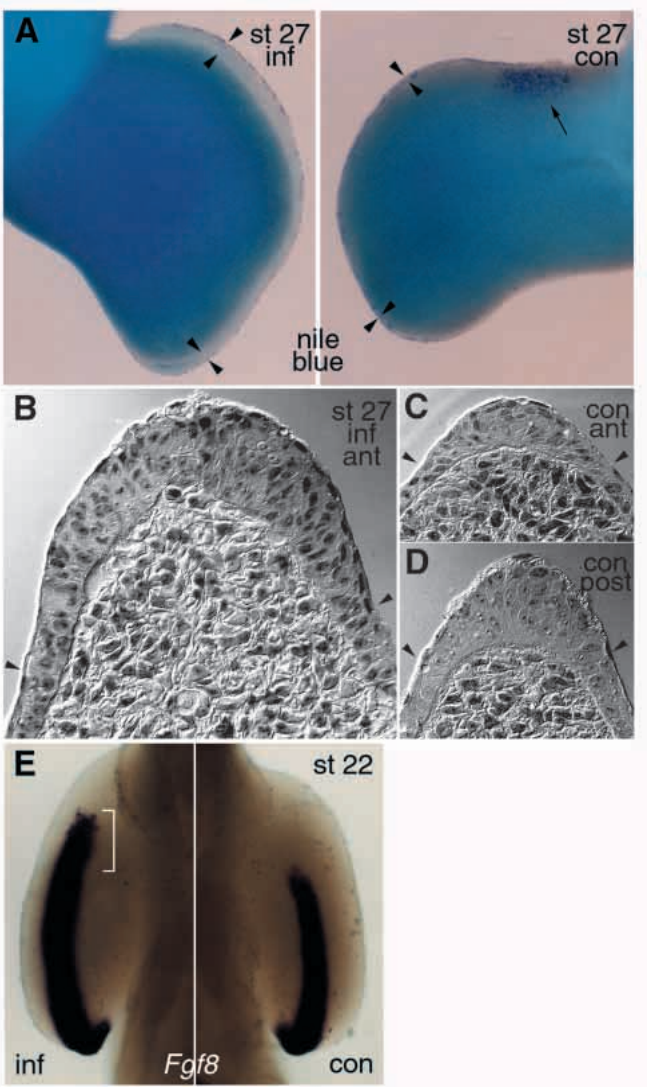

Fig. 4. Inhibition of BMP signaling at early stages results in increased AER height and extension of the AER anteriorly. (A,E) Ventral view, anterior towards top of panel; infected limb (inf) on left, control limb (con) on right. (A) Stage 27 limbs stained with Nile Blue to visualize cell death. In the RCAS-*Noggin*-infected limb (right), the AER along the entire anteroposterior axis is considerably taller than in wild type (left) (arrowheads delimit AER height). Cell death in the AER is similar in infected and control limbs, although the mesenchymal anterior necrotic zone (arrow) is absent in the infected limb. The distal and superficial ectoderm that shows some Nile Blue staining is the periderm layer covering the AER. (B-D) Thin plastic transverse sections through the AER of infected (B; anterior side) and control (C,D; anterior and posterior sides, respectively) limbs of similar stage and phenotype to those in A. In the *Noggin*-infected limb, the AER is taller and the borders between the AER and the non-AER ectoderm are located more dorsally and ventrally than normal (B-D, same magnification; B,C, chosen sections come from comparable levels along the anteroposterior axis of control and infected limbs; arrowheads mark approximate AER borders). (E) In *Noggin*-infected limbs, the AER is anteriorly extended as visualized by the expression of *Fgf8* (stage 22; bracket indicates anterior extension).

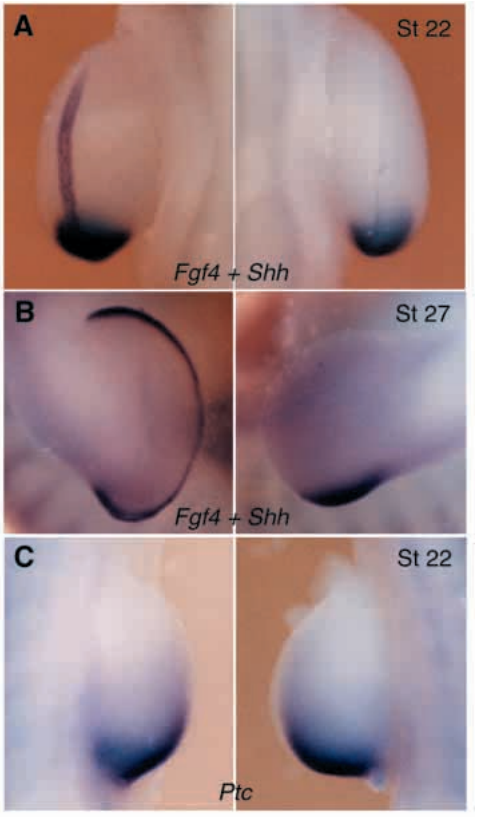

Fig. 5. *Noggin* misexpression induces ectopic expression of *Fgf4* in the absence of ectopic SHH signaling. RCAS-*Noggin* was injected into the limb field at stage 14 and the embryos processed for double RNA in situ hybridization for *Fgf4* and *Shh* at stage 22 (A) and stage 27 (B). *Shh* expression is restricted to its normal domain in the posterior mesenchyme (A) or is greatly reduced (B) in *Noggin*-infected limbs while *Fgf4* is expressed at high levels throughout the AER of infected versus control stage 22 and 27 limbs (A,B). In addition, in infected limbs the level of *Fgf4* expression is increased in the AER posteriorly. (C) *Ptc* expression is similar in stage 22 *Noggin*-infected and control limbs confirming the absence of ectopic SHH signalling. In all panels, anterior at top; infected limb on left, control limb on right. A, ventral view; B,C, dorsal views.

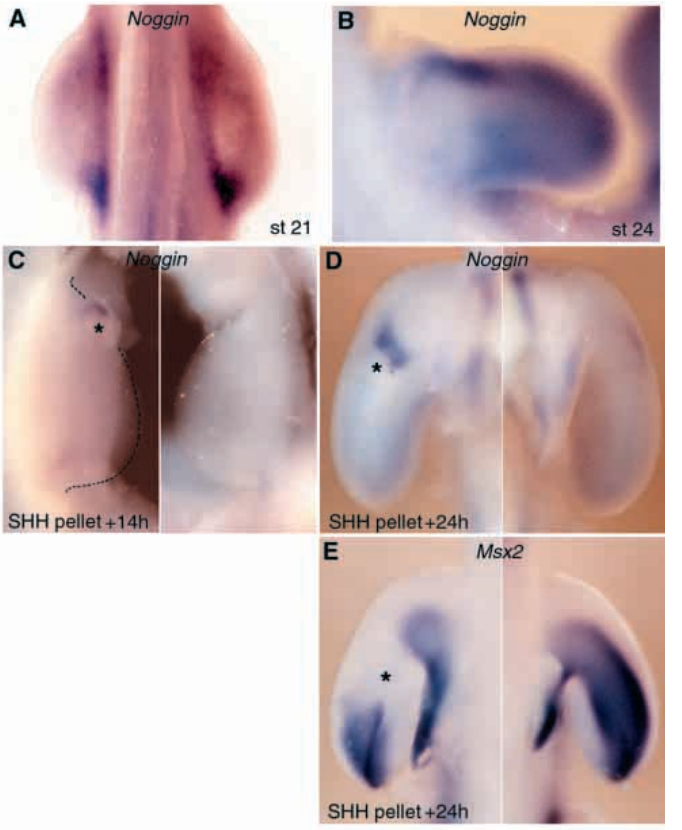

Fig. 6. *Noggin* expression is induced by ectopic SHH. Endogenous *Noggin* expression in stage 21 (A) and stage 24 (B) limbs. (C-E) Expression of *Noggin* (C,D) and *Msx2* (E) 14 hours (C) or 24 hours (D,E) after application of SHH-expressing cell pellet (*) to the anterior limb margin. (C-E) The contralateral control limb is on the right. *Noggin* expression is upregulated in mesenchyme proximal to the pellet whereas *Msx2* expression is downregulated both proximal and distal to the SHH pellet. *Noggin* transcription is induced to significantly higher levels than normal as endogenous transcripts are barely detectable in these samples.

This morphological change mimics what could be an effect of unrestricted AEMF activity. To examine this possibility, we assayed the expression of AEMF candidate genes, *Fgf10* and *Shh* (see two sections below). We found, however, that *Fgf10* expression pattern was unaltered in the infected limbs at stage 21, when the normal distribution of *Fgf10* transcripts is still posteriorly biased (data not shown).

An additional structural difference in the AER of infected limbs was noted: the AER extended significantly further anteriorly than normal at all stages and, in the early limb bud, almost to the body wall as visualized by *Fgf8* mRNA (Fig. 4E). This extension was reflected by expression of other AER markers (*Msx2* [Fig. 8C], *Bmp2*, *Bmp4*, *Bmp7*, *Notch1*; data not shown).

Cell number is increased in the AER of *Noggin*-infected limbs

To better understand the Noggin-induced AER changes, we looked for changes in cell number within the AER. *Noggin*-infected wings or legs at stage 27 contained a significantly higher number of nuclei in their AER relative to stage-matched controls, as outlined in Table 1. Intriguingly, in these samples, the increased nuclei number cannot be attributed to changes in proliferation. Both infected and control limbs showed about 10% BrdU-labeled nuclei. The extent of cell death in the AER at stages 24 and 27 was variable and thus not a consistent aspect of the phenotype. However, in many cases, apoptosis was absent in the mesenchymal anterior and posterior necrotic zones as examined by whole-mount TUNEL or Nile Blue staining at these stages (Fig. 4A and data not shown). This is in agreement with the proposed role for BMP signaling in these apoptotic regions (Yokouchi et al., 1996). The increase in nuclei number may result from a gradual or a stage-specific increase in cell division or decrease in cell death that we did not detect. Fig. 4B shows that, in the infected AER, the extra nuclei are distributed both in height, resulting in a taller AER, and in width as the borders between the AER and the non-AER ectoderm are

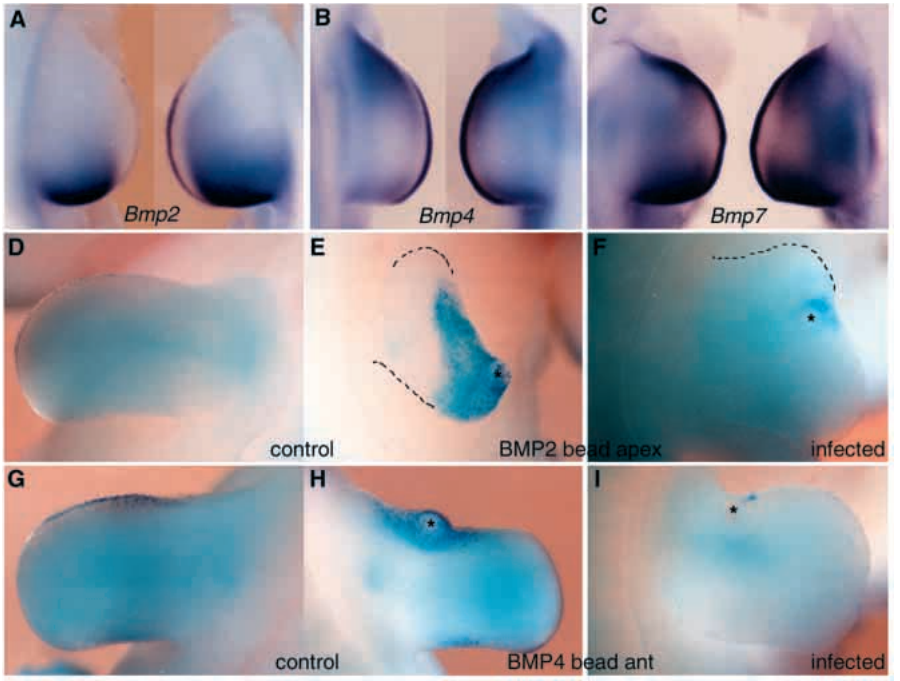

Fig. 7. *Bmp* expression is upregulated following *Noggin* misexpression, but virally expressed Noggin blocks both increased endogenous and exogenous BMP signal. (A-C) Stage 22 limbs hybridized with digoxigenin-labeled probes for (A) *Bmp2*, (B) *Bmp4* and (C) *Bmp7*. *Noggin*-infected limbs are on the right side of each panel and contralateral uninfected control on left side. In all cases, *Bmp* expression is upregulated in the AER and mesenchyme but remains largely confined to the normal domain of expression. Application of a BMP2 (E,F) or BMP4 (H,I)-soaked bead (*) to the apical (E) or anterior (H) margin of an uninfected limb causes extensive cell death as detected by Nile blue staining (contralateral limb shown in D,G) whereas BMP-induced and developmentally programmed cell death are greatly reduced or absent in *Noggin*-infected limbs (F,I). (A-I) Anterior at top, dorsal views.

located more dorsally and ventrally than normal. Therefore, there could also have been a change in the number of cells allocated to the AER.

Fgf4 transcripts are ectopically expressed in the AER of infected limbs

Fgf4 transcripts are normally restricted to the posterior half of the AER (Laufer et al., 1994; Niswander et al., 1994). In *Noggin*-infected limbs, *Fgf4* mRNA was also detected in the anterior half of the AER (Fig. 5A,B 100% of cases). Similarly, when *Noggin*-expressing cells were introduced locally under the AER anteriorly, *Fgf4* expression expanded anteriorly (data not shown). In addition, *Fgf4* expression was stronger than normal along the entire anteroposterior axis of the AER (Fig. 5A,B). Similar results were obtained following restriction of dnBMPR-IB expression to the ectoderm, as the AER was anteriorly extended and ectopic *Fgf4* expression observed.

SHH signal transduction is not ectopically activated

SHH is a key signal from the ZPA (Riddle et al., 1993) and introduction of SHH at the anterior margin of the limb bud induces *Fgf4* expression in the anterior AER (Laufer et al., 1994; Niswander et al., 1994). It also causes the anterior AER to adopt a posterior AER morphology (L. N., unpublished observations). Conceivably, Noggin-induced changes in the AER could result from ectopic expression of *Shh*. This was not so in infected limbs (examined from stages 22 to 27, Fig. 5A,B). Furthermore, in many cases (81% at stage 27) the normal posterior domain of *Shh* expression was greatly reduced or undetectable in infected limbs, while *Fgf4* continued to be highly expressed throughout the expanded AER (Fig. 5B). To determine whether signal transduction downstream of SHH was ectopically activated, we examined a number of putative downstream targets. *Patched* (*Ptc*) encodes the receptor for SHH and is also one of its transcriptional targets (Ingham, 1998). In stage 22 *Noggin*-infected limbs, a time when *Fgf4* is already ectopically expressed in 100% of the cases, *Ptc* was expressed in its normal domain (Fig. 5C). In addition *Bmp2*, *Hoxd11* and *Hoxd13*, genes activated in response to SHH signaling in the anterior margin (Laufer et al., 1994), were also not ectopically activated (Fig. 7A and not shown). These data strongly indicate that SHH signaling is not ectopically induced in *Noggin*-infected limbs. Moreover, they show that, in the presence of Noggin, SHH is not needed to induce ectopic *Fgf4* expression or to maintain endogenous *Fgf4* expression in the posterior AER. One possibility is that, in the normal situation, SHH overrides the negative BMP signal by inducing *Noggin* expression.

Table 1. Cell number is increased in the AER of Noggin-infected limbs

	Anterior third of AER	Posterior third of AER
Control wing	21 (13/24 min/max)	43 (36/48 min/max)
Noggin wing	78 (72/83 min/max)	70 (53/93 min/max)
Control leg - embryo 1	37	44
Control leg – embryo 2	42	49
Noggin leg – embryo 1	67	98
Noggin leg - embryo 2	69	92

Numbers reflect the average number of nuclei in the AER/section.

Noggin expression is induced by ectopic SHH

Noggin mRNA is normally detected at low levels throughout the stage 20 to 22 limb bud with higher levels in the ventral posterior proximal region (Fig. 6A). At stage 24, predominant expression at the anterior and posterior limb margins is observed, with lower expression level distally (Fig. 6B). We examined whether *Noggin* is induced in response to implantation of cells expressing SHH under the AER anteriorly. *Noggin* mRNA was induced 14 hours after SHH

application (Fig. 6C; *n*=4). It was expressed at even higher levels 24 hours after treatment (Fig. 6D; *n*=2). At both time points, the level of induced expression was significantly higher than that found normally. Moreover, in response to SHH application, *Msx2* expression was downregulated in an overlapping pattern to that of *Noggin* (Fig. 6E; *n*=5). None of these changes were observed following application of control cell pellets (not shown).

Expression of *Bmp*s and their potential downstream targets, *Msx* genes, in the absence of BMP signaling

We sought to determine whether BMPs regulate their own expression. We found a marked difference in the level of expression of *Bmp2*, *Bmp4* and *Bmp7* between injected and control limbs at stage 22 and 24: in the presence of Noggin, these genes were upregulated in the mesenchyme and AER (Fig. 7 and data not shown). *Bmp4* and *Bmp7* mRNA were largely confined to their normal location, whereas *Bmp2* transcripts were detected in an anteriorly expanded mesenchymal domain. These data indicate that BMPs do not positively control their expression in the limb at the stages studied. To confirm the ability of Noggin to block this increased endogenous BMP signal, we implanted BMP-soaked beads into *Noggin*-infected and uninfected limbs. Implantation of a BMP2- or BMP4-soaked bead into the anterior or apex of an uninfected limb resulted in extensive cell death (Macias et al. 1997; Zou et al. 1997a; Fig. 7E,H). However, similar application to a *Noggin*-infected limb bud resulted in little or no cell death (Fig. 7F,I). These results indicate that the amount of Noggin produced following RCAS-*Noggin* misexpression is sufficient to not only inhibit endogenous BMP signaling (including the increase in BMP expression) but to also antagonize exogenously supplied BMP.

In a number of developmental models, *Msx1* and *Msx2* are putative downstream targets of BMP (Vainio et al., 1993; Graham et al., 1994; Liem et al., 1995). Experiments in the chick limb in ovo indicate that *Msx1* expression is under both AER-dependent and -independent control (Ros et al., 1992; Fallon et al., 1994). *Msx2* expression in the anterior mesenchyme is AER-independent (Ros et al., 1992). We therefore sought to examine whether BMP signaling regulates *Msx* 1 and *Msx2* expression in the limb. These genes were differentially affected in *Noggin*-infected limbs (Fig. 8). At stage 22, *Msx1* transcript distribution in the progress zone was normal, although the expression level was slightly reduced (Fig. 8A). However, by stage 24, *Msx1* transcripts were undetectable in the posterior progress zone, while still present in the anterior mesenchyme (Fig. 8B). For *Msx2*, although AER expression persisted at stage 22, its mRNA was dramatically downregulated in the anterior and posterior mesenchyme (Fig. 8C), regions suggested to correspond to the anterior and posterior necrotic zones. By stage 24, *Msx2* expression was absent from the mesenchyme and greatly reduced in the AER (Fig. 8D). These results highlight the complexity of *Msx* transcriptional control. The AER expression of *Msx2* displays an intermediate sensitivity to the loss of BMP signaling. On the contrary, the anterior domain of *Msx1* expression is in part independent of BMP signaling, whereas the anterior *Msx2* domain and the posterior domains of both *Msx1* and *Msx2* require BMP activity for continued expression.

DISCUSSION

BMP signaling negatively regulates AER structure and function

Here we have used *Noggin* misexpression to study the roles of BMPs during limb development. Our results indicate that BMP signaling regulates AER structure and function throughout all stages. In response to Noggin, the anterior part of the AER is extended towards the body wall, is taller and expresses *Fgf4*, a gene that is normally posteriorly restricted in the AER. This occurs in the absence of ectopic Hedgehog signaling. Moreover, the AER posteriorly also appears taller than normal and expresses increased levels of *Fgf4* mRNA even in the absence of SHH. Thus, in the presence of a BMP inhibitor, SHH signaling is not required to induce *Fgf4* mRNA in the anterior AER, to maintain *Fgf4* expression in the posterior AER or to maintain the tall posterior AER morphology. As Noggin blocks BMP signaling, these results indicate that BMPs normally act as repressive signals along the entire anteroposterior axis controlling AER morphology and gene

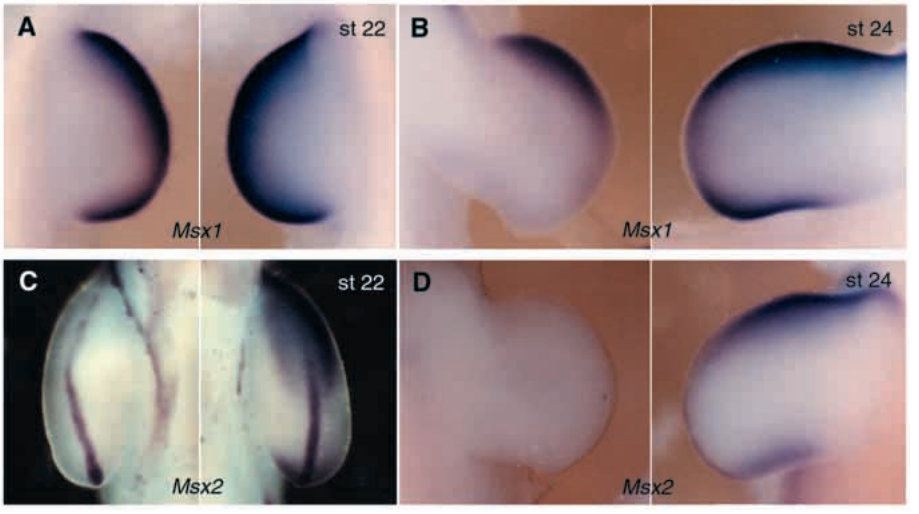

Fig. 8. *Msx1* and *Msx2* domains of expression are differentially affected by *Noggin* misexpression. (A-D) Infected limb on left in each panel, control on right, anterior at top and dorsal views except for C. Whole-mount RNA in situ hybridization with digoxigenin-labeled probes for (A,B) *Msx1*, (C,D) *Msx2*. (A) At stage 22, *Msx1* expression is slightly reduced, in particular in the posterior progress zone. (B) By stage 24, *Msx1* is lost in the posterior domain but maintained, although at lower levels, in the anterior mesenchyme. (C) At stage 22, *Msx2* transcription is strongly downregulated in the anterior and posterior mesenchymal domains but continues to be expressed in the AER, including the anteriorly extended portion. (D) By stage 24, *Msx2* expression is lost in the mesenchyme and greatly reduced in the AER.

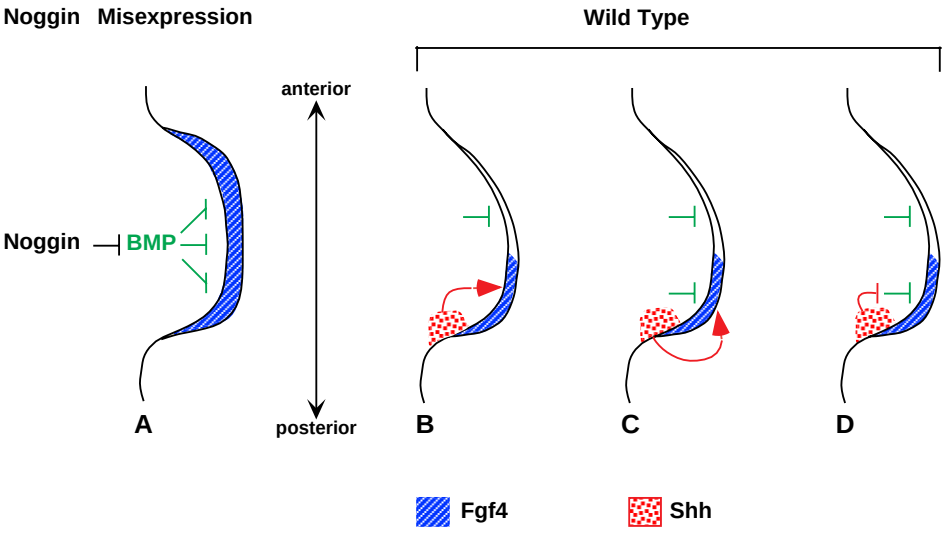

Fig. 9. Models for regulation of early AER function. (A) Summary of the observations. *Noggin* misexpression causes the AER along its anteroposterior extent to become taller than normal and *Fgf4* expression (blue), which is normally restricted posteriorly in the AER, to be induced in the AER anteriorly. *Fgf4* is also expressed at higher levels than normal throughout the AER. Noggin acts by blocking BMP signaling (in green) involved in the negative regulation of AER structure and function. The BMP inhibitory signal may arise from the AER, the mesenchyme, or both. Several models could account for these results. (B) The BMP inhibitory signal originates only anteriorly in the limb bud and a positive signal, most likely SHH (red stippling), originates posteriorly. (C,D) The BMP inhibitory signal is widely distributed in the limb. SHH in the posterior mesenchyme positively regulates *Fgf4* expression and AER morphology either independently of BMP signaling (C), or by inhibiting the BMP inhibitory signal (D). Our results favor model D.

expression (Fig. 9A). Furthermore, our results indicate that BMPs are required for AER regression as ectopic expression of *Noggin* or *dnBmpR-IB* inhibits AER regression and maintains *Fgf* expression and distal proliferation. Thus, BMP signaling appears to limit distal limb growth by promoting AER regression.

Consistent with our results, gene targeting studies in mice also indicate a role for BMPs in regulating AER function. *Bmp7*-deficient embryos possess an anteriorly extended AER, and these embryos and *Bmp4* heterozygous mutants display preaxial polydactyly thought to be due to additional growth on the anterior side of the limb (references in Hogan, 1996; Dunn et al., 1997). Similarly, *Noggin*-infected limbs exhibit an anteriorly extended AER and additional anterior tissue growth (see discussion below). Our results indicate that the knock-out phenotypes are due to reduced BMP signaling and hence weakened negative regulation of AER function

BMP signaling is required for AER regression

It has been suggested that an imbalance between proliferation and cell death, favoring cell death, is the mechanism leading to AER regression (Vaahtokari et al., 1996; Ferrari et al., 1998). As BMPs can mediate apoptosis, it could be that in our experiments Noggin maintains the late AER by inhibiting this BMP cell death signal. However, apoptosis is unlikely to be the sole explanation for the mechanism of AER regression. Indeed, interposing a graft of quail ectoderm subjacent to the AER of the chick leg bud results in the incorporation of cells of quail origin into the AER and subsequently into the claws (Saunders et al., 1976; J. Saunders, personal communication). This observation indicates that some cells of the AER persist after its regression. Whether BMPs control AER regression by mediating apoptosis, negatively regulating proliferation, or other unknown mechanism awaits further investigation.

In *Noggin*-infected limbs, the persistent AER continues to express AER-specific markers, in particular *Fgf4* and *Fgf8*. Mesenchyme proliferation and excess distal soft tissue are observed in regions underlying the *Fgf*-expressing AER. Lethality following early widespread *Noggin* misexpression and the potent effect of Noggin on chondrogenesis, necessitated minimal infection of the embryo to study AER regression. Consequently, the excess soft-tissue phenotype is relatively limited. Nonetheless, the close correlation between continued *Fgf* expression and continued mesenchyme proliferation suggests that the duration of *Fgf* expression in the AER is a critical factor in controlling the extent of limb growth.

During late stages of normal limb development, *Noggin* is expressed in cartilage condensations where it locally antagonizes BMP function necessary for cartilage formation. *Noggin*$^{-/-}$ mice exhibit expanded regions of chondrogenesis, inhibition of joint formation and digit shortening (Brunet et al., 1998). With respect to the studies reported here, *Noggin* is expressed at the digit tips in the condensing cartilage (Brunet et al., 1998; Capdevila and Johnson, 1998; Merino et al., 1998). This is of interest as the AER overlying the digits regresses later than the AER over the interdigital region. This pattern of *Noggin* expression could in part regulate the onset of AER regression by modulating the negative BMP signal. Although this has not been analyzed, it would be interesting to determine whether premature AER regression contributes to the digit shortening observed in *Noggin*$^{-/-}$ mice.

BMPs play an earlier role in regulating AER morphology and *Fgf4* expression

We envision three possible models to explain the early structural and molecular asymmetry in the AER. In the first model (Fig. 9B), a negative signal (BMP) originates anteriorly and a positive signal (most likely SHH) originates posteriorly in the limb bud. This model, however, is not consistent with the overall *Bmp* expression pattern or with our results, which indicate that posterior AER morphology and *Fgf4* expression are affected in *Noggin*-infected limbs. Therefore, Fig. 9C,D illustrate a repressive signal throughout the anteroposterior aspect of the limb. In Fig. 9C, SHH acts independently to positively regulate posterior AER morphology and *Fgf4* expression. Introduction of SHH anteriorly into the limb is

sufficient to induce *Fgf4* expression and alter AER morphology, however in the presence of Noggin, these changes occur independently of *Shh* expression. Therefore, we favor the third model in which SHH in the posterior mesenchyme acts by inhibiting the BMP inhibitory signal (Fig. 9D). One possibility is that the endogenous BMP inhibitor is Noggin. Although the *Noggin* expression pattern does not correlate with that of *Shh*, we found that *Noggin* transcripts are highly induced by application of SHH to the anterior mesenchyme. Moreover, *Msx2*, a presumed BMP target, is downregulated by Noggin overexpression, as well as by SHH application. These results suggest that ectopic SHH represses BMP signaling via induction of Noggin and are thus consistent with our model of SHH action in the posterior mesenchyme. In addition, a number of other BMP inhibitors have been recently described (Piccolo et al., 1996; Zimmerman et al., 1996; Hsu et al., 1998) and these may repress the negative BMP signal in response to endogenous SHH.

Molecular basis of AEMF activity

The effect of *Noggin* misexpression is most evident on the AER anteriorly as, in the normal limb, the BMP repressive signal appears to be largely overridden posteriorly. Previous experimental manipulations of the chick limb were interpreted such that the mesenchyme produces a signal posteriorly, the AEMF, that maintains the tall shape of the posterior AER (Zwilling, 1956; Saunders and Gasseling, 1963). Our results indicate that this activity modulates the BMP repressive effect. SHH, as indicated above, or a downstream effector could function as the AEMF. Although FGF10 is a candidate and its expression is regulated by SHH (Martin, 1998), no change in *Fgf10* expression was seen in response to Noggin.

It is possible that FGFs from within the AER modulate the BMP effect. Previous studies demonstrated that FGF4 antagonizes the negative effect of BMP on limb mesenchyme proliferation (Niswander and Martin, 1993). In addition, *Fgf*s and *Fgf* receptors are expressed in the AER (Martin, 1998) and may serve an autocrine role in positively regulating AER morphology. Differing levels of FGF could differentially influence AER structure. *Fgf4* is expressed in the AER posteriorly whereas other *Fgf*s are expressed throughout the AER. This posterior bias of FGF levels may be part of the mechanism by which the taller morphology of the posterior AER is maintained. In our experiments, *Fgf4* induction anteriorly was observed approximately 1 day earlier than the tall anterior AER shape. This is consistent with a link between *Fgf4* expression and increased AER height. In the later AER, *Fgf4* transcription normally ceases prior to AER regression. Similarly, it is possible that loss of FGF4 tips the balance in favor of BMP thus allowing negative signals to predominate and resulting in AER regression (see also Macias et al., 1996).

Events underlying the Noggin-induced limb shape

The hand and foot plates of *Noggin*-infected limbs are wider than normal along the anteroposterior axis. The most prominent change was observed on the anterior side although widening posteriorly was also seen. This wide limb shape is due in part to changes in proliferation of the anterior subridge mesenchyme (as analyzed by BrdU incorporation, data not shown), most likely a consequence of the anterior extension of the AER and ectopic and prolonged *Fgf4* expression. This could as well reflect the repression of BMP signaling that may inhibit cell proliferation (Niswander and Martin, 1993). Lack of cell death in the anterior and posterior necrotic zones also likely contributes to the wide shape. In this respect, loss of the *Msx2* mesenchymal expression domains associated with these cell death regions (Yokouchi et al., 1991) may be mechanistically relevant to the loss of the BMP-mediated apoptosis.

A wide limb bud shape is reminiscent of that seen in a number of mouse and chick limb mutants in which *Fgf4* is expressed anteriorly in the AER, whereas *Shh* is or is not ectopically activated in the anterior mesenchyme (references in Niswander, 1997; Rodriguez et al., 1996). In these mutants, extra digits form on the anterior side of the limb, the polarity of which correlates with *Shh* expression. In limbs with widespread *Noggin* misexpression, the number of digits and their polarity can not be determined due to the accompanying dramatic inhibition of chondrogenesis.

Fgf4, a target of BMP-mediated negative regulation

Strikingly, in both early and late *Noggin*-infected limbs, *Fgf4* transcription is deregulated. In early infected limbs, the level of *Fgf4* mRNA is increased throughout the entire AER (Fig. 5A,B), although an ectopic Shh pathway is not induced, indicating that *Fgf4* expression is actively repressed in the AER anteriorly and posteriorly. In older uninfected limbs, *Fgf4* mRNA is downregulated well before final AER regression, indicating regulation of its transcription is independent of AER regression. In *Noggin*-infected limbs, *Fgf4* transcription is prolonged in the late stage AER and we propose that this is not linked to inhibition of AER regression. Hence, the effect of Noggin on *Fgf4* expression in the AER at all stages suggests that the spatially and temporally restricted *Fgf4* expression pattern is due to either direct or indirect negative regulation by BMP. The existence of a negative control is supported by analysis of *Fgf4* promoter elements by creation of transgenic mice (Fraidenraich et al., 1999). These experiments identified a repressive element that limits *Fgf4* expression to the posterior AER. In its absence, *Fgf4* is expressed throughout the AER.

Negative and positive roles for BMPs in the limb

Bmp2, *Bmp4* and *Bmp7* are expressed in the limb mesenchyme and throughout the AER, and one of the receptors (*BmpRII*) is also expressed throughout the AER. In our studies, we cannot distinguish whether the negative regulation of the AER is mediated by BMP signaling from the AER, the mesenchyme or both. It is possible that different BMPs (homodimers or heterodimers) may have different effects on limb development, acting either negatively or positively. Indeed, in *Xenopus*, BMP heterodimers and homodimers differentially affect gene expression (Suzuki et al., 1997; Nishimatsu and Thomsen, 1998). Duprez et al. (1996b) have suggested a positive role for BMP2 in relaying SHH polarizing activity, based on the ability of cells expressing BMP2 implanted in the anterior margin of the limb to induce ectopic *Fgf4* and *Hoxd13* expression, and sometimes digit **2** duplications. However, a BMP-soaked bead applied under the anterior AER leads to cell death and limb truncations (Macias et al., 1997; Zou et al., 1997a) or normal digit formation (Francis et al., 1994). This difference is likely related to the manner in which exogenous BMP is delivered (BMP-expressing cells versus recombinant protein). Our results reported here indicate that blockade of global BMP activity

results in activation of *Fgf4* expression, rather than in its extinction as would be predicted from the Duprez results. Nonetheless, it is still conceivable that BMP2 or other BMP protein combinations may positively influence *Fgf4* expression. In support of this, in RCAS-*Noggin*-injected embryos, the posterior progress zone expression of *Msx1* previously reported to require AER signals, is downregulated indicating a positive interplay between FGF and BMP in the posterior mesenchyme. This, together with the loss of endogenous *Shh* expression and the concomitant downregulation of *Hoxd13* expression (data not shown), in response to Noggin, supports the idea that some aspect of BMP signaling has a positive action in the posterior part of the limb bud.

Noggin specifically and efficiently inhibits BMP signaling

Noggin misexpression is very effective in blocking BMP functions in ovo as determined by (1) dramatic downregulation of *Msx1* and *Msx2* expression, (2) complete inhibition of chondrogenesis, and (3) loss of cell death in the anterior, posterior and interdigital apoptotic regions; all processes shown to involve BMP signaling. *Bmp2*, *Bmp4* and *Bmp7* are themselves expressed at higher levels than normal in *Noggin*-infected limbs; however, they are unlikely to signal as the activity of additional BMP exogenously supplied is still blocked. Nevertheless, we cannot rule out that BMP heterodimers are formed, which are not inhibited by Noggin, and that these heterodimers have activities unrelated to known BMP functions.

Our results with Noggin are corroborated by studies of dominant-negative BMPR misexpression in the limb, which also leads to ectopic and prolonged *Fgf* expression (reported here), decreased apoptosis and inhibition of chondrogenesis (Kawakami et al., 1996; Yokouchi et al., 1996; Zou and Niswander, 1996; Zou et al., 1997b). Interestingly, widespread misexpression of dnBMPR-IB causes only distal truncations of the autopodial cartilage elements, although *BmpR-IB* is expressed in all limb cartilage condensations, whereas Noggin completely inhibits all chondrogenesis in the limb. Noggin is secreted and therefore can act cell non-autonomously whereas dnBMPR-IB must be present at sufficiently high levels within the appropriate cell in order to block wild-type receptor signaling. As Noggin binds multiple BMPs and blocks specifically, and with very high efficiency, known BMP functions, *Noggin* misexpression can serve as an effective means to address potentially redundant BMP actions.

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