

BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb

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SUMMARY

Dorsoventral (DV) patterning of the vertebrate limb requires the function of the transcription factor Engrailed 1 (EN1) in the ventral ectoderm. EN1 restricts, to the dorsal half of the limb, the expression of the two genes known to specify dorsal pattern. Limb growth along the proximodistal (PD) axis is controlled by the apical ectodermal ridge (AER), a specialized epithelium that forms at the distal junction between dorsal and ventral ectoderm. Using retroviral-mediated misexpression of the bone morphogenetic protein (BMP) antagonist Noggin or an activated form of the BMP receptor in the chick limb, we demonstrate that BMP plays a key role in both DV patterning and AER induction. Thus, the DV and PD axes are linked by a common signal. Loss and gain of BMP function experiments show that BMP signaling is both

necessary and sufficient to regulate EN1 expression, and consequently DV patterning. Our results also indicate that BMPs are required during induction of the AER. Manipulation of BMP signaling results in either disruptions in the endogenous AER, leading to absent or severely truncated limbs or the formation of ectopic AERs that can direct outgrowth. Moreover, BMP controls the expression of the MSX transcription factors, and our results suggest that MSX acts downstream of BMP in AER induction. We propose that the BMP signal bifurcates at the level of EN1 and MSX to mediate differentially DV patterning and AER induction, respectively.

Key words: Apical ectodermal ridge, Bone morphogenetic protein, Dorsal-ventral, Engrailed 1, Chick, Limb, Msx

INTRODUCTION

Dorsoventral (DV) patterning and growth of the vertebrate limb both require signals from the limb ectoderm to the underlying mesenchyme. Transplantation studies indicate that just before the emergence of the limbs, the information providing DV patterning is encoded within the ectoderm which then imposes DV patterning onto the distal mesoderm (Chen and Johnson, 1999). Within the last decade, the molecular basis of DV patterning has begun to unfold. Engrailed 1 (EN1) in the ventral ectoderm represses the expression of *Wnt7a*, thus restricting *Wnt7a* mRNA to the dorsal ectoderm. WNT7A induces the expression of *Lmx1* in the dorsal mesenchyme, ultimately leading to specification of dorsal fates (Chen et al., 1998; Loomis et al., 1996; Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). However, our understanding of DV patterning is incomplete. For example, the role of EN1 in DV patterning is not entirely mediated through its regulation of *Wnt7a* expression (Loomis et al., 1998). Additionally, it appears EN1 and WNT7A are not the only molecules expressed in the ectoderm that control DV patterning, as in the double knockout, *Lmx1b* is still expressed in the distal dorsal posterior mesenchyme (Chen and Johnson, 1999).

Furthermore, cell marking experiments in the mouse and chick demonstrate that the limb ectoderm is divided into D and V compartments before limb formation (Altabef et al., 1997; Kimmel et al., 2000; Michaud et al., 1997) but the molecules that serve to establish these compartments are yet unknown. Therefore it is important to identify new components in the pathway that regulates DV limb patterning.

PD elongation of the limb depends on the AER, a specialized group of pseudostratified columnar epithelial cells located along the DV border of the developing limb bud. The AER stimulates proliferation of the underlying mesenchyme through the secretion of numerous fibroblast growth factors (FGFs), one of which is *Fgf8*, an early marker of the AER (Martin, 1998). Failure of AER formation or maintenance results in absence or truncation of the limb, respectively. Formation of the AER is a complex process, involving signals from both the mesenchyme and the ectoderm. Tissue grafting experiments have shown that a signal from the limb mesoderm induces AER formation in the overlying ectoderm (Carrington and Fallon, 1984; Kieny, 1960; Kieny, 1968; Saunders and Reuss, 1974) and experimental and genetic studies have identified *Fgf10* and its receptor FGFR2 as molecules responsible for this aspect of AER morphogenesis (Arman et al., 1999; Min et al., 1998;

Ohuchi et al., 1997a; Ohuchi et al., 1997b; Sekine et al., 1999; Xu et al., 1998; Yonei-Tamura et al., 1999). Although the inductive signal comes from the mesenchyme, the position of the AER is thought to be directed, at least in the chick, by an ectodermal signal (Crossley et al., 1996; Fraser and Abbott, 1971; Goetinck, 1964; Kieny, 1960), such that ectodermal cells express *Fgf8* and form an AER at a specific position along the DV axis (Cohn et al., 1995; Crossley et al., 1996). It appears that there may be differences between mouse and chick in the mechanism setting the AER position, as the AER progenitors (cells expressing *Fgf8* but still cuboidal in shape) are initially detected throughout the ventral ectoderm in mouse, whereas they are already roughly located at the DV border in the chick (Crossley and Martin, 1995; Crossley et al., 1996) (Fig. 1A). We will refer to AER induction as the step in the chick that results in the formation of cuboidal AER precursors that express *Fgf8* and are positioned near the DV boundary. The subsequent formation of a morphologically distinct AER (dome-shaped, presence of a pseudostratified columnar epithelium) that involves compaction of AER precursors in both mouse and chick, and the establishment of AER borders at least in mouse, will be referred to as AER maturation. Later events such as AER maintenance will not be considered.

Because the AER resides at the interface between dorsal and ventral ectoderm, and because several mutants (chick *limbless* and *wingless*, and mouse *legless*) are defective in both DV patterning and AER induction or maintenance (Bell et al., 1998; Grieshammer et al., 1996; Ohuchi et al., 1997a; Ohuchi et al., 1997b; Ros et al., 1996), DV polarity of the ectoderm and AER formation may be linked at some level. Interestingly, the primary defect in the aforementioned mutants resides in the ectoderm, and ventral limb ectoderm markers are not expressed. Thus, if the ventral ectoderm contains crucial information for both AER formation and DV patterning, the loss of ventral ectoderm activity in the above mutants could explain their observed phenotypes. In further support of a link, experimental juxtaposition of dorsal and ventral limb ectoderm results in the formation of an ectopic AER at the new dorsal and ventral ectoderm boundary (Laufer et al., 1997; Tanaka et al., 1997).

Interestingly, however, the two genes known to govern DV patterning in the limb ectoderm, *En1* and *Wnt7a*, are not required for AER induction, although EN1 is necessary for AER maturation (Cygan et al., 1997; Loomis et al., 1996; Loomis et al., 1998; Parr and McMahon, 1995). This paradox could be resolved if one postulates that the initial signal that links DV patterning and AER induction regulates these two processes by activating separate pathways and that EN1 acts downstream of this signal in the DV patterning branch. Two pieces of evidence are in agreement with this proposal. First, the chick mutant *eudiplopodia* exhibits ectopic AERs on the dorsal side of the limb, whereas both *En1* and *Wnt7a* transcripts are localized normally (Laufer et al., 1997). Second, EN1 is not the most upstream signal in ventral patterning as its misexpression does not alter the ectodermal DV compartment boundary (Altabef et al., 2000). Thus, there may exist upstream of EN1, a molecule that is crucial for AER induction and DV patterning.

During vertebrate limb development, BMPs are expressed in dynamic patterns and have been implicated in multiple processes. These include a negative role in the maintenance of

the AER after it has formed, anteroposterior patterning, chondrogenesis and programmed cell death (Drossopoulou et al., 2000; Dudley et al., 1995; Dunn et al., 1997; Kawakami et al., 1996; Luo et al., 1995; Macias et al., 1997; Pizette and Niswander, 1999; Pizette and Niswander, 2000; Yokouchi et al., 1996; Zou and Niswander, 1996; Zou et al., 1997). Yet, little is known of the molecular mechanisms by which BMPs mediate such a wide range of developmental effects, although the answer lies in part in the access to different sets of transcription factors that elicit different transcriptional responses depending on the developmental history of the cell.

We report two novel roles for BMPs in vertebrate limb development. We show that BMPs are expressed in the early ventral limb ectoderm and that BMP signaling is required for both DV patterning and AER induction, providing a molecular link between these processes. We demonstrate that BMP controls DV patterning by acting upstream of EN1. Moreover, our data suggest that BMP regulates AER induction through a separate pathway (i.e. non-EN1) that involves the MSX transcription factors.

MATERIALS AND METHODS

RNA in situ hybridization was performed as referenced in Zou et al. (Zou et al., 1997) with antisense probes prepared as referenced in (Dealy et al., 1993; Pizette and Niswander, 1999; Vogel et al., 1995). Anti-chicken EN1 and anti-viral gag (AMV3C2) antibodies were obtained from Developmental Studies Hybridoma Bank. RCAS viruses (Hu et al., 1998; Logan et al., 1997; Pizette and Niswander, 1999; Vogel et al., 1995; Yang and Niswander, 1995; Zou et al., 1997) were prepared as described by Morgan and Fekete (Morgan and Fekete, 1996). Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992) and by counting somite number. Except for RCAS-*Lmx1* virus, which was injected into the presumptive limb field, all other viruses were laid on top of the blastoderm beneath the vitelline membrane at stage 7-10. At the time of AER formation this led to widespread infection of the limb ectoderm, except in cases when the virus was diluted. It is however important to note that the infection does not stay restricted to the ectoderm, owing to spread of the virus, and that the mesenchyme ultimately becomes infected.

RESULTS

Bmps are expressed in the ventral limb ectoderm and regulate DV patterning

During early stages of chick limb development, *Bmp2*, *Bmp4* and *Bmp7* are expressed in the mesenchyme in an unrestricted manner along the DV axis, and in the AER once formed (Fig. 1M-O) (Francis et al., 1994; Francis-West et al., 1995). Importantly, we found here that these Bmps are expressed in the early chick limb in the ventral ectoderm, coincident with EN1 and in a complementary pattern to that of *Wnt7a* in the dorsal ectoderm (Fig. 1A-C,G-I) at the time the ectoderm provides DV information to the underlying mesenchyme (Geduspan and MacCabe, 1989). In the mouse, *Bmp2* expression has also been reported in the early ventral limb ectoderm (Lyons et al., 1990).

To test the potential role of BMPs in DV patterning, we altered BMP signaling by misexpressing the secreted BMP

antagonist *Noggin* (Zimmerman et al., 1996, Pizette and Niswander, 1999), or a constitutively activated BMP receptor (BMPRIA, BMPRIB) (Zou et al., 1997). We infected the presumptive limb ectoderm at stages 7-10 with RCAS viruses encoding these proteins, and examined DV molecular markers at later stages (stages 18-27). We found that *Noggin* misexpression resulted in partial or total absence of EN1 in ectoderm on the ventral side of the limb (compare Fig. 2D with 2A; 100% of limbs lacked EN1 in some or all ectoderm cells on the ventral side in correlation with viral expression; $n=8$ limbs) and ectopic *Wnt7a* and *Lmx1* expression in ectoderm and mesenchyme on the ventral side, respectively (compare Fig. 2E,F with 2B,C; *Wnt7a* 48%, $n=33$ limbs; *Lmx1* 100%, $n=49$ limbs). Thus, based on molecular criteria, loss of BMP signaling dorsalizes the limb. Conversely, constitutively activated *BmpRIA* or constitutively activated *BmpRIB* misexpression resulted in ectopic expression of EN1 in ectoderm on the dorsal side of the limb (100%, $n=28$ limbs) and downregulation of *Wnt7a* (89%, $n=56$ limbs) and *Lmx1* (100%, $n=8$ limbs) in or underlying regions of ectopic EN1 expression, respectively (Fig. 2H-J and not shown; viral expression [GAG] shown in Fig. 2G,K). Thus, increased BMP signaling ventralizes the limb. As EN1 expression is regulated by BMP and as gain or loss of function of BMP (this study) and EN1 (Cygan et al., 1997; Logan et al., 1997; Loomis et al., 1996; Loomis et al., 1998) have similar effects on *Wnt7a* and *Lmx1* expression, our data thus indicate that BMPs are ventralizing factors that act upstream of EN1. This epistatic relationship is supported by the fact that misexpression of *En1* does not affect expression of *Bmp2*, *Bmp4* and *Bmp7* in the non-AER ectoderm (data not shown). In addition, it appears that ectodermal but not mesenchymal BMP signaling is likely to be involved, as the expression of *Bmps* in the mesenchyme is unrestricted along the DV axis and ectodermally limited misexpression of the cell autonomous constitutively activated *BmpR* is sufficient to produce the described phenotypes (Fig. 2H-K).

To look for other indications of DV patterning changes, we examined the expression of sonic hedgehog (*Shh*), a gene crucial for anteroposterior patterning (Riddle et al., 1993), which is positively regulated by WNT7A (Parr and McMahon, 1995; Yang and Niswander, 1995). In the chick, *Shh* RNA is normally largely restricted to dorsal posterior mesenchyme (Fig. 2L). *Noggin* virus infection targeted to the ectoderm resulted in expansion of *Shh* into ventral posterior mesenchyme (Fig. 2M; 33%, $n=12$ limbs), similar to what is observed after loss of *En1* function (Cygan et al., 1997) or misexpression of *Wnt7a* (not shown; 100%, $n=10$ limbs) or *Lmx1* (Fig. 2N; 44%, $n=18$ limbs). Interestingly, in *Noggin*-infected limbs, ventral expansion of *Shh* expression (33%) and ectopic *Wnt7a* (see earlier results, 48%) occur at a lower frequency than ectopic *Lmx1* expression (100%). In addition, *Wnt7a*, but not *Lmx1*, misexpression always induced ectopic *Shh* expression. This suggests that LMX1 does not directly mediate the positive regulation of *Shh*

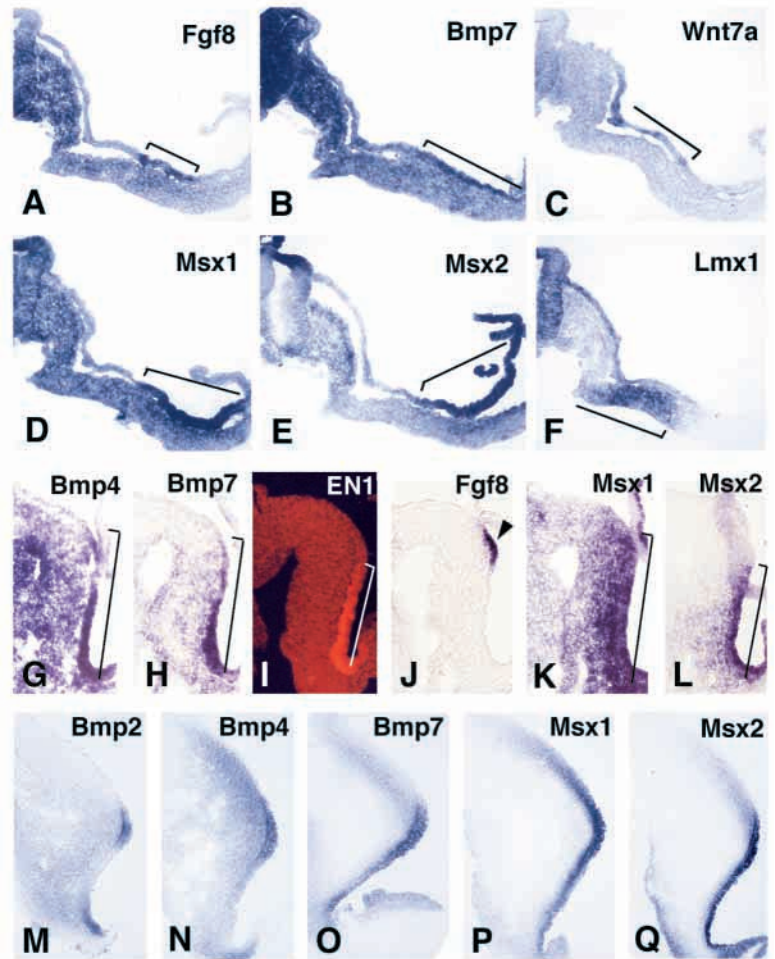


Fig. 1. Time course of *Bmp* and *Msx* RNA expression in ventral ectoderm of the chick early limb bud. RNA in situ hybridization of alternate sections from the hindlimb region of (A-F) an early stage 17 (29 somite) embryo and (G-L) a late stage 17 (32 somite) embryo; (M-Q) whole-mount of late stage 18 embryos which were subsequently cryosectioned (forelimbs). Hybridization with probes as indicated and immunolocalization of EN1 protein (I). Brackets outline the domains of expression in the ectoderm (except in F, where the bracket indicates mesenchymal domain), arrowhead in J indicates position of the AER. (A-F) Dorsal (medial) towards the left and ventral (lateral) to the right. (G-L and M-Q) Dorsal at top, ventral at bottom. In N-Q, expression on the dorsal side is in the mesenchyme, not the ectoderm.

expression by WNT7A in the limb, consistent with the decreased expression of *Shh* described for *Wnt7a*^{-/-}, but not for *Lmx1*^{-/-} mouse embryos (Chen et al., 1998; Parr and McMahon, 1995). Together, our results indicate that BMP signaling is necessary and sufficient to regulate genes whose differential expression is critical for proper DV patterning, as well as a gene restricted by DV patterning signals.

Constitutively activated BMPR misexpression causes AER disruptions as well as ectopic *Fgf8* expression in dorsal ectoderm

In addition to the alterations in DV patterning, we observed alterations in AER formation caused by gain or loss of BMP function. Widespread ectodermal misexpression of constitutively activated *BmpR* (as determined by in situ or immunohistochemistry for RCAS virus, Fig. 2K and not

shown) led to a partial or complete loss of the AER, as revealed by expression of the AER marker *Fgf8* (Fig. 3A-C and Fig. 2H). Because the AER is necessary for limb outgrowth, the consequences included absent, or severely truncated or notched limbs (Fig. 3B, 100%, $n=52$ limbs).

Our previous studies using a different infection protocol that targets later processes in limb development showed that constitutively activated BMPR virus injection leads to degeneration of the AER (Zou et al., 1997), presumably owing to the negative regulation of AER maintenance by BMP (Pizette and Niswander, 1999). Our infection protocol targets early limb stages when the AER is being induced. Indeed, using this early infection protocol, we find partial or complete absence of *Fgf8* expression at early stage 17, when *Fgf8* is normally first activated in AER precursors in the hindlimb (Fig. 3C; $n=10$ limbs from 29-30 somite stage embryos). This indicates that AER induction rather than maintenance is affected. In addition, no ectopic cell death as assayed by TUNEL staining was observed at stages 17 and 18 in constitutively activated BMPR-infected limbs ($n=8$ limbs). Thus, misregulation of BMP signaling is detrimental to AER induction but not through a cell death mechanism. Moreover, ectodermal BMP signaling is likely to be involved, as AER disruptions were observed even when constitutively activated BMPR misexpression was still limited to the ectoderm at the time of analysis (for example, see Fig. 2H).

Strikingly, we found that misexpression of constitutively activated *BmpR* also resulted in ectopic *Fgf8* expression in the dorsal limb ectoderm, in samples with or without disruptions of the endogenous AER (Fig. 3F,G,J,N; $n=14$, 86% as assayed by whole-mount RNA hybridization at stages 17-19). No ectopic expression was ever observed in the ventral ectoderm (Fig. 3I) where *Bmps* are normally expressed. These ectopic patches of *Fgf8* could be extensive but often were found as clumps of a few cells scattered throughout the dorsal ectoderm (compare Fig. 3F with 3G which are left and right sides of the same embryo). *Fgf8* misexpression was noted at stages 17-19 and corresponded to regions of viral expression (Fig. 3J,K and not shown).

In constitutively activated BMPR-infected samples analyzed at later stages we did not note the presence of ectopic *Fgf8*, AERs or outgrowth. As shown in the previous section, constitutively activated BMPR virus infection also results in strong misexpression of EN1. EN1 misexpression has been shown to cause loss of *Fgf8* expression and AER disruptions (Kimmel et al., 2000; Laufer et al., 1997; Logan et al., 1997; Rodriguez-Esteban et al., 1997). Indeed, we noted a correlation in that ectopic *Fgf8* expression was observed in regions that displayed no or low levels of ectopic EN1 (compare Fig. 3J with 3M or 3N with O). Thus, ectopic *Fgf8* expression/AER formation may not be sustained, and endogenous AER formation may be disrupted, owing to concomitant induction of EN1 by constitutively activated BMPR.

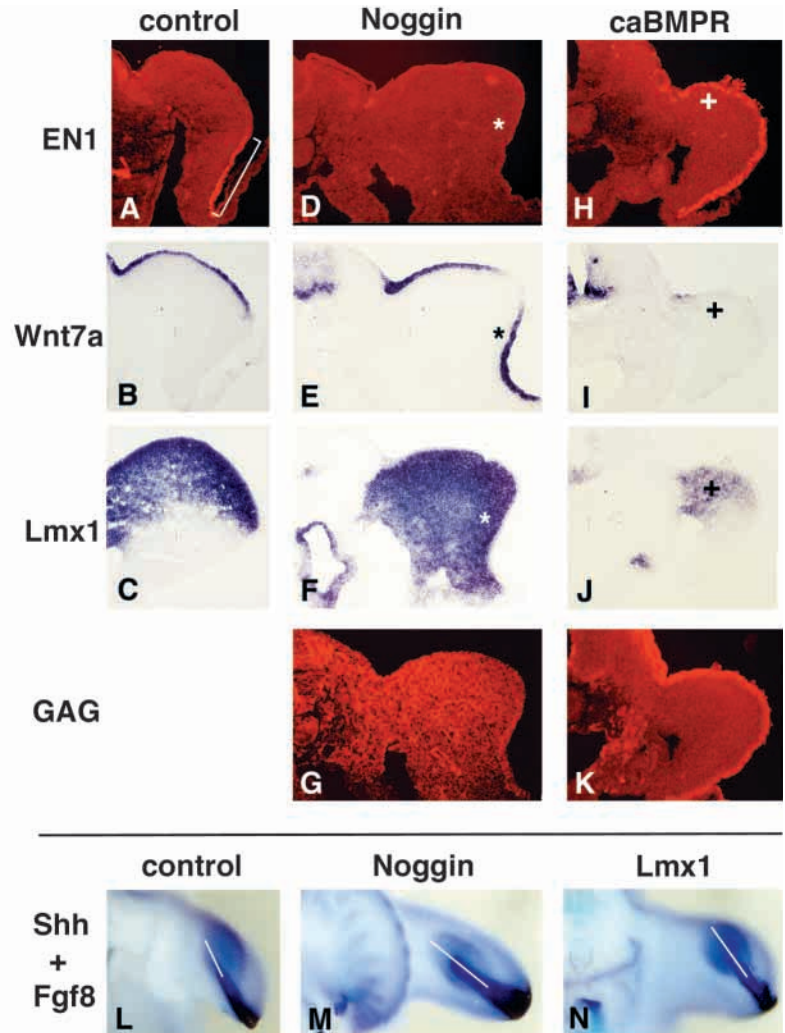
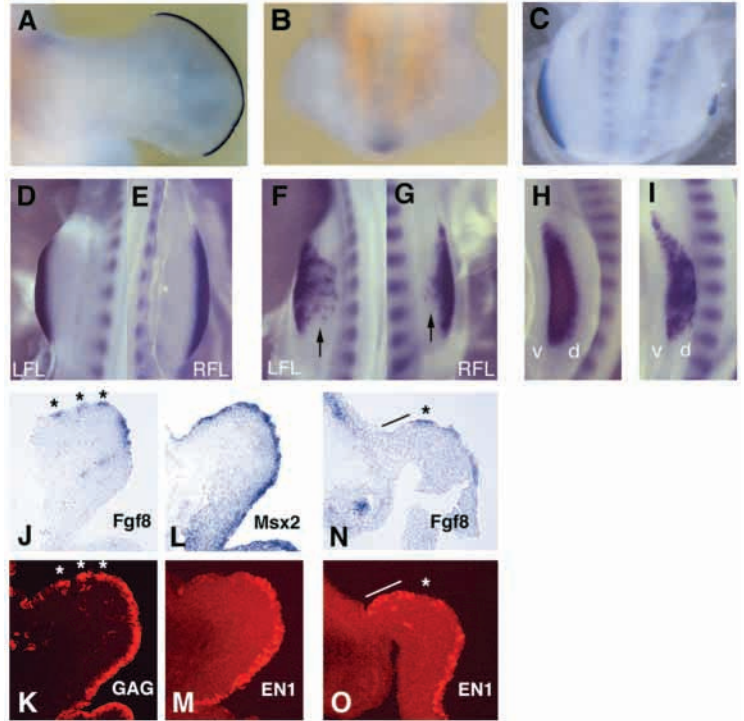


Fig. 2. Dorsoventral patterning is altered by loss or gain of BMP function. Alternate sections of (A-C) control uninfected stage 20 limb, (D-G) *Noggin*-infected stage 20 limb and (H-K) constitutively activated *BmpRIB*-infected stage 19 limb. *Noggin* misexpression results in dorsalized limbs due to loss of EN1 and ectopic *Wnt7a* and *Lmx1* expression on the ventral side (marked by asterisks in D-F), whereas constitutively activated *BmpRIB* misexpression ventralizes the limb because of EN1 misexpression and downregulation of *Wnt7a* and *Lmx1* on the dorsal side (marked by + in H-J). Note that in H-K, there is no morphological AER. (G,K) Anti-GAG antibody to reveal virus distribution. (L-M) Whole-mount double RNA in situ hybridization for *Fgf8* in the AER and *Shh* in the mesenchyme. (L) Control, (M) *Noggin*-infected, (N) *Lmx1*-infected stage 24 limbs. The white line marks the midline of the AER and the DV interface. After dorsalization of the limbs due to misexpression of *Noggin* or *Lmx1*, *Shh* expression is ventrally expanded.

Noggin misexpression causes AER disruptions as well as ectopic *Fgf8* expression in ventral ectoderm

Interestingly, we found that *Noggin* misexpression also led to AER disruptions and ectopic *Fgf8* expression. Widespread *Noggin* misexpression interfered with AER induction as shown by absence of *Fgf8* expression and the presence of truncated or notched limbs (49%, $n=238$ limbs; Fig. 4A,B,G). *Fgf8* transcripts were not detected at stage 17, when the AER first forms (Fig. 4D,E). Moreover, we noted that ectopic outgrowths capped by ectopic AERs were formed following more limited

Fig. 3. AER formation is altered by gain of BMP function. (A-I) Whole-mount RNA in situ hybridization for *Fgf8* expression in the AER. (A) Uninfected stage 27 hindlimb. (B) Constitutively activated *Bmpr1B*-infected stage 27 hindlimb. In B, the limbs are severely truncated, owing to the absence of the AER and *Fgf8* expression. (C) Constitutively activated *Bmpr1B*-infected stage 17 hindlimb. *Fgf8* expression is normal in the left hindlimb but is almost absent in the right hindlimb. (D,E,H) Uninfected stage 18 embryo and (F,G,I) constitutively activated *Bmpr1B*-infected stage 18 embryo; right and left forelimbs (RFL and LFL) viewed from the dorsal (D-G) and lateral (H,I) side. Note ectopic *Fgf8* expression is limited to dorsal ectoderm (arrows in F,G) and not ventral ectoderm (I; same limb as shown in F). Alternate sections of constitutively activated *Bmpr1B*-infected stage 19 forelimb (J-M) and stage 18 hindlimb (N,O). *Fgf8* is ectopically expressed in the dorsal ectoderm (asterisks in J,N), where it correlates with viral GAG expression (K) and *Msx2* (L). In another sample, ectopic *Fgf8* expression is not observed in regions of high EN1 expression (line in N,O).



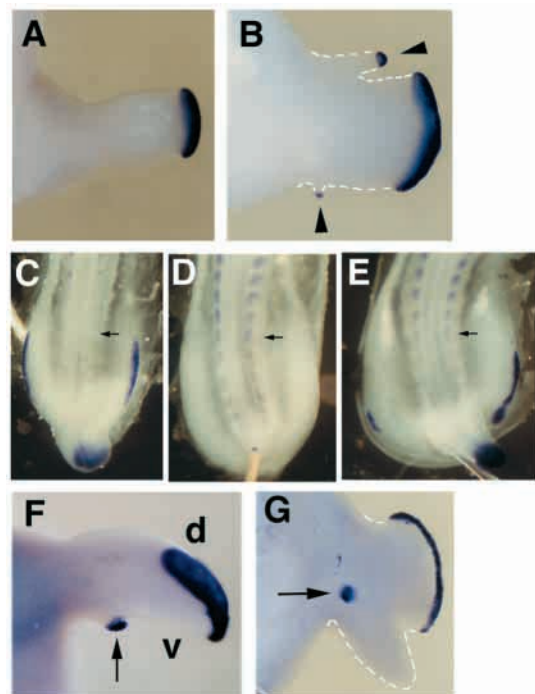
misexpression of *Noggin* (achieved by dilution of the virus, salt and pepper viral pattern at the time of AER formation, not shown). These ectopic AERs were present only on the ventral *Bmp*-expressing side of the limb (visualized by *Fgf8* expression, Fig. 4F,G, 6%, $n=36$ limbs). This aspect of the phenotype could be due to the *Noggin*-mediated downregulation of *En1* expression in the ventral ectoderm or in the AER (see Fig. 2D), as *En1* mutant mouse embryos also form ectopic AERs in the ventral ectoderm that are presumed to arise due to a defect in AER maturation. However, the overall *Noggin* effects cannot be explained solely by the loss of *En1* expression as, in mouse embryos that lacked *En1* function, the endogenous AER is not disrupted, *Fgf8* is expressed, and the limbs are not truncated (Loomis et al., 1996). These results, and those derived from constitutively activated *BMPR* misexpression, indicate that the AER phenotypes generated through manipulation of *BMP* signaling are complex, and suggest a role in AER maturation via *EN1* regulation, as well as an *EN1*-independent role for *BMP* in AER induction. Therefore, we conducted further studies to determine whether *BMP* acts through an *EN1*-independent pathway to mediate AER induction.

BMP regulates *Msx* expression in the ventral limb ectoderm

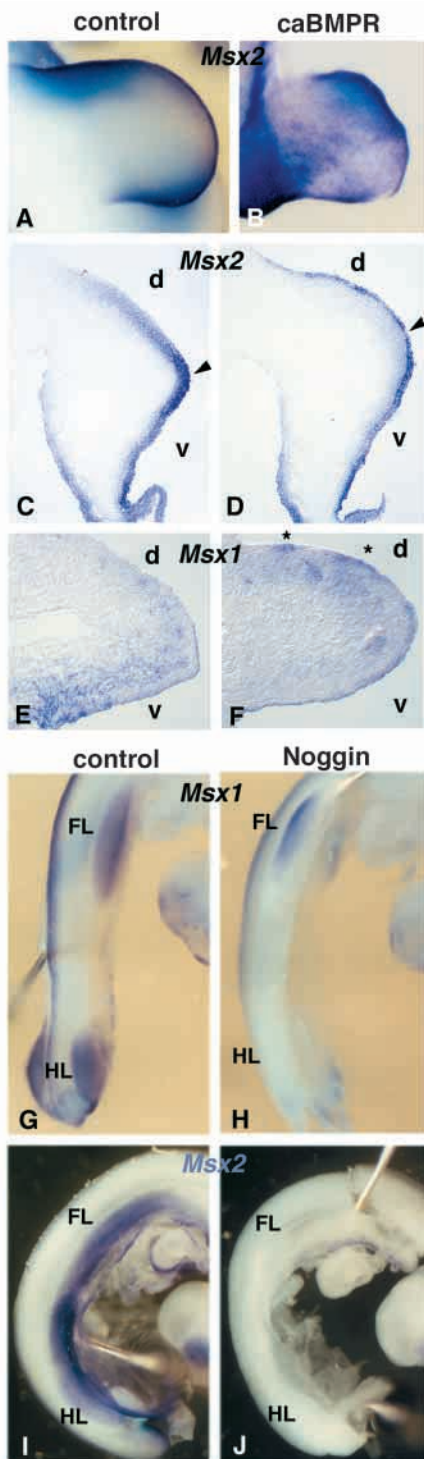
To explore the molecular pathway by which *BMPs* control AER formation, we focused on the *Msx* family of transcription factors. *MSX* are targets of *BMP* signaling in a number of

tissues including the limb and, in some developmental contexts, *MSX* is the transcriptional mediator of the *BMP* signal (Bei and Maas, 1998; Graham et al., 1994; Maeda et al., 1997; Pizette and Niswander, 1999; Suzuki et al., 1997). We found that *Msx1* and *Msx2* are expressed in an overlapping pattern with *Bmp4* and *Bmp7* in the early ventral limb ectoderm and AER as it forms (stage 16 and 17; compare Fig. 1D,E,K,L,P,Q with 1B,G,H,M-O) (Davidson et al., 1991; Yokouchi et al., 1991). These genes are also expressed in the

Fig. 4. AER formation is altered by loss of *BMP* function. (A,B) *Noggin*-infected stage 27 hindlimbs; in B, arrowheads point to remnant AER marked by *Fgf8*, broken lines outline the notched regions. (C-E) Stage 17 embryos showing the hindlimb region, arrows point to somite 28. (C) Uninfected and (D,E) *Noggin*-infected embryos. In D, *Fgf8* expression is not detected in either limb; in E, there is a small region of *Fgf8* expression in the left limb and, in the right limb, the anterior region of *Fgf8* expression is absent and the posterior region is disrupted. (F,G) Two examples of *Noggin*-infected hindlimbs where arrows mark ectopic AERs on the ventral (v) side; in G, the limb is also notched. Anterior is towards the bottom, posterior is towards the top, and G, which is a ventral view.



limb mesenchyme. By stage 18 *Bmp4* becomes restricted to the AER, whereas *Bmp2*, *Bmp7* and *Msx1* RNAs are starting to be downregulated in the ventral ectoderm and are still expressed strongly in the AER and most proximal ventral ectoderm (Fig. 1M-P; *Msx2* RNA starts to fade in ventral ectoderm at stage 19, Fig. 1Q and Fig. 5C,D). By stage 20, *Msx1* is almost absent from the ventral ectoderm and AER, whereas *Msx2*, *Bmp2*, *Bmp4* and *Bmp7* remain in the AER and in the most proximal ventral ectoderm (Fig. 5E and not shown).



Misexpression of constitutively activated *BmpR* induced ectopic *Msx1* and *Msx2* expression in ectoderm on the dorsal side of the limb (Fig. 5B,D,F, Fig. 3L; 100%, $n=24$ limbs in both cases). Ectopic dorsal expression of *Msx1* and *Msx2* was already detected by stage 17 when the AER is starting to form. Conversely, *Noggin* misexpression repressed ectodermal *Msx1* and *Msx2* expression by stage 17 (Fig. 5H,J; 15%, $n=24$ and 65%, $n=23$ for *Msx1* and *Msx2*, respectively). Mesenchymal *Msx* expression was also lost, and at later stages this could in part be due to the absence of the AER because AER signaling has been shown to maintain *Msx* expression (Davidson et al., 1991; Fallon et al., 1994; Ros et al., 1992). Therefore, MSX proteins are good candidates as downstream effectors of BMP signaling in AER induction.

Msx participates in AER induction

To examine the consequences of altered *Msx* expression directly, we infected the presumptive limb ectoderm at stage 7-10 with RCAS-*Msx1*. This resulted in AER disruption (18%, $n=22$ limb, not shown, identified by gaps in *Fgf8* staining) and the formation of ectopic AERs in the dorsal limb ectoderm where *Msx* genes are not normally expressed (Fig. 6A-D; 9% by visualization of sustained ectopic outgrowth, $n=288$ limbs). No correlation was observed between the presence of the ectopic AERs and the gaps in the endogenous AER. Interestingly, analysis of sectioned tissue showed that 75% of the limbs exhibited ectopic *Fgf8* expression in individual or small clusters of cells in the dorsal ectoderm ($n=12$, Fig. 7A; analyzed at stages 18-20). These phenotypes appear to be mediated by ectodermal MSX activity, as they were observed in samples displaying viral infection restricted to the ectoderm at the time of marker gene analysis (Fig. 7D). This difference in frequency (75% versus 9%) may reflect the inability of a small number of *Fgf8*-positive cells to support outgrowth, or a requirement for a limiting factor to maintain these *Fgf8*-positive cells.

Because it has been previously shown in the limb that mesenchymal *Msx* misexpression induces mesenchymal *Bmp* expression (Ferrari et al., 1998), one possibility is that the *Msx*-induced phenotypes are the consequence of deregulated *Bmp* expression. However, ectopic *Bmp* expression was not observed in the non-AER ectoderm of *Msx*-infected limbs (data not shown) indicating that the regulation of *Bmp* expression by MSX is context dependent. Therefore, our data indicate that BMP regulates *Msx* expression and MSX in turn acts to direct ectopic AER formation. Our data also show that deregulated *Msx* expression interferes with proper formation of the endogenous AER. Further understanding of the role of MSX

Fig. 5. Changes in BMP signaling alter *Msx* expression.

(A-F) Constitutively activated *BmpRIB* misexpression induces ectopic *Msx2* and *Msx1* expression in dorsal ectoderm (B,D,F) compared with uninfected limbs (A,C,E). Asterisks in F highlight ectopic *Msx1* expression in dorsal ectoderm. (G-J) *Noggin* misexpression represses *Msx* expression (H,J) compared with uninfected limbs (G,I). (H) *Msx1* is not expressed in the hindlimb in either the ectoderm or mesenchyme, whereas, in this sample, it is still detected in the forelimb. (J) *Msx2* is not expressed in the forelimb, hindlimb and flank ectoderm or mesenchyme. (A,B) Stage 24 forelimbs shown in whole-mount; (C,D) stage 19 and (E,F) late stage 20 forelimbs shown in section. (G,H) Stage 18 (37 somite) embryos; (I, J) stage 17 (32 somite) embryos.

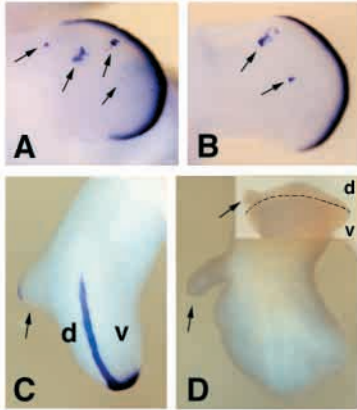


Fig. 6. *Msx1* misexpression promotes AER formation. (A-D) *Msx1* misexpression induces ectopic AER formation in dorsal ectoderm as detected by ectopic *Fgf8* expression (arrows in A-C) and ectopic outgrowths (arrows in C, D). (A,B) Stage 24, dorsal view, (C) stage 27, dorsal towards the left, and (D) stage 30, dorsal view. Inset indicates outgrowth occurs from the dorsal aspect of the limb, the broken line highlights the AER. d and v indicate dorsal and ventral sides, respectively.

in endogenous AER formation will have to await the double knockout of *Msx1* and *Msx2*, which show functional redundancy in the mouse embryo (Satokata et al., 2000).

EN1 and MSX appear to be regulated independently by BMP

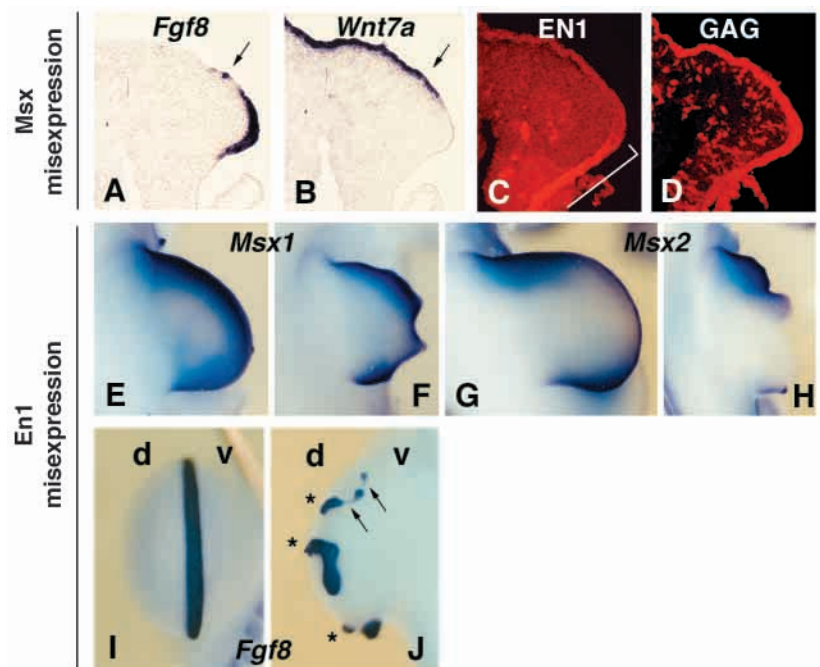
The above results indicate that BMPs regulate both *Msx* and *En1* expression and that these appear to mediate BMP effects on AER induction and DV patterning, respectively. However, EN1 has been suggested to be involved in AER formation, and thus we sought to determine whether MSX and EN1 act independently by examining whether they influence each others expression.

First, we examined the expression of EN1 in RCAS-*Msx* infected limbs displaying dorsal ectopic AERs. *Msx* misexpression did not affect the expression of EN1 ($n=6$), nor did it modify the pattern of expression of *Wnt7a* in non-AER ectoderm ($n=14$) or that of *Lmx1* in the mesenchyme ($n=11$) (Fig. 7A-D and not shown). *Wnt7a*, however, was downregulated in regions of ectopic *Fgf8* expression, consistent with its normal exclusion from the AER. MSX therefore triggers AER formation without changing DV patterning, in a process independent of EN1. In addition, *En1* is not expressed in the ectopic AERs indicating that these can form independently of EN1.

Next, we looked for changes in the pattern of *Msx* expression in RCAS-*En1* infected limbs. Misexpression of *En1* led to AER disruptions and downregulation of *Wnt7a* and *Lmx1* expression, as previously reported (Logan et al., 1997; Laufer et al., 1997; Rodriguez-Esteban et al., 1997), yet there was no ectopic expression of *Msx1* and *Msx2* in dorsal ectoderm (Fig. 7E-H; $n=19$ and 20 limbs, respectively). Thus, in this context, it appears that EN1 is not upstream of MSX, and is consequently not responsible for the dramatic induction of *Msx* expression by BMP signaling.

En1 misexpression has also been reported to give rise to dorsal ectopic AER formation at low frequency (Laufer et al., 1997; Logan et al., 1997; Rodriguez-Esteban et al., 1997). Using the same technique of misexpression with viruses of equivalent titer, we observed differences in the position of ectopic *Fgf8* expression between *Msx1*- and *En1*-infected limbs. Misexpression of *Msx1* resulted in small groups or individual *Fgf8*-expressing cells in a 'salt and pepper' pattern scattered throughout the dorsal ectoderm and in the formation of ectopic AERs not correlated with disruption of the endogenous AER (Fig. 6, Fig. 7A). By contrast, in our hands, the ectopic AERs formed by *En1* misexpression were always in close proximity to areas where the endogenous AER was disrupted or were connected to the endogenous AER by regions of *Fgf8* expression (Fig. 7J; 93%, $n=56$ limbs displayed

Fig. 7. MSX and EN1 act in separate pathways to promote AER formation and DV patterning, respectively. (A-D) *Msx1* misexpression does not disrupt DV patterning. Alternate sections from a stage 19 limb bud detected for (A) *Fgf8* RNA, (B) *Wnt7a* RNA, (C) EN1 immunolocalization (bracket) and (D) virus with anti-GAG antibody. GAG staining in the mesenchyme reflects expression in blood vessels. *Wnt7a* is downregulated only in regions of ectopic *Fgf8* expression (arrows in B and A, respectively) as *Wnt7a* is normally not detected in the AER. (E-H) *En1* misexpression does not alter *Msx* expression, except in cases of AER loss, where *Msx* expression, which marks the AER, is lost. *Msx1* expression in stage 23 uninfected (E) and *En1*-infected (F) limbs. *Msx2* expression in stage 24 uninfected (G) and *En1*-infected (H) limbs. (I,J) *Fgf8* expression in uninfected (I) and *En1*-infected (J) limbs. In J, arrows indicate faint *Fgf8* staining, asterisk indicates dorsal displacement of the *Fgf8*-positive AER. Note that the dorsally displaced AER fragments are often still connected to the midline AER by a line of *Fgf8* expression.



disruptions in the endogenous AER and/or an irregular border; two of these limbs also had ectopic AERs). In addition, in *En1*-infected limbs, patches of *Fgf8* expression could be located on either the dorsal or ventral side of the presumptive DV midline. Thus, the *En1*-induced ectopic AERs appear to arise as misplaced fragments of the endogenous AER, consistent with the genetic evidence that demonstrates EN1 serves to position AER borders but is not involved in AER induction (Kimmel et al., 2000). On the contrary, our results suggest that *Msx*-induced ectopic AERs arise de novo, i.e. independently of the endogenous AER. Nevertheless, the low frequency of obtaining *En1*-induced ectopic AERs hindered us from analyzing *Msx* expression in the ectopic AERs, and therefore it remains to be determined whether *Msx* expression is altered in this context.

DISCUSSION

BMP signaling regulates DV limb patterning

We demonstrate that BMP signaling regulates both DV patterning and AER induction in the embryonic chick limb. In terms of DV patterning, ectodermal BMP signaling is necessary and sufficient to trigger a cascade of gene regulation through EN1. This serves to limit expression of *Wnt7a* and *Lmx1* to the dorsal aspect of the limb, and the differential expression of *En1*, *Wnt7a* and *Lmx1* is known to be crucial for later DV morphogenesis of the limb. Additional evidence for this novel role for BMP comes from the targeted knock-out of the mouse BMP receptor-1A in the ventral limb ectoderm, which exhibits defects in DV patterning and also in AER formation (Ahn et al., 2001).

Not all aspects of DV patterning are EN1-dependent (Chen and Johnson, 1999). Since BMP acts upstream of *En1*, it is conceivable that BMP also influences DV patterning in an EN1-independent mechanism, and a comparison of the mesenchyme DV patterning (tendons and muscles) at later stages following misexpression of *En1*, constitutively activated *BmpR* or *Noggin* solely in the ectoderm may have helped to clarify this. However, early lethality of the embryos (~E6) and spread of the virus into the mesenchyme, where BMPs affect muscle and tendon morphogenesis (Amthor et al., 1998; Pizette and Niswander, 2000), prevented such an analysis. Future methods to circumvent this problem include the introduction of a replication-defective virus exclusively into the limb ectoderm or the study of DV morphogenesis in a conditional knockout of the BMPs or their receptors in the non-AER ectoderm. Nonetheless, BMP regulates the only known ventral patterning determinant, EN1, and hence is required for at least the EN1-dependent specification of ventral pattern.

BMP signaling regulates AER induction

Our results show that BMP signaling is also necessary for induction of the AER. Manipulation of BMP signaling results in disruptions in the endogenous AER leading to absent or severely truncated limbs. Alterations of BMP signaling also result in induction of ectopic *Fgf8* expression and, in the case of *Noggin* misexpression, the formation of ectopic AERs that can direct outgrowth. *Bmp* genes are expressed in the ventral limb ectoderm at the time of AER induction and the effects of alterations in BMP signaling on *Fgf8* expression and AER

formation occur at the time of induction. It is possible that ectopic *Fgf8* expression also represents a displacement of *Fgf8*-positive cells. Our data are not definitive and both induction and displacement could contribute to ectopic *Fgf8* expression. However, we favor the induction model, based on our analysis of early stage 17 hindlimbs in which *Fgf8* appears to be misexpressed in many more cells than would normally be *Fgf8* positive in AER precursors or the definitive AER. Moreover, our *Noggin* results and the results of Ahn et al. (Ahn et al., 2001) demonstrate that BMP signaling is required for *Fgf8* expression and AER formation.

It therefore appears that the level of BMP signaling and/or the localization of the BMP signal are important in AER induction and we propose that AER induction requires a boundary of BMP signaling in the ectoderm. *Bmp* genes are expressed in the ventral ectoderm where they overlap the region of *Fgf8* expression located near the DV border. Limited constitutively activated BMPR misexpression results in ectopic *Fgf8* expression on the dorsal side of the limb, where *Bmp* genes are not normally expressed. Limited *Noggin* misexpression which causes loss of BMP signaling presumably in small patches of ventral ectoderm results in ectopic *Fgf8* expression and outgrowths on the ventral side. Thus, BMP mislocalization is sufficient to induce ectopic *Fgf8* expression. This suggests that a boundary of BMP-signaling and non-signaling cells is important for AER induction. Furthermore, deregulation of BMP signaling is incompatible with endogenous AER formation. The seeming paradox of AER loss resulting from either loss or gain of BMP function can be explained mechanistically as a disruption in an endogenous boundary between BMP signaling and non-signaling cells. Widespread expression of constitutively activated BMPR or *Noggin* would disrupt the boundary resulting in loss of the AER and subsequent defects in outgrowth. Establishment of a correlation between the borders of viral infection and location of the ectopic *Fgf8*/AERs or gaps in the endogenous AER was however complicated by the transient nature of these borders, owing to the spread of the replication competent virus. Definitive proof of our hypothesis will rely on a different technology allowing the generation of stable ectopic boundaries.

It is also likely that BMP from the ventral ectoderm acts in concert with a signal derived from the dorsal ectoderm to induce an AER, because even prior to formation of a morphological AER, chick *Fgf8* is normally expressed near the DV border and not throughout the ventral ectoderm where *Bmps* are expressed (Crossley and Martin, 1995) (this study). Other studies have suggested that AER formation relies on a boundary of expression of radical fringe (*Rfng*) which is normally present in the dorsal ectoderm and AER (Laufer et al., 1997; Rodriguez-Esteban et al., 1997), although loss of function experiments in the mouse have not confirmed this role (Moran et al., 1999). To address whether *Rfng* and BMP signaling converge to control AER formation, we sought to study *Rfng* expression in limbs infected with constitutively activated BMPR or *Noggin* retroviruses. Unfortunately, although *Rfng* transcripts were readily observed in the AER, we were unable to detect differential expression between dorsal and ventral ectoderm in control or infected limbs, and thus were unable to test this hypothesis. WNT signaling has also been implicated in AER formation. WNT7A is not needed for

endogenous AER formation (Parr and McMahon, 1995) although ectopic *Wnt7a* in the absence of *En1* causes ventral expansion of the AER, perhaps by altering the movement of AER precursors (Cygan et al., 1997; Loomis et al., 1998). In our study, *Wnt7a* expression was altered in response to changes in BMP signaling, although it is unclear whether this is a direct effect on *Wnt7a* or indirect through the regulation of *En1* expression. It was not possible to determine whether Noggin induction of ectopic ventral AERs requires ectopic WNT7A expression or whether constitutively activated BMPR induced ectopic dorsal *Fgf8* requires downregulation of *Wnt7a*. Another type of WNT signaling is implicated in endogenous AER formation through the activation of TCF1/LEF1 transcription factors, as well as the regulation of *Lef1* expression (Galceran et al., 1999; Kengaku et al., 1998). It is conceivable that Wnt genes other than *Wnt7a* may be affected by alterations in BMP signaling. However, our preliminary results show no changes in *Lef1* expression following misexpression of *Noggin* or *Msx*. Thus, WNT and BMP may operate in independent pathways. One possibility is that WNT is part of the signaling cascade in the limb mesoderm that induces the overlying ectoderm to form an AER. Indeed, recent studies suggest that *Wnt2b* and *Wnt8c* are signals necessary for limb initiation through the regulation of *Fgf10* expression in the limb mesenchyme (Kawakami et al., 2001). Thus, the relationship between BMP and dorsal signals (perhaps WNT and Rfng) in AER induction remains unclear. It will be of great interest in the future to learn how these different pathways are coordinated to ensure proper AER formation.

BMP appears to act in the ectoderm to control AER induction

Our results are consistent with BMP signaling acting in the ectoderm, not the mesenchyme, to control *Fgf8* expression and AER induction. *Bmp2*, *Bmp4* and *Bmp7* are expressed in the early pre-limb ectoderm and, when confined to the ectoderm, the cell-autonomous constitutively activated BMPR is capable of inducing ectopic expression of *Fgf8* in the ectoderm. Thus, we suggest that BMPs act as molecular determinants in the ectoderm for AER induction. However, as Noggin is secreted and as these *Bmp* genes are also expressed in the limb mesenchyme, we cannot exclude an influence on mesenchymal signaling involved in AER formation.

BMPs may act as a permissive signal to establish competence of the ectoderm to respond to the mesenchymal inducer and express *Fgf8*. In the chick it appears that an additional signal from the dorsal ectoderm would act in conjunction with BMP to limit *Fgf8* expression to the distal domain of *Bmp* expression. Alternatively, BMP could be an instructive signal but, in this case, a second factor on the ventral side would be postulated to restrict *Fgf8* to the DV border.

It is not clear whether the initial domain of *Fgf8* expression and the establishment of AER borders are regulated differently between chick and mouse. Mouse *Fgf8* expression is activated over a broad domain of ventral ectoderm cells and then apparently *Fgf8* expression becomes stabilized in cells in proximity to the DV border whereas more ventrally localized cells turn off *Fgf8*. In chick, *Fgf8* appears more limited to a subset of the ventral ectoderm. Another possible difference is

that chick AER precursors arise in both dorsal and ventral ectoderm (Altabef et al., 1997). Although similar studies have not been done in mouse, the data to date indicate that mouse AER precursors reside in the ventral ectoderm. Whether these represent real differences in the mechanism of AER induction will require further study to answer.

EN1 in AER formation and downstream of BMPs

EN1 is expressed in the ventral ectoderm and then also in the ventral half of the AER. EN1 is involved in formation of the AER; however, it appears to mediate only a subset of this complex process. In an elegant set of studies by A. Joyner and colleagues performed in the mouse (Loomis et al., 1996; Loomis et al., 1998; Cygan et al., 1997; Kimmel et al., 2000), it was found that EN1 is not required for *Fgf8* expression or PD outgrowth. Instead, EN1 appears required to set the DV midline border in the AER and to direct the compaction of ventral ectoderm cells towards this DV border to generate a morphologically distinct AER. Hence, EN1 does not play a role in AER induction but in AER maturation.

Misexpression of *En1* virus can result in dorsally localized *Fgf8*-expressing ectopic AERs (Laufer et al., 1997; Logan et al., 1997; Rodriguez-Esteban et al., 1997) (this study). The mechanism by which these ectopic AERs arise is controversial and has been suggested to be due to a role for EN1 in AER induction or to abnormal AER positioning through an effect on radical fringe expression (see above). The recent genetic studies of Kimmel et al. (Kimmel et al., 2000) further support a role in AER positioning. They found that misexpression of *En1* at moderate levels throughout the AER resulted in displacement and fragmentation of the AER. Our *En1* misexpression results are consistent with the previous suggestion by Kimmel et al. (Kimmel et al., 2000) that *En1* virally induced ectopic AERs could represent a displacement of the endogenous AER, as in our relevant samples the ectopic *Fgf8* expression was contiguous with but displaced from, or in close proximity to, the endogenous AER. In this respect, it is possible that ectopic *Fgf8* elicited by activated BMPR in part results from misexpression of EN1. However, ectopic *Fgf8* was not correlated with gaps or displacement of the endogenous AER. It is perhaps surprising that we did not observe dorsal displacement of the endogenous AER after activated BMPR misexpression, given the mouse and chick EN1 misexpression results. This presumably reflects the differences in the mechanisms via which these molecules act: activated BMPR induces *Msx1* and *2*, *Fgf8* and *En1* expression whereas EN1 appears to exert its effect on *Fgf8* through regulation of *Wnt7a* (also see below for discussion of level of EN1 expression). In further agreement, ectopic AERs that are present in the *eudiplopodia* mutant or that form in dorsal ectoderm following *Msx* misexpression do not appear to require EN1 (Laufer et al., 1997) (this study). This demonstrates that ectopic dorsal AERs can arise independently of EN1.

It is likely that the misregulation of EN1 expression contributes to other AER phenotypes generated by manipulation of BMP signaling. For example, overexpression of EN1 is likely to be detrimental to endogenous AER formation. Kimmel et al. (Kimmel et al., 2000) have shown that high levels of EN1 abrogate AER formation and *Fgf8* expression. In this respect, the absence or the gaps in the

AER we observed after constitutively activated BMPR misexpression may partly result from alterations in the level of EN1 expression as constitutively activated BMPR misexpression induces strong EN1 expression. In addition, one of the features of the *En1* knockout phenotype is that a secondary AER can form at the ventrally displaced border of the endogenous AER, owing to a problem in maturation of the endogenous AER (Loomis et al., 1996; Loomis et al., 1998). It is therefore possible that the ventral ectopic AERs that form in Noggin-infected limbs arise because of the downregulation of EN1 expression that is also observed in response to Noggin. It is however important to note that an alternate explanation for these two phenotypes (gaps in the endogenous AER and ectopic ventral AERs) is the misregulation of *Msx* expression. We cannot distinguish between these possibilities at the present time.

Nonetheless, there is an aspect of the AER phenotypes caused by modulation of BMP signaling that a modification in EN1 expression cannot account for. Indeed, in Noggin-infected embryos, the lack of AER induction cannot be explained by a lack of EN1 expression because *En1* mutant mouse embryos form an AER, albeit morphologically abnormal, that expresses *Fgf8* (Loomis et al., 1996). Taken together, these observations suggest that BMP acts through an EN1-independent pathway to mediate *Fgf8* expression and AER induction.

MSX as a transcriptional mediator of AER induction downstream of BMP

Bmp and *Msx* genes are co-expressed in the ventral limb ectoderm, and then throughout the AER. Loss or gain of function experiments indicate that BMP signaling is critical for the regulation of *Msx* expression at the time of AER induction. Furthermore, ectopic ectodermal MSX induces ectopic *Fgf8* expression and the formation of ectopic AERs that can promote outgrowth. These ectopic AERs only arise in the dorsal ectoderm where *Msx* is not normally expressed and without any change in EN1 expression. Constitutively activated BMPR also induces ectopic *Fgf8* expression in dorsal ectoderm apparently through induction of *Msx* expression. However, constitutively activated BMPR does not result in ectopic outgrowths, presumably because it also induces EN1 and elevated levels of EN1 could have prevented AER formation. Thus, BMP signaling through MSX, but independent of EN1, appears to regulate AER induction. Another interesting feature of *Msx* misexpression is that it also results in gaps in the endogenous AER. Similar to BMP, the MSX effects are most readily explained by a disruption in the endogenous boundary between MSX-expressing and non-expressing cells.

BMP signaling appears to independently control DV patterning and AER induction

Our results indicate that BMP signaling regulates both DV patterning and AER induction. Thus, BMP signaling provides a molecular link between the DV and PD axes. Previous establishment of a link between DV patterning and AER formation came from the observation of the loss of markers of the ventral ectoderm and the lack of AER formation or maintenance in *limbless*, *legless* and *wingless* mutants (Grieshammer et al., 1996; Ros et al., 1996; Bell et

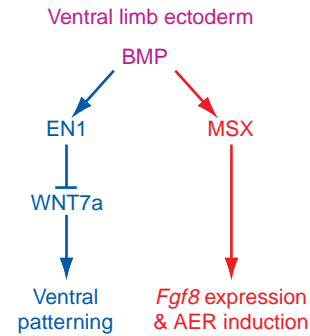


Fig. 8. Model for BMP signaling in the ventral ectoderm in the control of both DV patterning and AER formation. See text for details.

al., 1998; Ohuchi et al., 1997). However, as previously discussed, this link lies upstream of the known DV patterning genes *En1* and *Wnt7a*. Based on our results, we predict that a loss of BMP signaling contributes to the phenotype observed in these mutants and thus it will be interesting to examine the expression of BMP signaling components in these mutants.

Moreover, our results indicate that DV patterning and AER induction are likely to be separately regulated by BMP signaling. Crenshaw and colleagues reach a similar conclusion in their studies of the conditional BMPR-IA knockout (Ahn et al., 2001). Independent regulation of these two processes is also evident in the *eudiplopodia* mutants which display ectopic dorsal AERs but DV patterning and *En1/Wnt7a* expression are normal (Laufer et al., 1997). In this respect, there is an intriguing similarity between the phenotype generated by *Msx* misexpression and that observed in the chick limb mutant *eudiplopodia*. It is tempting to speculate that the molecular underpinnings of the *eudiplopodia* phenotype may involve a deregulation of *Msx* expression. Previous studies also suggested that DV patterning and AER induction can be independently regulated, although the molecular mechanism was unclear (Laufer et al., 1997; Loomis et al., 1996; Parr and McMahon, 1995; Rodriguez-Esteban et al., 1997). Our studies suggest a molecular mechanism whereby DV patterning and AER induction are integrated via BMP signaling, yet separately controlled through the regulation of two transcriptional targets *En1* and *Msx1/Msx2* (Fig. 8). Indeed, MSX can induce AER formation but does not influence DV patterning, whereas EN1 regulates DV patterning but does not participate in AER induction, although once the AER has been induced, EN1 is involved in positioning its borders. Thus, we suggest that MSX and EN1 function independently of one another downstream of BMPs to differentially direct AER induction and DV patterning, respectively.

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