BMPs mediate lateral inhibition at successive stages in feather tract development

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SUMMARY

The spacing of feather buds in a tract is thought to arise from the interaction between an inducing signal from the dermis and an inhibitory signal generated in the nascent buds. Local BMP-2 expression in the ectoderm precedes the formation of the ectodermal placodes, which are the first morphological sign of bud differentiation. We have altered the activity of BMP-2 or BMP-4 in the ectoderm of the feather field by expressing them or their inhibitor noggin using retroviral vectors. These experiments demonstrate that BMP-2 is necessary and sufficient to mediate the lateral inhibition that positions buds in a tract. After buds are initiated, BMP-2 and BMP-4 continue to inhibit the adoption of bud fates and help to specify the size and shape of the bud. They may do so in part by their regulation of Fgf receptor expression in both the ectoderm and dermis. Additional insights into pattern formation in the feather bud can be inferred from the effects of altered BMP activity on bud morphogenesis.

Key words: feather bud, lateral inhibition, Bone Morphogenetic Protein, pattern formation

INTRODUCTION

The feather tract is a classic example of a sequentially generated, reiterated pattern in embryonic development. The morphological events in bud formation have been described in detail (Wessells, 1965; Mayerson and Fallon, 1985). Feathers form in discrete tracts or pterylae at consistent positions in the embryo. The first morphological indication of tract formation is a general thickening of the dermis. This dense dermis forms throughout the tract and does not distinguish between presumptive bud and interbud regions. The first indication of bud specification is the local thickening of the ectoderm to form a disc-like structure called the epidermal placode. Dermal cells then migrate to form a ‘dermal condensation’ beneath the placode and subsequently proliferate to form a protruding feather bud (Desbiens et al., 1991). Both ectoderm and dermis proliferate in a controlled fashion such that the bud outgrowth becomes biased to the posterior (reviewed by Chuong, 1993).

By recombining ectoderm and dermis at different stages of development, it has been possible to dissect the series of inductive interactions between these layers that orchestrate bud formation (Cairns and Saunders, 1954; Saunders and Gasseling, 1957; Novel, 1973; reviewed in Sengel, 1976). The dense dermis is either instructive or permissive for the initiation of bud formation and can induce epidermal placodes in ectoderm from normally apteric regions. The ectodermal placode in turn induces the localized dermal condensations and is required to maintain them in early stages. The dermal condensation induces additional changes in the epidermal placode and reciprocal signaling between these structures results in the coordinated outgrowth of the bud.

The formation of a feather tract entails the morphogenesis of buds in a regular, two-dimensional array. Within each tract, a single row of buds is initiated in rapid sequence to begin tract formation. Additional rows are formed consecutively parallel to this primary row until a regular array of buds fills the tract. The prevailing model for the spacing of buds is illustrated in Fig. 1. A positive inducing or permissive factor propagates laterally through the dermis while lateral inhibition from the previously specified buds influences the response to this activity to localize the formation of ectodermal placodes (Ede, 1972; Davidson, 1983; reviewed in Sengel, 1976). As shown in the figure, the sequential addition of buds within a row also contributes to the regularity of spacing. As a result of the progressive initiation of buds, each row represents a tightly spaced progression of developmental stages along the anterior/posterior (A/P) axis, while each file of buds reflects a more broadly spaced progression of developmental stages distributed along the mediolateral axis (Fig. 2L).

Prior to placode formation, it appears that the potential to form placode is generally distributed through the ectoderm. Experiments to date have not distinguished the point at which lateral inhibition first acts. Although the epidermal placode is the first morphological indication of a localized response, it could result either from a localized inductive activity in the dermis or by a restricted response in the ectoderm to a more generally expressed inducer in the dermis. Members of the hedgehog, BMP, Fgf, Wnt and TGF-β families as well as their...
cognate receptors are expressed during feather development and are candidates to mediate these interactions (Patstone et al., 1993; Nohno et al., 1995; Marigo et al., 1996; Song et al., 1996; Ting-Berreth and Chuong, 1996a). Genes involved in local lateral inhibition processes—Notch-1, Notch-2, Serrate-1, Serrate-2 and Delta-1 (Chen et al., 1997; Crowe et al., 1998; Viallet et al., 1998) and Lunatic fringe (see below)—are also expressed in the feather bud. Among these molecules, Shh and FgfS have a positive role in feather formation. Local application of exogenous Fgfs to cultured skin explants can cause the formation of feather buds in normally apteric regions of wild-type skin and in the defective pterylae of the scaleless mutant, which do not normally make most feathers (Song et al., 1996; Widelitz et al., 1996). Forced expression of Shh can cause the formation of ectopic feather buds in apteric regions or premature formation of feather buds in the feather tracts as well (Ting-Berreth and Chuong, 1996b; Morgan et al., 1998). However, in contrast to other reports (Jung et al., 1998), we find that Shh is not expressed until after the placode and dermal condensation have formed and does not appear to play a role in the early specification or positioning of these structures (see Morgan et al., 1998 for further discussion). While all of these signals promote feather bud formation, overexpression of Delta-1 in chick skin has complex effects on feather bud development, which include suppression of bud formation (Crowe et al., 1998; Viallet et al., 1998).

In examining the expression of these and other signaling molecules, we found that BMP-2 is among the earliest genes identified to date that are expressed locally during feather bud formation and presage the distinction between placode and interplacodal fates. BMPs are expressed at a number of sites in the developing vertebrate embryo where epithelial-mesenchymal interactions occur and play a role in tooth, kidney, lung, limb, eye, heart, skin and hair development (Vaino et al., 1993; Luo et al., 1995; Gañán et al., 1996; Zou and Niswander, 1996; Neubüser et al., 1997). BMP-2 and BMP-4, and their Drosophila homologue decapentaplegic, are thought to act as instructive morphogens whose level of activity imposes fate on responsive cells and can act over a distance of many cell diameters from the site of synthesis (reviewed by Neumann and Cohen, 1997; Dosch et al., 1997). It may be noteworthy that BMP promotes the formation of epidermis at the expense of more specialized neurctoderm in early frog development (Wilson and Hemmati-Brivanlou, 1995).

In this work, we document the dynamic expression patterns of BMP-2 and BMP-4 during the earliest stages of feather bud initiation and subsequent development. Retroviral vectors were used to manipulate the activity of BMP-2 and BMP-4 in the chick embryonic ectoderm in various patterns at different stages of feather development. The subsequent changes in the expression of the endogenous feather bud markers and gross phenotypic effects due to altered BMP activity demonstrate that BMPs act as inhibitors of placode formation, which contribute to the spacing of buds within a tract as well as subsequent patterning of each individual bud.

**MATERIALS AND METHODS**

**Virus constructs and infection protocol**

RCAS retroviruses encoding mouse BMP-4 and human BMP-2 were kindly provided by P. Brickell and P. Francis-West (Duprez et al., 1996). The RCAS-noggin retrovirus was the generous gift of R. L. Johnson (Capdevila and Johnson, 1998). The RCAS vector RCASBP(A) encoding human BMP-2, mouse BMP-4 and chick noggin were used to generate stocks of viral inoculum of 5×10⁷, 5×10⁸ and 3×10⁸ infectious units/ml, respectively, and prepared for microinjection as described (Morgan and Fekete, 1996). For timing of inoculation, embryos were staged according to Hamburger and Hamilton (1951). Timing of harvest is described in days of incubation at 37.64°C in a Petersime model 1 incubator. Day 6 of incubation is prior to the appearance of feather rudiments, while day 7 corresponds to the appearance of the primary row and day 8 embryos have several rows of buds in the femoral and dorsal tracts. The number of feather rudiments observable in the figures provide the most accurate assessment of the stage of development of the feather tracts. Approximately 50 to 100 nanoliters of viral suspension was delivered between the amniotic membrane and the ectoderm, in ovo, at stages 22-24. Eggs were resealed with tape and incubated at 37.64°C until harvest.

For each viral preparation, the timing of exogenous gene expression was characterized by analysis of viral transcripts by whole-mount in situ hybridization. As such, there may be some delay between the detection of transcripts and the expression of the encoded protein. Extensive analysis of infection shows that, at day 6 of incubation, prior to the appearance of feather rudiments, viral transcripts are rarely observed except in occasional isolated cells. By day 7, scattered foci of infection comprised of groups of 10 or more cells are observed in the flank ectoderm dispersed from the site of injection. For the BMP-2 virus, 45 embryos infected, 6 were harvested just prior to tract formation (day 6), 4 were harvested at the onset of tract formation (day 7) and 33 were harvested at day 8. Representative samples are shown in Fig. 4. Significant expression of exogenous BMP is not observed until day 7 of incubation, and was observed in all embryos harvested at day 7 and day 8. The BMP-4 virus shows a similar time course of expression, although this higher titer virus preparation was also used to generate more extensive patches of infection. In total, the expression of viral transcripts was examined in 196, 45 and 49 embryos infected with BMP-4, BMP-2 or noggin-encoding viruses, respectively.

To characterize normal gene expression patterns, whole-mount in situ analysis was performed on a minimum of 20 uninfected embryos. In addition, infection with viruses that either lack an inserted gene product or contain unrelated inserts had no effect on the expression of these markers (not shown). 281 embryos infected with BMP-4, 45 infected with BMP-2, and 49 infected with noggin were used to analyze effects on endogenous gene expression. Because each embryo has multiple foci of infection, the number of sampled data points is 10- to 100-fold higher. The phenotypes shown are representative of those observed in all infected embryos and these phenotypes were not observed in control embryos. The number of infected embryos analyzed with each marker were: Cek-3 (64), Cek-1 (48), chick BMP-2 (86), Wnt7a (68), Shh (23), Lunatic fringe (15), chick BMP-4 (12). An additional 66 embryos were analyzed for the expression of other markers not shown in this study. Alterations of their expression were consistent with the conclusions reached below.

Morphological effects were scored on embryos harvested at day 12 of incubation. The effects illustrated in Fig. 3 were observed in 11 of 11 embryos infected with BMP4 virus, while the spectrum of defects demonstrated in Fig. 8 were observed in 18 of 18 embryos infected with noggin virus and none of these defects were observed in 16 control embryos harvested in conjunction with these experiments.

**Whole-mount in situ hybridization**

Protocols were modified from those previously described by reduction of protease K treatment to 5 minutes at room temperature at a concentration of 3.3 μg/ml (Burke et al., 1995). For sequential in situ analysis, embryos were hybridized simultaneously with digoxigenin- and FITC-labeled riboprobes. After detection of the dig-labeled probe,
samples were photographed prior to detection of the FITC-labeled probe with the same substrate (BCIP/NBT). Photographs of the first and second detections were compared to reveal additional signal from the second hybridization. Control embryos hybridized only with the digoxigenin-labeled probe but taken through both detection protocols revealed some increase in signal intensity following the second detection, but no change in the pattern of the original signal was observed. After whole-mount in situ detection of RNA, some samples were cryosectioned to further characterize expression patterns. After the final dehydration cycle, samples were rehydrated into PBS, then TBS, and infiltrated with 25% sucrose, 10 mM Tris, pH 7.5, followed by overnight infiltration at room temperature in OCT embedding compound. Samples were embedded in fresh OCT, frozen at −20 °C, and cryosectioned at 10 µm.

Probe templates for chick BMP-2, BMP-4, Wnt7a, Shh and PRD4 were as described in Noramly et al. (1996). Cek-1 and Cek-3 probe templates were as described in Song et al. (1996) and were kindly provided by P. Goetinck. The Lunatic fringe probe was provided by C. Tabin (Laufer et al., 1997). The coding sequences for human BMP-2, mouse BMP-4 and chick noggin were subcloned into Bluescript pSK (Stratagene) to generate templates for probes to detect the viral transcripts.

RESULTS

Normal expression of BMP-2 and BMP-4 in the developing feather

The pattern of BMP-2 expression in the skin reflects the appearance of feather rudiments in discrete tracts (Fig. 2A). The expression of BMP-2 observed at later stages in this process is consistent with previous reports (Nohno et al., 1995; Widelitz et al., 1997). However, prior to bud formation, BMP-2 is activated in the ectoderm of the forming feather field in a diffuse pattern that does not distinguish between presumptive bud and interbud regions (Fig. 2B,F). This diffuse expression increases locally in discrete spots in the ectoderm to demarcate the future placodes prior to their morphological differentiation (Fig. 2B,C,G). The epithelium then thickens and stratifies to form the ectodermal placode within the range of elevated BMP-2 expression while expression of BMP-2 in the adjacent ectoderm is decreased (Fig. 2H). The ectodermal placode induces the underlying dermal cells to aggregate into the dermal condensation, which expresses BMP-2 from the earliest stages of its formation (Fig. 2I,J). As development proceeds, BMP-2 expression is restricted to the anterior region of the bud in both the ectoderm and dermis (Fig. 2D (arrow) and P). The orientation of rows, files, and the A/P axis in a femoral tract are indicated in Fig. 2L. Unlike BMP-2, BMP-4 is not expressed in the ectoderm during these stages and is first detectable in the forming dermal condensation (Fig. 2K). The expression of BMP-4 in the dermis is similar to that of BMP-2 throughout these stages (Fig. 2M,N) and also becomes restricted to the anterior dermis of the bud at mid-bud stages (Fig. 2O).

Forced expression of BMP-2 and BMP-4 causes inhibition of feather formation

The localized ectodermal expression of BMPs at the onset of both placode and dermal condensation formation suggested that they play important roles in feather induction. This hypothesis was tested by forcing the expression of BMP-2 and BMP-4 in developing chick skin using the RCASBP(A) replication competent retroviral vectors. When the amniotic cavity over the mid-dorsum was inoculated with high titer viral preparations at stage 23 of development, patches of infection are observed at day 7 of incubation, while by day 8 most of the feather tract is infected. Using this protocol, infection and
exogenous gene expression is largely confined to the ectoderm through day 8 of development (Fig. 6F and data not shown). In embryos injected with BMP-2 or BMP-4 virus and harvested at 12 days of incubation, there were large areas of the tracts where feather formation was suppressed (compare Fig. 3A and B, arrowheads). These areas were localized to the lateral regions of the feather field while the feathers that did form were located in medial regions of the field and were abnormal in appearance (Fig. 3B). Abnormal feathers over a broad region of the tract shared similar morphology. Instead of long, tapering filaments (Fig. 3C), the deformed feathers were bulbous and wider at the distal tip than at the base, resembling baseball bats (Fig. 3D). Because of the mediolateral progression of development within a field, infection initiated at a given time in more lateral regions will affect an earlier stage of bud development than a corresponding infection in a more medial region. Thus, these results imply that, up to a specific stage, forced BMP-2 and BMP-4 expression can suppress bud development. After that stage, forced expression of BMP-2 and BMP-4 no longer suppresses bud development but leads to consistent patterning defects within the bud itself. Forced expression of BMP-2 or BMP-4 had identical effects in this and all assays reported below and the term BMPs will be used to refer to these two proteins.

To examine suppression of bud development more closely, injection of a lower titer virus preparation was employed to generate small patches of BMP-2 expression in the forming field. The time course of exogenous gene expression in these experiments is shown in Fig. 4A-C and diagrammed in Fig. 4D. Prior to tract formation at day 7, viral transcripts are observed in only a few isolated cells. As feather tract formation begins at day 7, small groups of cells expressing the viral transgene are observed scattered throughout the ectoderm. Additional exogenous gene expression is detected at day 8. Depending on their relative position within the forming tract, similar foci of infection will initiate exogenous BMP expression at different stages of tract development. The distribution of transcripts normally found in the feather placode such as BMP-2, BMP-4, BMP-7, Shh and Wnt7a (Crowe et al., 1998; Nohno et al., 1995; Widlitz et al., 1997) were examined during the early stages of bud specification to characterize the inhibition mediated by BMP-2. Local infection led to gaps in the normally regular hexagonal array of feathers and none of these placode markers were expressed in the gap (Fig. 4 and data not shown). Subsequent detection of the exogenous BMP-2 transcripts confirmed that the regions where bud formation was suppressed correlated with the areas of infection (Fig. 4E-J (yellow outlines) and data not shown). These areas of suppression could be observed at the very lateral edge of the feather tract, implying that BMP-2 can suppress the earliest steps in placode formation. The earliest localization of endogenous gene expression prior to placode formation was not observed in infected regions (e.g. Fig. 4I,J). Sections through the infected areas confirmed that there were no morphological signs of feather bud development in the absence of the examined feather bud markers. The ectoderm remained a simple cuboidal epithelium and no dermal condensations were observed. These sections also showed that the dense dermis underlying the infected areas was normal and that the block to feather initiation caused by BMP-2 occurs after dense dermis formation but prior to placode specification (Fig. 5E,K).

Medial to areas of local suppression, the spacing and development of buds in uninfected regions appeared normal.

![Fig. 2. BMP expression in the feather field.](image-url)
expression was not limited to the developing feather fields. Ectopic BMP-2 or BMP-4 expression outside of the feather fields is sufficient to induce Cek-3 in ectoderm where it is not normally expressed (Fig. 5M,N).

**Forced expression of noggin causes loss of interbud regions**

To better assess the role of BMP in feather field patterning, we sought to reduce BMP activity during tract formation. A retrovirus was used to express the BMP inhibitor noggin which sequesters endogenous BMP-2 and BMP-4 in complexes incapable of activating their signaling pathways (Holley et al., 1996; Zimmerman et al., 1996). Infection with this virus caused phenotypes complementary to those caused by forced expression of BMP-2. Local expression of noggin in the ectoderm leads to increased commitment to the placodal fate at the expense of interfollicular areas. Areas of noggin expression in more medial regions where buds were already forming resulted in buds that were abnormally large relative to controls (Figs 6B (arrow) and 7B). Noggin expression also resulted in the fusion of adjacent placodes (Fig. 6B (arrowheads),D,E). The pattern of fusion was clearly biased such that a bud was most often fused with the immediately anterior bud in the row more lateral to it (Fig. 6D (arrows),E). In the extreme margins of the feather field, patches of noggin expression led to the fusion of several buds into a single region expressing placodal markers (Fig. 6D (arrowhead),E). In this case, fusion of buds within a row was common. Sections through such a region showed broad areas of the ectoderm assume the pseudostratified, columnar morphology of the placode (Fig. 7G-J). The effects of noggin on the expression of feather bud markers was confined to the feather field. No activation of any subset of the gene expression associated with placode formation in regions outside of the feather field was observed (Figs 6B,C and 7B,C and data not shown). For example, the embryo in Fig. 7C shows a large patch of infection that spans the border of the feather tract (white arrow), but BMP-2 expression is only induced on the side of the border where feathers are normally forming at this time (Fig. 7B). These observations demonstrate that noggin has no activity in the absence of the BMPs, which are only expressed in the feather tracts. Furthermore, BMPs are not required to prevent the lateral spread of placode induction across the border of a tract. Sections through the skin confirmed that viral infection and noggin expression is confined to the ectoderm (Fig. 6F).

**Effects of noggin on the A/P polarity of the feather bud**

Although the initial expression of BMP-2 and the other markers employed in this study is radially symmetric within the nascent placode, a subsequent step in bud morphogenesis is the development of restrictions in gene expression along the A/P axis (e.g. Figs 2D, 7A). BMP-2 expression is localized to the anterior ectoderm and dermis while Cek-1 and BMP-4 are expressed in the anterior dermis (Fig. 2O,P and data not shown; Noji et al., 1993; Widelitz et al., 1997). In contrast, Lunatic fringe, Cek-3 and Wnt7a are expressed at high levels in the posterior ectoderm, while Notch-1, Delta-1 and Serrate-1 show preferential expression in the posterior dermis (Chen et al., 1997; Crowe et al., 1998; Noji et al., 1993; Widelitz et
al., 1997). In noggin-infected regions, the A/P axis of the bud forms normally in both enlarged buds and those fused with their lateral neighbors. Wnt7a, Cek-3 and Lunatic fringe are all restricted to the posterior of maturing buds, including in the regions that have been converted to placode from a normally interplacodal fate (Figs 6B,D, 7D,E). When noggin expression caused the fusion of placodes with their anterior neighbors, posterior restrictions of gene expression were observed. In this case, one group of genes including Wnt7a and Cek-3 are restricted to a stripe of cells in the posterior of the enlarged bud similar in width to that observed in a normal bud (Figs 6B, 7D). Lunatic fringe exhibits a posterior bias across the enlarged placode but is expressed in an expanded region reflecting the expanded anterior-posterior length of the placode (Fig. 7G-H). Although restrictions in gene expression along the A/P axis occur for most genes examined, endogenous BMP-2 and BMP-4 are not downregulated normally in the posterior of noggin-infected buds (Fig. 7B and data not shown).

**Noggin feather phenotypes**

Widespread infection of the skin with the BMP virus led to either suppression of buds or a characteristic ‘baseball bat’ morphology (Fig. 3). In contrast, similar infection with the noggin virus led to a spectrum of feather phenotypes in 12-day-old embryos (Fig. 8D). A gradation in the phenotypes correlated with the developmental ages of the feathers in the field. In the lateral areas of the feather tracts there were large flattened outgrowths with irregular thickened edges (Fig. 8C) which appear to arise from the fused placodes shown in Fig. 6D,E. The most medial areas containing the oldest buds often had feather buds that were pyramidal in shape, with large bases tapering to a point at the distal tip (Fig. 8A), or were otherwise thicker than normal. The areas of the feather field between these two phenotypes contained three-pronged feathers, reminiscent of a fleur-de-lis (Fig. 8B). In more medial regions, the taller, middle lobe curls towards the base of the feather. In these feathers, the lateral outgrowths were oriented consistently in the field such that the axis through the three lobes of the bud corresponds to the mediolateral axis and the lobes were aligned along the A/P midline of the bud. The expression and activity of Shh in bud development is consistent with a role in organizing the distal outgrowth of the bud (Ting-Berreth and Chuong, 1996b; Morgan et al., 1998). In embryos infected in this fashion, Shh is expressed in a spot at the distal tip of each outgrowth (Fig. 8E). In younger embryos, enlarged buds with three spots of Shh can

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**Fig. 4.** Suppression of early feather bud markers by exogenous BMP-2. (A–C) The time course of exogenous BMP expression is shown. After infection at stage 23, human BMP-2 transcripts encoded by the virus were detected in embryos harvested at day 6, day 7 and day 8 of incubation. (A) Few viral transcripts are observed prior to feather tract development at day 6. (B) By day 7 when tracts are beginning to form, scattered patches of infection are observed in the ectoderm. (C) By day 8, patches of robust expression are observed. (D) The timing of exogenous BMP expression is shown schematically. (E,G) Shh expression in BMP-2-infected embryos at day 8 of incubation reveals local suppression of feather buds. Removal of a single bud (E, yellow arrows), or even large patches of buds (G, yellow outlines) does not interfere with the lateral propagation of the field (shown schematically in D). Buds form in normal spacing medial to (left) the areas of suppression. Disruption to the regular array is seen lateral to (right) the areas of suppression. (I) Endogenous BMP-2 is not expressed in the suppressed areas (yellow outlines). (F,H,J) Subsequent detection of the virally encoded hBMP-2 transcript reveals that the areas of bud suppression correspond to the areas of infection. Even at the lateral edges of the tract where bud patterning is in its earliest stages, the endogenous expression of BMP-2 is suppressed by exogenous BMP (I, J red outlines).
DISCUSSION

Sequential roles for BMPs in feather morphogenesis

The dynamic expression patterns of BMP-2 and BMP-4 and the effects of altering their activity in the ectoderm of the feather field demonstrate that these genes play several roles during the morphogenesis of the tract. These experiments suggest three distinct roles for BMP in specifying sites of bud initiation, preventing recruitment to the epidermal placode fate after initiation centers are fixed, and later contributions to morphogenesis of the bud itself.

Fig. 5. Effects of exogenous BMP on FGF receptor expression. The expression of Cek-1 in control (A,F) and mBMP-4-infected thighs (B,D,E) at day 8 of incubation. (A,F) Cek-1 is expressed throughout the dense dermis in the early feather tract and its expression is increased in the dermal condensation of the forming bud (F, arrowhead). (B) In infected embryos, expression of Cek-1 is suppressed in lateral regions of the tract (arrowhead). (C) Subsequent detection of exogenous mBMP-4 transcripts demonstrate that these areas of suppression underlie regions of mBMP-4 expression in the ectoderm. (D) Cek-1 expression in an infected embryo where the broken arrow indicates the plane of section shown in E, arrowheads indicate regions of suppression. (E) These regions (arrowheads) show reduced or absent Cek-1 expression when compared to a section through a corresponding region of a control embryo (F). Dermis in these regions is not detectably different in structure from the adjacent uninfected regions or the control embryo. The expression of Cek-3 in control (G,L) and mBMP-4-infected embryos (H,J,K,M) at day 8 of incubation. (G,L) Cek-3 is expressed throughout the ectoderm of the early feather tract and is suppressed in all but the posterior margin of the ectodermal placodes as they form. (H) In infected embryos, Cek-3 expression persists in regions where bud formation is suppressed (arrowhead). Subsequent detection of exogenous mBMP-4 transcripts confirms infection in this region (I, arrowhead). (J) Whole-mount view of Cek-3 expression in an infected embryo where the broken arrow indicates the plane of section though the suppressed area (arrowhead). Strong and uniform Cek-3 expression persists in the ectoderm of this region (K, arrowhead). (L) In an uninfected embryo, Cek-3 is not expressed at high levels in the ectoderm adjacent to the forming tracts. (M) In infected embryos, Cek-3 expression is induced in the ectoderm of the apteric regions and lateral to the forming buds (arrowheads). Subsequent detection of the viral transcripts confirms infection in this region (N, arrowheads).

Spacing of buds within a tract

The generation of buds in an orderly array occurs by the interaction of an inductive signal propagating laterally through the dermis with inhibitory signals from previously generated epidermal placodes (Fig. 1). The expression of BMP-2 in the nascent placode and its function as an inhibitor of placodal fate suggest it as a candidate for the inhibitor that defines the spacing of buds in this model. The effects of changing BMP-2 activity on the two components of this model can be considered separately. The first component is the positive factor. This activity does not appear to be affected by BMP because neither increases nor decreases in BMP activity affect the progression of induction across the field. When feather buds are suppressed locally by forced BMP-2 expression, the temporal progression of bud formation lateral to this region...
proceeds normally. It could be argued that the inductive wave propagates around the area of suppression by spreading along the A/P axis distal to the area of infection (Fig. 9). However, this would predict that, when the formation of several buds in a row is suppressed (e.g. Figs 9B, 4G, I), the placodes forming lateral to it would show a developmental progression along the A/P axis as the inductive wave spread in this direction (diagrammed in Fig. 9C, lower panels). The dynamic expression of placodal markers during bud development is sufficient to distinguish between individual buds in the normal array. However, we see no evidence of a temporal lag along the A/P axis lateral to areas of suppression (see Figs 9, 4 and data not shown). Thus, the localized expression of the markers examined in this study and the morphological differentiation of the placode, both of which are prevented by forced expression of BMP-2, are not required for the lateral propagation of inductive activity in the field.

The second component of the model is a local inhibitor expressed in the foci of the presumptive buds, which diffuses radially from this point to inhibit response to the inducer. The expression of BMP-2 and its activity as a suppressor of placode formation are consistent with this role. The initial localization of BMP-2 expression in the feather field is the earliest change detected to date that demarcates the presumptive epidermal placode. This local expression occurs in groups of cells separated from each other by many intervening cells. The ability of BMPs to act over such distances make them attractive candidates for this signal (Lecuit et al., 1996; Nellen et al., 1996; Singer et al., 1997).

Because spread of the inducing wave is independent of BMP activity, distortions in the spacing of buds within a field caused by local expression of BMP will be subtle. Inhibition can shift the formation of a focus at most a fraction of a row since larger shifts will appear as deletion or fusion of buds. Lateral to the region of perturbed BMP expression, the patterning activity of the induced buds will rapidly result in a regular field again. As a result, the most obvious consequence of a local distortion of spacing will be the appearance of branched or irregular files and rows lateral to the disturbance (Fig. 1B). The effect of altering BMP activity on the spacing of buds is seen most clearly when it is reduced locally by infection with the noggin virus. Buds shifted in position by 1/2 row can be observed in Fig. 7B,E (arrowheads), and branched files are observed as well (Figs 6B, 7B,E). Bud formation is also suppressed lateral to regions of exogenous BMP expression leading to the altered spacing diagrammed in Fig. 1. This effect is clear in Fig. 4G in the area lateral to infection where the files are crooked and branched. More subtle disturbances are seen in Fig. 4I and in the forming placodes in Fig. 5. The level of BMP transcript accumulation achieved with the virus is similar to that of endogenous BMP observed during early placode formation and appears to exert patterning effects over limited distances outside the area of infection. Because of the mediolateral progression of bud development, altered spacing is observed lateral to regions of infection. Medial to areas of infection, exogenous BMP added to that expressed from previously

![Fig. 6. Forced expression of noggin causes enlarged or fused feather buds.](image)

The expression of Cek-3 in control (A) and noggin-infected chick thighs (B,C) at day 8 of incubation. Fused feathers form in regions corresponding to areas where developmentally younger feathers are found (B, arrowheads), whereas enlarged buds are formed in regions where more mature feathers are observed (B, arrow). Subsequent detection of exogenous noggin transcripts confirms infection through the feather field, with strong expression in the regions of fused and enlarged buds (C). (D) The expression of Lunatic fringe in a noggin-infected 8-day-old chick thigh. Fused feather rudiments are of two types: buds that are fused to a younger neighboring bud located to the anterior-lateral side of the feather (arrows) and buds that are fused to neighbors on all sides (arrowhead). (E) The expression of endogenous BMP-2 in the dorsal feather field of a noggin-infected embryo. Buds are fused in files pointing away from the midline and fuse with neighbors within a row at the edges of the tract. (F) Section through a noggin-infected embryo that has been detected for the presence of viral transcripts reveals infection is limited to the ectoderm. (G) A schematic representation of the timing of infection and the range of bud phenotypes and fusions seen. The earlier the infection, the more extensive the bud fusion. Infections in or near a day 7 feather field are shown on the left and the corresponding phenotypes in the day 8 feather field are shown on the right. The primary row is marked and field propagation occurs to the left as marked by the long arrow. The relative timing of noggin expression is inferred from the relative mediolateral position of phenotypes observed and is indicated by the relative position of the infection foci (shaded blue) in the diagram. A postulated timing of noggin expression with respect to bud morphogenesis is also indicated as inferred from the position of phenotypes observed with different levels of infection.
formed placodes will suffice to suppress bud formation locally but will have little effect on bud spacing (e.g. Fig. 4E,F,J). These changes demonstrate that ectodermally expressed BMP can specify the position in which adjacent buds form by functioning as a lateral inhibition signal at this stage.

One prediction of this model is that increased levels of BMP expression should result in broader regions of bud suppression around the expressing cells. Our ability to modulate expression levels in vivo using retroviral expression is limited. However, Jung et al. (1998) have recently shown that exogenous BMP applied to in vitro skin cultures on ion exchange beads causes suppression of bud formation around the beads for a distance that varies with the dose applied. In these in vitro experiments, it is difficult to distinguish whether BMP plays a role in specifying the position of bud initiation, or whether the observed effects of exogenous protein reflect a later activity to suppress further development of previously initiated buds. This is because the spacing of buds in these explants, although relatively normal, is not as regular as that observed in vivo and the subtle spacing changes that distinguish between these two roles cannot be reliably scored. Nevertheless, based on these observations, a similar role for BMP-2 in the spacing of buds was postulated by these authors. They also suggest that high levels of BMP within the feather rudiment may serve as an activator of bud development. However, no such activity was revealed by reducing BMP activity with Noggin in our experiments.

**BMP inhibits recruitment to the placodal fate**

The effects of noggin expression suggest that BMP continues to inhibit the placodal fate after the positioning of feather rudiments has been specified. In more medial regions of the tract, buds are enlarged at the expense of interfollicular fates, but the same number of buds are found within a given area (e.g. Fig. 6A,B). This process can lead to the fusion of adjacent buds, although in most cases the morphology suggests normally spaced initiation foci within the fused structures. The period when the positions of inductive foci become fixed may correlate with the formation of the ectodermal placode and the shift in inductive activity from the dermis to this structure.

The pattern of fusion between adjacent buds suggests that the ability to participate in placode formation is lost in a sequential fashion. Buds are most likely to fuse with the bud more lateral and anterior to it (Fig. 6D,E). Because of the posterior-to-anterior progression of bud formation in these tracts, this is the ‘younger’ of the two equidistant buds. This pattern of fusion between buds suggests that the ectoderm becomes refractive to placode formation in the brief period between the formation of
adjacent buds within a row. Fusion between buds within a row is rare at this stage and individual oversized buds tend to be broader along the mediolateral axis than the A/P axis as well. These observations imply that the requirement for BMP to promote the interplacodal fate is lost first in the ectoderm anterior and posterior to the bud and then in the lateral region.

**The role of Notch signaling**

Based on the fact that forced expression of Delta-1 can suppress bud formation, as well as the early expression of Notch pathway components in the feather tract, it has been suggested that Notch signaling mediates the initiation of buds in an evenly spaced pattern within the tract (Crowe et al., 1998; Viallet et al., 1998). To date we have failed to detect any localized gene expression presaging the position of the presumptive placode prior to the local upregulation of BMP-2 in the ectoderm (Fig. 2). This change occurs in large groups of cells that are separated from adjacent pre-placodes by many cell diameters. While BMPs have been shown to act over such distances, the lateral inhibition mediated by the Notch receptor and its ligands acts on immediately abutting cells and is less likely to function at this step which appears to require signaling over many cell diameters. Although Delta-1 is expressed in the dermis prior to placode formation, it is uniformly distributed in the dense dermis (Crowe et al., 1998; Viallet et al., 1998). The timing of its subsequent localization compared to that of BMP-2 has not been adequately examined and may be

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**Fig. 8.** Noggin induced feather phenotypes. (A-C) A number of different feather phenotypes are seen in a day 12 chick embryo. The type of phenotype corresponds with the timing of infection during development. (A,D) Large, wide, often pyramidal feathers form in regions where more mature feathers are observed in control embryos. (B,D) Younger noggin-infected buds formed three-pronged feathers. The orientation of the three prongs relative to one another is consistent and follows the A/P boundary of the localized mid-bud markers. The A/P axis is indicated by the white arrow. (C,D) Flattened, wide disorganized outgrowths are localized to areas where the youngest feathers are found in control embryos. (E) Shh is seen in the apex of all three lobes of the trilobed buds in a day 12 chick embryo infected with noggin. (F) Alternative models for limiting Shh expression (blue) to the center of the bud by either lateral inhibition from the periphery (I) or from the center of the bud (II) are shown at left and the consequences of enlarging the bud are shown at right. The anterior half of the bud is indicated in yellow and predicted spots of shh which are not observed are shown in red.

**Fig. 9.** The inductive wave propagates through areas of bud suppression. The dynamic expression of lunatic fringe distinguishes buds at different stages of development in control (A) and BMP-4-infected (B) limbs. The buds lateral to an area of suppression show similar patterns of Lunatic fringe expression (B, arrows). Two alternative mechanisms for propagation of the inductive wave past a region of inhibition are diagrammed (C). The initial infection pattern is diagrammed at the right in purple, while the changing expression of Lunatic fringe in the feather placodes is diagrammed in green. Successive stages of development are shown in the middle and right panels as the morphogenetic wave in the dermis (red) progresses through the field. If the inductive wave propagates through an area of suppression, the outcome shown above is expected (a). Lateral to the area of suppression, the buds within a row exhibit co-ordinated changes in Lunatic fringe expression reflecting their similar developmental age. The lower panels (b) show the possibility that the morphogenetic wave is blocked in an area of suppression but spreads along the A/P axis after it has passed the area of infection. In this case, there is a temporal lag in the changes of gene expression within rows lateral to the area of suppression. This was not observed in injected embryos irrespective of the size of the region of bud suppression.
informative in ordering the roles of these two signaling pathways in bud development.

However, experiments with the scaleless mutant demonstrated that localization of Delta-1 expression to the feather primordia occurs only after signaling from the dermis (Viallet et al., 1998). In the scaleless mutant, a defect in the ectoderm largely prevents the response from signals in the dermis that is required for the initiation of the ectodermal placode (Abbott and Asmundson, 1957). Feather tract development is arrested and Delta-1 is expressed uniformly through the dermis of the defective pterylogae in scale-less embryos. However, recombination of scaleless dermis with wild-type ectoderm leads to the formation of normal feather tracts and results in the localization of Delta-1 expression to the forming dermal condensations (Viallet et al., 1998). In addition, local application of exogenous FGF can replace this signal from the dermis to promote bud development and Delta-1 localization to the dermal condensation (Song et al., 1996; Viallet et al., 1998). However, forced expression of Delta-1 suppresses the rescue of scaleless feathers by FGF (Crowe et al., 1998) arguing that this block is exerted downstream of initial patterning signals from the ectoderm. These experiments suggest that Delta/Notch acts locally to mediate or reinforce cell fate decisions in response to localized cues from the ectoderm. The suppression of feather buds by precocious and ectopic expression of Delta-1 (Crowe et al., 1998; Viallet et al., 1998) might be expected even if the normal role of Notch signaling occurs after the specification of initiation centers, during the recruitment of cells to the placode or dermal condensation and stabilization of those fates. The development of the bud depends on mutual signaling between the placode and dermal condensation and, in its absence, both signaling centers regress (reviewed in Sengel, 1976). Clearly, restriction in the expression of Delta-1 is important for normal development of the bud and, in its absence, any initial patterning is not stabilized. Although further examination of the role of Notch signaling in bud development is warranted, we suggest that Notch signaling is more likely to be required downstream of the initial specification of buds mediated in part by BMP-2.

The A/P axis of the bud

It does not appear that BMP-2 or BMP-4 play a role in generating the initial A/P asymmetry of the bud. The expression of Wnt7a is normally restricted despite forced expression of BMP-4 in the posterior of the bud where it is not normally found (not shown). In noggin-infected buds, both Wnt7a and Cek-3 are expressed in a stripe of cells of normal width at the posterior of the bud irrespective of the size of the enlarged placode (Figs 7D, 6B (arrowheads), respectively). This would be expected if the signals inducing or maintaining gene expression arise in the interfollicular area and can activate over a fixed distance into the posterior of the bud. Lunatic fringe expression is also restricted to the posterior portion of the oversized bud but, in this case, the domain of expression is expanded in response to the enlargement of the bud (see Fig. 7G,H). These results suggest that one group of genes is activated by signals from the interplacodal region, while the other more directly partitions the area of the placode. This latter group may be responsible for restricting the response of the former to the posterior bud margin as well.

The normal restriction of BMP-2 and BMP-4 expression to the anterior half of the bud does not occur in noggin-infected buds (Fig. 7B and data not shown). One explanation for this observation is that BMP regulates its own expression and that active BMP signaling represses its own transcription. If this is the case, the persistent expression of BMP in the anterior of the bud may reflect a lack of responsiveness to this protein in these cells. Such a block to BMP signaling in the cells actively expressing the protein could explain the paradoxical observation that this inhibitor of bud formation is highly expressed in the bud itself from the earliest stages of its development.

Interactions between BMPs and Fgfs

These results refine the model presented in Fig. 1 by suggesting that the inductive signal from the dermis induces both an activator and inhibitor in the ectoderm, which promote and inhibit the placodal fate, respectively. While within the placode the activator dominates, BMP signals more effectively to surrounding cells. Although it remains possible that BMP acts purely by inhibiting the activity of this ectodermally expressed activator, the apparent lack of any early placodal gene expression in regions of exogenous gene expression suggests that it directly inhibits the dermal inductive signal as well. The identity of the inducing signal from the dermis remains unclear although our preliminary results and the phenotype of the Left-1 mutant mouse suggest that a Wnt may play this role (Kratochwil et al., 1996). Although it is possible that an Fgf mediates this induction, an appropriately expressed member of the family has not been identified. The experiments to date suggest that Fgfs expressed in the nascent placode promote both placodal formation and perhaps dermal condensation as well. When Fgf-soaked beads are used to induce feather buds in wild-type or scaleless apteric skin, individual buds are frequently induced around the bead (Song et al., 1996; Widlitz et al., 1996; Viallet et al., 1998). However, a factor acting downstream of the lateral inhibitor that mediates spacing in this model would be expected to induce formation of placode in an uninterrupted ring around the bead. This result implies that Fgf must both activate and overcome lateral inhibitory mechanisms. Consistent with this model, exogenous Fgf induces the expression of BMP-4 (Jung et al., 1998), although its effect on BMP-2 expression in the ectoderm has not been examined.

The inhibitory effect of BMP on placodal recruitment may result from direct interference with Fgf signaling since it appears to regulate the expression of Fgf receptors in both the ectoderm and dermis. Fgf receptor-2 is normally suppressed as the placode forms and this downregulation may be necessary for differentiation. During myoblast differentiation, Fgf receptor-2 is expressed in the myoblast but is normally downregulated upon differentiation and forced expression of the receptor in myoblasts prevents their fusion into myotubes (Itoh et al., 1996). BMP-2 is sufficient to induce and maintain the expression of Fgf receptor-2, Cek-3, in the skin and this may explain its inhibitory effect on placode recruitment.

Throughout this study, the infections employed alter gene expression in the ectoderm. Because this results in alterations in ectodermal placode formation, we cannot determine whether changes in dermal development are the direct effects of BMP signaling to this tissue or reflect indirect effects of changes in
the ectoderm. However, the suppression of Cek-1 expression in dermis beneath regions infected with the BMP virus cannot readily be explained by effects on placode formation. Rather, this observation implies that BMP produced in the ectoderm is directly effecting gene expression in the dermis as well. During normal development, suppression of Cek-1 in interfollicular dermis correlates with the loss of ability to participate in bud formation. BMP from the placode or dermal condensation may mediate this suppression and contribute to lateral inhibition in the dermis as well as the ectoderm.

**Related insights into bud patterning**

The enlarged buds caused by noggin infection provide further insights into the patterning of the bud. When trilobed buds are induced by infection with the noggin virus, all three lobes express Shh at the distal tip (Fig. 8E). This region of Shh expression appears to demarcate, and perhaps mediate, the activity of an organizer that co-ordinates outgrowth of the bud. BMP-2 could directly restrict Shh expression to the center of the bud. Alternatively, Shh expression might normally be restricted to the center of the placode by a lateral inhibition mechanism independent of BMP-2 (Fig. 8F). If inhibition radiates from the center of the bud, then an enlarged bud would free cells at the edges of the placode from lateral inhibition and result in secondary spots of Shh expression observed at the periphery of buds in the noggin-infected embryo. This model predicts the observed transition from abnormally shaped single lobed buds to trilobed buds. It is striking that all three lobes of these tri-partite feather buds are consistently arrayed along the mediolateral axis of the bud. Additional lobes are not formed at the anterior and posterior of the bud as predicted by a simple radially inhibition model (red spots in Fig. 8F). The normal expression of Shh and the ectopic expression associated with these additional lobes arises at the A/P midline at the border of Lunatic fringe expression. The importance of this border in generating an organizer for directed outgrowth is supported by the observations that manipulations that cause the formation of bilobed buds result in two borders of Notch/Delta expression within the bud, while manipulations that suppress oriented outgrowth result in the absence of a border of Notch/Delta expression in the bud (Chen et al., 1997).

**Conclusions**

The experiments reported here confirm key aspects of a lateral inhibition model of feather tract morphogenesis and identify BMP-2 as a necessary and sufficient inhibitor to specify the spacing of buds in this system in conjunction with a bud-promoting activity. The role of BMP as an inhibitor of bud fates extends to restricting the size of the placode after buds are initiated. This latter function is distinct from its role in spaceing the bud. The coordinated expression of Fgf receptor expression. BMPs antagonize the activity of Fgfs in several epithelial/mesenchymal interactions in the embryo and the opposing effects of these signals from the apical ectodermal ridge during limb bud formation and from odontogenic ectoderm during tooth patterning may be directly analogous to their functions in feather bud formation (Niswander and Martin, 1993; Gañán et al., 1996; Neubüser et al., 1997). It seems likely that antagonistic interactions between these two signaling pathways may constitute a subroutine in embryonic development widely employed to pattern the embryo. The ability to resolve initial patterning events from the subsequent role of BMPs in restriction of the placode and morphogenesis of the bud are pivotal to the dissection of their function in feather tract morphogenesis. Fgfs, TGF-βs, Wnts and Notch signaling all contribute to patterning the feather tract and this is an ideal system to dissect the interactions between these growth factors that generate pattern in many tissues of the developing embryo.

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