DEVELOPMENT AND DISEASE

Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion

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SUMMARY

Our expression studies of bone morphogenetic proteins (BMPs) and Noggin (a BMP antagonist) in the embryonic chicken face suggested that BMP signals were important for closure of the upper lip or primary palate. We noted that Noggin expression was restricted to the frontonasal mass epithelium but was reduced at the corners of the frontonasal mass (globular processes) just prior to fusion with the adjacent maxillary prominences. We therefore performed gain- and loss-of-function experiments to determine the role of BMPs in lip formation. Noggin treatment led to reduced proliferation and outgrowth of the frontonasal mass and maxillary prominences and ultimately to the deletion of the maxillary and palatine bones. The temporary block in BMP signalling in the mesenchyme also promoted epithelial survival. Noggin treatment also upregulated expression of endogenous BMPs, therefore we investigated whether increasing BMP levels would lead to the same phenotype. A BMP2 bead was implanted into the globular process and a similar phenotype to that produced by Noggin resulted. However, instead of a decrease in proliferation, defects were caused by increased programmed cell death, first in the epithelium and then in the mesenchyme. Programmed cell death was induced primarily in the lateral frontonasal mass with very little cell death medial to the bead. The asymmetric cell death pattern was correlated with a rapid induction of Noggin in the same embryos, with transcripts complementary to the regions with increased cell death. We have demonstrated a requirement for endogenous BMP in the proliferation of facial mesenchyme and that mesenchymal signals promote either survival or thinning of the epithelium. We furthermore demonstrated in vivo that BMP homeostasis is regulated by increasing expression of ligand or antagonist and that such mechanisms may help to protect the embryo from changes in growth factor levels during development or after exposure to teratogens.

Key words: Bone morphogenetic protein, Cleft lip, Chicken embryo, Noggin, Craniofacial, Beads, Programmed cell death, Msx1, Msx2, Fgf8, Shh

INTRODUCTION

The basis for cleft lip is the failure of individual components of the upper embryonic face (facial prominences) to meet and fuse at the appropriate time. The primary palate consists of those facial prominences that come together to form the upper lip. Failure of fusion to occur in the human primary palate results in a cleft in the upper lip that extends into the nostril. However, the midline of the upper lip is intact in patients with cleft lip. In contrast, clefts of the secondary palate occur later in development than cleft lip and are because of the failure of fusion of the palatal shelves. Cleft palate (CP) results in a communication with the nasal cavity and is a midline defect. Cleft lip, with or without cleft palate (CL/P), is one of the most common birth defects in humans (approximately 1 in 800 live births) and is genetically distinct from isolated CP (Bear, 1976; Fraser, 1980).

Relatively few candidate genes have been linked to nonsyndromic CL/P (Schutte and Murray, 1999), although the genetic contribution to nonsyndromic orofacial clefts has been estimated to be between 20 and 50% (Wyszynski et al., 1996). Use of mouse strains with an increased liability to form cleft lip has led to the identification of at least two loci that are linked to cleft lip (Juriloff et al., 2001). Other genetic approaches have rarely identified genes that cause cleft lip. The majority of orofacial clefts in transgenic or knockout mouse embryos involve the secondary palate or are midline clefts between the medial nasal prominences (Beverdam et al., 2001; Diewert and Lozanoff, 2002; Francis-West et al., 1998; Lohnes et al., 1994; Richman and Mitchell, 1996; Schorle et al., 1996; Zhang et al., 1996). This mouse data further supports the idea that clefts of the secondary palate are caused by different genes than clefts of the primary palate, but also highlight that other approaches are required to
identify signals that regulate lip closure. While the chicken embryo has a naturally occurring cleft of the secondary palate, and therefore is not similar to mammals, the avian primary palate closely resembles the mammalian primary palate and therefore is an excellent model in which to study the signals that control fusion of the lip.

Fusion of the avian primary palate occurs primarily between the corners of the frontonasal mass [globular processes, see Romanoff (Romanoff, 1960)] and the anterior-medial maxillary prominences at the base of the nasal slit (Fig. 1D,H,L,Q) (see Will and Meller, 1981). In order for fusion to occur the facial prominences need to grow out sufficiently to make contact. Outgrowth of the prominences depends on proliferation within the facial mesenchyme and directed expansion (McGonnell et al., 1998; Minkoff and Kunz, 1977; Minkoff and Kunz, 1978; Patterson and Minkoff, 1985; Peterka and Jelinek, 1983). The epithelium provides a key signal required for outgrowth (Richman and Tickle, 1989; Wedden, 1987).

Once the frontonasal mass and maxillary prominences meet, a double layer of epithelium must be removed. A combination of processes is used to eliminate the epithelial seam in the primary palate: some cells are removed via apoptosis whereas the inner basal epithelial cells undergo epithelial-mesenchymal transformation (Sato, 2000; Sun et al., 2000). However, the signals that control these processes are not known. After the epithelium is removed, the mesenchyme invades and proliferates to fill out the remaining grooves in a process called ‘merging’.

After fusion has occurred in the primary palate, the ossification of intramembranous bones occurs (Romanoff, 1960), whereas chondrogenesis begins prior to fusion (Matovinovic and Richman, 1997). Grafting experiments in the chicken embryo demonstrate that the frontonasal mass gives rise to the premaxillary bone, prenasal cartilage and egg tooth (Richman and Tickle, 1989; Wedden, 1987), whereas the maxillary prominence appears to form several bones (Richman and Tickle, 1989). Manipulations to the maxillary prominence need to grow out sufficiently to make contact. Where embryo position permitted, the presence of the maxillary prominence or with [35S]UTP labelled radioactive probes as previously described (Rowe et al., 1991). Chicken probes were generously provided by the following individuals: Fgf-8, J. C. Ispizua Belmonte; Msx1, Msx2, S. Wedden; Bmp2, Bmp4, P. Francis-West; Bmp7, B. Houston; Noggin, R. Johnson; Tbx2, Shh, C. Tabin.

**MATERIALS AND METHODS**

**In situ hybridization**

In situ hybridization was performed in wholemount (Shen et al., 1997) or with [35S]UTP labelled radioactive probes as previously described (Rowe et al., 1991). Chicken probes were generously provided by the following individuals: Fgf-8, J. C. Ispizua Belmonte; Msx1, Msx2, S. Wedden; Bmp2, Bmp4, P. Francis-West; Bmp7, B. Houston; Noggin, R. Johnson; Tbx2, Shh, C. Tabin.

**Bead preparation**

Afigel Blue beads (BioRad) 150 μm in diameter were dried and then soaked in 0.66-1.0 mg ml⁻¹ of human recombinant BMP2 (Genetics Institute) or 1.05 mg ml⁻¹ of human recombinant Noggin (Regeneron). There were no differences in the cell death responses with the range of concentrations of BMP2 used, so the data were grouped together. For the gene expression and programmed cell death experiments, a single BMP2 soaked bead was implanted in the corner of the frontonasal mass at stage 24. A subset of embryos was treated with a BMP2 bead placed in the anterior maxillary prominence at stage 24 and used for analysis of skeletal defects.

Several different Noggin treatments were tested. The embryos used for the wholemount in situ, BrdU and TUNEL data had one bead placed in the anterior maxillary prominence and one bead placed in the globular process of the frontonasal mass at stage 22, followed by a second set of two beads added to the same locations at stage 26 (stage 22 and 26 embryos are separated by 24 hours). A subset of embryos was treated with other conditions and used for analysis of the skeleton. Where embryo position permitted, the presence of the beads was checked on the subsequent day.
Skeletal staining, BrdU staining and TUNEL reaction

Embryos were skinned and then fixed in 100% ethanol for four days followed by 100% acetone for four days. Embryos were stained in Alizarin Red and Alcian Blue solution and cleared in glycerol as described (Plant et al., 2000).

Embryos were treated with BrdU for two hours beginning 10 hours after the first two Noggin beads were placed. Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and embedded in wax. Sections were treated with 2 M HCl for 30 minutes at 37°C and proteinase K 5 μg ml⁻¹ at 37°C for 10 minutes, blocked and then incubated for one hour at 37°C with anti-BrdU (Becton-Dickinson, 1:30). The secondary biotinylated anti-mouse antibody (1:500, ABC kit, Vectastain) and avidin-biotin complex (ABC kit, Vectastain) were applied for one hour each. Detection was with diaminobenzidine (DAB). Sections were counterstained with Hoechst 33258 (5 μg ml⁻¹ in PBS) to visualize the nuclei. Cell counts were made on DAB-stained and Hoechst-stained views to calculate the percentage of proliferating cells. A paired t-test was performed to determine relative differences in proliferation between treated and untreated mesenchyme.

TUNEL reaction was done on serial sections from the BMP2 treated embryos as described in Shen et al. (1997). Counts of TUNEL positive cells were made on at least two adjacent sections from a particular region of an embryo. Wherever possible, sections were selected that included the globular process of the frontonasal mass and the medial or anterior maxillary prominence on both the treated and untreated sides. Regions of interest were defined as frontonasal mass corner epithelium – the epithelium covering the globular process; caudal epithelium – the epithelium at the caudal edge of the lateral third of the frontonasal mass; corner mesenchyme – the frontonasal mass mesenchyme within the globular process; lateral third and future mesenchymal bridge region – this region was seen only in deeper sections of stage 25 (eight hours post-bead treatment) in which there was a connection between the maxillary prominence and frontonasal mass globular process. As the absolute number of dead cells is difficult to determine in areas with large numbers of dying cells, we placed the results into the following five categories: 0-5, 5-10, 10-50, 50-100 and greater than 100 (Table 1A-D).

Table 1. A quantification of programmed cell death induced by BMP2 in defined regions of the embryonic face

<table>
<thead>
<tr>
<th>Region</th>
<th>4-5h (n=44)</th>
<th>7-8h (n=60)</th>
<th>12h (n=7)</th>
<th>24h (n=6)</th>
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<tr>
<td>A. FNM corner epithelium</td>
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<td>0-5</td>
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<td>B. FNM caudal epithelium</td>
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<td>C. FNM corner mesenchyme</td>
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<td>D. Lateral third of FNM mesenchyme and future mesenchymal bridge region</td>
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Epithelial stripping

Epithelium was stripped by first applying a solution of Nile Blue Sulfate (Yang and Niswander, 1995) and then immediately peeling the blistered epithelium using a tungsten needle.

Acridine Orange staining

Embryos were rinsed in PBS, put into a solution of 500 pg ml⁻¹ of Acridine Orange in PBS for 10 minutes at room temperature, rinsed in PBS for 10 minutes and viewed under fluorescence illumination.

RESULTS

Expression of BMPs and Noggin in the region of lip fusion

We began our studies with a detailed examination of BMP and Noggin expression in the future mesenchymal bridge region prior to and at the beginning of lip fusion (fusion occurs between stages 28.5 and 30) (Hamburger and Hamilton, 1951; Sun et al., 2000; Will and Meller, 1981). Of the three BMPs examined, Bmp2 transcripts were most abundant in the mesenchyme involved in fusion of the lip, the globular processes of the frontonasal mass and anterior or medial corner of the maxillary prominences (Fig. 1A,C,D). Bmp2 expression also overlapped regions with the highest proliferation in the frontonasal mass and maxillary prominences (McGonnell et al., 1998; Peterka and Jelinek, 1983). Bmp7 and Bmp4 were predominantly localized to the epithelium in the zone of fusion and therefore may act in a paracrine manner on the mesenchyme (Fig. 1E,G,I,K,H,L). The data on Bmp2 and Bmp4 expression confirm and extend the results of Francis-West et al. (Francis-West et al., 1994) whereas Bmp7 data has not been reported previously. We also examined the expression of Noggin, in order to give us insight into the local control of BMP activity. Noggin was expressed specifically in a strip of
epithelium at the caudal edge of the frontonasal mass (Fig. 1M,N,P). The expression of Noggin overlapped that of Bmp4, which was also expressed in a narrow strip of frontonasal mass epithelium (Fig. 1F). Other molecules that were more widely expressed in the epithelium but also overlapped Noggin transcripts included Bmp7 (Fig. 1J) and Shh (Fig. 1O). The rostral edge of the Shh expression domain coincided with the boundary of Noggin and Bmp4 expression and marks the interface between stomodeal and surface epithelium (Fig. 1N,O). Interestingly, just prior to fusion, Noggin transcripts were reduced at the corners of the frontonasal mass (Fig. 1P). These changes in expression directly preceded the onset of fusion.

Clefts of the primary palate and defects in maxillary derivatives are induced by exogenous Noggin

We next applied Noggin protein to the globular process of the frontonasal mass and anterior-medial maxillary prominence, where Bmp2 was expressed, in order to examine the role of BMPs in outgrowth of the facial prominences and removal of the epithelial seam. We also chose to implant Noggin-soaked beads into the mesenchyme so that we could examine the effect of decreased BMP signalling in the mesenchyme on the overlying epithelium.

We first determined the stage at which endogenous BMPs are required for the fusion process. Application of beads at stage 22 did not affect development of the beak (n=8/8). It was necessary to reapply Noggin 24 hours after the first beads were implanted in order to induce skeletal pattern changes. One bead placed at stage 22 in the maxillary prominence followed by two beads placed in the globular process of the frontonasal mass at stage 26 induced changes in morphology of the palatine and maxillary bones (n=5/5 for both bones). The most common changes in morphology were bifurcations of the maxillary bone (compare Fig. 2A with 2C) and the loss of the distal extension of the palatine bone (compare Fig. 2B with 2D). Application of three beads over a 24-hour period infrequently caused

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**Fig. 1.** Expression of BMPs and Noggin at the time of fusion of the primary palate. (A-D) Expression of Bmp2, (E-H) expression of Bmp4, (I-L) expression of Bmp7, (M,N,P,Q) expression of Noggin. Plane of section for midsagittal sections (B,F,J,N,O) is the broken line in (A), plane of section for frontal sections is the broken line in B. (O) Expression of Shh. Bottom row was photographed in brightfield; silver grains appear black on a white background. (A,C) Expression of Bmp2 is high in the facial mesenchyme at the zone of fusion (arrows in A). Some epithelial signal can also be seen in (D). (E,G,H) Bmp4 is expressed primarily in epithelium in the maxilla and frontonasal mass. (I,K) Bmp7 is expressed in all epithelia at high levels as well as the inferior mesenchyme of the frontonasal mass (arrows). (M) Noggin is expressed at the caudal edge of the frontonasal mass at stage 24, including the lateral corners (globular processes, arrow); however, at stage 28 expression is lost at the corners (arrow, P). (F,N) Sagittal sections close to the midline show that Noggin and Bmp4 distinguish the frontonasal mass epithelium from that of stomodeal and general surface epithelium, whereas Shh is expressed in stomodeal epithelium up to the edge of the oral epithelium (O). The edges of the Noggin expression domain in relation to those of Bmp4 and Shh are indicated by arrowheads (F,O). (D,H,L) Higher-power views of the region of contact between the frontonasal mass and maxillary prominence highlight epithelial expression of all three Bmps and no expression of Noggin (Q). Scale bars: 500 μm for dark field images; 100 μm for brightfield images. Abbreviations: fnm, frontonasal mass; lnp, lateral nasal prominence; md, mandibular prominence; mx, maxillary prominence.
externally visible notches or clefts in the upper beak (n=3/14), whereas application of four or five beads over a similar period (stage 22 and stage 26) caused externally visible clefts of the upper beak in a majority of embryos (Fig. 2E, n=10/13). During morphogenesis, embryos treated with four beads had a reduction in size of the maxillary prominence and globular process compared to the contralateral side (Fig. 2G, stage 28). Prior to extension of the beak, the Noggin-induced cleft resembled that of human; there was a cleft between the frontonasal mass and the maxillary or lateral nasal prominences extending into the nasal pit (Fig. 2H, stage 30). Clefts were therefore caused by the inability of the frontonasal mass and maxillary prominences to make contact.

The changes in the maxillary prominence predicted the skeletal defects. On the side of treatment, the maxillary bone was absent and the palatine bone was either
very thin or completely absent (Fig. 2F, n=10/12). Other derivatives of the maxillary prominence were less affected (jugal, quadratojugal and pterygoid). In human cleft lip there are deficiencies in the maxillary bone often necessitating bone grafts (Ross, 2002). Despite the effects on the side of the beak, all the midline structures derived from the frontonasal mass formed in Noggin-induced cleft embryos (Fig. 2E,F; n=10/10; premaxilla, prenasal cartilage and egg tooth). Similarly, non-syndromic CL/P in humans continue to form the nose, nasal septum and the premaxilla. Thus the Noggin-induced clefts were a reasonable phenocopy of human CL/P.

**Noggin treatment suppresses proliferation of the facial mesenchyme**

The early morphology data suggested that endogenous BMPs regulate outgrowth of the facial mesenchyme, however the reduced growth may have been because of either decreased cell proliferation or increased cell death. TUNEL reaction showed there was no increase in programmed cell death (7/7; data not shown). In contrast, proliferation was significantly decreased 12 hours after the first set of beads (Fig. 2I,J; stage 24, n=3, 31% reduction, P=0.043 in the frontonasal mass; 39% reduction, P=0.002 in the maxilla). Greater reduction in maxillary proliferation is consistent with other studies showing that proliferation in the maxillary prominences remains high for a longer period of time than in other parts of the face (Minkoff and Kuntz, 1977; Minkoff and Kuntz, 1978). We conclude that endogenous BMP positively controls proliferation of the facial mesenchyme.

**Noggin effects on the mesenchyme are because of a block in BMP signalling**

In order to confirm that Noggin had blocked BMP activity, we examined expression of muscle segment homeobox *Msx1*. *Msx1* expression is commonly increased following exogenous BMP treatment (Barlow and Francis-West, 1997; Vainio et al., 1993; Wang et al., 1999) and decreased following Noggin treatment (Montero et al., 2001; Tucker et al., 1998). We found that *Msx1* expression was decreased in the maxillary and frontonasal mass mesenchyme six hours after the first beads were implanted (Fig. 2K, stage 22+, 3/3). The second set of beads maintained the lower expression levels of *Msx1* in the maxillary prominence; however, *Msx1* expression was restored somewhat in the frontonasal mass (Fig. 2L; stage 28, 3/3). There was no change in *Msx1* expression in embryos treated with PBS-soaked beads (6/6, data not shown) or on the contralateral side. Noggin treatment led to a relatively greater suppression of *Msx1* expression in the stage 28 maxillary prominence than in the frontonasal mass, similar to the proliferation data. It is possible that *Msx1* is downstream of endogenous BMPs in facial mesenchyme and may be a downstream mediator of BMP-stimulated proliferation.

**Noggin treatment induces rapid and sustained expression of Bmp7**

Blocking BMP signalling with Noggin has been shown to deregulate the cellular machinery that controls BMP synthesis. This is especially evident when Noggin containing virus is misexpressed in the chicken limb bud (Capdevila and Johnson, 1998; Pathi et al., 1999; Pizette et al., 2001; Pizette and Niswander, 1999). In addition, we have shown that Noggin soaked-beads increase BMP expression, 24 hours after implantation (Lee et al., 2001). We examined the effect of Noggin on BMP expression at shorter time intervals. Similar to our previous results, we found that increased *Bmp7* expression was easier to detect than *Bmp2*. In addition, we found that *Bmp7* was induced six hours after beads were implanted (Fig. 2M; n=7/8, stage 22+), and is maintained for at least 36 hours (Fig. 2N; n=2/2) 12 hours after the second set of beads was placed (stage 28). The early increase in *Bmp7* may not have functional significance because expression of *Msx1* was simultaneously decreased (Fig. 2K). However, the sustained increase in *Bmp7* after most of the protein has been released from the bead may account for the recovery of gene expression of *Msx1* that is observed after implanting the second set of beads (Fig. 2L). Noggin treatment neither increased nor decreased endogenous Noggin expression (n=6/7).

**Blocking BMP signalling in the mesenchyme promotes survival of the frontonasal and maxillary epithelium**

It was possible that blocking BMP signalling in the mesenchyme would indirectly affect the epithelium. We therefore looked at programmed cell death, thickness of the epithelium and expression of cell death and cell survival genes in the epithelium of the globular process and medial maxillary prominence. Acridine Orange staining was used to globally label the drying cells in the epithelium and subjacent mesenchyme. Noggin treatment led to a qualitative reduction in staining near the bead (Fig. 3B, n=6/7). Furthermore, when we examined sections through the frontonasal mass we found that the thickness of the epithelium was increased near the bead (compare Fig. 3C with D, n=3/4). Similarly, overexpression of Noggin retrovirus in limb mesenchyme also induces an overgrowth of the apical ectodermal ridge in limb buds (Pizette and Niswander, 1999). Accompanying the inhibition of cell death and increased epithelial thickness, *Msx2* expression was downregulated in the epithelium (Fig. 3E, n=5/5) and *Fgf8* expression was increased in globular process epithelium, but not induced ectopically in anterior maxillary epithelium (Fig. 3F).

One layer of the double-layered epithelial seam is derived from the medial maxillary prominence and one from the frontonasal mass globular process. *Shh* is a specific marker for the maxillary epithelium within the epithelial seam because the globular process epithelium does not express this gene (Fig. 3G). The normal time course of *Shh* expression includes a decrease in the medial maxillary prominence epithelium at stage 29 when fusion has just begun (Helms et al., 1997). We therefore treated embryos with Noggin and then examined *Shh* expression prior to fusion at stage 26 and at stage 29, after the time when *Shh* should be downregulated. We found that *Shh* expression was normal compared to the contralateral side in the stage 26 embryos (Fig. 3G, n=3/3). However at stage 29 expression was increased in both the globular process and in the intraoral surface of the maxillary prominence compared to the contralateral side (Fig. 3H, n=4/4). Thus decreased BMP signalling in the mesenchyme of the frontonasal mass promotes survival of the globular process epithelium via increased *Fgf8* and ectopic *Shh* expression, whereas in the maxillary prominence, blocking BMP signalling leads to increased...
epithelial survival, mediated by increased and prolonged Shh expression.

**Ectopic BMP2 application induces defects in maxillary prominence derivatives**

Because Noggin treatment increased endogenous BMP expression, we needed to determine whether the effects of Noggin could be replicated with exogenous BMP. A BMP2 soaked-bead was placed into the frontonasal mass mesenchyme in the position where upregulation of BMP was observed. Dose-response experiments showed that it was necessary to soak beads in 0.66–1 mg ml⁻¹ BMP2 to obtain reproducible skeletal and soft tissue changes (data not shown). However, unlike with Noggin, it was only necessary to implant one bead soaked in BMP2 into the mesenchyme. In control experiments, beads placed in contact with intact epithelium had no effect on development (n=6/6, data not shown); thus effects that were seen were primarily because of effects on the mesenchyme.

**Fig. 3.** Effects of Noggin on epithelial survival. (A) Brightfield and (B) fluorescence view of a Noggin-treated embryo showing a decrease in Acridine Orange staining near the bead (arrowheads). (C) Treated and (D) control sides of the frontonasal mass showing an increased thickness in the epithelium (arrow in C, stage 26). (E) Decrease in expression in epithelium and mesenchyme six hours after the first set of beads (arrow, stage 22+). (F) Increase in size and intensity of expression domain near bead (arrow), 12 hours after second set of beads, stage 28. (G) No change in Shh expression 12 hours after the first set of beads were applied (stage 24, arrow). (H) Increase in Shh expression in the epithelium (arrowheads) of the globular process (upper arrow) and of the anterior-medial maxillary prominence (stage 29). On the contralateral side there is no expression of Shh in the globular process or in the anterior-medial maxillary prominence (lower arrow). Scale bars: 0.5 mm for A,B,E-H; 100 μm for C,D. Abbreviations: b, bead; fnm, frontonasal mass; mx, maxillary prominence.

**Fig. 4.** Effects of BMP2 on facial morphogenesis. (A) SEM showing the reduction in size of the frontonasal mass (arrow). (B) Loss of the maxillary bone (asterisk) and the abnormal articulation of the palatine bone to the jugal bone (arrow). (C) Upregulation of Msx1 expression in frontonasal mass (arrow) and maxillary prominence. (D) Expression of Msx2 encompasses one-third of the maxillary prominence (lower arrow) and the lateral third of the frontonasal mass (upper arrow). Normal expression domain of Msx2 in the maxilla is restricted to a small patch at the posterior edge of the prominence (arrowhead). (E) Downregulation of Shh expression in the epithelium of the stomodeum (arrows). (F) Slight decrease in Shh expression after only 3 hours of BMP2 treatment (stage 24). Note the lack of expression in the globular process on the contralateral side. (G) Decrease in expression of Fgf-8 around the treated nasal pit (arrow). Position of bead is shown with asterisk in D-G. Scale bars: 500 μm for A,C-G (bar in C applies to C-E); 3 mm for B. Abbreviations: fnm, frontonasal mass; mx, maxillary prominence; mxb, maxillary bone; md, mandibular prominence; j, jugal; p, palatine bone.
Some aspects of the BMP2 phenotype were indeed similar to those produced by Noggin. There was a reduction in outgrowth of the globular process by stage 28 (Fig. 4A, 24 hours post-bead implantation, n=3/3), ultimately resulting in a notch on the side of the upper beak (7/7, Fig. 4B and data not shown). Similar to Noggin treatment, the midline frontal nasal mass derivatives formed in all specimens (Fig. 4B, premaxilla, premaxillary, nasal septum and egg tooth, n=7/7). Some of the skeletal defects were also similar to Noggin-treated embryos. The maxillary bone and the maxillary process of the palatine bone were usually absent (n=7/7). However, unlike the Noggin-treated embryos, the majority of the palatine bone was present, as was the jugal bone. In order to determine whether the milder effects on the palatine bone were because of the fact that the BMP2 bead was not implanted into the maxillary prominence, a subset of embryos was treated with a bead placed directly in the medial maxillary prominence. All had identical skeletal phenotypes to the embryos treated with beads placed in the globular process of the frontal nasal mass (n=4/4). The entire maxillary bone was deleted along with the distal end of the palatine bone. Beads soaked in PBS or protein diluent affected neither outgrowth of the globular process nor craniofacial morphology (n=5/5).

**BMP2 has opposite effects to those of Noggin on Msx1 and Msx2 expression**

There were enough similarities in the BMP2 and Noggin phenotype to suggest the possibility that both phenotypes were mediated by similar molecular responses. We therefore examined Msx1 and Msx2, which are normally induced by BMP2, 4 and 7 (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997; Mina et al., 2002; Wang et al., 1999). We found that expression of both Msx1 and Msx2 was expanded in the lateral third of the frontal nasal mass and Msx2 was ectopically induced in the anterior maxillary prominence (Msx1, n=2/3 at six hours, Fig. 4C; Msx2, n=6/6 at three hours; Msx2, n=3/3 at six hours, Fig. 4D). The exogenous BMP2 was biologically active and able to rapidly increase Msx1 and Msx2 expression, the exact opposite of the effects of Noggin. Bioactivity of the BMP2 was also confirmed by examining expression of the same two genes expressed in the epithelium as were studied in the Noggin-treated embryos. Both Shh and Fgf8 were downregulated by BMP2 (Shh 6/6, 3–6 hours post-bead implantation, Fig. 4E,F; Fgf8 3/3, 3–6 hours after bead implantation, Fig. 4G). Thus the similarity in the phenotypes of Noggin- and BMP2-treated embryos cannot be explained by the induction of BMP by Noggin.

**Ectopic BMP2 in the mesenchyme induces apoptosis first in the epithelium and then in the mesenchyme**

BMPs induce programmed cell death in many model systems, including the face (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997; Mina et al., 2002; Shigetani et al., 2000; Wang et al., 1999). We therefore investigated the possibility that induction of cell death explained the skeletal defects produced by BMP2. Indeed, cell death was greatly increased and this occurred soon after bead placement (Fig. 5A,B) with a peak at 12 hours (Fig. 5G,H; Table 1A-D). Programmed cell death was induced primarily lateral to the bead (Fig. 5A,B,D,E) but by 12 hours, the time at which programmed cell death had peaked, there was a narrow band of TUNEL positive cells 100 μm medial to the bead. (Fig. 5G,H). There was also increased cell death in the mesenchymal bridge between the frontonasal mass and the maxillary prominence, which may explain why maxillary derivatives were affected (Fig. 5D,E). We noticed that despite equal diffusion of the protein in all directions, cell death in the frontal mass did not include the midline where the premaxillary will differentiate (Fig. 5H, n=7/7 had ~5 dead cells in the central mesenchyme, 12 hours post-bead implantation).

The non-treated side of the embryo develops normally as determined by skeletal and gene expression analyses (Fig. 4A-G). We have determined that there are slight increases in programmed cell death in the globular process mesenchyme and epithelium at stage 28 on the contralateral side (Table 1A,C,D). By stage 29 there are approximately 10–20 dead cells in the zone of fusion (data not shown). Therefore by stage 28, localized programmed cell death is beginning to increase in the primary palate and this is a part of the normal fusion process. Similar increases in programmed cell death in the globular process and medial maxillary prominence to those seen on the contralateral side were observed in control, PBS-treated embryos (data not shown).

Another observation made during the TUNEL analysis was the increase in cell death in the treated epithelium at six hours that preceded the substantial increase in cell death in the mesenchyme observed at 12 hours. One possible explanation for this time difference may have been that the epithelium is required to maintain cell survival of the mesenchyme. We therefore mechanically stripped frontonasal mass epithelium using Nile Blue Sulfate. A localized increase in programmed cell death was observed in the denuded region of mesenchyme (Fig. 5J, n=5/5). Control embryos treated with Nile Blue Sulfate but not stripped had no increase in programmed cell death (n=3/3). Thus the effects of BMP2 on the epithelium may

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**Table 2. Induction of Noggin in BMP2-treated specimens**

<table>
<thead>
<tr>
<th>Hours post bead implantation</th>
<th>Increase in Noggin expression in FNM epithelium</th>
<th>Increase in Noggin expression in FNM mesenchyme medial to the bead</th>
<th>Increase in Mx epithelium</th>
<th>Increase in NP epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-5</td>
<td>3/3</td>
<td>3/3</td>
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<tr>
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<td>24</td>
<td>5/5</td>
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<td>1/6</td>
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</tbody>
</table>

FNM, frontonasal mass; Mx, maxillary prominence; NP, nasal pit.
have had similar effects to removal of the epithelium and may have exacerbated cell death in the mesenchyme.

A Noggin-BMP autoregulatory loop

We next addressed the asymmetric induction of programmed cell death in the frontonasal mass. The possibility we investigated was that BMP2 induces Noggin and that Noggin protects the mesenchyme from excessive cell death. BMP2, BMP4 and BMP7 induce Noggin expression in micromass culture of chicken chondrocytes (Kameda et al., 1999), in mouse mandibular organ cultures (Stottmann et al., 2001) and in explant cultures of mouse limb buds (Nifuji and Noda, 1999), respectively. In order to compare the distribution of Noggin transcripts to the areas with induced programmed cell death, we probed adjacent sections to those analysed for cell death with the Noggin probe. We found rapid and strong induction of Noggin in the epithelium and mesenchyme (Fig. 5C,F; Table 2). A detailed analysis showed a gradient of expression was induced in the mesenchyme medial to the bead with higher levels of Noggin further from the bead (Fig. 5C,F,I,L). Continued expression of Noggin was detected 24 hours after the bead was implanted (Fig. 5L), a time when programmed cell death had returned to normal levels (Fig. 5K).

These observations suggest that the lower concentrations of BMP2 found further from the bead or at longer time intervals from implantation may be low enough to be antagonized by the increased Noggin expression. Expression of Noggin was also induced ectopically in the nasal pit and in the maxillary epithelia (Fig. 5F). However, the increase in Noggin expression in the epithelium at four hours (Fig. 5C) and six hours does not appear to be sufficient to protect the epithelia from programmed cell death because the same epithelia later have many TUNEL positive cells (Fig. 5D,E).

In order to determine the functional significance of the increased Noggin expression, we re-examined the patterns of expression of Msx1 and Msx2. If the induced Noggin expression was being converted to protein, we should only see increased expression of Msx1 and Msx2 in areas with low Noggin expression such as maxillary prominence mesenchyme or lateral frontonasal mass mesenchyme. Furthermore, the programmed cell death patterns should overlap the expression domains of the two Msx genes. We observed that the upregulation of Msx genes (Fig. 4C,D) overlapped the areas with increased cell death in the maxillary and frontonasal mass prominences and that the timing of expression changes preceded the large increase in cell death.
observed at 12 hours. Thus the medial position of the Noggin transcripts may have helped to restrict Msx1 and Msx2 expression to the lateral mesenchyme of the frontonasal mass. In the maxillary prominence, there was no increase in Noggin expression in the mesenchyme; therefore Msx2 was induced in this region (Fig. 4D).

**DISCUSSION**

We have determined that endogenous BMPs have several important roles in the fusion of the lip. First, BMP signalling is required to stimulate mesenchymal cell proliferation and directed outgrowth of the facial prominences. Second, decreased BMP activity in the mesenchyme regulates cell survival in the epithelium and consequently increases epithelial thickness. However, we have also documented that a BMP autoregulatory loop is induced by either exogenous Noggin or exogenous BMP. Because the bead only releases protein over a limited distance and over a limited time, the autoregulatory loop may help to restore BMP homeostasis.

**Endogenous regulation of Noggin**

Our expression studies showed for the first time that Noggin transcripts were present in the face and were restricted to frontonasal mass epithelium during the predifferentiation stages of morphogenesis. Furthermore, Noggin was specifically downregulated at the corners of the frontonasal mass one day prior to fusion. This suggests that signals taking place within the globular processes (equivalent to the tips of the medial nasal prominences in mammals) are different than elsewhere in the frontonasal mass.

We have shown that BMP2 can induce Noggin, however it is not possible to say whether BMPs in the globular process mesenchyme play the same role in vivo. We did not see any evidence for a change in Noggin expression in embryos treated with Noggin. We acknowledge however, that slight changes in Noggin expression may have been below the level of detection of our in situ hybridization experiments. It may be that other types of mesenchymal signals may be required in addition to BMPs to control Noggin expression. There is evidence of FGF2 being present in facial mesenchyme (Richman et al., 1997) as well as several FGF receptors (Mina et al., 2002; Wilke et al., 1997). Studies on the morphogenesis of digits have shown that FGF2 can downregulate Noggin whereas BMP2 upregulates Noggin (Merino et al., 1998). Thus it is possible that FGFs together with BMPs could be regulating expression of Noggin in the frontonasal mass (Fig. 6B).

**Epithelium is required for cell survival in the mesenchyme**

Prior to the present studies, we had shown that epithelium has a supportive role in promoting outgrowth (Richman and Tickle, 1989). However, in those earlier studies we had not identified the specific roles of the facial epithelium in outgrowth. More recent work from others has shown that removal of frontonasal epithelium decreases proliferation (Hu and Helms, 1999). We provide new evidence from epithelial stripping experiments in vivo, that facial epithelium is also required for cell survival in the mesenchyme. A similar result was described in organ culture, although specific markers for programmed cell death were not used in these studies (Minkoff, 1991; Saber et al., 1989). The signals that originate in the epithelium could include FGFs, SHH and BMPs (Fig. 6B). We have previously shown that FGF2 and FGF4 can partly rescue outgrowth of denuded frontonasal mass mesenchyme (Richman et al., 1997), demonstrating that epithelially derived FGFs may be one type of signal required for mesenchymal cell survival.

**Regulation of cell survival and programmed cell death within the epithelium**

One can divide the secreted proteins expressed in the epithelium of zone of fusion (Fig. 6A) into those that normally promote cell death (Bmp2, Bmp4, Bmp7) (Barlow and Francis-West, 1997; Mina et al., 2002; Wang et al., 1999) (our own data) and those that promote cell survival: Fgf-8 (Trumpp, 1999); Shh (Ahlgren and Bronner-Fraser, 1999; Ohkubo et al., 1999). FGFs and BMPs are expressed in the epithelium (Fig. 6B). Shh is expressed in the mesenchyme (Fig. 6B). FGFs and BMPs are expressed in the mesenchyme (Fig. 6B). Shh is expressed in the mesenchyme (Fig. 6B).
Msx2
Msx1
BMP receptor signalling leads to ectopic expression of
In a different experiment we showed that increased
increased programmed cell death in the mesenchyme.
morphogenesis.
proliferation and programmed cell death during facial
links between BMP signalling, Msx expression, cell
output from our present study, support the
date from our previous study, coinciding with the decrease in Noggin and Shh
expression.
Altering BMP levels in the mesenchyme affects
epithelial fate
In the present study, we show that by changing levels of BMP
signalling in the mesenchyme, cell survival or cell death in the
epithelium is promoted. However, a third possibility that was
not investigated is that BMPs control epithelial-mesenchymal
transformation. BMPs are members of the TGFβ growth factor
family [Transforming growth factor (Hogan, 1996)] and
TGFβ3 has been shown to promote epithelial-mesenchymal
transformation in the secondary palate (Sun et al., 1998).
BMPs control epithelial-mesenchymal transformation of the
neural crest into ectomesenchyme (Sela-Donenfeld and
Kalcheim, 1999). The use of different TGFβ family members
in the primary and secondary palate to induce transformation of
epithelium would be consistent with genetic distinctiveness of
CL/P and isolated CP.
BMP signalling is required for Msx expression
outgrowth and cell survival of the mesenchyme
In addition to a role for mesenchymal BMPs in promoting
epithelial removal by various mechanisms, our data shows that
endogenous BMPs are critical for proliferation of the
mesenchyme and outgrowth of the facial prominences. A
similar result was recently described for the developing
chicken brain (Ohkubo et al., 2002). Noggin-containing virus
caued a decrease in proliferation and hypoplasia of the
telencephalon and optic vesicles. However, we extended
these results by correlating failure of outgrowth of facial
prominences with a decrease in expression of Msx genes.
The overexpression of Noggin virus in the limb bud
cailed a similar downregulation of Msx1 and Msx2 and
inhibited outgrowth (Pizette and Niswander, 1999). Our
novel finding that decreased Msx gene expression is
associated with a decrease in cell proliferation, suggests the
downstream targets of these two transcriptional repressors
(Catron et al., 1996) may include regulators of cell
proliferation.
We show that exogenous BMP2 induced rapid Msx1 and
Msx2 expression and that the increase occurred prior to the
increased programmed cell death in the mesenchyme.
In a different experiment we showed that increased
BMP receptor signalling leads to ectopic expression of
Msx1 and Msx2 (Ashique et al., 2002). These data,
together with the data from our present study, support the
links between BMP signalling, Msx expression, cell
proliferation and programmed cell death during facial
morphogenesis.

Exogenous BMPs can elicit cell death or cell
proliferation in facial mesenchyme
We showed that Noggin decreased proliferation and outgrowth; however, in the reciprocal experiment we did not stimulate
outgrowth but instead increased cell death. Our data differs
from that of other studies in which beads soaked in 10-fold
lower concentrations of BMP2 placed in the posterior stage 24
maxillary prominence stimulate proliferation and duplicate
bones (Barlow and Francis-West, 1997). In addition, clefts are
not induced and there is only localized cell death immediately
around the bead. Differences in the bead position and soaking
concentrations for BMP2 may account for the differences in
results. In our study, we wanted to study orofacial clefting so
the beads were placed close to the area where fusion will take
place rather than near the posterior maxillary prominence, as
described in Barlow and Francis-West (Barlow and Francis-
West, 1997). The bead position could be critical because
medial and lateral mandibular mesenchyme has different
responses to exogenous BMP2 or BMP7 (Barlow and Francis-
West, 1997; Mina et al., 2002). It is equally possible that low
concentrations of BMPs are required for proliferation, whereas
higher concentrations result in increased programmed cell
death. However, our data on younger embryos showed that low
concentration beads placed into the edge of the frontonasal
mass induce programmed cell death and deletion of bones and
cartilages rather than ectopic or duplicated skeletal elements
(M. Eblaghie and J. M. Richman, unpublished results). We
hypothesize that the globular process and anterior maxillary
prominences respond to increases in BMP levels by increased
programmed cell death rather than with increased proliferation.
This idea is consistent with the overlap of high expression of
BMPs with regions that have increased programmed cell death.
For example, low BMP expression was seen in the centre of
the frontonasal mass and lower levels of programmed cell
death were also seen in this region. Areas with higher BMP
expression, such as the groove between the lateral nasal and
maxillary prominences, also have an increased number of
dying cells (see also Shen et al., 1997).

Similarity of phenotypes produced by Noggin and
BMP suggest a dual origin for the maxillary bone
We have shown that the maxillary bone is deleted in both
Noggin- and BMP-treated embryos. The reason the same bone
was affected was that cells that make a major contribution to
the maxillary bone were targeted in both experiments. In the
Noggin-treated embryos, we demonstrated a significant effect
on maxillary proliferation, which fits the skeletal phenotype. It
is curious, however, that in embryos treated with BMP2, beads
implanted into the frontonasal mass or maxillary prominence
deleted the maxillary bone and left the frontonasal mass
derivatives unaffected. This leads us to suspect that the
maxillary bone has a dual contribution from the frontonasal
mass and the anterior-medial maxillary prominence. A fate
map of the primary palate is needed to resolve this question.
Nonetheless, the manipulations we have developed to induce
cleft lip suggest that one of the ossification centres for the
maxillary bone lies directly in the zone of fusion.

BMPs are required for skeletal patterning because Noggin-
treated embryos had several changes in size and shape of
maxillary prominence derivatives. Moreover, the decrease in
size of the palatine bone in Noggin-treated embryos is
complementary to the observations of others who have shown that BMP leads to duplications of the palatine bone (Barlow and Francis-West, 1997). We conclude that because the changes in shape in maxillary bones were not secondary to the abnormal fusion of the prominences, BMP signalling is required for intramembranous bone patterning. Finally, by targeting the maxillary bone in the chicken embryo model system, we have demonstrated that phenotypes not unlike human cleft lip can be produced.

**Genes and signals involved in mammalian primary palate formation**

It is clear that lip fusion can occur in the absence of Noggin (Brunet et al., 1998) or Chordin (Bachiller et al., 2000). Double homozygous knockouts of Noggin and Chordin have a severe reduction in head development that do not permit analysis of primary palate fusion (Bachiller et al., 2000). However, compound heterozygous-null knockouts have been made of Chordin and Noggin and these embryos have no clefts (Stottmann et al., 2001). Thus the evidence seems to suggest that it is not essential to have Noggin for lip fusion. However, it is not known whether Noggin is expressed in the murine medial nasal prominence in a similar way to the chicken frontonasal mass (Stottmann et al., 2001).

Compared to the BMP antagonists, stronger evidence exists for genes that lie in the BMP signalling pathway as having a role in mammalian cleft lip. Haploinsufficiency of human *MSX1* is correlated with cleft lip and palate (van den Boogaard et al., 2000). *Msx1* knockout mice do not have cleft lip but have cleft secondary palate and tooth agenesis (Satokata and Maas, 1994). *Msx2<sup>−/−</sup>* embryos have calvarial defects but no clefts (Satokata et al., 2000). The double knockout of *Msx1* and *Msx2* has a more severe phenotype that includes bilateral cleft lip and palate (Y. Chen, personal communication), demonstrating that there is some functional redundancy in the area of lip fusion between the two Msx genes. Preliminary analysis of the secondary palates of *Msx1<sup>−/−</sup>* mice shows regional decreases in cell proliferation in anterior palate mesenchyme (Zhang et al., 2001). In the interdigital region of *Msx1/Msx2<sup>−/−</sup>* embryos, programmed cell death does not occur, leading to webbing (Chen and Zhao, 1998). Therefore it is likely that programmed cell death in the primary palate will also be affected in *Msx1/Msx2<sup>−/−</sup>* homozygous mice. Closer inspection of *Msx1/Msx2<sup>−/−</sup>* embryos may reveal variation in the phenotypes, ranging from bilateral cleft lip to microforms of cleft lip. Microforms may include persistent epithelial seam because of decreased programmed cell death, or a thin mesenchymal bridge because of decreased mesenchymal cell proliferation.

The search for causative genes involved in human CL/P has revealed candidates such as *TGFα* and *methylenetetrahydrofolate reductase* (*MTHFR*) (Schute and Murray, 1999). However, these studies are by no means exhaustive and it is possible that genes directly involved in BMP signalling will be associated with human CL/CP.

**Embryonic regulation of BMP homeostasis**

One of the most striking results of our study was the manner in which the embryo can respond to external perturbation of BMP signalling. Endogenous mechanisms were rapidly activated in order to restore normal signalling. Although there are reports of embryos in vivo responding to exogenous BMPs by activating a feedback loop (Merino et al., 1998), we have shown that BMP induces lower levels of Noggin close to the bead and higher levels further from the bead, whereas Noggin induces high *Bmp* levels closer to the bead. Furthermore, we showed by examining downstream signalling that the response is at first overwhelmed by the exogenous protein and finally that the endogenous response remains at a high enough level to ultimately antagonize the ever-diminishing concentrations of exogenous protein. This ability to regulate BMP signalling may apply to normal development and also may play a protective role in cases of teratogen exposure, some of which increase BMP expression (Rodriguez-Leon et al., 1999).

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**REFERENCES**


