

Bmp7 regulates branching morphogenesis of the lacrimal gland by promoting mesenchymal proliferation and condensation

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Summary

The lacrimal gland provides an excellent model with which to study the epithelial-mesenchymal interactions that are crucial to the process of branching morphogenesis. In the current study, we show that bone morphogenetic protein 7 (Bmp7) is expressed with a complex pattern in the developing gland and has an important role in regulating branching. In loss-of-function analyses, we find that *Bmp7*-null mice have distinctive reductions in lacrimal gland branch number, and that inhibition of Bmp activity in gland explant cultures has a very similar consequence. Consistent with this, exposure of whole-gland explants to recombinant Bmp7 results in increased branch number. In determining which cells of the gland respond directly to Bmp7, we have tested isolated mesenchyme and epithelium. We find that, as expected, Bmp4 can suppress bud extension in isolated epithelium stimulated by Fgf10, but interestingly, Bmp7 has no discernible effect. Bmp7 does,

however, stimulate a distinct response in mesenchymal cells. This manifests as a promotion of cell division and formation of aggregates, and upregulation of cadherin adhesion molecules, the junctional protein connexin 43 and of α -smooth muscle actin. These data suggest that in this branching system, mesenchyme is the primary target of Bmp7 and that formation of mesenchymal condensations characteristic of signaling centers may be enhanced by Bmp7. Based on the activity of Bmp7 in promoting branching, we also propose a model suggesting that a discrete region of Bmp7-expressing head mesenchyme may be crucial in determining the location of the exorbital lobe of the gland.

Key words: Bone morphogenetic protein, Bmp, Branching morphogenesis, Lacrimal gland,

Introduction

The lacrimal gland is a structure that develops by branching morphogenesis. Its accessibility and ease of culture combined with the availability of convenient reporter constructs render it an excellent system for studying the branching process. Lacrimal gland development begins when the primary bud invaginates from the conjunctival epithelium at the temporal extremity of the eye (Govindarajan et al., 2000; Makarenkova et al., 2000). This invagination extends as a tubular sinus into the periorbital mesenchyme where it branches in two locations to form the intra- and exorbital lobes of the mature gland (Govindarajan et al., 2000; Makarenkova et al., 2000). The main function of the lacrimal gland is to produce secretions that lubricate and protect the ocular surface. Dysfunction of the lacrimal gland leads to the absence of corneal lubrication and, in severe cases, can result in ulceration and blindness.

In previous studies (Govindarajan et al., 2000; Makarenkova et al., 2000), it has been shown that fibroblast growth factor 10 (Fgf10) is expressed in temporal periorbital mesenchyme and has activity as an inducer of the lacrimal gland. In vitro inhibition studies show that Fgf10 promotes proliferative responses in lacrimal gland epithelium through Fgfr2IIIb

(Makarenkova et al., 2000), a result consistent with the involvement of this pathway in many examples of branching morphogenesis (Arman et al., 1999; Cancilla et al., 1999; Sekine et al., 1999; Ohuchi et al., 2000). The epithelial expression of *Pax6* in lacrimal gland (Kammandel et al., 1999; Makarenkova et al., 2000) has also implied a developmental role for this transcription factor and indeed, *Pax6*^{+/Sey-1Neu} mice show vestigial glands (Makarenkova et al., 2000). The absence of any change in the pattern of expression of *Fgf10* in periorbital mesenchyme of *Pax6*^{+/Sey-1Neu} mice suggests that *Pax6* might act as a lacrimal gland competence factor in conjunctival epithelium (Makarenkova et al., 2000).

The process of branching morphogenesis (Hogan, 1999) requires an exchange of signals between epithelium and mesenchyme. The end result is the reiterative production of a series of epithelial buds that have a characteristic distribution. Signaling molecules of the bone morphogenetic protein (Bmp) family have previously been implicated in the regulation of branching morphogenesis. For example, Bmp4 has a role in development of the lung (Bellusci et al., 1996), kidney (Raatikainen-Ahokas et al., 2000) and prostate gland (Lamm et al., 2001). In each case, Bmp4 appears to modulate

branching by suppressing proliferation of the epithelial component of the structure. Bmp7 has also been identified as an important regulator of branching morphogenesis. In the kidney, Bmp7 promotes growth and survival of the metanephric mesenchyme (Dudley et al., 1999), a result consistent with the effect of low doses of Bmp7 on the mIMCD-3 cell model of collecting duct morphogenesis (Piscione et al., 1997). Similarly, *Bmp7*-null mice have deficiencies in development of the submandibular gland (Jaskoll et al., 2002). An assessment of the role of Bmps in branching morphogenesis is complicated by their overlapping and dynamic expression patterns, their ability to heterodimerize (Suzuki et al., 1997), the variety of receptor heterodimers through which they can signal (Massague, 1998) and the possibility of activity modulation by secreted antagonists (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996; Cho and Blitz, 1998; Cancilla et al., 2001).

In the current study, we have investigated the function of Bmp7 in branching morphogenesis of the lacrimal gland. We show that *Bmp7* is expressed with a complex pattern in the developing gland and that *Bmp7*-null mice have distinctive reductions in lacrimal gland size and branch number. Consistent with a role for Bmp7 in promoting branching is the observation that whole gland explant cultures exposed to recombinant Bmp7 show an increased number of lacrimal gland buds, while those exposed to the inhibitors noggin and follistatin show decreased budding. Explants also show that Bmp7 does not have a discernible effect on isolated epithelium but does stimulate a response in mesenchymal cells in the form of increased cell division, aggregation and upregulation of cadherins, connexin 43 and α -smooth muscle actin. These data suggest that mesenchyme is the primary target of Bmp7 and, in turn, that the effect of Bmp7 on epithelial responses is indirect. These data also suggest that through its activity in stimulating mesenchymal condensation, Bmp7 may be important for formation of signaling centers. This model can explain both the mesenchymal responses to Bmp7 and the gland development deficiency of the *Bmp7*-null mice.

Materials and methods

Mouse lines

P6 5.0-lacZ reporter carrying a *Pax6* based reporter transgene (Williams et al., 1998) were used as described previously (Makarenkova et al., 2000) to visualize the epithelial component of the lacrimal gland. Genotyping of *Bmp7^{m1Rob}* animals was carried out essentially as described (Dudley et al., 1995). Genotyping of *Bmp7^{lacZ}* animals (Godin et al., 1998) was performed by staining a small tail biopsy with X-gal according to established techniques (Song et al., 1996).

Lacrimal gland reporter gene visualization and explant cultures

Lacrimal glands were visualized in whole-mount embryos by dissecting away the skin overlying the gland and then X-gal stained using established techniques (Song et al., 1996). Whole gland explant cultures were prepared from embryos between embryonic days (E) 15.5 and 17.5. Lacrimal glands were excised and placed into collagen gel in a four-well plate (Nunc). Glands were cultured at 37°C and 5% CO₂ in growth medium consisting of CMRL-1066 (Gibco BRL) supplemented with heat inactivated 10% fetal calf serum (FCS), glutamine, non-essential amino acids and an antibiotic-antimycotic

(Gibco BRL) antibiotics. Explants were cultured in either serum-free defined medium (Zuniga et al., 1999) or in the CMRL-1066+10% FCS supplemented with 100 ng/ml Bmp7 (R&D systems). For experiments with Bmp inhibitors, explants were cultured with or without either noggin-conditioned medium or 100 ng/ml recombinant noggin (R&D systems) or follistatin. *Xenopus* noggin concentrated in conditioned medium was used as described previously (Lamb et al., 1993). After 48 hours in culture, tissues were fixed and photographed or stained with X-gal (Song et al., 1996).

Culture of lacrimal gland epithelium

Lacrimal gland epithelium was isolated and cultured as described previously (Makarenkova et al., 2000). Briefly, the isolated epithelium was placed in the center of one well of a four-well plate and covered with Matrigel or collagen gel. Once the gel had set, 300 μ l of defined medium was added into the well. For some experiments, an in vitro epithelial bud extension assay was performed as previously described (Weaver et al., 1999). In these assays, heparin acrylic beads (Sigma) were loaded with recombinant Fgf10 (R&D systems) and placed ~100–150 μ m from the anterior part of the epithelial bud. Explants were cultured in defined medium alone or in the same medium supplemented with either recombinant Bmp4 or Bmp7 (R&D systems).

Mesenchyme cultures

Lacrimal gland mesenchyme cultures were prepared in a similar manner to limb bud micromass cultures (Vogel and Tickle, 1993). Lacrimal glands were isolated from E15.5–16 embryos and placed in 2% trypsin solution on ice for 1 hour. The tissue was then placed in medium containing serum to stop the enzyme reaction and then the epithelium was mechanically separated from the surrounding mesenchyme using fine needles. The mesenchyme was triturated and then centrifuged at 400 *g* for 5 minutes. Cells were re-suspended in defined medium and plated at a density of 10,000 cells in 10 μ l on Poly-L-Lysine and laminin-coated coverslips (Biocoat) in Nunc four-well plates and left to attach for 1 hour. Once the cells had attached, 300 μ l defined medium or defined medium containing 1–100 ng/ml Bmp7 was added to each well. Cultures were maintained for 6, 24, 48 or 72 hours in an incubator at 37°C with 5% CO₂ in air before being fixed and immunolabeled. For mesenchyme cell proliferation assays, the culture medium was replaced after 24 hours with DMEM containing 1% FCS and 10 μ M BrdU with or without Bmp7 for 2 hours prior to fixation. Cells were fixed and labeled as described previously (Brennan et al., 2000).

Quantification

The degree of lacrimal gland development in explants was quantified simply by counting the number of terminal epithelial buds (acini). All error bars shown represent standard errors. Statistical significance of data was determined using the Student's *t*-test with the exception of Fig. 4L, which required the unpaired *t*-test, and of Fig. 5A, which was better suited to the Mann-Whitney U test. *P* values of less than 0.05 were considered to be significant and this was the case (as indicated in legends and Figures) for all data shown. For Fig. 3, the length of the lacrimal gland from the temporal fornix of the conjunctiva to the most distal end of the epithelial component of the gland was measured in mm (by placing a mm scale within the images of gland preparations). As budding of the lacrimal first occurs at E13.5, we defined the length as zero and the acinus number as one. In this case, statistical significance was determined using the unpaired *t*-test.

Preparation of tissue sections for immunostaining

Lacrimal glands were dissected and fixed in 4% paraformaldehyde for 1 hour on ice, washed in PBS and dehydrated through graded ethanols. Polyester wax (Polysciences) was melted at 37°C and diluted with an equal volume of ethanol. Lacrimal glands were then placed in the ethanol:wax mixture in small glass vials and left until the tissue had

sunk to the bottom of the vial. Half the volume of the mixture was then removed and replaced with fresh wax until the tissue was impregnated with 100% wax. The tissue was then placed into moulds filled with fresh wax and left to set at room temperature before being stored at 4°C. Sections (7 or 10 µm) were cut using a Leica microtome and mounted onto Superfrost Plus microscopy slides (Fisher). Prior to immunostaining, the sections were de-waxed in 100% ethanol and dried at room temperature.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Nieto et al., 1996). The *Fgf10* probe has been described previously (Bellusci et al., 1997).

Antibodies and immunostaining

For immunostaining, the following primary antibodies were used: rabbit polyclonal antibody to Connexin 43 (Cx43) (GAP 15) (amino acids 131-142) was diluted to 1:200; mouse anti-BrdU antibody (Dakopatts) diluted 1:100 in PBSBT (PBS containing 1% BSA and 0.2% Triton); mouse anti- α -smooth muscle actin (Clone 1A4 Sigma) diluted 1:500; rabbit polyclonal pan-cadherin (Sigma) diluted 1:500; mouse anti human Ki-67 (Novocastra laboratories, Newcastle upon Tyne, UK) diluted 1:250; mouse anti chick Pax6 (Developmental Hybridoma Studies Bank, Iowa, USA) diluted 1:500. All antibodies were diluted in TBST (TBS containing 1% Triton). All incubations with primary antibodies were carried out at 4°C overnight except for BrdU antibody which was added for 1 hour at room temperature. Primary antibodies were revealed by either Alexa-, Rhodamine-, Cy3 or Texas Red-conjugated goat anti-mouse or goat anti-rabbit immunoglobulins (Molecular Probes Eugene, OR). Nuclei were counterstained with either OliGreen (Molecular Probes Eugene, OR) at a dilution of 1:5000 for 5 minutes or with Hoechst 33258. Labeled preparations were viewed on a Zeiss Axioplan microscope and digital images obtained with a Sony DKC5000 camera. Confocal images were obtained on a BioRad laser-scanning confocal microscope.

Results

Bmp7 is expressed in developing lacrimal gland

As a first step in determining whether *Bmp7* might be involved in lacrimal gland development, we assessed the expression pattern of *Bmp7* in both the induction and branching phases using heterozygous gene-targeted mice in which the *lacZ*-coding region has been inserted into the *Bmp7* gene (Godin et al., 1998). We have confirmed, using *Bmp7* probe whole-mount in situ hybridization on wild-type glands, that the *Bmp7^{lacZ}* expression pattern reflects that of the endogenous gene (data not shown). At E13.0, when the lacrimal bud has not yet formed on the temporal side of the eye, *Bmp7^{lacZ}* expression is observed in the periocular epithelium and in the periocular mesenchyme (Fig. 1A). Interestingly, mesenchyme adjacent to the site of lacrimal gland budding appears to have only low level *Bmp7^{lacZ}* expression (Fig. 1A, broken yellow lines). Sectioning confirms that at E13.0, a triangular domain of mesenchyme (Fig. 1B, arrow) located temporal to the invaginating presumptive conjunctival epithelium (Fig. 1B) expresses *Bmp7^{lacZ}* at a low level. At this stage, the presumptive eyelid tissue (Fig. 1B, asterisks) also express *Bmp7^{lacZ}*.

In mice that carry both the *P6 5.0-lacZ* reporter (a marker of lacrimal gland epithelium) as well as the *Bmp7^{lacZ}* allele, the lacrimal bud is observed extending into a defined region of *Bmp7^{lacZ}*-expressing mesenchyme at E14.5 (Fig. 1C, arrows). Sectioning confirms that at E14.5, the lacrimal bud (Fig. 1D,

lb) is extending into the *Bmp7^{lacZ}* mesenchymal expression domain (Fig. 1D, *mes*). At higher magnification (Fig. 1E), it can be observed that the lacrimal bud (Fig. 1E, *lb*) including the epithelial component (Fig. 1E, *ep*) is negative for *Bmp7^{lacZ}* expression. One day later at E15.5, according to sections from *Bmp7^{lacZ}* embryos (Fig. 1F) and whole-mounts from *P6 5.0-lacZ*, *Bmp7^{lacZ}* embryos (Fig. 1G), extension of the lacrimal bud into the lacrimal mesenchyme has continued. At this stage, some glands show the first signs of secondary budding and branching.

As development proceeds through E17.0, *Bmp7^{lacZ}* expression is observed in the lacrimal gland mesenchyme (Fig. 1H) with the strongest expression associated with the distal tips of the epithelial buds (Fig. 1H, red arrows) and with the midsection of the lacrimal duct (Fig. 1H, *ld*). Sectioning of whole X-gal-stained glands from E17.5 *Bmp7^{lacZ}* embryos showed that *Bmp7^{lacZ}* expression is observed not only in the mesenchyme (Fig. 1I, broken line) but also in some cells of the gland epithelium. This is apparent in the epithelium of the lacrimal duct (Fig. 1I, *ld*) and in the terminal epithelial buds (Fig. 1I, e.g. *ep*). Expression of *Bmp7^{lacZ}* in condensing mesenchyme adjacent to epithelial buds (Fig. 1I, arrowheads) is consistent with the patterns observed in both E17.0 (Fig. 1H) and E17.5 (Fig. 1J) *Bmp7^{lacZ}* whole-mount glands and is confirmed by higher-power magnifications of the lacrimal duct (Fig. 1K), an extended region of budding epithelium (Fig. 1L) and a terminal epithelial bud (Fig. 1M). A higher magnification view of the *Bmp7^{lacZ/+}* lacrimal gland at E18.5 in excised whole-mount (Fig. 1N) also indicates that cells at the tip of developing branches are more strongly positive than at the branch points. This pattern of expression can be contrasted with the uniform X-gal labeling observed throughout the epithelium of the *P6 5.0-lacZ* mouse (Fig. 1O). Although sporadic epithelial cells express *Bmp7^{lacZ}*, the overall pattern corresponds to the mesenchymal component of the so-called signaling centers (Hogan, 1999).

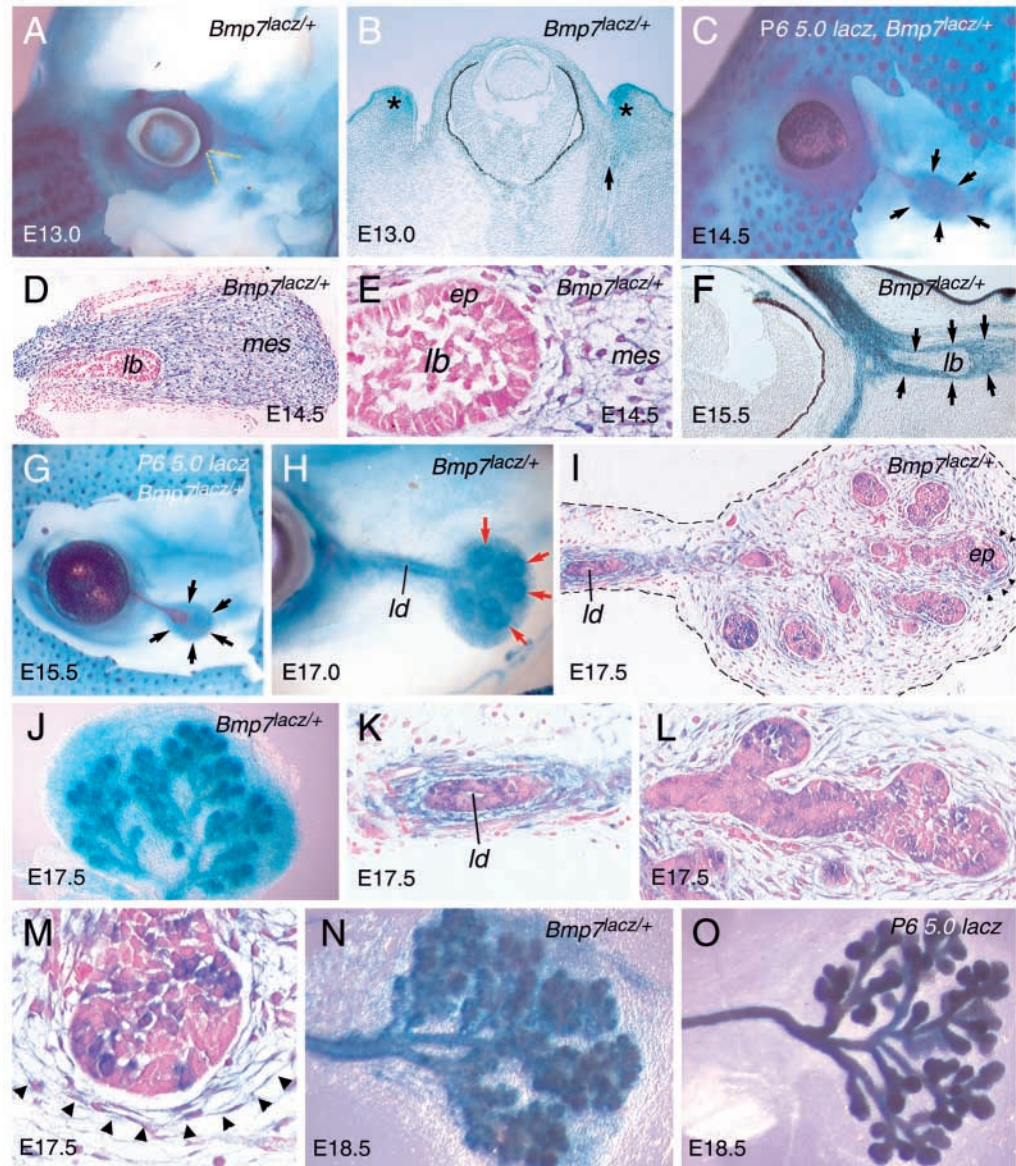
Bmp7 is essential for normal branching morphogenesis of the lacrimal gland

To determine whether *Bmp7* functions in development of the lacrimal gland, we examined a second line of mice that carry a conventional targeted allele (designated *Bmp7^{m1Rob}*) of the *Bmp7* gene (Dudley et al., 1995). These animals were crossed with the *P6 5.0-lacZ* reporter to the F2 generation to produce *Bmp7^{m1Rob/m1Rob}*, *P6 5.0-lacZ*, and *Bmp7^{m1Rob/+}*, *P6 5.0-lacZ* embryos. These could be stained with X-gal for easy visualization of gland structure (Makarenkova et al., 2000). Despite the microphthalmia that is characteristic of some *Bmp7*-null mice (Dudley et al., 1995; Wawersik et al., 1999) formation of the first lacrimal bud occurred with normal timing (E13.0-13.5) and in the normal location on the temporal side of the eye (compare Fig. 2A with 2B). By E19.5, the wild-type gland consists of two lobes: a small intraocular lobe derived from a single branch of the proximal duct (Fig. 2C, io and blue arrowhead); and an extensively branched exorbital lobe (Makarenkova et al., 2000) (Fig. 2C, xo). By contrast, the lacrimal gland in *Bmp7*-null animals showed varying degrees of deficiency. In some cases, both intraorbital (Fig. 2D, blue arrowhead) and exorbital lobes (Fig. 2D, xo) were near normal in size while in others, the gland was severely vestigial (Fig. 2E and F). The distribution of buds and branches in the lacrimal

Fig. 1. *Bmp7* is expressed in developing lacrimal gland. All preparations are stained with X-gal. (A,C,G,H) The dermis overlying the developing lacrimal gland has been removed. (D,E,I,K,L-M) Paraffin wax-embedded sections counterstained with nuclear Fast Red. (A) E13.0, *Bmp7^{lacZ/+}* mouse. The region of mesenchyme through which the lacrimal gland will grow does not stain for *Bmp7^{lacZ}* expression (broken yellow lines). (B) Section through eye region of E13.5 *Bmp7^{lacZ/+}* embryo showing *Bmp7*-positive presumptive eyelid mesenchyme (asterisks) and periorbital mesenchyme (arrow). The section plane shown does not correspond to the *Bmp7^{lacZ}*-negative region in A. (C) Whole-mount *Bmp7^{lacZ/+}*, *P6 5.0-lacZ* embryo at E14.5 showing epithelial bud extending into *Bmp7^{lacZ}*-positive mesenchyme (arrows).

(D) Section through E14.5 *Bmp7^{lacZ/+}* lacrimal gland showing lacrimal bud (*lb*) and mass of mesenchymal cells (*mes*). The epithelium of the lacrimal bud is negative for *Bmp7^{lacZ}* expression; the mesenchyme is positive (blue X-gal staining). (E) As in D, but at higher magnification to show X-gal-negative epithelium (*ep*) and positive mesenchyme (*mes*).

(F) The eye region of E15.5 *Bmp7^{lacZ/+}* embryo showing lacrimal bud (*lb*) extending into *Bmp7^{lacZ}*-expressing mesenchyme (arrows). (G) As in C but at E15.5. (H) Whole-mount *Bmp7^{lacZ/+}* embryo at E17.0 when secondary branching is apparent. The highest levels of *lacZ* expression are observed in the condensing mesenchyme surrounding the tips of the epithelial buds (red arrows) and the lacrimal duct (*ld*). (I) Section through E17.5 *Bmp7^{lacZ/+}* lacrimal gland showing *Bmp7^{lacZ}* expression throughout mesenchyme (surrounded by broken line) in some cells of the ductal (*ld*) and bud epithelium (*ep*) and in mesenchyme adjacent to epithelial bud tips (arrowheads). (J) Dissected whole-mount preparation of E17.5 *Bmp7^{lacZ}* lacrimal gland showing general low level *Bmp7^{lacZ}* expression in mesenchyme and higher levels adjacent to epithelial bud tips. (K) Higher magnification of the lacrimal duct shown in I to indicate *Bmp7^{lacZ}*-expressing epithelial and mesenchymal cells. (L) Higher magnification of extended epithelial region in *Bmp7^{lacZ}* lacrimal gland showing distribution of X-gal-positive cells. (M) Section through terminal *Bmp7^{lacZ/+}* epithelial bud showing X-gal-positive condensing mesenchyme (arrowheads). (N) Whole-mount *Bmp7^{lacZ/+}* lacrimal gland at E18.5. (O) E18.5 *P6 5.0-lacZ* lacrimal gland to show contrasting pattern of epithelial expression.



glands of *Bmp7*-null mice was also abnormal; they were often observed in the primary duct region (Fig. 2F-H, red arrowheads). With dissection of glands from *Bmp7^{m1Rob/m1Rob}*, *P6 5.0-lacZ*, embryos, it was clear that absence of *Bmp7* did not prevent formation of the mesenchymal sac (Fig. 2H, arrows). The degree to which development of the lacrimal gland was affected did not correlate with the variable degree of microphthalmia observed in *Bmp7*-null mice (Fig. 2D-G, mi), indicating that the two structures develop independently. Quantification of the gland length and the number of branches

or acini in the lacrimal gland indicated that in the *Bmp7*-null mouse, while there was not a significant reduction in the overall length of the gland ($P=0.7$, $n=6$), the number of branches and acini was significantly reduced ($P<0.01$, $n=4$) (Fig. 3A,B). These results demonstrate that *Bmp7* is required to establish the appropriate number and position of lacrimal gland branches.

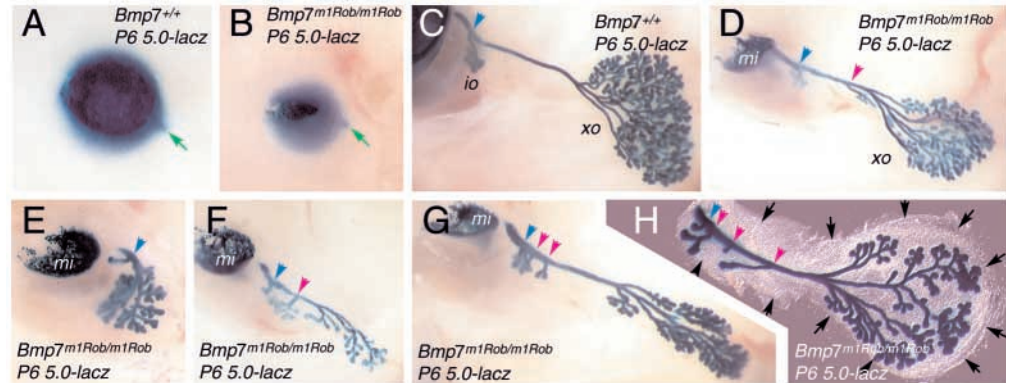
As a further test of *Bmp* function in lacrimal gland development, we took advantage of the *Bmp* antagonists noggin and follistatin. Both Noggin and follistatin bind *Bmp2*,

Fig. 2. *Bmp7* is required for normal development of the lacrimal gland.

All preparations are stained with X-gal. (A) E13.5 *P6 5.0-lacZ* mouse and (B) *Bmp7^{m1Rob/m1Rob}, P6 5.0-lacZ* mouse both showing normal formation of the primary lacrimal bud. (C) Wild-type E19.5 *P6 5.0-lacZ* mouse showing normal lacrimal gland branching pattern. The gland is composed of a small intra-orbital lobe (*io*) (blue arrowhead) and a large exorbital lobe (*xo*).

(D-H) Lacrimal glands in E19.5 *Bmp7^{m1Rob/m1Rob}, P6 5.0-lacZ* mice

showing the position of the intraorbital lobe (blue arrowheads), misplaced buds and branches (purple arrowheads) and in some cases (E,F) loss of lobe distinctions. The degree to which the lacrimal gland was affected did not correlate with the degree of microphthalmia (*mi*). (H) Lacrimal gland dissected from E19.5 *Bmp7^{m1Rob/m1Rob}, P6 5.0-lacZ* mouse (black arrowheads indicate the extent of gland mesenchyme).



Bmp4 and *Bmp7*. However, *Noggin* binds *Bmp2/4* more strongly than it binds *Bmp7*, while *follistatin* binds *Bmp7* more strongly than *Bmp2/4* (Yamashita et al., 1995; Zimmerman et al., 1996; Bailemans and Van Hul, 2002). To determine whether these antagonists could modulate development of the gland, we treated a series of whole-gland explant cultures with recombinant human *noggin* or *follistatin*. Explants were established from either E16.5 or E17.5 mice, and cultured with and without the *Bmp*-binding proteins for a period of 48 hours. We observed that application of either *noggin* or *follistatin* resulted in suppression of branching, as indicated by the reduced number of acini in the treated samples [compare Fig. 4A with B and C (*noggin*) and D with E (*follistatin*)]. The difference in the number of acini was quantified and shown to be statistically significant for both *noggin* (Fig. 4F) and *follistatin* (Fig. 4G) treatment.

A reduction in bud number in lacrimal gland explants treated with *Bmp* inhibitors is consistent with suppression of bud formation observed in the lacrimal gland of *Bmp7*-null mice. However, the morphology of acini in *noggin*/*follistatin* treated glands was very different from that observed in the *Bmp7* null. Specifically, in the *Bmp7* null, while there were fewer acini, those that existed were of normal size and shape. By contrast, the acini in *noggin* and *follistatin*-treated glands were much larger. We reasoned that this might be a consequence of the activity of additional *Bmp* family member ligands that could be inhibited by *noggin* and *follistatin* in explants but were unaffected in the *Bmp7* null.

To investigate this possibility, we performed *noggin* inhibition studies on gland explants established at E16.5 from *Bmp7^{m1Rob/m1Rob}, P6 5.0-lacZ* embryos. Again, whole gland explants were cultured for 48 hours in the presence or absence of *noggin* and as before, a quantitatively significant reduction in the number of acini was observed (Fig. 4L). Interestingly, the acini that did form in *Bmp7^{m1Rob/m1Rob}* *noggin*-treated glands (Fig. 4K) were larger than those in the untreated controls (compare Fig. 4I with 4K) as had been observed in earlier inhibition experiments. When combined, these data indicate that at least one other *Bmp* family member functions in lacrimal gland development and is most probably involved in regulating epithelial cell proliferation.

Lacrimal gland mesenchyme is a target for *Bmp7* activity

As it was possible that the lacrimal gland branching defect in *Bmp7^{lacZ}* mutant mice could reside in either epithelium or mesenchyme, we performed a series of gain-of-function experiments using explant cultures of whole glands or isolated gland epithelium and mesenchyme.

When whole-gland explants were established at E15.5 and cultured for 48 hours in the presence or absence of recombinant *Bmp7*, we observed a modest but statistically significant increase in the number of branches formed following *Bmp7* treatment (Fig. 5A). This was in agreement with assessment of the *Bmp7*-null mice indicating that the role of *Bmp7* is to promote branching of the epithelium.

To assess the action of *Bmp7* on the epithelial component of the gland, we performed a bud extension assay that has been used previously to characterize the responses of lung endoderm (Weaver et al., 2000). In both the lung and the lacrimal gland, *Fgf10* is an endogenous stimulus for the epithelial proliferation and chemotaxis that drive epithelial extension (Makarenkova et al., 2000; Weaver, 2000). In the lung, *Bmp4* can suppress *Fgf10* mediated proliferation and as a result, suppress epithelial extension (Weaver et al., 2000). We used *Fgf10*-soaked heparin-sepharose beads to stimulate extension of epithelial

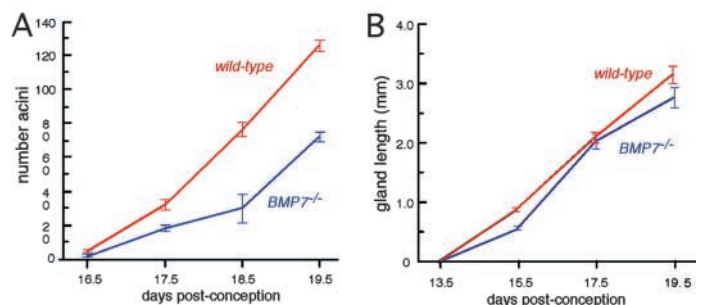
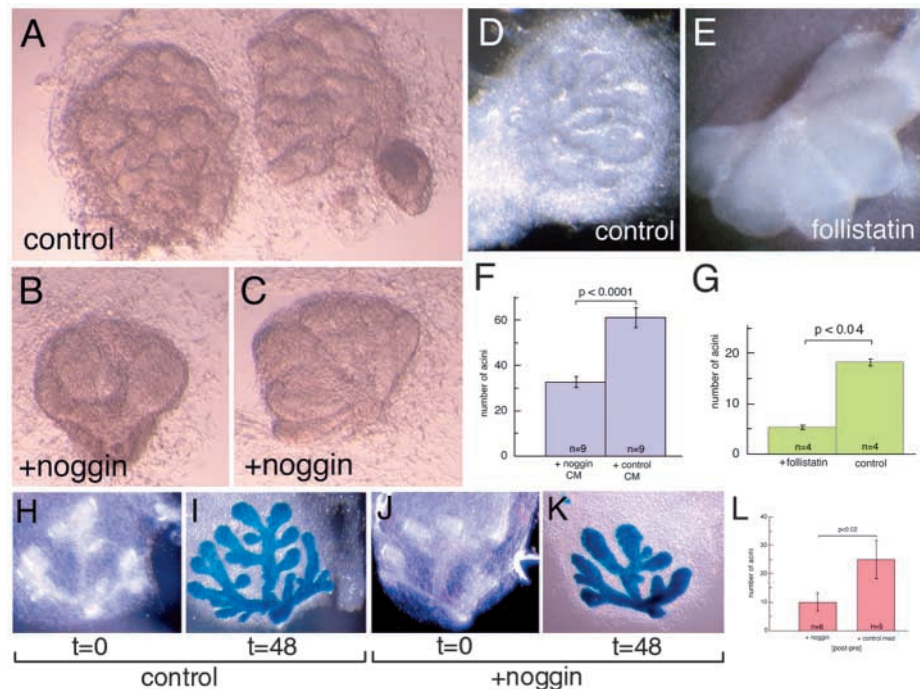


Fig. 3. *Bmp7* is required for lacrimal gland budding. Quantification of the number of acini (A) and total gland length (B) in control (red lines) and *Bmp7^{m1Rob/m1Rob}* mice (blue lines) at different embryonic stages. Error bars represent standard error.

Fig. 4. Inhibition of Bmps in explant cultures suppresses new bud formation. Lacrimal gland explants that are untreated (A,D) or treated with noggin (B,C) or follistatin (E) at 100 ng/ml. Inhibition of Bmps (B,C,E) causes a dramatic decrease in bud number and an increase in bud size. All explants are shown at the same magnification. (F) Quantification of the decrease in bud number at E17.5 + 48 hours of noggin treatment. (G) Quantification of the decrease in bud number at E16.5 + 48 hours of follistatin treatment. (H-K) Lacrimal gland explants from *P6 5.0-lacZ*, *Bmp7^{m1Rob/m1Rob}* mice either untreated (H,I) or treated with noggin for 48 hours (J,K). H and J are taken at *t*=0 hours; I and K at *t*=48 hours where the glands have been stained with X-gal. (L) Quantification of the decrease in bud number at E16.5 + 48 hours of noggin treatment of *P6 5.0-lacZ*, *Bmp7^{m1Rob/m1Rob}* lacrimal glands.



explants from the lacrimal gland, and assessed the effect of adding either Bmp4 or Bmp7 to the media of these cultures.

E15.5 mesenchyme-free primary bud explants were established in collagen gel 100–150 μ m from an Fgf10 bead and allowed to respond over a 48 hour period. Control explants were grown in defined medium only and showed the expected extension response (Fig. 5B). The addition of Bmp4 to the media suppressed growth and extension of the lacrimal gland epithelial explant in response to an Fgf10-soaked bead in the same manner as has been observed with lung epithelium (Weaver et al., 2000) (Fig. 5B). Interestingly however, addition of Bmp7 to media did not suppress extension or growth towards the Fgf10-soaked bead (Fig. 5B).

These data, combined with the knowledge that *Bmp7* is expressed in gland mesenchyme, suggested that mesenchyme might be the crucial target of Bmp7 activity. To examine this possibility, we isolated mesenchymal cells from wild-type embryos at E15.5 and determined whether there were distinctive responses to 100 ng/ml Bmp7 in low density micromass cultures. In control cultures, mesenchymal cells were evenly distributed over the plating area even after 48 hours (Fig. 6A). By contrast, Bmp7 treatment caused dramatic changes in cell morphology and distribution. After 12 hours, the cells became elongated and formed multiple small aggregates; in 24–48 hours, these small cell aggregates had formed larger clusters (Fig. 6B). As we did not observe increased levels of apoptosis or a decrease in cell number in the Bmp7-treated cultures (data not shown), this changed distribution presumably required migration.

Bmp7 has been shown to act in a dose-dependent manner to stimulate proliferation of kidney mesenchyme (Piscione et al., 2001). To assess the possibility that this was also the case in the lacrimal gland, we performed BrdU labeling of Bmp7-treated and control cultures using both low (1 ng/ml) and high (100 ng/ml) doses of Bmp7. We found that Bmp7 induces cell

proliferation and that the effect was dose dependent, with the highest levels of division being observed in cultures treated with high concentrations of Bmp7 (Fig. 6C). Mesenchymal proliferation and aggregation (condensation) are cell responses characteristic of the signaling centers that are critical for branching morphogenesis.

In a number of systems, mesenchymal condensation is associated with increased expression of the cadherin adhesion molecule family as well as junctional proteins such as connexin 43 (Cx43) (Minkoff et al., 1994; Haas and Tuan, 1999). To determine whether the Bmp7-stimulated aggregation of mesenchyme was associated with formation of cell contacts, we immunolabeled mesenchymal cell cultures for the gap junction protein Cx43 and for cadherins. This showed that the cells found in aggregates upregulated Cx43 (compare Fig. 6D with 6E) and cadherins (compare Fig. 6F with 6G) in response to Bmp7.

As many glandular structures contain cells that express smooth-muscle α -actin (α -SMA), we also determined whether Bmp7 might influence expression of this marker. In Bmp7-stimulated mesenchyme cultures, we observed an upregulation of α -smooth muscle actin (α -SMA) immunoreactivity in most cellular aggregates (Fig. 6H,I) and positive cells showed a network of well organized stress fibers (Fig. 6I). Only a few α -SMA-positive cells were detected in control cultures even after 72 hours in vitro and stress fibers were not observed (Fig. 6H). Interestingly, explanted lacrimal glands exposed to 100 ng/ml Bmp7 for 48 hours do not show a changed distribution of α -SMA but do show an upregulation of this marker in cells associated with the epithelium-mesenchyme boundary (Fig. 6J,K).

These data implied that in the *Bmp7* null gland, we might observe reduced proliferation as well as reduced expression of cadherins and α -SMA. To test this, we performed immunofluorescence detection for cadherins and α -SMA in

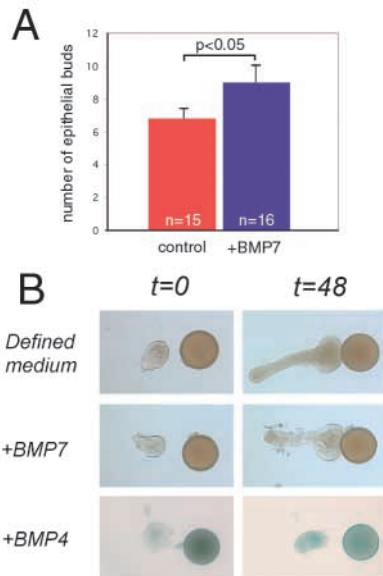


Fig. 5. Bmp7 induces the formation of epithelial branches in explant culture but does not suppress Fgf10-mediated epithelial bud elongation. (A) E15.5 whole lacrimal gland explants treated with 100 ng/ml Bmp7 for 48 hours contained more epithelial branches than control explants. The error bars are standard errors. The significance value of $P < 0.05$ is calculated according to the Mann-Whitney U test. (B) Left column, epithelial explants adjacent to Fgf10-soaked beads at $t = 0$ hours; right column, the same explants at $t = 48$ hours. Epithelial explants treated with Bmp7 resemble control cultures, whereas those treated with Bmp4 show suppression of the epithelial growth and elongation that is normally observed in response to Fgf10.

wild-type and *Bmp7* null glands (Fig. 7). We also double-labeled with anti-Pax6 antibodies to identify epithelial cells. So that we could objectively compare levels of immunoreactivity, sections from wild-type and mutant embryos were processed and labeled in the same experiment, images were acquired digitally under identical lighting conditions and figure panels were adjusted en masse in the same digital image file. Cadherin immunoreactivity in wild-type glands shows the anticipated junctional pattern in Pax6-positive epithelial cells (Fig. 7A). By contrast, although Pax6 nuclear labeling is easily detected, *Bmp7*-null glands show a consistently reduced level of cadherin immunoreactivity (Fig. 7B). Similarly, when detecting α -SMA in both wild-type (Fig. 7C,E) and *Bmp7*-null glands (Fig. 7D and F) Pax6 and α -SMA positive cells are found in smaller numbers in the mutant (compare 7C with 7D, arrowheads). The α -SMA expression level in the positive cells of mutant glands is also very much lower. This is obvious when observing wild-type (Fig. 7E) and *Bmp7*-null (Fig. 7F) glands at higher magnification. Interestingly, the α -SMA positive cells are also positive for the Pax6 epithelial marker. An assessment of proliferation was performed using BrdU-labeling (Fig. 7G). This showed that in both the mesenchyme and epithelium of *Bmp7*-null glands, there were statistically significant reductions in the level of proliferation. The reduction was more dramatic in the mesenchyme. Given that Fgf10 has been identified as a proliferative stimulus for gland epithelium, we performed in situ hybridization for Fgf10 in both wild-type and *Bmp7* mutant glands (Fig. 7H,I). This indicated that Fgf10

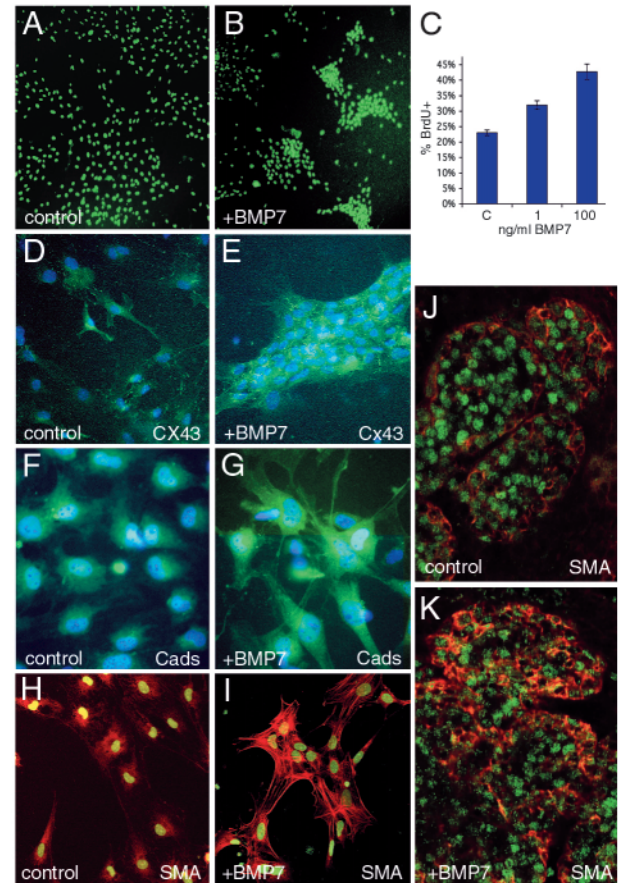


Fig. 6. Bmp7 induces mesenchymal aggregation and proliferation. Mesenchyme from E15.5 lacrimal glands was isolated and cultured for 48 hours in either defined medium (A,D,F,H) or with 100 ng/ml Bmp7 (B,E,G,I). (A,B) Cultures labeled with the nuclear dye Oligreen. Bmp7 treatment results in a striking aggregation of the cells (B). (C) Treatment of lacrimal gland mesenchyme with Bmp7 for 24 hours results in a dose-dependent increase in proliferation as indicated by the increased number of BrdU-labeled cells. Mesenchyme cultures were immunolabeled for connexin 43 (D,E), cadherins (F,G) or α -smooth muscle actin (H,I). (D-G) Immunoreactivity is shown in green, while nuclei are counterstained blue with Hoechst. (H,I) Immunoreactivity is shown in red, the nuclei in green. In all three cases, an increase in immunoreactivity was observed in Bmp7-treated cultures and associated with aggregated cells. (J,K) Confocal images of whole, wild-type, explanted lacrimal glands labeled for α -SMA (red) and Pax6 (green) after growth in the absence (J) or presence (K) of 100 ng/ml Bmp7. Bmp7 increased the level of expression of α -SMA.

expression levels were not noticeably different in the absence of Bmp7, suggesting an alternative explanation for reduced epithelial proliferation levels.

These data indicate that the response of explanted whole glands and isolated gland mesenchyme to Bmp7 is consistent with responses observed in the *Bmp7* mutant. Interestingly, however, for both cadherins and α -SMA, the immunoreactivity in both wild-type and *Bmp7* null was associated with the epithelialized component of the gland and was found only at low levels in surrounding mesenchyme. This observation raises the interesting issue of the origin of epithelial cells in this system.

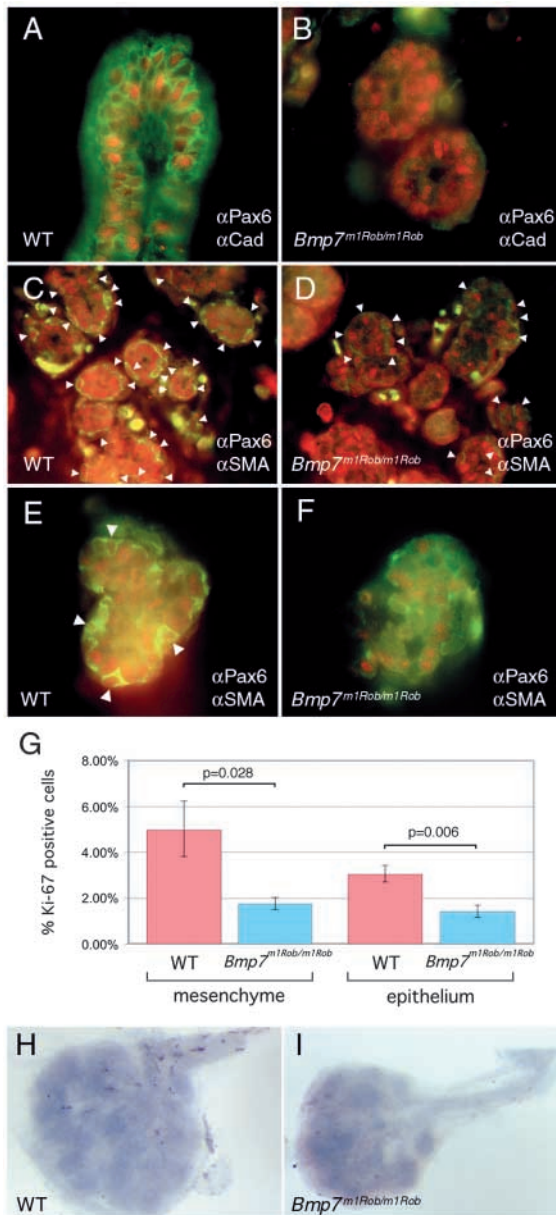


Fig. 7. *Bmp7*-null lacrimal glands show reduced cadherin and α SMA expression as well as reduced proliferation. Sections of wild-type (A,C,E) and *Bmp7*^{m1Rob/m1Rob} (B,D,F) Day of birth lacrimal glands. (A,B) Pax6 (red) and pan-cadherin (green) immunolabeling. (C-F) Pax6 (red) and α SMA (green) immunolabeling. Comparing A with B, it can be seen that the level of cadherin immunoreactivity is greatly reduced in the absence of *Bmp7*. (C,D) α SMA-positive cells (white arrowheads) are fewer in number and stain at lower intensity in the absence of *Bmp7*. (E,F) Higher magnification of wild-type (E) and *Bmp7*^{m1Rob/m1Rob} sections, showing reduced number of α SMA-positive cells and their reduced labeling intensity. (G) Graphs showing that, compared with wild-type (red bars), the *Bmp7*^{m1Rob/m1Rob} lacrimal gland (blue bars) shows a reduced number of Ki67 immunoreactive cells in both mesenchyme and epithelium. Error bars indicate s.e.m. and significance values are calculated according to Student's *t*-test. Day of birth glands were analyzed. (H,I) In situ hybridization performed on lacrimal glands from wild-type (H) and *Bmp7*^{m1Rob/m1Rob} (I) E17.5 embryos using a probe for *Fgf10*.

lacrimal gland epithelium and mesenchyme has suggested that the primary target of *Bmp7* is the mesenchyme and that epithelial responses are indirect. The activity of *Bmp7* in stimulating branching, combined with the expression pattern in the developing head, suggests a mechanism by which the lobe pattern arises.

Bmp7 is required for normal morphogenesis of the lacrimal gland

Several lines of evidence indicate that *Bmp7* has an important function in regulating the formation of buds and branches in the lacrimal gland. Morphological assessment of gland development in wild-type and *Bmp7* null mice supports this proposal as there are many fewer buds and branches in *Bmp7* null glands. Interestingly however, gland length is not significantly affected by the absence of *Bmp7*, and this suggests that *Fgf10*-driven primary and secondary branch extension occurs independently of *Bmp7*. This is also consistent with unchanged *Fgf10* expression observed in the *Bmp7* null. The appearance of primary lacrimal gland buds in all *Bmp7* null mice also indicates that *Bmp7* is not required during the inductive phases of lacrimal gland development and this, in turn, suggests that there may be distinct bud induction mechanisms employed at different stages of gland formation.

There are also likely to be multiple *Bmp* family members active during lacrimal gland development. Exposure of whole gland explants to the *Bmp* inhibitors noggin and follistatin produces fewer buds in a response that is consistent with *Bmp7* function. However, the buds that do form have an unusual morphology in that they are larger than normal. This response is not a result of inhibition of *Bmp7* activity as it is also seen when *Bmp7*-null glands are treated with noggin and follistatin and is a phenotype absent from the *Bmp7*-null mice. This is a clear indication that other *Bmp* family members are active in suppressing epithelial proliferation during lacrimal gland development. We speculate, based on evidence from the lung (Weaver et al., 2000), kidney (Raatikainen-Ahokas et al., 2000) and prostate (Lamm et al., 2001) that one of them is *Bmp4*.

Previous studies have demonstrated the importance of *Bmp7* for the development of branched structures. In the kidney system, *Bmp7* is involved in the early developmental stages in

Discussion

In the current study, we have assessed the role of *Bmp7* in development of the mouse lacrimal gland. We show, using a *Bmp7*^{lacZ} allele (Godin et al., 1998) that *Bmp7* has a dynamic expression pattern within the mesenchyme of the gland. At E14.5, when the primary epithelial bud has entered the mesenchymal sac, the mesenchyme but not the epithelium expresses *Bmp7*^{lacZ}. From E15.5 and beyond, the strongest *Bmp7*^{lacZ} expression is observed in mesenchymal cells that correspond to the signaling centers crucial for budding and branching (see below). Supporting an important role for *Bmp7* in lacrimal gland branching morphogenesis is the distinctive, albeit variably penetrant gland deficiency observed in the *Bmp7*-null mice. This gland deficiency is consistent with the results of explant experiments where *Bmp* inhibition reduced, and *Bmp7* oversupply increased, the number of branches and buds that formed. Ex vivo analysis of the responses of isolated

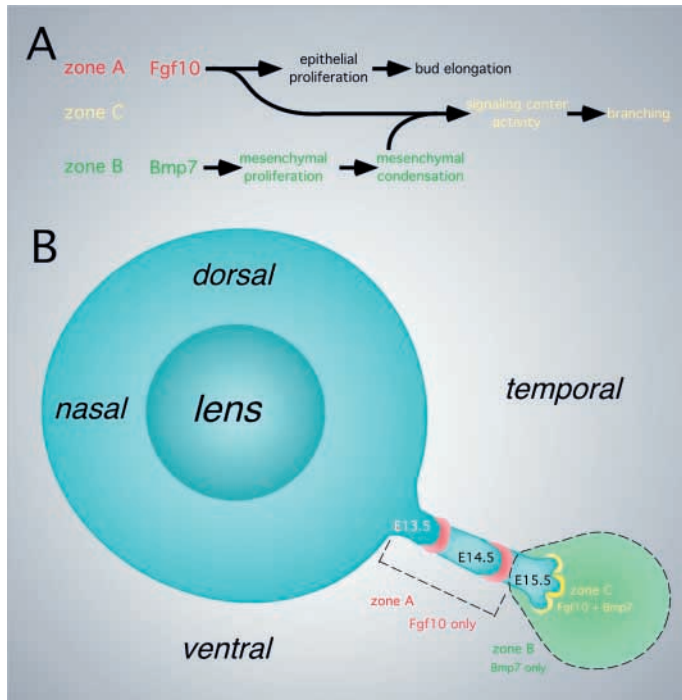


Fig. 8. A model describing Bmp7 function in lacrimal gland development. We propose that in the lacrimal gland, through stimulation of mesenchymal proliferation and aggregation, Bmp7 promotes formation of signaling centers and, as a result, branching morphogenesis (A). We also suggest that the pattern of lobe formation in the lacrimal gland can be explained in part by the combined action of Fgf10 and Bmp7 where Fgf10 is a stimulus for bud elongation and Bmp7 for branching (A,B). During the initial 48 hours of lacrimal gland development, we suggest that the primary bud extends and does not branch because the epithelium is exposed only to Fgf10 (A,B, zone A). By contrast, we propose that upon entering the defined region of Bmp7-expressing mesenchyme, epithelium will branch because it is exposed to Bmp7 (A,B, zone C). It is possible that this regulation of branching is mediated not by the absolute presence or absence of Bmp7, but by different Bmp7 levels.

mediating epithelial-mesenchymal interactions and stimulating survival of metanephric mesenchyme (Dudley et al., 1999). Although our experiments do not reveal a function for Bmp7 in stimulating mesenchymal survival, the positive effect of Bmp7 on lacrimal gland branching may be analogous to its function in the kidney. It has also been recorded that *Bmp7*-null mice have deficiencies in development of the submandibular gland (Jaskoll et al., 2002) and this too is consistent with the general effect of Bmp7 in the lacrimal gland system.

Bmp7 stimulates lacrimal gland branching by signaling mesenchymal responses

In attempting to understand the origin of the lacrimal gland development defect, we performed a series of explants of whole glands and of isolated mesenchyme and epithelium. Consistent with the phenotype of *Bmp7*-null mice, whole lacrimal glands exposed to Bmp7 showed a statistically significant increase in the number of epithelial buds. This indicates that Bmp7 is necessary for the formation of normal bud number and in the context of the explant assay, is sufficient to stimulate new bud formation. Furthermore, an Fgf10-driven bud extension assay (Weaver et al., 2000) was used to demonstrate that gland epithelium did not discernibly respond to Bmp7 (but did to Bmp4). By contrast, exposure of isolated mesenchyme to Bmp7 resulted in distinctive responses that included proliferation, aggregation and differentiation. These data argue that the primary target of Bmp7 in lacrimal gland development is the mesenchyme and that deficient epithelial responses are an indirect consequence of defective mesenchymal function.

The *in vitro* response of mesenchyme to Bmp7 is distinctive with dose-dependent increases in proliferation and the formation of cellular aggregates. Increased proliferation is

presumably important for providing the numbers of cells required for expansion of the gland. The formation of aggregates is accompanied by upregulation of cadherin family adhesion molecules and the junctional protein Cx43; both have been associated with formation of condensed mesenchyme (Minkoff et al., 1994; Haas and Tuan, 1999). These data demonstrate that in response to Bmp7, gland mesenchymal cells form close contacts characteristic of the tissue structures found in the lacrimal gland.

However, the nature of the gland components that Bmp7-stimulated mesenchyme represents is currently open to interpretation. Two models can be proposed. In the first, we can suggest that the major function of Bmp7 is to facilitate the formation of signaling centers (Fig. 8). In the context of branching morphogenesis (Hogan, 1999) signaling centers are defined as small groups of closely associated cells that are able to secrete factors and respond to stimuli in a manner that directs the process of branching. Close association of a critical number of cells in signaling center-like structures is thought to be essential for providing the necessary level of signaling [the so-called community effect (Gurdon, 1988)] required for some developmental processes. Signaling centers are proposed to include both epithelial and mesenchymal cells in the region of the developing bud tip. Reiterative formation of signaling centers is crucial for the progression of a single epithelial bud into a multi-branched mature organ. The shape of signaling centers is also thought to influence the shape of the growing tissue (Hogan, 1999).

A number of factors suggest that one function of Bmp7 may be to enhance the formation of signaling centers. These include the spatial and temporal coincidence of *Bmp7* expression with the signaling center, and that characteristics of the signaling center (proliferation, cell aggregation and condensation) are stimulated by Bmp7. In addition, most obviously, the *Bmp7*-null mice have reduced numbers of signaling centers as indicated by reduced bud number in the gland. Furthermore, the aberrant location of many buds in the lacrimal gland of *Bmp7*-null mice suggests that signaling center distribution is aberrant.

In an alternative, and arguably more speculative, model, we can propose that Bmp7 may function to stimulate a mesenchymal to epithelial transition. This developmental strategy has precedents in the formation of branched structures, notably in the kidney (Stark et al., 1994), where epithelial cells of the ureteric bud produce signals that stimulate mesenchymal

cells to condense and form the epithelial cells of the nephron. Suggestive evidence for this model in the context of the lacrimal gland comes from the observation that markers expressed at high levels in the epithelial component of the lacrimal gland (Connexin 43, cadherins and smooth muscle actin, Fig. 7) are upregulated when isolated mesenchymal cells are stimulated with Bmp7. In the wild-type gland, we also show, using the epithelial marker Pax6, that cadherin and α -SMA-positive cells have epithelial character. Thus, it is possible that the high expression level of Bmp7 found in the signaling centers is crucial to stimulate proliferation and a mesenchymal-epithelial transition that supplies the expanding gland with epithelial cells. Clearly, an assessment of the mesenchymal-to-epithelial transition model will require additional work developing strategies for fate-mapping different components of the gland.

Localized Bmp7 may be crucial for patterning of the lacrimal gland

A striking feature of structures that arise through branching morphogenesis is the reproducibility of the primary branching pattern and, as a consequence, the placement of organ lobes. The lung is a good example (Hogan et al., 1997), but this is also true for the lacrimal gland where both intraorbital and exorbital lobes arise and the timing and placement of branch initiation is highly reproducible. Interestingly, our current analysis suggests a mechanism by which Bmp7 can determine the location of the exorbital lobe of the gland (Fig. 7).

In Bmp7^{lacZ} embryos, it is clear that Bmp7 expression is absent from the periorbital mesenchyme adjacent to the initial lacrimal gland bud. However, strong Bmp7 expression is observed in a discrete patch of mesenchyme located between the eye and the pinna of the ear. After Fgf10-driven bud extension (Govindarajan et al., 2000; Makarenkova et al., 2000), during which the lacrimal gland epithelium extends away from the temporal side of the eye, the epithelium reaches and invades the Bmp7-expressing region of mesenchyme. Only when the epithelial bud has invaded does epithelial branching occur. When combined with evidence that Bmp7 is an important stimulus for branching, this sequence of events suggests that migration of the epithelial bud into a Bmp7 positive mesenchymal domain may determine the timing and location of primary branching. Further supporting this model is the loss of defined lobe structure in Bmp7-null mice. Conceivably, other steps in lacrimal gland development, such as formation of the intraorbital lobe and secondary branching, may be regulated by similar, Bmp7-dependent mechanisms.

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