

FGF-regulated BMP signaling is required for eyelid closure and to specify conjunctival epithelial cell fate

Jie Huang¹, Lisa K. Dattilo¹, Ramya Rajagopal¹, Ying Liu¹, Vesa Kaartinen³, Yuji Mishina⁴, Chu-Xia Deng⁵, Lieve Umans^{6,7}, An Zwijsen^{6,7}, Anita B. Roberts⁸ and David C. Beebe^{1,2,*}

There are conflicting reports about whether BMP signaling is required for eyelid closure during fetal development. This question was addressed using mice deficient in BMP or TGF β signaling in prospective eyelid and conjunctival epithelial cells. Genes encoding two type I BMP receptors, the type II TGF β receptor, two BMP- or two TGF β -activated R-Smads or the co-Smad Smad4 were deleted from the ocular surface ectoderm using Cre recombinase. Only mice with deletion of components of the BMP pathway had an 'eyelid open at birth' phenotype. Mice lacking *Fgf10* or *Fgfr2* also have open eyelids at birth. To better understand the pathways that regulate BMP expression and function during eyelid development, we localized BMPs and BMP signaling intermediates in *Fgfr2* and *Smad4* conditional knockout (CKO) mice. We found that *Fgfr2* was required for the expression of *Bmp4*, the normal distribution of Shh signaling and for preserving the differentiation of the conjunctival epithelium. FGF signaling also promoted the expression of the Wnt antagonist *Sfrp1* and suppressed Wnt signaling in the prospective eyelid epithelial cells, independently of BMP function. Transcripts encoding *Foxc1* and *Foxc2*, which were previously shown to be necessary for eyelid closure, were not detectable in *Smad4*^{CKO} animals. c-Jun, another key regulator of eyelid closure, was present and phosphorylated in eyelid periderm cells at the time of fusion, but failed to translocate to the nucleus in the absence of BMP function. *Smad4*^{CKO} mice also showed premature differentiation of the conjunctival epithelium, conjunctival hyperplasia and the acquisition of epidermal characteristics, including formation of an ectopic row of hair follicles in place of the Meibomian glands. A second row of eyelashes is a feature of human lymphedema-distichiasis syndrome, which is associated with mutations in FOXC2.

KEY WORDS: Eyelid closure, Conjunctival cell fate, c-Jun nuclear transport, BMP signaling, FGF signaling, Mouse

INTRODUCTION

Eyelid formation from the surface ectoderm and the underlying periocular mesenchyme involves four processes: eyelid specification, growth, closure and re-opening. In mice, eyelid specification begins by embryonic day (E) 9, when the expression of the transcription factor *Foxl2* defines the future location of the eyelids dorsal and ventral to the globe (Swindell et al., 2008). At E11.5, invagination of the dorsal and ventral periocular ectoderm signals the beginning of the period of eyelid growth. The resulting eyelid folds grow towards each other across the surface of the eye between E11.5 and E15.5. At E15.5, a projection of the outer, peridermal layer of the ectoderm extends from the eyelid margins across the cornea until the periderm extensions meet and fuse. The two lids separate at 'eye opening' on about postnatal day 10 (P10) (Findlater et al., 1993).

The closing eyelids are constituted by a loosely organized mesenchyme and the overlying epithelium. The eyelid epithelium differentiates into the palpebral epidermis (outer surface of the eyelid) and the palpebral conjunctiva (inner surface of the eyelid). The palpebral conjunctiva is continuous with the bulbar conjunctiva (the epithelium covering the anterior periphery of the globe), which is continuous with the corneal epithelium on the most anterior surface of the globe (Fig. 1).

After eyelid closure, the palpebral epidermis differentiates as part of the skin. Stratification and keratinization begin, and the regularly spaced hair follicles of the eyelashes form at the margins of the lids. However, the conjunctival epithelium does not stratify until after the eyelids re-open and it remains non-keratinized throughout life. The mature conjunctival epithelium contains abundant goblet cells, which produce mucus that is important for the properties of the tear film. Soon after birth, the Meibomian glands, which produce a lipid component of the tears, form by in growth of the conjunctival epithelial cells near the inner surface of the lid margin (Findlater et al., 1993).

Defects in eyelid growth or fusion may cause the eyelids to be open at birth (EOB). A surprising number of genes and signaling pathways are required for eyelid closure. An EOB phenotype is seen in mice with germline deletion of activin β -B (*Inhbb*), MEK kinase1 (*Map3k1*), c-Jun N-terminal kinase (*Mapk8*), c-Jun (*Jun*), the epidermal growth factor (EGF) family members HB-EGF (*Hbegf*) and transforming growth factor α (*Tgfa*), and their receptor (*Egfr*), fibroblast growth factor 10 (*Fgf10*), its receptor (*Fgfr2*), the forkhead transcription factors *Foxc1* and *Foxc2*, and the Wnt antagonist *Dkk2* (Gage et al., 2008; Kidson et al., 1999; Kume et al., 1998; Li et al., 2001; Li et al., 2003; Lueteteke et al., 1994; Lueteteke et al., 1993; Miettinen et al., 1995; Mine et al., 2005; Smith et al., 2000; Takatori et al., 2008; Tao et al., 2005; Vassalli et al., 1994;

¹Department of Ophthalmology and Visual Sciences and ²Department of Cell Biology and Physiology, Washington University, St Louis, MO 63130, USA.

³Developmental Biology Program, Childrens Hospital Los Angeles, Departments of Pathology and Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA 90027, USA. ⁴Molecular Developmental Biology Group, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA. ⁵Genetics of Development and Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. ⁶Laboratory Molecular Biology (Celgen), Department for Molecular and Developmental Genetics, VIB, B-3000 Leuven, Belgium. ⁷Laboratory Molecular Biology (Celgen), Center for Human Genetics, KU Leuven, B-3000 Leuven, Belgium. ⁸Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

* Author for correspondence (e-mail: Beebe@vision.wustl.edu)

Weston et al., 2004; Zenz et al., 2003; Zhang et al., 2003). Previous studies suggested that activin β -B promotes eyelid closure by activating a Smad-independent cascade involving MEK kinase1, Jun N-terminal kinase (JNK) and c-Jun (Takatori et al., 2008; Weston et al., 2004; Zhang et al., 2003). EGF family members contribute to periderm migration by activating the ERK signaling pathway (Mine et al., 2005). Upstream of the EGF cascade, c-Jun increases EGF receptor expression (Li et al., 2003; Zenz et al., 2003). FGF10 controls eyelid epithelial proliferation and periderm migration by stimulating the expression of activin β -B and TGF α , and by modulating the expression of sonic hedgehog (*Shh*) (Tao et al., 2005). The administration of a short-acting Shh antagonist at E9 results in EOB (Lipinski et al., 2008). Recently, mice lacking the Wnt antagonist *Dkk2*, showed EOB, indicating that Wnt activity must be properly tuned during eyelid development.

It has not been clear whether bone morphogenetic protein (BMP) signaling plays a role in eyelid closure. An EOB phenotype was detected in one mouse strain in which *Bmpr1a* was conditionally deleted in the ectoderm by using a keratin 14-driven Cre-recombinase transgene (Andl et al., 2004). However, mice overexpressing the BMP antagonist noggin under the control of the human K14 or K5 promoters had eyelid defects, but no EOB phenotype (Plikus et al., 2004; Sharov et al., 2003). Mice overexpressing the inhibitory Smad Smad7, driven by the bovine K5 promoter, did have an EOB phenotype (He et al., 2002). However, whether this phenotype was attributable to blocking TGF β , activin, or BMP signaling has not been clarified. In addition, overexpression of BMP signaling antagonists or deficiencies in the BMP signaling pathway cause other epithelial defects that may indirectly result in EOB. For example, in some of these cases, the epidermal, conjunctival and corneal epithelia were hyperplastic, and sweat glands transdifferentiated into hair follicles (He et al., 2002; Plikus et al., 2004).

To clarify the possible function of BMP signaling in eyelid development, we conditionally deleted two type I BMP receptors, two of the BMP-activated R-Smads or the co-Smad Smad4 in the prospective eyelid epithelium beginning on E9. In each case, the mice showed normal eyelid formation and adequate growth, but the eyelid epithelia did not fuse, resulting in an EOB phenotype. Deletion of the sole type II TGF β receptor or the two activin- and TGF β -activated R-Smads did not interfere with eyelid closure. Further analysis suggested that Fgf10 from the mesenchyme activates Fgfr2 in the lid ectoderm. Fgfr2 signaling modulates Shh levels, resulting in *Bmp4* expression in the mesenchyme. FGF signaling also inhibits Wnt signaling in the eyelid ectoderm, independently of its effects on BMP expression. BMPs are required for the expression of the transcription factors Foxc1 and Foxc2 in the ectoderm, the nuclear translocation of activated c-Jun in periderm cells, the proper timing of conjunctival epithelial differentiation and the establishment of conjunctival epithelial cell fate. In the absence of BMP signaling, ectopic hair follicles formed on the inner edges of the eyelid at the expense of the Meibomian glands, a feature of human lymphedema-distichiasis syndrome.

MATERIALS AND METHODS

Mice genotyping and mating

The following genetically modified mice were used in this study: Le-Cre (Ashery-Padan et al., 2000), *Acvr1* flox (Dudas et al., 2004), *Bmpr1a* flox (Gaussin et al., 2002), *Smad4* flox (Yang et al., 2002), *Smad1* flox (Huang et al., 2002), *Smad5* flox (Umans et al., 2003), *Smad2* flox (Piek et al., 2001), *Smad3* germline knockout (Roberts et al., 2006), *Fgfr2* floxed (Yu et al., 2003), *Tgfb2* floxed (Chytil et al., 2002), presenilin 1 floxed (Yu et al., 2001), presenilin 2 germline knockout (Steiner et al., 1999) and TOPGAL,

a Wnt reporter strain (DasGupta and Fuchs, 1999). Matings between mice that were homozygous for the floxed allele, only one of which was Cre-positive, resulted in litters in which about half of the offspring were Cre-positive (conditional knockout, CKO). The others were Cre-negative (wild type). Noon of the day when the vaginal plug was detected was considered as embryonic day (E) 0.5 of development. Embryos were collected at the desired stages ($n=3$ to 5 for each genotype and stage).

Histology

Embryo heads were fixed in 4% paraformaldehyde/PBS overnight at 4°C, dehydrated through a series of ethanol concentrations, embedded in paraffin and sectioned at a thickness of 4 μ m. For morphological studies, sections were stained with Hematoxylin and Eosin (Surgipath, Richmond, IL, USA).

In situ hybridization

Frozen sections were fixed in 4% paraformaldehyde/PBS, treated with proteinase K (10 μ g/ml), post-fixed in 4% paraformaldehyde/PBS and acetylated in triethanolamine-acetic anhydride solution. Samples were pre-hybridized in 50% formamide, 5 \times SSC, 5 mM EDTA, 1 \times Denhardt's, 100 μ g/ml heparin, 0.3 mg/ml yeast tRNA and 0.1% Tween-20, incubated in the same solution with riboprobes overnight, washed with 0.2 \times SSC, blocked in 10% lamb serum and incubated with anti-digoxigenin antibody overnight. The color reaction was developed using NBT and BCIP in the dark. After the reaction was completed, the slides were washed in PBS, fixed in 4% paraformaldehyde/PBS and mounted in 100% glycerol.

Digoxigenin-labeled riboprobes were synthesized from cDNA generated from RNA isolated from wild-type E15.5 eyelids using the following PCR primer pairs:

Foxc1, 5'-CCAGAAAGTGTTCCAAAAGC-3' and 5'-GAAACCACC-CCAGACTAATG-3';

Foxc2, 5'-GCCACCTCTGGTATCTGAAC-3' and 5'-CTGGGCAA-GAACAAAATAGCC-3';

BMP4, 5'-TGGTAACCGAATGCTGATGG-3' and 5'-GGCGACGG-CAGTTCTTATTC-3';

Sfrp1, 5'-ATCCCCCTCTTTCTGCCTTAG-3' and 5'-GAAATACC-TCTGGGCACTTG-3';

Dkk2, 5'-TTTACAAAGTGGGTTCCCTTG-3' and 5'-CTCCATTTT-CACATCACAAAGC-3'.

Probe for patched 1 was a kind gift from Dr David Ornitz (Washington University, St Louis, MO, USA). Gene expression patterns were compared between CKO and wild-type littermates and each in situ hybridization was performed at least twice.

Immunofluorescence staining

Frozen sections were warmed to room temperature and then fixed in 4% paraformaldehyde/PBS. After three washes in PBS, the samples were treated with 3% H₂O₂ in methanol to quench endogenous peroxidase activity, blocked in 5% goat serum/0.1% Triton X-100, incubated in primary antibody overnight, washed and processed with tyramide amplification. The antibodies for pSmad1/5/8 and p-c-Jun were from Cell Signaling Technology (Danvers, MA, USA). The keratin 14 and keratin 10 antibody was from Covance Research Products (Denver, PA, USA). The keratin 4 antibody was from Sigma-Aldrich (St Louis, MO, USA). The Dkk2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

X-gal staining

Staged embryos expressing a *lacZ* reporter gene were fixed 4% in paraformaldehyde/PBS at 4°C for 30 minutes, washed twice in PBS with 2 mM MgCl₂, 0.02% NP-40/0.01% deoxycholate (DOC), and stained with X-gal solution [5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 1 M MgCl₂, 0.02% NP-40/0.01% DOC NP-40, 1 mg/ml X-gal in PBS] for 5 hours at 37°C, post-fixed with 4% paraformaldehyde for 1 hour, cryoprotected and, when required, 10- μ m sections were prepared.

BrdU and TUNEL staining and quantification

Pregnant female mice were injected with 50 mg/kg of a mixture of 10 mM BrdU (Roche, Indianapolis, IN, USA) and 1 mM 5-fluoro-5'-deoxyuridine (Sigma, St Louis, MO, USA) and sacrificed after 1 hour. A monoclonal anti-BrdU antibody (diluted 1:250; Dako, Carpinteria, CA, USA) was used with

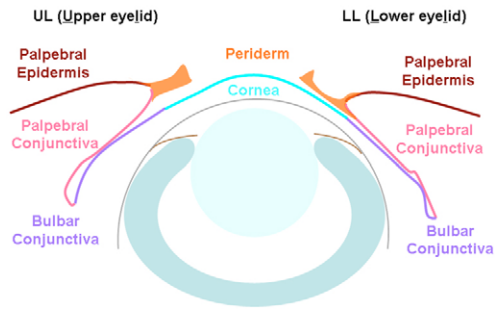


Fig. 1. Wild-type mouse eyelid anatomy at E15.5. The components of the normal ocular epithelia are color coded: bulbar conjunctiva, purple; palpebral conjunctiva, pink; palpebral epidermis, dark red; periderm, orange; cornea, blue.

a Vectastain Elite Mouse IgG ABC kit. Sections were counterstained with Hematoxylin. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) was done with an Apoptag kit (Chemicon, Temecula, CA, USA). The deparaffinized slides were treated with 3% H₂O₂ in methanol for 30 minutes, followed by proteinase K treatment (20 µg/ml) for 15 minutes. Slides were incubated with TdT enzyme in equilibration buffer for 1 hour at 37°C. The reaction was terminated with wash buffer provided by the manufacturer for 10 minutes at room temperature. Anti-digoxigenin-peroxidase conjugate was added for 30 minutes at room temperature, followed by DAB and H₂O₂ treatment. Slides were counterstained with Hematoxylin.

BrdU and TUNEL-positive cells were counted in the ocular surface epithelia at E14.5 in 4-5 sections from each embryo (wild type, $n=6$; *Smad4*^{CKO}, $n=8$). The means and standard error (s.e.m.) were calculated from the pooled data. Differences were considered significant if $P < 0.05$, as determined by Student's *t*-test.

RESULTS

BMP signaling is required for mouse eyelid closure

To study the functions of BMPs in early eye development, we used Cre recombinase to delete floxed alleles of key components of the BMP signaling pathway. Transgenic Cre expression was driven by a promoter that is first expressed at E9 in the lens placode and in the ectoderm that later differentiates into the ocular surface epithelia (Le-Cre) (Ashery-Padan et al., 2000). The ocular surface epithelia targeted by the transgene include the palpebral epidermis, palpebral conjunctiva, bulbar conjunctiva and corneal epithelium (Ashery-Padan et al., 2000) (Fig. 1). Le-Cre mice were mated to mice with floxed alleles of two of the three type I BMP receptors (*Acvr1* and *Bmpr1a*), two of the BMP-activated R-Smads (*Smad1* and *Smad5*), the two activin/TGFβ-activated R-Smads (*Smad2* and *Smad3*), the sole type II TGFβ receptor (*Tgfb2*) or the co-Smad *Smad4*. Matings were between Cre-positive homozygous flox and Cre-negative homozygous flox animals, assuring that about half of the offspring were Cre-positive and no animals received two copies of the transgene.

In each knockout targeting the BMP pathway (*Acvr1/Bmpr1a*, *Smad1/5* and *Smad4*), Cre-positive animals had an eyelid-open-at-birth (EOB) phenotype (Fig. 2B-D). Offspring with conditional deletion of one allele of the BMP pathway genes (*Acvr1/Bmpr1a*, *Smad1/5* and *Smad4*; not shown), both *Tgfb2* alleles (Fig. 2G), or both *Smad2* and *Smad3* alleles (data not shown) had normal-appearing, closed eyelids at birth and normal-appearing conjunctival epithelium between E15.5 and birth. By examining embryos between E16.5 and birth, we found that eyelids from the Cre-positive embryos

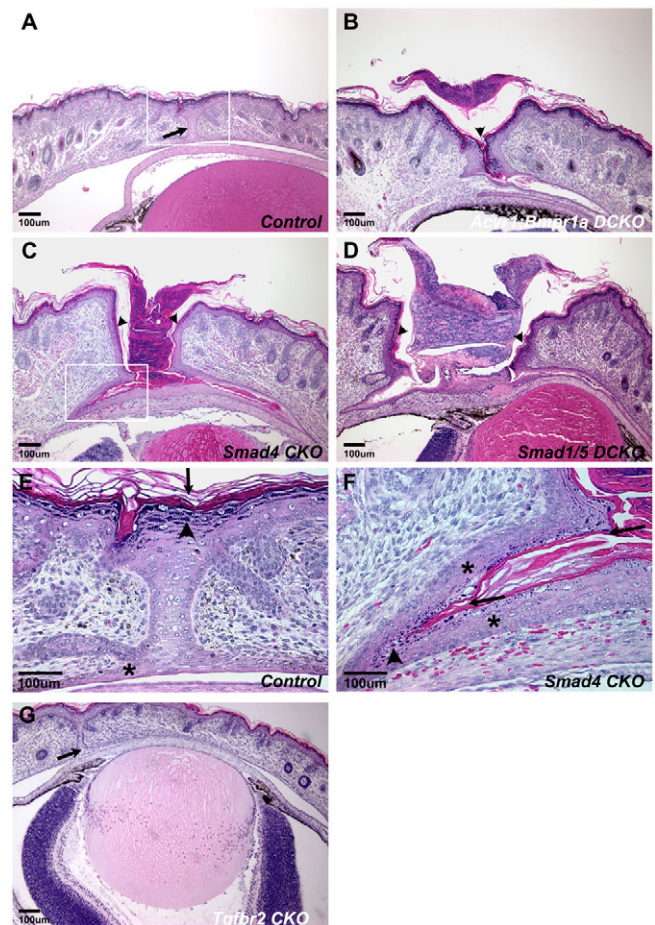


Fig. 2. Eyelid defects in mice with deficiencies in BMP signaling.

(A-G) Hematoxylin and Eosin staining of frontal eye sections from wild-type (A,E), *Acvr1;Bmpr1a*^{DCKO} (B), *Smad4*^{CKO} (C,F), *Smad1/5*^{DCKO} (D) and *Tgfb2*^{CKO} (G) mice at postnatal day 3 (P3). Wild-type eyelids are fused (arrow in A), whereas eyelids deficient in BMP signaling in the ectoderm are separate (arrowheads in B-D). (E,F) Higher magnification views of insets in A and C. (E) Eosin-stained keratin (arrow) and Hematoxylin-stained keratinocytes (arrowhead) in wild-type eyelid palpebral epidermis. (F) Ectopic Eosin-stained keratin-like protein (arrow) and ectopic Hematoxylin-stained keratinocyte-like cells (arrowhead) in *Smad4*^{CKO} conjunctiva. Asterisks in E and F illustrate the hyperplasia in the *Smad4*^{CKO} conjunctiva. (G) *Tgfb2*^{CKO} neonate eyelids are fused (arrow). Scale bars: 100 µm.

with EOB never closed, indicating that the phenotype resulted from the failure of eyelid closure, not from premature eyelid opening (not shown). Because only knockouts in the canonical BMP-Smad pathway had an EOB phenotype, in the remainder of the studies described we show only the phenotype of *Smad4*^{CKO} mice to represent the function of BMP signaling in eyelid closure. The 'control' eyelids shown are all from homozygous flox, Cre-negative littermates.

Besides the EOB phenotype, eyes with BMP signaling deficiencies showed hyperplasia and what appeared to be keratinization of the conjunctival epithelium. As opposed to the two-layered epithelium seen in wild-type embryos, the conjunctival epithelium comprised several cell layers, including a stratum granulosum with dark, Hematoxylin-stained keratohyalin granules, and an eosinophilic stratum corneum, which suggested keratinization (Fig. 2E,F).

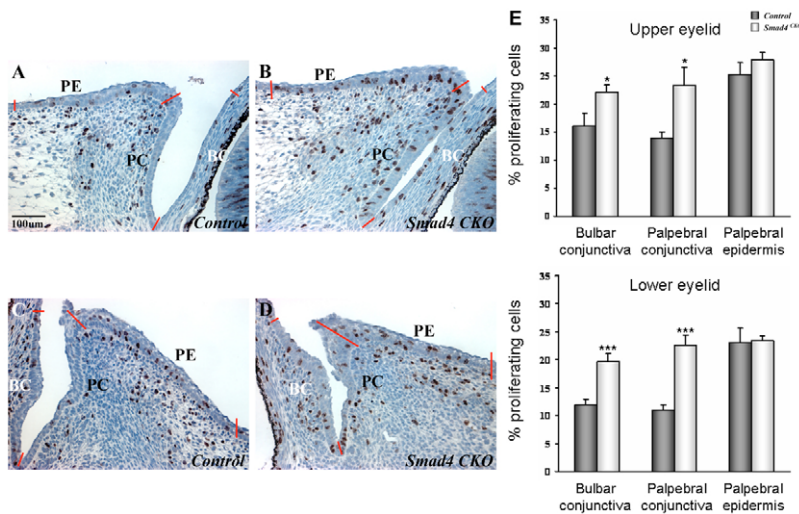


Fig. 3. Cell proliferation in *Smad4*^{CKO} eyelid epithelia at E14.5. (A–D) BrdU staining in frontal eye sections from wild-type upper eyelid (A), wild-type lower eyelid (C), *Smad4*^{CKO} upper eyelid (B) and *Smad4*^{CKO} lower eyelid (D). Red lines mark the approximate boundaries of the bulbar and palpebral conjunctiva and the palpebral epidermis. (E) Percentage of BrdU-labeled cells in each compartment. Compared with wild-type animals, the BrdU labeling index in *Smad4*^{CKO} bulbar and palpebral conjunctival epithelia was significantly increased. The y-axes indicate the mean percentage of BrdU incorporation in each area assayed. Error bars represent the s.e.m. **P*<0.05, ****P*<0.001. B, bulbar conjunctiva; PC, palpebral conjunctiva; PE, palpebral epidermis. Scale bar in A: 100 μ m.

EOB caused by interruption of BMP signaling is not due to decreased cell proliferation or increased cell death

We investigated whether loss of BMP signaling contributed to the failure of eyelid closure by affecting cell proliferation or cell death. At E12.5, BrdU and TUNEL labeling were similar in wild-type and *Smad4*^{CKO} eyelids (not shown). However, a significant increase in BrdU labeling occurred in the conjunctival epithelia of *Smad4*^{CKO} eyes at E14.5 (Fig. 3A–E). This observation was consistent with the conjunctival hyperplasia seen at later stages. No increase in BrdU labeling was detected in the palpebral epidermis (Fig. 3A–E) and no change was seen in programmed cell death in any of the ocular surface epithelia (not shown).

BMP signaling activates the expression of transcription factors that are required for eyelid closure

Expression of the forkhead transcription factors *Foxc1* and *Foxc2* is required for eyelid closure (Kidson et al., 1999; Kume et al., 1998; Smith et al., 2000). *Foxc1* mRNA was present in wild-type upper and lower eyelid epithelia (Fig. 4A), but undetectable in *Smad4*^{CKO} eyelids (Fig. 4C). Similarly, in wild-type embryos, *Foxc2* mRNA was expressed in palpebral conjunctival epithelial cells (Fig. 4D), but could not be detected in *Smad4*^{CKO} palpebral conjunctiva (Fig. 4F).

BMP signaling is required to promote the translocation of c-Jun into the nuclei of migrating periderm cells

c-Jun and the signaling cascade that leads to its phosphorylation are required for eyelid closure (Li et al., 2003; Zenz et al., 2003). In wild-type mice, a shelf of periderm cells begins to extend from the margin of the eyelids at E15.0 (Fig. 5A), covering much of the cornea by E15.5 (Fig. 5B). The nuclei of these periderm cells were strongly stained by an antibody to phosphorylated c-Jun (Fig. 5A,B, insets). In the *Smad4*^{CKO} eyelid epithelium, the appearance of the lid margin was comparable to that of wild type at E15.0 (Fig. 5C), although, by E15.5, fewer migrating periderm cells were present than in control eyes (Fig. 5D). In the *Smad4*^{CKO} embryos, the levels of phosphorylated c-Jun appeared to be lower than in wild-type periderm cells and p-c-Jun staining was present in the perinuclear cytoplasm, but not in the nuclei (Fig. 5C,D, insets). Thus, BMP

signaling is required for the full activation of c-Jun and for its translocation into the nucleus to exert its function as a transcription factor.

BMP expression and function is regulated by FGF signaling during eyelid closure

Fgf10 signaling via *Fgfr2* is essential for eyelid growth and closure (Li et al., 2001; Tao et al., 2005). To determine whether there is a relationship between FGF and BMP signaling, we deleted *Fgfr2* in the prospective eyelid epithelium using Le-Cre. Mice deficient in *Fgfr2* in the ectoderm showed an EOB phenotype, as described previously (Garcia et al., 2005), and deficiencies in BMP expression and function. In wild-type lower eyelids at E15.5, *Bmp4* mRNA was expressed in a cluster of mesenchymal cells underlying the palpebral conjunctival epithelium (Fig. 6A, arrows), but *Bmp4* transcripts were undetectable in the lower eyelids of *Fgfr2*^{CKO} mice (Fig. 6C). *Bmp4* mRNA was expressed in two groups of mesenchymal cells in the wild-type upper eyelid: in a cluster corresponding to those found in the lower eyelid (Fig. 6A, arrows) and in a cluster underlying the palpebral epidermis (Fig. 6A, arrowheads). In the *Fgfr2*^{CKO} upper eyelid, *Bmp4* mRNA accumulation in the mesenchyme underlying the palpebral conjunctiva was not affected, but *Bmp4* transcripts were not detectable in the mesenchyme underlying the palpebral epidermis (Fig. 6B).

A previous study found that Fgf10 maintains *Shh* expression in the eyelid mesenchyme (Tao et al., 2005). We found that hedgehog function in the mesenchyme, as measured by the expression of the hedgehog receptor patched 1 (*Ptch1*), a direct target of Shh signaling, was remarkably similar to the distribution of *Bmp4* transcripts (Fig. 6D). Moreover, the pattern of residual *Ptch1* expression in the *Fgfr2*^{CKO} eyelid was similar to the pattern of residual *Bmp4* expression. Although *Ptch1* expression was preserved in the upper lid ectoderm and mesenchyme, it diminished greatly in the lower lid ectoderm and mesenchyme of *Fgfr2*^{CKO} mice (Fig. 6E). *Ptch1* expression was not affected in *Smad4*^{CKO} mice (Fig. 6F).

In agreement with the dependence of *Bmp4* expression on *Fgfr2*, nuclear staining for phosphorylated Smad1/5/8, the receptor-activated Smads that transduce BMP signals, was strong in wild-type epithelial cells (Fig. 6G,H), but greatly diminished in the upper and lower eyelids of *Fgfr2*^{CKO} mice (Fig. 6I,J). As in the *Smad4*^{CKO} eyelids, *Foxc1* and *Foxc2* mRNA was not detectable in *Fgfr2*^{CKO}

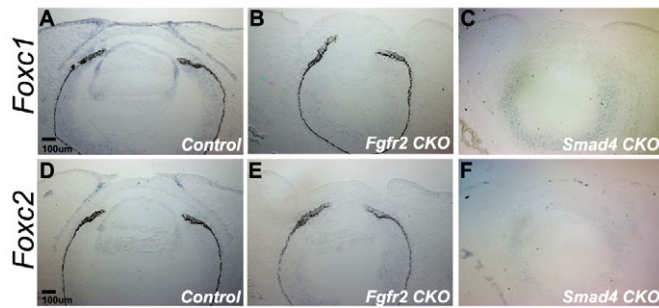


Fig. 4. *Foxc1* and *Foxc2* transcripts are greatly reduced in *Fgfr2*^{CKO} and *Smad4*^{CKO} eyelids. (A-C) In situ hybridization for *Foxc1* mRNA on frontal eye sections from wild-type (A), *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) eyelids at E15.5. In wild-type eyelid (A), *Foxc1* mRNA was detected in the palpebral epithelium, palpebral conjunctiva, bulbar conjunctiva, periderm and retina. In *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) eyelids, *Foxc1* mRNA was detectable only in the retina. (D-F) In situ hybridization of *Foxc2* mRNA on frontal eye sections from wild-type (D), *Fgfr2*^{CKO} (E) and *Smad4*^{CKO} (F) eyelids. In wild type eyelids (D), *Foxc2* mRNA was detected in the palpebral conjunctiva and retina at E15.5. In *Fgfr2*^{CKO} (E) and *Smad4*^{CKO} (F) eyelids, *Foxc2* mRNA was detectable only in the retina. Scale bars: 100 μm.

conjunctival epithelia (Fig. 4B,E). Thus, FGF signaling controls eyelid closure, at least in part, through the activation of BMP signaling.

Activation of *Fgfr2* suppresses Wnt signaling and promotes the expression of *Sfrp1* in a BMP-independent manner

Loss of *Dkk2*, a Wnt signaling antagonist, causes EOB, revealing that excessive Wnt signaling can prevent eyelid closure (Gage et al., 2008). To further assess the regulation of Wnt pathway signaling in the ocular surface epithelia, we produced *Fgfr2* and *Smad4* conditional knockouts in the ectoderm in a TOPGAL background, in which canonical Wnt signaling activates a β -galactosidase reporter transgene (DasGupta and Fuchs, 1999). In wild-type (Cre-negative) eyelids at E15.5, β -galactosidase staining was abundant in hair follicles of the epidermis and in the conjunctival epithelium in a band near the edge of the upper eyelid. Weaker staining was present in a band along the edge of the lower eyelid (Fig. 7A,D). In *Fgfr2*^{CKO} eyes, TOPGAL reporter activity increased in intensity and spread over the conjunctival epithelium of the upper and lower eyelids (Fig. 7B,E). In *Smad4*^{CKO} eyelids, β -galactosidase expression was not increased, but had a different distribution from wild type. Instead of localizing in a continuous band in the peripheral conjunctival epithelium, staining was present in an extra row of ectopic hair follicles in the upper and lower eyelids (Fig. 7C,F). A double row of eyelashes is called distichiasis. Because distichiasis occurs in humans and mice haploinsufficient for FOXC2 (Fang et al., 2000; Kriederman et al., 2003), this finding is consistent with our observation that BMP signaling was required for *Foxc2* expression.

Although loss of *Dkk2* expression causes EOB (Gage et al., 2008), *Dkk2* mRNA or protein expression was not affected in *Fgfr2*^{CKO} or in *Smad4*^{CKO} eyelid epithelial cells (Fig. 8A-F). FGF signaling modulates *Shh* expression in the eyelid and, in other tissues, hedgehog signaling induces the expression of the Wnt antagonist, secreted frizzle-related protein 1 (*Sfrp1*) (He et al., 2006; Katoh and Katoh, 2006). We examined the levels of *Sfrp1* mRNA in

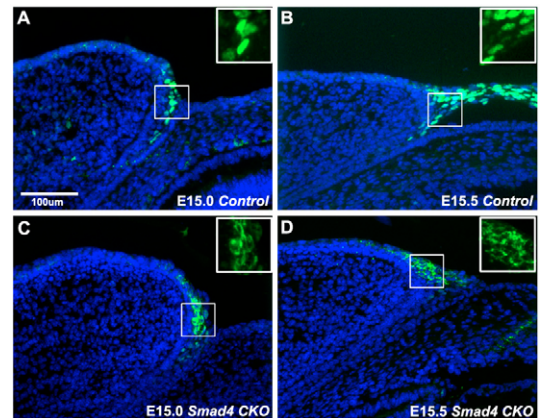


Fig. 5. The nuclear localization of phosphorylated c-Jun requires *Smad4*. Immunostaining for phosphorylated c-Jun (p-c-Jun, green) on frontal eye sections from wild-type E15.0 (A), wild-type E15.5 (B), *Smad4*^{CKO} E15.0 (C) and *Smad4*^{CKO} E15.5 (D) embryos. (A) In wild-type eyelids, a small number of migrating periderm cells are present at E15.0. Staining for p-c-Jun is present in the nuclei of these cells (inset). (B) By E15.5, the number of periderm cells has increased and they have begun to migrate over the cornea. Staining for p-c-Jun is present in the nuclei of these cells (inset). (C) At E15.0, periderm cells in *Smad4*^{CKO} eyelids appeared similar in number to wild type. However, p-c-Jun staining was restricted to the cytoplasm around the nuclei (inset). (D) At E15.5, fewer periderm cells were present in *Smad4*^{CKO} eyelids than in wild type. Staining for p-c-Jun was weaker than in wild-type periderm cells and was still restricted to the perinuclear cytoplasm (inset). Insets show p-c-Jun staining without the nuclear counterstain (blue). Scale bar in A: 100 μm.

wild-type, *Fgfr2*^{CKO} and *Smad4*^{CKO} eyelids. In wild-type eyelids, *Sfrp1* mRNA was expressed in the entire eyelid epithelium, with strongest expression in the conjunctival epithelia (Fig. 8G). Consistent with the effects of FGF and BMP signaling on TOPGAL activity, *Sfrp1* transcripts were undetectable in *Fgfr2*^{CKO} eyelids (Fig. 8H), but were present at normal levels in *Smad4*^{CKO} eyelids (Fig. 8I). Thus, FGF signaling suppresses Wnt signaling, at least in part, through the activation of *Sfrp1*. However, the control of *Sfrp1* expression by *Fgfr2* is independent of BMP signaling.

Although the BMP-Smad pathway is not involved in suppressing Wnt signaling, it appears to be required to suppress ectopic hair follicle formation from the conjunctival epithelial cells at the inner margin of the eyelid (Fig. 7C). In its absence, the uniform band of Wnt signaling near the eyelid margin was replaced by a row of eyelash follicles (Fig. 7C,F). These observations suggest that BMP signaling maintains the pattern of Wnt pathway activity required for the differentiation of the Meibomian gland progenitor cells at the inner margins of the eyelids.

BMP signaling suppresses the differentiation of conjunctival epithelial cells prior to eyelid closure and specifies conjunctival epithelial cell fate

The conjunctival epithelium in mice deficient in BMP signaling developed features that were reminiscent of epidermis, including keratinization and ectopic hair follicles. We, therefore, examined the levels of the epithelial cell differentiation marker keratin 14 (K14) at E15.0, before eyelid closure, and at E15.5, during closure. We also stained for a specific epidermal differentiation marker, keratin 10 (K10), and a conjunctival epithelial differentiation marker, keratin 4 (K4), at E17.5, after eyelid closure. In wild-type mice, the conjunctival

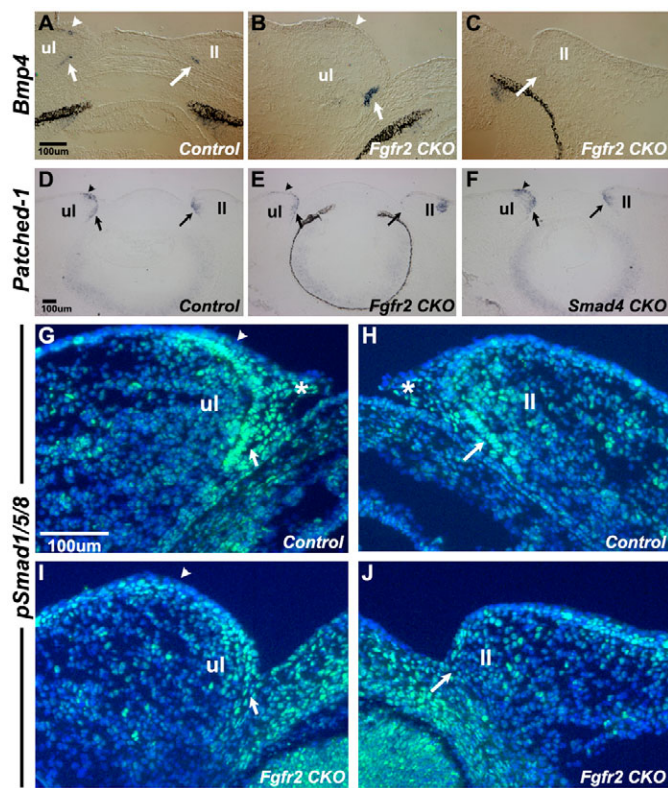


Fig. 6. Signaling from *Fgfr2* promotes *Bmp4*, patched 1 expression and BMP signaling in the eyelid.

(A–C) In situ hybridization for *Bmp4* transcripts at E15.5 in frontal eye sections from wild-type upper and lower eyelids (A) and *Fgfr2*^{CKO} upper (B) and lower (C) eyelids. (A) In the wild-type eyelids, *Bmp4* transcripts were present in a cluster of mesenchyme cells underlying the palpebral conjunctiva (arrows) and the palpebral epidermis (arrowhead). (B) In the upper eyelids of *Fgfr2*^{CKO} mice, *Bmp4* transcripts were detected in the mesenchyme underlying the palpebral conjunctiva (arrow), but not in the mesenchyme underlying the palpebral epidermis (arrowhead). (C) In the lower eyelids of *Fgfr2*^{CKO} mice, *Bmp4* transcripts were not detected in the mesenchyme underlying the palpebral conjunctiva (arrow). (D–F) In situ hybridization for *Ptch1* transcripts in frontal eye sections from wild-type (D), *Fgfr2*^{CKO} (E) and *Smad4*^{CKO} (F) eyelids. In wild-type (D) and *Smad4*^{CKO} eyelids (F), *Ptch1* transcripts were present in clusters of mesenchyme cells and the overlying palpebral conjunctiva (arrows) and palpebral epidermis (arrowhead). (E) In the upper eyelids of *Fgfr2*^{CKO} mice, *Ptch1* transcripts were decreased in the mesenchyme and the overlying palpebral conjunctiva (arrow), and were barely detectable in the mesenchyme and the overlying palpebral epidermis (arrowhead). In the lower eyelids of *Fgfr2*^{CKO} mice, *Ptch1* transcripts were undetectable in the mesenchyme and the overlying palpebral conjunctiva (arrow). (G–J) Immunostaining for phosphorylated Smad1/5/8 (pSmad1/5/8) in frontal eye sections from wild-type upper (G), wild-type lower (H), *Fgfr2*^{CKO} upper (I) and *Fgfr2*^{CKO} lower (J) eyelids. (G) In the wild-type upper eyelid, nuclear pSmad1/5/8 staining was seen in the eyelid mesenchyme, the overlying palpebral conjunctiva (arrow), the palpebral epidermis (arrowhead) and the periderm (asterisk). (H) In the wild-type lower eyelid, pSmad1/5/8 staining was seen in the eyelid mesenchyme, the overlying palpebral conjunctiva (arrow) and the periderm (asterisk). (I) In the upper eyelid of *Fgfr2*^{CKO} embryos, pSmad1/5/8 staining was reduced in the palpebral conjunctiva (arrow) and greatly reduced in the palpebral epidermis (arrowhead). (J) In the *Fgfr2*^{CKO} lower eyelid, pSmad1/5/8 staining was greatly reduced in the palpebral conjunctiva (arrow), upper eyelid; ll, lower eyelid. Scale bars: 100 μ m.

epithelium first expressed K14 at about the time of eyelid closure (Fig. 9A). However, in conjunctival epithelial cells deficient in BMP signaling, expression of K14 was precocious (Fig. 9C), with the mutant conjunctiva differentiating at the same time as the epidermis. In wild-type E17.5 eyes, K10 is expressed by epidermis, and K4 is expressed by conjunctiva (Fig. 9D,G). However, in *Smad4*^{CKO} conjunctival epithelium, we found ectopic K10 expression, with K4 staining detected only in a few residual cells (Fig. 9F,I), suggesting that most mutant conjunctival cells transdifferentiated into epidermal cells. Although conjunctival differentiation in *Fgfr2*^{CKO} eyes was premature, as determined by K14 expression (Fig. 9B), the transdifferentiation of conjunctiva to epidermis was not evident, because *Fgfr2*^{CKO} conjunctiva did not express K10 (Fig. 9E,H). It seems possible that residual BMP signaling in the *Fgfr2*^{CKO} conjunctiva maintained conjunctival cell fate, but was unable to suppress the premature differentiation of this tissue.

DISCUSSION

Smad-dependent BMP signaling is required for eyelid closure

Conditional deletion of the genes encoding two of the three type I BMP receptors, two of the three BMP-associated R-Smads or the co-Smad Smad4 in the eyelid epithelia resulted in an EOB phenotype, precocious conjunctival epithelial differentiation and the transdifferentiation of conjunctiva to epidermis, including ectopic eyelash follicle formation and epidermis-specific keratin expression. Although activin signaling is important for eyelid closure, a previous investigation indicated that activin β -B signals are transduced to c-Jun by MEKK1 and JNK, not through the canonical Smad pathway (Zhang et al., 2003). Our results are consistent with this interpretation, as deletion of the activin/TGF β -stimulated R-Smads Smad2 and Smad3 did not cause an EOB phenotype. Deletion of the TGF β type II receptor also did not show an eyelid phenotype, indicating that the Smad4 phenotype was due to defects in signaling through the canonical BMP pathway, and not in another of the TGF β superfamily pathways that share Smad4 function.

The EOB phenotype in eyelids lacking epithelial BMP receptors and BMP-activated R-Smads is consistent with the results of *Bmpr1a* deletion using a K14-Cre transgene (Andl et al., 2004) and the overexpression of the inhibitory Smad Smad7 using the bovine K5 promoter (He et al., 2002). The inability of K14-driven noggin overexpression to cause EOB may be due to the late expression of K14 in the conjunctiva, just as eyelid closure is occurring, giving insufficient time for noggin to prevent the activation of BMP receptors. EOB in mice deficient in BMP signaling was not due to decreased cell proliferation or increased cell death in the palpebral epithelia. On the contrary, increased cell proliferation was found in the conjunctival epithelia, consistent with the hyperplasia observed in the BMP receptor knockouts or when Smad7 is driven by the K14 promoter (Andl et al., 2004; He et al., 2002). Thus, BMPs normally inhibit the proliferation and differentiation of the conjunctival epithelium, and specify conjunctival epithelial cell fate.

FGF-regulated BMP signaling is required for the nuclear localization of phosphorylated c-Jun and the transcription of *Foxc1* and *Foxc2*

The phosphorylation and function of c-Jun in the eyelid epithelium depends, at least in part, on Smad-independent signaling by activin β -B (Zhang et al., 2003). However, in *Acvr1*;*Bmpr1a*^{DCKO}, *Smad1/5*^{DCKO} and *Smad4*^{CKO} eyelids, c-Jun appeared to be more weakly phosphorylated than in wild type and failed to translocate into the nuclei of periderm cells. Previous studies showed that

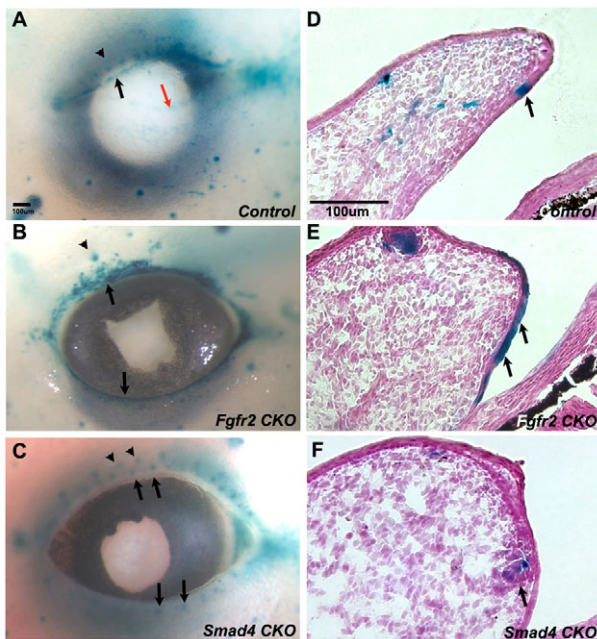


Fig. 7. The activity of the canonical Wnt pathway in the eyelid is modified in different ways by FGF and BMP signaling.

(A-C) Whole-mount X-gal staining from the TOPGAL reporter in wild-type (A), *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) eyelids at E15.5. (D-F) Frontal sections of the eyes shown in A-C. (A) A prominent band of Wnt activity is present in the upper (black arrow) and a weaker band in the lower eyelid margins (red arrow) of wild-type eyes. Wnt activity is also present in eyelash follicles (arrowhead) and in epidermal hair follicles. (B) Wnt activity is uniformly increased in the upper and lower eyelids of *Fgfr2*^{CKO} mice (black arrows) and is present in epidermal hair follicles (arrowhead). (C) Wnt activity is present in the epidermal hair follicles (arrowhead) and in ectopic patches in the upper and lower eyelids of *Smad4*^{CKO} mice (black arrows). (D) Frontal sections reveal that Wnt activity is located near the anterior edge of the palpebral conjunctiva of wild-type eyelids (arrow). (E) In *Fgfr2*^{CKO} eyelids, Wnt activity expands into the palpebral conjunctiva (arrows). (F) In *Smad4*^{CKO} eyelids, Wnt activity is present in ectopic hair follicles (arrow), corresponding to the location of the band of cells with high Wnt activity in the wild-type palpebral conjunctiva. Scale bars: 100 μ m.

Smads can bind to c-Jun when it is not associated with DNA, that c-Jun is phosphorylated after treatment of cells with TGF β and that Smad-c-Jun complexes promote AP-1-dependent transcription (Liberati et al., 1999; Qing et al., 2000; Verrecchia et al., 2001; Zhang et al., 1998). Our data suggest that BMPs, acting through the canonical R-Smad/Smad4 pathway, cooperate with activin β -B to promote maximal phosphorylation of c-Jun, and that association with the R-Smad/Smad4 complex mediates the translocation of phosphorylated c-Jun to the nucleus. To our knowledge, this is the first report suggesting that activated Smads are required for the nuclear translocation of c-Jun. Further studies are required to determine whether this function of BMP signaling is important in other examples of epithelial fusion, such as closure of the neural tube and ventral closure of the optic cup, for which proper function of the JNK pathway is essential (Xia and Karin, 2004).

BMP signaling was required for the accumulation of Foxc1 and Foxc2, transcription factors shown in previous studies to be required for eyelid closure (Kidson et al., 1999; Kume et al., 1998; Smith et al., 2000). Foxc1 and Foxc2 are also expressed in pericocular

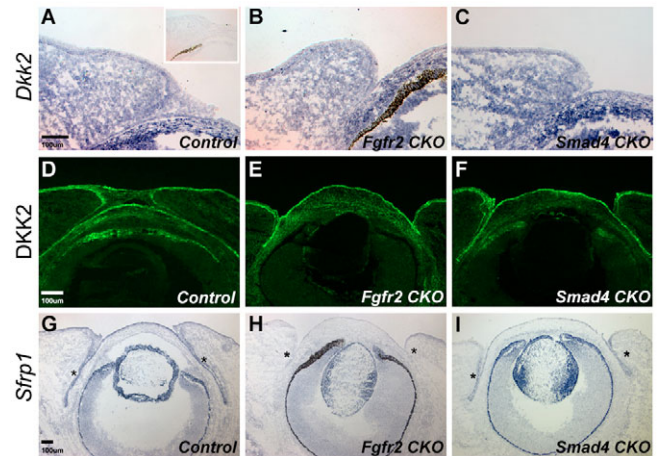


Fig. 8. The effects of FGF and BMP signaling on *Dkk2* and *Sfrp1* expression in the eyelid.

(A-C) In situ hybridization for *Dkk2* transcripts in frontal sections from wild-type (A), *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) embryos at E15.5. (D) *Dkk2* transcript levels (blue) were strong in the eyelid epidermis, bulbar conjunctiva and corneal stroma, with weaker staining in the palpebral conjunctiva, periderm and mesenchyme in wild type. A sense probe control is shown in the inset. (B,C) No significant change in *Dkk2* mRNA level was detected in *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) embryos at E15.5. (D-F) Immunostaining for DKK2 protein in frontal eye sections from wild-type (D), *Fgfr2*^{CKO} (E) and *Smad4*^{CKO} (F) embryos at E15.5. DKK2 protein in wild-type and CKO embryos was similar to the in situ hybridization results. (G-I) In situ hybridization for *Sfrp1* transcripts in frontal sections from wild-type (G), *Fgfr2*^{CKO} (H) and *Smad4*^{CKO} (I) eyelids. In wild-type (G) and *Smad4*^{CKO} (I) eyelids, *Sfrp1* transcripts were present in the conjunctiva (asterisks), retina, retinal pigment epithelium and lens. In *Fgfr2*^{CKO} embryos (H), *Sfrp1* transcripts were readily detected in the retina, retinal pigment epithelium and lens, with low levels in the conjunctiva and palpebral epithelium. Scale bars: 100 μ m.

mesenchyme (Gage et al., 2005) and limb bud mesenchyme (Nifuji et al., 2001), and treatment with Bmp4 and Bmp7 increases the transcription of Foxc2 in limb bud mesenchyme in organ culture (Nifuji et al., 2001). The function of Foxc1 and Foxc2 in eyelid closure is not clear. However, it has been shown that Foxc1 and Foxc2 control somitogenesis by regulating Notch signaling (Kume et al., 2001). Foxc transcription factors also directly stimulate the production of the Notch ligand Dll4, to activate Hey2 accumulation in vascular endothelial cells (Hayashi and Kume, 2008). In experiments undertaken for another study, we found that deletion in the surface ectoderm of the presenilins *Psen1* and *Psen2* resulted in an EOB phenotype (see Fig. S1 in the supplementary material). Because, in most tissues, presenilin activity is required for Notch activation, Foxc1 and Foxc2 might promote Notch signaling during eyelid closure. However, the presenilins are also required for the activity of other transmembrane proteins. For this reason, the function of Notch signaling in eyelid closure requires further exploration.

BMPs do not mediate all effects of FGF signaling in eyelid development

Although the functions of BMPs that were identified in this study were dependent on *Fgfr2*, BMPs do not mediate all effects of FGF signaling in eyelid development. *Fgfr2* is required in the palpebral ectoderm to suppress Wnt signaling, at least in part by inducing the Wnt antagonist *Sfrp1*. Loss of BMP signaling does not affect the expression of *Sfrp1*.

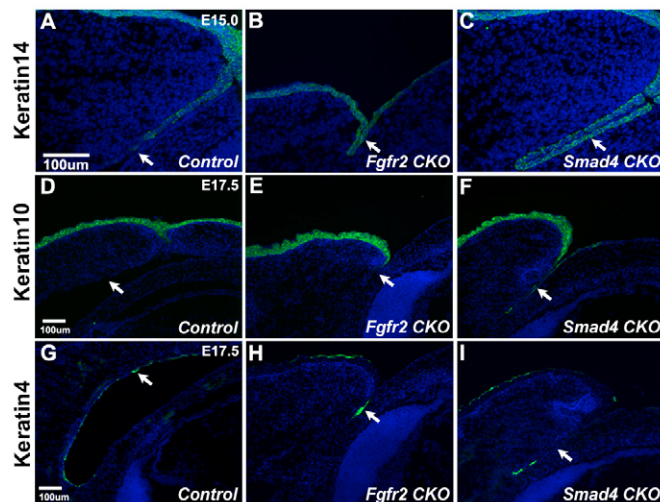


Fig. 9. Abnormal eyelid epithelial differentiation in *Fgfr2*^{CKO} and *Smad4*^{CKO} mice. (A–C) Immunostaining for keratin 14 (K14) in frontal sections from wild-type (A), *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) embryos at E15.0. In wild-type eyelids (A), K14 staining was weak in the palpebral conjunctiva and was not detected in the bulbar conjunctiva (arrows). In *Fgfr2*^{CKO} (B) and *Smad4*^{CKO} (C) eyelids, K14 staining was strong and uniform in the palpebral and bulbar conjunctiva (arrows). (D–F) Immunostaining for keratin 10 (K10) in frontal sections from wild type (D), *Fgfr2*^{CKO} (E) and *Smad4*^{CKO} (F) embryos at E17.5. In wild-type eyelids (D), K10 staining is observed only in the epidermis; it is negative in the conjunctiva (arrow). In *Fgfr2*^{CKO} (E), K10 is found in epidermis and a few cells of the palpebral conjunctiva, although the majority of conjunctival cells do not express K10 (arrow). In *Smad4*^{CKO} (F), K10 is detected in the epidermis and in the majority of conjunctival cells (arrow). (G–I) Immunostaining for keratin 4 (K4) in frontal sections from wild-type (G), *Fgfr2*^{CKO} (H) and *Smad4*^{CKO} (I) embryos at E17.5. In wild-type eyelids (G), expression of K4 is continuous in the conjunctiva (arrow). In *Fgfr2*^{CKO} (H), K4 is found in most conjunctival cells (arrow). In *Smad4*^{CKO} (I), K4 is only detected in a few conjunctival cells; most conjunctival cells do not express K4 (arrow). Scale bars: 100 μ m.

This arm of the FGF signaling pathway might involve Shh, as expression of *Shh* in the eyelid is modulated by Fgf10 (Tao et al., 2005), and the expression of *Ptch1*, an Shh receptor that is a direct target of Shh signaling, was greatly decreased in the lid ectoderm and the underlying mesenchyme in eyelids lacking *Fgfr2* in the ectoderm.

Wnt signaling must be suppressed in the palpebral epithelia to effect eyelid closure (Gage et al., 2008) (this study). However, a band of Wnt signaling activity is normally present in the palpebral conjunctiva near the margins of the upper and lower eyelids. These cells appear to include the precursors of the Meibomian glands. BMP signaling is required to maintain this domain of Wnt signaling and Meibomian gland cell fate (Plikus et al., 2004) (this study). Thus, FGF signaling is required to suppress Wnt signaling and promote BMP signaling, yet BMPs maintain local Wnt signaling in the Meibomian gland precursor cells. The factors that specify the location and extent of BMP and Wnt signaling in Meibomian gland formation remain to be studied.

Mice deficient in BMP signaling provide a model for the human disease distichiasis

In mice deficient in BMP signaling, conjunctival epithelial cells in both eyelids formed an extra row of eyelashes, a characteristic called distichiasis. This phenotype is similar to that of mice that

overexpress noggin in the ectoderm, in which ectopic eyelashes are formed at the expense of the Meibomian glands (Plikus et al., 2004). Human distichiasis syndrome is characterized by the presence of an aberrant second row of eyelashes in place of the Meibomian glands (Fox, 1962). As a consequence, patients have Meibomian gland dysfunction, corneal irritation, conjunctivitis and photophobia. Most families presenting with distichiasis have lymphedema in common, or lymphedema-distichiasis (LD) syndrome (OMIM 153400). LD syndrome is an autosomal dominant disease caused by mutations in *FOXC2*. *Foxc2* heterozygous mice mimic LD syndrome, demonstrating distichiasis and hyperplasia of lymphatic vessels and lymph nodes (Kriederman et al., 2003). The distichiasis seen in our studies is consistent with a dependence of *Foxc2* expression on BMP signaling.

Dkk2-null mice also develop distichiasis, with decreased *Foxc2* expression in the eyelids (Gage et al., 2008). *Dkk2* expression is promoted by the transcription factor Pitx2, which functions in the neural crest-derived eyelid mesenchyme. Surprisingly, we detected *Dkk2* mRNA and protein in the eyelid epithelia and in the mesenchyme. This observation suggests that another pathway regulates *Dkk2* expression in the ectoderm. Excessive Wnt signaling, whether resulting from defects in the Pitx2→*Dkk2* pathway (Gage et al., 2008), or the Fgf10→*Fgfr2*→*Sfrp1* pathway, appears to be sufficient to suppress *Foxc2* expression, prevent eyelid closure and cause distichiasis. Because loss of BMP signaling also leads to distichiasis, it seems possible that excessive Wnt pathway activity inhibits *Bmp4* expression in the eyelid mesenchyme or the function of the BMP pathway in the conjunctival epithelium. These possibilities remain to be tested.

BMP signaling is required for normal conjunctival epithelial cell fate

Different temporal and spatial expression of keratin intermediate filaments is an important aspect of the differentiation and function of many epithelia (Kurpakus et al., 1994). In wild-type eyelids, the palpebral epidermis expresses K14 before eyelid closure, whereas the conjunctival epithelia begin expressing K14 as the eyelids close. However, in mice deficient in BMP signaling, the conjunctival epithelium expressed K14 at the same time as the epidermis. In addition, conjunctival cells expressed K10, which is normally restricted to the epidermis, and K4 expression was reduced. Together with the transdifferentiation of the Meibomian gland precursor cells to hair follicles, these observations suggest that BMP signaling normally prevents conjunctival cells from adopting the epidermal cell fate (Fig. 10A).

The cross-talk between FGF and BMP signaling may be mediated by Shh

Previous studies and the results described here reveal complex epithelial-mesenchymal interactions in eyelid development. Activation of *Fgfr2* in the surface ectoderm by Fgf10 from the underlying mesenchyme (Tao et al., 2005) is required for the localized expression of *Bmp4* in the palpebral mesenchyme. The signal from the epithelium that promotes *Bmp4* expression in the mesenchyme is likely to be Shh, as *Shh* is expressed in the eyelid margin from E13.5 and active Shh signaling (indicated by *Ptch1* expression) has an expression pattern similar to that of *Bmp4* in the wild-type eyelid. Moreover, the pattern of residual Shh signaling in the *Fgfr2*^{CKO} eyelid is similar to the pattern of residual *Bmp4* expression. These observations, together with the fact that preventing BMP signaling did not alter Shh function, suggested that Shh mediates the Fgfr2-dependent cross-talk between epithelium and mesenchyme.

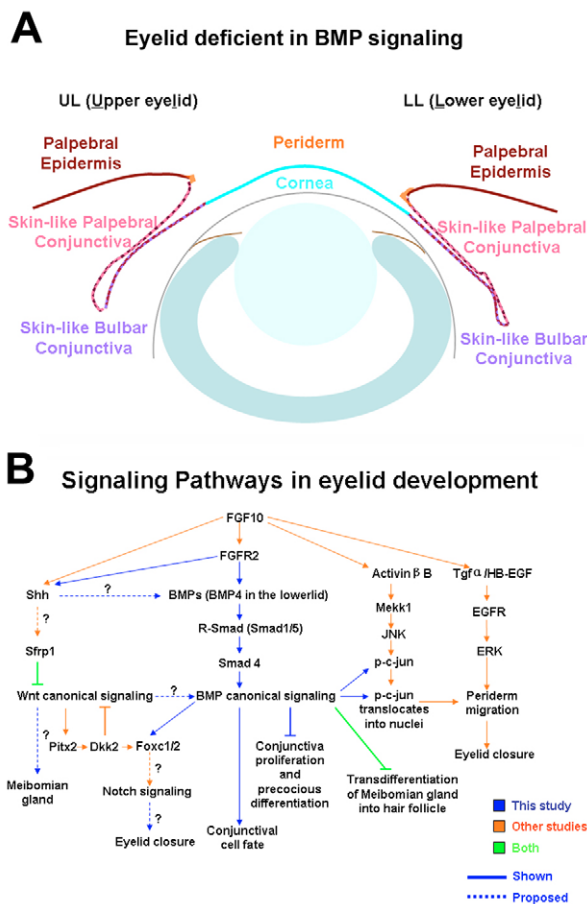


Fig. 10. Summary of the role of BMP signaling and its interaction with the other signaling pathways known or proposed to function in mouse eyelid development. (A) In eyelids that are deficient in BMP signaling, the conjunctival epithelial cells express epidermis-specific keratin 10 and form hair follicles near that lid margin. (B) The network of known or proposed signaling pathways controlling eyelid development. Observations made in this study are indicated by blue arrows, previous findings by orange arrows and findings confirmed in this study by green arrows. Our model suggests that epidermal cell fate is the default pathway and that BMP signaling is required for prospective conjunctival epithelial cells to suppress the epidermal differentiation pathway and become conjunctival cells. BMP signaling is not required to initiate the migration of periderm cells at the lid margins, but is required for the expansion of these cells across the corneal surface. During this process, BMP signaling is required for the expression of *Foxc1* and *Foxc2*, and for the full activation (phosphorylation) of c-Jun. BMP-dependent formation of active R-Smad4 complexes is required for the translocation of p-c-Jun into the nuclei of periderm cells, where it has been reported to increase the expression of the epidermal growth factor receptor.

Bmp4 signals to the overlying ectoderm, as demonstrated by the phosphorylation of the BMP-dependent R-Smads in the eyelid epithelia. Phosphorylated R-Smads are also seen in the mesenchyme, suggesting that BMPs may affect additional targets there. In an additional level of complexity, the *Bmp4* and *Ptch1* expression patterns and Wnt activity were of different magnitude and location in the upper and lower eyelids, and *Bmp4* and *Ptch1* expression were differentially affected in the upper and lower eyelids by deletion of *Fgfr2*.

The signaling pathways involved in eyelid closure

In addition to the functions of the FGF, BMP, Shh and Wnt pathways examined in this study, germline deletion showed that activin β -B, TGF α , HB-EGF and the EGF receptor are each required for eyelid closure (Luetteke et al., 1994; Luetteke et al., 1993; Miettinen et al., 1995; Vassalli et al., 1994). Notch signaling might also be involved (see Fig. S1 in the supplementary material). The pathways activated by these morphogens interact in a remarkably complex web to assure the proper migration and fusion of a small population of periderm cells (Fig. 10B). Further studies are needed to fully define the functions and interactions of these pathways. Such studies will provide a more complete understanding of eyelid fusion and, perhaps, other morphogenetic events that depend on epithelial fusion, such as closure of the neural tube, the optic fissure, the lens vesicle and the palatal shelves.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/cgi/content/full/136/10/1741/DC1>

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