BMP6 Regulates Corneal Epithelial Cell Stratification by Coordinating Their Proliferation and Differentiation and Is Upregulated in Pterygium

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The corneal epithelium (CE) is a non-keratinized, stratified, squamous epithelial tissue that serves as a barrier between the external environment and the rest of the eye. In the mouse, one- to two-cell-layered neonatal CE begins to stratify around eyelid opening at postnatal day (PN)-12, forming five to six cell layers by PN-20.1–4 Mature CE is continuously renewed as the superficial cells are lost regu- larily due to normal aging or are frequently damaged by physical, biological, and chemical insults. Both initial stratification and homeostatic renewal of the CE depend on limbal epithelial–mesenchymal transition (EMT) that culminated in squamous metaplasia, suggesting that the interplay between KLF4 and TGF-β signaling plays a key role in regulating the CE phenotype.5,15 Despite this progress, our knowledge of signaling molecules and regulatory networks that control CE cell proliferation and differentiation during initial stratification and homeostasis remains incomplete.

Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, play important roles in various aspects of development including epidermal stratification.16–19 BMPs are classified into different subgroups (BMP2/4, BMP5/6/7/8a/8b, BMP9/10, and BMP12/13/14/15) based on sequence similarity and functions.20,21 BMPs initiate signaling by binding the heterotetrameric transmembrane signaling molecules and regulatory networks that control CE cell proliferation and differentiation during initial stratification and homeostasis remains incomplete.

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Purpose. Proper balance between cell proliferation and differentiation is essential for corneal epithelial (CE) stratification and homeostasis. Although bone morphogenetic protein-6 (BMP6) is known to be expressed in the CE for over 25 years, its function in this tissue remains unknown. Here, we test the hypothesis that BMP6 promotes CE cell stratification and homeostasis by regulating their proliferation and differentiation.

Methods. We employed postnatal day-12 (PN-12), PN-14, PN-20, and PN-90 mouse eyes; human corneal limbal epithelial (HCLE) cells; and ocular surface fibrovascular disease pterygium tissues to evaluate the role of BMP6 in CE proliferation, differentiation, and pathology by RT-qPCR, immunoblots, and/or immunofluorescent staining. Cell proliferation was quantified by immunostaining for Ki67.

Results. Coincident with the mouse CE stratification between PN-12 and PN-20, BMP6 was significantly upregulated and the BMP6 antagonist Noggin downregulated. Mature CE retained high BMP6 and low Noggin expression at PN-90. BMP6 and its receptors BMPR1A and BMPR2 were upregulated during in vitro stratification of HCLE cells. Consistent with its anti-proliferative role, exogenous BMP6 suppressed HCLE cell proliferation, downregulated cyclin-D1 and cyclin-D2, and upregulated cell-cycle inhibitors Krüppel-like factor 4 (KLF4) and p21. BMP6 also upregulated the desmosomal cadherins desmoplakin and desmoglein in HCLE cells, consistent with its pro-differentiation role. Human pterygium displayed significant upregulation of BMP6 coupled with downregulation of Noggin and cell-cycle suppressors KLF4 and p21.

Conclusions. BMP6 coordinates CE stratification and homeostasis by regulating their proliferation and differentiation. BMP6 is significantly upregulated in human pterygium concurrent with downregulation of Noggin, KLF4, and p21.
receptor complex of two subunits each of type I and type II receptors. Canonical BMP signaling involves phosphorylation of the type I receptor by BMP-bound type II receptor, which in turn triggers intracellular signaling via phosphorylation of receptor-regulated SMAD1, 5, and 8 (R-SMADs). Phosphorylated SMAD1/5/8 forms a complex with the co-SMAD (SMAD4) and translocates to the nucleus, where it regulates gene expression in association with cofactors. BMP signaling is also mediated by non-canonical pathways involving the mitogen-activated protein kinase cascade and is fine-tuned extracellularly (e.g., by Noggin) and intracellularly (e.g., by FKBP12 and inhibitory SMADs), as well as by co-receptors such as endoglin in the plasma membrane. BMP signaling plays a key role in anterior eye development and homeostasis as evidenced by (1) the inhibition of eyelid opening upon overexpression of Noggin, (2) regulation of clonal growth of limbal epithelial progenitor cells by BMPs, and (3) the need for suppression of BMP signaling during transdifferentiation of CE cells to epidermal cells.

Many components of BMP signaling with key roles in epidermal stratification are also expressed in the adult cornea. In this report, we focused our attention on corneal functions of BMP6, as it (1) has no known corneal functions, although its expression was documented over 25 years ago; (2) regulates a wide range of biological processes, including cell proliferation and iron homeostasis, which are dysregulated in fibrovascular proliferative disorders such as pterygium; and (3) is significantly upregulated in conjunctival scar tissue consistent with its potential involvement in pterygium. In the epidermis, strong and uniform overexpression of BMP6 inhibited suprabasal keratinocyte proliferation in neonates, whereas a weak and patchy expression induced hyperproliferation in postnatal stages, suggesting that BMP6 regulates cell proliferation and differentiation in a context-dependent manner. Despite many structural and functional similarities between the epidermis and the cornea, it is not known if BMP6 plays a similar role in regulating CE proliferation and differentiation. Here, we have attempted to fill this gap by evaluating the expression and function of BMP6 during CE stratification. We report that mouse CE stratification is accompanied by a significant upregulation of BMP6 coupled with a downregulation of its antagonist Noggin and that exogenous BMP6 suppresses the proliferation of in vitro cultured human corneal limbal epithelial (HCLE) cells and initiates their differentiation. We also found that BMP6 is significantly upregulated in human pterygium concurrent with downregulation of Noggin. Collectively, our results suggest that BMP6 plays an important role in CE cell stratification by regulating their proliferation and differentiation and that the expression of BMP6 and its antagonist Noggin is dysregulated in pterygium.

Collection and Processing of Human Normal Corneas and Pterygium Samples

Normal human corneas were sourced from donor corneal tissues rejected for transplants, following the procedures approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Dece- dents. Human pterygium samples were collected following the Institutional Review Board-approved protocol. Three patients with pterygium complications were recruited for the study (Supplementary Table S1). They did not undergo any pre- or postoperative treatment during surgical removal of pterygium. Pterygium was graded based on the position of the advancing edge as follows: grade 0, normal (no pathology); grade 1, pterygium head located between the limbus and a point midway between the limbus and the pupillary margin; grade 2, pterygium head located at a point midway between the limbus and the pupillary margin; and grade 3, pterygium head located within the pupillary area.

Cell Culture and BMP6 Treatment

HCLE cells (a generous gift from Dr. Ilene Gipson, Harvard University) were maintained in complete keratinocyte serum-free medium (KSF; Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown to 70% confluence in complete KSF and treated with 50 ng/mL BMP6 (R&D Systems, Minneapolis, MN, USA) for 24 hours in KSF without supplements. HCLE cells were cultured in 12-well plates and stratified as previously described. Briefly, the cells were grown to confluence in a 1:1 mixture of KSF and low-calcium (0.3-mM) Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific) until they reached confluence. The cells were then stratified in high-calcium (1-mM) DMEM/F-12 with 10% fetal calf serum (FCS) and 10 ng/ml epidermal growth factor for 3 days.

Total RNA Isolation and RT-qPCR

Corneal epithelium was isolated by incubating mouse eyeball with Dispalpe for 2 hours at 37°C. Total RNA was isolated from mouse CE, HCLE cells, or human pterygium tissues using the EZ-10 Spin Column Total RNA Miniprep Kit (Bio Basic, Inc., Amherst, NY, USA). Isolated RNA was converted into cDNA using mouse Maloney leukemia virus reverse transcriptase (Promega, Madison, WI, USA). SYBR Green RT-qPCR gene expression assays were performed in duplicate a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using appropriate endogeneous controls. The sequence of oligonucleotide primers used for quantitative reverse transcription PCR (RT-qPCR) is presented in Supplementary Table S2.

Immunoblots

The source and details regarding the antibodies used in this study are listed in Supplementary Table S3. HCLE cells were lysed in radiimmunoprecipitation assay buffer and clarified by centrifugation. An equal amount of total protein in the supernatant was separated on 4% to 12% gradient polyacrylamide gels using 3-(N-morpholino)propanesulfonic acid or 2-(N-morpholino)ethanesulfonic acid buffer and blotted onto polyvinylidene fluoride membranes. The membranes were blocked for 1 hour at 23°C, incubated overnight at

Materials and Methods

Animals

All experiments with mice were performed following the University of Pittsburgh Institutional Animal Care and Use Committee guidelines and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Studies were performed with the C57BL/6J strain of mice between PN-12 and PN-90.
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RESULTS

BMP6 Expression Is Elevated During CE Stratification

As the CE stratified into a multilayered structure between PN-12 and PN-20, the number of Ki67+ proliferative cells decreased significantly, and the expression of pro-differentiation transcription factor KLF4 increased (Fig. 1). Concomitantly, BMP6 expression increased moderately between PN-12 and PN-14, increased highly significantly between PN-14 and PN-20, and remained high in mature corneas at PN-90 (Fig. 2i). In contrast, Noggin expression was significantly decreased between PN-12 and PN-20 and remained low at PN-90 (Fig. 2ii). Increased expression of BMP6 during CE stratification was further confirmed by immunofluorescent staining with anti-BMP6 antibody (Fig. 2ii). Collectively, these results suggest that BMP6 plays a role in the decreased proliferation and increased differentiation accompanying CE stratification.

Increased Expression of BMP6 and Its Receptors in Stratified HCLE Cells

To establish the relationship between BMP6 and CE cell stratification, we employed HCLE cells stratified in vitro as described previously.51,52 Differentiation of HCLE cells under these culture conditions was confirmed by increased expression of pro-differentiation transcription factor KLF4 and CE differentiation markers DSG and DSP in the stratified HCLE compared with the unstratified cells (Fig. 3i). The stratified HCLE cells also displayed a robust 80-fold increase in BMP6 mRNA expression compared with the unstratified cells (Fig. 3iiA). Consistent with these results, immunoblots revealed a 29-fold increase in BMP6 protein expression in the stratified HCLE cells (Figs. 3ii B-C). Transcripts encoding BMP6 receptors BMPR1A and BMPR2 also were significantly upregulated in the stratified HCLE cells compared with the unstratified cells (Fig. 3iii). Together, these results provide evidence of increased expression of BMP6 and its receptors upon stratification of HCLE cells in vitro.

BMP6 Inhibits HCLE Cell Proliferation

Next, we evaluated the effect of exogenous BMP6 on HCLE cell proliferation by treating them with BMP6 (50 ng/mL). Immunostaining with anti-Ki67 antibody revealed that the number of Ki67+ cells decreased in BMP6-treated cells by more than 50% compared with the control group (Figs. 4i, 4ii). RT-qPCR revealed that the expression of Noggin was significantly increased in BMP6-treated cells.
FIGURE 2. BMP6 expression increased with a concurrent reduction in BMP6 antagonist Noggin expression in stratifying mouse CE cells. (i) RT-qPCR revealed a modest increase in BMP6 expression between PN-12 and PN-14 and a robust increase at PN-20 that was maintained at PN-90 ($n = 6$). In contrast, Noggin expression was high at PN-12 and decreased significantly in the stratifying (PN-14 and PN-20) and mature (PN-90) CE ($n = 6$). All data are presented as mean ± 1SEM. Corresponding P values are shown. (ii) (A) Control (no primary antibody). Immunofluorescent stain showed increased expression of BMP6 at PN-20 (C) and PN-90 (D) compared with PN-12 (B). CS, corneal stroma. Scale bar: 40μm ($n = 3$).

HCLE cells, suggesting that the well-established feedback regulation of BMP signaling is operational in HCLE cells (Fig. 4iii).23–25,27 Next, we evaluated the effect of 50-ng/mL BMP6 on the expression of cell-cycle regulators cyclin-D1 and cyclin-D2 (G1/S transition promoters) and p21 (cyclin inhibitor). BMP6 treatment resulted in a significant decrease of cyclin-D1 and cyclin-D2 and an increase in p21 transcripts (Fig. 5i). Consistent with these results, immunofluorescent staining with anti-cyclin-D1/-D2/-D3 and anti-p21 antibodies revealed decreased expression of cyclin-D and increased nuclear p21 in BMP6-treated HCLE cells compared with the controls (Fig. 5ii). Collectively, these results indicate that BMP6 exerts an anti-proliferative effect on HCLE cells in a cyclin- and p21-dependent manner.

**BMP6 Promotes HCLE Cell Differentiation**

The significant increase in BMP6 expression during the mouse CE stratification between PN-12 and PN-20 (Fig. 2) and in stratified HCLE cells (Fig. 3) suggested a pro-differentiation role for BMP6. To evaluate this further, we treated 70% confluent HCLE cells with 50 ng/mL of BMP6 for 24 hours and quantified the expression of CE differentiation markers. RT-qPCR revealed increased expression of KLF4, DSG, and DSP transcripts in BMP6-treated HCLE cells (Fig. 6i). Immunoblots confirmed the 1.3- and 1.8-fold increase in expression of desmosomal cadherins DSG and DSP, respectively, in BMP6-treated HCLE cells (Fig. 6ii). Consistent with these results, immunofluorescent staining revealed increased nuclear expression of KLF4 and membranous expression of DSG and DSP in BMP6-treated HCLE cells compared with the controls (Fig. 6). Taken together, these results establish the pro-differentiation effect of BMP6 on HCLE cells.

**Elevated BMP6 Expression Coupled with Downregulation of Noggin in Human Pterygium**

Pterygium refers to a group of conjunctival fibrovascular degenerations with diverse etiologies that resemble pre-squamous metaplasia and affect vision by progressively encroaching into the cornea.53–55 Considering that (1) pterygium is associated with suppression of cyclin-dependent kinase inhibitor p21,56 (2) BMP6 is upregulated in scarred conjunctival tissue46 and its upregulation is associated with tumor aggressiveness,57 and (3) the data presented thus far indicate that BMP6 regulates CE cell proliferation and differentiation in a cyclin- and p21-dependent manner, we tested whether or not BMP6 is altered in pterygium.
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**FIGURE 4.** Exogenous BMP6 decreased HCLE cell proliferation and upregulated *Noggin* expression. (i) (A) Control (no primary antibody). (B, C) Immunofluorescent stain showed Ki67+ cells in the control and BMP6-treated HCLE cells (*n* = 3). Scale bar: 50 μm. (ii) Effect of BMP6 on HCLE cell proliferation, presented as percentage control and BMP6-treated HCLE cells (body). (iii) Immunofluorescent stain showed Ki67 expression. (values are shown. *n* = 3). Corresponding *P* values are shown.

RT-qPCR revealed significant upregulation of *BMP6* transcripts coupled with significant downregulation of transcripts encoding cell cycle suppressors *KLF4* and *p21*, as well as *Noggin*, in pterygium tissues compared with the normal CE (Fig. 7i). Consistent with these results, immunofluorescent staining revealed a robust increase in BMP6 expression in pterygium compared with the control (Fig. 7ii). Collectively, these results provide evidence that BMP6 expression is elevated in human pterygium concomitant with the downregulation of cell proliferation suppressors *KLF4* and *p21* and the BMP6 antagonist *Noggin*.

**DISCUSSION**

Proper formation and function of the CE, a stratified squamous tissue at the front of the eye, is essential for normal refraction and transmission of the incident light to the retina, a prerequisite for normal vision. Despite this importance, molecular mechanisms and signaling networks that regulate CE stratification during initial development and in adult CE homeostasis have not been adequately studied. In this report, we have demonstrated the following: (1) BMP6 expression is significantly elevated in stratifying mouse CE and in HCLE cells undergoing stratification in vitro; (2) BMP6 antagonist *Noggin* expression is downregulated in stratifying mouse corneas and HCLE cells; (3) BMP6 serves as an anti-proliferative agent by downregulating cyclin-D1 and cyclin-D2 and upregulation of cell-cycle inhibitor *p21*; (4) BMP6 acts as a pro-differentiation factor by promoting the expression of *KLF4*, *DSP*, and *DSG*; and (5) BMP6 is significantly upregulated in human pterygium, coupled with the downregulation of *Noggin*. Collectively, these results suggest that BMP6 is a key contributor to CE stratification and that its expression is dysregulated in ocular surface pathologies such as pterygium.

Although TGF-β1 and TGF-β2 play crucial roles in regulating cellular proliferation and differentiation in a diverse array of tissues, including the cornea, similar studies on other members of the TGF-β superfamily including the BMP subfamily are limited. Previously, we reported that mouse CE-specific ablation of *KLF4* results in TGF-β-mediated EMT and squamous metaplasia-like changes in the mouse CE. In the developing mammalian epidermis, BMP–FGF signaling mediates Wnt-induced epidermal stratification. BMP6 expression coincides with epidermis stratification, which it influences by regulating cell proliferation and differentiation. BMP2 and BMP6 regulate *p57* expression and cell growth arrest and promote terminal differentiation in human epidermal keratinocytes. The data presented here demonstrate that, like epidermis, the CE also displays increased BMP6 expression during stratification. To the best of our knowledge, our current report provides the first demonstration of BMP6 promoting CE stratification by suppressing CE proliferation and facilitating differentiation, and this study sets the stage for further investigation into the interplay among different members of the TGF-β superfamily in this process.

Proper balance between CE cell proliferation and differentiation is crucial for its initial stratification during early postnatal stages and homeostasis in the adult stage. Any
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FIGURE 6. BMP6 increased the expression of KLF4, DSP, and DSG. (i) RT-qPCR showed increased expression of differentiation markers KLF4, DSP, and DSG in stratified BMP-6-treated HCLE cells compared with the control. (ii) Immunoblots and the corresponding histograms showed increased DSP and DSG levels in BMP6-treated HCLE cells compared with the control. All data are presented as mean ± 1 SEM (n=3). Corresponding P values are shown. (iii) Immunofluorescent staining showed increased expression of KLF4, DSP, and DSG in BMP6-treated HCLE cells compared with the control. Scale bar: 50 μm. See Supplementary Figure S1 for a complete image of representative immunoblots.

imbalance in CE proliferation and differentiation results in ocular surface complications. Data presented in this report indicate that BMP6 tilts the balance toward stratification by suppressing cell proliferation through downregulation of cell-cycle suppressors KLF4 and p21 and NOG expression in human pterygium tissues compared with the healthy control. All data are presented as mean ± 1 SEM (n=3). Corresponding P values are shown. Immunofluorescent staining showed increased expression of KLF4, DSP, and DSG in BMP6-treated HCLE cells compared with the control. Scale bar: 40 μm.

Pterygium is a fibrovascular disease of the ocular surface with dysregulated cell proliferation and differentiation. The increased levels of BMP6 in pterygium are contradictory to its role in normal CE, reminiscent of TGF-β having contradictory roles in normal and pathological conditions. However, given that BMP6 regulates both proliferation and differentiation and is upregulated in different tumors and fibrotic conjunctival scars, its increased expression in pterygium comes as no surprise. BMP6 upregulates Noggin expression, which in turn inhibits BMP6 activity, resulting in a negative feedback loop that regulates BMP6 signaling. Our discovery of BMP6 upregulation with concomitant downregulation of Noggin suggests that this negative feedback regulation is disrupted in pterygium as in other proliferative pathologies such as prostate cancer. Previous reports of large-scale gene expression comparisons between pterygia and normal tissues failed to detect any change in BMP6 expression. One potential reason for this is that pterygia with diverse etiologies display unique gene expression patterns. Thus, additional studies are needed to validate BMP6 involvement in pterygium.

Although pterygia accumulate high levels of iron, the underlying mechanisms are unknown. Stocker’s line, a subepithelial brownish line in front of the invasive apex of the pterygium, is presumed to contain elevated iron depots. Because pterygia accumulate high levels of iron that may play a pathogenic role by generating oxygen-free radicals and because elevated iron levels induce BMP6
expression in the liver, which in turn activates hepcidin involved in iron metabolism. It would be worthwhile to determine if iron accumulation is responsible for the upregulation of BMP6 in pterygium as reported here. Similarly, it would be important to determine if the expression and/or function of hepcidin, a primary target of BMP6 that is involved in iron homeostasis, is altered in pterygium.

To summarize, increased expression of BMP6 during mouse CE and HCLE cell stratification coupled with the anti-proliferative and pro-differentiative effects of BMP6 on HCLE cells suggest that BMP6 coordinates CE stratification by regulating their proliferation and differentiation. These data strongly support a role for BMP6 in CE stratification and reveal that BMP6 expression is dysregulated in pathological conditions such as pterygium. Whether dysregulated BMP6 expression is a common occurrence in pterygium remains to be determined by testing additional disease specimens. Also, the physiological relevance of disrupted BMP6 signaling in pterygium should be further evaluated.

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