Bves Is Expressed in the Epithelial Components of the Retina, Lens, and Cornea

Anna N. Ripley,1,2,3,4 Min S. Chang,5 and David M. Bader1,2,3,4

PURPOSE. To demonstrate the expression pattern and subcellular localization of Bves/Pop1a protein, a newly identified cell adhesion molecule, during eye development and corneal regeneration.

METHODS. Staged embryonic and adult eyes were assayed using fluorescence immunohistochemistry to detect the Bves protein. A human corneal epithelial (HCE) cell line was used as a model to examine Bves localization during corneal growth and regeneration, with and without antisense morpholino treatment.

RESULTS. The data detail the expression and localization of Bves protein before, during, and after differentiation of the eye. In these analyses, Bves was localized to an apical–lateral position in the initial epithelial primordia of the eye. Later, Bves became localized to specific cell types and subcellular domains in the retina, lens, and cornea, indicating changes in Bves expression in the differentiated eye. Finally, an in vitro model of corneal wound healing showed that Bves staining was missing at the epithelial surface during cellular migration across the wound, but it reappeared at points of cell contact during the reinitiation of epithelial continuity. When epithelial sheets were treated with Bves antisense morpholinos to inhibit Bves function, disruption of epithelial integrity was observed. After injury, similar treatment resulted in an acceleration of cell movement at the wound surface but regeneration of an intact epithelium was ultimately impeded.

CONCLUSIONS. Taken together, these studies suggest that Bves is expressed in epithelial elements of the developing eye and may have a role in corneal epithelial growth and regeneration.

(Invest Ophthalmol Vis Sci. 2004;45:2475–2483) DOI: 10.1167/iovs.04-0013

Development of the eye requires a complex series of differentiative events. Establishment of the three main eye primordia—the retina, lens, and cornea— involves movement and reshaping of embryonic epithelia, and additional contributions of mesenchymal cells derived from head mesoderm and neural crest are also essential for proper eye development.1–4 A shared property in the development of the retina, lens, and cornea is that each structure originates in part from ectodermally derived epithelium. In all three cases, the original epithelium undergoes dramatic morphogenetic changes that depend on proper reshaping of the original cell layer while maintaining epithelial polarity and integrity. The formation and reshaping of epithelia is dependent on cell–cell interaction through a complex set of adhesion molecules.5–8 In large part, tight adherens, and desmosomal junctions are responsible for maintaining cell adhesion and polarity in mature epithelia,9 10 but components of these structures often intermix during epithelial morphogenesis.6 7 Resolution of the distribution of adherent proteins during eye development is essential for an understanding of their function in this process. Although it is clear that epithelial adhesion and movement are critical for eye development, little is known about the role of specific cell adhesion molecules in eye morphogenesis.

The present study explores the role of a novel cell adhesion molecule, Bves/Pop1a, in the formation of the retina, lens, and cornea. We originally isolated Bves (blood vesse/epicardial substance) from a heart-specific subtraction screen and showed expression in the epicardium and coronary smooth muscle using our first generation of antisera.11 Subsequently, Andree et al.12 identified two or three genes they termed popeye in chickens, mice, and humans. The bves transcript and gene are identical with pop1a. In their study, analysis of RNA expression showed that the bves/pop1a transcript is expressed at high levels in cardiac and skeletal muscle by in situ hybridization and RNA blot analyses. DiAngelo et al.13 demonstrated protein expression in cardiac muscle of the developing chicken using a monoclonal antibody. The Human Gene Nomenclature Committee, National Center for Biotechnology Information (NCBI), and Mouse Genome Informatics have designated this gene product as “Bves,” and we use this term henceforth in this report.

Our previous data suggest that Bves regulates cell–cell interaction in a calcium-independent, homophilic manner when transfected into nonadherent cells.14 15 The molecule has three conserved transmembrane domains that are essential for insertion and/or retention in the membrane. As first suggested by Thomas Brand’s group, recent biochemical analysis confirms that Bves has a short extracellular N terminus with a long intracellular C-terminal tail.16 Although Bves proteins share no homology with any other known protein family, the members within the family and across species are highly conserved in amino acid sequence.11 12 17 In a recent study, Andree et al.18 observed no developmental abnormalities or phenotypes after ablation of the pop1 gene. However, they noted a delay in skeletal muscle regeneration in these mice and suggested that this may be due to a problem in cell–cell interaction. Thus, although in vitro analysis of cell behavior suggests a role in cell–cell adhesion or interaction, the role of this gene family in development and repair is only now being investigated.

There are several lines of evidence to suggest that Bves expression extends beyond heart and skeletal muscle. First, RT-PCR analysis in Andree et al.12 shows a broad distribution of expression in the developing heart, skeletal muscle, lung, kidney, brain, and spleen of the mouse embryo but not to the levels detectable by in situ or RNA blot analysis. In addition, although not noted in the text, mRNA expression is clearly seen in the endoderm of the developing gut by in situ hybri-
ization (Andree et al. 12, Fig. 4). In addition to these studies, numerous expressed sequence tags (ESTs) have been reported for nonstriated cell types in many organisms (NCBI). Still, in contrast to our studies, a recent report was unable to demonstrate the presence of Bves in the PEO, the anlagen of the epicardium, and its descendants.19 In contrast, Vasavada et al. 20 have recently reported that Bves is transiently detected with their monoclonal antibody in the developing epicardium further implicating this protein in epithelial function. Using a second set of monoclonal, polyclonal, and single-chain antibodies, our group has reported Bves expression in other epithelial structures as well as skeletal muscle and cardiac muscle 14,15 and in the three embryonic germ layers of the developing chicken embryo.21

The data from these studies led us to examine the expression pattern of Bves in developing embryos, with special reference to epithelia that contribute to organs or organ systems. The eye provides a unique developmental system in which its three major and divergent components, the retina, lens and cornea, are derived from epithelial sources. These epithelia display complex morphogenetic movements that are essential for generating the adult structure. In addition, in the case of the cornea, regeneration of the epithelium entails the migration of adjacent surviving cells over the wound to replace lost cells and serves as an example of epithelial morphogenesis in the adult organism. In the present study, we show that Bves is expressed in all three epithelial progenitor populations of the eye but in unique subcellular locations. In addition, using an in vitro model of corneal wound healing, we observed that Bves staining is lost at the surface of cells advancing from the wound edge. Later, as epithelial continuity is established, Bves is again seen at the cell surface. In an initial effort to elucidate Bves function in the eye, we conducted an antisense morpholino study to “knock down” Bves protein expression in cultured corneal epithelium. This methodology has been an effective method for disruption of protein function.22–24 We observed that morpholino treatment of uninjured corneal epithelium led to irregularities in cellular sheet formation. On injury, morpholino-treated corneal cells move more rapidly into the wound area compared with nontreated cells. Still, these cultures did not produce an intact monolayer of epithelial cells in the wound area compared with control cultures. We discuss the implications of these experiments in reference to the possible role of Bves in cell–cell interaction and epithelial integrity.

**Materials and Methods**

**Embryo and Tissue Collection and Cell Culture**

The experimental animals used adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. White Leghorn fertilized embryos were obtained from Truslow Farms (Chesterton, MD) and incubated in a high-humidity 37°C chamber. Embryos were collected and staged according to Hamburger and Hamilton.25 Staged Black Swiss mice were killed and the eyes removed for tissue sectioning. An SV40-immortalized human corneal epithelial cell line was obtained from the laboratory of Araki-Sasaki et al.26 and cultured at 37°C in a defined serum-free keratinocyte medium (Invitrogen-Gibco, Grand Island, NY).

**Antibodies**

The B846 polyclonal antiserum was developed in rabbits (Bio-Synthesis, Lewisville, TX) against the C-terminal tail of Bves1 (DPTLND-KKKKLEQOMS; amino acids 266-283 of mouse bves) followed by affinity purification with a peptide-conjugated column.21 This serum has been shown to be reactive with the Bves protein produced from the cloned mRNA and cross-reacts with several avian and mammalian species (Ripley A, Bader D, unpublished data, 2003). In addition, the antisemur has been shown to be specific for Bves protein by immunopeptide competition and transfection analysis.22 Monoclonal antibodies against various junctional complex proteins were purchased as follows: β-catenin (C-7207; Sigma-Aldrich, St. Louis, MO), E-cadherin (877-232-8995; BD-Transduction Laboratories, Lexington, KY), ZO-1 (33-9100; Zymed, South San Francisco, CA), and 3F11, anti-Pop1/Bves (Hybridoma Bank; National Institutes of Health, Bethesda, MD).

**Immunohistochemistry**

Embryos and tissue were collected in PBS and incubated in 30% sucrose for 2 hours. Samples were embedded in optimal cutting temperature (OCT) compound, frozen in dry ice/ethanol bath, and 5-μm sections were cut on a cryostat. Sections were fixed in 70% methanol for 5 minutes, rinsed with PBS, permeabilized with 0.25% Triton X-100 and blocked in 2% bovine serum albumin/PBS for 1 hour at room temperature. Sections were co-incubated with the B846 Bves antibody and antibodies directed against other junctional proteins overnight at 4°C. The sections were washed three times with PBS, and incubated with appropriate secondary antibodies (Jackson Laboratories) and 4′,6-diamino-2-phenylindole (DAPI) for at least 4 hours at room temperature in the dark. Images were captured on a fluorescence microscope (Olympus Corp. of America, Lake Success, NY) with a digital camera (Magnafire image-processing software; Optronics, Goleta, CA). Negative control experiments included incubation with no primary antibody, nonreactive primary antibody, and peptide competition. For peptide competition, the 3′ DNA sequence coding for the C-terminal 289 amino acids were cloned in-frame into pGEX and used to produce recombinant protein by published techniques.13 Purified protein was reacted with affinity-purified antisera in PBS at a 100-, 50-, or 10-fold molar excess along with no added peptide for 4 hours at 4°C. After this reaction, the solution was transferred to sections of day-7 chick eyes and incubated overnight at 4°C. Sections were then processed for indirect immunofluorescence by standard techniques detailed earlier. Images are captured and processed identically for comparison between control and experimental groups. Corneal epithelial cells were grown on glass chamber slides, rinsed with PBS, fixed in 70% MeOH and permeabilized with 0.1% Triton X-100. Similar peptide competition experiments as described earlier were conducted on cultured corneal epithelial cells to determine the level of background staining in this particular experimental method. Immunofluorescence analysis of these cells was completed as described for tissue sections.

**RT-PCR Analysis**

RNA was isolated (TRizol reagent; Invitrogen-Gibco, Grand Island, NY) from whole eyes and hearts at selected stages and subjected to RT-PCR analysis. The entire eye was dissected, and thus the values presented can only be ascribed to expression in the entire eye and not to any specific cell type within the structure. Tissues were sterilely isolated and extracted according to Osler and Bader.21 RNA concentrations were quantified and reacted with primers for GAPDH and Bves. Sequences for these primers and PCR conditions are given in Osler and Bader.21 No RT controls were conducted to ensure the RNA-dependence of the reaction. Products were analyzed on 1% agarose gels.

**Morpholino “Knockdown” of Bves Expression and Wound Healing**

SV40-immortalized human corneal epithelial cells were plated at a seeding density of 20,000/cm². Typically, the cells reached full confluence by day 6. Injury was induced by a scratch injury or by application of modified drill press under sterile conditions.27–29 Cultures were returned to the incubator and grown for selected periods. Cultures were fixed and processed for immunofluorescence as described earlier. Two morpholino antisense oligonucleotides for human bves1 were synthesized and applied to cultures using the manufacturer’s methods (Gene Tools, Philomath, OR). The sequence of the morpholino that gave the strongest phenotype was 5′-ATCTTCTTTATACCTG-GATGTGCAG-3′. Control morpholinos recommended by the manufac-
Bves was expressed in the CNS and during optic vesicle formation. (A) Dotted line: the plane of the section shown in the accompanying micrograph (bottom left). Immunohistochemical analysis showed Bves expression (red, arrow) in the epithelial lining of the chick neural tube at stage 7. The position of the notochord is shown (arrowhead). The schematic in (A) represents this stage. (B) Schematic representation of a stage-9 chicken embryo in the longitudinal plane illustrating the formation of the optic vesicle. The pink box in the schematic represents the area of the section shown in the micrograph (bottom right). Bves expression (green, arrow) in the outpocketing of the diencephalon during early optic vesicle formation of a stage-9 chick shows the apical position in this staining in the epithelium. Inset: A higher-power view is given to show the apical position of Bves in the neuroectoderm. Nuclear stain: DAPI (blue).

RESULTS

Bves Expression in the Eye

In an initial survey of Bves expression during embryogenesis, we determined that Bves staining was observed in elements of...
the developing eye, most prominently in its epithelial precur-
sors of the cornea, lens, and retina (Fig. 1A). These data led us
to conduct an analysis of Bves expression in the eye at selected
stages of development. In the following studies, we primarily
used the chick as a developmental model for eye formation,
because of the ease in obtaining embryos, but we also con-
ducted similar analyses on mice and frogs.

To demonstrate that bves mRNA was present in the devel-
oping eye, RT-PCR analysis was conducted on selected stages
of eye development in the chick. Heart tissue was also analyzed
as a positive control for expression. RT-PCR analysis was cho-

A series of peptide competition experiments were con-
ducted to test specificity of B846 for Bves. As seen in Figure
1Ca, antiserum B846 reacts with cells of the developing retina
day 7 of embryonic development (to be discussed in more
detail later). Peptide competition at 100-M excess completely
eliminated B846 antibody reactivity in sections of the day-7
retina (Fig. 1Cb). Lesser concentrations of competing peptides
also blocked anti-serum reactivity. No primary antibody control
results also showed no staining for these sections (Fig. 1Cc).

The Retina

The following description of Bves expression concentrates on
the developing chicken; a similar protein distribution was seen
in the embryonic mouse eye. Bves protein was detected in
central nervous system (CNS) cells adjacent to the central canal
(Fig. 2A) and was sustained in this layer throughout the CNS
and in the diencephalon as it evaginated to form the optic
cup. This staining was confined to the apical aspect of the epithelium adjacent to the lumen of the optical vesicle (Fig. 2B, inset). Next, strong expression was seen in the apical surfaces of the inner and outer epithelial layers as they merged to form the mature retina (Figs. 3A–H). Close inspection (Figs. 3B, 3C) showed that Bves was present in both the inner and outer layers of the optic cup. Thus, Bves staining was observed continuously in an apical position throughout the course of retinal cup formation and differentiation even though

the entire eye was dissected, and thus the resultant reaction
products reflect expression throughout the eye at the indicated
stage. As seen in Figure 1B, a discrete reaction product of the
predicted size was obtained with RNA isolated from day-2 head
region and day-6, -18, and -21 embryonic chicken eyes. (It was
impossible to dissect specific eye-forming regions with cer-
tainty.) A product at the same mobility was observed using
RNA isolated from hearts at selected stages of development but
was not observed in control experiments without RT (data not
shown). The GAPDH reaction serves as a positive control for
RNA input (Fig. 1B). It should be noted that abundance of
product from the eye is similar to that observed from the heart
in this assay system. Andree et al. also demonstrated rela-
tively high levels of RT-PCR product in tissues other than heart and striated muscle.

A product at the same mobility was observed using
RNA isolated from hearts at selected stages of development but
was not observed in control experiments without RT (data not
shown). The GAPDH reaction serves as a positive control for
RNA input (Fig. 1B). It should be noted that abundance of
product from the eye is similar to that observed from the heart
in this assay system. Andree et al. also demonstrated rela-
tively high levels of RT-PCR product in tissues other than heart and striated muscle.

To demonstrate that bves mRNA was present in the devel-
oping eye, RT-PCR analysis was conducted on selected stages
of eye development in the chick. Heart tissue was also analyzed
as a positive control for expression. RT-PCR analysis was cho-

FIGURE 3. Bves localization during formation and apposition of inner and outer retinal layers. (A–D) Bves expression (green) during inner and outer optic cup apposition in stage-18 chick embryos. Phase contrast is overlapped in images (D, H, L) to illustrate pigment localization in the RPE (arrows). Note that pig-
ment granules did not accumulate in the section shown in (D). (E–H) Bves (green) expression after the optic cup layers had fused in the day-8 chick embryo. (I–L) Bves (green) continued to be expressed in the differen-
tiated retinal layers: photoreceptors (arrowhead) and RPE (ar-
row) derived from the epithelial inner and outer optic cup layers in the day-19 chick. Bves (green) ex-
pression overlapped with the adher-
ens junction marker β-catenin (red),
during (M–O) and after (P–R) optic
cup apposition. Yellow: overlap of
Bves and β-catenin in the merged im-
ages (O, R). Note that β-catenin stain-
ing was broader than that of Bves.
Nuclear stain: DAPI (blue). Scale bar:
(A, E, I) 100 μm; (B, F, J) 25 μm; (D,
H, I, M–R) 10 μm.
The Lens

The lens was derived from surface ectoderm where Bves was broadly expressed before any morphologic evidence of lens induction (data not shown). Bves staining was observed on the inner surface of the lens vesicle (i.e., the apical-lateral surface of this epithelium; diagram in Figs. 4A, 4B). Again, extensive but not complete overlap of Bves staining with adherent junction markers, in this case E-cadherin, were observed in the apical position of the lens vesicle epithelium (Figs. 4B–D). The next stage of lens development was characterized by the growth or extension of primary fibers that filled the lens cavity (Fig. 4E). As cellular extension proceeded, high levels of Bves were seen at the advancing edge of the primary fibers that course anteriorly during their differentiation to obliterate the lens vesicle cavity (Fig. 4E). After the lens vesicle cavity was obliterated, Bves continued to be expressed in the anterior undifferentiated epithelial cells (Fig. 4H). After these cells migrated past the bow region and begin to elongate, the expression of Bves was not seen on the lateral surface of the elongating lens fibers, but may remain localized at the apical regions of these cells, even during their pronounced elongation (Fig. 4H).

Our previous studies have shown that Bves colocalizes with adherens junctions proteins, such as E-cadherin and β-catenin during epithelial sheet formation in vitro. To determine whether this is a common feature in diverse embryonic epithelia, colocalization studies with immunochemical markers of cell adhesion molecules were conducted. We found that Bves had extensive overlap of staining with anti-β-catenin, a marker of the adherens junctions in the apical-lateral region of the optic cup epithelia both before (Figs. 3M–Q) and after epithelial apposition (Figs. 3P–R). This overlap was confined to the apical-most portion of the cell as staining of adherent junction proteins extended more basally. It should be noted that additional, nonoverlapping staining of β-catenin was also observed in the RPE.

**FIGURE 4.** Bves expression during lens formation in the chick. (A) Depiction of lens morphogenesis and development. Bves (green, B) and E-cadherin (red, C) expression overlapped (yellow, D) in the apical region of the epithelial lens vesicle at stage 18. (E–G) As the posterior lens epithelium pushed up into the vesicle lumen and began to elongate, Bves (green, E) expression remained apical and colocalized (G, yellow) with E-cadherin (red, F). (A, purple box) Area of the day-3 lens shown in (E–G). As the posterior lens at day 4 differentiated into fiber cells, Bves (green) expression was restricted to the anterior epithelium. Expression of E-cadherin in the differentiating lens (I, red) and overlap with Bves (J, yellow). A higher-power magnification of the anterior lens epithelium demonstrates the extensive but not complete overlap of Bves and E-cadherin staining. Nuclear stain: DAPI (blue).

Scale bar: (B–D, H–J) 100 μm; (E–G) 50 μm; (K–M) 10 μm.

**FIGURE 5.** Bves localization in corneal epithelium. (A) Immunohistochemical analysis of Bves expression (green) in the anterior half of the 6-week-old mouse eye. The lens was lost in this preparation. Higher magnifications in (B) and (C) of the corneal epithelium indicated by the yellow box in (A). (C, arrow) Punctate staining of Bves in the basal and wing cell layers of the corneal epithelium. (D, E) Higher magnification of the corneal edge indicated by the pink box in (A). Bves was expressed in the corneal epithelium but not the sclera. (F, G) Sections obtained through a chick cornea at hatching. Bves expression in this corneal epithelium was similar to that in the mouse. Nuclear stain: DAPI (blue).

Scale bar: (A) 100 μm; (B, G) 20 μm; (C, E) 10 μm; (D) 40 μm; (F) 50 μm.
FIGURE 6. (A) Bves was expressed at the cell surface of human corneal epithelial (HCE) cells as they made contact during epithelial sheet formation. Immunohistochemical analysis of Bves expression in HCE cells at low density showed limited peripheral staining. (AB) Bves staining was seen along regions where cells made contact and was prominent at the surface of cells within nascent epithelial sheets (AC), but not along the free edge of these sheets (arrowheads). At confluence (AD), Bves was present around the entirety of epithelial cells. Intracellular staining (arrowheads) was absent in some sheet cells. Membrane and intracellular stainings were lost after peptide competition (AE). Nuclear stain: DAPI (blue). (B) Dynamic expression of Bves in wound healing of HCE cells. A confluent sheet of HCE cells was scratched, and Bves expression (green) was analyzed at selected times. Initially, cells at the surface lost Bves staining on the free surface (BA, arrows). Next, cells without prominent Bves staining moved into the wound area (BB). At higher power (BC), the difference in the location of Bves staining in adjacent intact epithelium (arrows) and wound area cells (arrowheads) in a 2-day culture were visible. Nuclear stain: DAPI (blue). Scale bar: (AA, AC) 10 μm; (AB, BC) 5 μm; (AB, AD, AE) 15 μm; (BA, BB) 20 μm.

Although E-cadherin staining was observed in this same region, it was more broadly expressed through the lens fibers (Figs. 4E-J). High-power magnification of a day-4 embryo showed overlap of staining in the anterior lens epithelium (Figs. 4K–M). Bves continued to be expressed in the differentiated layers in the anterior epithelium and apically in the primary fibers.

The Cornea

Bves was broadly expressed in surface ectodermal cells before corneal induction and differentiation in the chicken (Figs. 1, 3A, 4H; arrow) and mouse (data not shown). This staining was not confined to regions of the presumptive cornea and was present in much of the ectoderm of the head. When the surface ectoderm became stratified as seen in this 6-week-old mouse preparation, Bves staining was seen in the corneal epithelium, but was absent in the primary and secondary corneal stroma, endothelium, and sclera (Fig. 5). The same restriction of Bves staining to corneal epithelium was observed in the chick (Figs. 5F, 5G). Bves staining within the corneal epithelium varied from basal to apical strata (Figs. 5B, 5C). In the deep layers, prominent punctate staining around the periphery of cells was observed, whereas rather homogeneous reactivity was observed around the entirety of cells in the intermediate or wing cell layers (Fig. 5C). Finally, as cells orient themselves perpendicular to the apical–basal axis in the most superficial region, strong homogeneous staining was seen throughout these cells.

Corneal Epithelial Sheet Formation and Regeneration

Because our previous studies suggested that Bves may be one of the first proteins to traffic to points of cell–cell contact in forming epithelia and play a role in cell–cell interaction, we sought to determine the localization of Bves in corneal epithelial formation and regeneration.

After initial plating of HCE cells, Bves was not observed at high levels at the surface of nonconfluent cells but was instead present in the cytoplasm (Fig. 6AA). Cytoplasmic localization of Bves is a common property of this protein in single cells and in forming and nascent epithelium.11,12,14,15 This cytoplasmic staining was clearly above background when compared with peptide blocking (Fig. 6AE) or no primary antibody (data not shown) control results. As epithelial cells made contact, Bves staining was observed at points of cell contact as epithelial sheets began to form (Figs. 6AA–6AC). Bves staining was seen along the surfaces of cells where cell–cell contact was established and was absent from free surfaces. Later, Bves was present around the entirety of cells in confluent epithelial sheets (Fig. 6AD). It is important to note that Bves staining was also noted intracellularly in confluent sheets. Both membrane and intracellular stainings were eliminated with peptide competition (compare Fig. 6AD with 6AE).

To assess a possible role for Bves in this process, localization of Bves in corneal wound healing in vitro was analyzed. Confluent corneal sheets were wounded and allowed to heal by using standard scratch or press protocols. As predicted, Bves staining was not observed on the free surface of surviving cells at wound edge but remained at the surface of uninjured cells (Fig. 6BA). Next, cells at the wound surface, either individually or in clusters, moved into the free space and Bves staining was lost on the surface of these cells (Fig. 6BB). With time, the gap between wound surfaces was filled with migrat-
Morpholino treatment disrupted corneal epithelial sheets. Numerous disruptions in corneal epithelial sheets were detected with Bves morpholino antisense treatment. These regions showed disruption of Bves staining (A), whereas control morpholino (B) and no treatment (C) showed continuous Bves staining after 4 days in culture. Nuclear stain: DAPI (blue). Scale bars, 10 μm.

A

B

C

Morpholino treatment disrupted corneal epithelial sheets. Numerous disruptions in corneal epithelial sheets were detected with Bves morpholino antisense treatment. These regions showed disruption of Bves staining (A), whereas control morpholino (B) and no treatment (C) showed continuous Bves staining after 4 days in culture. Nuclear stain: DAPI (blue). Scale bars, 10 μm.

In an initial effort to elucidate the possible role of Bves in maintaining epithelial integrity in the cornea and its ability to regenerate, we used a morpholino knockdown strategy on cultured corneal cells. Cultures were plated at 2 × 10³/cm² seeding density and treated with Bves antisense or control morpholinos at day 3 (50% confluence) and again at day 5 (80% confluence) to access whether inhibition of Bves function would affect epithelial integrity. Cultures were monitored daily for possible variation between control and experimental groups. During these studies, it became readily apparent that Bves antisense morpholino-treated cultures retained numerous gaps in the epithelial sheet (Fig. 7A). These gaps were not observed in great numbers in control morpholino or non-treated cultures at comparable time points. Immunochemical analysis of protein expression and epithelial morphology demonstrated loss of Bves staining in regions of epithelial discontinuity (Fig. 7A). In these regions, Bves staining was either absent or diminished. In contrast, control cultures exhibited the standard peripheral pattern of Bves staining (Figs. 7B, 7C).

To determine whether morpholino inhibition of Bves protein had an effect on the wound-healing process, control, and experimental cultures were press injured to create a standard wound area and monitored for cell migration and injury repair. The initial wound area was measured for all samples. This area was layered on phase images obtained from control and experimental groups to visualize the percent of wound covered (Fig. 8; Table 1). During the first day of wound repair, it was apparent that cells in cultures with Bves antisense morpholino treatment moved into the wound area more rapidly than those of control morpholino and no-treatment cultures. The amount of "healing" or filling in of the defect was followed for 3 days after injury. After 24 hours, cultures treated with Bves antisense morpholino filled 64% ± 5% (SE) of the defect, whereas the control morpholino treatment and no treatment covered 54% ± 9% and 55% ± 8% of the wound, respectively. Statistical analysis demonstrated a significant difference (P < 0.05) with the anti-Bves morpholino compared with both control morpholino and no-treatment groups. This difference remained statistically significant over the 3-day period. As predicted, no significant difference was observed between the control morpholino and no-treatment groups. Still, after this initial acceleration in wound healing, regenerated epithelial sheets in the experimental group were not as highly organized as those in control groups with significant gaps in the regenerated epithelium.

An essential element in the development of the eye is the movement of epithelia. Clearly, it is necessary to identify and characterize new players in this process to elucidate the underlying mechanisms that regulate eye morphogenesis. Our initial interest in analyzing Bves expression in the eye was to test our overall hypothesis that Bves regulates epithelial adhesion and movement during organogenesis. Early in development, Bves is localized to the apical regions of epithelial precursors in the cornea, lens, and retina. Later in morphogenesis and in the adult, Bves is redistributed in a cell type-specific manner. Finally, morpholino knockdown experiments with a cultured human corneal cell line suggest that Bves may play a role in epithelial movement during corneal sheet formation and regeneration in vitro.

Quantification of corneal epithelial regeneration. The original wound area in cells treated with Bves morpholino (A), control morpholino (B), and no treatment (C) are shown in pink. The movement or growth of cells into this area at 24 hours was seen respectively in these same cultures (D–F). Scale bars, 50 μm.
From that of the experimental groups and given as zero.

The average area of cell growth of these groups at selected time points is presented. Growth of the no-treatment group is subtracted from that of the experimental groups and given as zero.

Bves is present in the apical region of epithelia during the initial morphogenetic period of eye development. In the case of the retina, Bves is localized to the apical regions of the neural and pigmented retinas before and at the time of optic cup apposition in the chick (Fig. 3C) and mouse (data not shown). Later when the definitive layers of the retina are formed, Bves expression persists but is redistributed within specific layers of the differentiated retina. In addition, it colocalizes with components of the adherens and tight junctions. This should not be surprising as proteins of tight, adherens and gap junctions have been shown to colocalize during epithelial formation and movement. During the stratification and differentiation of retinal and corneal epithelia, Bves localization varies in the specific epithelial strata. In the case of the lens, Bves expression is retained at the apical portion of the anterior epithelium, possibly participating in maintaining this structure as an epithelium but permitting lateral cellular movement that is necessary for replenishing primary fibers. The expression of Bves appears to be lost after the lens epithelial cells differentiate into lens fibers. This is seen in the sharp demarcation of Bves expression at the lens bow region. The redistribution of Bves in the adult retina, lens, and cornea may also suggest a related but second function for this protein in the differentiated eye.

Bves is expressed in the surface ectoderm before corneal induction. As the corneal placode forms, Bves is expressed in these cells but is not restricted to them and is seen in the adjacent, noncorneal ectoderm. Still, as seen in Figure 5, Bves protein quickly became restricted to the cornea during its stratification as expression was lost at the corneal–scleral junction. Cornea-specific expression of other gene products, such as keratin 12, has been observed during the stratification stage of development.

Our current in vitro studies on the formation and regeneration of corneal epithelium suggest a role for Bves in wound healing. In models of epithelial wounding, cellular migration plays a key role in resurfacing the denuded epithelium. Cellular movement into a wound gap is accomplished by two methods: lamellipodial extension of cells into wound and actin-filament purse string closure. Both methods of epithelial wound closure have been described in the cell culture monolayer and animal models of wounding. A key difference in these two modalities of wound closure is the degree of polarity retained by the migrating cells. In actin-filament wound closure, the epithelium migrates as a sheet—pulled by an actin filament, which runs from cell to cell at the wound’s edge. These cells retain their apical and basolateral orientation. In contrast, cells that migrate by lamellipodial extension lose their polarity and cell–cell contract. In our wound cell culture, we also observed two distinct morphologies exhibited by cells filling the wounded region. In the first morphology, cells delaminated in a disorganized fashion from the wound edge; in the second, the cells appeared to extend into the wound gap in smooth curved fingerlike projections retaining expression of Bves and other junction proteins. We suspect that these two healing morphologies represent healing by extension of lamellipodia and by actin-filament closure, respectively. As we disrupted Bves expression with morpholino treatment, we noted an associated increase in the percentage of wound area covered. One interpretation of these results is that impairment of Bves leads to disruption of cell–cell adhesion or interaction at the wound surface, resulting in an early release of cells into the wound. A second interpretation is that disruption of Bves results in a disruption of actin-filament formation at the wound’s edge with a subsequent increase in the number of cells extending into the wound by lamellipodial extension. Given that treatment with E-cadherins blocking antibody leads to disruption of actin filament formation and that Bves expression precedes other adhesions molecule, it is possible that the disruption Bves expression could lead to a disruption actin-filament formation.

The present data must be viewed in light of recent studies on the popeye gene family as a whole must be conducted to delineate the actions of these proteins in development and repair.

Acknowledgments

The authors thank Bettina Wilm for helpful comments on the manuscript and Megan Osler for assistance in the morpholino antisense oligonucleotide experiments.

References


