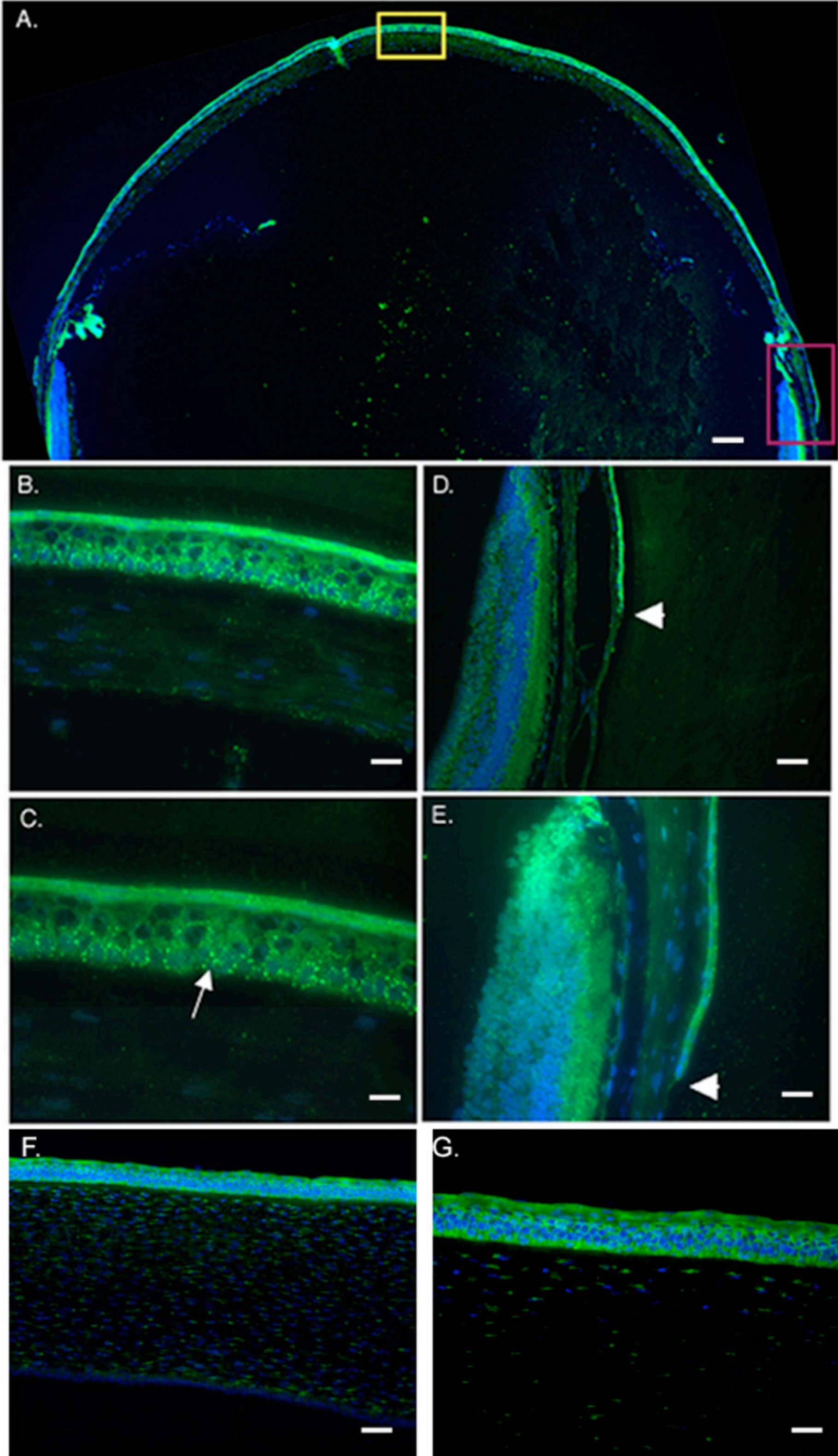
Figure 36. Bves localization in corneal epithelium. (A) Immunohistochemical analysis of Bves expression (green) in the anterior half of the 6 week mouse eye (scale bar = 100 um). The lens was lost in this preparation. (B, C) Higher magnification (scale bars = 20 and 10 um) of the corneal epithelium indicated by the yellow box in (A). The white arrow in C indicates the punctate staining of Bves in the basal and wing cell layers of the corneal epithelium. (D, E) Higher magnification (scale bars = 40 and 10 um) of the corneal edge indicated by the pink box in (A). Bves is expressed in the corneal epithelium but not the sclera. (F, G) Sections are through a chick cornea at hatching. Bves expression in this corneal epithelium is similar to that seen in the mouse. (F**,** scale bar = 50 um; G**,** scale bar = 20 um**)** DAPI (blue) was used to stain nuclei.


A.
B.
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Corneal epithelial sheet formation and regeneration

Human corneal epithelial lines (HCE) have been used as models to study epithelial growth and wound healing *in vitro* (Lambiase et al., 2000; Pancholi et al., 1998). As 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 (Wada et al., 2001), we sought to determine the localization of Bves in corneal epithelial formation and regeneration.

After initial plating of HCE cells, Bves is not observed at high levels at the surface of non-confluent cells but is instead seen in the cytoplasm (Figure 37A). Cytoplasmic localization of Bves is a common property of this protein in single cells and in forming and nascent epithelia (Andree et al., 2000; Reese et al., 1999; Wada et al., 2001; Wada et al., 2003). This cytoplasmic staining is clearly above background when compared to peptide blocking (Figure 37E) or no primary antibody (data not shown) controls. As epithelial cells make contact, Bves staining is observed at points of cell contact as epithelial sheets begin to form (Figure 37A-C). Bves staining is seen along the surfaces of cells where cell-cell contact is established and is absent from free surfaces. Later, Bves is present around the entirety of cells in confluent epithelial sheets (Figure 37D). It is important to note that Bves staining is also seen intracellularly in confluent sheets. Both membrane and intracellular staining are eliminated with peptide competition (compare Figure 37D and 37E).

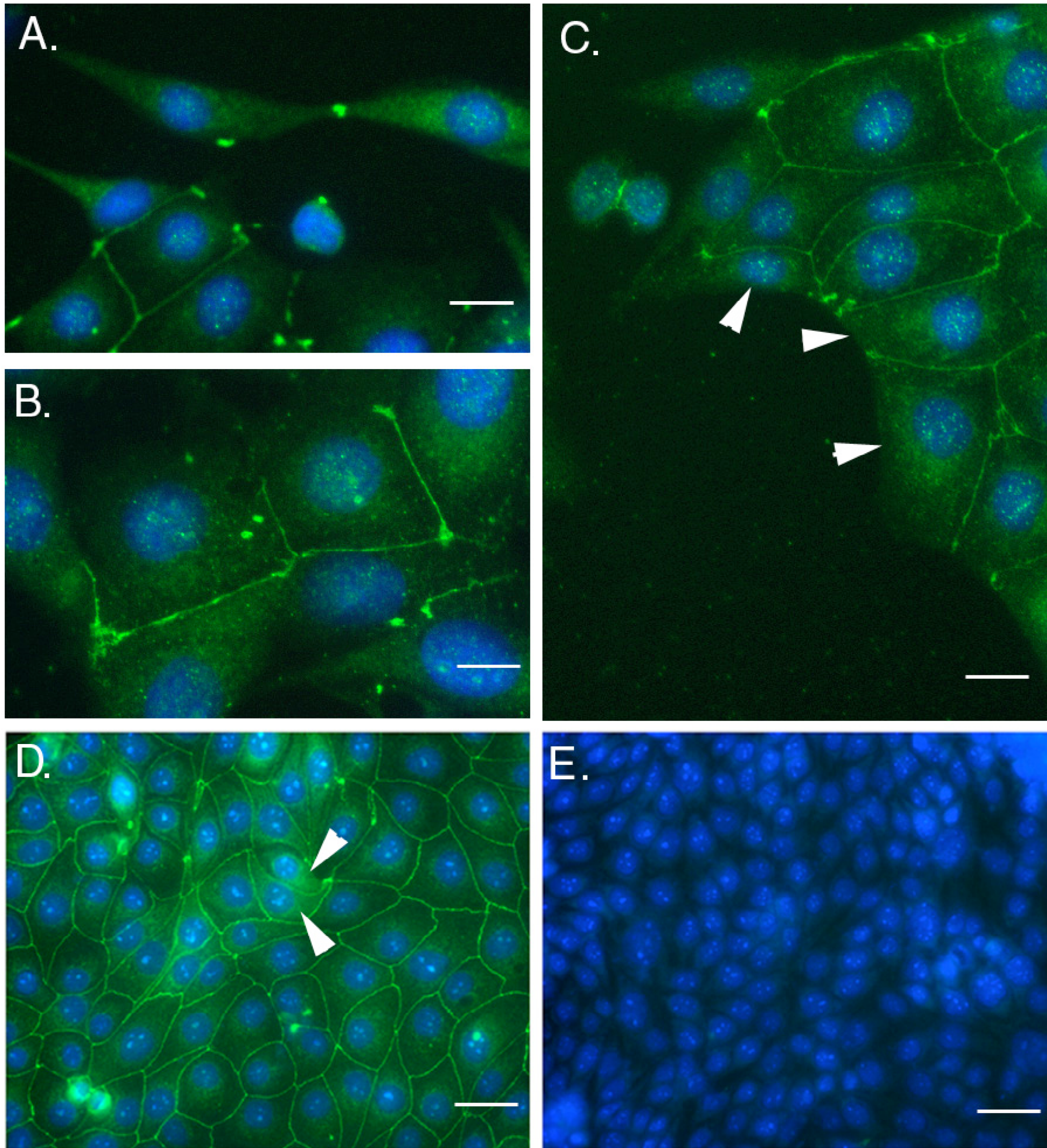

Figure 37. Bves is expressed at the cell surface of human corneal epithelial (HCE) cells as they make contact during epithelial sheet formation. (A) Immunohistochemical analysis of Bves expression in HCE cells at low density (scale bar = 10 um) shows limited peripheral staining. (B) Bves staining is seen along regions where cells make contact (scale bar = 5 um). (C) Bves staining is prominent at the surface of cells within nascent epithelial sheets but not along the free edge of these sheets (arrowheads, scale bar = 10 um). (D) At confluence (scale bar = 15 um), Bves is seen around the entirety of epithelial cells. Note that intracellular staining (arrowheads) is observed in some sheet cells. (E) Membrane and intracellular staining are lost after peptide competition (scale bar = 15 um). DAPI (blue) was used to stain nuclei.

Corneal wound healing is a dynamic process characterized by modulation of cell adhesion, motility and division. 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 using standard scratch or press protocols. As predicted, Bves staining is not observed on the free surface of surviving cells at the wound edge but remains at the surface of uninjured cells (Figure 38A). Next, cells at the wound surface, either individually or in clusters, move into the free space and Bves staining is lost on the surface of these cells (Figure 38B). With time, the gap between wound surfaces is filled with migrating cells that lack peripheral Bves staining (Figure 38C). As cells fill the wound area, Bves staining is again seen around each corneal cell.

In an initial effort to elucidate the possible role of Bves in maintaining epithelial integrity in the cornea and its ability to regenerate, we employed a morpholino knockdown strategy on cultured corneal cells. Cultures were plated at 2 x $10^4/cm^2$ seeding density and treated with *Bves* anti-sense 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* anti-sense morpholino-treated cultures retained numerous gaps in the epithelial sheet (Figure 39A). 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 (Figure 39A). In these regions, Bves staining was either absent or diminished. In contrast, control cultures exhibited the standard peripheral pattern of Bves staining (Figure 39B-C).

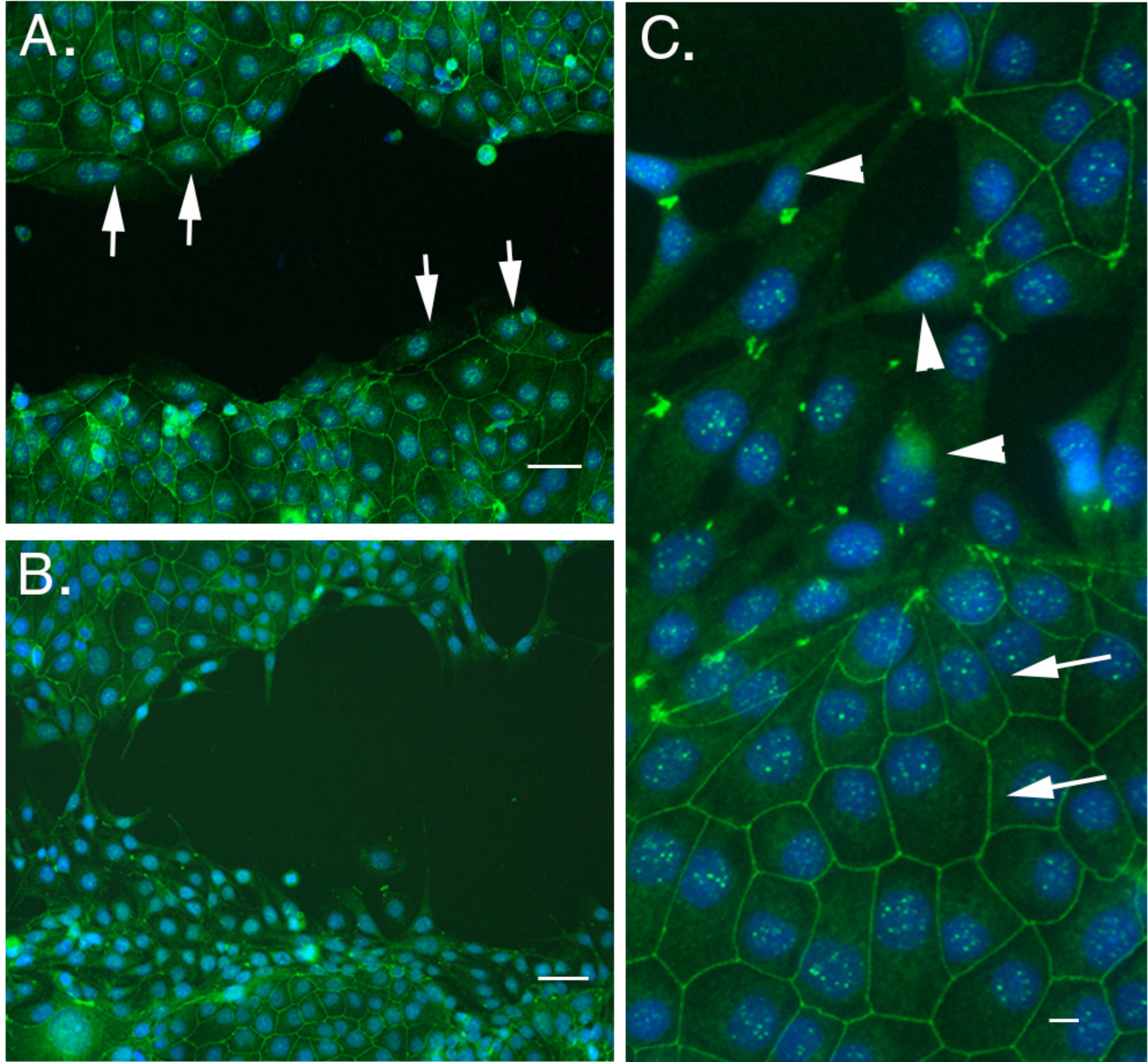

Figure 38. Dynamic expression of Bves in wound healing of HCE cells. (A-C) A confluent sheet of HCE cells was scratched and Bves expression (green) was analyzed at selected times. (A) Initially, cells at the surface lose Bves staining on the free surface (arrows, scale bar = 20 um). (B) Next, cells without prominent Bves staining move into the wound area (1 day post-wound, scale bar = 20 um). (C) At higher power, the difference in the location of Bves staining in adjacent intact epithelium (arrows) and wound area cells (arrowheads) can be seen (2 day post-wound, scale bar = 5 um).

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 movement or growth of cells into the wound area (Figure 40). During the first day of wound repair, it was apparent that cells in cultures with *Bves* anti-sense morpholino treatment moved into the wound area more rapidly than those of control morpholino and untreated 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* anti-sense morpholino filled 64% (SE $\pm$ 5%) of the defect, while the control morpholino treatment and no treatment covered 54% (SE $\pm$ 9%) and 55% (SE $\pm$ 8%) of the wound respectively (Table 1). Statistical analysis demonstrated a significant difference ($p < 0.05$) with the anti-Bves morpholino as compared to both control morpholino and no treatment groups. This difference remained statistically significant over the three day period. As predicted, no significant difference was observed between the control morpholino and untreated groups. Still, after this initial acceleration in wound healing, regenerated epithelial sheets in the experimental group were not as highly organized as those in controls with significant gaps in the regenerated epithelium.

Discussion

An essential element in the development of the eye is the movement of epithelia. Regulation of cell/cell adhesion is critical for proper orientation and modeling of epithelia during numerous embryonic events. Clearly, it is necessary to identify and characterize new players in