The Corneal Epithelial Barrier and Its Developmental Role in Isolating Corneal Epithelial and Conjunctival Cells From One Another

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PURPOSE. During development, the corneal epithelium (CE) and the conjunctiva are derived from the surface ectoderm. Here we have examined how, during development, the cells of these two issues become isolated from each other.

METHODS. Epithelia from the anterior eyes of chicken embryos were labeled with the fluorescent, lipophilic dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). DiI was placed on the epithelial surface of the developing anterior eye and its diffusion was monitored by fluorescence microscopy. Concomitant morphologic changes in the surface cells of these epithelial were examined by scanning electron microscopy. Immunofluorescence was used to analyze the expression of cytokeratin K3, ZO-1, N-cadherin and Connexin-43 and the function of gap junctions was analyzed using a cut-loading with the fluorescent dye rhodamine-dextran.

RESULTS. Prior to embryonic day 8 (E8), DiI placed on the surface of the CE spreads throughout all the epithelial cells of the anterior eye. When older eyes were similarly labeled, dye diffusion was restricted to the CE. Similarly, diffusion of DiI placed on the conjunctival surface after E8 was restricted to the conjunctiva. Scanning electron microscopy showed that developmentally (1) physical separations progressively form between the cells of the CE and those of the conjunctiva, and (2) by E8 these separations form a ring that completely encompasses the cornea. The functional restriction of gap junctions between these tissues did not occur until E14.

CONCLUSIONS. During ocular development, a barrier to the diffusion of DiI forms between the contiguous CE and conjunctiva prior to the differential expression of gap junctions within these tissues.

Keywords: anterior eye, development, epithelium

Development of the vertebrate eye involves the determination and differentiation of a number of different epithelial tissues, including those of the cornea and those of the surrounding limbus and conjunctiva. It is thought that the development of each of these tissues involves a series of sequential interactions. For example, formation of the corneal epithelium (CE) results from an induction of surface ectoderm by the underlying lens,[1] which itself is formed through a previous interaction between the surface ectoderm and the underlying neuro-ectodermal–derived optic vesicle.[2] In addition, many structures within the anterior eye, including the corneal stroma (CS) and the CE, form through successive waves of neural crest cell migration. The fate(s) of these neural crest cells themselves are thought to be dependent on lens-derived molecular cues.[1,3]

A number of previous studies[4–6] have identified some of the molecular factors and signaling pathways involved in these processes, such as fibroblast growth factor (FGF) and Wnt/β-catenin. However, little is known about how these cues, in particular, secreted factors, remain spatially isolated from adjacent developing tissues and structures. If specific cues are not restricted to their target tissue, they could interfere with the development and differentiation of adjacent tissues. Studies on a variety of developing systems in a number of species, ranging from *Drosophila* to mice, have suggested the involvement of mechanisms ranging from gradients of diffusible signaling molecules[7] to ionic coupling.[8] However, in most studies involving the differentiation of multiple tissues, including those dealing with development of the anterior eye, spatial restriction of the signaling processes involved is not addressed.

One mechanism that has been proposed is the differential expression of Connexin-43 (CX43) in the anterior eye. During early mammalian development, CX43, a component of gap junctions that can allow the transfer of cytosolic components from cell to cell, is broadly expressed throughout the undifferentiated ocular surface. As development progresses and the cornea and conjunctiva begin to differentiate, CX43 is differentially expressed within these tissues. Although CX43 remains in the conjunctiva and is strongly expressed in the CE, it is absent at the border between them at the limbus. This mechanism is thought to isolate the cells that reside there from signals in the surrounding tissues and to play a role in their maintenance in an undifferentiated state. Although the developmental restriction of CX43 within the ocular surface epithelia has been described in the rat,[9] neither its expression

nor its role in isolating the cornea has been analyzed in the embryonic chicken.

In a previous study on the developmental innervation of the embryonic chicken cornea to visualize nerves, we used labeling with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), which is a fluorescent, lipophilic molecule that intercalates into the cell membrane of cells it contacts.[10] Also, if a DiI-labeled cell is in contact with one that is unlabeled, the dye diffuses from the labeled cell to the unlabeled cell, rendering it also labeled. During these studies, we observed that, while DiI labeling of the CE cells occurred, the cellular labeling remained restricted to the CE, with none of the label translocating into the surrounding conjunctiva, suggesting that its movement was somehow inhibited.

In the present study, we advanced this observation on the restriction of DiI diffusion and shown that (1) a developmentally regulated barrier to the diffusion of DiI forms in chicken at embryonic day 8, and (2) the formation of the barrier precedes the functional, differential expression of CX43 by 6 days. Also, using scanning electron microscopy (SEM), we linked this inhibition of diffusion with changes in cell morphology that suggest a mechanism involving the physical isolation of the CE, forming what we term the CE barrier.

METHODS

Eggs

Chicken eggs (White Leghorn) were obtained from Hyline (Elizabethtown, PA, USA) and incubated at 38°C. Embryos were removed, rinsed in Hank's balanced saline solution (HBSS), and staged both by chronological time of incubation and by the criteria of Hamburger and Hamilton.[11] All animal work was approved by the Tufts University Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

DiI Labeling of Anterior Eyes

Briefly, embryonic chicken embryos were removed from eggs, staged, and killed by decapitation. The heads were placed in HBSS (ThermoFisher, Waltham, MA, USA), and a small crystal of DiI (Molecular Probes, Eugene, OR, USA) was placed on the center of the corneal surface of the left eye using a fine tungsten needle without damaging the epithelium. The right eye served as an unlabeled control. The head was then placed in freshly made paraformaldehyde (4% in 0.1 M PBS, pH 7.4) and kept at 37°C in the dark for various times up to 2 months, depending on the experiment (see Results). Eyes were photographed under a fluorescent dissecting stereomicroscope (SMZ 1500; Nikon Instruments, Melville, NY, USA) equipped with a real-time charge-coupled device camera (SPOT Flex; Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Eyes were also frozen in optimal cutting temperature compound (TissueTek; Sakura Finetek USA, Inc., Torrance, CA, USA) and serial sectioned at 10 μm using a cryostat. The sections were then labeled with Hoechst dye to stain the nucleus, coverslipped, and visualized under a microscope (N400; Nikon Instruments).

Scanning Electron Microscopy

For analyses by SEM, anterior eyes were removed and fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were then post-fixed in 1% osmium tetroxide, washed, dehydrated in ethanol, and critically point dried using an Edwards Auto 306 Vacuum Evaporator (Edwards Vacuum, Crawley, West Sussex, UK). The samples were sputter coated with palladium–gold and imaged using an ISI DS130 scanning electron microscope (International Scientific Instruments, Inc., Milpitas, CA, USA).

Immunohistochemistry

For immunohistochemistry (IHC) of tissue sections, anterior eyes were dissected in HBSS, embedded fresh in OCT, and stored frozen at −80°C. For frozen sectioning, 10-μm serial sections were cut using a cryostat. Sections were mounted on poly-L-lysine–coated slides and were then air dried (2 hours at room temperature) and stored (at −20°C). For fluorescence IHC, the sections were fixed in acetone, rinsed (three times in PBS), and then incubated in blocking solution (10% heat-inactivated sheep serum in PBS with 0.1% Triton X-100) for 1 hour at room temperature. For labeling, CX43 and CK3 (AE5) antibody (Abcam, Cambridge, MA, USA) were used at a dilution of 1:500 in 1% block solution diluted in PBS; ZO-1 antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) was used at 1:400 in 1% block solution; N-cadherin antibody (Cell Signaling, Danvers, MA, USA) was used at 1:100 in 1% block solution; and type V collagen monoclonal antibody was used undiluted.[12] Control corneas were incubated either in blocking solution without primary antibody or in the AC-9 antibody to type X collagen.[13] After incubation in primary antibody (overnight at 4°C), slides were washed (three times for 5 minutes each in PBS) and then incubated (for 1 hour at room temperature in the dark) in anti-rabbit (for CX43 and ZO-1) or anti-mouse (for CK3, N-cadherin, and type V collagen) secondary antibody conjugated to Alexa-fluor (ThermoFisher; diluted 1:500 in PBS). Slides were washed three times in PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and coverslipped.

Cut Loading

Analyses of functional gap junctions in the embryonic chicken cornea were performed using a modified procedure based on previous studies investigating gap junctions in the rabbit.[14] Briefly, chicken embryos from E6 to E14 were killed, and a nonpenetrating incision of approximately 1 mm in length was made using a #10 scalpel blade within the cornea parallel to the corneal conjunctival border. The eye was then irrigated with a solution of HBSS containing 0.5 mg/mL dextran-rhodamine (Invitrogen, Carlsbad, CA, USA) during cutting and subsequently for 5 minutes. The control group was incised and irrigated with HBSS alone. Two groups were irrigated with Rhodamine-dextran 3000 or rhodamine-dextran 70000 ($n = 5$ for all groups). The eye was rinsed twice with HBSS and then embedded in OCT for cryostat sectioning (as described above).

RESULTS

Detection of the CE Barrier by DiI Labeling

In a previous study involving the developmental regulation of corneal innervation,[10] corneal nerves were labeled with DiI to retrograde label the axons and trace the nerves back to their origin in the trigeminal ganglion. For the previous studies and those presented here, the DiI labeling was achieved by placing crystals of DiI on the surface of fixed corneas (Fig. 1A). In these corneas, we observed that in addition to labeling the nerves (Fig. 1B, arrowheads), the DiI also spread throughout the cells of the CE (Fig. 1B), suggesting that the cells of the CE are in close contact with one another. This is consistent with previous reports demonstrating the presence of cell junction components within the CE, including tight junctions at the surface.[15,16]
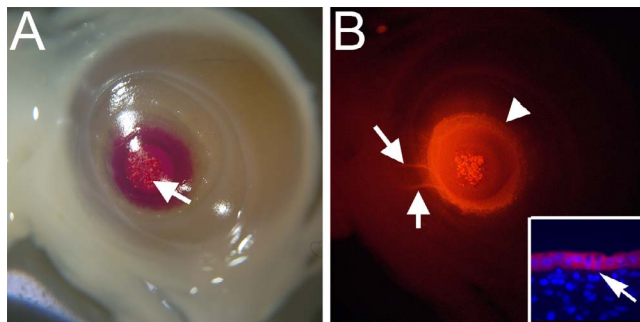
FIGURE 1. DiI labeling in embryonic chicken cornea. Brightfield (**A**) and immunofluorescent (**B**) images of the same DiI-labeled E10 cornea. *Arrow* in **A** shows crystals on CE surface. *Arrowhead* in **B** indicates sharp border of DiI diffusion (*red*); *arrows* in **B** show DiI-labeled nerves. *Inset* in **B** shows a section of DiI (*red*)-labeled cornea with nuclei stained with DAPI (*blue*); *arrow* in *inset* indicates Bowman's layer separating CE (above) from stroma (below).

When DiI-labeled corneas were sectioned and viewed by fluorescence microscopy, the labeling of the corneal cells was observed to be restricted to those of the CE, with none being detected in the cells of the corneal stroma (inset in Fig. 1B). This absence of transfer of the DiI from the CE to the CS is most likely due to Bowman's layer, which separates these two areas of the cornea (inset in Fig. 1B, arrow). As Bowman's layer is a cell-free extracellular matrix, this lack of transfer of the DiI is consistent with cell–cell contact being necessary for transfer of the dye to occur. Also, this observation eliminates any possibility of the observed results being obscured by spurious, non–cell contact-mediated diffusion of the dye.

Of importance for the present study, we also observed that lateral diffusion of the DiI label ended abruptly where the corneal epithelium transitioned into the conjunctiva (Fig. 1B, arrowhead). One possibility for this loss of the fluorescent signal at the CE border was that it reflects a decreasing gradient of diffusion of the dye, from its origin at the crystal of DiI to its termination where the fluorescent signal is no longer detectable. The other possibility is that a barrier to diffusion exists at the border between the CE and the conjunctiva.

To test these possibilities, experiments were done in which diffusion was examined when smaller and larger crystals were placed at the center of the CE on eyes from the same stage embryos. When small crystals of DiI were placed at the center of the CE, the fluorescent signal produced gradually decreased, as would be expected for a gradient, but it terminated before reaching the CE border as shown in sectioned corneas (Fig. 2A, note the consistent thickness of the CE and smooth Bowman's layer). However, when large crystals of DiI were used for labeling (i.e., size routinely used in the labeling experiments or larger), the signal produced was consistently strong and rather than show a gradual decrease, as would be predicted for a gradient, and it always terminated abruptly at the CE border (Fig. 2B). For these studies, we defined the CE border as the point at which the smooth, regular appearance of Bowman's
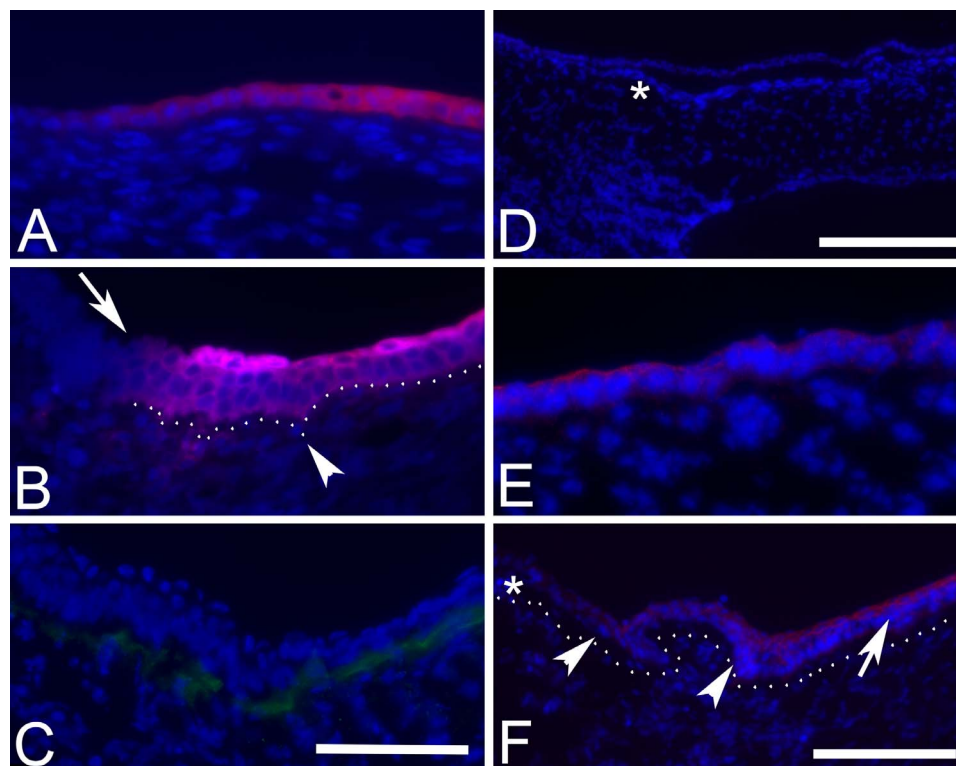

FIGURE 2. Analysis of DiI diffusion in chick cornea. (**A**) Section of DiI (*red*)-labeled E9 cornea in which one small crystal placed on the CE surface shows gradual diffusion of red fluorescence within CE. (**B**) DiI diffusion from a large crystal of DiI in section of E13 cornea at the CE–conjunctival border shows an abrupt loss of red fluorescence (*arrow*) just peripheral to the end of Bowman's layer (*arrowhead*). *Dashed line* indicates Bowman's layer separating the epithelium from the stroma below. Section in **C** of E13 CE–conjunctival border immunolabeled for type V collagen (*green*) shows termination of Bowman's layer as the *green label* is lost. (**D**) Low-magnification image corneal–conjunctival border marked in the underlying stroma by *-labeled cytokeratin K3 (*red*) shows absence of label in the ocular surface at E8. **E** shows E10 cornea labeled for K3 (*red*) with heterogeneous staining in the apical layers of the corneal epithelium. At E17 (**F**), K3 (*red*) marks the corneal epithelium (*arrow*); at the corneal–conjunctival junction, label is lost in the basal cell layer (*arrowheads*) and completely lost more peripherally (*). Nuclei stained with DAPI (*blue*) in all images. *Scale bar* in **C** denotes 50 μm and is the same for **A**–**C** and **E**. *Scale bar* in **D** denotes 200 μm; *scale bar* in **F** denotes 100 μm.
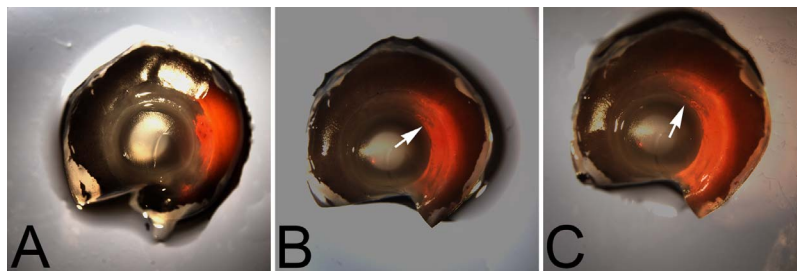
FIGURE 3. DiI diffusion in the conjunctiva. DiI crystals placed on the conjunctival surface of an E10 anterior eye and imaged after 1 (**A**), 2 (**B**), and 4 weeks (**C**). Images are brightfield with overlayed red fluorescence to show DiI. *Arrows* in **B** and **C** show DiI diffusing along the CE–conjunctival border but into CE.

layer transitions into the curved, undulating region basal to epithelium (i.e., forming the palisades of Vogt). The palisades of Vogt have been used by many other studies for determining the location of the junction between the corneal and conjunctival epithelium.[17,18] In sections of DiI-labeled corneas, we observed that the fluorescent label terminated within this transitional area. As shown in Figure 2B, the DiI label terminates within the epithelial layer (arrow), just lateral to the end of Bowman's layer (arrowhead and dashed line). We further defined Bowman's layer using an antibody against type V collagen that specifically labels Bowman's layer[19] within the cornea (Fig. 2C). Initially we attempted to use this antibody on DiI-labeled tissues, but the fixation used during the DiI labeling rendered the epitope inaccessible. However, the comparison of sections from anterior eyes of the same age labeled for type V collagen (green labeling in Fig. 2C) to sections of DiI-labeled corneas (Fig. 2B) confirmed the location of the CE barrier.

Although the palisades of Vogt are one feature used here and in other studies to determine the location of the corneal–conjunctival transition, we also sought further confirmation of this location using differentiation markers to further define the transition between these tissues. The cytokeratin pair K3/K12 has been used in many studies as a specific marker of corneal epithelial differentiation.[20,21] Here, we used a monoclonal antibody against K3 to differentiate where the border of the cornea was in relationship to the barrier to DiI diffusion. Consistent with the original description by Schermer et al.,[20] and as shown here in Figures 2D through 2F, K3 initially appears within the ocular surface at embryonic day 10. At embryonic day 8 (Fig. 2D), there is no immune reactivity anywhere in the ocular surface of this lower magnification image (asterisk in Fig. 2D is in the stroma underlying the epithelium at the junction between cornea and sclera). At E10, K3 begins to be expressed in the central corneal epithelium and is strongest in the apical cells (Fig. 2E). At this time, labeling is heterogeneous within the corneal epithelium, with areas showing varying levels of staining. As developmental differentiation of the cornea progresses though later stages (as shown in Fig. 2F, which is an E17 cornea at 4 days prehatching), K3 labeling becomes more consistent through all the cell layers of the corneal epithelium, from the basal cells through the wing cells and apical cells (arrow in Fig. 2F). Although K3 labeling is strong in the corneal epithelium, at the corneal–conjunctival transition, K3 expression is lost, particularly in the basal cells (arrowheads in Fig. 2F). More peripherally (asterisk in Fig. 2F), K3 labeling is completely lost. This is consistent with the previous descriptions of mammalian developmental expression of the corneal-specific cytokeratin pair K3/K12. Importantly for the studies presented here, this transition from the cornea to conjunctiva as indicated by K3 occurs at the point where Bowman's layer transitions from smooth to undulating, that is, at the palisades of Vogt (as indicated by the dashed line drawn along Bowman's

layer in Fig. 2F). These data confirm the location of the corneal-conjunctival transition.

These results, when taken together, show that DiI can diffuse among the cells of the CE, but also suggest that a barrier is present that restricts diffusion into the surrounding conjunctiva at the corneal–conjunctival border. We refer to this as the CE barrier.

Inhibition of Bi-Directional Diffusion by the CE Barrier

We next examined whether the barrier is unidirectional (i.e., preventing diffusion only from the CE into the conjunctiva) or whether it is bi-directional (i.e., also preventing diffusion from the conjunctiva into the CE). To determine this, crystals of DiI were placed on the surface of the bulbar conjunctiva, and then diffusion of the dye was followed.

As shown in Figure 3A, after the DiI was allowed to diffuse for 1 week, the fluorescent label spread through the conjunctiva and approached the corneal border. However, after 2 (Fig. 3B) and 4 weeks (Fig. 3C), the DiI, rather than entering the cornea, instead continued to spread through the conjunctiva, forming a semicircular ring following the border of the cornea (arrows in Fig. 3C). This blocking of diffusion from the conjunctive into the cornea, when coupled with the blocking from the CE into the conjunctiva as described above, shows the barrier to be bi-directional.

Temporal Formation of the CE Barrier

One possibility for the CE barrier is that it is involved in corneal development. If so, formation of the barrier should itself be developmentally regulated. To determine this, we examined the diffusion of DiI at different stages of development. At E6, the youngest stage examined, diffusion from DiI crystals placed on the epithelial surface at the center of the eye (arrows in Fig. 4A) did not remain restricted to the cornea. Instead, as shown in Figure 4B, dye became dispersed throughout the surface of the anterior eye. This result was confirmed in sections of anterior eyes (Fig. 4C) that show the DiI (red) spreading throughout the CE (arrows), and into the presumptive bulbar conjunctiva, well past the point overlying the developing ciliary body (arrowheads).

When this experiment was performed using a somewhat older embryo at E7 (Fig. 4D) in most of the eyes examined (12 of 20), the DiI still diffused throughout the surface epithelium, including into the bulbar conjunctiva (arrows in Fig. 4D). However, in some eyes, the labeling was now restricted to the CE (8 of 20 embryos). By E8 (Figs. 4E, 4F), the restriction of label to the CE was clear cut, occurring in all embryos examined ($n = 20$). Therefore, formation of the CE barrier is developmentally regulated, and temporally this occurs between E7 and E8.
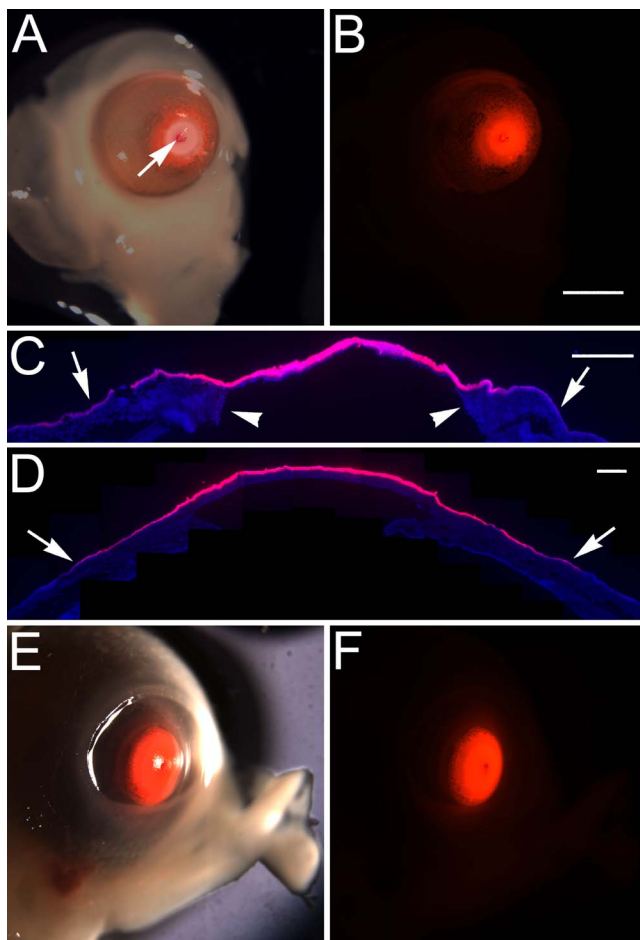
FIGURE 4. Temporal analysis of DiI diffusion. DiI (*red*) placed on the corneal surface in whole mounts of the head of the embryo was imaged by brightfield with red fluorescent overlay (**A**) and fluorescent channel alone (**B**) to show DiI diffusion throughout anterior eye at E6. In **C**, 20× images and a section E6 DiI-labeled cornea were stitched together to show DiI diffusion from the cornea into the conjunctiva (*arrows*), past the developing ciliary body (*arrowheads*). Similarly, merged images at E7 (**D**) show DiI still able to diffuse into conjunctiva (*arrows*). **E** and **F** are DiI-labeled E8 whole mounts imaged similarly to **A** and **B** but show DiI-restricted to the CE. *Scale bar* in **B** (also for **A**, **E**, and **F**) denotes 500 μm; *scale bars* in **C** and **D** denote 200 μm.

Scanning Electron Microscopy Analysis of the CE Barrier

To further examine the development of the CE barrier, we used SEM, which allowed examination of large areas of the ocular surface at high resolution. At the earliest stage (E6) examined by SEM, morphologically the surface of the eye is an uninterrupted sheet of cells, as can be seen in the micrograph shown in Figure 5A. Also, this uniform morphology is consistent with the DiI observations at this stage, which showed diffusion throughout the entire epithelial surface the anterior eye (as described above). However, a day later (at E7), the epithelial surface has become interrupted by fenestrations located along the periphery of the cornea (arrows in Fig. 5B). Also, some of these fenestrations have become elongated and have begun to contact one another (arrowhead in inset, higher power).

Subsequently, these fenestrations become more abundant and continue to elongate laterally, and by 1 day later at E8 (Fig. 5C) they have fused together and formed a continuous ring of separated cells that circumscribe the cornea. The completion of this continuous ring of separated cells dovetails with the stage (E8) when the barrier to DiI diffusion is established between the cornea and conjunctiva, suggesting that cellular separation is involved in the formation of, and possibly the mechanism responsible for, the CE barrier.

To investigate the cause of the separations observed in the SEM studies, immunohistochemistry was performed with the ZO-1 antibody (Figs. 5D, 5E), as the separations suggested changes in cell adhesions, and the expression of tight junctions has previously been shown in the ocular surface epithelia.[15] However, as shown in Figure 5E, red, punctate labeling indicative of tight junctions was observed at the apical borders of cells throughout the ocular surface, in both the presumptive corneal (arrow) and conjunctival epithelia (arrowhead), as well as the region in between them (asterisk in Figs. 5D, 5E). As this was observed at E8, the time point in which the barrier is established, it suggests that tight junctions may not be involve in formation of the barrier. However, to fully determine this, other junction components need to be tested (see Discussion).

Connexin-43 Expression in the Embryonic Chicken Anterior Eye

At present, we do not know what molecular components are involved in the formation of the CE barrier. However, studies by others have suggested that the differential expression of the gap-junction protein CX43 is involved in defining zones of differentiation in the ocular epithelium of developing rat and rabbit embryos.[9] Furthermore, because cell–cell communication can occur through gap junctions, this differential expression of CX43 may also limit the diffusion of molecules and thereby affect the development of the ocular epithelia. Therefore, we examined whether changes in the expression of CX43 might be involved in formation of the CE barrier in the chicken embryo.

To determine the expression of CX43 in the anterior eye of the chicken during embryonic development, we used immunohistochemistry to label sections of anterior eyes at daily intervals from embryonic day 4 through embryonic day 15. As shown in Figure 6A, we observed the same pattern of expression that had been described previously in other species. CX43 was initially expressed broadly as punctate labeling (shown in red in Fig. 6A at E8), throughout the ocular epithelia, in both the presumptive conjunctival (arrow) and corneal epithelium (arrowhead). Then as development progressed, the pattern of CX43 labeling changed, such that CX43 was strongest within the corneal epithelium (arrowhead in Fig. 6B) and lost in the epithelium just peripheral to the border of the conjunctiva and cornea (arrows in Fig. 6B). Again, the border between the corneal and conjunctival epithelia was identified by the change in Bowman's layer (marked with an asterisk in Fig. 6B), as described above. Spatially, this abuts the location of the corneal barrier to DiI diffusion. Temporally, however, this restriction of CX43 expression was not observed until E14, 6 days after the DiI corneal barrier formed. We further evaluated the developmental differentiation of this region using an antibody to N-cadherin, which has been used by others as a maker for corneal limbal stem cells.[17,22] As shown in Figure 6B, a pocket of N-cadherin–positive cells (green-labeled cells) develops within the region abutting, and just peripheral to the junction in the CX43-negative region. This spatial correlation is consistent with the hypothesis that CX43 is involved with development of the limbal stem cell niche. However, because CX43 loss occurs at a later developmental time point in this region, CX43 is likely not involved in the formation of the DiI CE barrier.
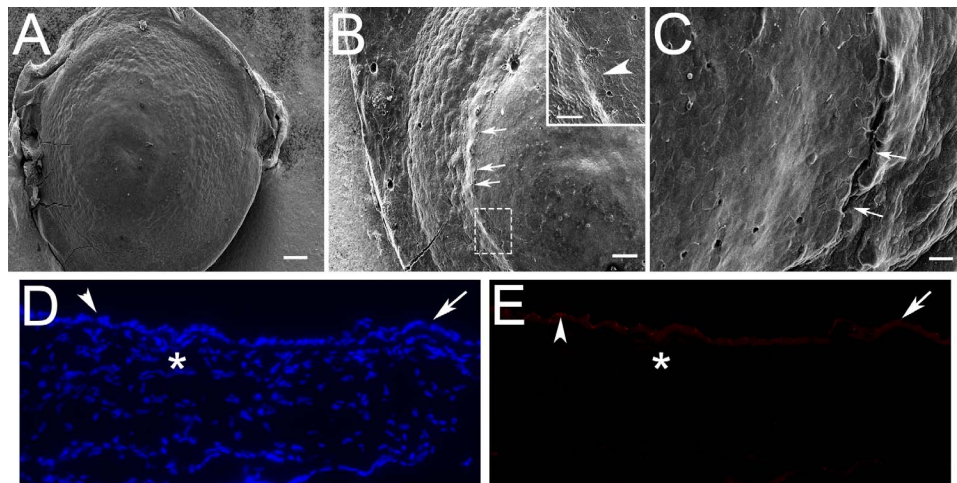
FIGURE 5. Scanning electron microscopy of anterior eye surface from E6 through E8 and ZO-1 expression. Anterior eyes were imaged en face by SEM at E6 (**A**), E7 (**B**), and E8 (**C**). *Inset* in **B** is higher power of region enclosed by *dashed box. Arrows* in **B** show fenestrations at E7 that elongate into connections at E8 (*arrows* in **C**) at the corneal–conjunctival border. **D** and **E** are the same section labeled for nuclei with DAPI (*blue* in **D**) and ZO-1 (*red* in **E**), with an * marking the position of the corneal–conjunctival transition. Note no difference in *red* punctate labeling between apical cells in the conjunctival (*arrowhead*) versus corneal (*arrow*) epithelium. *Scale bars* in **A** denote 213 μm; in **B** denote 111 μm; in *inset* in **B** denote 48 μm; and in **C** denote 23 μm.

We further tested the function of gap junctions during eye development using a modified cut-loading procedure.[14] For this, fluorescent rhodamine-dextran was loaded into the CE, and diffusion through gap junctions was then analyzed in corneal sections. In eyes loaded with rhodamine-dextran with a molecular weight of 70 kDa, which is too large to pass through gap junctions, the fluorescent label did not spread from the cut-loading site (data not shown). Sections of eyes loaded with rhodamine-dextran 3K, which is small enough to diffuse through gap junctions, demonstrated the spread of the dye from cell to cell (Figs. 6C, 6D). At E8, the developmental time that the CE barrier to DiI diffusion was first observed, rhodamine-dextran 3K spread from the CE (arrowhead in Fig. 6C) into the conjunctival cells (arrow in Fig. 6C) at E8, likely

due to the presence of functional gap junctions and consistent with the CX43 immunohistochemistry results described above. It was not until E14, the same stage we observed the loss of CX43 labeling at the corneal–conjunctival border, that rhodamine-dextran 3K was restricted from entering this region. As shown in Figure 6D, the fluorescent label can be seen within cells of the cornea (arrowheads in Fig. 6D), but is absent in cells, particularly in the basal cells (arrows in Fig. 6D) of the corneal–conjunctival border (asterisk in Fig. 6D). Some label did appear to spread through gap junctions in the apical cells in this region. Again, however, these results show that the restriction of cell–cell communication through gap junctions within the anterior eye forms at a stage well after the formation of the DiI CE barrier.
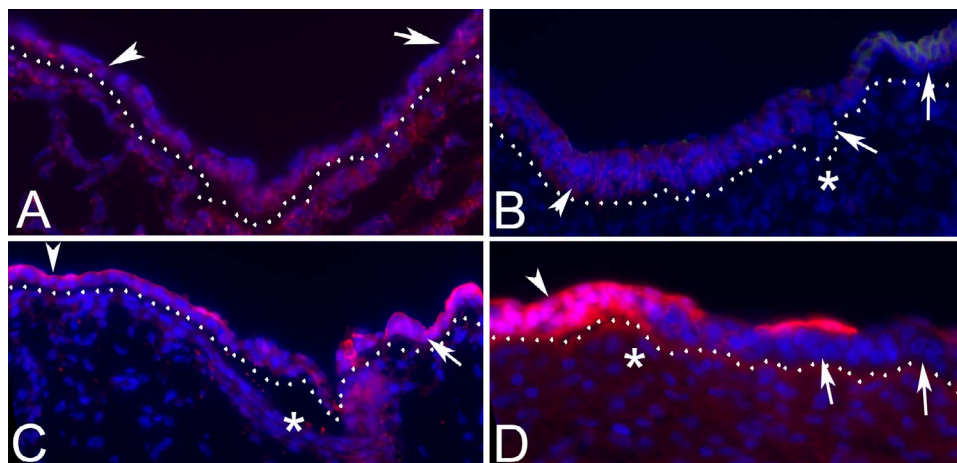

FIGURE 6. Gap junctions in the embryonic chicken anterior eye. Sections of the corneal–conjunctival border at E8 (**A**) and E14 (**B**) were immunolabeled for CX43 (*red*) with nuclei stained with DAPI. *Arrowhead* and *arrow* in **A** show labeling throughout the corneal epithelium and conjunctiva (respectively) at E8, but at E14 (**B**), label is lost in the conjunctiva (*arrows* in **B**) peripheral to the corneal–conjunctival border (*). Note strong punctate *red label* central to border at E14 but absent peripherally. *Green label* in **B** shows cells labeled for the limbal stem cell marker N-cadherin, peripheral to the junction. Cut loading with rhodamine-dextran 3000 at E8 (**C**) and E14 (**D**) show diffusion of the dye (*red*) through gap junctions from the CE (*arrowhead*) to the conjunctiva (*arrowhead*) through the corneal–conjunctival junction (*) at E8 in **C**. At E14 (**D**), rhodamine-dextran 3000 does not diffuse into the basal layers of limbal epithelium (*arrows*) from the CE (*arrowhead*), peripheral to the corneal–conjunctival border (*). *Dashed line* in all image shows the location of the basement membrane separating the epithelium from the underlying stroma.

DISCUSSION

Here, we showed the formation of a barrier to the diffusion DiI within the developing ocular surface. By SEM, this barrier appears to be the result of physical separation between cells at the junction of the CE and the conjunctiva. In the chicken embryo, these separations form between E7 and E8 and restrict the diffusion of DiI between the conjunctiva and the CE. The timing of the formation of this barrier to DiI diffusion correlates with other developmental events in the anterior eye that also occur at E8. For example, both we[23] and others[24] have reported previously that this is the stage when nerves from the surrounding peri-corneal nerve ring enter the cornea. It is also when the corneal and conjunctival epithelia begin to change morphologically, as observed here by SEM, and after this cornea begins to differentiate, as shown by K3 labeling. Although it is known that diffusible factors from the lens (e.g., semaphorin 3A) and the mesenchyme underlying the ectoderm (such as Wnt3A) are involved in these processes,[24,25] the mechanisms that restrict the diffusion of factors such as these remain unknown.

As described above, DiI is a lipophilic dye that is able to spread between cells when their membranes are in close contact. In the case of the cornea, Bowman's layer, which is acellular, prevents the diffusion of DiI into the corneal stroma. Similarly, our results suggest that at the corneal barrier, DiI diffusion is restricted at E8 by the development of separations between the epithelial cells. This barrier could potentially limit the spread of diffusible factors, such as Wnt and Sema3A that are thought to be involved in the differentiation of the anterior eye. The exact mechanisms for how these factors diffuse through the tissue is unclear, but without mechanisms to limit their spread, they could alter the differentiation of an inappropriate target tissue.

One such mechanism that has also been proposed to limit diffusion during eye development is through gap junctions.[9] It has been reported that prior to differentiation of the epithelia of the ocular surface, CX43, a component of gap junctions, is expressed throughout these tissues. However, as development progresses, CX43 becomes restricted to the CE and is down-regulated at the corneal–conjunctival border. Within the CE, it is likely that communication can occur through these gap junctions, as evidenced by spread of the dye Lucifer yellow[14] and rhodamine dextran (Fig. 6D). As we have now shown here in the embryonic chicken, and others have shown in the adult rabbit,[14] one mechanism through which these dyes are prevented from spreading from cells within the CE to those of the limbus may involve the absence of functional gap junctions within the limbus. This mechanism also creates a barrier at the corneal–conjunctival border (i.e., the limbus). However, as we have observed here, in the developing chicken eye the CE barrier forms at E8, whereas the barrier formed by the lack of gap junctions does not form until E14, almost 1 week later in development. Therefore, it is unlikely that gap junctions play a role in the initial formation of the DiI CE barrier. Our immunohistochemical localization of the corneal differentiation marker K3 and the gap junction marker CX43 showed that just peripheral to the corneal–conjunctival junction, as defined by the palisades of VOGT and K3, CX43 expression was lost. These data, together with the spatial and temporal appearance of the stem cell marker N-cadherin within this region, are consistent with the previously described hypothesis that gap junctions regulate cell–cell communication within the limbal stem cell niche wolosin. The DiI barrier abuts this region centrally; however, it is unclear if or how it plays a role in CX43 expression in this region.

It is currently unclear what mechanisms lead to the restriction of CX43 and establishment of the gap junction barrier in the corneal limbus. Peng et al.[26] proposed a mechanism where the differential expression of microRNAs (miRs-103/107) negatively regulates the formation of gap junctions at the limbus. This miR pair also had differential effects on E-cadherin and in regulating aspects of cell–cell adhesion, which have been proposed to help maintain the integrity of the stem cell niche. Our SEM studies are consistent morphologically with these effects, as we observed changes indicative of alterations in cell–cell contacts where the barrier forms at the corneal–conjunctival junction. It is unknown if, or when, this miR pair appears in the developing chicken anterior eye. Therefore, this miR pair could also be involved in the formation of the DiI CE barrier.

Understanding the changes in cell–cell contacts that mediate the DiI CE barrier will be necessary to understand its direct effects on anterior eye development. To our knowledge, no spatial or temporal changes in tight junctions, desmosomes, or adherens junctions have been described by immunohistochemistry,[15,16] and no differences were observed in previous electron microscopy studies of the developing embryologic chick cornea.[27,28] We also observed no differences in the pattern of ZO-1 labeling, a marker for tight junctions, within the ocular surface at the time of its formation. The function of cell junctions can vary in response to their molecular constituents. There are more than 40 potential components of tight junctions, and changes in their expression can alter the permeability through this junction.[29] Most of these cell adhesion structures, and their components, have not been fully investigated during the embryonic development of the anterior eye. Current studies are testing the expression of many of these components at the corneal–conjunctival junction during early ocular development.

The temporal and spatial correlation between the formation of the corneal barrier and these events suggests its involvement in the differentiation of the anterior eye. However, this mechanism may not be limited to the border of the corneal and conjunctival epithelia. This border has been described as an "epithelial transition zone," where two similar, but functionally different, epithelia meet at an abrupt border.[30] Other examples include the z-line of the gastro-esophageal border and the white line of Hilton separating the zona hemorrhagica and zona cutanea within the anal canal.[31–33] Within these tissues, it is thought that diffusible factors are responsible for the differentiation of the abutting epithelia. However, similar to the anterior eye, it is unknown how these factors remain restricted from one another so that two distinct epithelia are formed. Therefore, future studies will be necessary to determine further the function of the CE barrier during eye development, as well as those of potential similar mechanisms within other epithelial tissues.

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