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EphA2/Ephrin-A1 Mediate Corneal Epithelial Cell Compartmentalization via ADAM10 Regulation of EGFR Signaling

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PURPOSE. Progenitor cells of the limbal epithelium reside in a discrete area peripheral to the more differentiated corneal epithelium and maintain tissue homeostasis. What regulates the limbal-corneal epithelial boundary is a major unanswered question. Ephrin-A1 ligand is enriched in the limbal epithelium, whereas EphA2 receptor is concentrated in the corneal epithelium. This reciprocal pattern led us to assess the role of ephrin-A1 and EphA2 in limbal-corneal epithelial boundary organization.

METHODS. EphA2-expressing corneal epithelial cells engineered to express ephrin-A1 were used to study boundary formation in vitro in a manner that mimicked the relative abundance of these juxtamembrane signaling proteins in the limbal and corneal epithelium in vivo. Interaction of these two distinct cell populations following initial seeding into discrete culture compartments was assessed by live cell imaging. Immunofluorescence and immunoblotting was used to evaluate the contribution of downstream growth factor signaling and cell-cell adhesion systems to boundary formation at sites of heterotypic contact between ephrin-A1 and EphA2 expressing cells.

RESULTS. Ephrin-A1-expressing cells impeded and reversed the migration of EphA2-expressing corneal epithelial cells upon heterotypic contact formation leading to coordinated migration of the two cell populations in the direction of an ephrin-A1-expressing leading front. Genetic silencing and pharmacologic inhibitor studies demonstrated that the ability of ephrin-A1 to direct migration of EphA2-expressing cells depended on an a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and epidermal growth factor receptor (EGFR) signaling pathway that limited E-cadherin-mediated adhesion at heterotypic boundaries.

CONCLUSIONS. Ephrin-A1/EphA2 signaling complexes play a key role in limbal-corneal epithelial compartmentalization and the response of these tissues to injury.

Keywords: Ephrin-A1, EphA2, EGFR, boundary, wound healing

The anterior surface of the eye functions as a barrier to the external environment and protects the delicate underlying tissues from injury. Central to this protection are the corneal, limbal, and conjunctival epithelia. The limbal basal epithelium is enriched in stem cells (SCs) that are interspersed among their progeny, the transit amplifying (TA) cells.^{1,2} These two cell types are intimately associated with one another and contribute to homeostasis and repair by giving rise to more differentiated cells of the suprabasal limbal epithelium and the entire corneal epithelium.¹⁻³ A distinct boundary is evident between the limbal and corneal epithelium that has been extensively described by well-defined molecular, biochemical, and morphologic features. These studies have yielded important clues about a variety of factors that help maintain tissue organization and homeostasis in the anterior segmental epithelium of the eye. During wound healing, the highly organized boundary between limbal and corneal epithelium is

disrupted as limbal progenitor cells are recruited into the central cornea to restore tissue integrity and barrier function. Given the functionally integrated nature of the limbal-corneal epithelium that is collectively engaged under conditions of tissue repair, it seems likely that cell-cell adhesion and communication mechanisms contribute to the organization of the limbal-corneal epithelial boundary zone to help maintain tissue homeostasis.^{1,4}

Eph receptors are a large family of transmembrane receptor tyrosine kinases that mediate cell-cell communication by interacting with their membrane associated ephrin ligands. Ligand binding triggers a cascade of tyrosine phosphorylation events within the cells expressing Eph receptors. Signaling through Eph receptors and their ephrin ligands participate in a variety of physiologic activities such as differentiation, migration, and tissue boundary formation.⁵⁻⁹ For example, ephrin-A family ligands have been shown to promote keratinocyte

differentiation and inhibit migration largely by acting on EphA2.^{7,10,11} Accordingly, EphA2 loss is associated with increased tumor growth in the skin of mice.¹² Systemic disruption of Eph/ephrin signaling using a recombinant fusion protein approach also led to increased epithelial cell proliferation in mouse skin and gut, suggestive of a role for this cell-cell communication pathway in activation of stem cell compartments.^{8,13,14} Further support for this possibility comes from work in the intestinal epithelium where EphB receptors help maintain the organization of stem cell compartments at the base of crypts in concert with ephrin-B ligands that are concentrated in the more differentiated cells that populate the villi of this simple epithelium.¹⁵ Whether EphA family members or their ephrin-A ligands play similar roles in epithelial stem cell compartment organization is not known.

Expression of EphA1, EphA2, EphA3, ephrin-A1, and ephrin-A2 has been detected in mouse corneal epithelium where their roles remain somewhat unclear.¹⁶ Using a human corneal epithelial culture system, we previously delineated a role for EphA2/ephrin-A1 signaling complexes in regulating cell migration.⁷ In particular, we showed that increasing ephrin-A1 ligand using either gene overexpression or a recombinant ligand mimetic protein restricted the migration of cultured human corneal epithelial cells. In the present study, we extended our analysis of this cell-cell communication pathway in corneal epithelial cells *in vitro* and *in vivo* in attempt to understand if EphA2 or ephrin-A1 contribute to the establishment and maintenance of the boundary between limbal and corneal epithelium.

Herein, we report that ephrin-A1 ligand is concentrated in the limbal region of the human and mouse anterior segmental epithelia, whereas EphA2 is mainly localized in the more differentiated corneal epithelium. This led us to hypothesize that ephrin-A1 and EphA2 signaling complexes in corneal epithelial cells regulate tissue patterning events that contribute to a functionally integrated limbal-corneal epithelial compartment. Reconstitution of this boundary in a corneal epithelial cell coculture system illustrated a key role for ephrin-A1 in directing the migration of EphA2-expressing cell populations upon heterotypic cell-cell contact. Our studies further revealed the involvement of a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) in cell-cell junction stability and the regulation of EGFR signaling downstream from ephrin-A1 and EphA2. Modulation of ADAM10 and epidermal growth factor receptor (EGFR) signaling elicited by Eph/ephrin-mediated cell-cell communication help further define the complex mechanisms governing tissue homeostasis and wound healing in epithelial barrier tissues like the cornea.

METHODS

Cell Culture

The telomerase-immortalized limbal derived human corneal epithelial cell line, hTCEpi, was obtained from Drs. Cavanaugh and Robertson (UT Southwestern, Dallas, TX, USA)¹⁷ and maintained in Keratinocyte Serum Free Media (Invitrogen, Carlsbad, CA, USA) supplemented with 2.5 µg/mL human recombinant epidermal growth factor 1-53 (EGF 1-53), 50 µg/mL bovine pituitary extract (BPE), 0.25 µg/mL amphotericin B (Mediatech, Manassas, VA, USA), 10 µg/mL gentamicin (Sigma-Aldrich Corp., St. Louis, MO, USA), and 0.15 mM CaCl₂ as described before.⁷ For collective cell migration studies, the calcium concentration was raised to 0.3 mM to help stabilize cell-cell adhesion.⁷

Gene Expression and Silencing

Retroviral transduction was used to manipulate ephrin-A1 levels in hTCEpi cells. A full-length human ephrin-A1 cDNA was obtained from Waldemar Debinski, MD, PhD, (Wake Forest University Medical Center, Winston-Salem, NC, USA)¹⁸ and subcloned into the pLZRS-Linker vector.¹⁹ Retroviral supernatants were generated and used to transduce hTCEpi cells as previously described.²⁰ To silence receptor expression, siRNA oligonucleotide duplexes (20 nM) targeting EphA2 (Invitrogen) or Ephrin-A1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) along with a scramble control were transiently transfected into hTCEpi cells using Dharmafectin-1 (Dharmacon, Lafayette, CO, USA) as described.¹⁰

Silicone Chamber Coculture Confrontation Assay

Ephrin-A1-overexpressing hTCEpi cells or EphA2-expressing control cells were differentially labeled with Vibrant DiI (red; Invitrogen) or Vibrant DiO (green; Invitrogen) before dissociation by trypsin for replating. The green and red dye-labeled cell suspensions were seeded to confluency (70 mL of 0.9 million cells/mL per compartment; 60,000 cells/22 mm²) in discrete culture compartments with a 500-µm silicone separation (Ibidi, Fitchburg, WI, USA) to prevent intermixing of cell populations. This differential seeding was used to mimic the limbal and corneal expression patterns of ephrin-A1 and EphA2, respectively. Silicone chambers were removed 24 hours after plating to allow for migration and initiate confrontation between the two distinctly labeled cell populations. Time lapse imaging of ephrin-A1 and EphA2 hTCEpi cells confronting one another was recorded via a BioStation CT (Nikon, Melville, NY, USA) imaging system using 6 × 6 tiling from images acquired using a 4× objective every 1 to 2 hours for 48 hours. Still images were used to calculate the deviation from midline or % deviation from starting point after removing the silicone chamber using ImageJ software (National Institutes of Health, Bethesda, MD, USA).²¹ Percent deviation from the midline was assessed by the area covered by red or green cells after removal of the silicone chamber. Reversal of migration was assigned a negative value for these calculations. Quantification of confrontation was measured by % deviation from migration front (% deviation from start) of red labeled cells (Control red or Ephrin-A1 red). Each experiment was repeated at least three times, and the area covered by differentially labeled cells was represented as mean values ± SEM.

In some experiments, cells were preincubated for 2 to 5 hours in the presence of the inhibitors such as G1254023X (5 mM),²² AG-1478 (1 mM; Sigma-Aldrich Corp.), U0126 (10 mM; Cell Signaling Technology, Danvers, MA, USA), LY294002 (20 mM; Cell Signaling Technology), or Y-27632 (10 mM; Sigma-Aldrich Corp.) before removal of the silicone barrier. After removal of the silicone chamber, fresh medium with inhibitor was added to the culture system and maintained throughout the confrontation imaging period.

Corneal Debridement

Epithelial debridement of mouse corneas was performed as described previously.²³ All experimental procedures complied with the rules specified in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Briefly, 1.5-mm central corneal wounds were generated in 7- to 8-week-old BALB/c mice using a dull blade to remove the corneal epithelium. The mice were allowed to heal for 12, 24, and 48 hours before eyes were enucleated for whole mount staining (see below). To collect samples for protein and RNA from these injured corneas, five to eight corneas were scraped

at each time point with a dull blade and snap frozen for protein detection (see below) or total RNA isolation for quantitative real time PCR as described before.⁷

Western Blot Analysis

Protein lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.0, 150 mM NaCl, 1% Nonident-P 40, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, containing 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail; Roche, Indianapolis, IN, USA) from hTCEpi cells and uninjured control or injured corneas, and subjected to Western blot analysis as previously described.^{7,24} Briefly, 5 to 25 µg protein lysate was separated by SDS-PAGE and probed with the following primary antibodies: mouse monoclonal antibody against EphA2 (D7; Millipore); rabbit polyclonal antibodies against ephrin-A1 (V18; Santa Cruz Biotechnologies), pSer897-EphA2 (Cell Signaling Technology), E-cadherin (HECD1; Abcam, Cambridge, MA, USA), and ERK1/2 (Cell Signaling Technologies) and GAPDH (Santa Cruz Biotechnologies) as loading controls.

Immunocytochemistry and Immunohistochemistry

To assess the impact of altered EphA2 or ephrin-A1 expression in hTCEpi cells, mono-cultures of these variously engineered cell populations were grown on glass coverslips and then fixed in 4% paraformaldehyde in PBS for 5 minutes. For coculture studies aimed at assessing changes at a heterotypic boundary, hTCEpi cells were plated on glass coverslips with a silicone divider that was subsequently removed to initiate confrontation; these cocultures were fixed in 4% paraformaldehyde in PBS 48 hours after removal of the silicone barrier. The cells on glass coverslips were then permeabilized in 0.1% Triton X-100 for 5 minutes and processed for immunofluorescence staining as previously described²⁵ using a goat antibody against EphA2 (AF3035; R&D Systems, Minneapolis, MN, USA), rabbit polyclonal antibody against ephrin-A1 (V18; Santa Cruz Biotechnologies), or a mouse monoclonal antibody against E-cadherin (HECD1; Abcam) with detection using an Alexa Fluor-488 or -555 or 647-nm conjugated donkey anti-goat, anti-rabbit or anti-mouse antibody (Invitrogen). 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain nuclei. Images were acquired using a 20× or 40× 0.5 EC Plan-Neofluar objective on an epifluorescence microscope system (AxioVision Z1; Carl Zeiss, Thornwood, NY, USA) fitted with an Apotome slide module and a digital camera (AxioCam MRm, Carl Zeiss).

Frozen sections (5 µm) of optimal cutting temperature compound (OCT)-embedded human and mouse corneas were fixed in 4% paraformaldehyde, blocked in 10% donkey serum in PBS, and incubated overnight with the following primary antibodies: a goat anti-human EphA2 (AF3035) or a goat anti-mouse EphA2 (AF639; R&D Systems), a rabbit anti-ephrin-A1 (V18), a rabbit polyclonal antibody against ADAM10 (Abcam), or a mouse monoclonal antibody against E-cadherin (HECD1; Abcam). Detection of primary antibodies was carried out using appropriate secondary antibodies conjugated to Alexa-555 or Alexa-488 (Invitrogen). The slides were incubated with DAPI and mounted using Gelvatol. Imaging of the samples were performed using a 20× 0.5 EC Plan-Neofluar objective on the Zeiss epifluorescence microscope system described above.

Whole-mount staining of mouse corneas was performed as described previously.²⁴ Briefly, 1.5-mm corneal wounds were generated in BALB/c mice using a dull blade. Anterior segment of the eyes were dissected and fixed in 1% formalin in PBS, MgCl₂, and EGTA and then permeabilized in MeOH:DMSO (4:1) before incubation in MeOH. Samples were rehydrated in

sequential gradient of Triton X-100:MeOH mixture and then blocked in 10% serum PBS before incubation with primary antibodies overnight. The primary antibodies were fluorescently coupled for detection using Alexa-555 or -488 secondary antibodies overnight, and the corneas were incubated with DAPI and mounted in Gelvatol.

Statistical Analysis

The data are shown as mean values ± SEM. Statistical significance was determined via unpaired *t*-test and ANOVA. Parameters with *P* < 0.05 are considered significant. All experiments were repeated at least in triplicate.

RESULTS

Spatiotemporal Expression of Ephrin-A1 and EphA2 in Human and Mouse Corneal Epithelium

There is a sharp transition between basal cells of the limbal epithelium and the more differentiated basal cells of the corneal epithelium, which is referred to as the limbal-corneal epithelial junction.^{1,4} Given the role of Eph/ephrins in cell segregation and boundary formation⁹ and our previous data showing a role for EphA2 and ephrin-A1 in corneal epithelial cell migration,⁷ we examined the expression patterns of this receptor-ligand system in various zones (i.e., limbus, limbal-corneal junction, central cornea) of the human cornea using frozen tissue sections (Fig. 1A). Ephrin-A1 staining was present throughout the limbal epithelium and extended into the corneal/limbal epithelial junction. Ephrin-A1 expression was also detectable in the corneal epithelium but at lower levels. In contrast, the expression of EphA2 was concentrated in the corneal epithelium (Fig. 1A, upper) and the most superficial layers of limbal epithelium. This reciprocal expression pattern of EphA2 and ephrin-A1 in human corneal and limbal epithelia, respectively, mirrored our observations in mouse ocular anterior segmental epithelium where ephrin-A1 was concentrated in the limbal epithelium (*arrow*) and EphA2 was prominent in corneal epithelium (Fig. 1B).

Superficial corneal epithelial debridement wounds disrupt the organization of the limbal-corneal boundary as limbal epithelial progenitor cells are rapidly recruited into the central corneal epithelium to repair and restore tissue barrier function.^{26–28} We examined EphA2 and ephrin-A1 mRNA levels and distribution in wounded corneas of mice (Fig. 2) as a means to assess the regulation of this cell-cell communication pathway in response to epithelial tissue damage in the eye.^{24,26,29,30} During corneal epithelial regeneration, EphA2 immunoreactivity increased throughout the cornea (Figs. 2A, 2C) in a manner that corresponded with elevated EphA2 mRNA transcript levels (Fig. 2F). Although ephrin-A1 mRNA levels did not markedly change under these conditions (Fig. 2F), ephrin-A1 immunoreactivity extended outside of the limbal epithelium and was apparent in clusters of cells present proximal to the wound edge (Figs. 2B, 2C, dotted lines outline the wounded area; arrowheads represent ephrin-A1-positive cell clusters). The appearance of ephrin-A1-positive cell clusters corresponded to areas of increased EphA2 immunoreactivity in damaged corneal epithelium (Fig. 2A, arrows represent EphA2 enriched areas near the wound edge). Whole-mount co-immunostaining of EphA2 (green) and ephrin-A1 (red) along the entire length of cornea revealed substantial overlap in receptor and ligand distribution in the injured corneal epithelial tissue (Fig. 2C). Protein lysates from these injured

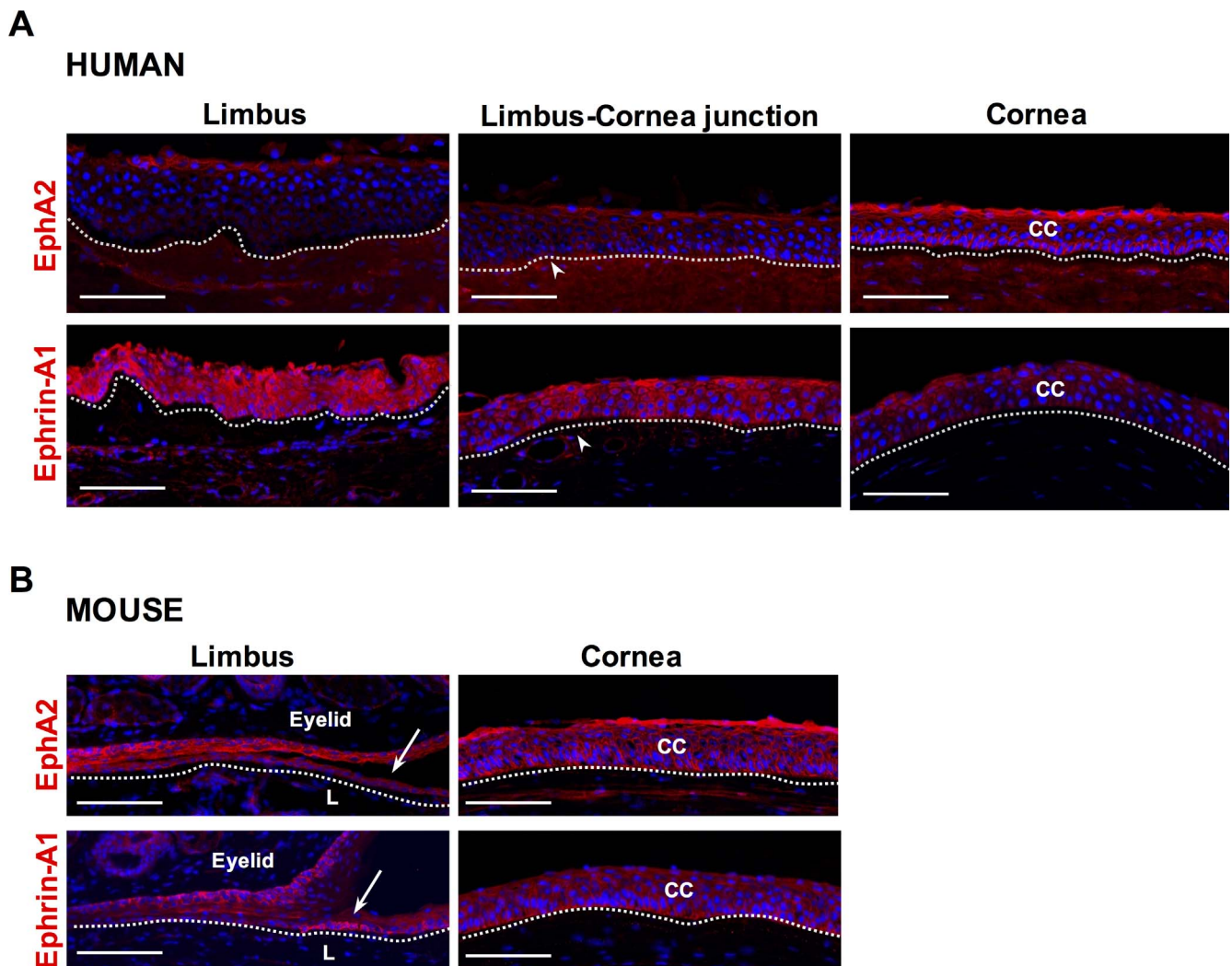


FIGURE 1. Reciprocal regulation of ephrin-A1 and EphA2 expression in human and mouse cornea. Frozen corneal tissue sections from human cadavers (**A**) and wild-type Balb/C mice (**B**) were immunostained with antibodies against EphA2 or ephrin-A1 (red, bottom). DAPI (blue) was used to highlight nuclei. (**A**) Arrowheads indicate the limbus-cornea junction where the limbus ends and the cornea begins. (**B**) Mouse eyelids are marked as a reference point for limbal tissue orientation. Arrows show concentrated ephrin-A1 staining and paucity of EphA2 staining in the limbus. White dotted lines demarcate the basement membrane region. CC, central cornea; L, limbus. $n = 3$. Scale bar denotes 100 μm .

corneas showed a transient elevation of EphA2 that was highly phosphorylated at Serine 897 (pS897-EphA2), which is a form of EphA2 that is commonly found in migratory cells (Figs. 2D, 2E, 12 hours).¹¹ Total and pS897-EphA2 levels returned to baseline coincident with increased ephrin-A1 expression in the corneal epithelium at later time points (Figs. 2D, 2E). These observations indicate that ephrin-A1 and EphA2 are concentrated in limbal and corneal epithelium under steady-state conditions and are dynamically redistributed to areas of tissue repair on injury.

Reciprocal Regulation of Ephrin-A1 and EphA2 Expression in Cultured Human Corneal Epithelial Cells

To interrogate the role of ephrin-A1 in limbal epithelial cells, we used a retroviral gene delivery system to increase the levels of this ligand in a limbal-derived corneal keratinocyte line (hTCEPi) that expresses high levels of EphA2.⁷ Ectopic expression of ephrin-A1 in hTCEPi cells reduced the expres-

sion and junctional distribution of EphA2 (Figs. 3A, 3B). Conversely, siRNA-mediated knockdown of ephrin-A1 led to an increase in the expression and border localization of EphA2 indicating that even low levels of ephrin-A1 are capable of limiting EphA2 expression in corneal epithelial cells (Figs. 3C, 3D). Additional evidence for reciprocal regulation of EphA2 and ephrin-A1 in corneal epithelial cells was obtained following knockdown of EphA2, which led to a concomitant increase in ephrin-A1 levels (Figs. 3C, 3D).

In hTCEPi cells expressing high levels of ephrin-A1, where EphA2 is subsequently downregulated (Figs. 3A, 3B), the calcium-dependent cell-cell adhesion molecule, E-cadherin, was enhanced at cell-cell borders. This occurred without major changes in total protein expression suggestive of increased junctional stability at these ephrin-A1-enriched cell-cell contact sites. In contrast, knockdown of both EphA2 and ephrin-A1 (siEphA2+siEphrin-A1) reduced E-cadherin distribution at cell-cell contacts in a manner similar to ephrin-A1 knockdown alone (siEphrin-A1), providing additional support for a role for ephrin-A1 in maintaining junctional stability (Fig. 3B). These observations in a corneal epithelial

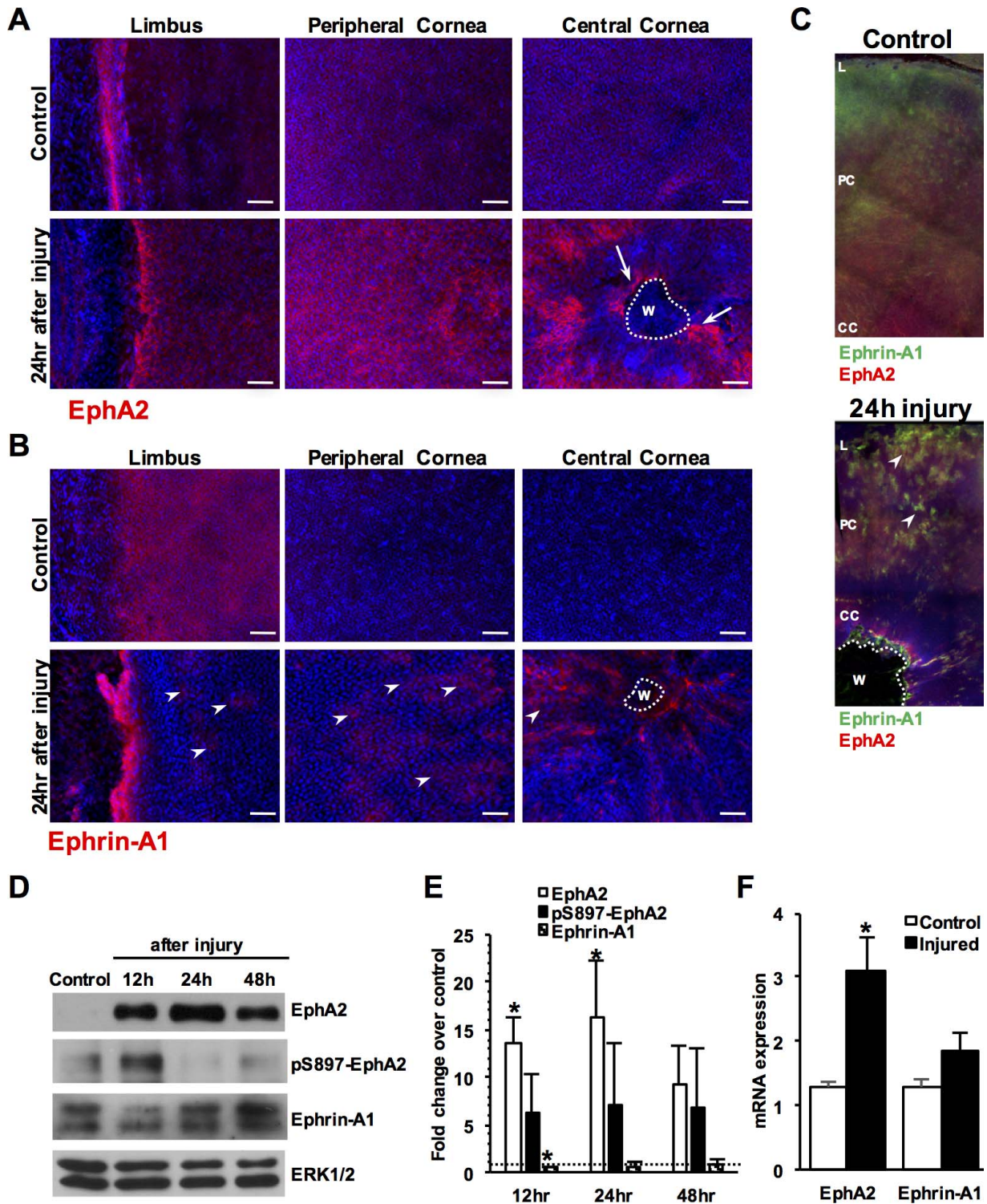


FIGURE 2. Ephrin-A1 is redistributed into the cornea of injured mouse eyes. Whole mounts of mouse anterior segmental epithelium in uninjured (Control) eyes and following central corneal wounding (24 hours after injury). Immunostaining was performed for (A) EphA2 (red) and (B) Ephrin-A1 (red). Scale bar denotes 80 μ m. W, wound opening. Arrowheads show clusters of ephrin-A1-expressing cells in the cornea. Arrow represents EphA2-enriched areas near the wound edge. White dotted line marks the wound edge. $n = 3$. (C) Whole mounts of control (upper) and injured (lower) mouse corneas that were dual stained for EphA2 (red) and ephrin-A1 (green). Confocal stitched images show the entire cornea. L, limbus; PC, peripheral cornea; CC, central cornea; W, wound opening. White dotted line marks the wound edge. (D) Immunoblotting of EphA2, pS897-EphA2, or ephrin-A1 in samples isolated from wounded corneas. ERK1/2 was used as a loading control. (E) Densitometry results of Western blots represented in D are shown as fold changes over levels present in uninjured, control corneas. $*P < 0.05$, $n = 3$. (F) Real time-quantitative PCR measurement of mRNA transcript levels for EphA2 and ephrin-A1 in samples isolated from wounded corneas. $*P < 0.05$, $n = 4-6$.

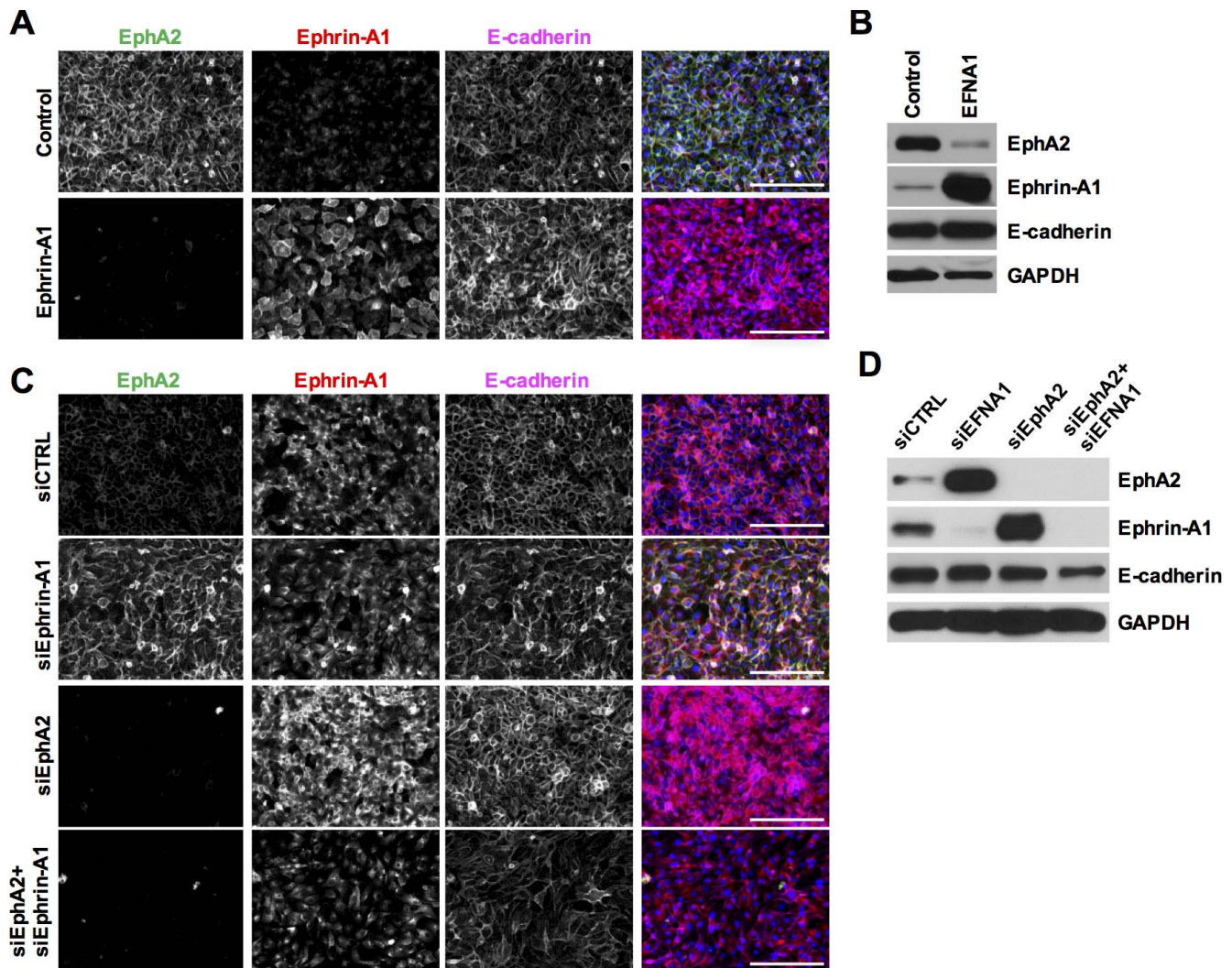


FIGURE 3. Reciprocal regulation of ephrin-A1 and EphA2 expression in corneal epithelial cell cultures. (A) Immunostaining of EphA2 (green), ephrin-A1 (red), and E-cadherin (magenta) in mono-cultures of hTCEPi cells transduced with an empty control or an ephrin-A1 cDNA construct. Representative images from $n = 3$. (B) Immunoblotting for total EphA2, ephrin-A1, or E-cadherin in hTCEPi cells overexpressing ephrin-A1 (EFNA1). GAPDH was used as a protein loading control. Representative blots from $n = 4$. (C) Immunostaining of EphA2 (green), ephrin-A1 (red), and E-cadherin (magenta) in hTCEPi cells knocked down for ephrin-A1 (siEphrin-A1), EphA2 (siEphA2), or both proteins (siEphA2+siEphrin-A1). Scale bar denotes 100 μm . Representative images from $n = 3$. (D) Immunoblotting for total EphA2, ephrin-A1, or E-cadherin in hTCEPi cells with siRNA targeted knockdown of ephrin-A1, EphA2, or double knockdown. GAPDH was used as a protein loading control. Representative blots from $n = 3$.

cell culture system where ephrin-A1 and EphA2 levels were experimentally manipulated shared key features with the reciprocal expression pattern of this receptor-ligand system observed in the cornea in vivo (Fig. 1).

Ephrin-A1 and EphA2 Compartmentalization in a Limbal-Corneal Epithelial Cell Coculture Model

We took advantage of a silicone chamber coculture apparatus and fluorescent tracer dyes to separate EphA2- and ephrin-A1-expressing corneal epithelial cells as a means to study the roles of this receptor-ligand system at a reconstituted limbal-corneal epithelial-like boundary (Fig. 4A). Distinct populations of ephrin-A1- or EphA2-expressing cells were differentially labeled with green or red fluorescent dyes and plated in these respective chambers to prevent intermixing at the time of seeding. Removal of the silicone barrier allowed these two cell populations to migrate toward one another and establish a heterotypic boundary zone (Figs. 4A, 4B). When EphA2-

expressing control cells were plated on both sides of the chamber (Control green versus Control red), the differentially labeled cells underwent extensive intermingling at sites of initial contact (Figs. 4B, 4C; Supplementary Movie S1). In contrast, ephrin-A1-overexpressing cells (EFNA1 red) plated on one side of the chamber induced a repulsive response in adjacent EphA2 expressing cells (Control green) at heterotypic points of contact (Figs. 4D, 4E). In particular, EphA2-expressing control cells switched the direction of migration following contact with ephrin-A1 resulting in coordinated migration of both cell populations in the direction of the ephrin-A1 leading front (Figs. 4B, 4D, 4E).

At the interface between ephrin-A1- and EphA2-expressing cells, a distinct boundary was observed between these discrete cell populations. (Supplementary Movie S2). There was a marked reduction in E-cadherin immunoreactivity at the boundary between ephrin-A1- and EphA2-expressing cells (Fig. 5A; Control: Ephrin-A1), suggestive of reduced cell-cell adhesion. These observations in a corneal epithelial coculture

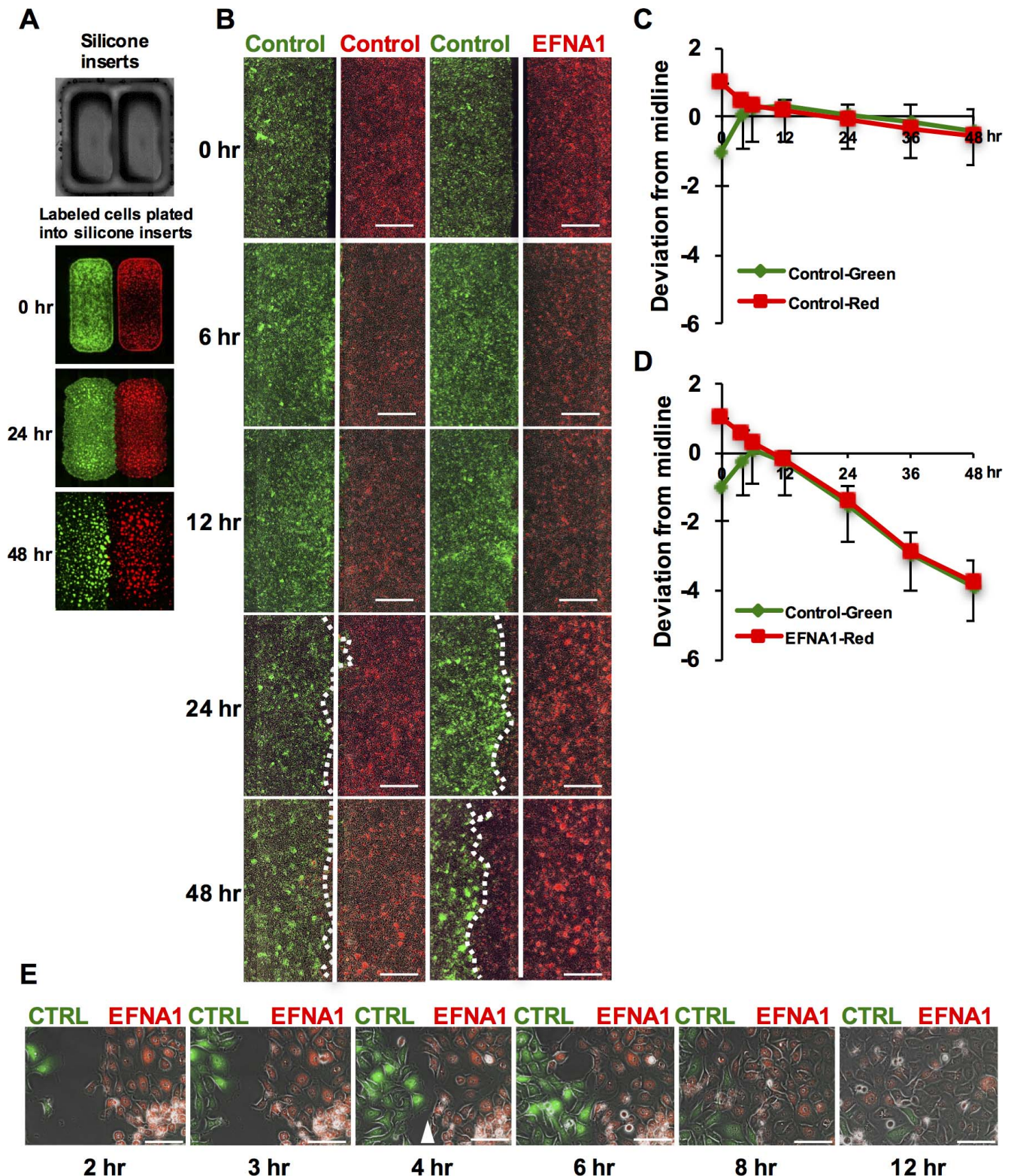


FIGURE 4. EphA2/Ephrin-A1 signaling complexes in a heterotypic cell confrontation coculture model. (A) hTCEpi cells were differentially labeled with fluorescent cell trackers (*red* and *green*) and seeded into discrete culture compartments using a silicone chamber confrontation apparatus. After removal of the silicone divider, live cell imaging was used to monitor cell confrontation for 48 hours. Snapshots of 0, 24, and 48 hours are shown. (B) EphA2-expressing control cells (Control, *green*) confronting “like” control cells (Control, *red*) are presented on the *left*, while control cells (Control, *green*) confronting “unlike” ephrin-A1-overexpressing cells (EFNA1, *red*) are presented on the *right*. After removal of the silicone divider, time-lapse imaging was used to examine the formation and organization of the epithelial boundary between these two cell populations. *White solid lines* mark the midline where the silicone divider was present prior to removal. *Dotted lines* indicate the boundary between the two cell populations after initiation of confrontation. Snapshots of 0, 6, 12, 24, and 48 hours are shown. *Scale bar* denotes 1 mm. (C, D) *Line graphs* showing the migrating front of control cells (Control, *green*) confronting control (Control, *red*) (C) or ephrin-A1 (EFNA1, *red*) (D) overexpressing cells normalized with respect to the midline. Negative values on the *y-axis* represent the reversal of migration initiated by ephrin-A1. *n* = 3 (D). (E) Higher-magnification images from early time points (2, 3, 4, 6, 8, and 12 hours) of ephrin-A1-overexpressing cells (EFNA1, *red*) confronting control cells (CTRL, *green*). *Arrowhead* points to the initial confrontation area at 4 hours. *Scale bar* denotes 200 μ m.

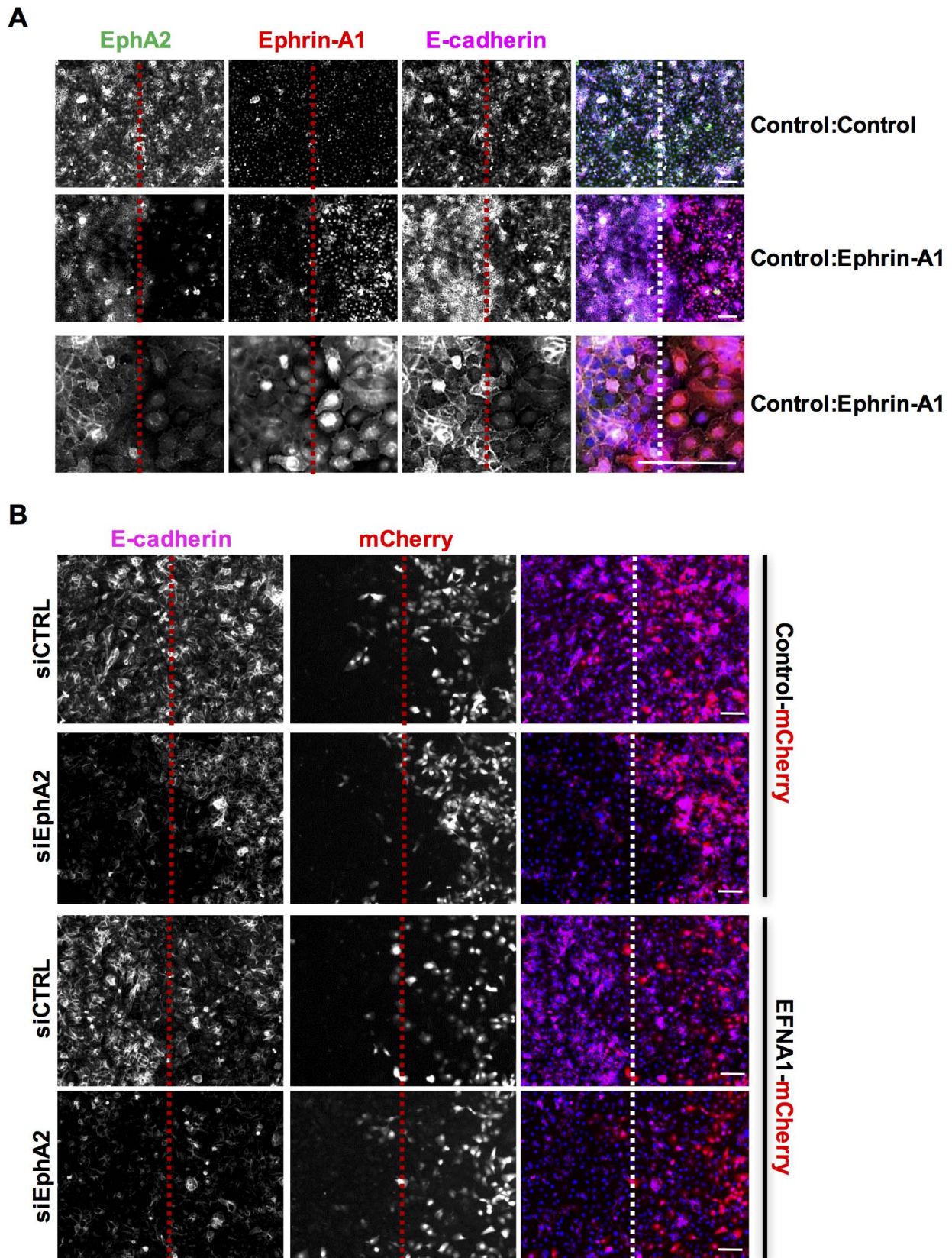


FIGURE 5. Cell-cell border localization of E-cadherin is reduced at EphA2/Ephrin-A1 boundaries. **(A)** Immunofluorescent staining of EphA2 (green), ephrin-A1 (red), and E-cadherin (magenta) in cells present at the boundary of Control:Control- or Control:Ephrin-A1-expressing cell cocultures 48 hours after removal of the silicone barrier. A magnified view of the boundary is shown below in control cells confronting ephrin-A1-expressing cells. Dotted lines indicate the boundary between the two different cell populations 48 hours after initiation of confrontation. $n = 4$. Scale bar denotes 80 μm . **(B)** Control or ephrin-A1-expressing cells were transduced to express mCherry (Control-mCherry or EFNA1-mCherry, respectively) to differentiate these cell populations from control cells transfected with siControl (siCTRL) or siEphA2. Immunostaining of E-cadherin (magenta) was performed in cocultures 48 hours after initiation of confrontation. Dotted lines indicate the boundary between the two different cell populations at 48 hours. $n = 3$.

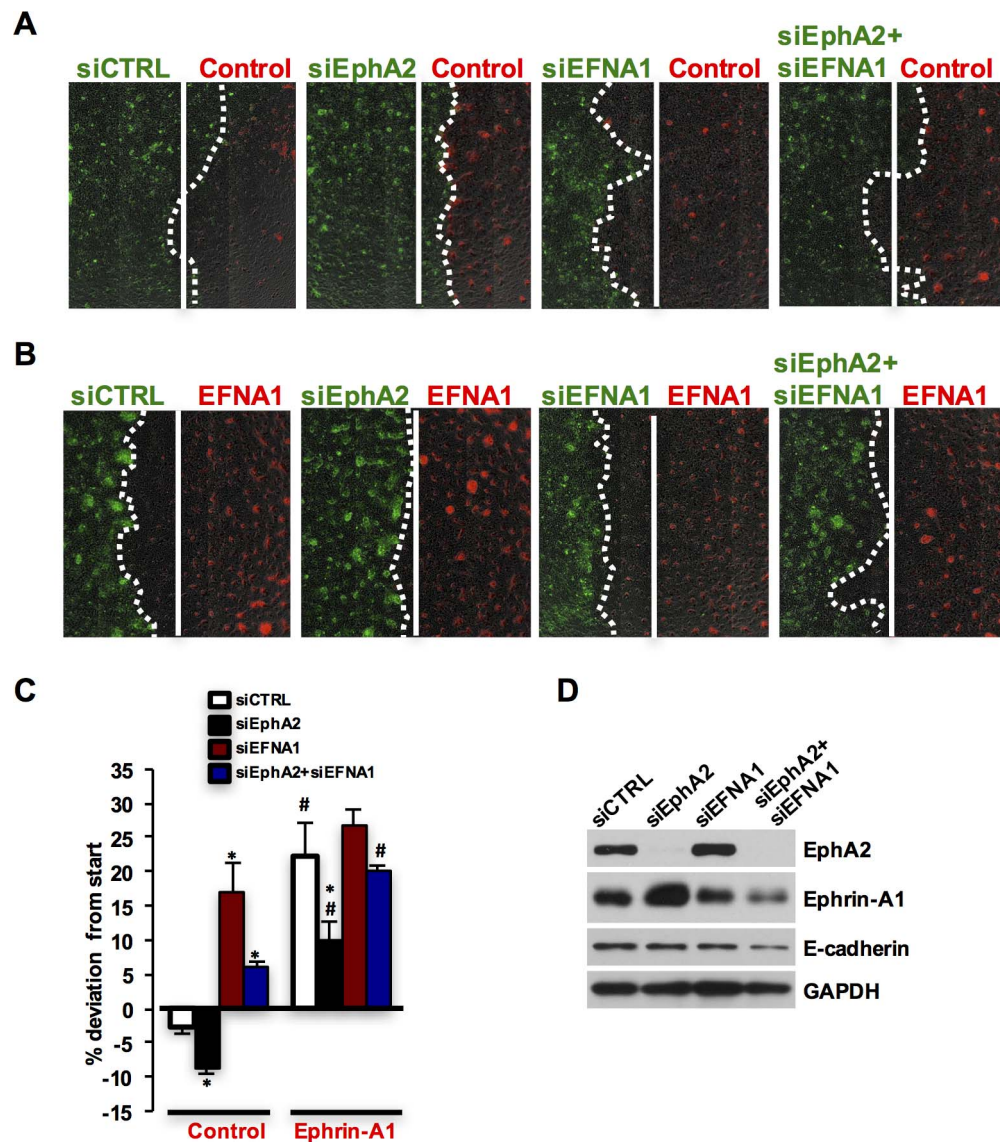


FIGURE 6. Ephrin-A1-induced boundary formation requires EphA2. (A) Control (Control, red) or (B) ephrin-A1-expressing (EFNA1, red) cells are shown at 48 hours after initiation of confrontation with cells transfected with siControl (siCTRL, green), siEphA2 (green), siEphrin-A1 (siEFNA1, green), or double siRNA (siEphA2+siEFNA1, green) oligonucleotides. Solid white lines mark the midline where the silicone divider was present at the time of removal. White dotted lines indicate the boundary between two cell populations 48 hours after initiation of confrontation. (C) Quantification of confrontation response in A and B as measured by % deviation from migration front of red-labeled cells (Control, red or Ephrin-A1, red). * $P < 0.05$ versus siCTRL; # $P < 0.05$, Ephrin-A1, red versus Control, red, $n = 3$. (D) A representative Western blotting showing the levels of EphA2 or ephrin-A1 after siRNA depletion in lysates harvested at the end of the experiment (96 hours after siRNA transfection).

system highlight the ability of ephrin-A1-expressing cells to impact the adhesion, polarity, and directed migration of EphA2-expressing cells. Such a finding has implications for corneal epithelial repair mechanisms in vivo as ephrin-A1 distribution extends out of the limbus and into fields of EphA2-expressing cells present in the cornea following wounding (Fig. 2).

Ephrin-A1 Requires EphA2 in Adjacent Corneal Epithelial Cell Fields to Direct Migration

Although EphA1, EphA2, and EphA3 are each present in mouse corneal epithelium,¹⁶ EphA2 is a preferred receptor for ephrin-A1 and modulates many of its downstream actions in epithelial cells.³¹ siRNA-mediated gene silencing (Supplementary Fig. S1) was used to study the requirement of EphA2 in the repulsive

response mediated by ephrin-A1 at heterotypic cell-cell boundaries. In particular, EphA2 expression was knocked down (siEphA2) in cells confronting either control (Control-mCherry) or ephrin-A1-overexpressing cells (EFNA1-mCherry). The reduced levels of EphA2 in corneal epithelial cells normalized E-cadherin border localization at areas of heterotypic contact with ephrin-A1-expressing cultures (Fig. 5B; siEphA2 versus EFNA1-mCherry). In addition, the reversal in cell migration initiated by ephrin-A1-expressing cells was diminished when EphA2 was no longer present at these boundaries (Figs. 6A–6C; siEphA2 green versus EFNA1 red). These findings demonstrated that EphA2 is required for ephrin-A1-mediated reversal of cell migration at heterotypic points of contact.

Knocking down EphA2 in corneal epithelial cells induced a concomitant increase in ephrin-A1 levels (Fig. 3; Supplementary Fig. S1), which provided an opportunity to test the

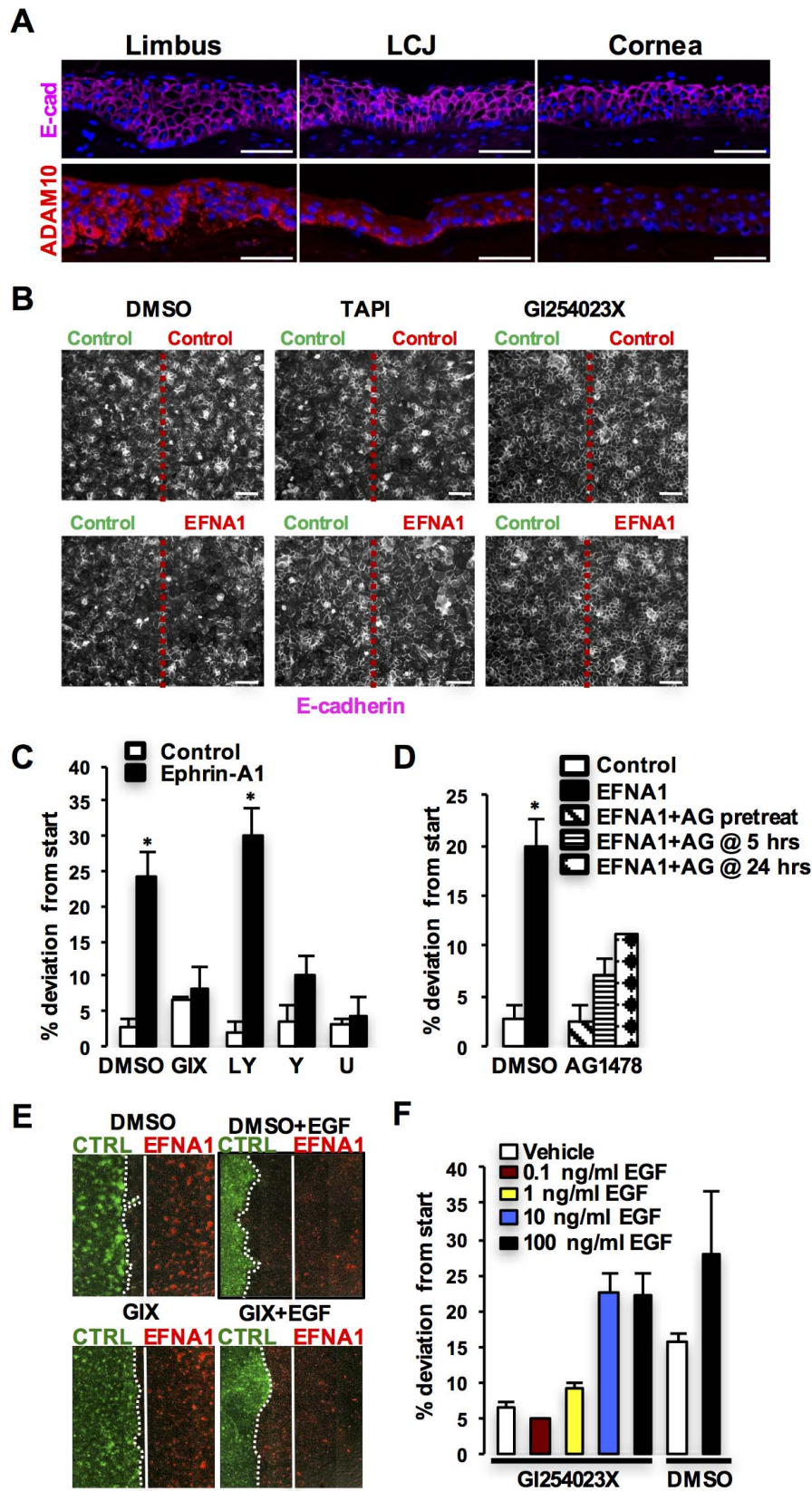


FIGURE 7. ADAM10 mediates Ephrin-A1/EphA2 boundary organization via EGFR signaling. (A) E-cadherin (E-cad; top) and ADAM10 (bottom) immunofluorescence staining in human anterior segmental epithelium. Scale bar denotes 100 μ m. (B) E-cadherin staining of control cells (Control, green) confronted by “like” control cells (Control, red) or ephrin-A1-expressing cells (EFNA1, red) confronted by “unlike” control cells (Control, green; bottom) in the presence of general MMP inhibitor, TAPI, or a specific ADAM10 inhibitor, GI254023X (GIX). Red dotted lines indicate the boundary between the two cell populations 48 hours after initiation of confrontation. Scale bar denotes 80 μ m. (C) Quantification of confrontation

experiments at 48 hours in cocultures treated with DMSO, GIX, LY294002 (LY), Y27632 (Y), or U0126 (U). * $P < 0.05$, $n = 3-4$. (D) Quantification of confrontation experiments at 48 hours using ephrin-A1-expressing cells (EFNA1) in contact with “unlike” control cells that had been treated with DMSO or the EGFR inhibitor, AG1478 (AG). Cells were either pretreated before the initiation of confrontation for 1 hour (AG pretreat) or treated with inhibitor 5 (AG @ 5 hrs) or 24 hours (AG @ 24 hrs) after initiation of confrontation. (E, F) Various concentrations of EGF (0.1, 1, 10, or 100 ng/mL) were added to the culture medium of these ephrin-A1 and control cell cocultures after pretreatment with GIX for 5 hours. Images (E) and quantification (F) are shown 48 hours after confrontation. *Solid white lines* mark the midline where the silicone divider was present at the time of its removal. *White dotted lines* indicate the boundary between the two different cell populations 48 hours after initiation of confrontation. $n = 3-4$.

importance of endogenous ephrin-A1 in boundary formation independent of ectopic overexpression. Under these circumstances, EphA2-deficient corneal epithelial cells disrupted E-cadherin distribution (Fig. 5B, upper) and led to a reversal in migration upon confrontation with EphA2-expressing control cells (Fig. 6A, siEphA2 green versus Control red). The elevation of ephrin-A1 in EphA2-deficient cells (Fig. 3) was, in part, responsible for this phenotype as dual silencing of EphA2 and ephrin-A1 prohibited the ability of these cells to alter the migration of control cultures at heterotypic contact points (Figs. 6A, 6C; siEphA2+siEFNA1 versus Control red). Collectively, these data indicate that a finely tuned balance exists between the expression levels of EphA2 receptor and ephrin-A1 ligand in corneal epithelial cells that may play a role in governing the organization of the limbal-corneal epithelial boundary.

ADAM10 Mediates Ephrin-A1/EphA2 Boundary Organization via Regulation of Growth Factor Signaling

The ability of Eph/ephrins to regulate epithelial stem cell compartments or tumor cell boundaries has been associated with modulation of E-cadherin junctional stability by metalloproteinases (MMPs), including ADAM family members.³² Specifically, EphB receptor tyrosine kinase recruitment of ADAM10 has been shown to weaken E-cadherin-dependent epithelial cell-cell junctions to modulate sorting between distinct cell populations expressing ephrin-B ligands.¹⁵ ADAM10 also interacts with EphA receptors and ephrin-A ligands.³³⁻³⁵ The destabilization of E-cadherin observed at ephrin-A1/EphA2 boundaries in corneal epithelial cells led us to investigate the role of ADAM10 in this process.

We first determined the distribution of ADAM10 and E-cadherin in human corneal epithelium. Immunofluorescence staining of ADAM10 in the human eye showed a strong signal in the limbal epithelium with a reduced gradient toward the corneal epithelium (Fig. 7A, lower). Interestingly, E-cadherin immunoreactivity at cell-cell borders was weakest at the limbal-corneal epithelial junction (LCJ; Fig. 7A, upper) where ephrin-A1 and EphA2 receptors are both present (Fig. 1A), sharing features with the reduced junctional staining observed in heterotypic cocultures where ephrin-A1 expressing cells came into contact with EphA2 expressing cells (Fig. 5A).

To study the possible contribution of ADAM10 to ephrin-A1-mediated destabilization of E-cadherin at heterotypic contacts, we used a general MMP inhibitor (i.e., TAPI) in addition to a more specific ADAM10 inhibitor (GI254023X; GIX)²² in these coculture systems. Addition of these inhibitors prevented E-cadherin destabilization as assessed by robust junctional immunostaining at EphA2/ephrin-A1 interfaces (Fig. 7B). ADAM10 recruitment to junctions has also been shown to aid in cell compartmentalization via extracellular cleavage of E-cadherin.¹⁵ However, we failed to detect shed fragments of E-cadherin in conditioned culture medium collected from EphA2 and ephrin-A1 corneal epithelial cocultures (data not shown). Therefore, we considered the possibility that growth factor signaling pathways impacted by ephrin-A1 ligand stimulation of EphA2 may contribute to the regulation E-cadherin at

heterotypic contacts. To this end, we examined the role(s) of signaling pathways that are known to destabilize cell-cell adhesion.^{36,37} Inhibition of PI3K/AKT signaling by addition of LY294002 to the coculture system delayed cell migration in general, but this treatment did not significantly alter cell repulsion or boundary formation (Fig. 7C). In contrast, treatment of cocultures with inhibitors of Rho/ROCK1/2 (Y-27632) or MEK1/2 (U0126)-dependent signaling pathways prevented the reversal in cell migration triggered by ephrin-A1 at heterotypic contact sites (Fig. 7C).

In addition to direct effects on E-cadherin cleavage, ADAM10 acts as a sheddase for EGFR ligands to regulate signaling through this receptor tyrosine kinase.^{38,39} Notably, EGFR signaling is an important pathway that becomes activated during corneal epithelial wound healing⁴⁰ and also operates upstream of MAPK-ERK1/2 and Rho-ROCK signaling. EGFR activation is further capable of destabilizing E-cadherin-dependent junctions.^{41,42} Consequently, we tested whether an inhibitor of EGFR (AG1478) would interfere with ephrin-A1-mediated boundary formation. Consistent with this notion, EGFR inhibition prevented ephrin-A1/EphA2 boundary formation in corneal epithelial cell cocultures (Fig. 7D).

Collectively, these data suggested that both ADAM10 and EGFR are important for the maintenance of ephrin-A1/EphA2 boundaries in corneal epithelial cells. To assess whether ADAM10 sheddase activity contributed to the effects of EGFR on ephrin-A1-mediated boundary formation, we attempted to restore this cellular process in the presence of an ADAM10 inhibitor by supplying excess EGFR ligand into the culture system. Under these conditions, the addition of exogenous EGFR ligand would presumably bypass a need for ADAM10-dependent sheddase activity and delivery of endogenous ligands into the cellular microenvironment. Accordingly, exogenous delivery of EGF was sufficient to restore ephrin-A1-mediated boundary formation under conditions of ADAM10 blockade (Figs. 7E, 7F). Taken together, these studies suggest that ADAM10-dependent shedding of EGFR ligand contributed to the formation of an ephrin-A1/EphA2 boundary in a limbal-corneal epithelial coculture system.

DISCUSSION

Juxtacrine signaling through Eph receptors and ephrin ligands regulate tissue patterning and boundary formation throughout development and continue to be important for homeostasis of adult tissues.⁹ What constitutes and controls the boundary between limbal and corneal epithelia has been under intense scrutiny for some time.^{1,4} Surprisingly, the Eph/ephrin signaling pathway has not been studied extensively in this context. We previously showed that EphA2/ephrin-A1 signaling complexes regulate corneal epithelial cell migration and implicated increases in ephrin-A1 expression as part of a pathologic response in delayed wound healing observed in association with diabetes.⁷ However, not much is known about the roles of Eph/ephrin in establishing and maintaining the limbal-corneal epithelial boundary under homeostatic conditions. We now demonstrate that a reciprocal expression gradient of ephrin-A1 and EphA2 exists in the limbal and

corneal epithelium that likely contributes to the organization of these two distinct cellular compartments (Fig. 1).

Numerous studies have revealed the presence of a sharp boundary between limbus and cornea.^{4,43,44} The striking difference in stroma underlying the corneal and limbal epithelia is a prime example, with a corneal stroma that is relatively acellular, avascular and compact versus a highly vascularized, cellular, and loosely organized limbal stroma.^{45,46} The limbal–corneal epithelial boundary is equally dramatic as evidenced by the expression and/or lack of expression of a variety of proteins. For example, enolase, which is highly expressed in limbal epithelial basal cells abruptly ceases in the corneal epithelial basal cells.⁴⁷ Conversely, miR-184 expression, which is high in corneal epithelial basal cells, terminates at the limbal epithelial interface.⁴⁸ Calcium-linked, epithelial differentiation protein (CLED) and early epithelial differentiation protein (EEDA) are two proteins with corneal epithelial compartmentalization that sharply stop at the limbal epithelium.^{49,50} Numerous other examples of such limbal–corneal boundary-driven expression patterns can be found in the literature.^{2,51} In all of these instances, it appears that a single cell separates expression versus nonexpression. Not surprisingly, perturbation of the corneal epithelium easily disrupts these biochemical and/or morphologic boundaries indicative that compartmentalization of the limbal and corneal epithelium is in a constant state of flux. Our work indicates that Eph/ephrin signaling contributes to such fluidity.

A boundary between the conjunctiva and limbus also exists.^{52,53} No clearly distinct staining pattern for ephrin-A1 and EphA2 was noted between limbal and conjunctival epithelium in the human eye (Fig. 1A); however, in mouse eye, a sharp ephrin-A1 staining in limbal epithelium seems to segregate conjunctival epithelium from corneal epithelium (Fig. 1B). This restricted expression pattern of ephrin-A1 in the limbal epithelium can also be disturbed in disease conditions of the eye such as pterygium⁵⁴ and diabetes,⁷ accentuating the need for the preservation of boundaries between compartments of a healthy anterior segmental epithelium.

The corneal epithelium undergoes constant regeneration to replace superficial cell loss and to repair the barrier in response to environmental insults. During the early recovery period following an experimentally induced wound, we observed increased expression of unligated EphA2 (pS897-EphA2) in injured mouse corneas that corresponds with increased migratory response in a variety of other cell types.¹¹ Early response to injury is correlated with increased EGFR signaling in corneal epithelium,⁴⁰ which might contribute to the increased levels of EphA2 seen in corneal epithelium after injury.⁵⁵ For example, elevated EGFR signaling in human squamous carcinoma cell lines leads to increased EphA2 expression.⁵⁶ A resultant increase in EphA2/ephrin-A1 signaling among heterogeneous cell populations created a repulsive response leading to segregation of oncogenic ras transformed cells with highest EphA2 levels from the remaining, untransformed cell population.⁵⁷ A similar situation seems to exist in healthy tissues where corneal epithelial cells expressing abundant EphA2 respond to ephrin-A1-expressing cells that emanate from the limbal epithelium to enhance regeneration and repair mechanisms after injury (Figs. 1–4; Supplementary Movies S1, S2). The response of cells harboring ephrin-A1- to EphA2-expressing cells in culture resembles features of corneal injury in vivo with recruitment of limbal progenitor cells into the central cornea.^{27,28} If we assume that these culture conditions already represent a wounded state, with heightened EphA2 expression in corneal epithelial cells, a reversal of migration when confronted by ephrin-A1 expressing cells could be one of the drivers of epithelial sheet movement necessary for wound closure.

A balance of EphA2 and ephrin-A1 protein levels appears necessary to maintain a limbal–corneal epithelial boundary, at least in a cell coculture model. For example, EphA2 depletion leads to increased ephrin-A1 expression and generates a repulsive response toward cells expressing EphA2 (Fig. 6C). Any alteration in the levels of EphA2, such as what happens after injury, likely changes the distribution of ligand expressing cells in a similar manner. As such, initial increases in EphA2 expression after injury might reduce overall ephrin-A1 levels in corneal epithelial cells to enrich for an unligated, highly migratory form of EphA2 (Fig. 2D). This change could help drive the ephrin-A1-expressing TA cells to sites of injury.

Junctional stability between two segregated populations of cells is dynamic and requires constant regulation.⁵⁸ The interface between EphA2/ephrin-A1-expressing cells exhibited destabilized E-cadherin containing junctions in corneal epithelial cocultures. Our results show a role for ADAM10 in the regulation of E-cadherin disassembly at these heterotypic contact points leading to a repulsive response initiated by ephrin-A1 (Figs. 7B, 7C). Similarly, Ephrin-A5/EphA3-dependent axon repulsion requires ADAM10 activity³⁵ through interactions between the ligand binding domain of EphA3 and ADAM10.³⁴ Although ADAM10 has been shown to cleave the E-cadherin extracellular domain⁵⁹ in response to ephrins, we were unable to detect any change in the level of E-cadherin cleavage products in cocultures incubated in the presence or absence of an ADAM10 inhibitor. ADAM proteins are also involved in shedding of HB-EGF, TGF- α , TNF- α , and the Notch ligand, Delta.^{60–62} Corneal epithelial wounds have been shown to initiate an ADAM17-dependent EGFR ligand shedding⁶³ to activate a migratory wound repair response. Our data suggest that EphA2 recruitment of ADAM10 to the confrontation site could also be important for the shedding of EGFR ligands to mobilize corneal epithelial cells, which are dependent on EGFR signaling for their migration. Elevated EGFR signaling may also trigger a positive feedback loop on the expression of EphA2 in these activated corneal epithelial cells in a manner that contributes to increased level of EphA2 in injured corneas.

Our data suggest that ephrin-A1 and EphA2 signaling complexes in the cornea are important for establishing a functionally integrated limbal–corneal epithelial compartment. The balance between EphA2 and ephrin-A1 at the limbal–corneal epithelial junction may help determine, in part, the steady-state homeostasis of this boundary, as well as a successful response to injury. A change in this reciprocal expression pattern between receptor and ligand could lead to defects in wound healing as seen in diabetic corneas and opens up the possibility of therapeutically targeting the EphA2/ephrin-A1 axis for improving corneal health.

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