The Role of Endogenous Epidermal Growth Factor Receptor Ligands in Mediating Corneal Epithelial Homeostasis

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Purpose. To provide a comprehensive study of the biological role and therapeutic potential of six endogenous epidermal growth factor receptor (EGFR) ligands in corneal epithelial homeostasis.

Methods. Kinetic analysis and dose response curves were performed by using in vitro and in vivo wound-healing assays. Biochemical assays were used to determine receptor expression and activity. Human tears were collected and quantitatively analyzed by multianalyte profiling for endogenous EGFR ligands.

Results. Epidermal growth factor receptor ligands improved wound closure and activated EGFR, but betacellulin (BTC) was the most efficacious promoter of wound healing in vitro. In contrast, only epidermal growth factor (EGF) promoted wound healing in vivo. Human tears from 25 healthy individuals showed EGFR ligands at these average concentrations: EGF at 2053 ± 312.4 pg/mL, BTC at 207 ± 39.4 pg/mL, heparin-binding EGF at 44 ± 5.8 pg/mL, amphiregulin at 509 ± 28.8 pg/mL, transforming growth factor-α at 84 ± 19 pg/mL, and epiregulin at 52 ± 15 pg/mL.

Conclusions. Under unwounded conditions, only EGF was present at concentrations near the ligand's K_d for the receptor, indicating it is the primary mediator of corneal epithelial homeostasis. Other ligands were present but at concentrations 11- to 7500-fold less their K_d, preventing significant ligand binding. Further, the high levels of EGF and its predicted binding preclude receptor occupancy by exogenous ligand and can explain the discrepancy between the in vitro and in vivo data. Therefore, therapeutic use of EGFR ligands may be unpredictable and impractical.

Keywords: EGFR, EGF, betacellulin, HB-EGF

The human cornea plays a critical role in the refraction of light onto the retina as well as protecting the eye against external agents, such as microbes, viruses, and debris. Maintenance of this multilayered tissue is imperative for proper vision; perturbation of corneal integrity is the second leading cause of blindness worldwide.[1,2] Within the cornea, the epithelial layer provides the first line of defense against environmental insults and its structural integrity is a key component to this function.

Physical injury is a common cause of corneal epithelium disruption; however, frequently nontraumatic alterations in corneal homeostasis arise as a consequence of a systemic disease, an eye disease, or from a side effect of a locally or systemically administered drug. For instance, reoccurring corneal abrasions and erosions are prevalent among patients suffering from diabetes mellitus[3] and patients taking Erlotnib for lung cancer[4] or Cetuximab for colorectal cancers.[5] Despite the frequent occurrence of corneal wounds, there are no clinically available drugs to promote corneal wound healing. Understanding the molecular mechanisms that regulate the maintenance of a fully functional corneal epithelium can lead to treatments that will alleviate patient discomfort, reduce the opportunity of infection, and limit the potential for loss of sight.

Superficial corneal epithelial wound healing is a quick and efficient assay used to track the processes that lead to a fully differentiated epithelial layer. Changes in corneal epithelial homeostasis can often occur slowly, with subtle changes happening over long periods of time. However, when the epithelial layer is removed, one can easily track cell migration, proliferation, and differentiation that is necessary to re-establish epithelial thickness and restore homeostasis. Study of this process may lead to better understanding of the cellular mechanisms involved in establishing and maintaining healthy corneal epithelial homeostasis.

While many growth factors and cytokines have been shown to contribute to corneal wound healing,[6–10] epidermal growth factor receptor (EGFR) signaling has been found to be both necessary and sufficient for corneal epithelial migration, proliferation, and differentiation.[4,6,11] The EGFR is a prototypic tyrosine kinase receptor and is part of a larger family of ErbB receptors. It shares characteristics with the three other members, ErbB2, ErbB3, and ErbB4, which include extracellu-

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lar ligand binding sites, intracellular kinase domains, and tyrosine-rich regions. Epidermal growth factor receptor activation is characterized by ligand binding that triggers dimerization of receptors, activation of the kinase domain, and subsequent transphoshorylation of the tyrosine residues within the receptor dimer. These phoshotyrosines serve as docking sites for downstream signal proteins (effectors), whose activities integrate to induce the previously discussed changes in cell biology: migration, proliferation, and differentiation.

Despite strong experimental evidence indicating the EGFR's role in corneal epithelial homeostasis, the clinical use of one of its ligands, epidermal growth factor (EGF), to promote wound healing has yielded mixed results. Several studies suggest improved healing,[12,13] whereas others show no significant improvement.[14,15] This has led us to investigate the role of other ligands in corneal wound healing. Along with EGF, there are six additional endogenous EGF-like ligands that are known to bind EGFR: betacellulin (BTC), transforming growth factor-α (TGFα), amphiregulin (AR), heparin-binding EGF (HBE), epiregulin (EPR), and epigen. Although many studies use EGF to stimulate the EGFR experimentally, there are studies that suggest HBE elicits better wound healing responses,[16,17] and TGFα and HBE are upregulated in response to corneal wounding.[17,18] To date, there has not been an inclusive study assessing the role of these other EGFR ligands in corneal epithelial homeostasis and wound healing.

This work provides a comprehensive examination of six EGFR ligands, using both in vitro and in vivo assays. This study identified BTC as the most efficacious mediator of in vitro corneal wound healing. However, in vivo analysis revealed that EGF is better at promoting corneal epithelial wound healing. An analysis of human tears indicated that while multiple EGFR ligands were detected, EGF is the only ligand that is present in concentrations that would predict a significant level of receptor occupancy. Further, the high levels of endogenous EGF likely prevent binding of other EGFR ligands. Thus, we conclude that the high level of basal EGF in tear fluid promotes EGFR-mediated corneal epithelial homeostasis and precludes the pharmacologic use of EGFR ligands therapeutically.

Materials and Methods

Cell Culture

Human telomerase-immortalized corneal epithelial cells (hTCEpi) were obtained from Geron Corp. (Menlo Park, CA, USA) and described previously.[19] Cells were maintained in growth media (Defined Keratinocyte Media with growth supplement; Life Technologies Corp., Grand Island, NY, USA) containing 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in 5% CO_2. Human corneal epithelial cells (HCECs) were cultured, as previously described,[20] from corneas that were unusable for transplantation (Oklahoma and Kentucky Lion's Eye Banks). Cells were plated on fibronectin (AthenaES, Baltimore, MD, USA)–coated tissue culture dishes and maintained in growth media at 37°C in 5% CO_2. Use of human tissue adhered to the tenets of the Declaration of Helsinki.

Ligand Treatment, Cell Lysates, and Immunoblotting

Cells were plated and grown to from 90% to 100% confluency and treated with equivalent molar concentrations of recombinant human EGFR ligands as noted in the figure legends. Ligand sources were as follows: EGF and BTC from Prospec (Rehovot, Israel); AR and HBE from R&D Systems (Minneapolis, MN, USA); and TGFα from Leinco Technologies (St. Louis, MO, USA). Cell lysates were generated as previously described.[21] Cells were harvested in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride), soluble protein concentration was assessed by BCA assay (Pierce, Rockford, IL, USA), and samples were diluted in SDS-sample buffer. Equivalent amounts of protein (indicated in the figure legends) were separated by SDS-PAGE, transferred to nitrocellulose, and detected with the indicated antibody. Antibody sources were as follows: EGFR (SC-03) from Santa Cruz (Dallas, TX, USA); EGFR phosphotyrosine 1068 and 998 (pY1068, 2234; pY998, 2641) from Cell Signaling (Danvers, MA, USA), and α-tubulin (T-6199) from Sigma-Aldrich Corp. (St. Louis, MO, USA).

In Vitro Wound-Healing Assay

This model was originally developed by Barbara Safiejko-Mroczka at the University of Oklahoma Department of Zoology (Norman, OK, USA). Silicone elastomer base (Sylgard 184 Elastomer; Dow Corning, Midland, MI, USA) was made per manufacturer's directions. Five 2-mm diameter silicone plugs/well were placed directly onto the bottom of a 6-well tissue culture plate and spaced at least 2 mm apart. Cells were plated at a concentration of 600,000 cells/well and incubated for 48 hours. Plugs were removed to create a 2-mm diameter acellular area and the remaining cells were washed twice with PBS. Serum-free media with or without growth factors (AR, BTC, EGF, HBE, or TGFα) or AG1478 (Cayman Chemical, Ann Arbor, MI, USA) was added. The acellular area was photographed at the indicated times with Nikon Eclipse Ti microscope with an ×4 objective by using NIS-Elements AR Acquisition software (Nikon Instruments, Inc., Melville, NY, USA). The remaining acellular area at each time point was quantified by using ImageJ software[22] (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Data were plotted as the percentage of the initial area that was covered (wound healed) at each time point, the percentage of the remaining wound at each time point (open wound), or as the fold change in wound closure.

Isolation of Human mRNA/Semiquantitative PCR

Ribonucleic acid was extracted as previously described[23] and modified.[24] Semiquantitative PCR was performed by using 1 U *Taq* polymerase (Crimson Taq; New England Biolabs, Ipswich, MA, USA) and 5 μL cDNA per 20-μl reaction. Reactions were run for 30 cycles (95°C for 30 s/59°C for 30 s/72°C for 40 s). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Polymerase chain reaction products were separated by using 3% agarose gel electrophoresis and stained with ethidium bromide before imaging.

Isolation of Mouse mRNA

Ribonucleic acid was isolated from mouse corneal epithelia, mouse heart (positive control), and human cornea epithelial cell line (hTCEpi) with RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following manufacturer's instructions. Messenger RNA was reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Life Technologies) as described by manufacturer. To determine whether ErbB mRNA was expressed in mouse corneal epithelia, we purchased predeveloped/validated Taqman assays (EGFR: MM00433023_M1; ErbB2: MM00658541_M1; ErbB3: MM01159999_M1; ErbB4: MM01256793_M1) from Life Technologies and followed the manufacturer's protocol. Polymer-

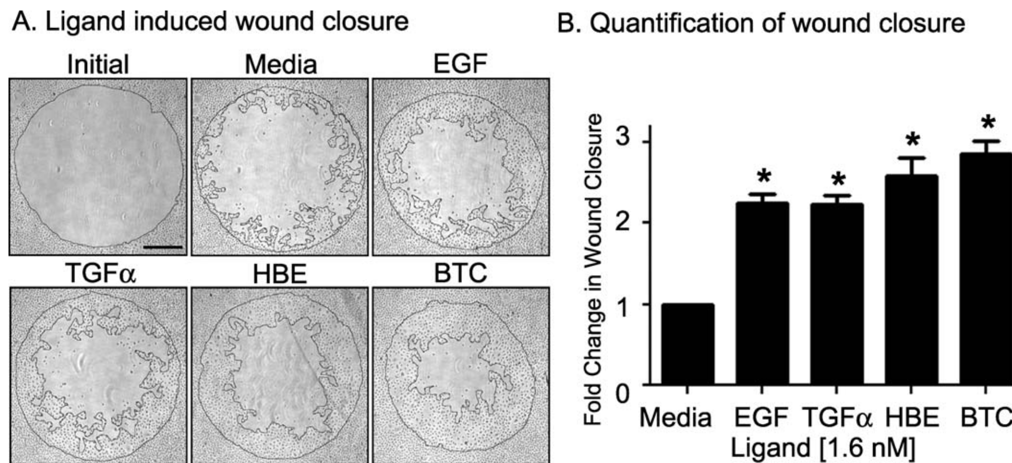

Figure 1. Epidermal growth factor receptor ligands promote variable wound-healing responses in vitro. (**A**) Human telomerase-immortalized corneal epithelial cells were plated around 2-mm-diameter silicone plugs that, when removed, created an acellular wound area to monitor healing. Cells were photographed at 0 and 16 hours after treatment with serum-free media without (Media) or with EGFR ligands (AR, EGF, TGFα, HBE, and BTC) at a concentration of 1.6 nM. Photographs were then used to trace, measure, and quantify the area of the initial wound (*outer circle*) and the remaining wound (*inner circle*). *Scale bar*: 500 μm. (**B**) Quantification of the fold change in wound healing elicited by each ligand compared to no ligand (Media), using ImageJ software. Each experiment yielded two to five data points per treatment and was performed three times. Data were analyzed by one-way ANOVA with a Tukey post hoc test. Each *bar* represents the mean ± SEM. $^*P < 0.001$ compared to no ligand (Media).

ase chain reaction products were run on a 3.5% Metaphor agarose (Lonza, Walkersville, MD, USA) gel and visualized with ethidium bromide.

In Vivo Mouse Corneal Wound Healing

Adult female C57BL6/J mice (Jackson Laboratory, Bar Harbor, ME, USA) between the ages of 8 and 10 weeks were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg; Butler Schein, Dublin, OH, USA). The central epithelium was demarcated with a 1.5-mm-diameter biopsy punch and removed with a 0.5-mm burr by using the AlgerbrushII (Alger Company, Inc., Lago Vista, TX, USA), taking care not to disrupt the basement membrane.[25] Eyedrops containing PBS with or without EGF, BTC, TGFα, AR, or HBE (16 nM) were applied to the wound. At each time point (0, 16, 24, 40 hours) the corneal wounds were visualized by using sterile fluorescein sodium ophthalmic strips USP (Fluorets, Chauvin Laboratory, Aubenas, France) dampened with sterile PBS. Wounds were examined and photographed at ×3 magnification with a stereoscopic zoom microscope (SMZ1000; Nikon, Tokyo, Japan) equipped with a digital sight DS-Fi2 camera (Nikon). The wound areas were measured by using ImageJ software. All treatment of animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC No. 12046).

Tear Collection and Analysis

Tears were collected from 25 self-identified healthy individuals with no history of ophthalmic problems, ranging in age from 22 to 45 years. Tear Flo test strips (HUB Pharmaceuticals, Rancho Cucamonga, CA, USA) were placed in the lower eyelid and remained until saturated (<10 minutes). Tear fluid was extracted from the strip by centrifugation and then frozen. Samples were sent for analysis of the presence of the indicated ligands by Multi-Analyte Profiling (Myriad RBM, Austin, TX, USA). Our investigation was conducted by following the tenets of the Declaration of Helsinki and was approved by the University of Louisville Institutional Review Board (IRB No. 13.0045). All subjects provided pretesting verbal and written informed consent.

Results

EGFR Ligands Significantly Improve In Vitro Wound Healing in Human Corneal Epithelial Cells

To examine the healing potential of other endogenous EGFR ligands, we used an in vitro wound-healing assay. Using immortalized corneal epithelial cells (hTCEpi), we created an initial acellular area (Fig. 1A, Initial) that can be used to monitor the rate of closure in response to recombinant human ligands. Shown are representative images for each ligand with the initial wound marked (Fig. 1A, outer line) along with the leading edge of cells in the acellular area (Fig. 1A, inner line). Quantification of the fold change in wound closure revealed that the EGFR ligands produced a range of effects that significantly improved wound closure compared to serum-free media alone (Fig. 1B). Of the ligands tested, HBE and BTC had the greatest effect on wound closure, prompting us to investigate them further.

BTC and HBE Are More Efficacious Mediators of Wound Healing Than EGF

To determine if BTC and HBE are pharmacologically better activators of wound healing, time course and dose response analyses were performed by using the in vitro wound-healing assay. Images taken every 4 hours were used to assess the percentage of remaining wound at each time point (Fig. 2A). Heparin-binding EGF and BTC showed a trend toward improvement over EGF at every time point, with a significant improvement by 16 hours (Figs. 2B, 2C). Dose response curves showed that all three ligands reached their E_{max} at 1.6 nM where BTC was the most efficacious followed by HBE then EGF (Fig. 2D). The half maximal effective concentration (EC_{50}) of EGF, HBE, and BTC is 0.07, 0.05, and 0.05 nM, respectively, indicating no difference in the potency of the

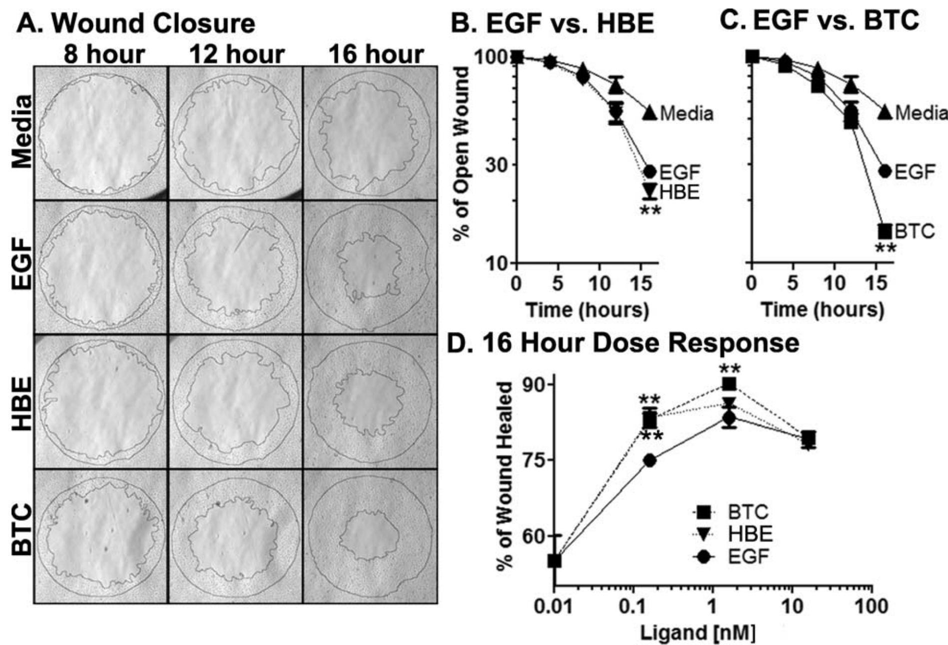

FIGURE 2. Betacellulin and HBE are more efficacious at promoting wound healing than EGF. Human telomerase-immortalized corneal epithelial cells were plated around 2-mm-diameter silicone plugs that, when removed, created an acellular wound area to monitor healing. (**A**) Cells were photographed at various time points after treatment with or without EGF, HBE, and BTC at a concentration of 1.6 nM. Photographs were then used to trace, measure, and quantify the area of the initial wound (*outer circle*) and the remaining wounds at 8, 12, and 16 hours (*inner circle*). (**B**) Quantification of wound healing mediated by EGF and HBE over time. (**C**) Quantification of wound healing mediated by EGF and BTC over time. (**D**) Quantification of ligand-mediated wound healing at varying concentrations of ligands after 16 hours. In all instances, each experiment yielded two to five data points per time point per ligand and was performed three times. Data were analyzed by using a paired Student's *t*-test. Each *graph point* represents the mean ± SEM. $^{**}P < 0.05$ compared to EGF.

ligands. This suggests that BTC is the most efficacious mediator of in vitro corneal wound healing with no change in potency.

BTC and EGF Work Through EGFR and Independently of ErbB4

Epidermal growth factor, HBE, and BTC are all activating ligands for the EGFR; however, BTC and HBE can also activate ErbB4, another ErbB receptor family member.[26,27] Additionally, ErbB4 has previously been reported to be expressed in the rat corneal epithelium.[28] On this basis, we wanted to determine if ErbB4 was playing a role in the wound-healing process.

We began by looking for the presence of ErbB4 protein. Immortalized cells can differ in their profile of gene expression; therefore, both primary HCECs and the immortalized hTCEpi cells were examined. Whole cell lysates of HCECs and hTCEpi cells were immunoblotted for the presence of ErbB4 (Fig. 3A); MCF-7 cell lysates were used as a positive control for ErbB4.[29] ErbB4 protein was not detected in either primary or immortalized corneal epithelial cells, but it was present in the MCF-7 control cells. It should be noted that in all cell types tested, there was a strong nonspecific band running below the expected ErbB4 protein. ErbB4 small interfering RNA (siRNA) experiments confirmed the higher band as ErbB4 since siRNA had no effect on the lower band, while it decreased the protein levels in the upper band in a dose-dependent manner (data not shown). Protein for all other ErbB family members was detected.

To confirm the absence of the receptor was not due to the limits of detection of the immunoblot, we followed up with a more sensitive semiquantitative PCR assay to determine if ErbB4 mRNA was present (Fig. 3B). ErbB4 mRNA was detected in the positive control MCF-7 cells, but not in the corneal immortalized cells or in primary corneal cells (four samples shown, seven samples tested). Messenger RNA was also detected for ErbB1, ErbB2, and ErbB3 at varying levels. Together, these data suggest that ErBb4 receptors are absent from human corneal epithelial cells. Therefore, HBE- and BTC-mediated wound closure is working independently of ErbB4.

Next, we wanted to determine whether HBE and BTC wound closure is mediated exclusively through the EGFR. Using the EGFR inhibitor AG1478, we monitored wound closure in the in vitro wound-healing assay. After 16 hours, there was complete inhibition of wound closure when AG1478 was added, independent of any ligand (Fig. 3C). Immunoblots of hTCEpi cells treated with EGF or BTC, along with varying concentrations of the inhibitor, showed that as AG1478 increased, the amount of EGFR phosphorylation decreased while total levels of EGFR were unchanged (Fig. 3D). Similar results were seen with HBE (data not shown). These data suggest that EGFR activity is required for EGF-, HBE- and BTC-induced wound closure in corneal cells.

EGF Is the Only Ligand That Improves In Vivo Wound Healing

With the improvement of wound healing seen in vitro, we wanted to see if the EGFR ligands would be more efficacious in vivo. In mice, we marked a 1.5-mm-diameter wound and removed the epithelium within the mark.[25] Eyedrops containing PBS with or without ligand were added to the wound. Photographs of the wound were taken at various time points, using fluorescein staining, and representative wounds at 24 hours are shown (Fig. 4A). Quantification of the wounds over time showed each ligand had variation in its kinetics of healing.

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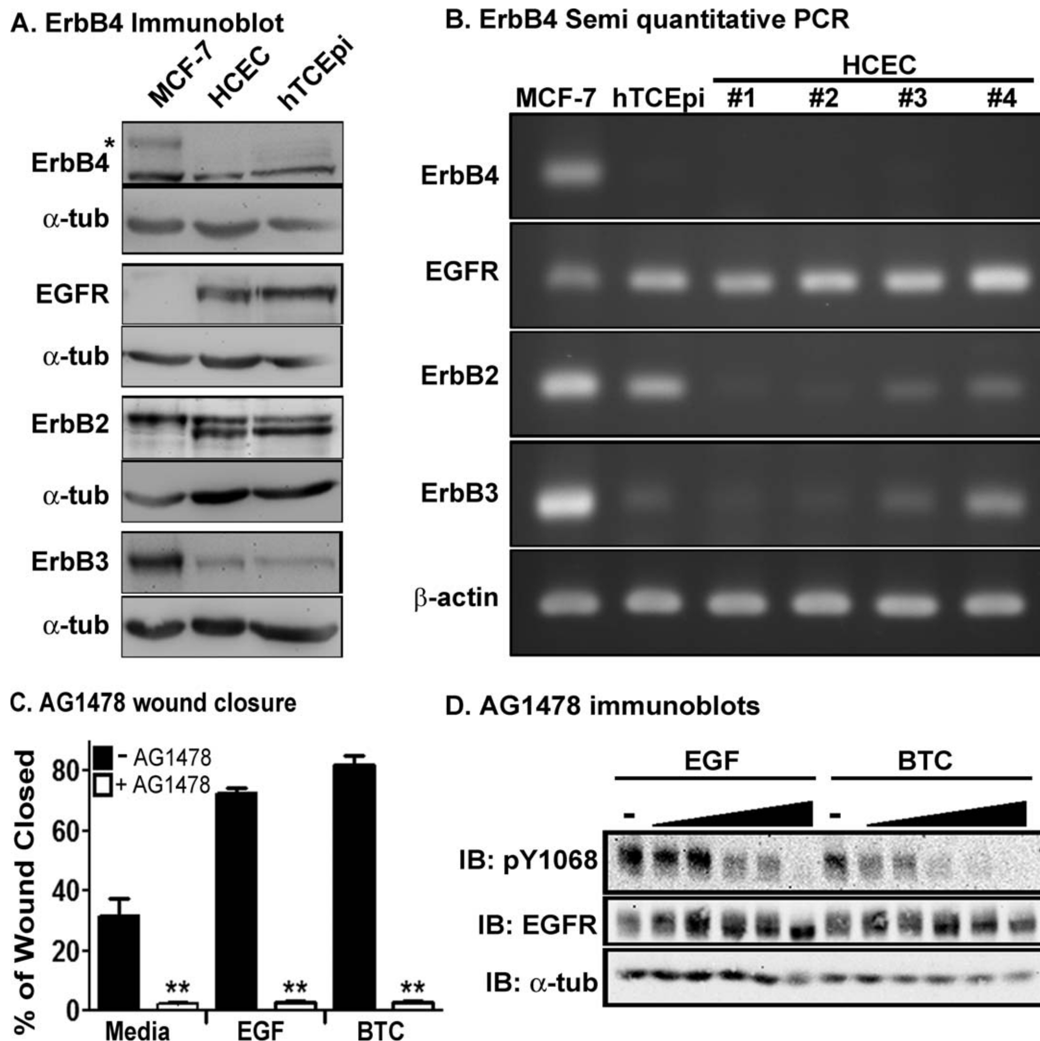

FIGURE 3. Betacellulin-induced epithelial migration works through EGFR and independently from ErbB4. (**A**) Cell lysates were prepared from MCF-7 cells, hTCEpi cells, or primary HCECs. Cell lysates (40 μg) were resolved by 6.0% SDS-PAGE and immunoblotted with antibodies for ErbB4, EGFR/ErbB1, ErbB2, ErbB3, or α-tubulin. Shown is a representative blot repeated at least three times. (**B**) Ribonucleic acid was harvested from MCF-7 cells, hTCEpi cells, or primary HCECs from four different donors (Nos. 1–4). Semiquantitative PCR was used to determine whether EGFR, ErbB2, ErbB3, ErbB4, or β-actin was present. Following the PCR reaction, samples were run on a 3% agarose gel, stained with ethidium bromide, and imaged. Shown are representative data from four of seven different donors. (**C**) Human telomerase-immortalized corneal epithelial cells wound healing was assayed and quantified as described previously. Cells were incubated for 16 hours with media containing no additions, EGF (1.6 nM), or BTC (1.6 nM) in the presence or absence of an EGFR inhibitor (AG1478 [1 μM]). Shown is the mean percentage of wound healing ± SEM ($n = 3$) $^{**}P < 0.01$ compared to no AG1478. Data were analyzed by using a paired Student's *t*-test. (**D**) After 2-hour incubation with 0, 0.01, 0.03, 0.1, 0.3, and 1 μM AG1478, hTCEpi cells were treated with 1.6 nM ligand for 15 minutes. Cell lysates were prepared and 20 μg resolved by 7.5% SDS-PAGE and immunoblotted with antibodies for EGFR site-specific phosphorylation (pY1068), total EGFR (EGFR), α-tubulin (α-tub).

Epidermal growth factor had a faster initial rate of healing that tapered off after 24 hours and HBE had a fairly consistent rate of healing over time, whereas BTC, TGFα, and AR had slower rates of healing to start but after 24 hours the rate improved (Fig. 4B). But a closer look at the 24-hour time point revealed that only EGF significantly improved wound healing (Fig. 4C). Other ligands had no statistically different effect from PBS.

EGFR Ligands Activate EGFR in Both Human and Mouse Cells

We next wanted to reconcile the differences in ligand efficacies in the in vitro and in vivo assays. One explanation is that there are differences in ligand–receptor interactions for the human cells used in in vitro experiments versus the mouse corneal epithelial cells in the in vivo analysis. Alternatively, there may be other growth factors present in the in vivo system that mitigate the effect of exogenous growth factors.

To address potential species differences, we determined whether the EGFR ligands were able to activate the EGFR in both human primary (HCEC; Fig. 5A) and immortalized (hTCEpi; Fig. 5B) corneal epithelial cells, as well as mouse embryonic fibroblast cells (MEFs; Fig. 5C). Using both primary and immortalized human cells, we can be certain that the receptors can be activated in a similar fashion even after the immortalization process. The MEF cells were used as control cells, as they are known to express the EGFR,[30,31] and are more amenable to culturing for dose response analysis than primary mouse corneal cells. Using an antibody specific to phosphorylation of tyrosine 1068 of the EGFR (pY1068), we detected a dose-dependent activation of the receptor by EGF, TGFα, HBE, and BTC in both human types of cells. We were unable to detect any phosphorylation in response to AR, even after a

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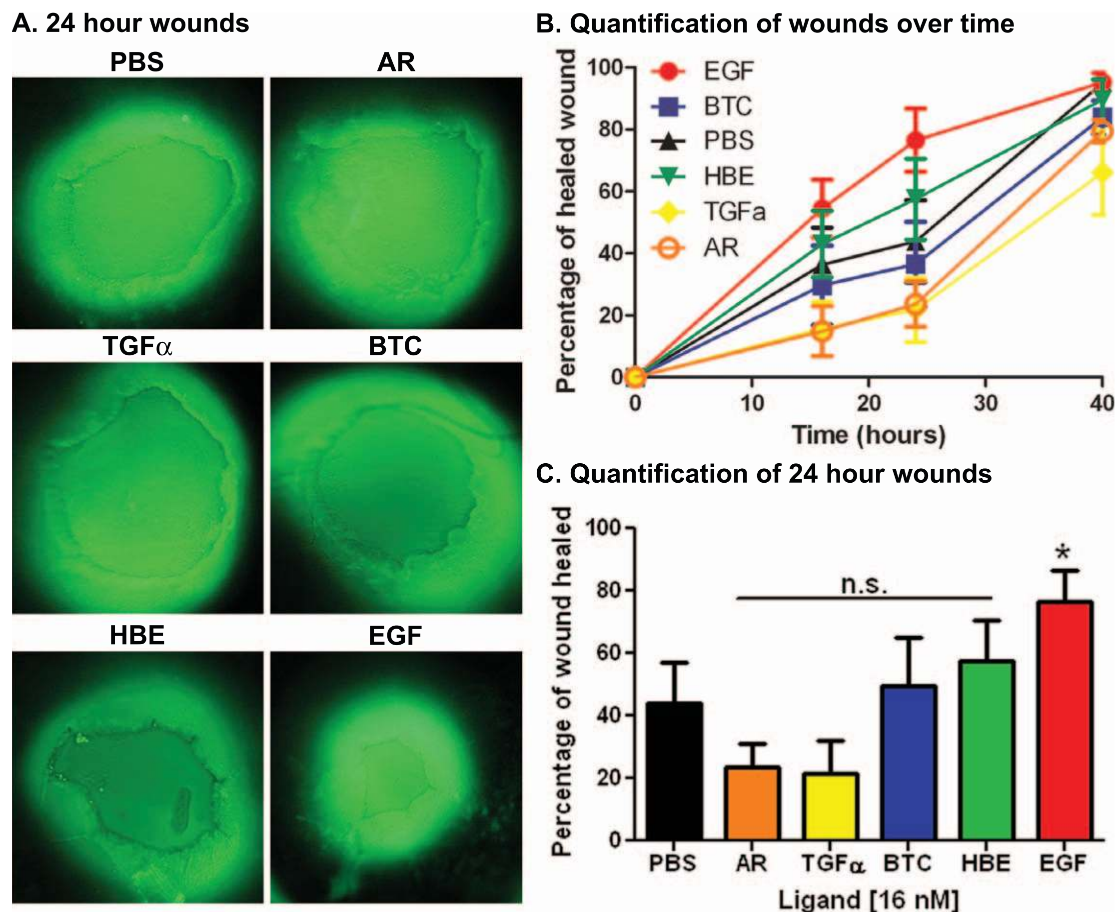

FIGURE 4. Epidermal growth factor improves in vivo corneal wound healing. Corneal epithelial layers in female C56BL6/J mice were removed within a 1.5-mm area as described in Materials and Methods. Wounds were imaged with fluorescein and treated with the indicated EGFR ligands. (**A**) Representative photographs of the corneal wounds 24 hours after wounding and treatment with the indicated ligand. (**B**) Quantification of wound healing over time after treatment with each ligand. (**C**) Quantification of the wounds at 24 hours. Data were analyzed by using a two-way ANOVA with a Bonferroni post hoc test. Each *graph point* represents the mean ± SEM. $^*P < 0.05$ compared to PBS.

long exposure. This is most likely due to the low predicted affinity of AR to the EGFR.[32] A minimum concentration of ~2 µg (22 nM) is necessary to detect AR-induced phosphorylation of the EGFR in hTCEpi cells (data not shown). In the long exposure image, there is a smear of phosphorylated EGFR at the higher doses. This is indicative of ubiquitylated receptor and is consistent with the degradation seen in the total EGFR protein. Furthermore, using an antibody that can detect phosphorylation of tyrosine 998 (pY998) in mice, we saw a similar pattern of dose-dependent phosphorylation in the MEF cells.

Since there have been reports indicating the presence of ErbB4 in rats,[28] which we did not see in our human cells, we next looked at the presence of the ErbB receptors in the mouse cornea. We extracted mRNA from mouse corneal epithelial cells and amplified it with real-time PCR. Mouse heart tissue was used as a positive control as all ErbB receptors have been shown to be present.[33] Messenger RNA levels of the EGFR, ErbB2, and ErbB3 were comparable to those in the heart. ErbB4 levels were present in the corneal epithelium, but at levels that were approximately 250-fold less than what were observed in the heart (Supplementary Fig. S1).

Additionally, protein sequences of the human and mouse ligands were compared by using BLAST alignments (Supplementary Fig. S2). The full-length precursor forms of each ligand were analyzed for amino acids that were identical (precursor identity) or a combination of identical and positive (different amino acid but similar characteristics, precursor positive). Of the ligands, EGF showed the least number of identical amino acids at only 66%. However, that number improved to 70% when we aligned only the EGF-like domain,[34] which is the portion that binds the EGFR. When comparing the EGFR, there is considerable identity between the human and mouse receptors. This suggests that, while there are species differences between the amino acid compositions of the proteins, they are not enough to interfere with ligand binding. This is supported by our data since EGF, with the lowest identity, can phosphorylate the EGFR at comparable levels in both species. Together these data suggest that the conflicting results seen with the in vivo and in vitro experiments are unlikely due to species differences, consistent with previous studies that show human ligands can activate mouse EGFRs in other cell types.[35,36]

EGFR Ligands Are Found in Varying Concentrations in Human Tear Samples

We next wanted to determine if high levels of endogenous EGFR ligands in tear fluid could be impacting the wound healing of the exogenous ligands. Owing to complexities of collecting mouse tears, we collected human tear samples. Previous reports on tears have only looked at the levels of

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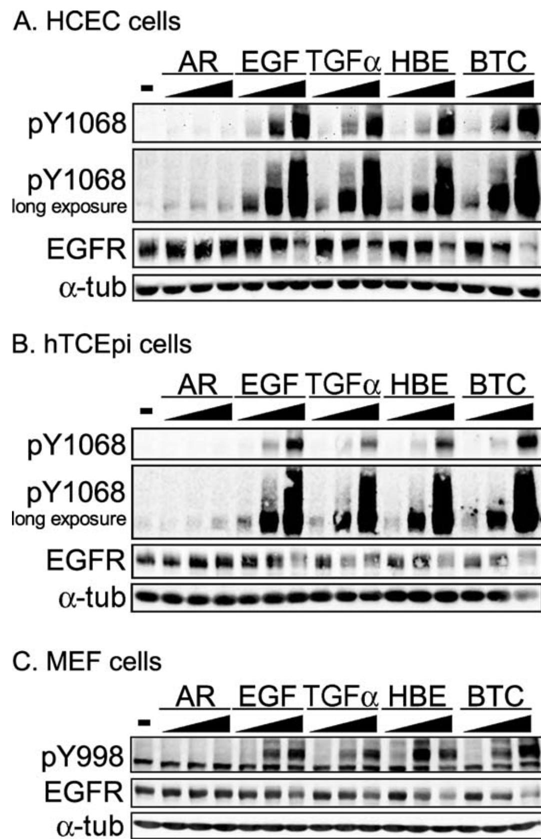

FIGURE 5. Epidermal growth factor receptor ligands can activate EGFR phosphorylation in a dose-dependent manner in human and mouse cells. (**A**) Primary HCECs, (**B**) hTCEpi and (**C**) MEFs were treated for 15 minutes with serum-free media without (Media) or with an EGFR ligand (AR, EGF, TGFα, HBE, and BTC) at concentrations of 0.16, 1.6, and 16 nM. Cell lysates (20–40 μg) were resolved by 7.5% SDS-PAGE and immunoblotted with antibodies for EGFR phosphorylation (pY1068 or pY998), total EGFR protein, and α-tubulin (loading control).

EGF[37,38] and TGFα[39] with no indication about the presence of the other EGFR ligands or the relative concentrations of the ligands. For this study, we used self-identified healthy volunteers (sample size = 25) with no prior history of ophthalmic problems. We collected the tears by using Tear Flo strips and used Multi-Analyte profiling to detect the levels of six EGFR ligands: AR, BTC, EGF, EPR, HBE, and TGFα (Fig. 6). Epigen, the seventh EGFR ligand, could not be tested owing to the absence of usable antibodies.

As expected with human samples, the concentration of growth factors was variable. However, we were surprised at the variability in presentation of individual growth factors. For instance, while EGF was found in every sample, EPR was present in only two samples (Fig. 6A). The number of ligands in each sample also varied; every sample had two or more ligands, but only one sample had all six of the ligands (Fig. 6B). There were no apparent trends to predict which combination of growth factors would be present.

To determine average concentration, only samples with detectable levels of that particular ligand were used in the calculation (Fig. 6C). Epidermal growth factor and TGFα had average concentrations of 2053 and 83.8 pg/mL (12.83 and 0.524 nM), respectively, which is in relatively close agreement with previously published data.[37–39] However, when one compares the individual ligand concentrations with the reported K_d, only EGF was present at a concentration sufficient to induce receptor activity (Fig. 6D) (sources for K_d values: BTC[40] using human ligand and mouse EGFR; TGFα,[41] HBE,[42] and EGF[43] using human EGFR and human ligand; AR and EPR[32] using predicted affinity, as experimental data was unavailable). Since these tear samples were taken from healthy, noninjured eyes, this suggests that EGF is the primary EGFR ligand that contributes to corneal epithelial homeostasis.

DISCUSSION

This is the first report that comprehensively examines endogenous EGFR ligands in promoting corneal epithelial homeostasis. Using both in vitro and in vivo assays, this study directly compared the therapeutic potential of EGFR ligands in corneal wound healing. With in vitro assays, all EGFR ligands improved wound closure, and BTC emerged as the most efficacious ligand for corneal wound healing. However, only EGF showed improvement in healing of in vivo corneal wounds. An analysis of growth factor levels in the tear fluid of healthy individuals not only sheds light on how corneal epithelial homeostasis is maintained, but also indicates why the use of exogenous growth factors yields mixed results when used clinically.

Using an in vitro wound-healing assay, we saw significant improvement of wound closure with all the EGFR ligands (Fig. 1). This was not a surprising result, since there have been many reports of EGFR ligands having effects in the processes involved in corneal wound healing, historically EGF,[35,44,45] and more recently, TGFα[20] and HBE.[17] But in each case, there are few data indicating how one ligand compares to other ligands, as these reports usually look at a single ligand at a time. By assessing several ligands simultaneously, we can establish the relative efficacy of the ligands and determine the best potential therapeutic candidates. Our studies showed HBE and BTC fared better than the other EGFR ligands and a more detailed analysis of these ligands revealed BTC could elicit the greatest wound-healing response (Figs. 1, 2).

Since HBE and BTC produced the greatest response, and both ligands can signal through ErbB4, we hypothesized that activation of this receptor population could be the mechanism behind the increased wound healing. However, we did not detect any ErbB4 protein or mRNA in immortalized or primary corneal epithelial cells (Fig. 3). These data indicate that ErbB4 is absent from human corneal cells, and therefore cannot mediate HBE- or BTC-driven effects of wound healing. Furthermore, pharmacologic inhibition of the EGFR completely abolished wound healing, indicating the EGFR is the primary mediator of EGF-, HBE-, and BTC-induced wound healing (Fig. 3). However, it cannot be overlooked that these data are in contrast to a report showing that ErbB4 is in the more basal layers of rat cornea and conjunctiva.[28] This group used the same antibody (SC-283; Santa Cruz) and this discrepancy could be either from nonspecific binding, similar to what we saw in our blots, or from species-dependent ErbB4 expression. The idea that there are species differences in ErbB4 expression is supported by the analysis of ErbB4 mRNA (Supplementary Data). ErbB4 mRNA was detected in mouse corneal epithelial cells, albeit ~250-fold less than what was observed in the mouse heart.

We predicted that HBE and BTC would also be more efficacious in mouse corneal wound healing. Surprisingly, only EGF showed any significant improvement and all of the other ligands were statistically indistinguishable from PBS treatment (Fig. 4). While we did detect ErbB4 mRNA in mouse cells, but not in human corneal cells, we do not know if that translates to active protein levels. Given that the addition of HBE and BTC,

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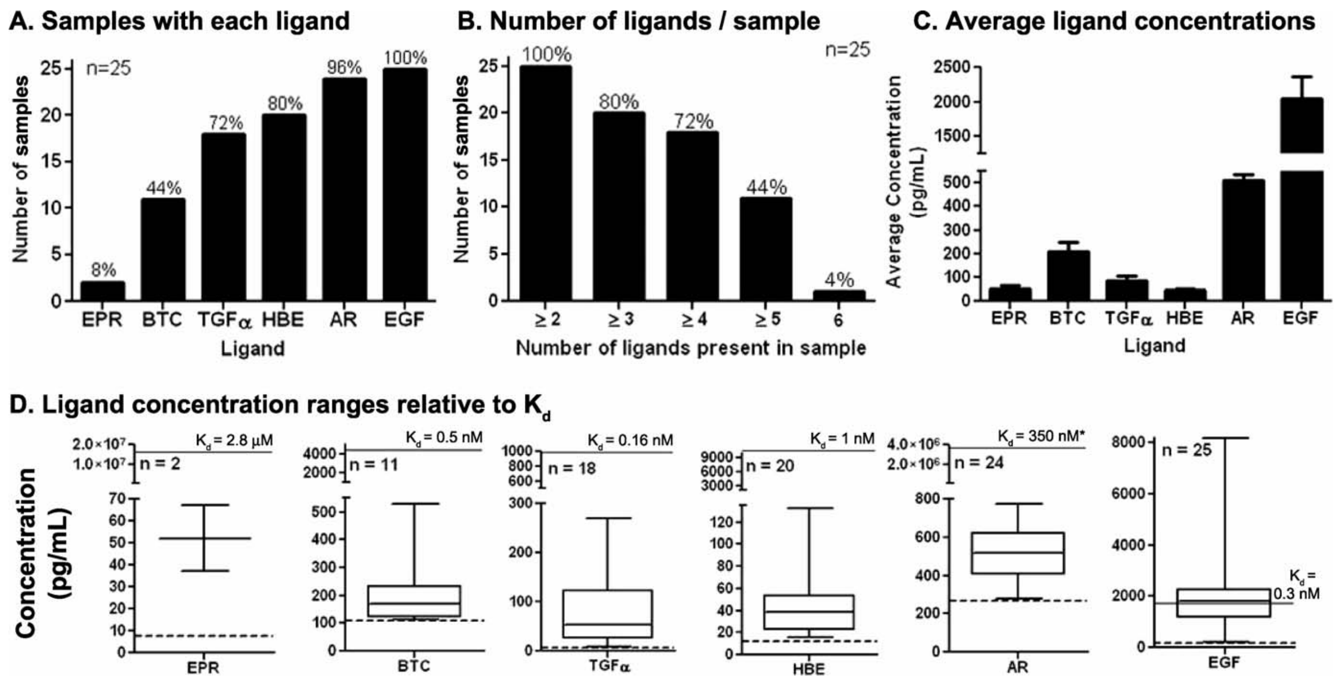

Figure 6. Analysis of endogenous EGFR ligands in human tear fluid. Tear Flo strips were placed in the lower eyelid of qualified and consented individuals and remained until saturated with tear fluid. The fluid was retrieved through centrifugation and analyzed by using multianalyte profiling. (**A**) The number of samples, out of 25, in which each ligand was detected. (**B**) The number of different ligands present within each sample. (**C**) Average concentrations of each ligand in the samples containing that ligand. (**D**) Range of ligand concentrations found in the samples, compared to the reported K_d. Concentrations were plotted by using a *box and whiskers graph*, where the *whiskers* indicate the highest and lowest sample concentration. The K_d for each ligand is represented with a *solid line* and the limit of detection for the assay is indicated with a *dotted line*. Sources for K_d values are noted in the text.

two ErbB4 activators, in the in vivo studies does not significantly change wound healing, even if the protein is present, our data suggest it does not play a role in mouse corneal wound healing. Alignment of the protein sequences of the ligands and the receptor revealed high levels of identity (Supplementary Table S1); all ligands were able to stimulate phosphorylation of both human and mouse EGFR when concentrations were at or above their K_d (Fig. 5). This is consistent with previous studies demonstrating human EGFR ligands are biologically active in other species, such as mice,[35] rabbits,[46,47] horses,[48] pigs,[17] and primates.[49] Therefore, we concluded that species differences could not account for the EGFR ligand differences between in vitro and in vivo studies.

Previously mentioned work examining EGFR ligands in corneal wound healing has relied heavily on in vitro or ex vivo experiments, which remove any endogenous ligand. Experiments done in vivo have only used EGF and are consistent with our data where EGF does show improvement. However, under these circumstances endogenous factors can affect exogenous ligands and attenuate their effectiveness. Therefore, we decided to look at endogenously produced EGFR ligands in the tear fluid. Earlier reports have looked at the levels of EGF and TGFα in human tears but not the occurrence of other EGFR ligands. Additionally, the aforementioned studies have used different methods of collection and analysis, making a true comparison of the two ligands difficult. Our study provided a comprehensive look at the presence and relative levels of EGFR ligands and allowed a direct comparison of multiple ligands in the same sample (Fig. 6).

Given the heterogeneity of the human population, the variability seen in our samples was not surprising. We did see consistency with EGF in that it was found in every sample and was usually the ligand of highest concentration. Yet, we saw no correlation between EGF levels and the presence or concentration of other ligands. For example, samples with low EGF did not show higher concentrations or increased number of other ligands.

When comparing the average ligand concentration in the tears to the concentration necessary to reach its K_d, we find that EGF is the only ligand present at values high enough to cause substantial receptor activation. This is consistent with EGF likely playing a role as a paracrine mediator of corneal epithelial homeostasis. While the other ligands are present, they are at such low levels that they are likely not contributing to paracrine EGFR stimulation. However, it does not preclude a role for these growth factors as mediators of autocrine or juxtacrine signaling. Regardless of the effects of these ligands in an in vitro system, EGF is in the tears at such high concentrations that to overcome the effects of EGF, the concentrations of any exogenous ligand would have to be exceedingly high. This suggests that using exogenous EGFR ligands for treatment of corneal epithelial wounds may be moot.

The limitations of this study must be considered. First, our sample size was relatively small. We assessed tears from 25 self-identifying healthy individuals and did not take into consideration other factors such as age, sex, contact lens use, allergies, or any other aspects that could potentially affect ligand concentrations. Second, the presence of EGFR ligands may be significantly altered in situations where the epithelium has been injured. Messenger RNA levels of TGFα, HBE, and AR, but not EGF, have been reported to change in response to corneal wounds, indicating that a wounding event may cause upregulation of EGFR ligands other than EGF.[11,18] Finally, further investigation is needed to identify the source of these ligands, as they may be produced by the corneal cells

themselves, by the lacrimal gland, as previously shown with EGF and TGFα,[39,50] or through cleavage of membrane-bound precursor growth factors on the epithelial cells. This information would benefit in the overall understanding of corneal homeostasis and repair.

These data argue that direct stimulation of the EGFR is an unreliable therapeutic target for restoration of a wounded cornea. However, given that EGFR activity is central to corneal epithelial homeostasis, it may be more effective to target other mechanisms that regulate EGFR signaling. One strategy would be to sustain activated receptors. Treatment with TGFα can divert stimulated EGFRs from degradation by recycling them back to the cell membrane[20]; this in turn leads to enhanced cell migration. However, given our in vivo work and tear fluid data, using TGF-α to stimulate the receptor would be ineffective. A more useful approach may be to antagonize molecules that regulate movement of the EGF–EGFR complex through the endocytic pathway to prevent degradation of the complex. Candidate molecules are the E3 ubiquitin ligase Casitas B-lineage lymphoma proto-oncogene (c-cbl)[51] (which ubiquitylates the EGFR, thereby targeting it for degradation), Tumor Suppressor Gene 101 (TGS101)[52] (which guides the ubiquitylated EGFR from the limiting membrane of the late endosome into the intraluminal vesicles), or the small-molecular-weight G-protein Rab7[21] (which regulates movement of the EGFR from the late endosome to the lysosome). The notion is that inhibiting these proteins will sustain the activity of EGFRs stimulated by the basal levels of EGF by causing the ligand–receptor complex to accumulate in the endocytic pathway.

An alternative strategy would be to directly activate the signaling pathways downstream of EGFR activation. Potential candidates that have been shown to influence corneal wound healing are effector proteins such as glycogen synthase kinase-3 (GSK3),[53] histone deacetylase 6 (HDAC6),[54] phosphatidylinositol-4,5-bisphosphate 3-kinase/protein Kinase B (PI3-K/Akt),[55] extracellular-signal–regulated kinases (ERKs), p38,[56] or nuclear factor κ-light-chain enhancer of activated B cells (NFκB),[57] transcription factors such as NFκB subtype–regulated CCCTC binding factor (CTCF),[57] and signaling intermediates such as reactive oxygen species[58] or lipoxin 4A.[56] Another option is to antagonize the activity of phosphatases that negatively regulate effectors that promote EGFR-mediated corneal homeostasis, such as mitogen-activated protein kinase phosphatase 1 (MKP-1/DUSP1) and dual-specificity phosphoprotein phosphatases 5 and 6 (DUSP5, DUSP6).[59,60]

Finally, since BTC is a more efficacious mediator of in vitro wound healing, understanding the mechanism behind its induction, especially compared to EGF, could reveal other potential targets useful for regulating EGFR signaling. An analysis of BTC–receptor interactions, BTC-induced receptor phosphorylation patterns, and effector activation may reveal additional targets not seen with EGF activation or provide evidence for further investigation into some of the aforementioned proteins.

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