Role of ErbB2 in Corneal Epithelial Wound Healing

Ke-Ping Xu, April Riggs, Yu Ding, and Fu-Shin X. Yu

Purpose. Human corneal epithelial cells (HCECs) were functionally depleted of erbB2 to elucidate its role in epidermal growth factor (EGF) receptor (EGFR) activation-dependent cell migration.

Methods. The retrovirus pBabe-5R, which encodes an erbB2 single-chain antibody with an endoplasmic reticulum (ER)–targeting sequence, and control pBabe-puro were used to infect THCE cells (an SV40-immortalized HCEC line). Several cell lines expressing 5R were selected along with a pBabe-puro control line. The depletion of erbB2 was verified by cell surface biotinylation of proteins, followed by streptavidin precipitation and subsequent detection of erbB2 by immunoblot analysis. Activation of erbBs was analyzed by immunoprecipitation using the phosphotyrosine antibody pY20, followed by Western blot analysis with erbB1 or erbB2 antibodies. Phosphorylation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3′-kinase (PI3K) was analyzed by Western blot with antibodies specific to phosphorylated proteins. Effects of erbB2 depletion on heparin-binding EGF-like growth factor (HB-EGF)–induced cell migration were determined by Boyden chamber migration assay and by scratch wound assay.

Results. Wounding induced erbB2 tyrosine phosphorylation. Expression of 5R encoding an erbB2 single-chain antibody with an endoplasmic reticulum–targeting sequence depleted the cell surface expression of erbB2 in HCECs. Wounding resulted in a rapid increase in the phosphorylation of erbB1 in both 5R-expressing and control cells, whereas wound-induced erbB2 phosphorylation in 5R-expressing cells was not detectable. Depletion of functional erbB2 attenuated the healing of scratch wounds in the presence of HB-EGF and impaired both chemotactic migration stimulated by HB-EGF and haptotactic migration toward a fibronectin-collagen I (3:1; FNC) coating mix. Expression of 5R affected both the intensity and the duration of wound-induced, EGFR-elicited ERK and PI3K activation. Inhibition of ERK and PI3K pathways in cultured porcine corneas impaired ex vivo epithelial wound healing.

Conclusions. ErbB2 serves as a critical component that couples erbB receptor tyrosine kinase to the migration machinery of corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2004;45: 4277–4283) DOI:10.1167/iovs.04-0119

The erbB family of receptor tyrosine kinases and their ligands have been shown to be involved in cell differentiation, proliferation, migration, and carcinogenesis.[1,2] There are four members of the erbB family: epidermal growth factor (EGF) receptor (EGFR) (also termed erbB1/HER1), erbB2/Neu/HER2, erbB3/HER3, and erbB4/HER4 (reviewed in Refs. 1,3,4). The EGFR ligands bind to the erbBs with different specificities. EGF, transforming growth factor-α, and amphiregulin bind exclusively to erbB1. Although neuregulin-1 and -2 bind only to erbB3, heparin-binding EGF-like growth factor (HB-EGF) binds to both erbB1 and -4.[1,5] Ligand binding drives receptor dimerization, leading to activation of the intrinsic tyrosine kinase and cross-phosphorylation of specific, C-terminal tyrosine residues (reviewed in Refs. 6,7) that provide docking sites for adaptor proteins, kinases, and phosphatases.[1,8,9] In this way, an array of downstream signaling cascades, including the Ras-Raf-MAP kinase pathway, the PI3K pathway, and the phospholipase C (PLC)-γ pathway, are then induced, depending on the identity of the erbB heterodimers, which is determined, in part, by the ligands.

erbB2 is most notable in that amplification of the erbB2 gene occurs in a variety of tumors, including 20% to 30% of breast cancer patients.[3] In fact, erbB2 amplification is used as an independent prognostic indicator of patient survival and is correlated with a number of adverse prognostic factors in breast cancer.[10,11] In vitro, in human breast cancer cell lines, ligand-dependent activation of erbB2 has been shown to promote cellular motility and invasiveness. ErbB-2 is closely related to EGFR/erbB-1, but unlike EGFR, erbB-2 is a ligandless receptor. Hence, erbB-2 is active only in the context of erbB heterodimers. Experimental data show that c-erbB-2 is the preferred dimerization partner of all erbB receptors[12] and increases the affinity of differentiation factor binding,[13] thereby amplifying and/or prolonging the signals elicited by growth and differentiation factors.[14–16] ErbB2-containing heterodimers also contribute to increased cell proliferation, migration, and resistance to apoptosis.[17] Recent studies have also provided evidence for erbB2 in re-epithelialization and epithelial wound healing.[18,19] Because erbB2 is widely expressed and is almost always present in the context of other erbB members, it has been difficult to determine the specific role of erbB2 signals in a given biological response.

We recently reported that wounding of epithelial cells resulted in EGFR activation in cultured porcine corneas and cultured human corneal epithelial cells (HCECs).[20] We showed that proteolytic release of HB-EGF generates an autocrine ligand for EGFR activation after wounding, and ectodomain shedding of HB-EGF and EGFR phosphorylation constitute initial signaling steps for corneal epithelial wound healing.[20] Furthermore, wound-induced EGFR activation elicits the ERK/MAPK pathway, the inhibition of which hampers epithelial wound closure. Thus, our studies and those of others[21] show that EGFR activation and subsequent intracellular signaling are required for corneal epithelial wound healing. However, the relative physiological importance of each erbB member remains unclear.

In this report, we used cells functionally devoid of erbB2 to directly study its role in eliciting intracellular signaling and in regulating cell migration during corneal epithelial wound healing. We provide evidence that erbB2 activation by erbB family receptors and their ligands is a fundamental event necessary for epithelial cell migration. Furthermore, we show that the impaired migratory response may be related to the reduced sig-

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naling of MAPK and PI3K, which resulted from erbB2 depletion in HCECs.

Materials and Methods

pBabe-5R and control plasmid pBabe-puro were kindly provided by Nancy E. Hynes (Friedrich Miescher Institute, Basel, Switzerland).[14] HB-EGF was purchased from R&D Systems (Minneapolis, MN). DMEM-F12 and defined keratinocyte-serum-free medium (SFM) were from Invitrogen-Gibco (Carlsbad, CA). Keratinocyte basal medium (KBM; lacking growth factors) was from BioWhittaker (Walkersville, MD). Fibronectin-collagen (FNC; 3:1 mixture) coating mix was from Athena Environmental Service, Inc. (Baltimore, MD). The EGFR inhibitor AG1478, the MEK inhibitor PD98059, and the PI3K inhibitor LY294002 were from Calbiochem (San Diego, CA). Monoclonal antibodies against ERK2 and phosphorylated ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT (also termed protein kinase B, a major substrate of PI3K) and anti-phospho-AKT were from Cell Signaling (Beverly, MA). Rabbit anti-erbB2 antibody-17 was from Labvision (Fremont, CA). NHS-LC-biotin (succinimidyl-6-(biotinamido) hexanoate), streptavidin agarose, and an enhanced chemiluminescence detection system were from Pierce Biotechnology (Rockford, IL). A Boyden chamber (48 wells) and polycarbonate membranes (14-μm pores) were purchased from Neuroprobe (Cabin John, MD) and Osmonics, Inc. (Livermore, CA), respectively. All other chemicals were purchased from Sigma-Aldrich.

Cell Culture and Gene Transduction

THCE cells, an SV40-immortalized human corneal epithelial cell line generously provided by Kaoru Araki-Sasaki,[22] were grown in a 1:1 mix of DMEM-F12 and defined keratinocyte-SFM in a humidified 5% CO_2 incubator at 37°C. Gene transfer was achieved by a retrovirus-mediated process. pBabe-5R and empty pBabe were used to transfect PT67 packaging cells, and puromycin-resistant cells were pooled. Conditioned media (24-hour culture) collected from the pooled PT67 cells were filtered through a 0.45-μm pore size syringe filter. The viral supernatants were mixed with defined keratinocyte-SFM (1:1). This mixture, supplemented with polybrene (8 μg/mL), was added to recipient cells that had been plated at 5×10^5 cells per 100-mm dish the day before. THCE cells were cultured in viral supernatants for 12 hours followed by 12 hours in defined keratinocyte-SFM. After three cycles of infection, the cells were subjected to selection in 2 μg/mL puromycin for 14 to 28 days. Individual clones for 5R infection were selected. For control infection, a pool of puromycin-resistant cells was obtained. The selected cells were then maintained in a defined keratinocyte-SFM/DMEM-F12 mix.

Cell Surface Biotinylation

The selected 5R and control cell lines cultured in 100-mm dishes were rinsed twice with PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then incubated with freshly prepared NHS-LC-biotin diluted in the same solution (1 mg/mL) for 5 minutes at room temperature. The reaction was quenched with 50 mM NH_4Cl, and the cells were lysed with a solution containing 1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA, and 0.2% BSA supplemented with protease inhibitors. Cell extracts were centrifuged to remove detergent-insoluble material, and detergent-soluble supernatant was incubated with immobilized streptavidin-agarose for 16 hours at 4°C to bind biotinylated proteins. Proteins bound to the agarose slurry were solubilized with Laemmli SDS sample buffer. The samples were then analyzed by SDS-PAGE and immunoblotted with anti-erbB2 or anti-EGFR antibodies.

Determination of EGFR, erbB2, ERK1/2, and AKT Phosphorylation

EGFR and ERK phosphorylation were assessed as described.[23] ErbB2 phosphorylation was determined by immunoprecipitation with pY20 antibody against tyrosine-phosphorylated proteins, followed by Western blot analysis with anti–erbB2 antibody. Phosphorylation of AKT, a major cellular substrate of PI3K, was determined by a phospho-AKT–specific antibody.

Western Blot Analysis

The cell lysates or samples immunoprecipitated in 1× SDS sample buffer were applied to a 5% to 15% gradient polyacrylamide gel and electrophoresed. The separated proteins were then transferred to a nitrocellulose membrane. The efficiency of protein transfer was assessed by staining the nitrocellulose membranes with ponceau S. The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour and then incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. Nitrocellulose membranes were washed three times with TBST and then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour. An enhanced chemiluminescence detection system was used to visualize the labeled protein bands.

Scratch-Wound Assay

5R-expressing or control pBabe cells were seeded on 12-well plates coated with FNC. After reaching subconfluence, the cells were starved with KBM overnight and wounded with a sterile 0.1- to 10-μL pipet tip (TipOne; USA Scientific, Ocala, FL) to remove cells by two perpendicular linear scratches. After washing away suspended cells, the cells were refed with KBM in the presence of HB-EGF (50 ng/mL). The progress of migration was photographed immediately or 24 hours after wounding, near the crossing point, with an inverted microscope equipped with a digital camera (SPOT; Diagnostic Imaging, Sterling Heights, MI).

Boyden Chamber Analysis for Cell Migration

A Boyden chamber was used to measure the migratory response of 5R cells to HB-EGF. Cells expressing 5R or control cells were starved overnight, detached by MEM containing 0.05% trypsin and 0.53 mM EDTA, and washed with 10% FBS in PBS to neutralize the trypsin. Polycarbonate membranes (14-μm pores; Osmonics Inc.) were coated with human FNC on the surface facing the lower chambers. A Boyden chamber (48 wells) was used, and the bottom wells were filled with KBM containing 50 ng/mL HB-EGF as a chemoattractant. The cells (3.6×10^5 per well) were placed into each of the top wells above the filter. The chambers were then incubated at 37°C in 5% CO_2 for 3 hours. After incubation, cells on the top of the filter were removed by scraping. The filter was then stained with a modified stain (Diff-Quik; Data International, Miami, FL). Epithelial cell migration activity was quantified as the number of migrated cells on the lower surface of the filter in six random fields of 400× magnification.

Corneal Wounding and Organ Culture

Porcine corneal organ culture and an ex vivo wound-healing assay were performed as described.[23] Briefly, a 4-mm epithelial wound made by demarcating an area on the central cornea was allowed to heal in a 5% CO_2 incubator at 37°C for 48 hours. To determine the role of EGFR-mediated signaling pathways, PD98059 or LY294002 were added to the MEM during organ culture. After 48 hours of incubation, the corneas were treated with Richardson's stain,[24] to mark the remaining wound area, and then photographed.

Results

ErbB2 Phosphorylation Induced by Epithelial Wounding

Activation of erbB2 through dimerization with other ligand-bound ErbB members results in phosphorylation of tyrosine residues in its cytoplasmic domain.[25,26] There are five known

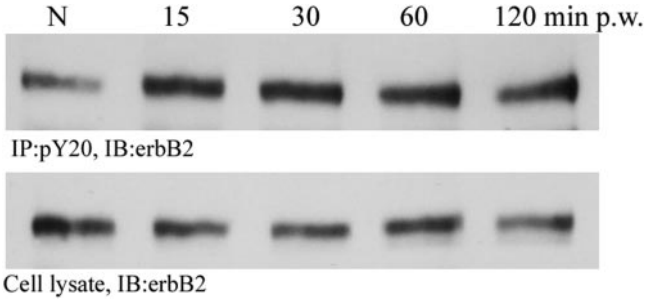

FIGURE 1. Time course of tyrosine phosphorylation of erbB2 in wounded THCE cells. THCE cells were cultured in 100-mm dishes and starved overnight. Cells were extensively injured by sequence comb scratching and incubated for different time points after wounding (p.w.). Wounded THCE cells were lysed, and 600 μg cell lysate from each time point was immunoprecipitated (IP) with 10 μg agarose-conjugated pY20 (phosphotyrosine-specific) antibodies, subjected to SDS-PAGE, and immunoblotted with anti-erbB2 antibody (*top*). To determine the cellular level of erbB2, 20 μg cell lysate from each sample was subjected to Western blot analysis with anti-erbB2 antibody (*bottom*). Results are representative of three independent experiments.

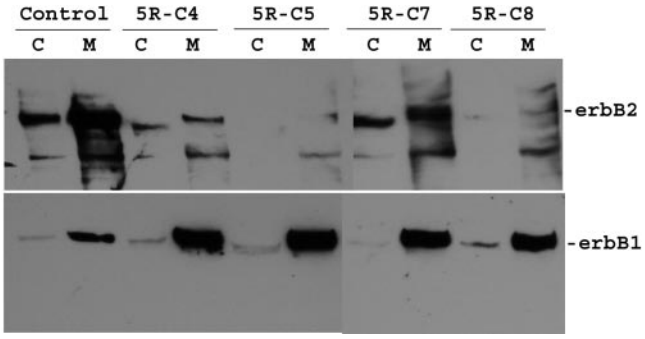

FIGURE 2. Detection of cell surface expression of erbBs by cell surface biotinylation in pBabe-5R cell lines. pBabe-puro (Control) and pBabe-5R cell lines (C4, C5, C7, and C8) cultured in 100-mm dishes were labeled with NHS-LC-biotin for 5 minutes at 37°C. Cell lysates were either directly analyzed by Western blot analysis (C) or precipitated by streptavidin-conjugated agarose (M), followed by Western blot analysis with erbB2 (*top*). After stripping of the erbB2 antibody, the membrane was reprobed with the EGFR antibody (*bottom*). Little or no erbB2 was precipitated as cell surface protein from 5R-expressing cells, indicating that erbB2 is functionally depleted in these cells. Results are representative of three independent experiments.

ErbB-2 tyrosine phosphorylation sites, and these phospho-tyrosines serve as high-affinity binding sites for docking proteins that transduce signals into the cells.[17,27] Thus, the phosphorylation of erbBs is indicative of the activation of the receptors. In a preliminary experiment, we used Western Blot analysis and detected abundant expression of erbB2 in THCE cells. Figure 1 shows the time course of tyrosine phosphorylation of erbB2 in wounded THCE cells. There was a low but detectable erbB2 phosphorylation in THCE cells cultured in KBM. Scratch wounding induced erbB2 phosphorylation that was observed 15 minutes after wounding and the erbB2 phosphorylation remained elevated 2 hours after wounding (Fig. 1, top). The increase in erbB2 phosphorylation in wounded THCE cells was not related to the total amount of erbB2 detected in cell lysates of the same samples (Fig. 1, bottom).

Depletion of Cell Surface Expression and Functional Inactivation of erB2 in THCE Cells

To investigate the involvement of erbB2 in wound-induced EGFR signaling, we used retroviral infection of THCE cells with a vector that directs the expression of an erbB2-specific single-chain antibody genetically engineered to prevent erbB2 transit through the endoplasmic reticulum (referred to as 5R).[28,29] Several clones were selected along with an empty vector (pBabe-puro)-generated cell line. To determine whether the expression of 5R depletes erbB2 from the cell surface, pBabe-5R and pBabe-puro cell lines were biotinylated with cell membrane-impermeable NHS-LC-biotin, and the labeled proteins were precipitated (Fig. 2). In the control pBabe-puro–infected cells, erB2 was detected in cell lysates (C), and a large amount of erbB immunoreactivity was detected in the precipitates of the cell surface proteins (M). The amount of biotinylated erbB2 was almost undetectable in the 5R lines C5 and C8 and was greatly reduced in lines C4 and C7. Furthermore, the levels of erbB2 in cell lysates were also reduced in C4, C5, and C8, but to a lesser extent in C7. In all cell lines, erbB1 was detected as a cell surface protein.

Using three methods, [^{3}H]thymidine incorporation, cell counting at each passage, and a cell proliferation assay (CellTiter 96 Aqueous; Promega, Madison, WI), to determine cell proliferation, we found that the basal cell proliferation rate for the cell lines expressing 5R or pBabe-puro–transduced remained unchanged. This observation is consistent with that reported for 5R-transfected T47D mammary carcinoma cells.[14,30]

To determine whether expression of 5R retards erbB2 activation, 5R and control cells were subjected to scratch wounding followed by detection of erbB phosphorylation (Fig. 3). In control pBabe-transduced cells there was detectable basal erbB2 phosphorylation in growth factor-starved THCE cells, and wounding increased erbB2 phosphorylation. In 15-minute postwound 5R-expressing cells, no erbB2 phosphorylation was detected in all 5R cell lines (5R-C4, and -C5 are shown), indicating the functional inactivation of erbB2 in these cells. Significantly, wound-induced erbB1 phosphorylation was detectable in both control and 5R-expressing cells (also see Fig. 6).

Effects of ErbB2 Functional Inactivation on Cell Migration

Two approaches, an in vitro scratch wound-healing assay and the Boyden chamber migration assay, were used to assess the role of erbB2 on HCEC migration. The scratch wound-healing

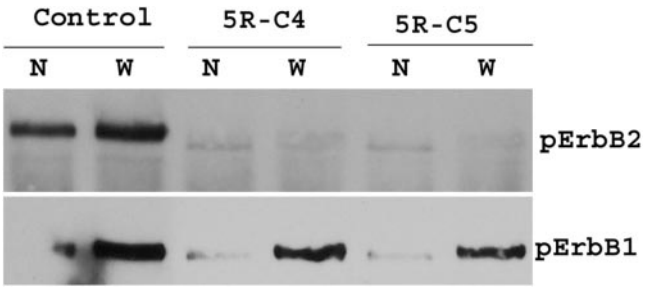

FIGURE 3. Wound-induced tyrosine phosphorylation of erbBs in pBabe-5R cell lines. pBabe-puro (control) and 5R-transfected cells (5R-C4 and 5R-C5 selected) were cultured in 100-mm dishes and starved overnight. Cells were extensively injured and further cultured for 15 minutes. Wounded cells (W), and noninjured, control cells (N), were lysed, and 600 μg protein was subjected to immunoprecipitation with pY20 antibodies, followed by individual Western blot analysis with erbB2 and -1 antibodies (after erbB2 antibody was stripped). Wound-induced erbB2, but not erbB1, phosphorylation (activation) was inhibited by 5R expression in THCE cells. Results are representative of three independent experiments.

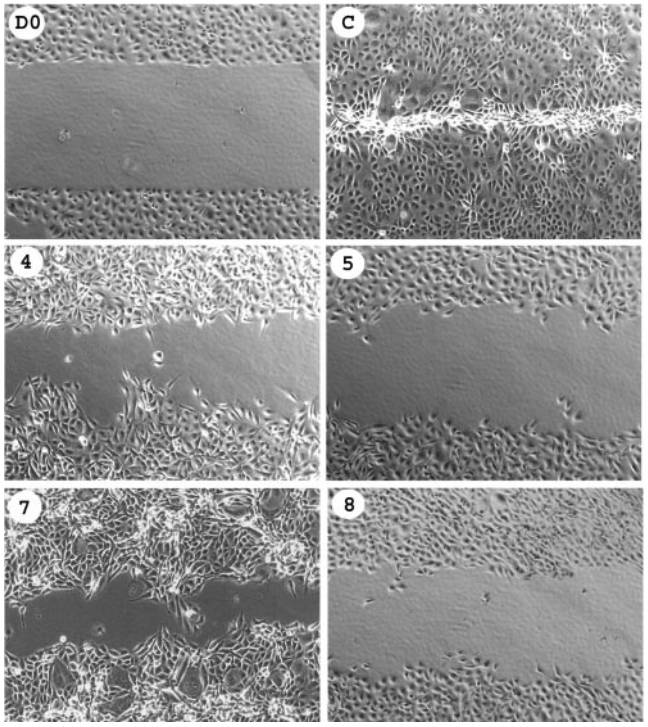

FIGURE 4. Effects of 5R expression on healing of scratch wounds. Growth factor–starved, 5R-expressing cells (4, 5, 7, and 8 for 5R lines C4, C5, C7, and C8, respectively) and a control pBabe-puro line (C) cultured in 12-well plates were injured with a sterile 0.1- to 10-μL pipet tip at day 0 (D0). The wounded cultures were allowed to re-epithelialize for 24 hours at 37°C in the presence of HB-EGF (50 ng/mL). Photomicrographs represent one of three samples performed each time. Functional depletion of erbB2 in THCE cells delayed healing of scratch wounds.

assay (Fig. 4) is considered to be an in vitro model for epithelial cell migration occurring during wound healing.[31] Confluent dishes of cells were wounded by scraping with a pipette tip, creating a space free of cells (Fig 4, panel D0). A scratch wound in the control cells was closed after 24 hours in the presence of HB-EGF (Fig. 4, panel C). A significant delay in the migration of the 5R-expressing cells into the empty space was observed, with a large portion of the wound remaining to be filled 24 hours after wounding (Fig. 4, panels 4, 5, 7, 8).

To quantify potential changes in the migration of cells devoid of functional erbB2 receptors compared with that of pBabe-transduced control cells, a Boyden chamber migration assay with FNC-coated polycarbonate membranes was performed. We first tested chemotaxis induced by HB-EGF. The presence of HB-EGF in the bottom chamber greatly increased the migration of control pBabe-transduced cells. Cells devoid of functional erbB2 receptors showed a significantly reduced chemotactic response to HB-EGF (Fig. 5), 28% and 22% of control cells for 5R-C7 and 5R-C8 ($P < 0.01$) cells, respectively. We then tested spontaneous haptotactic migration of HCECs toward FNC with no soluble EGFR ligands added. As shown in Figure 5, although control cells are able to migrate onto this substrate to some extent, the expression of 5R decreased cell migration onto FNC significantly, 20% and 25% of control cells for 5R-C7 and 5R-C8 ($P < 0.01$), respectively.

Together, these findings indicate that cell surface expression and activation of erbB2 is necessary for both HCEC chemotactic migration stimulated by HB-EGF and haptotactic migration toward FNC.

Effects of erbB2 Functional Inactivation on erbB1, ERK, and PI3K Activation

Activation of erbBs elicits a myriad of signaling events, including Ras/MAPK (ERK) and PI3K signaling cascades.[2,17,32] Using phospho-ERK and phospho-AKT (the major substrate of PI3K)-specific antibodies, we also assessed the activation of these two major signaling pathways during corneal epithelial wound healing (Fig. 6). Wounding induced persistent EGFR activation for more than 2 hours in control pBabe-puro–transduced cells and for a much shorter duration (<1 hour) in erbB2-depleted HCECs. Wounding also induced stronger ERK phosphorylation detectable at least 30 minutes after wounding in control cells, whereas a much weaker level of ERK phosphorylation was observed only in 15-minute postwound samples in erbB2-depleted cells. Similarly, functional depletion of erbB2 also greatly reduced the intensity and duration of AKT phosphorylation. Furthermore, AKT phosphorylation in 5R-expressing cells declined to a nondetectable level after a short period of increase (30 minutes).

Effects of ERK or PI3K Inhibition on Epithelial Wound Closure

To determine whether wound-induced ERK and AKT activation is necessary for epithelial wound healing, we treated wounded HCECs with EGFR (AG), ERK (PD), or PI3 K (LY) inhibitors (Fig. 7A). Inhibition of EGFR activation attenuated the wound-induced ERK1/2 and AKT phosphorylation to a level similar to or lower than that of control cells. As expected, the presence of PD98059 blocked wound-induced ERK1/2 phosphorylation and exerted a minimal effect on PI3K phosphorylation. Similarly, LY294002 inhibited wound-induced AKT, but not ERK1/2, phosphorylation. To assess the role of ERK and PI3K in mediating epithelial wound closure, we added PD98059 (50 μM) or LY294002 (10 μM) to corneal organ culture medium. The presence of these inhibitors attenuated epithelial wound closure (Fig. 7B).

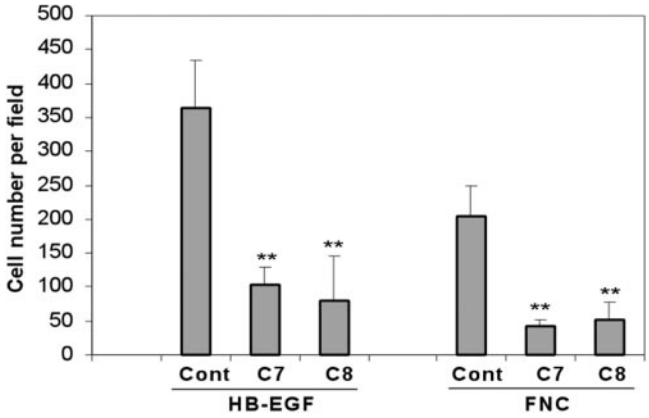

FIGURE 5. Effects of 5R expression on cell migration in a Boyden chamber assay. Growth factor–starved, 5R-expressing cells (lines C7 and C8) and control pBabe-puro line (pBabe C) cells were plated onto the top of the FNC-coated 14-μm pore, polycarbonate membrane and allowed to migrate for 3 hours in the presence or absence of 50 ng/mL HB-EGF. Subsequently, they were rinsed, fixed, stained, and counted. The number of cells per field is the average of four random fields in each well counted. The data represent the mean ± SD of counts in six wells. $^{**}P < 0.01$ (compared with results for pBabe control cells). Functional depletion of erbB2 in THCE cells retarded chemotactic migration stimulated by HB-EGF and haptotactic migration toward FNC.

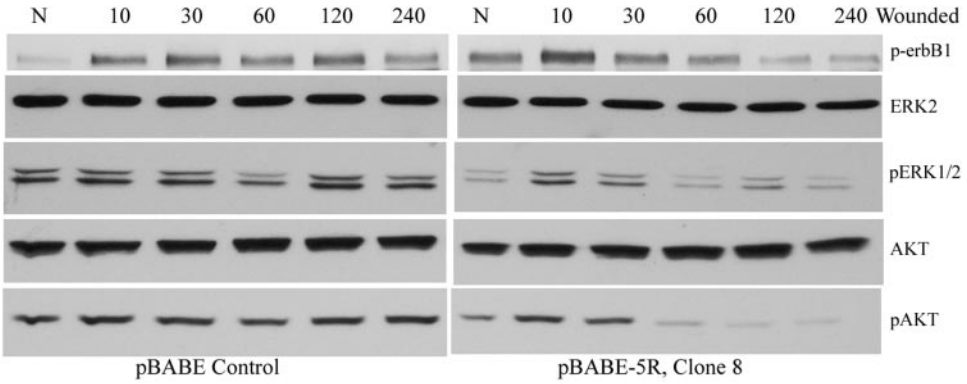

FIGURE 6. Effects of 5R expression on wound-induced EGFR, ERK1/2, and AKT phosphorylation. pBabe control (*left*) and 5R-expressing (clone-8, *right*) cells were extensively injured by sequence comb scratching and incubated for different time points after wounding (minutes) in KBM. To assess EGFR phosphorylation, wounded cells were lysed and subjected to EGFR immunoprecipitation, followed by Western blot with mouse anti-PY99 antibody (p-erbB1). To assess ERK and AKT phosphorylation, cell lysates of the same samples were subjected to Western blot analysis with anti-phospho-ERK1/2 (pERK1/2) or anti-phospho-AKT (pAKT). To normalize protein loading and determine the change of ERK2 and AKT in the cells, anti-ERK2 (ERK2) and anti-AKT (AKT) were used to probe the samples. The data are representative of results in two independent experiments.

DISCUSSION

In this study, we used human corneal epithelial cells depleted of cell surface erbB2 receptors to investigate the specific role of erbB2 in eliciting intracellular signaling pathways and in cell migration and wound healing. We provide evidence that erbB2 serves as a central signaling component in cell migration induced by the EGFR family of receptor tyrosine kinases. First, wounding of epithelial cells induced erbB2 phosphorylation. Second, whereas complete closure of a scratch wound was observed in THCE cells infected with control retrovirus within 24 hours in the presence of HB-EGF, wound healing was impaired in all four 5R cell lines devoid of functional erbB2 receptors in the scratch wound model. Furthermore, chemotactic migration of corneal epithelial cells was retarded in cells devoid of functional erbB2. Third, depletion of functional erbB2 greatly reduced both the intensity and duration of ERK and PI3K activation (phosphorylation), both of which are associated with induction of cell movement.[33,34] We reported recently that the inhibition of erbB1 kinase blocks wound-induced ERK activation and attenuates epithelial wound closure.[23] Together, our data suggest that erbB2 serves as an integral partner of the EGFR receptor signaling network and plays an essential role in mediating corneal epithelial migration and wound healing.

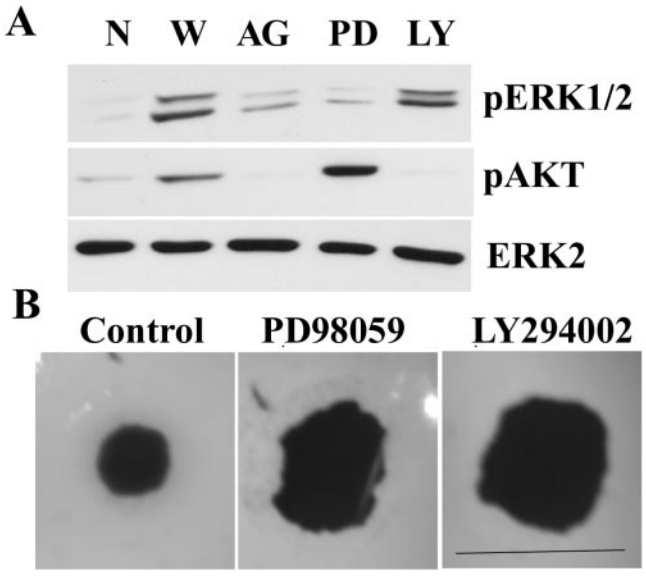

FIGURE 7. Requirement of wound-induced, EGFR-dependent ERK and PI3K pathways for epithelial wound closure. (**A**) Wound-induced ERK and PI3K activation is EGFR dependent. Growth factor–starved THCE cells were pretreated with 0.5 μM AG1478 (AG), 50 μM PD98059 (PD), or 10 μM LY294002 (LY) for 20 minutes and then wounded (W) by comb-scratching. Unwounded (N) cells served as the control. Cells were lysed 10 minutes after wounding and subjected to Western blot analysis with anti-phospho-ERK1/2 (pERK1/2) or anti-phospho-AKT (pAKT). Western blot analysis of ERK2 of the same samples was used to normalize protein loading. (**B**) ERK or PI3K inhibition retarded epithelial wound closure. A 4-mm epithelial wound in cultured porcine corneal cells was allowed to heal in MEM (control) or MEM containing MEK inhibitor (PD98059) or PI3K inhibitor (LY294002) for 48 hours. The cultured corneas were stained with Richardson's stain to show the area of the wound. Bar, 4 mm.

HCECs express multiple erbB members, including erbB2,[35–37] and their ligands.[21,38] These receptors undergo multiple heterodimerization and transactivation events after ligand binding,[39] thus making it difficult to link specific erbB receptor activation events to a given biological response, since, like other epithelial cells, HCECs express multiple erbBs.[35] In a recent study, we showed that HB-EGF is one of the endogenous EGFR ligands released after wounding. HB-EGF is known to bind erbB1 and -4. Using erbB1 antagonists, we and others[21,37] documented that erbB1 is required for wound-induced cell activation and epithelial wound closure. We observed in erbB2-depleted cell lines that erbB1 phosphorylation is induced by epithelial wounding while cell migration and wound healing are impaired in these cells. We and others observed that targeting EGFR (erbB1) by pharmacologic reagents inhibits corneal re-epithelialization in vivo[37] and in organ culture.[21] It appears that erbB1 in wounded corneal epithelial cells is necessary for recognition and interaction with the released ligand(s) induced by wounding; however, this interaction and subsequent activation of erbB1 is not sufficient for triggering cell migration. It has been reported that erbB1 facilitates cell migration through critical tyrosine residues in its cytoplasmic tail,[40] and this region of erbB1 may be dispensable for signaling, because it can associate with and signal through the erbB2 receptor.[41] Thus, erbB2 may serve as a link between erbB1 and the downstream signals that specifically affect the migration machinery of cells.

ErbB2 has been reported to contribute to increased cell proliferation.[17] Thus, the delay of wound closure in 5R-expressing cells may have been partially due to a decreased rate of cell proliferation. However, because the basal proliferation rate remains unchanged in these cells, we suggest that the inability of epithelial cells without functional erbB2 to close a wound properly is attributable, at least in a large part, to the loss of cell migration in these cells. This conclusion is based on results obtained in Boyden chamber migration assays, in which

HCECs showed chemotactic migration after exposure to HB-EGF, as well as haptotactic migration (to a low level) toward FNC. Depletion of cell surface erbB2 greatly retarded both types of cell migration. The retardation of haptotactic migration suggests that in addition to being involved in the erbB receptor signaling network, erbB2 may engage its downstream signals, which specifically impact the migration machinery of cells. For example, erbB2 has been reported to interact directly with integrins and with focal adhesion kinase. These interactions are required for cell migration and/or invasion in cultured cells.[42–44] Thus, erbB2 may function in a similar fashion in mediating corneal epithelial wound healing.

Activation of erbBs elicits a myriad of signaling events including Ras/MAPK (ERK) and PI3K.[2,17,32] EGF-induced activation of the ERK1/2 cascade has been well characterized. Studies by various groups have suggested a role for both ERK and PI3K pathways in corneal epithelial wound healing.[33,45–49] We showed that the inhibition of both ERK and PI3K in cultured corneas retarded epithelial wound closure, indicating that both signaling pathways are necessary for corneal epithelial wound healing ex vivo. In erbB2-depleted cell lines, wounding induces ERK and AKT phosphorylation to a certain level. However, both the intensity and the duration of the activation are much reduced in cells lacking erbB2 when compared with that of the control, indicating that a significant effect associated with erbB2 activation is enhanced and prolongs signaling, including the sustained activation of ERK and PI3K.[14,15] This effect on signaling may be due to the ability of erbB2 to enhance binding affinities for EGF peptides, leading to an increase in the half-life of receptor-ligand complexes—consistent with our observation of prolonged wound-induced erbB1 phosphorylation in the control, but not erbB2 depleted cells—and downstream signals that influence ERK and PI3K activities and the migration machinery of cells.[15] Thus, there appears to be a threshold of activities for ERK and PI3K necessary for triggering a migratory response, and the ability of erbB2 to enhance and prolong signaling events is critical for reaching this threshold in HCECs. Alternatively, the formation of integrin and PI3K in an erbB2 phosphorylation-dependent manner may be essential for cell migration, as shown for HaCaT keratinocytes on laminin-5.[42]

In summary, our findings demonstrate that erbB2 plays a central role in promoting corneal epithelial cell migration and wound healing. erbB2 serves as a fundamental signaling component that links EGFR ligands, erbB family receptor tyrosine kinases, to the migration machinery of epithelial cells by enhancing and prolonging ERK and PI3K signaling. These findings contribute to the understanding of how erbB family receptors and their ligands regulate signal transduction events associated with cell migration during wound repair.

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