Regulation of Cdc42 Expression and Signaling Is Critical for Promoting Corneal Epithelial Wound Healing

Swetha Pothula,[1] Haydee E. P. Bazan,[2] and Gudiseva Chandrasekher[1,3]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, South Dakota State University, Brookings, South Dakota
[2]Eye Center and Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana
[3]Department of Internal Medicine, Sanford School of Medicine of University of South Dakota, Sioux Falls, South Dakota

Correspondence: Gudiseva Chandrasekher, Department of Pharmaceutical Sciences, College of Pharmacy, South Dakota State University, Brookings, SD 57007; g.chandrasekher@sdstate.edu.

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Purpose. Cdc42, a member of Rho GTPases (guanosine triphosphatases), participates in cytokine- and growth factor–controlled biological functions in mammalian tissues. Here, we examined Cdc42 role in corneal epithelial wound healing and the influence of hepatocyte, keratinocyte, and epidermal growth factor (HGF, KGF, and EGF)–mediated signaling on Cdc42.

Methods. Epithelial wounds were created on the corneas of live rabbits by complete debridement and in rabbit corneal epithelial primary cultures through scratch injury. Cdc42 expression in cultures was suppressed using Cdc42 siRNA. Cdc42 activation was determined by pull-down assays with PAK-agarose beads. Cdc42 expression was analyzed by immunoblotting and immunofluorescence. Association of Cdc42 with cell-cycle proteins was identified by immunoprecipitation.

Results. In rabbit corneas, significant increase in Cdc42 expression that occurred 2 to 4 days after the injury coincided with wound closure, and by 8 days the expression reached near basal levels. Silencing of Cdc42 expression in cultures caused inhibition of wound closure as a result of 60% to 75% decrease in epithelial migration and growth. HGF, KGF, and EGF increased Cdc2 expression, activation, and its phosphorylation on ser71. Inhibition of growth factor–mediated PI-3K signaling resulted in the downregulation of Cdc42 expression and its phosphorylation. Increased association of cell-cycle proteins $p27^{kip}$ and cyclin-dependent kinase 4 (CDK4) with Cdc42; and phosphorylated Cdc42 with plasma membrane leading edges was also observed in the presence of growth factors.

Conclusions. Cdc42 is an important regulator of corneal epithelial wound repair. To promote healing, Cdc42 may interact with receptor tyrosine kinase–activated signaling cascades that participate in cell migration and cell-cycle progression.

Keywords: Cdc42, HGF, KGF, Rho-GTPases

The multilayered corneal epithelium undergoes continuous renewal to maintain optical clarity necessary for light refraction onto the lens and retina. Because of its location, corneal epithelium is also vulnerable to physical and chemical injuries, and impairment in epithelial wound repair produces ulceration and compromised vision. The wound-healing stages of cell migration and proliferation are influenced by ligands of receptor tyrosine kinases such as epidermal, hepatocyte, and keratinocyte growth factor (EGF, HGF, KGF); other cytokines such as TGF, TNF, and interleukins; and extracellular matrix proteins such as collagen, fibronectin, and laminin.[1–3] Levels of these compounds increase in the cornea in response to epithelial damage. The activation of their respective receptors on the plasma membrane triggers the communication between multiple intracellular signaling pathways. This cross-talk is important for coordinating cell migration and proliferation processes needed for wound closure and reestablishment of the multilayer epithelium.[1–6] Understanding the roles of specific signal mediators involved in the repair process would be beneficial for drug targeting to promote enhancement in the rate of wound healing.

Cdc42, which is also known as cell division cycle protein, is a GTP-binding protein belonging to the Rho family of small GTPases (guanosine triphosphatases). GTPases serve as molecular switches to control multiple biochemical pathways.[7,8] A wide variety of stimuli, including growth factors, cytokines, and intercellular or integrin-extracellular matrix interactions make these proteins transform from inactive (guanosine diphosphate [GDP]-bound) to active (GTP-bound) entities for intracellular signal transduction.[9,10] The active Rho GTPases interact with several downstream effector molecules such as p21-activated kinase (PAK), Wiskott-Aldrich syndrome protein (WASP), and IQ-motif-containing GTPase-activating protein (IQGAP) that participate in cellular processes.[11,12] Expression of Cdc42 has been linked to eye development and photoreceptor morphogenesis.[13,14] Deletion of Cdc42 in lens epithelium produced loss of fillopodia leading to defects in lens pit invagination.[15] Current research also indicates a prominent role for Cdc42 in cell-cycle progression and growth.[16,17]

Involvement of Rho and Rho kinases in regulating corneal epithelial migration and proliferation has been identified.[18–23] However, with regard to Cdc42, its role in corneal

epithelial wound healing has not been addressed. Previous studies from our laboratory and others demonstrated that HGF, KGF, and EGF activate PI-3K/Akt, mTOR, Erk, and p38 signaling cascades to promote corneal epithelial wound closure.[24–28] These pathways play an important role in the transmission of proliferative signals from membrane-bound receptors to the nucleus of the cells, and recent research emphasizes a key role of Cdc42 in coordinating those signals with cell-cycle machinery for cell-cycle progression.[16] Therefore, it is of relevance to investigate the participation of Cdc42-mediated signaling mechanisms in corneal epithelial cells and specific functional outcomes of such signaling. Here we study the effect of wound-healing–promoting growth factors on Cdc42 expression and signaling and the impact of changes in its expression during corneal epithelial wound repair in an animal model as well as in corneal epithelial primary cultures.

Methods

Reagents

HGF, KGF, and EGF were obtained from Millipore, Temecula, CA; Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) were from Atlanta Biological, (Lawrenceville, GA). All SDS-PAGE reagents were from Bio-Rad (Hercules, CA). Nitrocellulose membrane and enhanced Chemiluminescence (ECL) reagents were from Amersham (Arlington Heights, IL). The antibodies for $p27^{kip}$, Cdc42, cyclin-dependent kinase 4 (CDK4), β-actin, and Cdc42 activity assay kit were purchased from Millipore or Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from BD Pharmingen (San Diego, CA). Anti-phospho (ser71)-Cdc42/Rac1 antibody was purchased from Millipore or Epitomics (Burlingame, CA). LY294002 and wortmannin were obtained from Calbiochem, La Jolla, CA. Protein A agarose beads, Cdc42 siRNA and siRNA transfection kit were from Santa Cruz Biotechnology. The Biotinylated ECL Protein Markers Kit was from New England Biolabs (Beverly, MA).

Animal Experiments—In Vivo Rabbit Corneal Epithelial Injury

New Zealand white rabbits (6–8 weeks) weighing 4 to 6 lbs were purchased from Small-Stock Industries (Pea Ridge, AR). The animals were evaluated for general health by a veterinarian at South Dakota State University animal care facility prior to experimentation. Institutional Animal Care and Use Committee (IACUC) approvals were obtained for all experimental protocols. Rabbits were housed in a climate-controlled environment with a 12-hour light/dark cycle and were provided with free access to food and water during the experiment. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were placed under general anesthesia using isoflurane gas before the experiments. Prior to creating corneal injury, the eyes were topically treated with tetracaine hydrochloride (0.5% drops) to provide a local anesthetic effect. As described before[29] complete de-epithelialization was performed by gently scraping the corneal surface using a sterile No. 15 Bard-Parker scalpel blade (Cardinal Health, Dublin, OH) leaving the limbal epithelium intact. Regenerated epithelium from the wounded corneas of different animals was collected 1, 2, 4, and 8 days post injury. Fluorescein staining was employed to assess the de-epithelialization as well as re-epithelialization following the debridement. The amount of tissue regenerated after 1 and 2 days was found to be adequate (approximately 120–250 μg/cornea) to conduct Western immunoblotting experiments.

Corneal Epithelial Cell Culture and Preparation of Cell Extracts

Rabbit eyes were obtained from Pelfreze Biological (Rogers, AR). The eyes were shipped overnight on ice in Hanks' balanced salt solution (HBSS) containing antibiotic and antimycotic. Primary cultures of rabbit corneal epithelial cells (RCEC) were prepared as previously described.[30] Cultures were routinely grown in DMEM/F12 containing 10% FBS. First passage cells were used in all the experiments. Cell extracts were made after treating the cultures with ice-cold lysis buffer (20 mM Hepes, 2 mM $MgCl_2$, 2 mM EGTA, 2 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 0.15 M NaCl) for 10 minutes. The extracts were homogenized using a homogenizer and centrifuged at 20,000*g* for 20 minutes at 4°C. The supernatant was collected, and protein content of the extracts was determined by Bio-Rad dye-binding method.

Growth Factors and Inhibitors Treatment

RCEC at 70% to 80% confluence were starved overnight in DMEM/F12 with 0.25% FBS. Cells were then stimulated with HGF, KGF, or EGF (each 20 ng/mL) for short (0–10 minutes) or longer (24 hours) duration. Stock solutions of PI-3K inhibitors LY294002 and wortmannin were stored in dimethyl sulfoxide (DMSO) at −20°C and diluted in serum-free medium for the experiments. Since wortmannin is unstable in culture medium over longer times, we have used this compound for studies involving short-term incubations (0–10 minutes); whereas LY294002 was employed in incubations that were performed for 24 hours. Inhibitors were added to cells 30 minutes before incubating with the growth factors. After stimulation, cells were washed three times in ice-cold PBS and extracted with lysis buffer as described above.

Cdc42 Activation and Cdc42-GTP Pull-Down Assay

RCEC at 50% to 60% confluence were starved overnight and stimulated with HGF, KGF, or EGF for 5 to 10 minutes, and extracts as prepared above were analyzed for the levels of GTP-bound Cdc42 using Upstate-Millipore Cdc42 Assay Kit as per the manufacturer's protocol. Extracts containing equal amounts of protein (600 μg) from each experimental condition were incubated in microcentrifuge tubes with gentle end-to-end rotation at 4°C for 45 minutes with 30 μL of glutathione agarose beads conjugated with glutathione *S*-transferase (GST)-fusion protein corresponding to the PAK1-PBD (p21-binding domain of human p21 activating kinase-1) (Cell Biolabs, San Diego, CA). When activated, the GTP-bound form of Cdc42 associates with PAK-1. Active Cdc42 bound to agarose beads was pelleted by brief centrifugation (2 minutes, 2500*g*, 4°C). Beads were washed three times with 0.5 mL lysis buffer, suspended in SDS-PAGE sample buffer and subjected to Western immunoblotting for probing with anti-Cdc42 antibody.

Suppression of Cdc42 Expression—Cdc42 siRNA Transfection of Corneal Epithelial Cultures

RCEC were seeded in Petri dishes and allowed to grow to 50% confluence. Silencing of Cdc42-protein expression was performed using siRNA reagent kit (Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, 4 μL Cdc42 siRNA (40 pmol) and 5 μL transfection reagent were

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each diluted with 91 μL serum-free DMEM/F12, and the mixtures were incubated for 30 to 45 minutes at room temperature and then added to each culture incubated with serum-free DMEM/F12. Transfection with siRNA was continued for 12 to 16 hours. After that, medium was removed and cells were grown with fresh DMEM/F12 containing 1% fetal calf serum (FCS) until further use. Transfections with scrambled Cdc42 siRNA (negative controls) or transfection reagent only were also performed. Expression level of Cdc42 in transfected cultures was evaluated by Western immunoblotting.

Corneal Epithelial Wound Healing In Vitro

Corneal epithelial cultures transfected with Cdc42 siRNA and nontransfected control cultures grown in DMEM/F12 containing 1% FCS were employed for in vitro wound-healing assays. One to 2 days after Cdc42 siRNA transfection, the cultures were subjected to scratch injury using a sterile flat-headed scraper. Cells were gently removed by scraping from one end to the other end across the culture dish, and cultures were rinsed two times with DMEM/F12 to remove cell debris and loosely attached cells at the wound edges, and then incubated for another 24 hours. Images of cell cultures along the entire wound area at 0 hour (immediately after wounding) and after 24 hours were captured with a Zeiss-Axiocam digital camera (Oberkochen, Germany) fitted to a Zeiss fluorescence microscope and connected to a computer. Very fine permanent markings (that appeared as canyon-like structures in some cultures) were made on the plastic of the cultures at the wounded areas (away from cells) for the purpose of alignment of images taken at various areas at 0 and 24 hours. The extent of healing (cell migration and growth) at the wounded areas in Cdc42 siRNA transfected or nontransfected control cultures was determined by digital image analysis (Zeiss Axiovision software) by quantifying the areas covered with cells after 24 hours as compared with the same areas at 0 hour. The data collected from all images from each experimental condition were averaged and depicted as mean ± SD. In some experiments, the cells were harvested, and cell lysates were analyzed by Western immunoblotting to confirm the suppression of Cdc42.

Immunoprecipitation and Western Immunoblotting

Rabbit corneal epithelial cell (RCEC) extracts containing 600 μg protein were subjected to immunoprecipitation using anti-Cdc42 antibody (polyclonal). Extracts were treated with 15 μL antibody for 16 hours, and Cdc42-antibody complexes were separated by incubating with 30 μL protein A-agarose beads for 2 hours. The beads were pelleted by centrifugation, and pellets were washed three times with lysis buffer and suspended in SDS-PAGE sample buffer. The cell extracts (25–50 μg protein) or immunoprecipitates were subjected to SDS-PAGE in 12% to 15% gels. The proteins in the gels were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and then probed with specific antibodies as indicated in the results. The blots were incubated at room temperature with primary antibodies for 2 hours, washed six times with TBS (20 mM Tris HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) and further incubated for 2 hours with HRP-conjugated secondary antibodies. The membranes were treated with enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Amersham, IL). The protein bands of interest were identified after exposing the membranes in UVP-image analyzer (Upland, CA). Molecular sizes were determined by comparing with the mobility of biotinylated protein markers that were run simultaneously during electrophoresis. All protein extracts transferred to membranes were also blotted with anti-actin.

Immunofluorescence

RCEC were seeded at a density of 5000 cells/well, grown in four-well glass chamber slides (Labtek, Rochester, NY) to yield subconfluent cultures. Serum-starved cells were stimulated with growth factors for various time points as described in the results. Slides were fixed with 4% paraformaldehyde for 30 to 60 minutes and then permeabilized with 0.1% Triton X-100 in 0.1% Na-citrate solution for 30 minutes. Cells were blocked with 5% BSA in PBS for 1 hour at room temperature. They were further incubated with mouse monoclonal anti-Cdc42 (1:500) or anti-phospho (ser71)-Cdc42 antibodies overnight at 4°C. Slides were washed with PBS three times before treating with appropriate FITC or Alexa Fluor 555–conjugated secondary antibodies (1:500). Negative controls without primary antibody were also run simultaneously to rule out the nonspecific binding of fluorescent-tagged secondary antibodies.

Statistical Analysis

Statistical data are represented as the mean ± SD and analyzed using Student's *t*-test or ANOVA; $P < 0.05$ was considered statistically significant.

Results

Upregulation of Cdc42 Expression During Wound Healing In Vivo

In order to understand the relevance of Cdc42 to corneal epithelial cell physiology and wound healing, we first performed its expression analysis in regenerating epithelium as a function of time after complete debridement of epithelium of rabbit cornea. As shown in Figure 1, the expression of Cdc42 changed significantly with the progression of healing. As compared with levels in unwounded control corneas, the expression increased 2- to 3-fold between 2 and 4 days after the injury, and by day 8 the expression begin to decline. During the initial phase of healing (i.e., 24 hours after the injury), there was little or no change in its expression. This suggests that Cdc42 upregulation is important during the proliferative phase of epithelial wound repair, which is 2 to 4 days following injury, as cell growth is expected to be robust for the reestablishment of multilayer epithelium.

Downregulation of Cdc42 Expression Inhibits Corneal Epithelial Wound Healing

Since the expression of Cdc42 increased during wound healing, we investigated the relevance of its expression for the promotion of corneal epithelial wound healing by transfecting RCEC with Cdc42 siRNA and then subjecting the cultures to scratch wounding as explained in the Methods section. The siRNA transfected cultures showed a 50% to 60% decrease in Cdc42 level compared with control cells or cells treated with only transfection reagent (Figs. 2C, 2D) or transfected with scrambled siRNA (not shown). Twenty-four hours after the injury, the area of wound covered with new cells was significantly higher in the control cultures or cultures treated with only transfection reagent or transfected with scrambled siRNA (Fig. 2A) when compared with cultures transfected with siRNA, where a substantial portion of the wounded area (approximately 50%–60%) was found to be uncovered (Figs. 2A, 2B).

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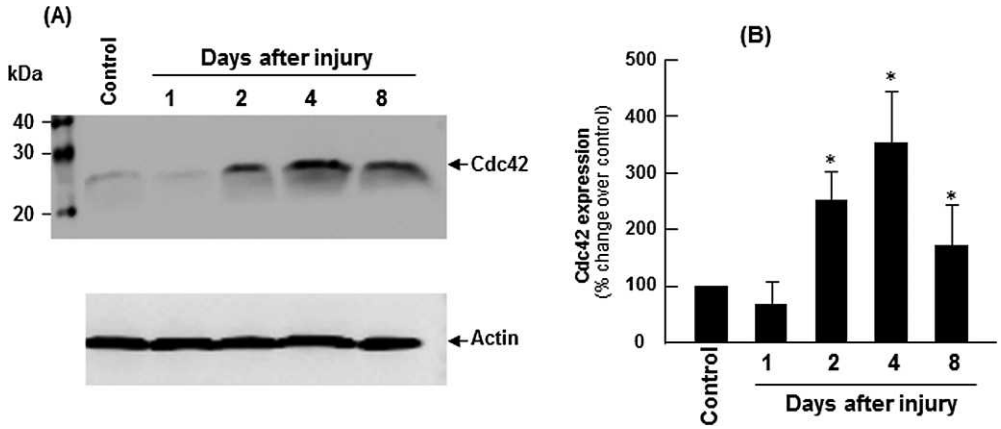

Figure 1. Changes in Cdc42 expression during in vivo wound healing after total corneal epithelial debridement. (**A**) Epithelium, regenerated at different days following the injury, was harvested as described in Methods, and equal amounts of protein (50 μg) for each condition were subjected to Western immunoblotting using monoclonal anti-Cdc42 antibody. Loading of the sample in each well was assured by reprobing the membrane with β-actin antibody (*lower panel*). (**B**) Quantification of Cdc42 levels by densitometry. The bars represent the mean ± SD of three different experimental samples that were collected from 3 to 5 rabbits at each time point (*$P < 0.05$ control versus various days after the injury as indicated).

Effect of Growth Factors on the Expression and Activation of Cdc42 in Epithelial Cells

HGF, KGF, and EGF are secreted by the cornea in response to epithelial injury and promote corneal epithelial wound healing.[1,2] To investigate the influence of these growth factors on the regulation of Cdc42 expression, we treated RCEC with HGF, KGF, or EGF (each 20 ng/mL) for 24 hours. There was an approximately 200% to 300% increase in Cdc42 level compared with untreated controls (Figs. 3A, 3B) as analyzed by Western immunoblotting. The elevation in Cdc42 expression after growth factor treatment was also evident from immunofluorescence study (Figs. 2C, 3C). Further, to investigate the activation status of Cdc42 in the presence of growth

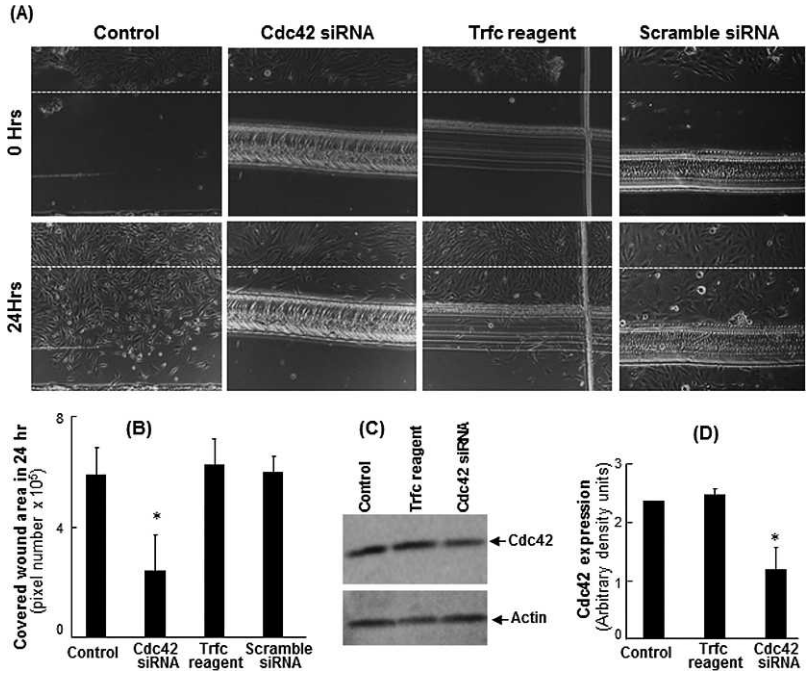

Figure 2. Effect of silencing of Cdc42 expression on corneal epithelial wound healing. RNA interference by siRNA transfection was performed in RCEC as described in Methods. The cells were treated with transfection reagent (Trfc) mixed with Cdc42 siRNA or Trfc alone for 16 to 20 hours. The cultures were incubated for an additional 24 hours in DMEM/F12 containing 1% FCS. After that, the cultures were harvested for determining Cdc42 expression levels by Western immunoblotting or subjected to scratch injury that caused the debridement of epithelium. Wounded cultures were further incubated in DMEM/F12 containing 1% FCS for 24 hours for healing to occur. (**A**) The images of wounded cultures captured at 0 hour and after 24 hours (same regions). The *white dashed lines* indicate the leading edge of the wound at 0 hour. (**B**) The quantification of wounded areas was performed by image analysis (Zeiss-Axiovision 4.5 software). Histograms for each experimental condition represent the covered wound area after 24 hours (measured from the 0-hour leading edge). The permanent, deep, canyon-like markings appearing on the culture dishes (*right three panels* in (**A**) did not cause impediment of cell growth. The details of image acquisition and quantification of wound healing are described in Methods. Cdc42 protein levels (**C**) in cultures were quantified by densitometry (**D**). Data shown in (**B**) and (**D**) are mean ± SD of three separate experiments (*$P < 0.05$ control versus various treatments as indicated).

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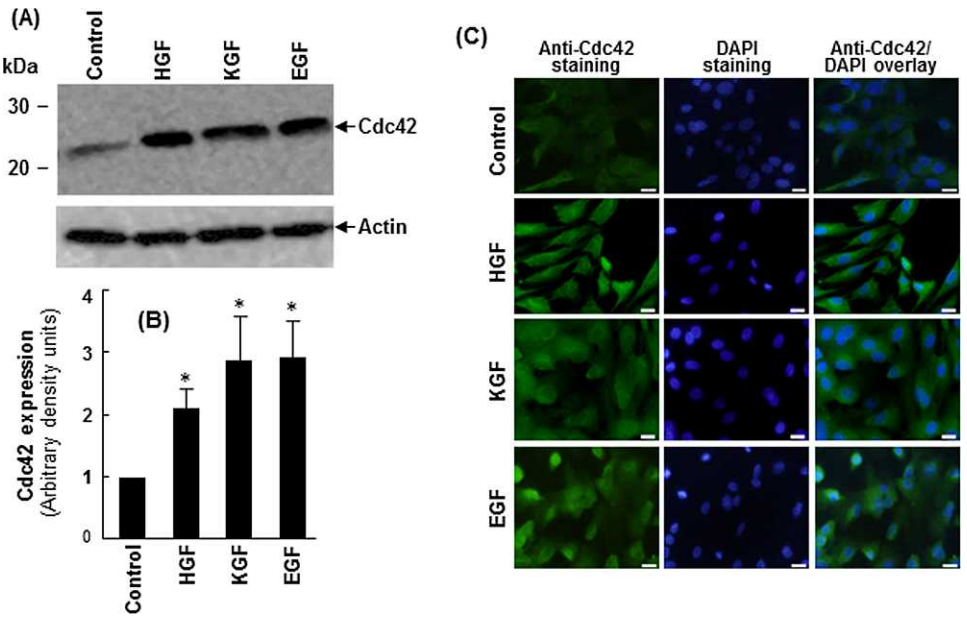

Figure 3. Effect of HGF, KGF, and EGF on the expression of Cdc42 in corneal epithelial cells. RCEC were cultured to 70% to 80% confluence. Cells were starved overnight in DMEM/F12 containing 0.25% FCS and stimulated with HGF, KGF, or EGF (each 20 ng/mL) for 24 hours. (**A**) Western blot probed with monoclonal anti-Cdc42 antibody (*upper panel*) or anti-actin antibody (*lower panel*). (**B**) Densitometric quantification of Cdc42 protein bands. Data represent mean ± SD of 4 to 5 different experiments; $^*P < 0.05$ control versus various treatments as indicated. (**C**) Images showing anti-Cdc42 immunofluorescence staining (*left row*). Nuclear fluorescence staining with DAPI is shown in the middle row. Overlay of Cdc42 immunofluorescence and nuclear fluorescence staining is depicted in the right row. Scale bar = 20 μM.

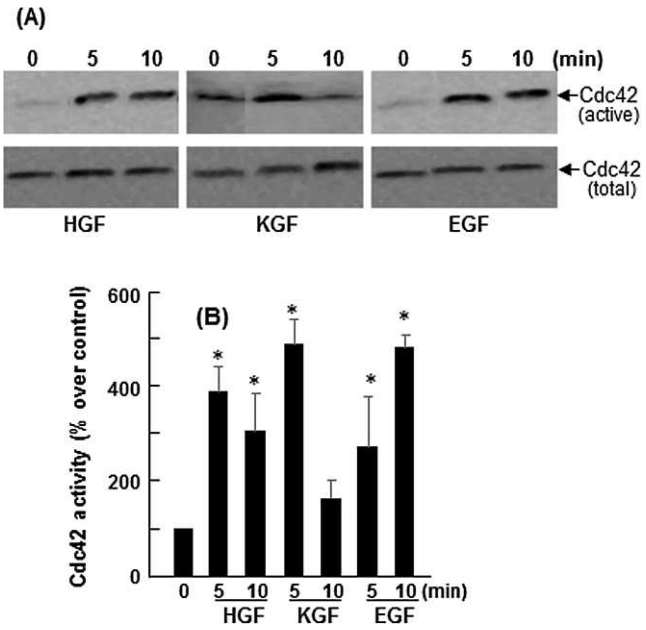

Figure 4. Effect of HGF, KGF, and EGF on Cdc42 activation in corneal epithelial cells. RCEC were cultured to 70% to 80% confluence. Cells were starved overnight in DMEM/F12 containing 0.25% FCS and stimulated with HGF, KGF, or EGF (each 20 ng/mL) for 5 and 10 minutes. Following the incubation, the cultures were harvested and assayed for Cdc42 activation (Cdc42-GTP pull-down assay) as described in Methods. Active Cdc42 (GTP-bound form) was separated from the assay mixture employing PAK-PBD beads that were subjected to SDS-PAGE and Western transfer. (**A**) Active Cdc42 bound to PAK-PBD beads as well as cellular extracts (containing total Cdc42) of each experimental condition were subjected to Western immunoblotting using anti-Cdc42 antibody. (**B**) Densitometric quantification of the Cdc42 protein bands. Data represent mean ± SD of three independent experiments ($^*P < 0.05$ control versus various treatments as indicated).

factors, the cells were stimulated with HGF, KGF, or EGF for 5 to 10 minutes, and the levels of GTP-bound Cdc42 associated with PBD-PAK1 were determined as explained in the Methods section (Fig. 4). The amount of active Cdc42 was found to be increased between 2- and 4-fold after treatment with growth factors (Figs. 4A, 4B). The amount of total Cdc42 remained unchanged under these conditions.

PI-3K Signaling Regulates Cdc42 Expression

We have previously demonstrated that HGF and KGF stimulate PI-3K/Akt signaling in corneal epithelium and promote wound healing.[25–28] To investigate if growth factor–dependent upregulation in Cdc42 expression is influenced by the PI-3K signaling cascade, RCEC were stimulated with growth factors in the presence and absence of PI-3K inhibitor LY294002. There was a significant decrease of Cdc42 expression when PI-3K activation was inhibited (Fig. 5), suggesting that Cdc42 lies downstream of the PI-3K pathway and growth factor–mediated increase in Cdc42 expression is regulated by PI-3K signaling cascade.

Effect of Growth Factors on Cdc42 (ser71) Phosphorylation

Cdc42 contains a region of amino acid sequence (residues 64–73, with serine at 71)[31] that can be specifically phosphorylated by Akt.[32] Since PI-3K activation influenced the expression of Cdc42, we evaluated Cdc42 (ser71) phosphorylation status in the presence of growth factors. Treatment of cells for 5 to 10 minutes with HGF, KGF, and EGF produced significant increase in the phosphorylation of serine 71 residue of Cdc42 (Figs. 6A, 6B). Further, the ability of these compounds to phosphorylate Cdc42 was inhibited in the presence of PI-3K inhibitor wortmannin (Fig. 6C) suggesting that PI-3K/Akt activation is also necessary for Cdc42 (ser71) phosphorylation.

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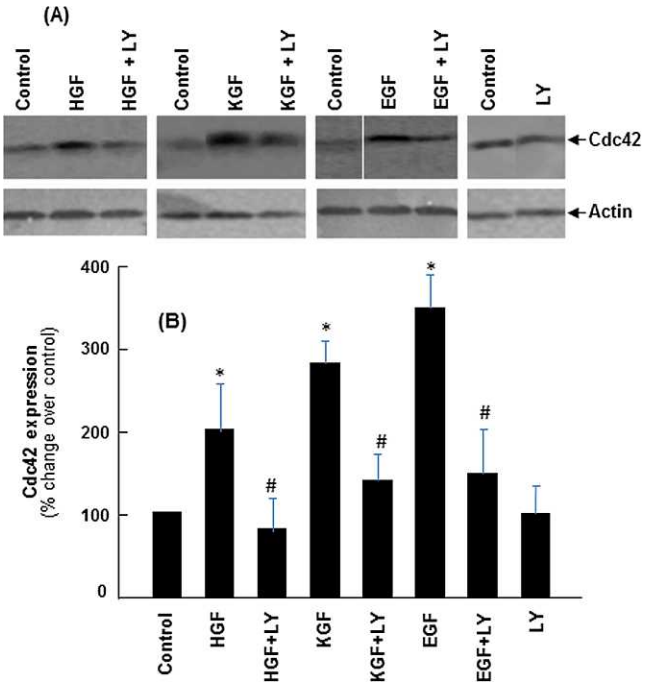

FIGURE 5. PI-3K signaling regulates Cdc42 expression. RCEC were starved overnight in DMEM/F12 containing 0.25% FCS and pretreated with PI-3K inhibitor LY294002 (LY, 20 μM) for 30 minutes prior to incubation with HGF, KGF, or EGF (each 20 ng/mL) for 24 hours. (**A**) Cellular extracts were subjected to Western immunoblotting and probed with anti-Cdc42 antibody. Blots were further probed with anti-actin antibody (*lower panel*) to determine sample-loading accuracy on SDS-PAGE gels. (**B**) Densitometric quantification of the Cdc42 protein bands. Data represent mean ± SD of three to five different experiments (*$P < 0.05$ control versus various treatments and #$P < 0.05$ growth factor versus growth factor with LY294002 as indicated).

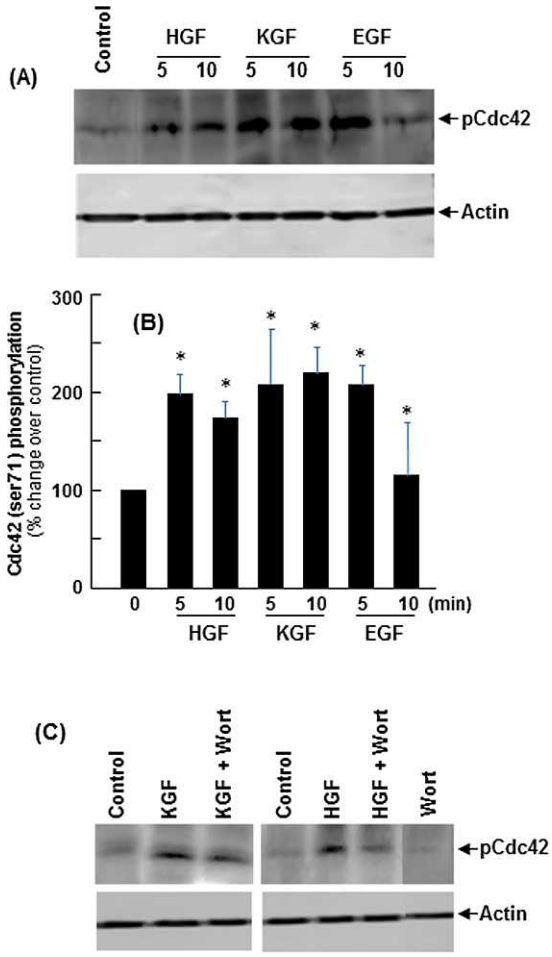

FIGURE 6. Effect of growth factors on the phosphorylation of Cdc42 in corneal epithelial cells. RCEC cultures grown to 70% to 80% confluence were starved overnight in DMEM/F12 containing 0.25% FCS and stimulated with HGF, KGF, and EGF at 20 ng/mL for 5 to 10 minutes. To determine the effect of PI-3K signaling on Cdc42 phosphorylation, the cultures were pretreated with wortmannin (wort, 200 nM) for 30 minutes before performing 10-minute incubation with growth factors. Following the incubations, the cultures were harvested for protein extraction and subjected to Western immunoblotting for probing with anti-phospho (ser71)-Cdc42 and anti-actin antibodies (**A** and **C**). Densitometric quantification of the phospho Cdc42 protein bands in **A** is shown in **B**. Data represent mean ± SD of three different experiments. (*$P < 0.05$ control verses various treatments as indicated).

Cell-Cycle Proteins Associate With Cdc42

The inhibition of wound healing in epithelial cultures expressing reduced levels of Cdc42 suggested that Cdc42 could be important in promoting cell division and growth. Multiple interactions between various cell-cycle proteins are necessary for the progression of the cell cycle. To investigate if Cdc42 is linked to cell-cycle proteins, we stimulated RCEC with growth factors, immunoprecipitated Cdc42 with anti-Cdc42 antibody, and then analyzed for the presence of different cell-cycle proteins in the immunoprecipitates. We found CDK4 and $p27^{kip}$ proteins in Cdc42 immunoprecipitates and an increase in the quantity of these proteins bound to Cdc42 after treatment with HGF, KGF, and EGF (Fig. 7), indicating possible interactions between these proteins. The cell-cycle inhibitor $p27^{kip}$ interactions with cyclin D and CDK4, and the cyclin E-CDK2 complexes play a key role during G1/S cell-cycle progression.[33] Binding of $p27^{kip}$ to Cdc42 could interfere with its interactions with cyclin and CDK complexes that lead to alteration in cell-cycle progression.

Localization of Phospho (ser71)-Cdc42 at Migratory Structures

To study the cellular localization of phospho (ser71)-Cdc42 after growth factor stimulation, RCEC were stimulated for 10 minutes with the growth factors and analyzed by immunofluorescence. Control cells showed no or very faint anti–phospho-Cdc42 immunostaining, but cells treated with HGF, KGF, and EGF showed significant increase in anti–phospho-Cdc42 immunostaining. Although, diffused staining was observed throughout the cell, dense rims of the fluorescent stain were found at the cell periphery, and much of this stain was punctuated at leading edges, which accumulate focal adhesions. These results suggest that serine 71 phosphorylation could cause the translocation of Cdc42 to the plasma membrane to facilitate cell migration.

DISCUSSION

In this study, we report that Cdc42 expression in RCEC increases significantly in the presence of HGF, KGF, and EGF. They also triggered the activation of Cdc42 as revealed by increased levels of GTP-bound Cdc42 in pull-down assays. The growth factors HGF, KGF, and EGF are secreted by lacrimal

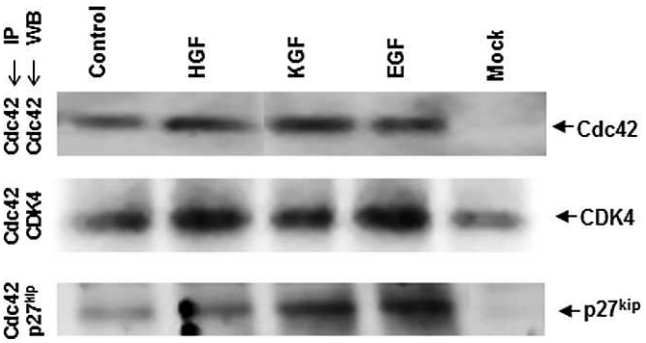

FIGURE 7. Association of cell-cycle proteins $p27^{kip}$ and CDK4 with Cdc42. RCEC were cultured to 70% to 80% confluence. Cells were starved overnight in DMEM/F12 containing 0.25% FCS and stimulated with HGF, KGF, or EGF (each 20 ng/mL) for 24 hours. Cellular extracts of different conditions containing equal amounts of protein (500 μg) were subjected to immunoprecipitation as described in Methods by treating with polyclonal anti-Cdc42 antibody. Lane denoted with "*Mock*" represents immunoprecipitation performed without cell extracts. Immunoprecipitates were subjected to Western immunoblotting, and membranes were probed for the presence different cell-cycle proteins using appropriate antibodies. The experiments were repeated two times.

gland and fibroblasts of corneal stroma in response to corneal epithelial injuries[34,35] and promote epithelium growth. Expression analysis data obtained from regenerating epithelium following complete de-epithelialization of corneas from rabbits indicated a gradual and robust increase in Cdc42 expression that coincided with the cell-proliferation phase of the wound-repair process. Increased cell proliferation begins 1 to 2 days after the injury (once the wound is covered) to restore the normal thickness of epithelium, and this phase lasts approximately a week in rabbits.[2,36] The level of Cdc42 showed a decline by postinjury day 8, suggesting that Cdc42 could play an important role during corneal epithelial cell growth. Further, with Cdc42 RNA interference experiments, we observed a significant delay in wound closure, presumably as a consequence of decreased Cdc42 expression. These findings suggest that Cdc42 participation could be important for epithelial wound repair. To the best of our knowledge, this is the first report showing the alterations in Cdc42 expression in epithelium during wound healing and how these alterations could impact wound repair. Other members of GTPases family, Rho and Rac, have been shown to influence cell migration and proliferation in corneal epithelium.[18–23]

Corneal epithelial wound healing is a complex process that involves three main phases: cell adhesion to the basement membrane, migration to cover the wound, and cell division and growth for multilayer organization. It is not possible to discern the relative importance of Cdc42 participation to cell migration or cell proliferation from the cell culture scratch–wound-healing model we employed in this study. However, as can be seen from the pattern of cell growth to cover the wound (Fig. 2), it appears that cell migration as well as proliferation influenced the healing process in cultures when Cdc42 expression was silenced, and it is likely that Cdc42 plays a major role in both the processes. Recent studies emphasize a key role for Rho GTPases in cell-cycle progression for the growth of many organs.[17,37–39] The cell cycle in eukaryotes consists of four distinct phases: the first gap (G1) phase, the DNA synthesis (S) phase, the second gap (G2) phase, and the mitotic (M) phase. Movement through the cell-cycle phases is driven by the interactions between cyclins, CDKs, and cyclin-dependent kinase inhibitors.[40] Rho GTPases are considered key contributors to the mitogen-stimulated G1-S phase progression. Interactions between cyclin E and CDK2, and cyclin D and CDK4/6 facilitate retinoblastoma protein phosphorylation resulting in G1-S phase transition.[17,33] CDK inhibitor $p27^{kip}$ can block cell-cycle progression by inhibiting cyclin E-CDK2 activity, whereas its binding to cyclin D-CDK4/6 complexes can induce progression to S phase.[33,41] In this study, we found increased association of $p27^{kip}$ with Cdc42 after growth factor treatment, indicating the possibility that sequestration of $p27^{kip}$ by Cdc42 could relieve the $p27^{kip}$-mediated effect on termination of cyclin E-CDK2 activity that is inhibitory to cell-cycle progression. Increased Cdc42 expression during wound healing, thus, could favor cellular growth. We have also found the association of CDK4 with Cdc42 after treatment with growth factors. Further studies are necessary to understand the significance of these interactions and the mechanism of action of Cdc42 in promoting cell proliferation.

Recent studies reveal that Cdc42 plays a key role in proliferation in several cell systems working through PI-3K downstream target S6 kinase (S6K-mTOR).[42,43] These studies report that S6K-induced expression of cyclin E during G1/S progression is influenced by Cdc42. S6K-mTOR is also involved in cell proliferation promoted through Cdc42 and its downstream effector molecule IQGAP.[44] Our previous studies have shown that HGF- and KGF-induced activation of S6K is dependent on PI-3K activation, and suppression of S6K activity by its specific inhibitor rapamycin blocks corneal epithelial wound healing.[25] In this study, we found that inhibition of PI-3K activation decreases Cdc42 expression. Cdc42 deficiency, which has also been shown to result in impaired B-lymphocyte proliferation, is attributed to decreased PI-3K/Akt activation caused by defective B-cell receptor signaling.[45,46] Further, mice deficient in Cdc42 in intestinal stem cells showed defective cell division.[47] Our study showing the inhibition of wound healing in Cdc42-deficient corneal epithelial cells, coupled with decreased Cdc42 expression as a result of inhibition of PI-3K (Fig. 5), thus substantiates a role for Cdc42 in corneal epithelial cell proliferation.

Increase in Cdc42 activity observed in the presence of growth factors can also stimulate cell migration to promote wound healing, and our in vitro model of epithelial-scrape wound healing shows that knocking down of Cdc42 increases the uncovered area after the wound, implicating that cell migration was also inhibited. Several studies in multiple cell systems have shown that Cdc42 is involved in chemotaxis and directed migration.[16] Cdc42/Rac induces the formation of lamellipodia, fillopodia, and membrane ruffles that result in actin cytoskeletal reorganization favoring cell migration. Our fluorescence immunostaining studies showed localization of intense Cdc42/Rac phospho serine 71 immunostaining, specifically at leading edges, which appeared to contain several focal adhesions after growth factor treatment (Fig. 8). Growth factors and extracellular-matrix proteins induce formation of focal adhesions, which facilitate cell migration, and we previously reported that HGF and KGF promote the migration of corneal epithelial cells.[27] Schoentaube et al. showed EGF-induced phosphorylation of Cdc42/Rac1 at serine 71 and binding of the phosphorylated form to PAK.[32] It should be noted that currently, with commercially available antibodies, we cannot distinguish whether phospho serine 71 immunostaining is contributed specifically by CDc42 or Rac. Nevertheless, our results suggest that in corneal epithelial cells, growth factor–mediated serine 71 phosphorylation of Cdc42/Rac may be important for cell migration and that phosphorylation at serine 71 may cause translocation of either or both of these proteins to the plasma membrane to interact with downstream effector molecules such as PAK and facilitate motility. Further, it has been reported that Akt kinase activity mediates serine 71 phosphorylation on Cdc42 and Rac.[32,48]

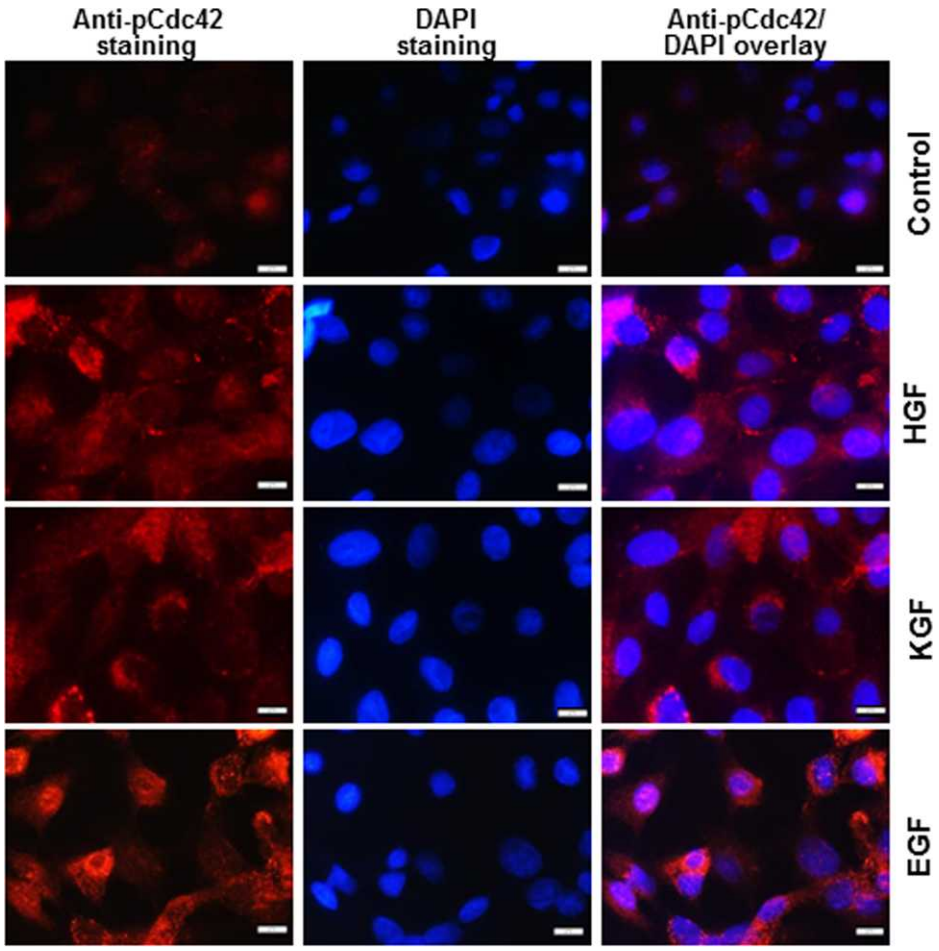

Figure 8. Effect of growth factors on the phosphorylation of Cdc42 in corneal epithelial cells. RCEC cultures grown to 70% to 80% confluence were starved overnight in DMEM/F12 containing 0.25% FCS and stimulated with HGF, KGF, or EGF at 20 ng/mL for 10 minutes. Following the incubation, the cultures were processed for immunofluorescence staining with anti-phospho (ser71)-Cdc42 antibody (*left row*) or nuclear staining with DAPI (*middle row*). Overlay of phospho (ser71)-Cdc42 immunofluorescence and nuclear fluorescence staining is depicted in the *right row*. Scale bar = 20 μM.

Our current study revealed that inhibition of PI-3K/Akt activation causes a significant decrease not only in Cdc42 expression (Fig. 5) but also in serine 71 phosphorylation (Fig. 6). Thus, a cross-talk between Cdc42/Rac and PI-3K/Akt signaling cascade is also important in growth factor–promoted cell migration. Involvement of Rac in fibronectin-promoted human corneal epithelial cell migration has been identified previously.[49] Cdc42-mediated induction of cell migration in corneal endothelial cells is also reported to be regulated by PI-3K.[50] Further, alteration in lens-fiber cell migration and elongation is attributed to disruption in membrane translocation of Cdc42 along with Rho and Rac.[51]

In conclusion, we report that Cdc42 expression is important for the growth of corneal epithelial cells during wound repair, and impairment in its synthesis is detrimental to wound healing. Growth factors that are secreted in response to corneal injuries increase Cdc42 expression. Further, our results suggest that HGF, KGF, and EGF-mediated signaling to regulate corneal epithelial cell-cycle progression and migration involves Cdc42. A detailed analysis of the links between Cdc42 and its interactions with downstream signaling molecules will provide a better understanding of Cdc42 cellular responses in corneal epithelial cells. It may be worthwhile targeting Cdc42-mediated signaling for the development of alternative treatment options for rapid regeneration of corneal epithelium in persistent epithelial defects that are difficult to heal.

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