FGF-2-Induced Wound Healing in Corneal Endothelial Cells Requires Cdc42 Activation and Rho Inactivation through the Phosphatidylinositol 3-Kinase Pathway

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PURPOSE. Acquisition of elongated cells with pseudopodia is observed when corneal endothelial cells (CECs) are simultaneously treated with basic fibroblast growth factor (FGF)-2 and RhoA inhibitors. This study was designed to determine whether these phenotypes are migratory and whether Cdc42 activation and RhoA inactivation are involved in cell migration.

METHODS. A scratch-induced directional migration assay was used to measure migratory rates. Activation of Cdc42 was determined by GTP pull-down assay. Transfection was performed using constitutively active (ca) or dominant negative (dn) Rho guanosine triphosphatase (GTPase) vectors.

RESULTS. Stimulation with basic FGF-2 alone resulted in a 43% recovery of the wound area, whereas CECs treated with FGF-2 and Y27632 (inhibitor of Rho-associated kinase) achieved an 84% recovery of the wound area with a fast migratory speed (0.72 μm/min). The synergistic effects of FGF-2 and Y27632 were completely blocked by LY294002 (PI 3-kinase inhibitor). Under these conditions, activation of PI 3-kinase and Cdc42 were observed in the migratory cells. The involvement of activated Cdc42 and inactivated Rho in endothelial migration was determined by transfecting CECs with ca- or dnRho GTPase vectors. A high migratory rate (0.52 μm/min) was seen in CECs expressing caCdc42, whereas endothelial migration was completely inhibited in CECs expressing caRho. When cells expressing caCdc42 were treated with FGF-2, migration reached the maximum rate (0.69 μm/min), similar to that observed in cells treated with FGF-2 and Y27632.

CONCLUSIONS. These findings suggest that endothelial migration is induced by activated Cdc42 and inactivated Rho via PI 3-kinase after FGF-2 stimulation and that Cdc42 activation is crucial for CECs to acquire the characteristic migratory phenotypes. (Invest Ophthalmol Vis Sci. 2006;47:1376–1386) DOI:10.1167/iovs.05-1223

The posterior corneal surface is lined by a monolayer of corneal endothelial cells (CECs) that rest on a self-made basement membrane (Descemet’s membrane). Corneal endothelium plays a critical role in maintaining corneal hydration and corneal transparency. Adult CECs are known to be mitotically inactive, and recent studies demonstrate that adult human CECs are arrested at the G1 phase of the cell cycle.¹ ² The characteristic antiproliferative behavior seems never to be challenged in the human eye. Endothelial cell loss is a life-long process. As the eye ages, the number of CECs decreases, never again equaling the number present at birth. Such progressive cell loss in corneal endothelium stresses the contact-inhibited monolayer of corneal endothelium and interferes with its pump function under physiologic conditions. To our benefit, corneal endothelium becomes resilient, although its condition is attenuated. Unlike other cell types, in which both cell proliferation and migration are major components of the repair mechanism, when corneal endothelium is injured, the cells in the wounded area do not regenerate by cell division but are replaced by the migration and spreading of existing cells. Thus, cell migration in the absence of cell division is the major mechanism in corneal endothelial wound healing, and such a characteristic repair mechanism is needed to regenerate endothelial functions fully, such as hydration and maintenance of transparency.

Cell migration is critical for a wide variety of physiologic and pathologic processes, including normal development, angiogenesis, inflammatory responses, wound repair, and tumor invasion.¹³–⁶ Cell migration within tissues requires the integration of key events in signaling, cytoskeletal reorganization, and adhesion processes. The Rho family of guanosine triphosphatases (GTPases), particularly Rho, Rac, and Cdc42, modulate many aspects of cytoskeletal function that occur during migration. Rac1 seems essential in most cells for the protrusion of lamellipodia at the leading edge and for forward cell movement; Cdc42 is needed to maintain cell polarity and to form filopodia; and RhoA is necessary to maintain substrate adhesions during cell movement and to produce a contractile force in the forward-migrating cell.⁷–¹⁵

Two key extracellular signals—soluble growth factors and extracellular matrix (ECM)—directly influence the cell’s decision to move or to stop.¹⁶–¹⁹ Cell migration, driven by growth factors and cytokines released coincidentally into the injury site, is particularly critical during early wound repair. In our previous study, we reported that interleukin (IL)-1β released by polymorphonuclear leukocytes causes a rapid activation of PI 3-kinase followed by de novo synthesis of basic fibroblast growth factor (FGF)-2 in CECs,²⁰ in addition to the preexisting FGF-2 that is stored in Descemet’s membrane.²¹,²² Through the action of PI 3-kinase, FGF-2 alters the cell morphology of CECs, causes the loss of stress fibers and focal adhesions and reorganizes actin cytoskeleton at the cortex.²²,²³ We also reported that, in the presence of inhibitors of RhoA or Rho-associated kinase (ROCK), FGF-2 further modulates the cellular morphology to a spindle shape with prominent pseudopodia. This dramatic change in cell morphology is completely blocked by the addition of LY294002 (a specific inhibitor of PI 3-kinase).²⁵ We assumed that the spindle-shaped cells with prominent processes that are induced by FGF-2 and Rho inhibitors are migratory endothelial cells.

In the present study, we used a scratch-induced directional cell-migration assay to demonstrate that each Rho GTPase exhibits a different migratory rate. The maximum healing rate...
was achieved by cells treated with a combination of FGF-2 and Rho inhibitors (C3 exoenzyme or Y27632: ROCK inhibitor) or by cells that expressed active Cdc42 in the presence of FGF-2 stimulation. The next levels of healing were achieved in cells that expressed active Cdc42, followed by CECs treated with FGF-2 alone. Cells that expressed active RhoA blocked cell migration, whereas cells that expressed active Rac1 only slightly facilitated migration. These results suggest that Cdc42 activation alone is sufficient to cause a very high level of cell migration in CECs and that FGF-2 further facilitate the migratory rate through the action of PI 3-kinase in these Cdc42-expressing cells. PI 3-kinase is well known to regulate cell polarization and migration via Rac and Cdc42.23-25,13,10,19,24 Our data further showed that elongated cells with pseudopodia, the alteration of which is a prerequisite to cell migration, were mediated by active Cdc42.

Materials and Methods

LY294002, mitomycin-C, and anti-hemagglutinin (HA) antibody were obtained from Sigma-Aldrich (St. Louis, MO); anti-Cdc42 monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-phosphorylated Akt (Ser473) and rabbit anti-Akt antibody from Cell Signaling Technology (Beverly, MA); rhodamine-phallolidin from Invitrogen (Carlsbad, CA); fluorescein isothiocyanate (FITC) and rhodamine-conjugated secondary antibodies from Chemicon (Temecula, CA); FGF-2 and Y27632 from Calbiochem (San Diego, CA); mounting solutions and biotinylated secondary antibodies from Vector Laboratories, Inc. (Burlingame, CA); constitutively active (ca) Rho GTPase plasmids (RhoG14V, RacG12V, and Cdc42G12V) and dominant negative (dn) Rho GTPase plasmids (RhoT19N, RacT12N, and Cdc42T12N) from the UMR cDNA Resource Center (Rolla, MO); and Akt/PKB (activated) and dnAkt1T17M plasmids from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Cultures

Rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR). Rabbit CECs were isolated and established as previously described.25 Briefly, the corneal endothelium-Descemet’s membrane complex was treated with 0.2% collagenase and 0.05% hyalurondase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. Primary cultures were maintained in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum and 50 μg/ml of gentamicin (DMEM-15) in a 5% CO2 incubator. First-passage CECs maintained in DMEM-15 were used for all experiments. Heparin (10 μg/ml) was added to cell cultures treated with FGF-2, since our previous study had showed that CECs require supplemental heparin if FGF-2 activity is to occur.21 In some experiments, pharmacologic inhibitors were used in the presence of FGF-2 stimulation. These were LY294002 (20 μM), or Y27632 (10 μM). The optimal concentration of each inhibitor was used as previously described.23

Cell Proliferation Assay

Cells (1 × 10^5/plate) were plated in 100-mm tissue culture dishes. After cells had been maintained with DMEM-15 for 16 hours, the medium was removed and replaced with fresh DMEM-15 and mitomycin-C (5 or 10 μg/ml) for 24 hours. At the end of the incubation period, the cells were washed with phosphate-buffered saline (PBS) to remove floating cells. The remaining attached cells were harvested using 0.1% trypsin containing EDTA (5 mM) and counted in a microscopic counting chamber (hemocytometer).

Scratch-Induced Directional Migration Assay

The cells were plated in six-well tissue culture dishes at a concentration of 1 × 10^5 cells and maintained in DMEM-15. After the cells reached 80% to 90% confluence, the tip of a micropipette was used to wound the cells, creating linear, cross-stripe scrapes 2 mm apart. The cells were washed with PBS to remove floating cellular debris and re-fed for an additional 24 hours with either serum-free medium (for use as a negative control) or experimental medium (DMEM-15, FGF-2 containing medium with a combination of LY294002, Y27632, or both). Wound closure or cell migration was photographed when the scrape wound was introduced and at designated times after wounding, using an inverted microscope equipped with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The speed of migration was quantitated by computer-assisted image analysis (SPOT ver. 2.1.2, Diagnostic Instruments, Inc.). The individual gaps were measured in each culture condition and at each time point, using this program, and the speed of migration was acquired by dividing the length of gap by the culture time. The residual gap between the migrating cells from the opposing wound edge was expressed as a percentage of the initial scraped area. All experiments were conducted in the presence of 5 μg/ml of mitomycin-C to inhibit cell proliferation.

Protein Preparation and Protein Determination

The cells were plated in 100-mm tissue culture dishes at a concentration of 3 × 10^5 cells, and wounds were created as just described, with a 10-mm separation between the linear scrape wounds. Migratory cells that moved into the wounded area were carefully scraped without contaminating the nonmigratory cells. These cells were washed with ice-cold PBS and then lysed with RIPA buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 minutes. The lysates were centrifuged at 15,000g for 10 minutes at 4°C. The resultant supernatant was concentrated to one-tenth of the original volume, using a two-step precipitation method with acetone: ice-cold acetone was gently added to the supernatant to achieve 70% saturation and the sample was incubated for 2 minutes at −20°C. After a brief centrifugation, the excessive acetone was removed by rotary vacuum evaporation. The precipitate was dissolved in water and precipitated again with 80% acetone for 30 minutes at −20°C. The sample was centrifuged at 15,000g for 10 minutes at 4°C, and the excessive acetone was removed by rotary vacuum evaporation. The resultant precipitates were dissolved in water. Protein concentration of the sample was assessed with the Bradford protein assay system (Bio-Rad Laboratories, Inc., Hercules, CA).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Proteins were separated using SDS-PAGE as described by Laemmli, using the discontinuous Tris-glycine buffer systems.26 Immunoblot analysis was performed as described previously,20,27 using a commercial system (ABC Vectastain; Vector Laboratories, Inc.). The proteins separated by SDS-PAGE were transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories, Inc.), and nonspecific binding sites of nitrocellulose membrane were blocked by 5% nonfat milk. The incubations were performed with primary antibodies (1:1000 dilution for Akt or phosphorylated Akt) and biotinylated secondary antibody (1:5000 dilution) and then with ABC reagent for 30 minutes. Membranes were treated with enhanced chemiluminescence (ECL) reagent (GE Healthcare, Piscataway, NJ) and exposed to ECL film. The relative density of the polypeptide bands detected on ECL film was determined using Gel-doc (Bio-Rad Laboratories, Inc.). Total Akt was measured to determine the level of the phosphorylated Akt in experimental conditions.

Transfection of CECs with Rho GTPases and Akt Expression Vectors

Mutant RhoA, Rac1, or Cdc42 plasmid containing HA tag and mutant Akt vector containing a Myc-His tag, driven by a CMV promoter, were used for transfection experiments. Cells (1 × 10^5/well) were seeded on six-well plates and maintained in culture until they reached 60% to 70% confluence. The cells were transiently transfected with ca-
dnRho GTPase or Akt plasmid using the transfection reagent (FuGENE 6; Roche Applied Science, Pleasanton, CA), according to the manufacturer’s instructions. After 8 hours, medium containing the transfection reagent was removed, and the cells were maintained further in DMEM-15 for 16 hours. Eukaryotic empty expression plasmid vectors pcDNA3.1 (for Rho GTPases) and pUSEamp (for Akt) were used for mock transfection.

Immunofluorescent Staining and Confocal Microscopy

Cells plated in four-well chamber slides were processed as described previously.20-23 Briefly, the cells were fixed and permeabilized, and nonspecific staining sites were blocked with bovine serum albumin. The subsequent incubation was performed with 2% bovine serum albumin in PBS, and all washes were performed at room temperature in PBS containing 0.1% Triton-X-100. The cells were incubated with anti-HA antibody (1:200 dilution for HA tagged Cdc42) for 1 hour at 37°C and then with FITC-conjugated secondary antibody (1:200 dilution) for 1 hour at 37°C in the dark. For Factin staining, the cells were incubated with rhodamine-phalloidin (1:300 dilution) at 37°C for 10 minutes. After extensive washing, the slides were mounted in a drop of mounting medium (Vectashield; Vector Laboratories Inc.). Control experiments, performed in parallel with the omission of the primary antibodies, did not show the activity. Antibody labeling was examined with a laser scanning confocal microscope (LSM-510; Carl Zeiss Meditec, Dublin, CA). The 1.8-μm optical slices were taken perpendicular to the cell monolayer (apical to basal orientation). A 488-nm argon laser was used in combination with a 499/509- to 530-nm excitation/emission filter set for fluorescein examination. For rhodamine, the 543-nm helium neon laser was used with a 543-nm excitation filter and a 560-nm emission filter. Image analysis was performed with the standard system operating software provided with the microscope. All illustrations were assembled and processed digitally (Photoshop 7; Adobe, San Jose, CA).

Expression and Purification of GST-PAK-CRIB Domain

Expression plasmids for GST fusion proteins with PAK-CRIB were generous gifts from John G. Collard.18 *Escherichia coli* BL21 cells transformed with GST-PAK-CRIB construct were grown at 37°C to an absorbance of 0.3. Expression of GST fusion protein was induced by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) at an absorbance of 1.0. After induction, the cells were collected, and lysed by sonication in bacterial lysis buffer (50 mM Tris-Cl [pH 8.0], 2.0 mM MgCl2, 2.0 mM dithiothreitol, 10% glycerol, 20% sucrose, 0.2 mM Na3S2O3, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, and 1 mM PMSF).16 Supernatant was recovered after centrifugation for 15 minutes at 14,000g. The total 9 mg of cleared cell lysates were split into three equal aliquots. As negative and positive controls for the pull-down, two of the aliquots were added to 100 μM GDP or GTPγS, respectively, and incubated for 15 minutes at 30°C with agitation, to deplete or enrich Cdc42-GTP. The third aliquot remained untreated and was kept on ice while the controls were loaded. PAK-CRIB beads were then added to each aliquot, and the reaction mixtures were incubated for 45 minutes at 4°C with gentle agitation. After the pull-down reaction, the supernatants were removed by brief centrifugation, and the precipitated proteins bound to the beads were subjected to immunoblot analysis with monoclonal antibody to Cdc42. Total Cdc42 was also determined to compare the level of the activated Cdc42 in respective experimental conditions. The relative density of the polypeptide bands detected on ECL film was then determined (Gel-doc; Bio-Rad Laboratories).

RESULTS

FGF-2-Mediated Wound Healing

Because treatment of cells with either DMEM-15 or FGF-2 containing DMEM-15 facilitates cell proliferation, both conditions possibly influence the migratory activity measured in the experiments. We therefore performed the migration assay in the presence of mitomycin-C and we first determined the inhibitory activity of mitomycin-C on CECs. Mitomycin-C at 5 μg/mL completely inhibited cell proliferation of CECs, whereas mitomycin-C at 10 μg/mL appears to be slightly cytotoxic (Table 1). We therefore chose 5 μg/mL as the concentration of mitomycin-C to block cell proliferation and to confirm that wound healing was completely attributed to the cell migration. Wound healing in tissue culture monolayers is a commonly used model for analyzing the molecular mechanism underlying cell migration. In this model, the wound-edge cells and those cells in the rows behind them migrate into the wound space and continue to move in an essentially unidirectional and synchronous fashion until they meet cells migrating toward them from the opposing wound edge. With this migration assay, subconfluent monolayers were scratched, washed, and then further cultured in serum-containing media (DMEM-15) with FGF-2 for 8 or 16 hours in the presence or absence of LY294002, Y27632, or a combination of the two inhibitors (Fig. 1). Migratory cells maintained in serum-free medium.

### Table 1. Concentration-Dependent Effect of Mitomycin-C on Cell Proliferation in CECs

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Incubation Time (h)</th>
<th>Number of Cells (×10^5)</th>
</tr>
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<tbody>
<tr>
<td>DMEM-15</td>
<td>0</td>
<td>2.37 ± 0.07</td>
</tr>
<tr>
<td>DMEM-15/mitomycin-C (5 μg/mL)</td>
<td>24</td>
<td>9.71 ± 0.19</td>
</tr>
<tr>
<td>DMEM-15/mitomycin-C (10 μg/mL)</td>
<td>0</td>
<td>2.21 ± 0.12</td>
</tr>
<tr>
<td>DMEM-15/mitomycin-C (10 μg/mL)</td>
<td>24</td>
<td>2.43 ± 0.15</td>
</tr>
</tbody>
</table>

Proliferation of CECs maintained with each culture condition and incubation time was investigated by counting the number of cells with a microscope counting chamber (hemocytometer). Data are representative of three experiments.

* Mitomycin-C at 5 μg/mL resulted in complete inhibition of cell proliferation without cytotoxicity.

Cdc42 GTPase Pull-down Assay

The cells were plated in 100-mm tissue culture dishes at a concentration of 3 × 10^6 cells. Wounds were created as stated earlier, with the cross-stripe scrapes placed 10 mm apart, and the cells were maintained under the experimental conditions. After the migratory cells were gathered, they were lysed with ice-cold GST-Fish lysis buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 2 mM MgCl2, 10% glycerol, 1% Nonidet P-40, and 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL aprotinin, and 1 mM PMSF).16 Supernatant was recovered after centrifugation for 15 minutes at 14,000g. The total 9 mg of cleared cell lysates were split into three equal aliquots. As negative and positive controls for the pull-down, two of the aliquots were added to 100 μM GDP or GTPγS, respectively, and incubated for 15 minutes at 30°C with agitation, to deplete or enrich Cdc42-GTP. The third aliquot remained untreated and was kept on ice while the controls were loaded. PAK-CRIB beads were then added to each aliquot, and the reaction mixtures were incubated for 45 minutes at 4°C with gentle agitation. After the pull-down reaction, the supernatants were removed by brief centrifugation, and the precipitated proteins bound to the beads were subjected to immunoblot analysis with monoclonal antibody to Cdc42. Total Cdc42 was also determined to compare the level of the activated Cdc42 in respective experimental conditions. The relative density of the polypeptide bands detected on ECL film was then determined (Gel-doc; Bio-Rad Laboratories).
DMEM-0 served as the negative control, while migratory cells maintained in DMEM-15 served as the positive control. It should be noted that rabbit CECs readily become nonresponsive when maintained in serum-free medium for 24 to 48 hours. Therefore, DMEM-15 was used as a positive control to demonstrate the basal migratory activity mediated by serum. In this scrape-wound assay over a 16-hour period (Fig. 1A), the cells maintained in DMEM-0 demonstrated negligible cell migration, while cells maintained in DMEM-15 re-covered approximately 30% of the wound area. LY294002 did not block cell migration, suggesting that serum-mediated cell migration is a PI 3-kinase-independent event. In contrast, cells maintained in DMEM-15 containing FGF-2 re-covered 43% of wound area, while the action of FGF-2 was completely abolished by LY294002. The enhanced wound healing mediated by FGF-2 was 50% greater than the serum-induced wound healing. In our previous study, we showed that CECs simultaneously treated with FGF-2 and inhibitors for Rho kinase pathways (C3 exoenzyme or Y27632) caused CECs to elongate and develop prominent pseudopodia. To determine whether the elongated CECs containing pseudopodia further acquire migratory potential, the scrape-wounded cultures were simultaneously treated with FGF-2 and Y27632. Under these conditions, 84% of the wound area was recovered with migratory cells, whereas the synergistic effect of FGF-2 and Rho inhibitor were completely blocked by LY294002 (Fig. 1A). Figure 1B also shows a time-dependent manner of healing in these scrape-wounded CECs. We further analyzed cell shape at the wound’s edge. Figure 1C demonstrates that the migrating cells at the leading edge had a different cell morphology. Cells treated with FGF-2 and Y27632 showed elongated cells with prominent processes. LY294002 reversed the migratory cell phenotypes to the spread cell morphology, similar to those maintained in DMEM-15.

The data are representative of results in three experiments. *P < 0.01.

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**FIGURE 1.** FGF-2 initiates and Rho inhibition further enhances cell migration during wound healing. First-passage cells were plated in six-well tissue culture plates at a cell density of $1 \times 10^5$ in DMEM-15 for 24 hours. Thereafter, a wound (scratch) was made at the middle of the wells, and free cells were removed by PBS washing. Media were then changed to designated fresh media containing mitomycin-C (5 μg/mL) to block cell proliferation and further incubated. Wound closure was photographed with SPOT (A) and quantified (B). Higher-magnification views of the scratched region after 16 hours of incubation are shown in (C). D-0, D-15, F-2, Y, and LY designate DMEM without serum, DMEM-15, FGF-2, Y27632, and LY294002, respectively. The data are representative of results in three experiments. *P < 0.01.
migrated at a speed of 0.42 μm/min, but the addition of LY294002 blocked the FGF-2-mediated cell migration. CECs treated with a ROCK inhibitor in the presence of FGF-2 stimulation showed the highest migration rate of 0.72 μm/min, and the PI 3-kinase inhibitor completely blocked the synergistic effect of FGF-2 and ROCK inhibitor, decreasing migration to the serum-induced level.

We further confirmed the involvement of PI 3-kinase in FGF-2-mediated cell migration. Cells between the initial wound site and the re-covered region were carefully removed and prepared for immunoblot analysis for both total Akt and phosphorylated Akt at Ser473. As shown in Figure 2, migratory cells maintained in DMEM-15 contained a very low level of phosphorylated Akt, which was lost by the addition of LY294002. Migratory cells stimulated with FGF-2 demonstrated a marked increase of PI 3-kinase activation, which is completely abolished by LY294002. Further enhancement of PI 3-kinase activation was observed in cells treated with both FGF-2 and Y27632, whereas the PI 3-kinase activation was completely abolished by LY294002. A control experiment was performed in parallel to show that nonmigratory cells taken from the contact-inhibited monolayer far from the wound’s edge demonstrated no activation of PI 3-kinase, even in the presence of serum (DMEM-15).

Our recent study showed that CECs transfected with either caCdc42 or dnRhoA demonstrated elongated cell morphology with prominent pseudopodia, similar to CECs treated with FGF-2 and Rho kinase inhibitors. These findings suggest that activated Cdc42 and inactivated RhoA are equally able to elicit the migratory cells with prominent processes. We therefore measured Cdc42 activity of the migratory cells obtained from the initial wound site and the re-covered region using a Cdc42-GTP pull-down assay. A detectable amount of Cdc42-GTP was seen in the migratory cells maintained in DMEM-15, whereas a marked increase in the amount of Cdc42-GTP was shown in the migratory cells stimulated with FGF-2 (Fig. 3). Simultaneous treatment of cells with FGF-2 and Y27632 resulted in a fivefold enhancement of Cdc42 activation compared with the level observed in CECs maintained in DMEM-15, whereas treatment with LY294002 completely abolished the Cdc42 activation. Thus, these findings confirm that FGF-2 facilitated cell migration of CECs by activating Cdc42 through the action of PI 3-kinase. Figure 3 further shows that inactivation of Rho facilitates Cdc42 activation. This event is also mediated by the PI 3-kinase pathway. In contrast, nonmigratory cells obtained from the contact-inhibited monolayer far from the wound’s edge demonstrated no Cdc42 activation.

### Cdc42-Mediated Wound Healing

The above finding that cell migration is mediated through the action of activated Cdc42 was further tested in CECs transiently transfected with caCdc42 or dnCdc42 mammalian expression vectors. Neither untransfected CECs nor cells trans-

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>Migratory Rate (μm/min)</th>
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<tbody>
<tr>
<td>D-0</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>D-15</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>D-15/LY</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>D-15/Y</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>D-15/FGF-2</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>D-15/FGF-2/LY</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>D-15/FGF-2/Y</td>
<td>0.72 ± 0.07*</td>
</tr>
<tr>
<td>D-15/FGF-2/Y/LY</td>
<td>0.23 ± 0.03</td>
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* Stimulation with both FGF-2 and ROCK inhibitor resulted in the fastest migration rate.
fected with empty vector showed positive staining with anti-HA antibody (Fig. 4). When cells expressing active Cdc42 were stained with phalloidin, F-actin staining was clearly observed in the protrusive processes of the migratory cells (Fig. 4, arrowheads). Cells expressing inactive Cdc42 were smaller and round and demonstrated F-actin staining in the cytoplasm.

Healing rates of these transfected cells were measured. The cells were transiently transfected with either caCdc42 or dnCdc42 for 24 hours, after which a scrape wound was introduced. Wound healing was assayed 8, 16, or 24 hours after scraping (Fig. 5). The wound area was decreased markedly in a time-dependent manner, regardless of the experimental conditions. In CECs transfected with empty vector maintained in DMEM-15, approximately 30% of the wound area was re-covered 24 hours after wounding (Fig. 5A). In CECs expressing active Cdc42, 60% of the wound area was re-covered 24 hours after wounding (Fig. 5B). Wound healing was assayed 8, 16, or 24 hours after scraping (Fig. 5). The wound area was decreased markedly in a time-dependent manner, regardless of the experimental conditions. In CECs transfected with empty vector maintained in DMEM-15, approximately 30% of the wound area was re-covered 24 hours after wounding (Fig. 5A). In CECs expressing active Cdc42, 60% of the wound area was re-covered 24 hours after wounding (Fig. 5B).
after wounding, but the healing rate of CECs expressing inactive Cdc42 was similar to that observed in cells transfected with empty vector. In cells expressing mutant Cdc42, healing rates were slightly enhanced in response to the additional FGF-2 stimulation (Fig. 5B): CECs expressing active Cdc42 in the presence of FGF-2 stimulation re-covered 65% of the wound area, whereas cells expressing inactive Cdc42 re-covered 40% of the wound area in response to FGF-2. Table 3 further demonstrates that active Cdc42 alone induced a very fast migration rate (0.52 μm/min) and that FGF-2 further facilitated the migration to the range of the maximum speed (0.69 μm/min).

We also examined the healing rates of CECs transfected with caRac or dnRac in the absence or presence of exogenous FGF-2 (Fig. 6, Table 3). Cells expressing caRac1 showed slightly enhanced cell migration (0.39 μm/min) and overall healing, whereas the cells expressing dnRac1 showed a healing rate similar to that observed in cells transfected with empty vector. Exogenous FGF-2 slightly facilitated cell migration and the subsequent healing rate in cells expressing mutant Rac1.

The effect of RhoA on cell migration was also determined in CECs transfected with either caRho or dnRho (Fig. 7, Table 3); activation of Rho greatly inhibited cell migration (0.12 μm/min) and subsequently blocked the healing process in CECs, in which 20% of the wound area was re-covered. Because the migration rate of CECs maintained in serum containing Rho-activating lysophosphatic acid was a rather consistent 0.25 to 0.26 μm/min, constitutively active Rho demonstrates a strong inhibitory activity on cell migration (0.12 μm/min). However, dnRho neither stimulated nor inhibited the healing. The migration and healing rates in cells expressing inactive Rho were similar to the levels induced by serum. This finding may reflect the fact that dnRho could not overcome the Rho activated by 15% serum. Exogenous FGF-2 affected the migratory rate in both Rho mutants: FGF-2 greatly antagonized the active Rho activity, elevating the migration rate from 0.12 to 0.21 μm/min. Cells expressing dnRho demonstrated a marked increase in the migration rate to 0.51 μm/min. Of interest, this further demonstrates that active Cdc42 alone induced a very fast migration rate (0.52 μm/min) and that FGF-2 further facilitated the migration to the range of the maximum speed (0.69 μm/min).

Subconfluent CECs were transfected with mutant Rho GTPases or mutant Akt vectors. After transfection, scratch-induced directional migration assay was performed. Control cells were treated with transfection reagent alone. Data are representative of three experiments.

Migration of cells expressing caCdc42 treated with FGF-2 reached the maximum rate.
The migration rate is similar to that observed in cells expressing caCdc42 in the absence of FGF-2 stimulation (0.52 μm/min), suggesting that FGF-2 further activates Cdc42 in these dnRho-expressing cells. Both the activation of Cdc42 and the inactivation of Rho are necessary for a high rate of cell migration and, subsequently, for rapid wound healing, whereas the contribution of Rac to the cell migration is insignificant in CECs. Because PI 3-kinase is absolutely essential for the production of the migratory phenotypes of CECs, we further investigated whether such action of PI 3-kinase was relayed through Akt pathways. The CECs were transiently transfected with either caAkt or dnAktK179M. There was a slight increase in cell migration in those cells expressing active Akt, but there was no decrease in the healing rate of cells expressing dnAkt mutant when compared with that observed in cells transfected with empty vector (Fig. 8A, Table 3). The migration rate caused by the active Akt did not equal the rate achieved by caCdc42. Of interest, exogenous FGF-2 did not affect the migratory rates in either caAkt- or dnAkt-expressing cells (Fig. 8B, Table 3), suggesting that Akt is not a major downstream effector molecule in FGF-2-mediated cell migration. Finally, we measured the Cdc42 activity of the migratory cells obtained from CECs expressing Cdc42 mutants (Fig. 8C). There was an almost threefold increase in the amount of Cdc42-GTP in the migratory and caCdc42-expressing cells when compared with the level of cells transfected with empty vector, whereas cells expressing dnCdc42 demonstrated a level of Cdc42-GTP similar to that observed in cells transfected with empty vector.

**Discussion**

Corneal endothelium functions as an active barrier to prevent leakage of the aqueous humor into the corneal stroma. Any leakage at the level of corneal endothelium leads to corneal edema and opacification and thus to severe visual impairment. Maintenance of proper barrier function requires an intact endothelial monolayer; therefore, the number of CECs becomes a critical issue. If too many cells are lost, a decline in corneal transparency ensues. Unlike vascular endothelial cells, which undergo rapid division during wound healing, the corneal endothelium is considered to be a nonreplicating tissue. Wound repair of corneal endothelium after in vivo injury appears to have two distinct pathways: the regenerative pathway, by which endothelial cells do not replicate but are replaced by migration and spreading of existing endothelial cells, and the nonregenerative pathway (or fibrosis), by which transformed endothelial cells not only resume proliferation but alter their cell morphology and collagen phenotypes, leading to the production of an abnormal ECM in the basement membrane environment. However, it takes an extreme injury, such as an alkali burn, to cause such corneal fibrosis.
Thus, cell migration in the absence of cell proliferation has been recognized as the major wound-healing mechanism by which endothelial function is resumed.

Cell migration is essential, not only during development, but throughout life, for processes such as wound repair and immune surveillance. Soluble growth factors and ECM are recognized as the two key extracellular signals that directly influence the cell’s decision to move. Among soluble factors or local signals that cause migration of a variety of cells, EGF and FGF-2 induce migration of CECs.$^{36,37}$ FGF-2 employs activated PI 3-kinase to induce migration,$^{36}$ whereas EGF uses protein kinase C (PKC) to enhance the migratory response.$^{37}$ However, the involvement of PKC during corneal endothelial migration is FGF-2 independent. At present, it is unknown how EGF and FGF-2, both of which activate their respective receptor tyrosine kinases, exert differential signal transduction.

Cell migration directed by extracellular cues elicits a variety of intracellular responses that include changes in the organization of actin and microtubule cytoskeletons. It is widely accepted that local activation of Rac or Cdc42 is a key event that regulates actin polymerization at the leading edge, to generate net forward movement. Our previous study$^{25}$ demonstrated that simultaneous treatment of CECs with FGF-2 and Rho inhibitors alters the cell shape from a polygonal to an elongated morphology with prominent pseudopodia. Such a characteristic cell shape was also observed in CECs transfected with either caCdc42 or dnRho.$^{26}$ We therefore postulated that these elongated cells with prominent pseudopodia are the migratory phenotype of CECs and that activation of Cdc42 and inactivation of Rho are equally essential for cell migration. In the present study, we used a scrape-induced directional migration assay to determine the serum’s effect on cell migration, because the presence of serum in the experiment is absolutely necessary. CECs maintained in serum-free medium for longer than 24 hours became unresponsive to the target growth factor being tested. We also used mitomycin-C to inhibit cell proliferation of CECs and to confirm that wound healing is completely attributed to the cell migration. CECs maintained in DMEM-15 demonstrated a migration rate of 0.25 $\mu m/min$, whereas cells maintained in the absence of serum showed a rate of 0.05 $\mu m/min$. Of interest, serum facilitates cell migration, although serum contains lysophosphatic acid, a potent Rho activator. Maximum cell migration of CECs was achieved under two conditions: (1) simultaneous treatment of cells with FGF-2 and Rho inhibitors; and (2) stimulation of caCdc42-expressing cells with FGF-2. CECs expressing caRho showed marked inhibition of serum-induced cell migration. These data indicate that both activation of Cdc42 and inactivation of Rho are needed to achieve maximum cell migration leading to a maximum healing rate and that FGF-2 alone is unable to generate such a maximum healing. Also of note is the finding that activated Cdc42 alone sufficiently facilitates cell migration, leading to a moderate level of wound healing; the migration rate achieved by active Cdc42 is greater than that achieved by FGF-2 stimulation alone.

Figure 7. Retardation of the CEC migratory rate by activated Rho. CECs were transiently transfected with mutant Rho and then maintained for 24 hours. Thereafter, a scratch wound was made in the middle of the wells, and free cells were removed by washing with PBS. Media were then changed to fresh DMEM-15 (A) or DMEM-15 with FGF-2 (B) containing mitomycin-C, and the cells were further incubated. Migration was analyzed as described in Figure 5. Data are representative of results in three experiments.
CECs expressing active Rac demonstrated a relatively low level of migration and low healing rates. FGF-2 does not further facilitate either the healing rate or cell migration in CECs expressing active Rac. This particular finding is important to link actin cytoskeleton structures to the migratory phenotypes of CECs. We reported that FGF-2 reorganizes actin cytoskeleton to the cortex and modulates cell shape from a polygonal to an elongated shape. Our recent study further demonstrated that FGF-2-mediated cortical actin mat is formed through activation of PI 3-kinase and subsequently through active Rac. However, the present study showed that the reorganization of actin at the cortex observed in CECs treated with FGF-2 alone...
does not sufficiently enhance the cell migration rate. Assuming that CECs require additional actin structures (i.e., pseudopodia) that enable cells to move forward, we measured the migratory rate of the spindle-shaped cells with pseudopodia. These cells, generated either by simultaneous treatment of CECs with FGF-2 and Rho inhibitor or by stimulation of catcdc42-expressing cells with FGF-2, demonstrated the maximum migration speed. Thus, it is likely that CECs require pseudopodia formation for cell migration and that the acquisition of such pseudopodia is the net outcome of the activation of Cdc42 and antagonizing Rho activity. Failure of FGF-2 to generate the migratory phenotypes in this in vitro migration assay may suggest that local concentration of the growth factor is critical to activate Cdc42 while inactivating Rho. Unlike the in vitro system, the in vivo injury may not face the same problem, because FGF-2 itself is a resident protein in Descemet’s membrane, and the growth factor is rapidly induced by IL-1β released either by inflammatory cells or by damaged cells during the wound-healing process. Thus, the regenerative ability of the host and the degree of inflammation play a key role in determining whether CECs become migratory or undergo mesenchymal transformation (subsequently leading to corneal fibrosis). In most cases of wound healing, however, CECs become migratory; thus, efficient cell migration, as the only mechanism for repairing the wound, is of great importance for re-covering the denuded basement membrane to maintain corneal clarity. Herein, we have shown that acquisition of pseudopodia is crucial for CECs to become migratory and subsequently to re-cover the denuded basement membrane for proper barrier function.

References


