Potential Functional Restoration of Corneal Endothelial Cells in Fuchs Endothelial Corneal Dystrophy by ROCK Inhibitor (Ripasudil)

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• PURPOSE: Rho-associated kinase (ROCK) inhibitors have been successfully used as a rescue strategy in eyes that failed to clear after descemetorhexis without endothelial graft for treatment of Fuchs endothelial corneal dystrophy (FECD). The functional mechanisms by which ROCK inhibitors modulate corneal endothelial cell regeneration in FECD patients have, however, not been clarified. Here, we analyzed the effect of the ROCK inhibitor ripasudil on corneal endothelial cells of FECD patients and normal donors using ex vivo tissue and in vitro cellular models.

• DESIGN: Experimental study: laboratory investigation.

• METHODS: This institutional study used endothelial cell–Descemet membrane lamellae from FECD patients (n = 450) undergoing Descemet membrane endothelial keratoplasty (FECD *ex vivo* model), normal research-grade donor corneas (n = 30) after scraping off central endothelial cells (*ex vivo* wound healing model), normal donor corneas (n = 20) without endothelial injury, and immortalized cell lines (n = 3) generated from FECD patients (FECD in vitro model). Descemet membrane lamellae were dissected into halves and incubated for 24-72 hours in storage medium with or without a single dose of 30 μM ripasudil. The effects of ripasudil on expression of genes and proteins related to endothelial cell proliferation, migration, functionality, and endothelial-to-mesenchymal transition were analyzed and complemented by functional assays on FECD cell lines.

• RESULTS: A single dose of ripasudil induced significant upregulation of genes and proteins related to cell cycle progression, cell-matrix adhesion and migration, as well as endothelial barrier and pump function up to 72 hours, whereas classical markers of endothelial-to-mesenchymal transition were downregulated in both FECD and normal specimens compared to unstimulated controls ex vivo. In addition to stimulation of proliferation and migration, ripasudil-induced changes in expression of functional signature genes could be also verified in FECD cell lines in vitro.

• CONCLUSIONS: These data support the concept that inhibition of ROCK signaling represents a potent tool in regenerative therapies in FECD patients through reactivation of cell proliferation and migration as well as restoration of endothelial pump and barrier function without inducing adverse phenotypic changes. (Am J Ophthalmol 2021;224:185–199. © 2020 Elsevier Inc. All rights reserved.)

FUCHS ENDOTHELIAL CORNEAL DYSTROPHY (FECD) represents the most common form of corneal endothelial dystrophy and one of the most common indications for corneal transplantation.[1] This condition typically occurrs around the age of 50 to 60 years and leads to excrescences of Descemet membrane, termed guttae, and progressive loss of corneal endothelial cells, which causes visual impairment due to corneal edema. Although surgical treatment by endothelial keratoplasty, for example, Descemet membrane endothelial keratoplasty (DMEK), continues to be the first-line therapy,[2] it carries a risk of postoperative failure and is limited by donor tissue availability.[3] Thus, development of novel treatment options for corneal endothelial repair without transplantation remains an urgent medical need.

Several lines of evidence suggested a potential regenerative capacity, involving either migration or proliferation or both, of corneal endothelial cells in FECD patients.[4,5] These include clinical reports on repopulation of graft-free areas after successful DMEK[6] as well as spontaneous

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corneal clearance after partial or complete DMEK graft detachment[7–11] and after inadvertent removal of patient's Descemet membrane.[12–14] These observations initiated multiple single-center studies on a novel surgical approach, that is, Descemet stripping without endothelial graft replacement, alternatively termed descemetorhexis without endothelial keratoplasty (DWEK) or Descemet stripping only (DSO).[15–22] The underlying rationale was to remove the central area of diseased endothelial cell–Descemet membrane complex to allow the healthier peripheral cells to repopulate the bare corneal stroma, by either migration or proliferation or both. Despite growing knowledge of the predictive factors of success, clinical outcomes of this approach have been variable and inconsistent.[2,23]

To improve outcomes, topical Rho-associated protein kinase (ROCK) inhibitors have been used successfully as a rescue strategy or an adjuvant therapy to accelerate endothelial wound healing.[24,25] In a prospective study using the ROCK inhibitor ripasudil as an adjuvant to DSO, Macsai and Shiloach showed a significantly faster recovery and higher endothelial cell density after 1 year in the DSO+ripasudil group compared to the DSO group.[26] In another prospective interventional case series, DSO supplemented with ripasudil proved to be safe and effective, resulting in corneal clearance in 95% of eyes after 4 weeks on average.[27] Relapse of corneal edema on halting of ripasudil treatment provided strong evidence of efficacy of the drug. Thus, DSO combined with ROCK inhibitor eye drops has emerged as a novel attractive surgical intervention in selected patients with mild to moderate FECD characterized by centrally located guttae and an adequate peripheral endothelial reserve.[26]

ROCK is a serine/threonine kinase and a major downstream effector of the small GTPase Rho, which is activated by wounding, growth factors, cytokines, and via integrin activation.[28] The 2 isoforms ROCK-I and ROCK-II phosphorylate various intracellular substrates, such as myosin light chain, and regulate a wide spectrum of fundamental cellular events, including adhesion, migration and proliferation, mainly via reorganization of the cytoskeleton. Since their discovery in 1995, ROCK inhibitors have been studied extensively as adjuvants in corneal endothelial cell cultivation and cell-based therapies.[29] Previous pioneering studies showed that inhibition of ROCK signaling stimulated cell adhesion, migration, proliferation, viability, and wound healing of corneal endothelial cells in in vitro, ex vivo, and in vivo animal models.[30–35] Clinically, the use of ROCK inhibitor eye drops resolved corneal edema and improved visual acuity in FECD patients after removal of the central endothelium by trans-corneal freezing.[36,37] It also supported reconstruction of the endothelial layer in cell-based therapies in patients with bullous keratopathy.[38]

Thus, there is a substantial amount of evidence to support the efficacy of topical ROCK inhibitors for regenerative therapies in select FECD patients with mild to moderate corneal endothelial dysfunction. However, minor concerns regarding adverse cell morphologic changes toward transition into a mesenchymal phenotype have been raised,[39] and the functional mechanisms by which ROCK inhibitors modulate corneal endothelial cell regeneration in FECD patients have not been clarified conclusively. Apart from this, the question remains whether the restored endothelial cells are functionally competent.

In this study, we analyzed the effects of ROCK signaling inhibition on the expression of genes related to proliferation, migration, functionality and endothelial-mesenchymal transition (EnMT) in corneal endothelial cells of FECD patients using ex vivo tissue and in vitro cell models. Normal human donor corneas subjected to circular scratch wounds were used in a complementary ex vivo model system to reproduce an endothelial wound healing response. We used the novel selective ROCK-I and ROCK-II inhibitor ripasudil hydrochloride hydrate (Glanatec ophthalmic solution 0.4%, Kowa Co Ltd, Nagoya, Japan), which has been approved for the treatment of ocular hypertension and glaucoma in Japan[40] and has been suggested as an effective agent for adjunctive treatment in FECD patients.[24,26,29]

Here, we showed that ROCK inhibition by ripasudil activates cell cycle progression and promotes cell adhesion and migration without inducing any adverse effects on phenotype in corneal endothelial cells of both FECD and normal subjects ex vivo, and verified the beneficial effects in FECD cell lines in vitro. Most importantly, ripasudil induced an upregulation of functional proteins relevant for endothelial pump and barrier function in all three ex vivo and in vitro models, suggesting a potential functional restoration of corneal endothelial cells in FECD.

METHODS

• **HUMAN TISSUES AND STUDY APPROVAL:** Central endothelial cell–Descemet membrane (EDM) lamellae, 8 mm in diameter, were prepared by manual stripping from FECD patients (n = 450) during DMEK surgery. Mean patient age was 69.7 ± 9.6 years (201 male, 249 female). Immediately after excision, the EDM scrolls were dissected into halves and incubated for 24-72 hours in 4 mL of CorneaMax storage medium (Eurobio, Les Ulis, France) without or with a single dose of ripasudil hydrochloride dihydrate (K-115) (Selleck Chemicals, Houston, Texas, USA) at 37°C. Following incubation, the paired specimens were subjected to RNA or protein extraction.

Normal donor corneas (n = 50) unsuitable for transplantation with appropriate research consent were obtained from CorneaGen (Seattle, Washington, USA) or Iowa Lions Eye Bank (Coralville, Iowa, USA) in Optisol solution (Bausch & Lomb, Rochester, New York, USA) and

shipped at 4°C. Mean donor age was 58.2 ± 10.3 years (27 male, 23 female). There was a mean storage time of 9 (9.3 ± 3.1) days. For the assessment of dose-dependent effects, EDM complexes were prepared by manual stripping from 20 intact corneas. For each experiment (n = 4), 5 EDM complexes were dissected into 4 equal parts, combined into sets of 4 pools, and incubated with 0, 10, 30, and 100 μM ripasudil for 24 hours. For the ex vivo wound healing model, the central endothelial cells of 30 corneas were damaged by a modified scratch assay technique across the central 5-mm zone of the cornea using a trephine for gentle demarcation and a Sinskey hook for abrasion of endothelial cells. Twenty of 30 wounded corneas were subjected to EDM stripping after wounding, and EDM scrolls were again dissected into halves and incubated for 48 hours in CorneaMax without or with 30 μM ripasudil before RNA extraction. Ten of 30 wounded corneas in toto were dissected into halves, incubated for 72 hours at 37°C in Dulbecco's Minimal Essential Medium (DMEM; Biochrome GmbH, Berlin, Germany) supplemented with 2% fetal calf serum (FCS) and 50 μg/mL gentamicin without or with 30 μM ripasudil before processing for immunohistochemistry.

Handling of donor tissues was in accordance with the principles of the Declaration of Helsinki for experiments involving human tissues and samples. Ethics approval for this laboratory investigation was obtained from the Institutional Review Board of the Medical Faculty of the University of Erlangen-Nürnberg (No. 138_18B), and informed research consent was obtained from all patients.

• **CELL CULTURE:** Three immortalized cell lines established from 3 FECD patients (iFECD 1 and 2: ages 62 and 79 years, 2 men; iFECD 3: age and sex unknown)[41] were cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin/amphotericin B (PSA) solution (PAN-Biotech, Aidenbach, Germany) on FNC (Athena Enzyme Systems, Baltimore, Maryland, USA)–coated culture plates. To test the effect of ripasudil, cells were grown in serum-deprived medium (DMEM containing 1% FCS and 1% PSA) for 6-96 hours.

Cell migration and cell proliferation assays were performed as described previously (Supplementary Methods).[42] For transmission electron microscopy, iFECD cells were cultivated without or with 30 μM ripasudil on plastic cover slips, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 2% buffered osmium tetroxide, and embedded in epoxy resin according to standard protocols. Ultrathin horizontal sections were stained with uranyl acetate and lead citrate and were examined with a transmission electron microscope (EM 906E; Carl Zeiss Microscopy, Oberkochen, Germany).

• **LABORATORY ANALYSES:** Real-time polymerase chain reaction (RT-PCR), Western blot analyses, and immunohistochemistry are described in Supplementary Methods. Primer sequences and antibodies are noted in Supplementary Tables S1 and S2, respectively.

• **STATISTICAL ANALYSIS:** Statistical analyses were performed using SPSS v.24 (IBM, Ehningen, Germany) and GraphPad Prism V8.3.0 (GraphPad Software, San Diego, California, USA) software. Data are expressed as mean ± standard deviation. Gaussian distribution of data sets was tested using the Kolmogorov-Smirnov normality test. The Mann-Whitney *U* test (for real-time PCR and Western blot analyses) and the 2-way analysis of variance for multiple comparisons (BrdU proliferation assays) were performed to assess statistical significance between groups. To examine the relation between ripasudil-induced changes in gene expression and patient age, Spearman correlation analysis was employed. A P value of $<.05$ was considered statistically significant.

RESULTS

• **DOSE-DEPENDENT EFFECTS OF RIPASUDIL:** To determine the appropriate concentration of ripasudil, EDM specimens stripped from normal donor corneas (n = 4 individual pools from 20 donors) were incubated with ripasudil at concentrations of 0, 10, 30, and 100 μM for 24 hours, before expression levels of selected marker genes related to cytoskeleton, cell adhesion, and migration were analyzed. Relative to untreated controls, expression levels of ACTB (beta-actin) and ACTA2 (alpha-smooth muscle actin) were consistently downregulated, whereas expression levels of ITGA2 (integrin α2), ITGA6 (integrin α6), ITGA10 (integrin α10), ITGB1 (integrin β1), LAMA5 (laminin α5), and LAMB1 (laminin β1) were upregulated by ripasudil at various concentrations (Supplementary Figure S1, A). In most cases, ripasudil showed the most pronounced effects on gene expression changes at a concentration of 30 μM, which was, therefore, used for all subsequent experiments.

To confirm efficacy of 30 μM ripasudil on endothelial cells, EDM specimens (n = 5) obtained from FECD patients were used to analyze phosphorylation of myosin light chain (MLC2), a direct target of ROCK action. Western blot analysis revealed a significant dephosphorylation of MLC2 (approximately −50%; $P < .01$) in response to ripasudil after 6, 12, and 24 hours indicating a potent inhibition of ROCK signaling in FECD specimens (Supplementary Figure S1, B).

• **EFFECT OF RIPASUDIL ON ENDOTHELIAL CELL CYCLE REGULATION AND PROLIFERATION:** Following incubation in 30 μM ripasudil for 24 hours, EDM specimens (n = 35) derived from FECD patients ex vivo showed a significant upregulation of genes driving cell cycle

progression, i.e. CCND1 (cyclin D1) and CCND2 (cyclin D2), CDK6 (cyclin-dependent kinase 6) and PCNA (proliferating cell nuclear antigen), whereas genes inducing cell cycle arrest, i.e. CDKN1B (cyclin-dependent kinase inhibitor 1B; $p27^{Kip1}$), CDKN2A (cyclin-dependent kinase inhibitor 2A; $p16^{INK4a}$) and CDKN2B (cyclin-dependent kinase inhibitor 2B; $p15^{INK4b}$), were significantly downregulated (Figure 1, A, top). Other cell cycle-related genes, including CCNE1 (cyclin E1), CDK2/3/4 (cyclin-dependent kinase 2/3/4), and CDKN1A/1C/2A/2C (cyclin-dependent kinase inhibitor 1A/1C/2A/2C), were not affected by ripasudil. On the protein level, Western blot analysis confirmed significant upregulation of the proliferation marker PCNA by ripasudil up to 72 hours of stimulation compared to untreated controls (Figure 1, A, bottom).

Similar changes in expression levels of cell cycle-related genes were also observed in EDM specimens derived from normal wounded corneas ex vivo (n = 12), with the exception of CDKN2A (Figure 1, B, top). Immunohistochemistry/confocal microscopy of corneal whole mounts confirmed increased protein expression of Ki-67 and PCNA in corneal endothelial cell nuclei after ripasudil treatment in the periphery of the wound (Figure 1, B, bottom).

The effect of ripasudil on cell proliferation was assessed by BrdU incorporation assay in vitro. For this purpose, we used an FECD cellular model using immortalized cell lines derived from FECD patients (iFECD cells).[41] Compared with untreated control cells, proliferation rates were significantly increased in iFECD cells incubated with 10 and 30 μM ripasudil for 43-91 hours (Supplementary Figure S2, A). Staining for the proliferation marker PCNA confirmed increased numbers of positively labeled nuclei in cells upon ripasudil treatment compared to controls (Supplementary Figure S2, B).

These data indicate that ROCK inhibition by ripasudil activates cell cycle progression and stimulates proliferation of corneal endothelial cells of FECD patients ex vivo and in vitro. However, it has to be noted that iFECD cells are immortalized and that the ripasudil-induced increase in proliferation rate may not directly translate to the G0/G1 arrested endothelial cells in vivo.

• **EFFECT OF RIPASUDIL ON ENDOTHELIAL CELL ADHESION AND MIGRATION:** Common mechanisms involved in cell migration include actin polymerization, increased cell-matrix adhesion, formation and breakdown of focal adhesion complexes, disassembly of intercellular junctions, and regulation of the proteolytic microenvironment.

Integrins are major surface receptors that regulate dynamic interactions between cells and extracellular matrix components, such as laminin, during migration.[43] In EDM specimens from FECD patients (n = 35), 30 μM ripasudil induced a significant upregulation of several integrin subunits, including integrin α1 (ITGA1), α2 (ITGA2), α4 (ITGA4), α6 (ITGA6), α10 (ITGA10), and β1 (ITGB1) after 24 hours of incubation, whereas expression levels of integrin α3 (ITGA3), αV (ITGAV), β4 (ITGB4), and β5 (ITGB5) were not affected (Figure 2, A, top left). Expression levels of integrin α5, α7, α8, α9, αL, αM, β2, β3, β6, β7, and β8 were below detection limit. In concert with integrin induction, ripasudil also induced significant upregulation of laminin chains α5 (LAMA5) and β1 (LAMB1), whereas expression levels of laminin chains β2 (LAMB2), γ1 (LAMC1), and γ2 (LAMC2) were not changed (Figure 2, A, top left). Laminin chains α1, α2, α3, α4, β3, β4, and γ3 were not detected in FECD specimens. Moreover, expression levels of ICAM1 (intercellular adhesion molecule 1), PTK2 (focal adhesion kinase 1, FAK1), PXN (paxillin), ACTB (β-actin), and SERPINE1 (plasminogen activator inhibitor 1, PAI-1) were significantly downregulated in FECD specimens on ripasudil treatment, whereas expression levels of NCAM1 (neural cell-adhesion molecule 1), PLAU (urokinase-type plasminogen activator), and LRP1 (LDL receptor related protein 1) were not affected (Figure 2, A, top right).

Upregulation of integrins was also confirmed on the protein level using Western blotting, as exemplified for integrin β1, up to 72 hours of stimulation with 30 μM ripasudil compared with untreated control (Figure 2, A, bottom). Activation/phosphorylation of FAK1, which plays a central role in cell adhesion and migration, was, however, suppressed in FECD specimens on ripasudil treatment (Figure 2, A, bottom).

Similar alterations in expression levels of integrin subunits, laminin chains, focal adhesion proteins, beta-actin and PAI-1 were also observed in EDM specimens derived from normal wounded corneas (n = 12), with the exception of ICAM1, after 48 hours of ripasudil treatment (Figure 2, B, top). Immunohistochemistry/confocal microscopy of corneal whole mounts confirmed increased protein expression of integrin α3 and β1 at the leading edge of migrating endothelial cells in the periphery of the wound after ripasudil treatment compared to untreated control (Figure 2, B, bottom).

In vitro, inhibition of ROCK signaling with 10 and 30 μM ripasudil significantly enhanced cell migration of iFECD cells compared with untreated control cells after 3, 6, 9, 24, 28, and 32 hours (Supplementary Figure S3, A). Enhanced cell migration was associated with a disassembly of actin stress fiber bundles in ripasudil-treated endothelial cells by light and electron microscopy (Supplementary Figure S3, A).

These data indicate that ROCK inhibition by ripasudil stimulates endothelial cell migration by coordinated effects on cell-matrix adhesion, cell-cell adhesion, focal adhesion contacts, and factors of the proteolytic plasminogen system, which are major mechanistic determinants of cellular migration.[44]

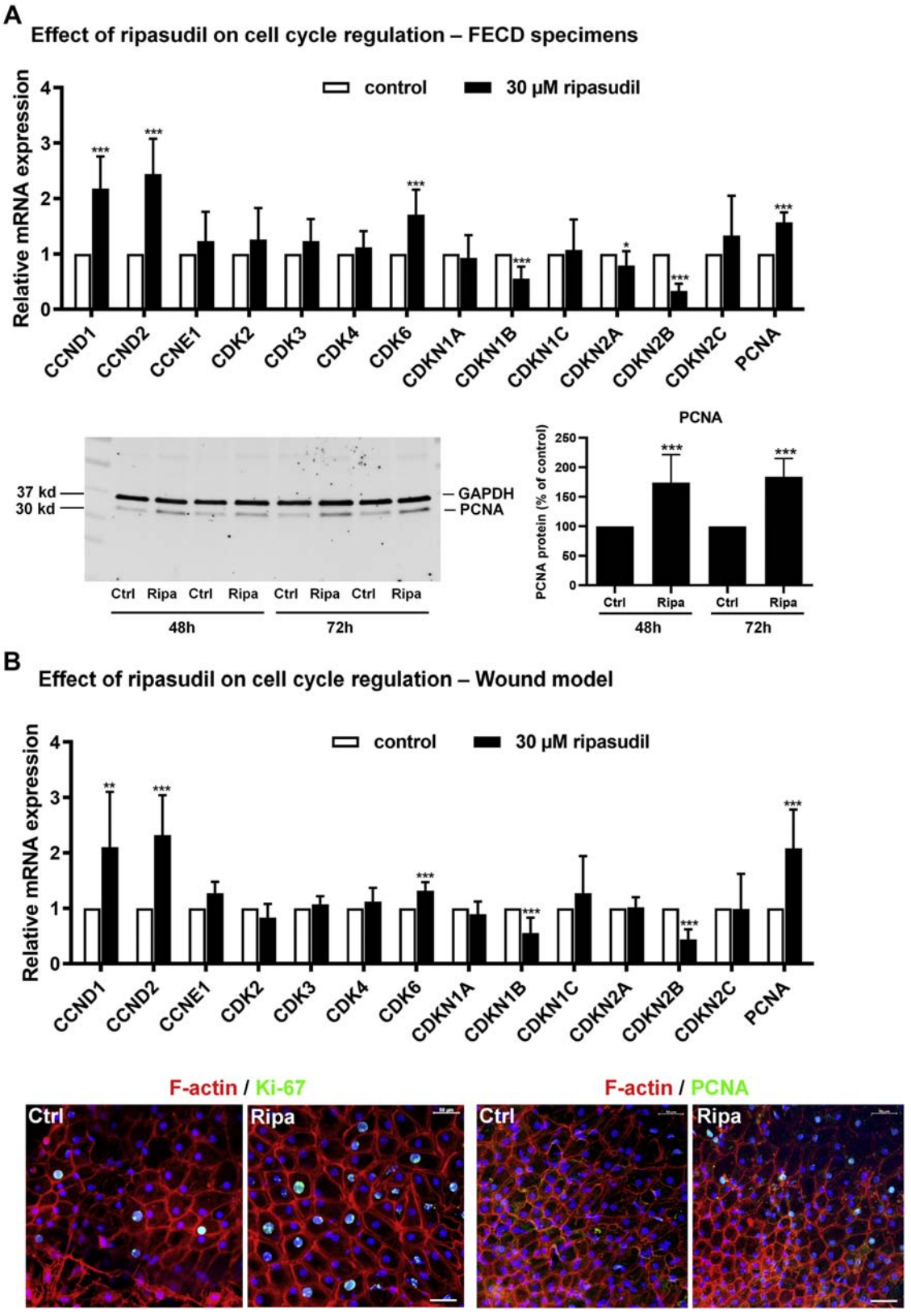

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FIGURE 1. Effect of ripasudil on cell cycle regulation ex vivo. Quantitative real-time polymerase chain reaction (qRT-PCR) primer assays showing relative expression levels of CCND1/D2/E1 (cyclin D1/D2/E1), CDK2/3/4/6 (cyclin-dependent kinase 2/3/4/6), CDKN1A/1B/1C/2A/2B/2C (cyclin-dependent kinase inhibitor 1A/1B/1C/2A/2B/2C), and PCNA (proliferating cell nuclear antigen) on exposure of endothelial cell–Descemet membrane complexes obtained from (A) Fuchs endothelial corneal dystrophy (FECD)

• **EFFECT OF RIPASUDIL ON ENDOTHELIAL-MESENCHYMAL TRANSITION:** The transition from a static to a motile phenotype during cell migration can also result in a phenotypic switch from an endothelial to a fibroblastic phenotype, termed EnMT, which has been also shown to be involved in the pathophysiology of FECD.[45]

In both endothelial cells from FECD (n = 35) (Figure 3, A, top) and normal wounded specimens (n = 12) (Figure 3, B, top), ripasudil at 30 μM downregulated critical genes involved in EnMT, that is, ACTA2 (α-smooth muscle actin), VIM (vimentin), and ZEB1 (zinc finger E-box binding homeobox 1) transcription factor after 24 and 48 hours, respectively. Expression levels of transcription factors SNAI1 and SNAI2 (Snail family transcriptional repressor 1 and 2) were additionally downregulated in FECD specimens. In both FECD and normal wounded specimens, ripasudil had no effect on expression levels of CTNNB1 (catenin-β1). Expression levels of other genes known to be involved in EnMT, that is, SNAI3, ZEB2, TWIST1 and 2 (twist family BHLH transcription factor 1 and 2), and CDH1 (E-cadherin), were below detection level.

Downregulation of vimentin and ZEB1 by ripasudil also could be confirmed on the protein levels in FECD specimens by Western blotting (Figure 3, A, bottom) and in normal wounded corneas by immunohistochemistry and confocal microscopy after 48 and 72 hours of incubation (Figure 3, B, bottom). Similarly, suppression of expression levels of both vimentin and ZEB1 could be demonstrated in iFECD cells on inhibition of ROCK signaling by 30 μM ripasudil compared with untreated control cells (Supplementary Figure S4).

These findings indicate that ROCK inhibition by ripasudil does not induce an EnMT phenotype in corneal endothelial cells of FECD and normal subjects.

• **EFFECT OF RIPASUDIL ON ENDOTHELIAL CELL FUNCTION:** The effect of ripasudil on endothelial cell functionality was determined by analyzing the expression levels of key proteins important for ion and water transport as well as barrier integrity, specifically the ATP1 (sodium-potassium pump), SLC4 (bicarbonate transporter), SLC16 (monocarboxylate cotransporter), AQP (aquaporin), CDH (cadherin), and TJP (tight junction protein) protein families.[46]

In EDM specimens from FECD patients (n = 35), 30 μM ripasudil upregulated several functional endothelial markers including AQP1 (aquaporin 1), SLC4A11 (sodium bicarbonate transporter-like protein 11), SLC16A7 (monocarboxylate transporter 2), ATP1A1, ATP1B1, ATP1B3 (sodium/potassium-transporting ATPase subunit alpha-1, beta-1 and beta-3), CA2 (carbonic anhydrase 2), CDH2 (N-cadherin), GJA1 (connexin-43), and TJP1 (tight junction protein ZO-1) (Figure 4, A, top) after 24 hours of incubation. Other functional proteins were not altered in expression (AQP11, SLC4A3, SLC4A4, SLC16A1) or not detected (SLC4A2, SLC4A7, ATP1A4, ATP1B2, AQP4, AQP5, AQP9). Significant upregulation of Na^+/K^+-ATPase, ZO-1, and N-cadherin in FECD specimens ex vivo also could be confirmed on the protein level by Western blotting up to 72 hours of incubation in ripasudil (Figure 4, A, bottom).

Ripasudil-induced alterations in expression levels of functionally relevant genes were also observed in EDM specimens from normal corneas after wounding (n = 12) (Figure 4, B, top). Immunohistochemistry/confocal microscopy illustrated increased protein expression of Na^+/K^+-ATPase and ZO-1, together with decreased expression of vimentin, in endothelial cells of corneal whole mounts on ripasudil treatment (Figure 4, B, bottom).

To confirm longer-term effects of ripasudil on functionality of FECD endothelial cells ex vivo, selected genes that showed expression changes after 24 hours were additionally analyzed after 48 and 72 hours following a single dose of 30 μM ripasudil. In consistency with the protein data, expression changes of all genes analyzed, that is, AQP1, SLC4A11, SLC16A7, ATP1A1, CA2, CDH2, GJA1, and TJP1, were consistently upregulated over the 3-day period (Figure 4, C). Finally, ripasudil-induced upregulation of functional genes, including AQP1, SLC4A11, GJA1, and TJP1, also could be confirmed in iFECD cell lines on inhibition of ROCK signaling by 10, 30, and 100 μM ripasudil in vitro (Supplementary Figure S5).

Altogether, these data indicate that a single dose of ripasudil can efficiently upregulate proteins relevant for endothelial cell pump and barrier function in FECD cells in a sustained manner up to 72 hours.

• **CORRELATION OF RIPASUDIL-INDUCED EXPRESSION CHANGES WITH AGE:** To assess potential effects of patient age on ripasudil-induced expression changes, we analyzed the correlation between age and expression levels of selected genes related to cell cycle progression and endothelial cell function, that is, CCND1, CCND2, CDKN1B, CDKN2B, GJA1, TJP1, AQP1, and ATP1A1, with age.

patients (n = 35) and (B) normal wounded corneas (n = 12) to 0 and 30 μM of ripasudil for (A) 24 and (B) 48 hours, respectively. Data are normalized to GAPDH and expressed as means ($2^{-\Delta CT} \times 1{,}000$) ± SD relative to untreated controls. *$P < .05$, **$P < .01$, ***$P < .001$; Mann-Whitney *U* test. A. Western blot analysis of PCNA protein expression in FECD specimens (n = 8) incubated without or with 30 μM ripasudil for 48 and 72 hours. Protein expression is normalized to the house-keeping gene GAPDH and is expressed as a percentage of the expression in controls. B. Expression of Ki-67 and PCNA protein (green fluorescence) in endothelial cell nuclei of normal wounded corneas incubated without or with 30 μM ripasudil for 72 hours by immunofluorescence labeling and confocal microscopy (F-actin counterstain in red, DAPI nuclear counterstain in blue; magnification bar = 50 μm). DAPI = 4′,6-diamidino-2-phenylindole, GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

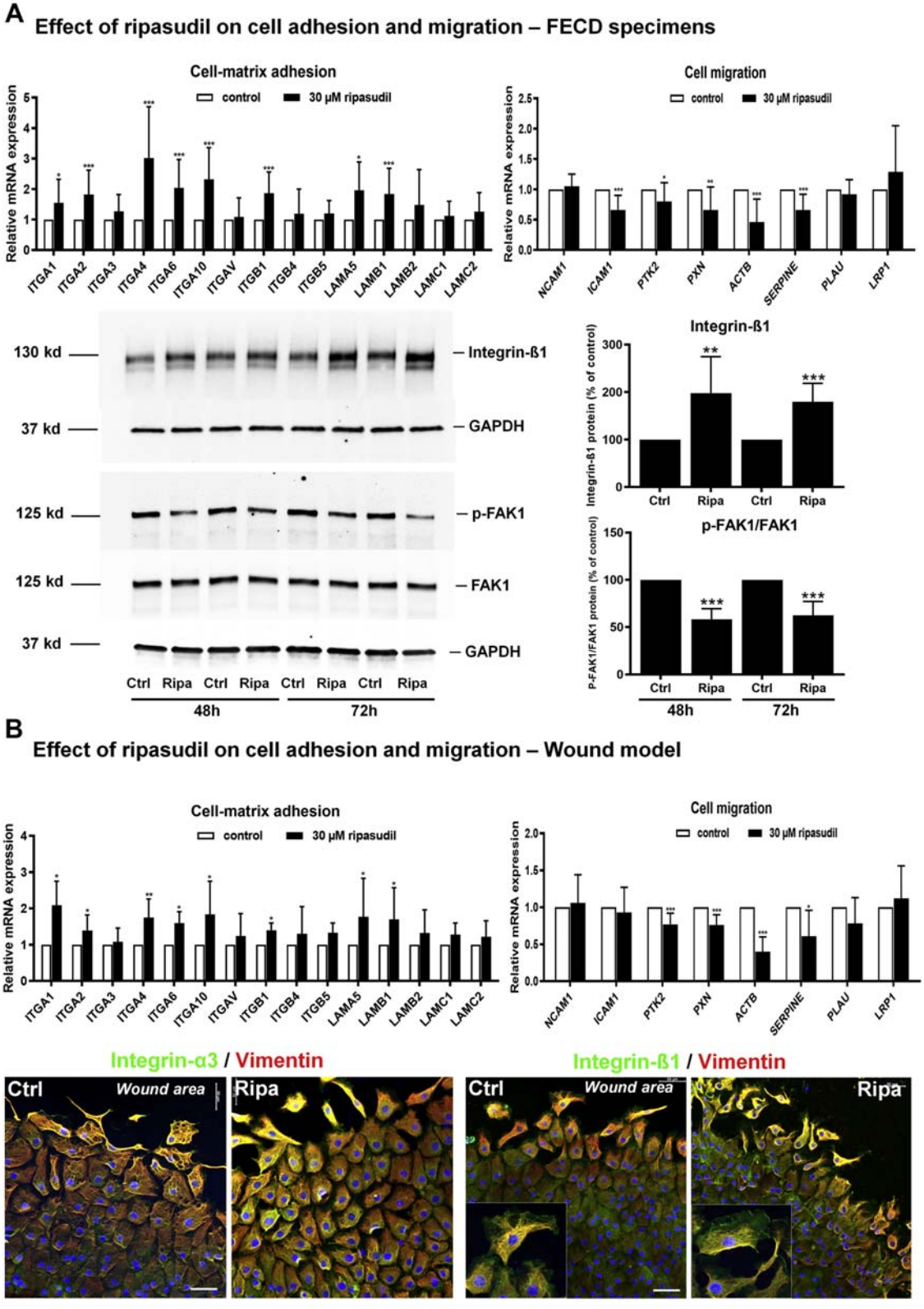

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FIGURE 2. Effect of ripasudil on cell adhesion and migration ex vivo. Quantitative real-time polymerase chain reaction (qRT-PCR) primer assays showing relative expression levels of ITGA1/A2/A3/A4/A6/A10/AV/B1/B4/B5 (integrin α1/α2/α3/α4/α6/α10/αV/β1/β4/β5), LAMA5/B1/B2/C1/C2 (laminin α5/β1/β2/γ1/γ2), NCAM1 (neural cell-adhesion molecule 1), ICAM1 (intercelular adhesion molecule 1), PTK2 (focal adhesion kinase 1, FAK1), PXN (paxillin), ACTB (β-actin), SERPINE1 (plasminogen activator inhibitor 1, PAI-1), PLAU (urokinase-type plasminogen activator), and LRP1 (LDL receptor related protein 1) on exposure of endothelial cell–Descemet membrane complexes obtained from (A) Fuchs endothelial corneal dystrophy (FECD) patients (n = 35) and (B) normal wounded corneas (n = 12) to 0 and 30 µM of ripasudil for (A) 24 and (B) 48 hours, respectively. Data are normalized to GAPDH and expressed as means ($2^{-\Delta CT}$ ×

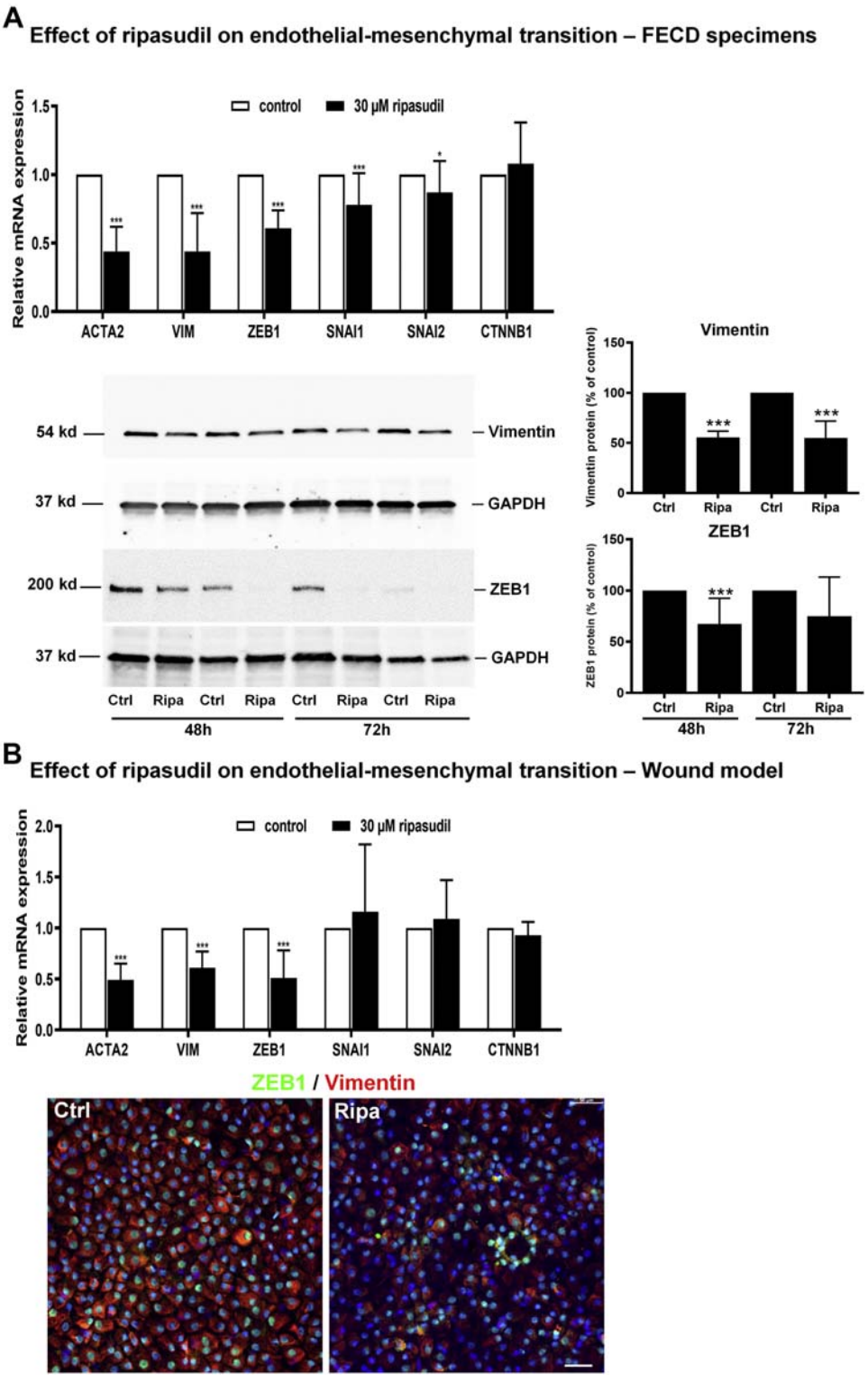


FIGURE 3. Effect of ripasudil on endothelial-mesenchymal transition ex vivo. Quantitative real-time polymerase chain reaction (qRT-PCR) primer assays showing relative expression levels of ACTA2 (α-smooth muscle actin), VIM (vimentin), ZEB1 (zinc

1,000) ± SD relative to untreated controls. *$P < .05$, **$P < .01$, ***$P < .001$; Mann-Whitney U test. A. Western blot analysis of integrin β1, FAK1, and phospho-FAK1 protein expression in FECD specimens (n = 8) incubated without or with 30 µM ripasudil for 48 and 72 hours. Protein expression is normalized to the house-keeping gene GAPDH and is expressed as a percentage of the expression in controls. (B) Expression of integrin α3 and integrin β1 (green fluorescence) in endothelial cells of normal wounded corneas incubated without or with 30 µM ripasudil for 72 hours by immunofluorescence labeling and confocal microscopy (vimentin counterstain in red, DAPI nuclear counterstain in blue; magnification bar = 50 µm). DAPI = 4′,6-diamidino-2-phenylindole, GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

However, fold changes in gene expression levels of ripasudil-treated FECD specimens compared with untreated controls were not significantly correlated with age (not shown).

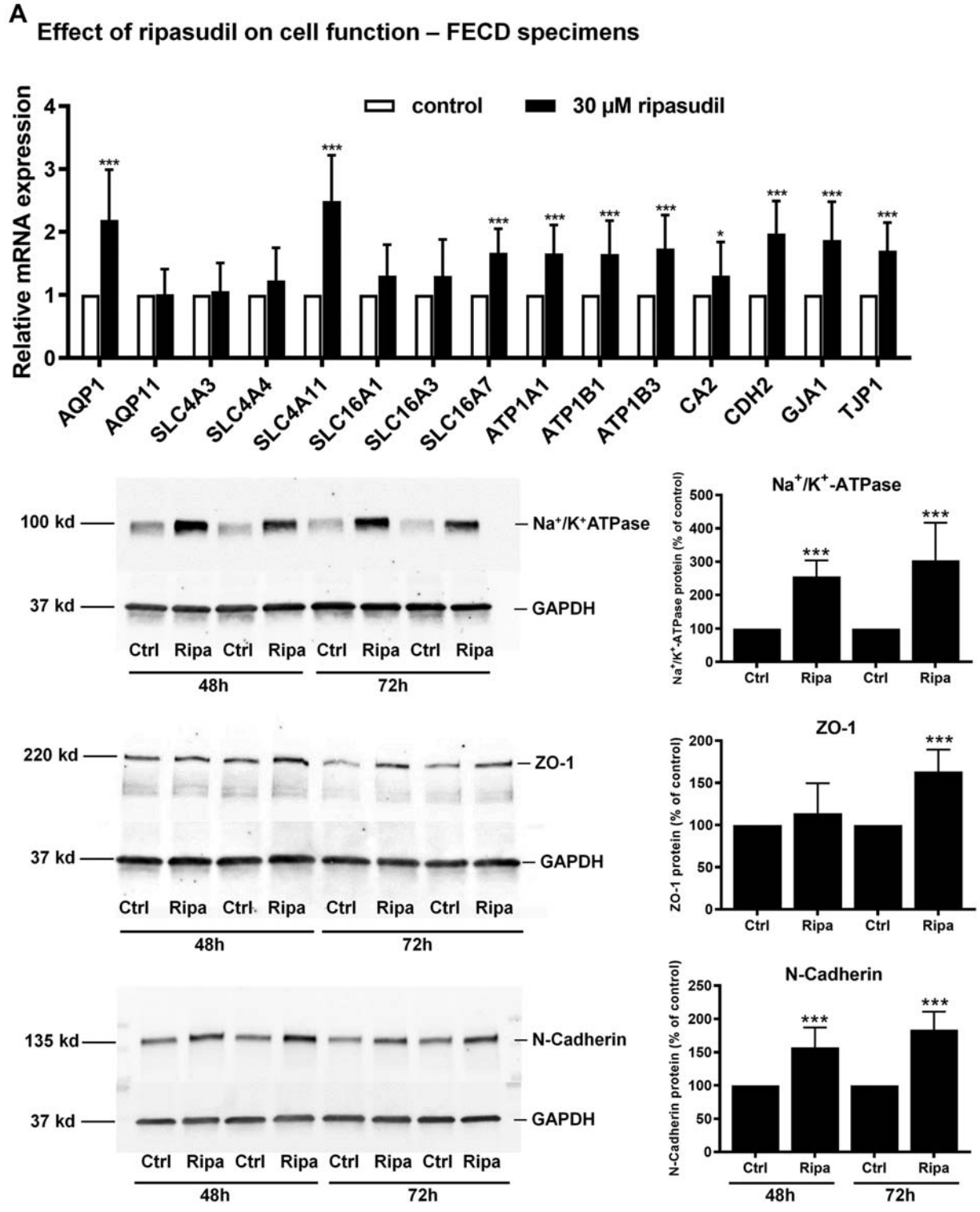
DISCUSSION

IN THE PRESENT STUDY, WE USED AN EX VIVO FECD TISSUE culture model, that is, stripped EDM complexes obtained from FECD patients during DMEK, to assess the effects of the ROCK inhibitor ripasudil on cell cycle regulation, migration, phenotype, and function of FECD endothelial cells. This ex vivo model was supplemented by 2 additional models, an in vitro FECD model comprising 3 immortalized corneal endothelial cell lines generated from central descemetorhexis specimens of FECD patients[41] and an ex vivo corneal endothelial wound healing model generated by scraping off the central endothelial cells before EDM stripping of normal donor corneas. We used ripasudil at a concentration of 30 μM, which showed the greatest modulating effects on expression levels of a panel of genes related to cytoskeletal dynamics, adhesion, and migration, and which has been used in other experimental studies.[31,47,48] This corresponds approximately to a concentration of 1.2%, which is 3 times the concentration of ripasudil 0.4% used in current clinical applications. However, whereas ripasudil 0.4% is clinically administered 4-6 times daily in FECD patients or twice a day in glaucoma patients, EDM specimens and cultured cells were incubated in a single dose of 30 μM ripasudil for up to 72 hours in the present study. Therefore, we think that the experimentally used ripasudil concentration by and large can be compared to the clinically applied dosage.

So far, it has not been clarified whether migration, proliferation, or both are the driving forces behind endothelial regeneration after DSO in FECD patients. Regeneration may be accomplished by a reservoir of progenitor-like cells in the periphery of the cornea, which are known to persist in FECD patients,[49] or by reactivated corneal endothelial cells.[4] The findings of this study indicate that ROCK inhibition by ripasudil both activates cell cycle progression and promotes cell adhesion and migration in corneal endothelial cells of FECD and normal subjects ex vivo. The stimulating effects on cell proliferation and migration could be also confirmed in iFECD cell lines in vitro, thereby confirming previous studies.[33] Activation of cell cycle progression was reflected by upregulation of cyclins D1 and D2 as well as downregulation of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p15^{INK4b}$, which all drive G1-to S-phase transition of endothelial cells.[50] These expression changes were accompanied by upregulation of the proliferation marker PCNA on the mRNA and protein levels. Thus, the present findings are consistent with previous studies showing that ROCK inhibition stimulated endothelial cell proliferation in vitro by downregulation of $p27^{Kip1}$ and upregulation of cyclin D1 and D3 via PI3K/AKT signaling favoring G1-to S-phase progression.[51] In addition, ripasudil appeared to stimulate corneal endothelial cell migration by coordinated effects on actin stress fiber depolymerization, cell detachment and cell-matrix readhesion, and regulation of the pericellular proteolytic microenvironment.[52] These effects were reflected by upregulation of several integrin subunits together with their extracellular ligands laminin α5 and β1,[53] and downregulation of beta-actin, ICAM-1, FAK1, Paxillin, and PAI-1. Inhibition of PAI-1, which is a critical determinant in regulation of matrix proteolysis during migration, has been shown to stimulate cell movement and migration.[44] Altogether, the present findings indicate that central corneal endothelial cells of FECD patients can be reactivated by ripasudil to achieve wound healing by both proliferation and migration.

Because ripasudil acts primarily on the cytoskeleton and induces actin reorganization, studies by Moloney considered that this reorganization may transform endothelial cells into a motile phenotype with EnMT-like changes.[39] During EnMT, endothelial cells adopt a fibroblastic phenotype, increase motility, disassemble cell-cell junctions, and express mesenchymal markers including vimentin and α-SMA. These changes are regulated by transcription factors such as ZEB and Snail.[54] Although a transient EnMT conversion is essential for cell migration and physiological wound healing, sustained activation of EnMT in pathologic conditions results in excessive deposition of a collagen-rich extracellular matrix, hence in fibrosis.[55] Because both uncontrolled EnMT and excessive deposition of extracellular material are known to occur in the pathophysiology of FECD,[45] attenuation of this phenotypic switch in FECD appears beneficial rather than detrimental.

finger E-box binding homeobox 1), SNAI1/2 (Snail family transcriptional repressor 1 and 2), and CTNNB1 (catenin-β1) on exposure of endothelial cell–Descemet membrane complexes obtained from (A) Fuchs endothelial corneal dystrophy (FECD) patients (n = 35) and (B) normal wounded corneas (n = 12) to 0 and 30 μM of ripasudil for (A) 24 and (B) 48 hours, respectively. Data are normalized to GAPDH and expressed as means ($2^{-\Delta CT} \times 1{,}000$) ± SD relative to untreated controls. *$P < .05$, **$P < .01$, ***$P < .001$; Mann-Whitney *U* test. (A) Western blot analysis of vimentin and ZEB1 protein expression in FECD specimens (n = 8) incubated without or with 30 μM ripasudil for 48 and 72 hours. Protein expression is normalized to the house-keeping gene GAPDH and is expressed as percentage of the expression in controls. (B) Expression of ZEB1 (green fluorescence) and vimentin (red fluorescence) in endothelial cells of normal wounded corneas incubated without or with 30 μM ripasudil for 72 hours by immunofluorescence labeling and confocal microscopy (DAPI nuclear counterstain in blue; magnification bar = 50 μm). DAPI = 4′,6-diamidino-2-phenylindole, GAPDH = glyceraldehyde 3-phosphate dehydrogenase.



Here, we show that inhibition of ROCK signaling by ripasudil downregulated mRNA and protein expression levels of the classical EnMT markers α-SMA, vimentin, ZEB1, and Snail1, emphasizing its potential to attenuate the EnMT process in endothelial cells of FECD patients. This is in line with reports showing that inhibition of ROCK signaling can block or reverse EnMT in corneal endothelial cells and other cell types in vitro.[56]

Apart from the demonstrated effects of ripasudil on cell cycle stimulation, adhesion, migration, and phenotype, the

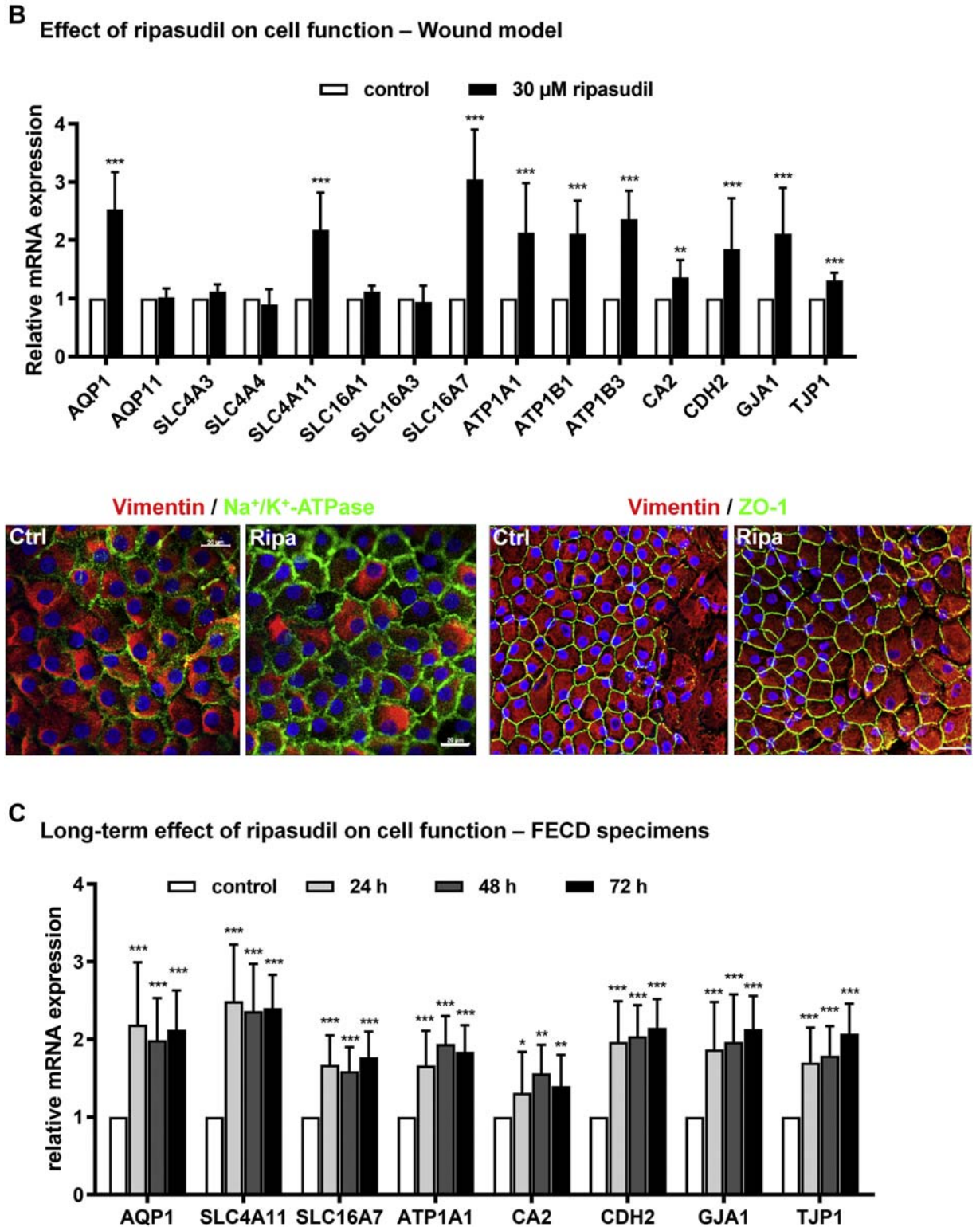


FIGURE 4. Effect of ripasudil on endothelial cell function ex vivo. Quantitative real-time polymerase chain reaction (qRT-PCR) primer assays showing relative expression levels of AQP1/11 (aquaporin 1/11), SLC4A3/4/11 (sodium bicarbonate transporter-like protein 3/4/11), SLC16A1/3/7 (monocarboxylate transporter 1/4/2), ATP1A1, ATP1B1, ATP1B3 (sodium/potassium-transporting ATPase subunit alpha-1, beta-1 and beta-3), CA2 (carbonic anhydrase 2), CDH2 (N-cadherin), GJA1 (connexin-43), and TJP1 (tight junction protein ZO-1) on exposure of endothelial cell–Descemet membrane complexes obtained from (A) Fuchs endothelial corneal dystrophy (FECD) patients (n = 35) and (B) normal wounded corneas (n = 12) to 0 and 30 μM of ripasudil for (A) 24 and (B) 48 hours, respectively. Data are normalized to GAPDH and expressed as means ($2^{-\Delta CT} \times 1{,}000$) ± SD relative to untreated

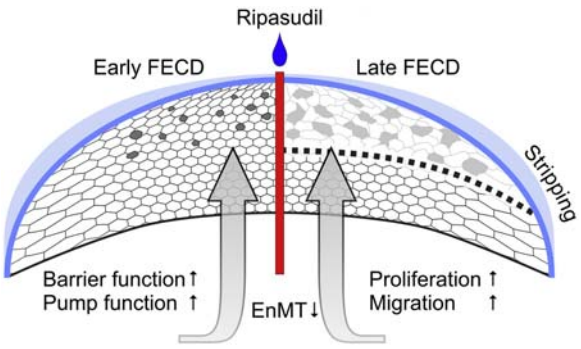


FIGURE 5. Schematic diagram of proposed effects of ripasudil on endothelial cell function in early and late stages of Fuchs endothelial corneal dystrophy (FECD). In early FECD, enhancement of endothelial pump and barrier function by ripasudil may reduce corneal edema and delay the indication for surgical intervention. In late FECD requiring Descemet stripping without endothelial keratoplasty, ripasudil may promote regeneration of the corneal endothelium by activation of cell cycle progression and cell migration without induction of endothelial-to-mesenchymal transition (EnMT).

question remains whether the restored endothelial cells are functionally competent. The barrier integrity of the corneal endothelium is maintained by intercellular junctions, including adherens and tight junctions formed mainly by N-cadherin and ZO-1. Pump functions are maintained mainly by Na^+/K^+-ATPase pump sites, supported by ion transporters such as bicarbonate transporters, monocarboxylate transporters (MCTs), and aquaporin water channels.[57] FECD is characterized by reduced endothelial cell density accompanied by a gradual decline in pump function, as reflected by reduced expression of Na^+/K^+-ATPase, MCT1 (SLC16A1), MCT4 (SLC16A3), and AQP-1.[46,58–60] In culture, however, FECD-derived cells showed a similar expression pattern of function-related proteins as those derived from healthy donors, suggesting a potential of recovery from their dysfunctional phenotype and regain of functionality in the absence of their pathologic environment, that is, Descemet membrane.[46] Here, we show that a single dose of ripasudil induced an upregulation of functional key proteins relevant for endothelial pump and barrier function in all 3 ex vivo and in vitro models. Specifically, it upregulated several subunits of Na^+/K^+-ATPase, AQP-1, SLC16A7 (MCT2), SLC4A11, and carbonic anhydrase 2, together with the junctional proteins ZO-1, N-cadherin, and connexin-43, both on the mRNA and protein levels up to 72 hours in endothelial cells of FECD patients as well as healthy donors. These findings are consistent with previous reports detailing an upregulation of Na^+/K^+-ATPase, ZO-1, and N-cadherin by various ROCK inhibitors, including Y-27632, thiazovivin, and ripasudil, in normal corneal endothelial cells in vitro and animal models in vivo.[31,36,56]

Taken together, the present study provides evidence that ROCK inhibition by ripasudil can activate cell cycle progression, promote cell migration, and enhance pump and barrier function without inducing adverse phenotypic changes in central corneal endothelial cells of FECD patients, which are most severely affected in advanced stages of disease. A single dose of 30 μM ripasudil was sufficient to induce and sustain expression changes for up to 72 hours independent of the age of patients. Notably, beneficial alterations in gene and protein expression occurred in the presence of a strong inhibitory environment created by cell-cell contact inhibition and a pathologically altered Descemet membrane with guttae. Thus, these findings support the notion that even central endothelial cells of FECD patients retain some regenerative potential and have the capacity to restore improved functionality in situ.[4,61,62]

Limitations of the study are that the FECD tissue specimens were not genotyped for the presence of *TCF4* (transcription factor 4) repeat expansions[1] impeding evaluation of an influence of the genetic background on ripasudil-mediated effects. Another limitation is that only 1 type of ROCK inhibitor was used, precluding generalized conclusions.

Nevertheless, based on the present findings, ripasudil has been verified as a potent tool in regenerative therapies in FECD patients, not only after acute damage of the corneal endothelium following DSO but also for preservation of the endothelium in early-stage FECD prior to surgical indications (Figure 5). In early FECD, enhancement of endothelial pump and barrier function may reduce corneal edema and delay the indication for surgical intervention. Such an alternative pharmacologic therapy could be facilitated

controls. *$P < .05$, **$P < .01$, ***$P < .001$; Mann-Whitney *U* test. A. Western blot analysis of Na^+/K^+-ATPase, ZO-1, and N-cadherin protein expression in FECD specimens (n = 8) incubated without or with 30 μM ripasudil for 48 and 72 hours. Protein expression is normalized to the house-keeping gene GAPDH and is expressed as percentage of the expression in controls. B. Expression of Na^+/K^+-ATPase and ZO-1 (green fluorescence) in endothelial cells of normal wounded corneas incubated without or with 30 μM ripasudil for 72 hours by immunofluorescence labeling and confocal microscopy (vimentin counterstain in red, DAPI nuclear counterstain in blue; magnification bar = 20 μm). C. qRT-PCR primer assays showing relative expression levels of AQP1, SLC4A11, SLC16A7, ATP1A1, CA2, CDH2, GJA1, and TJP1 following exposure of endothelial cell–Descemet membrane complexes obtained from FECD patients (n = 25) to 0 and 30 μM ripasudil for 24, 48, and 72 hours. *$P < .05$, **$P < .01$, ***$P < .001$; Mann-Whitney *U* test.

by incorporation of ROCK inhibitors into novel drug delivery systems, such as PLGA (*poly*-lactic/glycolic acid) microspheres, for sustained release and treatment of corneal endothelial disease.[63] Future studies will have to address the issue of long-term effects of ripasudil, particularly in the presence of detrimental guttae,[64] and potential correlation of ripasudil-mediated effects and responses with patient genotype.

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