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Elucidation of the migratory behaviour of the corneal endothelium

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Part II

Regenerative strategies for the treatment of Fuchs endothelial dystrophy

CHAPTER 4

Corneal endothelial wound healing: understanding the regenerative capacity of the innermost layer of the cornea

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Abstract

Currently, there are very few well-established treatments to stimulate corneal endothelial cell regeneration *in vivo* as a cure for corneal endothelial dysfunctions. The most frequently performed intervention for a damaged or dysfunctional corneal endothelium nowadays is corneal endothelial keratoplasty, also known as lamellar corneal transplantation surgery. Newer medical therapies are emerging and are targeting the regeneration of the corneal endothelium, helping the patients regain their vision without the need for donor tissue. Alternatives to donor tissues are needed as the aging population requiring transplants, has further exacerbated the pressure on the corneal eye banking system. Significant ongoing research efforts in the field of corneal regenerative medicine have been made to elucidate the underlying pathways and effector proteins involved in corneal endothelial regeneration. However, the literature offers little guidance and selective attention to the question of how to fully exploit these pathways. The purpose of this paper is to provide an overview of wound healing characteristics from a biochemical level in the lab to the regenerative features seen in the clinic. Studying the pathways involved in corneal wound healing together with their key effector proteins, can help explain the effect on the proliferation and migration capacity of the corneal endothelial cells.

Introduction

The corneal endothelium forms the innermost layer of the cornea and fulfills a key function in maintaining a relative state of dehydration, making it transparent.[1,2] This monolayer regulates corneal hydration through a well- established "pump-and-leak" mechanism. The dynamic balance between the passive barrier and active fluid pump of the endothelium is essential for maintaining the relatively dehydrated state of the stroma along with the correct alignment of stromal collagen.[3] When the corneal endothelium (CE) is compromised the leak/pump rate becomes unbalanced and the cornea becomes thick and cloudy. This swelling is known as corneal edema. The CE forms resistance to solutes and fluid permeability through paracellular transport routes, but allows the pas- sage of nutrients from the aqueous humor into the avascular cornea.[4,5] The "leaky" barrier is formed by junction proteins such as zonula occludens (ZO-1) and connexin-43, and the adhesion junction complex represented by cadherin isoforms.[5–7] The osmotic drive of the corneal stroma to swell is counteracted by removing excess stromal fluid. This fluid is removed by the activity of sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) pumps and bicarbonate-dependent Mg^{2+}-ATPase ionic pumps located mainly at the basolateral site of the cellular membrane.[5,8–12]

Traditionally, corneal endothelial cells (CEnCs) are not thought to have a significant capacity for *in vivo* regeneration.[13] They are highly differentiated and considered post mitotic and, in healthy individuals, show a gradual drop in endothelial cell density throughout life with an average cell loss of approximately 0.6% per year.[14] CEnCs are arrested in the G1 phase of mitosis and as a increased cell spreading with cells showing high polymorphism.[7,15,16] Age-related decline of the CE does not usually affect the critical barrier and pump function. In contrast, CEnC loss due to a pathology such as endothelial dystrophies, contact lens wear, previous refractive or intraocular surgeries, may lead to corneal decompensation.[17–20] When the CEnC density falls below a functional threshold (usually about 500 cells/mm^2), the pump and leak mechanism fails and the cornea swells.[21,22] In such cases, the gold standard treatment is to replace the ineffective endothelium with healthy, functional corneal endothelium by means of a corneal transplant.[15,23,24]

Over the past 2 decades, the procedure of choice to manage corneal disorders has shifted from penetrating keratoplasty (PK) to the more selective endothelial keratoplasty (EK).[25] The most selective form of EK is currently Descemet membrane endothelial keratoplasty (DMEK), where the endothelium is replaced with a single layer of donor cells.[26] Surgical outcomes are excellent, but the global donor shortage remains the major limitation for treatment.[27–32] This has led to the development of new therapeutic options.[33–36] New therapies aim to regenerate the corneal endothelium by inducing corneal endothelial wound healing which is known to occur through cell enlargement and migration rather than by cell proliferation.[37,38]

The mechanisms governing corneal endothelial cell migration pertain to the cytoskeleton and, in particular, to actin-based motility.[39] Corneal endothelium wound repair is accompanied by the appearance of actin which is involved in a dynamic process during cell movement. These actin filaments are involved in the formation of filopodia and lamellipodia, which will affect leading edge cell dynamics.[40,41] During wound healing, CEnC deposit fibronectin and laminin along the basement membrane.[42] These extracellular matrix molecules will act as guidance cues promoting signals associated with directed cell migration, including cytoskeletal reorganization.[43] Healthy remaining CEnC undergo cytoskeletal changes during the wound healing process. These changes consist of actin reorganization and cellular enlargement to form a polygonal cell shape to cover the damaged zone thereby rapidly restoring the barrier function. The process of these phenotypical changes during wound healing is known as endothelial-to-mesenchymal transition (EnMT). This process results in a disruption of the cellular monolayer and loss of cell-cell contact inhibition.[44] During EnMT the remaining cells lose their own function and shape and are converted to a fibroblast-like phenotype. The cells will break free of their neighboring cells and migrate individually along the Descemet membrane (DM) into the defected area resulting in a fast wound closure.[45–50] A hallmark of the EnMT is downregulation of the junctional protein E-cadherin and upregulation of cytoskeletal proteins such as fibronectin and vimentin concomitant with increased

expression of collagen type I genes (COL1A1 and COL1A2).[51] Also, mesenchymal transition marker genes such as snail family transcriptional repressor 1 (SNAI1), SNAI2, zinc finger E-box-binding homeobox 1 (ZEB1), and ZEB2 are known to regulate the expression of collagen type 1 and suppression of E-cadherins.47 Secretion of type I collagen can lead to retrocorneal membrane formation and corneal blindness.[52]

When CEnC receive a mitogenic cue, they activate cyclin proteins and cyclin dependent kinases (CDK). Both form cyclin/CDK complexes, which interact with the retinoblastoma protein known for its pivotal function in cell cycle progression.[8] The induction of cyclin D and E in combination with an inhibition of CDK-inhibitor cyclin-dependent kinase inhibitor 1B (p27kip1) are of particular importance in transitioning from the G1 phase to the S-phase of the cell cycle.[53]

Unraveling the processes involved in wound healing could lead to better insights in restoring the corneal endothelium. The goal of this review therefore is to compile what is currently known about the corneal endothelial wound healing process with special emphasis on involved pathways, biological modulators, and clinical implications.

Methods

The research strategy used for the pathway selection in the result section covered all relevant English papers concerning the corneal endothelial wound healing process. Research articles were selected in first line by title and abstract of the past decade (2011–2022). A total of 125 papers were found by using the query “Corneal endotheli*” AND Wound healing” OR “Repair” in PubMed. This selection was further refined manually through means of a screening table which was made to only select the most relevant papers in this field. This table was based on the activated pathway during corneal endothelial wound healing, the effector protein(s) involved in these processes and specific “marker” proteins involved in proliferation, migration, and endothelial-to-mesenchymal transition. We obtained 42 relevant papers concerning corneal endothelial wound healing. Following the literature study, the upstream and downstream proteins involved in corneal endothelial wound healing were connected into signaling cascades by using 2 online databases: Kyoto Encyclopedia of Genes and Genomes (KEGG) and Consensus Path Database (CPDB). For the clinical implication part in the result section, the following query in Pubmed was used: cornea and cell migration and endotheli*, endotheli* cell therapies. A total of 28 papers were selected between 2000 and 2021 based on their clinical relevance concerning wound healing aspects.

RESULTS

Main pathways involved in wound healing processes

In this section, we will summarize the key signaling pathways reported to date that are involved in corneal endothelial wound healing. These pathways are organized by various connections of specific effector proteins that typically form multi-tiered signaling cascades.

Rho/ROCK pathway

The most widely studied pathway involved in corneal endothelial wound healing is the Rho/Rho-associated coiled-coil containing kinase (Rho/ROCK) pathway (**Figure 1**).[54,55] Rho/ROCK pathway starting points are difficult to define, since different hormones, cytokines and growth factors can affect this cascade by regulating the upstream proteins.[56] Two important upstream regulators linked to corneal endothelial wound healing are guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), both of which are involved

in the activation of small G-proteins.[15,33,57–59] GEFs act as signal activators by catalyzing the exchange of small G protein-bound GDP to GTP. In contrast, GAPs will act as signaling suppressors by means of GTP hydrolysis.[60]

The most characterized small G protein is the Ras homolog family member A (RhoA).[61] This class of proteins act as molecular switches of downstream signaling pathways and hydrolyze guanosine triphosphate (GTP) to become active.[56,62] Phosphorylated RhoA will affect ROCK, which is a target molecule for cellular therapy and regenerative medicine.[63] ROCK signaling pathway is involved in many biological processes ranging from cell adhesion, migration and stress fiber formation to even the regulation of cell proliferation and apoptosis.[33,34,62,64]

The activation of ROCK sustains the activation of myosin light chain phosphatase (MLCP) and myosin light chain kinase (MLCK), which in turn activate the phosphorylation of myosin light chain (MLC).[64] Active ROCK causes MLCP inactivation so that the MLCK activity will outweigh the physiological balance of these 2 MLC regulators.[57] Phosphorylated MLC will cause actin polymerization and stress fiber formation in the cytoskeleton of CEnCs. Alternatively, the phosphorylation of LIM kinase (LIMK) by ROCK leads to an increase in phosphorylated cofilin. This actin binding protein will be inhibited upon phosphorylation, which results in a decrease of actin-depolymerizing activity. Consequently, a higher level of actin filament stabilization results in a higher contractile state during the wound healing process.[54,57,65] Consequently, the latter causes a higher level of actin filament stabilization which results in a higher contractile state during the wound healing process.

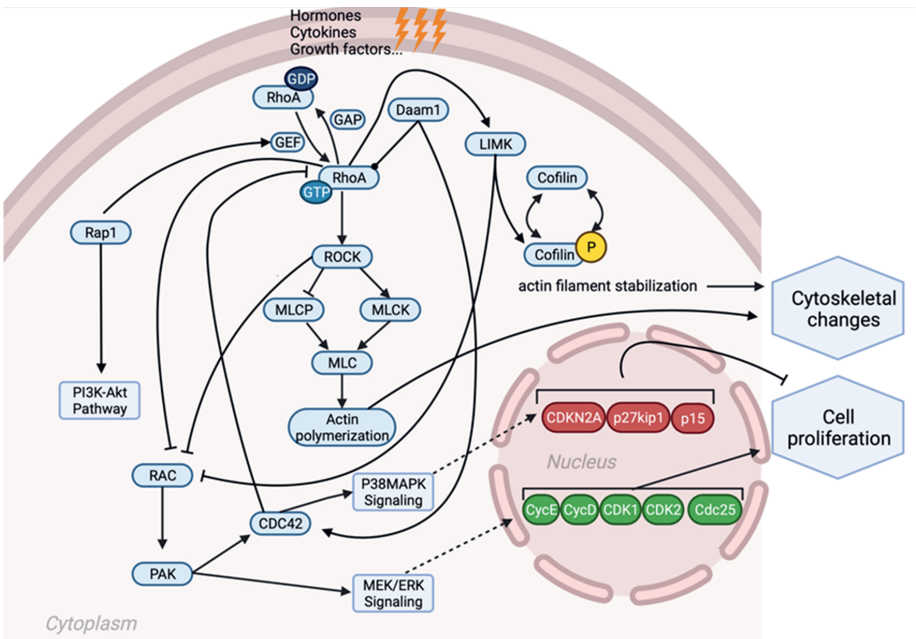

Figure 1| Rho/ROCK signaling pathway in a CEnC. Full lines represent either downstream stimulation (arrow) or inhibition (flathead). A rounded end line Indicates that it will form a complex. Dashed lines target the effector proteins without all the intermediate involved proteins.

Furthermore, LIMK, RhoA and ROCK can also block early G1-phase induction through inhibition of Ras-related C3 botulinum toxin substrate 1 (Rac) and cell division control protein 42 homolog (cdc42) resulting in low cyclin

D levels.[63,66,67] Rac and cdc42 are small GTPases which are key regulators of the p38 mitogen-activated protein kinases (p38MAPK) and mitogen activated ERK kinase-extracellular signal-regulated kinase (MEK-ERK) signaling pathways. Both pathways are known to regulate cell cycle progression.[57,61] Okumura et al. also showed the involvement of the phosphoinositol-3-kinase (PI3K) and protein kinase b (Akt) pathway when ROCK was not activated indicating the complex signaling route of this wound healing cascade.[57,68]

Furthermore, disheveled-associated activator of morphogenesis (Daam1) which is part of the winglessinkt (Wnt) pathway can form a complex with RhoA and directly stimulate its activity. Indirectly, Daam1 inhibits RhoA activity by cdc42 activation which creates a negative feedback loop. The cytoskeletal rearrangement together with decreased cyclin-cyclin kinase levels and increased cyclin kinase inhibition promote migration. Proliferation is therefore negatively affected upon activation of Rho/ROCK signaling thereby lowering the wound healing ability.[54,61,68]

Phosphoinositol-3-kinase (PI3K) and protein kinase b (Akt) pathway

The PI3K/Akt pathway is based on 2 key signaling proteins namely: phosphoinositol-3-kinase (PI3K) and protein kinase b (Akt) (**Figure 2**).[69] PI3K can be activated directly by small GTPase effector Ras-proximate-1 (Rap1), which acts as a cytosolic signaling transducer.[70] Rap1 can be activated by the binding of exchange protein directly activated by cAMP (EPAC) to the cytosolic cAMP that is released intracellularly. The release of cAMP occurs after the binding of pituitary adenylate cyclase activating polypeptide (PACAP) to their pituitary adenylate cyclase (PAC1) receptor.[15,71] Alternatively, PI3K can also be activated by focal adhesion kinase (Fak) that functions as an early mediator in integrin activation and the time-dependent generation of cell-ECM forces. During wound healing, the engagement of the integrin subunits activates protein kinase c (PKC).[33] This serine-threonine kinase is known to be upregulated in CEnCs at the wound edge thereby affecting the activity of Fak.[70–72]

PI3K phosphorylates phosphatidylinositol bisphosphate (PIP2) to generate the second messenger, phosphatidylinositol trisphosphate (PIP3) which signals to downstream effector Akt, a serine/threonine kinase implicated in the regulation of cell cycle progression and cell death.[68,73,74] The activation of Akt signaling by the transcription factor sex-determining region Ybox 2 (SOX2) alters the phosphorylation level of GSK-3ß targeting the b-catenin for ubiquitination and proteasome mediated degradation.[45] In confluent corneal endothelial monolayers, ß-catenin is bound to the cell

membrane while in a lower confluency degree more freely available cytosolic ß-catenin is present.[44,46] If there is no stabilization signal to suppress glycogen synthase kinase 3 beta (GSK-3ß) activity, the cytosolic ß-catenin is targeted for degradation by GSK-3b phosphorylation.[44,75] In this case, there is no internalization of ß-catenin into the nucleus to activate transcription factor 4 (TCF4) which can enhance cyclin D, E, cyclin dependent kinase 1 (CDK1) and breakdown p27kip1 and Cyclin Dependent Kinase Inhibitor 2A (CDKN2A).[45,46,70] As a result, there is no net effect on the cell cycle progression. SOX2 can also affect the GSK-3ß/ß-catenin system by interfering with the TCF4 binding site to promote cell proliferation.[45] SOX2 has an inhibitory effect on GSK-3ß thereby releasing ß-catenin to the cytosol which also promotes cell proliferation. Remarkably, ß-catenin induces morphogenic changes by enhancing EnMT related genes such as SNAI, SLUG, ACTA2 and ZEB1 contributing to aberrant ECM deposition and fibrosis.[44,47]

Akt activation also leads to forkhead box transcription factor FOXO3A (Fkhlr1) suppression which promotes cell survival over apoptosis (FOXO signaling pathway).[45] Additionally, activated Akt promotes the phosphorylation of cytosolic proline rich Akt substrate of 40 kDa (PRAS40) concomitant with unlocking the inhibition of tuberous sclerosis proteins 1 (TSC1) which results in the activation of mammalian target of rapamycin (mTOR) pathway.[76] Activated PRAS40 binds with rapamycin complex 1 (mTORC1) to stimulate ribosomal protein S6 kinase (S6K) which stimulates protein synthesis to become more metabolically active during wound closure. At

the same time activated PRAS40 as well as TSC will stimulate the mothers against decapentaplegic homolog 4 (SMAD4) transcription factor. The latter is known to activated EnMT related genes such as SNAI, SLUG, ACTA2 and ZEB1 causing enhanced motility and aberrant ECM deposition.[47,74]

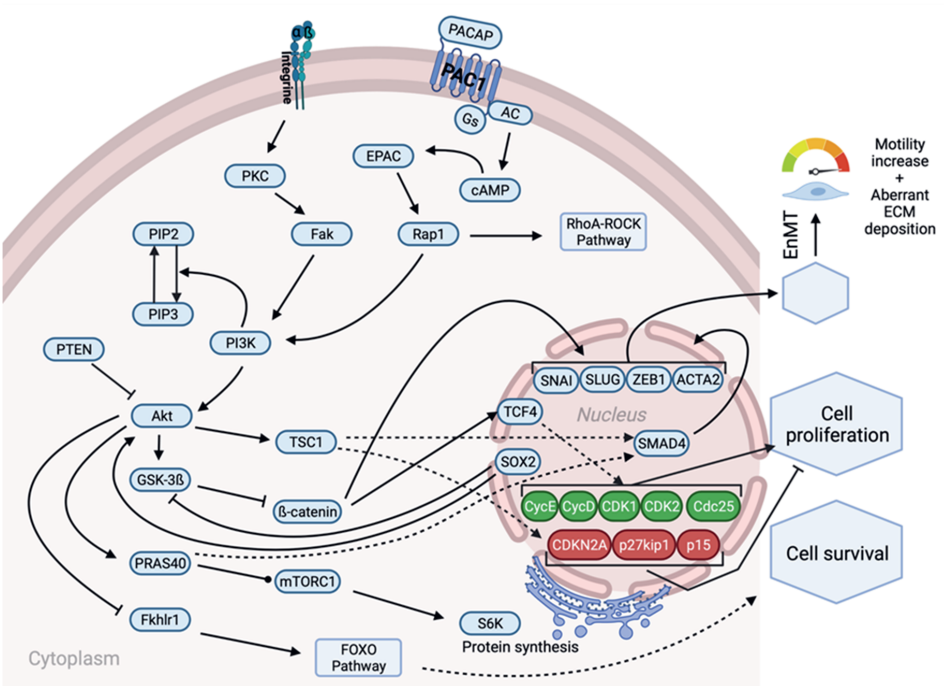

Figure 2| PI3K/Akt signaling pathway in a CEnC. Full lines represent either downstream stimulation (arrow) or inhibition (flathead). A rounded end line Indicates that it will form a complex. Dashed lines target the effector proteins and pathway without all the intermediate involved proteins.

Nuclear accumulation of p27kip1 is caused by the phosphatase and tensin homolog (PTEN) protein-induced inhibition of AKT phosphorylation.[77] Zhang et al. showed that inhibition of PTEN results in a stimulation of cell cycle progression as well as an increased migration behavior of CEnCs which emphasize the effect on wound healing.[77]

In general, PI3K and Akt modulate cell cycle progression, enhance cell survival, but may also cause EnMT induction with concomitant increase in motility together with aberrant ECM secretion. These different processes could be seen as characteristics for corneal endothelial wound healing.

Wingless-Inkt pathway

The Wnt pathway and can be subdivided in a canonical and noncanonical signaling cascade[44] (**Figure 3**). The canonical cascade passes signals in CEnCs through the activation of an atypic cell surface G-coupled receptor complex called Frizzled-Low density lipoprotein receptor related protein 5-6 (FzdLRP5/6).[59] This receptor complex inhibits GSK-3ß and leads to cytoplasmatic stabilization and nuclear transport of ß-catenin which enhances cellular proliferation abilities.[45,75]

The noncanonical Wnt pathway consists of different frizzled family receptor members such as Fzd5 and can be activated by Wnt5a ligand.[47,59] This protein will stimulate the cytoplasmatic protein Daam1 which form a complex with RhoA (Daam1-RhoA complex) to regulate cytoskeleton organization and cell migration through GTPases of the Rho-family, such as RhoA and Cdc42.[44,59] Activation of Cdc42 inhibits RhoA which, in turn, enhances cell migration through regulation of cofilin.[57] A higher level of dephosphorylated cofilin causes a faster modulation in actin turnover in cytoskeleton assembly resulting in a higher migratory state.[65]

In general, the Wnt pathway can alter cell proliferation and migration abilities by means of GSK-3ß regulation and Daam1 modulation respectively. Therefore, it is an important signaling cascade involved in corneal endothelial wound healing.

Transforming growth factor beta (TGF-) pathway

TGF-ß signaling pathway has been shown to regulate many cellular processes such as cell proliferation, differentiation, motility, adhesion, and programmed cell death (**Figure 3**). Earlier reports indicated the presence of 3 types of TGF-ß receptors in human corneal endothelium.[78] Moreover, Joyce et al. stated that those receptors were continuously exposed to latent TGF-ß2 that is found in aqueous humor, thereby preventing CEnCs to enter the G1-to-S transition phase of the cell cycle.[37,79] TGF-ß2 blocks the phosphorylation of p27kip1 which is a prerequisite for nuclear export of the inhibitor molecule for degradation.[77] Consequently, accumulation of p27kip1 molecules in the nucleus negatively regulate the CDK complexes that are necessary for cell cycle progression.[70] TGF-ß pathway activation together with contact inhibition are thought to be one of the main reasons which prohibit CEnC proliferation *in vivo*.[77] Additionally, the TGF-ß receptor can directly activate the downstream signaling Akt protein leading to loss of tight junctions and EnMT activation.

Joko et al. discovered that TGF-ß signaling caused an upregulation of the TGF-b-stimulated clone 22 (TSC-22) gene, encoding for a transcription factor that has cell proliferation suppressor properties.[76] TSC-22 binds to and modulates the transcriptional activity of Smad4 causing either an upregulation of cyclin-dependent kinase inhibitor 2B (p15) and thus inhibiting cell proliferation or enhances the expression of specific sets of target genes such as SNAI, ACTA2 and SLUG which trigger EnMT.[58,78,80]

Interestingly, TGF-β2 has been reported to induce wound healing by promoting CEnC migration through activation of p38MAPK rather than by stimulating cell proliferation because p38MAPK has shown to upregulate p27kip1 expression which promotes the G1 cell cycle arrest.[76] TGF-ß2 enhances CEnC migratory properties through mitogen-activated protein kinase 1 (MAPK1) activation.[37] This kinase affects the more downstream mitogen-activated protein kinase 3 and 6 (MAPK3/6) which modulates the phosphorylation of p38MAPK and promotes its translocation to the nucleus.[37,76]

In conclusion, TGF-ß can stimulate CEnC wound healing by enhanced migratory activities regulated through activation of p38MAPK.[76] However, TGF-ß signaling may force the CEnCs to adopt a fibroblastic like morphology causing aberrant collagen 1 and fibronectin deposition.[47,78] The presence of such changes in ECM production hampers the success of transplantation of cultivated cells *in vitro* due to this highly undesirable phenotype.[70,78]

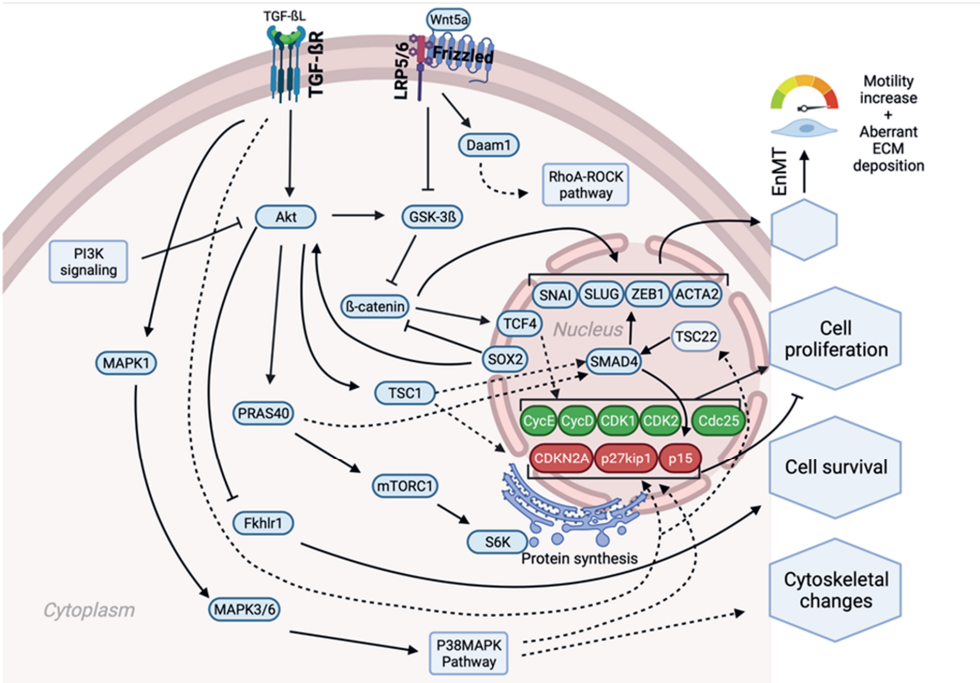

Figure 3| TGF-ß/Wnt signaling cascade in a CEnC. Full lines represent either downstream stimulation (arrow) or inhibition (flathead. Dashed lines target the effector proteins and pathway without all the intermediate involved proteins.

Clinical implications

Clinical scenarios that require corneal endothelial cell migration. Corneal opacification represents one of the prevalent causes of blindness, accounting for 4.2 million visual impaired patients of the worldwide blind population in 2019.[81–83] Corneal blindness may be caused by diseases such as Fuchs endothelial corneal dystrophy (FECD), (pseudophakic) bullous keratopathy, posterior polymorphous dystrophy, congenital hereditary endothelial dystrophy, or iridocorneal endothelial syndrome.[23] FECD is the most common form of corneal endothelial dystrophy with a regional prevalence that varies from 3.8% to 11% in individuals in the fifth or sixth decade of life. Moreover, it is the top indication for cornea transplantation worldwide.[30] FECD is a bilateral, sporadic, or autosomal dominant (inherited in some cases) dystrophy that progresses slowly and is characterized by deterioration of endothelial cells and development of basement membrane excrescences known as guttae.[84] Endothelial cell loss from FECD damages barrier function and if left untreated, central corneal edema will expand into the periphery leading to diffuse edema, bullous keratopathy, and eventually to subepithelial scarring.[85,86] However, corneal decompensation and bullous keratopathy can also result from corneal injury, iatrogenic damage by cataract surgery or other surgical procedures, or medication toxicity.[87–92] Corneal blindness from these cases is primarily treated by performing a corneal transplant.

For about ten decades, full thickness transplantation of the cornea was the gold standard for treating corneal disorders, while nowadays, EK has become the technique of choice. The 2 most successful endothelial keratoplasty techniques are Descemet stripping (automated) endothelial keratoplasty (DS(A)EK) and DMEK (Descemet membrane endothelial keratoplasty). The main difference between DMEK and DS (A)EK is the stroma-less graft in DMEK, which results in improved postoperative best-corrected visual acuity (BCVA) outcomes and a faster visual recovery.[93,94] Despite technique improvements and efforts to standardize

DMEK, postoperative complications have been reported including significant decline in endothelial cell density (ECD), especially during the early postoperative period, as well as graft detachment.[95–97]

Clinically, an important consideration is whether the host and/or recipient endothelial cells are capable of migrating to cover bare recipient stroma in the areas not covered by graft tissue. *In vivo* endothelial cell migration has been described in eyes with partially detached grafts resulting in corneal clearing, despite varying degrees of incomplete graft attachment[98,99] (**Figure 4**). Visual recovery was explained by endothelial cell migration or regeneration from either the donor or remaining recipient endothelial cells. Endothelial cell migration was also reported for grafts decentered in recipient eyes after DMEK or in patients who developed immune reaction episodes with endothelial precipitates detectable on the graft and on the bare stroma not covered by the DMEK graft.[100,101]

New insights in endothelial cell migration were also obtained after Quarter-DMEK surgery, that is, a modified DMEK-technique in which an untrephined full-sized DMEK graft is equally divided in quarters in order to treat 4 eyes with 1 donor cornea. In a first Quarter-DMEK cohort of 19 eyes, the central cornea underlying the graft cleared rapidly, while the peripheral bare stromal regions slowly improved over several months.[102–104] This suggested that donor endothelial cells migrated from the radial cut graft edges and induced corneal clearance in those areas. Cell migration from the round graft edge, on the other hand, was inhibited, possibly due to the arrangement of fibrillary bands of collagen in the graft periphery acting as a barrier for cell migration.[104] The lack of cell migration from the round graft edge resulted in localized longer standing corneal edema along the round graft edge.[102] Widespread cell migration from the cut graft edges may be responsible for the initially rapid decline in ECD observed after Quarter-DMEK surgery.[105] A follow up *in vitro* study carried out by the same group showed that alterations in the far peripheral area of Quarter-DMEK grafts were insufficient to trigger cell migration from the limbal graft edge.[106] This was attributed to progenitor cells located beneath the Schwalbe's line and which lack cytokinetic directional cues despite their exposure to free surface.[107]

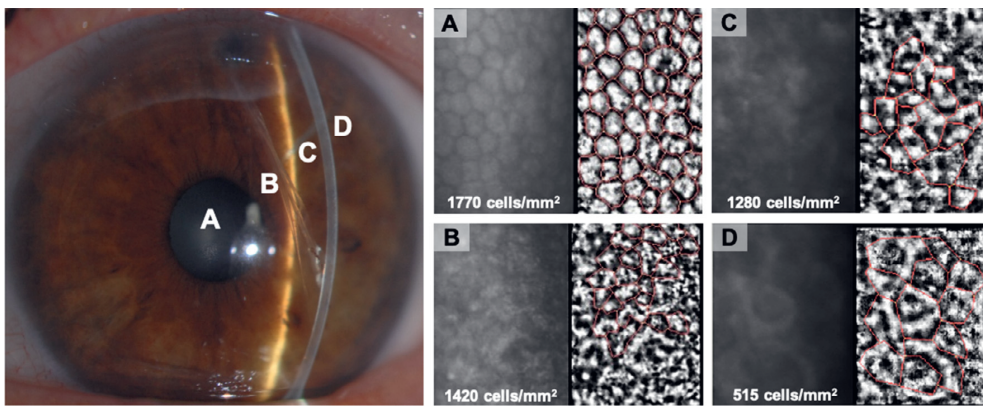

Figure 4| Slit-lamp and specular microscopy images of a DMEK graft at 8 months of follow-up. Although centrally the graft appeared detached, the area was visibly clear. Specular microscopy images taken at the indicated positions in the left image showed an endothelial cell density of 1770 cells/mm^2 in the corneal center (A), 1420 cells/mm^2 paracentrally (B), 1280 cells/mm^2 in the periphery (C), and 515 cells/mm^2 in the far periphery (D). Adapted from: "Descemet membrane endothelial transfer: "free-floating" donor Descemet implantation as a potential alternative to "keratoplasty". Dirisamer, M., Cornea, 2012.

Recently, another study reported complete recovery of corneal clarity and visual acuity when using cryopreserved full-thickness endothelial-free grafts for therapeutic PK.[108] Of the 18 out of 195 grafts showed

recovery of the corneal graft clarity within 1 year after graft transplantation. When corneal clarity was recovered, average ECD was 991 cells/mm^2 (range, 782-1531 cells/mm^2) and remained stable up to 2 years after surgery. The authors suggested that the endothelium may have regenerated by cell proliferation rather than cell migration and that cells originated from peripheral host.[108]

Experimental alternatives to transplantation techniques

Cell regeneration

In ophthalmology, ROCK-inhibitors play a role in the regulation of aqueous humor outflow by inducing relaxation of both the ciliary muscle and the actin cytoskeleton in the trabecular meshwork.109 Currently, Ripasudil (0.4% ROCK inhibitor) has market authorization in Japan for treating glaucoma or ocular hypertension.110 More recently, Netarsudil ophthalmic solution 0.02% was approved in the United States and the European Union for lowering elevated intraocular pressure (IOP).[34,111,112]

The first clinical study suggesting that *in vivo* proliferation of corneal endothelium could be stimulated by pharmaceutical treatment described the possibility of using ROCK-inhibitor eye drops subsequent to transcorneal freezing as an alternative to graft surgery in patients with corneal endothelial dysfunction.[113] Specular microscopy examination 18 months after the treatment showed small CEnCs present at a high cell density in the central part of the cornea. Two suggestions were made regarding the mechanism of action, namely the spontaneous cell remodeling and presence of endothelial progenitors in the peripheral cornea. In that experiment, it was not possible to conclude whether the endothelium improved due to the ROCK-inhibition application or whether the removal of the diseased cells was sufficient to induce wound healing.[113] Other case reports described that ROCK-inhibitors prevent the progression of bullous keratopathy in patients whose corneal endothelium was severely damaged by cataract surgery or rescue PK grafts from failure after an acute rejection episode.[63,114]

Surgical Techniques that require endothelial migration

Descemet stripping only (DSO) or Descemetorhexis without endothelial keratoplasty (DWEK) and acellular DM transplantation are experimental surgical strategies for treating central FECD that depend on the patient's own endothelial cells to grow and reform the barrier.[115–118] The surgery involves removal of the central 4-6 mm diseased endothelium and DM, that is, removal of central non-confluent guttae, to allow the centripetal migration and redistribution of the remaining healthy peripheral endothelial cells to cover either the bare stroma generated by DWEK/DSO or to re-populate the transplanted devitalized DM.[116,119] Transplanting a devitalized DM or trying to leave Descemet membrane's anterior banded layer intact during descemetorhexis may be beneficial for cell migration as this extracellular matrix contains important proteins and growth factors that are required for cellular process such as migration or proliferation [132].

Clinical case series evaluating DWEK/DSO for FECD have reported inconsistent results from total failure to complete recovery with central ECD in the range of 428-864 cells/mm^2 at the last reported follow-up visit (range: 6–24 months).[119–121] Observed corneal clearance time in successful DSO cases, which can most likely be linked to the speed to cell migration, varied between patients and was thought to be influenced by both surgical and patient factors. Overall, repopulation of the bare stroma and inducing corneal clearance can occur between 3 and 6 months postoperatively. This gives a timeframe for *in vivo* CEnC wound healing to happen.[119,121] (**Figure 5**).

When a topical ROCK-inhibitor such as Ripasudil or Netarsudil was administrated after DWEK/DSO, decreased clearance time together with an improved central ECD and an overall better cell architecture were found in eyes that received ROCK-inhibitor immediately after surgery.[66,122] Moreover, less loss of peripheral ECD was observed when ROCK-inhibitor was administrated immediately after surgery rather than waiting until later in

the healing course.[66] Hence, this may support the concept of endothelial cell proliferation in the ROCK-inhibitor treated group, as opposed to pure endothelial cell migration in the DWEK/DSO group. However, overall peripheral ECD decreased after DWEK/DSO regardless of whether ROCK-inhibitor had been administered. The repopulation of central bare stroma therefore most likely involved a combination of proliferation and migration of cells from the peripheral endothelium.[122]

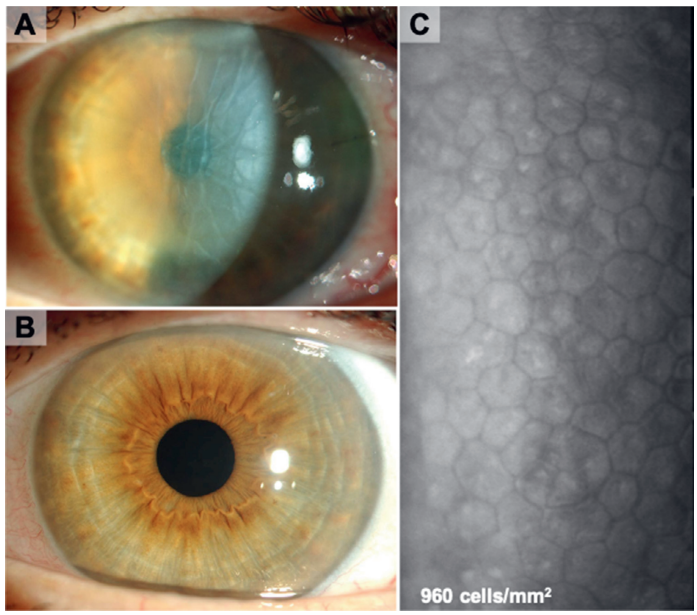

Figure 5| Slit-lamp and specular microscopy images of a cornea at 6 y post DSO. Although the posterior cornea appeared irregular (A) following 8 mm surgical descemetorhexis, the cornea clarity was restored within 12 weeks and maintained thereafter (B) with a central ECD of 960 cells/mm^2 (C).

The inability of the corneal endothelium to regenerate *in vivo* has been mainly attributed to strong contact inhibition cell behavior that in turn upregulates the p27Kip1 and prevents transition to the S-phase. Thus, corneal endothelial cells are not terminally differentiated but do possess proliferative potential. *In vitro* studies on corneal endothelium wound healing with ROCK-inhibitor showed that cell cycle progression is enhanced closer to the wound edge and stops once the wound is healed.[63,67,94] Studies of ROCK-inhibitor in human patients reported “pseudoguttae” visible on specular microscopy.[123] These ‘dark bodies’ were arising in the population of cells migrating to cover the descemetorhexis area but disappeared later, when the endothelium integrity was restored.[85,118] This led to the assumption that ROCK-inhibitor might have affected the distribution of actin microfilaments concomitant with inhibition of focal adhesion formation. Another important observation was the intercellular localization of the dark bodies rather than within the cell cytoplasm.[118] These clinical observations corroborate well with the in vitro findings that ROCK-inhibitor reduces cell adhesion through a loss of focal adhesion complexes and reduced expression of intracellular adhesion molecules.[112,124] Although there is little information about the success of DWEKDSO procedure in relation to patient factors, surgical factors such as avoiding constant pressure during DM scoring or stromal contact by overlaying an acellular DM to facilitate central cell migration and prevent posterior stromal scarring are better characterized.[116,117,125]

Because DWEK/DSO was shown to be more successful among patients suffering from FECD due to the presence of a larger cell reservoir to the periphery, transplanting a denuded DM together with the use of a topical ROCK-inhibitor might increase the success rate of DWEK/DSO.[117]

Cell- based therapies

Kinoshita et al. were the first to report on CEnC injection therapy in 11 patients (7 were FECD, 4 were BK) with a 5-year follow-up.[126] After removing the abnormal extracellular matrix and the degenerated CEnCs on the patient DM either by mechanical scraping or a 5 mm descemetorhexis, cultured human CEnCs were injected in combination with ROCK-inhibitor into the anterior chamber of the eye. ROCK-inhibitor was required as a supplement for the cell suspension to promote CEnC adhesion.[33,35] Since the number of injected cells was 106 in most treated eyes, proliferation is unlikely to occur despite the presence of ROCK-inhibitor, but it can be speculated that cell migration might have occurred in case of an initially uneven cell adherence on the posterior surface. Five years after the procedure, 10 out of 11 patients showed a restoration of the corneal function with a central ECD of 1257 ± 467 cells/mm^2 and no major adverse reactions directly related to the human CEnC injection therapy.[126] This promising preliminary study that merged an adjunct drug in cell-based therapies has offered a new perspective in the treatment of endothelial dysfunction.[126]

DISCUSSION

Currently, newly emerging alternatives to conventional EK are being investigated to compensate for the shortfall in global corneal graft tissue.[27–32] Within the field of regenerative medicine, new therapeutic strategies aim to regenerate the corneal endothelium through means of corneal endothelial wound healing and potentially pharmacologically stimulating this process.[61,62,77] Over the past decade, advances in our understanding of the biochemical and mechanical cues have help us better exploit the underlying wound healing process.

The main signaling pathways that govern normal corneal endothelial wound healing include the RhoA/ROCK, PI3K/Akt, Wnt, and TGF-ß pathways. These cascades carry on information of upstream/downstream relationships between interacting proteins. For example, Rap1 is a molecular switch that cycles between an active GTP-bound and inactive GDP-bound form and regulates the RhoA/ROCK and PI3K/Akt pathway. Because ROCK mediates various important cellular functions, inhibition of ROCK may affect multiple signaling pathways and will outweigh signaling cascades which act through the same effector proteins.[34] This crosstalk hypothesis may explain why ROCK-inhibition can have multiple biological effects such as enhanced proliferation or anti-apoptotic effects and why it is not only limited to cytoskeletal changes within the wound healing process.[62,66] The crosstalk of the Rho/ROCK pathway with other signaling cascades makes it challenging to unravel all involved processes in endothelial wound healing and more research is required to fully exploit the underlying biochemical processes for clinical applications.

Transcription factor SMAD4, which also is an indirect target of Akt, plays an important role in inducing EnMT thereby creating cytoskeletal changes, an increased motility and aberrant CEnC ECM deposition. However, strategies to overcome EnMT must not be accompanied by the impairment of cell migration during wound healing.[47] Sumioka et al. investigated the role of TGF-ß related signaling during corneal endothelial wound healing by avoiding the disruption of migration signals while blocking an unfavorable EnMT phenotype. This study indicates that it is possible to increase wound healing without inducing EnMT.[80]

Cyclins, cyclin-kinases and cyclin kinase inhibitors are the end staged effector proteins that regulate cell cycle progression and proliferation in CEnCs during wound healing. Enhancing these end-staged proteins can lead to a selective regulation of the proliferation rate instead of activating other upstream proteins that could adversely affect the normal CEnC phenotype.[13,38,53]

Endothelial wounds may trigger cells adjacent to the wound to lose their pericellular actin band pattern and later well-defined stress fibers fill their cytoplasm and persist until the wound is closed.[69] However, it was also reported that endothelial cells undergo directional migration into the wounded zone in the absence of an organized actin cytoskeleton and without stress fiber formation.[127,128]

Progenitor cells that are located in specific niches in the corneal far periphery may constitute an interesting target for regenerative therapies as they were shown to possess enhanced regenerative capacities.[107,129] However, knowledge about these cells and the involved processes is still limited.

This review provides an extensive overview over the signaling pathways involved in CEnC proliferation and migration. Knowledge about these pathways paves the way for pharmacological stimulation to effectively target these fundamental cell processes. This could lead to an effective topical treatment of corneal endothelial dysfunction either as a stand-alone treatment or in combination with surgical removal of diseased tissue. To conclude, basic research involved in corneal endothelial cell biology will act as a central anchor point for new therapies to treat corneal endothelial dysfunctions.

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