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# A Systematic Review on Cornea Epithelial-Stromal Homeostasis

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**Abstract**

**Introduction** This review aims to summarise the role of different cells, genes, proteins and lipid in regulating cornea epithelial-stromal homeostasis.

**Methods** We performed an Entrez PubMed literature search using keywords 'human', 'cornea', 'epithelial', 'stromal', 'homeostasis', 'fibrosis response', and 'pathogenesis' on 24th of September 2019, resulting in 35 papers, of which 18 were chosen after filtering for 'English language' and 'published within 10 years' as well as curation for relevance by the authors.

**Results** The 18 selected papers showed that corneal epithelial cells, fibroblasts and telocytes, together with genes such as Klf4, Pax6 and Id found in the cells, play important roles in achieving homeostasis to maintain corneal integrity and transparency. Proteins classified as pro-fibrotic ligands and anti-fibrotic ligands are responsible for regulating cornea stromal fibrosis and extracellular matrix deposition, thus regulators of scar formation during wound healing. Anti-inflammatory ligands and wound repairing ligands are critical in eliciting protective inflammation and promoting epithelial healing respectively. Protein receptors located on cellular membrane play a role in maintaining intercellular connections as well as corneal hydration.

**Discussion/ Conclusion** These studies prompt development of novel therapeutic strategies such as tear drops or ointments that target certain proteins to maintain corneal homeostasis. However, more *in vitro* and *in vivo* studies are required to prove the effectiveness of exogenous administration of molecules in improving healing outcome. Hence, more future investigation of the molecular pathways highlighted in this review will reveal novel therapeutic tools such as gene or cell therapy to treat corneal diseases.

## Introduction

The cornea, comprised of five layers namely the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium, is essential for clear vision. It helps refract two-third of light onto the retina through the pupil and lens. Also, the cornea acts as a physical barrier to protect internal ocular structures from external insults such as microbial, physical and chemical damages [1]. Given its superficial location, the cornea is externally exposed to the environment and hence prone to various diseases such as keratitis [2], keratopathy [3] and epithelial-stromal scarring [4]. These pathologies will hinder the transparency and smooth curvature of the cornea, which is maintained by keratocytes and collagen extracellular matrix (ECM) in the stroma [5], leading to a loss of refractive power and even ocular morbidity.

Since disrupted epithelial integrity and stromal opacification inhibit clear and focused vision, our cornea has its own homeostatic system to restore tissue transparency after wounding. In face of external disturbances, the cornea will initiate various mechanisms to develop protective inflammation and regulate cell migration and proliferation [6]. Epithelial cells will be continuously replenished by limbal stem cells to maintain an effective physical barrier [7]. Moreover, the cornea has its own dynamic process to repair injured cells through cell signalling cascades to prevent chronic inflammation and infection [8]. Hence, with the regulated immune response to injury, the cornea itself can have an improved healing outcome with minimal tissue changes [9]. Nevertheless, irreversible changes of the cornea such as corneal haze and loss of epithelial basement membrane may occur upon damage. This prompts studies to understand the molecular changes in the cornea so as to seek novel therapies to restore its homeostasis.

In this study, a systematic review of literature published within these 10 years was performed. This review focused on the pathways of how the cornea itself responds to external changes and maintains its constant condition. The responses of cornea upon scarring to permit protective inflammation were also discussed. We highlighted the cells, genes, proteins and lipid involved

in the epithelial and stromal layer that help restore the physiological status of the cornea. Potential therapeutic proteins or cells that can cure or alleviate corneal disorders were as well discussed.

## Methods

An Entrez PubMed search was conducted on the 24th of September 2019 using the search terms 'human', 'cornea', 'epithelial', 'stromal', 'homeostasis', 'fibrosis response', and 'pathogenesis'. Only articles published within 10 years and written in English language were included to ensure updated articles are reported. With these criteria, 35 articles were identified. Only *in vitro, ex vivo and in vivo* set-ups describing the mechanisms of the players or their antagonists involved in the homeostasis of the corneal epithelium and stroma were included. For example, papers concerning the pathways of epithelial-stromal injury and healing or process of stromal keratocytes differentiation were considered as relevant. Furthermore, only original articles were included, while review papers and meta-analyses were excluded. The resulting articles were then manually curated for subject relevance via abstract or full text by HLW and YKC independently, followed by a discussion on the relevance of identified papers. KCS made the arbitration when both HLW and YKC disagreed on paper selection, as well as confirmed the articles to be investigated in this study. HLW and YKC independently scored the selected papers for risk of biases, which were scored with "Low", "Moderate", or "High" for (1) whether a valid model was used to disrupt the cornea homeostasis, (2) sample randomization, (3) validate measurement, (4) blinded assessment of outcome and (5) appropriate statistical analysis, based on the Critical Appraisal Skills Programme (CASP). A study that satisfied all five, three to four, or less than three criteria was regarded as "Low", "Moderate", and "High" respectively. The relevant papers were stratified based on the cells,

genes, proteins and lipid involved in the epithelial-stromal homeostasis. Figure 1 describes the selection process for identified studies.

## Results

In total, 18 *in vitro*, *ex vivo* and *in vivo* studies were reviewed after manual curation **(see figure 1)**. Five papers were considered as having a low level of bias, such as the use of transgenic animals to evaluate the role of gene, while 13 were regarded as "moderate", including studies blocking certain signalling pathways. For analysis, we grouped our discussion of the finalized papers into the following headings 1) cells, **(see table 1)** 2) genes, **(see table 2)** 3) proteins, **(see table 3)** and 4) lipid **(see table 4)**.



### 1. Cell types

Corneal epithelial cells (CECs), fibroblasts and telocytes were found to contribute to cornea homeostasis.

*1.1 Corneal epithelial cells and fibroblasts*

Soluble cytokines secreted by fibroblasts were proven to regulate the proliferation of epithelial cells while growth factors secreted by epithelial cells were shown to affect corneal integrity [10]. In fact, the close interaction between human corneal epithelial cells (HCECs) and human corneal fibroblasts (HCFs) are crucial to corneal homeostasis and development [11]. Culturing epithelial cells on fibroblast-collagen mixture, 9 growth factors, namely fibroblast growth factor 7, hepatocyte growth factor, nerve growth factor (NGF), epidermal growth factor (EGF), sarcoma growth factor, transforming growth factor-α, β1, β2 and β3 expression level in HCFs were tested [12]. Among them, only the expression level of transforming growth factor-α (TGF-α) was increased significantly in HCFs co-cultured with CECs, with an increased level ranging from 12 to 152 times compared to those without CECs. Since TGF-α was found to

promote epithelial cell proliferation and migration, fibroblasts were suggested to play a role in influencing epithelial wound healing [13]. Also, stratification of epithelial layer was only observed when epithelial cells were seeded on HCF-collagen gel, hinting that TGF-α may be essential to maintain a mature corneal epithelial stratification.

Conversely, transforming growth factor-β (TGF-β), especially TGF-β1, 2 and 3, the mRNA expression was lower in HCFs co-cultured with CECs than that in HCFs alone. Since TGF-β was proven to inhibit CEC proliferation, its downregulation may induce minimal inhibition on cell growth and thus improve healing of the epithelium. [14] Thus, CECs interact with HCFs to maintain corneal homeostasis upon injury.

*1.2 Telocytes*

Telocytes, interstitial cells found in the stromal compartment in different organs, have been characterized with a small cell body with long telopodes, as well as podomers and podoms. [15] This cell type was believed to partake in intercellular signalling by releasing extracellular vesicles such as exosomes and ectosomes to communicate with other cell types [16]. Telocytes have been newly found in the corneal stroma to maintain a correct ECM organization. In a normal cornea, cells were found stained positive with markers of telocytes, CD34 and platelet-derived growth factor receptor α. Having long and thin cellular processes, $CD34^+/c\text{-}kit^+$ or $CD34^+/c\text{-}kit^-$ telocytes, were found in subepithelial or deeper stroma, illustrating that different telocytes subpopulations are densely populated in the stroma. However, in keratoconic cornea, $CD34^+$ and $c\text{-}kit^+$ stromal cells were decreased and telocytes were distributed in an uneven manner. A significant drop in telocytes was found compared to normal ones. Furthermore, telocytes from keratoconic cornea showed abnormal morphology such as loss of organelles, darkened cytoplasm and shrinkage of telopodes. Telocytes were suggested to play a critical role in maintaining a highly organized collagen matrix, thus contributing to high corneal

transparency and stability. In a keratoconic cornea, the loss of telocytes, in particular c-kit$^{+}$ subtype, leads to altered ECM arrangement and ultimately adversely affect the regeneration process, leading to the bulging of cornea into a cone-like shape.

## 2. Genes

Krüppel-like factor-4 (Klf4), Pax6 and Inhibitor of differentiation (Id) gene were indicated in maintaining the epithelial-stromal homeostasis.

### *2.1 Krüppel-like factor-4 gene*

Well-regulated tissue turnover and basal membrane integrity are crucial to the homeostasis of corneal epithelium [17]. Klf4 gene is a highly expressed transcription factor in the cornea and was found to play an integral role in the mature of mouse ocular surface [18]. It has been shown to play a role in regulating epithelial integrity and permeability. Doxycycline was administered to generate corneal epithelium-specific ablation of Klf4 in transgenic mouse. The number of epithelial cell layers and enlarged basal cells were increased in mice treated with 5 days of doxycycline, where superficial cells remained spherical [19]. In mice after 15 days of doxycyline adminstration, their cornea revealed a defective epithelial barrier function. Tight junction proteins ZO-1 and occludin levels in mice treated after 15 days of doxycycline were reduced compared to wild type (WT) mice, suggesting that tight junctions are defective due to the lack of Klf4.

The integrity of epithelial basement membrane highly relies on the expression of Klf4 gene. A decreased amount of laminin-332 expression was found in the corneal epithelial basement membrane of doxycycline-treated mice compared to that of WT mice. Yet, the expression of matrix metalloproteinase 9 (MMP9) in mice treated with doxycycline was increased, which may lead to the degradation of basement membrane. Expression of basement membrane components and epithelial markers in mice treated with doxycycline were reduced while that

of keratinized epithelia was increased. In addition, elevated cell cycle regulator Ccnd2 expression was observed, which led to the increase in epithelial layers in the cornea of Klf4 transgenic mouse. These results propose that Klf4 is important in maintaining corneal membrane integrity and homeostasis.

*2.2 Pax6 gene*

Pax6 gene was widely known to be expressed in eye development [20]. Absence of this gene will lead to ocular abnormalities such as aniridia-related keratopathy [21] and limbal epithelial stem cell deficiency [22]. Epithelial cells in transgenic mice with high Pax6 level were found to be irregular and had indistinct cell junctions with enlarged microvilli [23]. In the stromal layer, keratocytes from mice with low Pax6 level had larger vacuoles yet those with high Pax6 expression showed smaller ones, with both cells more highly innervated than WT keratocytes. Also, endothelial cells with low Pax6 level were bigger and slightly irregular in shape while those with high Pax6 level were highly irregular with unclear cell borders. This indicated that under or overexpression of Pax6 gene will lead to abnormal cell morphology, affecting cell adhesion and corneal hydration.

Additionally, PAX77$^{Tg/-}$ transgene, which has an over-expression of Pax6 gene, was revealed to rescue the altered ocular phenotype in Pax6 heterozygotes mice, normalizing corneal circumference and epithelial maintenance. By inducing PAX77 transgene, mosaic corneal stripe patterns were shown to be normal in heterozygotes, and putative deficiency in active limbal stem cell clones were normalized, reflecting that this transgene may be a therapy to restore corneal homeostasis.

*2.3 Inhibitor of differentiation gene*

Four Id genes (Id1- Id4) were identified as inhibitors of basic helix-loop-helix transcription factors, which contribute to Id proteins for the regulation of cell proliferation and differentiation [24]. The role of Id genes in cornea stromal homeostasis was studied using corneal fibroblasts culture [25]. In the study, Id1 and Id2 were found in human corneal epithelial and endothelial cells. HCFs even expressed all four Id genes but these genes were not found in myofibroblasts, which is a phenotypically differentiated HCF under fibrotic response.

Indeed, Id genes were discovered to be differentially regulated by TGF-β1 and bone morphogenetic proteins- 7 (BMP-7), a protein which was reported to oppose TGF-β1 activity [26]. Fibroblasts expressed Id genes in a time dependent manner under both TGF-β1 and BMP-7 treatment. After stimulation by TGF-β1 for 2-48 hours, Id 1-3 mRNA expression peaked at the second hour but decreased at longer time points. Id3 was especially found to have an increasing trend with TGF-β1 treatment at 24 and 48 hours. Similarly, Id 1-3 also showed gradually increased expression with BMP-7 treatment. Moreover, under co-stimulation of TGF-β1 and BMP-7, Id1 was significantly increased with low expression level of α-SMA in HCFs, proving the anti-fibrotic effects possessed by BMP7 in the presence of TGF-β1. Hence, Id genes may contribute to the modulation of corneal haze by controlling fibroblast activation or their de-differentiation.



## 3. Proteins

### 3.1 Pro-fibrotic ligands

TGF-β1 and connective tissue growth factor (CTGF) were found to promote fibrotic response in the stromal layer of the cornea.

#### *3.1.1 Transforming growth factor- beta 1*

TGF-β performs as a cytokine in promoting cornea development and homeostasis. Activation of TGF-β1 upon injury will disrupt the maintenance of corneal homeostasis, as previous research has shown that scar formation may be due to increased expression of TGF-β1 [27]. The endothelial layer of TGF-β transgenic mice at postnatal day 7 (P7) started to lose its cell-cell contact and detach from inner surface while the stroma became thicker. Concurrently, epithelial cells were found pooly differentiated. TGF-β transgenic mice at P14 demonstrated a strong retention of fluorescence staining on the corneal surface by fluorescein binding, showing the surface roughness and defective epithelial layer on the cornea [14]. In addition, as TGF-β1 is an important component in epithelial-to-mesenchymal transition (EMT), a marker for EMT, alpha-smooth muscle actin (α-SMA), was found in both corneal endothelial and epithelial layers at P16 mice, revealing that most cells have transformed into myofibroblast-like type, which lead to stromal opacification and impaired visual clarity. Endothelial cells even showed well-organized stress fibres between P21 and P30, suggesting that they may migrate as myofibroblasts into the stroma and impede normal vision. α-SMA expressing cells were firstly found in both endothelial and epithelial layers, subsequently in the stroma. Besides, *in situ* hybridizaiton resulted in a downregulation of E- and N-cadherin in TGF-β transgenic mice cornea while cadherin-11 was highly found in epithelial and stroma layers, which supported that the upregulation of TGF-β1 induces the formation of myofibroblasts in cornea. Thus, the study revealed that tight control of TGF- β1 secretion is crucial to maintain corneal homeostasis.

*3.1.2 Connective tissue growth factor*

CTGF, a protein of 349 amino acids, is a pivotal player in determining ECM remodelling and fibrosis after external disturbance [28]. CTGF-immunoreactivity was shown in corneal epithelium, especially in basal layers yet less prominent in superficial stratified layers [29]. CTGF was also found in cornea stromal keratinocytes and endothelial cells. Indeed, CTGF

interacts highly with TGF-β to induce the proliferation of telomerase-immortalized human cornea stroma fibroblast cells (THSFs) [30]. However, blocking CTGF action could hinder activation of TGF-β in promoting THSFs proliferation, which suggested that CTGF may act as a downstream mediator of TGF-β in controlling stromal fibrosis. CTGF production was further shown to be upregulated in TGF-β stimulated cells. After blocking the c-jun NH2 terminal kinase (JNK) pathway with JNK1 and JNK2 siRNA, CTGF mRNA level was shown to be drastically reduced. Moreover, less Phospho-JNK 1/2 was detected upon TGF-β stimulation in THSFs pre-treated with JNK1/2 siRNA. This indicated that JNK pathway regulates TGF-β-induced CTGF expression, leading to corneal fibrotic response upon injury. Hence, CTGF was found to promote corneal fibrosis under the influence of TGF-β.

### 3.2 Anti-fibrotic ligands

Hepatocyte growth factor (HGF) and Protein phosphate magnesium dependent 1A (PPM1A) were found to minimize fibrotic response in the stromal layer of the cornea.

#### *3.2.1 Hepatocyte growth factor*

Secreted by mesenchymal cells, HGF stimulates proliferation, morphogenesis and migration of most mammalian epithelial cells [31]. HGF has been proven to counteract TGF-β effects aforementioned to achieve anti-fibrotic function in different organs such as lung [32], liver [33] and heart [34]. The addition of TGF-β1 to HCFs for 24 hours was shown to increase mRNA expression of α-SMA [35], proving that TGF-β1 induces transformation of HCFs to myofibroblasts. In addition, HGF at 5-50 ng/mL could suppress TGF-β1 induced α-SMA expression, with 20 ng/mL HGF having the maximal inhibitory effect on keratocyte differentiation into myofibroblasts. A drastic drop in α-SMA was observed in HCFs with both TGF-β1 and HGF added compared to the cells with TGF-β1 only, indicating that HGF is effective in minimizing corneal stroma fibrosis.

To investigate how HGF alters the phenotype of myofibroblasts, HCFs were incubated with 5 ng/mL TGF-β1 for 24 hours, followed by another 24-hour treatment of 20 ng/mL HGF. This resulted in an obvious drop in α-SMA mRNA level and suggested that HGF may have the potential to revert myofibroblast phenotype to fibroblast one[36]. In addition, the stiffness of TGF-β1 treated HCF was increased. The increase in stiffness of HCFs could be greatly supressed by 20 ng/mL HGF, suggesting that the drop in rigidity may correlate to the amount of α-SMA expression since the formation of these contractile fibres was proven to contribute to increased cell stiffness [37].

*3.2.2 Protein Phosphatase Magnesium Dependent 1A*

Belonging to the serine threonine protein phosphatase family PPM1, PPM1A regulates cellular phosphorylation, differentiation and apoptosis by various signalling pathways such as TGF-β and p38-mitogen-activated protein kinase (MAPK) [38, 39]. The roles of PPM1A in corneal fibrosis and neovascularization were investigated via an ocular alkali burn model [40]. There was increased number of cells expressing high levels of α-SMA in PPM1A knockout mice, indicating scar tissue formation in the mice cornea. The stroma in these mice revealed more infiltrating inflammatory cells and proliferating cell nuclear antigen in dividing stromal cells, suggesting that PPM1A plays a role in restraining corneal inflammation. The PPM1A knockout mice also had a high expression of vascular endothelial growth factor (VEGF) in the cornea, which is associated with the formation of new blood vessels. Two days after alkali burn in PPM1A knockout mice, transcription of TGF-β-related genes in cornea such as MMP9 and VEGF were increased.

To understand the mechanism on how PPM1A controls the TGF-β signalling, phospho-p38 that has been indicated in the p38-MAPK pathway was investigated, as it was active in corneal keratocytes of PPM1A knockout mice even in control group. However, after TGF-β treatment,

cell phosphorylation in keratocytes from PPM1A knockout mice was significantly increased, showing that p38 is a preferred PPM1A substrate and its desphosphorylation regulates TGF-β signalling cascade. MMP9 and VEGF genes were activated in PPM1A-depleted keratocytes *in vitro*. Blocking p38-MAPK as well inhibited TGF-β to upregulate the expressions of MMP9 and VEGF. Thus, PPM1A was suggested to be crucial to corneal homeostasis via inhibiting the p38-MAPK pathway and interacting with TGF-β.

### 3.3 Anti-inflammatory ligands

Secreted lymphocyte antigen (Ly6)/ urokinase-type plasminogen activator receptor (uPAR)-related protein-1 (SLURP1) and vasoactive intestinal peptide (VIP) were found to reduce inflammatory response in the epithelial-stromal layer of the cornea.

#### *3.3.1 Secreted lymphocyte antigen (Ly6)/ urokinase-type plasminogen activator receptor (uPAR)-related protein-1*

SLURP1, a member of the Ly6 superfamily, is downregulated during infection to help develop protective inflammation and inhibit leukocyte infiltration [41]. Mutation of *Slurp1* gene is related to Mal De Meleda [42], which contributes to transgressive keratosis and keratoderma. SLURP1 was shown to have a suppressive effect on both the proliferation and migration of human corneal limbal epithelial (HCLE) cells [43]. In contrast, urokinase-type plasminogen activator receptor (uPAR), another Ly6 family member, was shown to play a role in regulating cell survival and invasion [44]. Its expression was upregulated during injury and its functions were dependent on ligands including urokinase-type plasminogen activator (uPA), which was confirmed to be one of the SLUPR1-binding proteins. SLUPR1 was suggested to hinder the stimulatory effect of uPA on cell migration. As a result, under normal condition, SLURP1 is hypothesized to be bound with uPA and inhibit the binding of uPA to uPAR, which maintains a stable ECM without inflammation. However, under injured conditions, uPA is free to bind to

uPAR since SLURP1 is downregulated for developing protective effects, hence promoting ECM degradation and inflammation. Therefore, SLUPR1 is critical to corneal homeostasis in controlling epithelial cells migration by acting as a soluble scavenger.

*3.3.2 Vasoactive intestinal peptide (VIP)*

VIP is a neuropeptide which can be found in corneal nerves and aqueous humour of human eyes. It can inhibit pro-inflammatory cytokine production and promote healing or growth of the cornea [45]. The increase in expression of growth factors like EGF and HGF, were shown in mice induced with *Pseudomonas aeruginosa* followed by VIP treatment [46]. VIP treated cornea had increased expressions of angiogenic molecules like VEGF-A and VEGF receptor 2 (VEGFR2), with histological examination showing greater vascularity in the peripheral cornea. All these may contribute to resistance to *Pseudomonas aeruginosa* keratitis. The increase in EGF, fibroblast growth factor (FGF), HGF, and VEGF-A was further confirmed at 5 days post infection in epithelium and stroma of VIP-added cornea.

Antimicrobial beta-defensins were also detected in infected cornea. After treated with VIP, the amount of mouse β-defensins 2 (mBD2) was increased in corneal epithelium and stroma at 5 days post infection. In addition, the VIP-treated samples had decreased bacterial counts at 1, 3 and 5 days post infection. Hence, this study supported the idea that VIP is involved in immune homeostasis and regulates other growth factors, thus facilitating corneal wound healing after keratitis.

3.4 Wound repairing ligands

Lumican and matrix metalloproteinase 12 (MMP12) were found to play a role in promoting wound healing in the cornea.

*3.4.1 Lumican*

Lumican, one of the proteoglycans in stroma, is expressed by mesenchymal fibroblasts. It binds to collagens and regulates collagen fibril growth [47]. Lumican-deficient mice were found to lose corneal transparency and this disorder was associated with abnormal collagen fibril architecture [48]. Among 2173 proteins identified via iTRAQ labelling, 113 corneal proteins were upregulated while 47 were downregulated in both lumican-expressing and lumican-deficient mice [49]. Basement membrane proteins including laminin-beta 2 and perlecan were found to be elevated in lumican-deficient mice while small leucine-rich repeat proteoglycans (SLRPs) such as decorin and biglycan were decreased. Collagen type II, which is normally absent in mammalian cornea, was detected with an increased amount in lumican-deficient mice. Collagen type IV, XII, XIV were also increased slightly; yet, the level of cytokeratin 8 dropped in the same study. In addition, the expressions of dermatopontin, insulin-like growth factor-binding protein 2 and fibromodulin were increased, but at the same time expressions of keratin 8, biglycan and keratocan were decreased. From the above protein changes, lumican is important in regulating ECM and cellular protein levels, thus disrupted corneal integrity and various ocular disorders such as keratoconus occur upon reduced production.

*3.4.2 Matrix metalloproteinase 12 (MMP12)*

MMP, a family of ECM proteinase, has been found to be a regulator in repairing process and remodelling. MMP12 was suggested to have protective effects on corneal fibrosis and maintaining stromal transparency by controlling immune cells [50]. After removal of the epithelial layer in mouse cornea, the MMP12 mRNA expression was increased at 2 hours post injury with a highest level observed at 8 hours [51]. Yet, MMP12 expression declined gradually after 4 days of injury, showing that MMP12 was expressed immediately after injury. MMP12 knockout mice had significant delay in epithelial closure at 8 and 18 hours after injury when compared to WT mice, revealing that MMP12 has a role in epithelial repair.

A slower migration rate in scratch wound assay was shown in epithelial cells of MMP12 knockout mice when compared to cells from WT mice *in vitro*. Only 25% of the wound recovered in MMP12 knockout epithelial cells yet WT mice cells had a 37% of recovered wound, which further proved that MMP12 is effective in promoting healing of the epithelial wound. Moreover, MMP12 knockout mice were found to have a drop in neutrophil infiltration in the anterior stroma when compared to WT mice, demonstrating that MMP12 positively recruits neutrophils upon epithelial injury to combat against infection.



3.5 Growth factors

Platelet-derived growth factor-BB isoform (PDGF-BB) and basic fibroblast growth factor (bFGF) promote corneal fibroblast migration and ECM synthesis during stromal repair.

*3.5.1 Platelet -derived growth factor- BB isoform and basic fibroblast growth factor*

Previous studies showed that PDGF-BB and bFGF are involved in corneal wound healing but their actions on ECM synthesis were not well understood [52, 53]. After scratch wound injury, PDGF-BB and bFGF-treated HCF wound had a similar healing pattern and the wounds were completely closed at day 10, while wound in serum free media had a slower healing rate [54]. In the same study, PDGF-BB treated cells also had a higher level of collagen III expression, which is a marker of repair matrix synthesis. Integrins subunits $\alpha 5$ and $\beta 1$ were highly expressed in PDGF-BB treated HCFs but not in bFGF-treated cells. The proteoglycan SDC4 had an increased mRNA expression in HCFs added with PDGF-BB and bFGF, suggesting that these growth factors upregulate new ECM synthesis. Unlike a homogeneous cytoplasmic distribution in normal situation, SDC4 was in the trailing edge of HCFs that were pre-treated with PDGF-BB. In addition, perlecan mRNA expression was increased in PDGF-BB and bFGF-treated cells upon injury, suggesting that these growth factors promote the secretion of

various proteins which may facilitate corneal repair. These proteoglycans are markers of non-fibrotic repair phenotype and are vital in maintaining corneal homeostasis.

3.6 Receptors

Vascular endothelial growth factor receptor-1 (VEGFR-1), Aquaporin-5 (AQP-5) and Transient receptor potential vanilloid subtype 1 (TRPV1) were found to be involved in maintaining the epithelial-stromal homeostasis.

*3.6.1 Vascular endothelial growth factor receptor-1*

In light of the involvement of VEGF in maintaining cornea avascularity, the role of VEGFR in regulating stromal fibroblast network and corneal integrity was investigated. A circuit of a communication network between fibroblasts has been found in the stroma, which is crucial to maintain corneal transparency [55]. To investigate the role of VEGFR-1 in forming intercellular networks, HCFs that did not form networks were studied. Among them, HCFs with high level of VEGFR-1 showed an increased number of cell interconnections yet HCFs with low level of VEGFR-1 did not show obvious response upon addition of exogenous VEGF. Also, inducing HCFs with high VEGFR-1 expression with VEGF-A and VEGF-C led to more cell migration. Nonetheless, addition of Avastin, an anti-VEGF agent, showed a negative result in cell motility, proving that VEGF controls fibroblast organization and migration. Hence, a well-regulated VEGFR-1 expression in fibroblast influences cell arrangement and thus visual clarity.

When fibroblasts were transfected with VEGFR-1 siRNA, the cell motility and network formation drastically decreased. However, transfection with VEGFR-2 and 3 siRNA did not show significant influence on network formation. Eventually, VEGFR-1 resulted in a decrease with increasing age, suggesting the loss of such a receptor leads to disorganized stromal network and hence poor vision in elderly.

*3.6.2 Aquaporin-5*

AQP water channels, responsible for water conductance across the plasma membrane, are highly found in lens to maintain homeostasis [56]. AQP5 transcripts expression and AQP5 protein were both found in mouse cornea and lens [57]. AQP5 expression was found to be low in keratocytes close to Bowman's membrane while corneal basal columnar epithelium had the highest abundance of AQP5. The AQP5 in stromal layer was suggested to maintain corneal hydration since epithelial water permeability was reduced and corneal thickness was surged in AQP5 knockout mice. Moreover, the recovery rate of epithelial cells of AQP5 knockout mice was significantly decreased after putting in a hypotonic condition when compared to cells of WT mice. AQP5 was demonstrated to be present in the cornea epithelium and the loss of AQP5 may lead to dry eye disease and corneal oedema [58].

Cyclic adenosine monophosphate (cAMP) was observed to regulate epithelial cells AQP5 expression by phosphokinase A (PKA) pathway. *In vitro* studies showed that when Madin-Darby Canine Kidney cells, which express AQP5, were exposed to a PKA agonist, mp-cAMP, the AQP5 plasma membrane localization dropped substantially. However, when a PKA antagonist, H-89, was added, the AQP5 membrane localization and abundance showed an obvious increase. This reflected that PKA-mediated regulation of AQP5s may be a therapeutic target for corneal disease.

*3.6.3 Transient receptor potential vanilloid subtype 1*

As a non-selective cation channel, TRPV1 was found to mediate inflammatory responses by releasing endogenous agonists and metabolites [59]. The role of TRPV1 in inhibiting corneal transparency upon wound healing was studied [60]. Reverse transcriptase-dependent generation of products for TRPV1 were found in HCFs and HCECs along membrane perimeter and perinuclear regions. Addition of capsaicin, a selective TRPV1 agonist, resulted in a 2-fold

$Ca^{2+}$ concentration rise in HCFs and such an increase was blocked by adding capsazepine, a TRPV1 antagonist, showing that TRPV1 expression was functional. Moreover, induction of capsaicin on TRPV1 led to a rise in $Ca^{2+}$, resulting in MAPK phosphorylation in both HCECs and HCFs. Capsaicin-induced terminal kinase phosphorylation was found absent with capsazepine on TRPV1. In cells transduced with TRPV1 siRNA, capsaicin-induced MAPK activation was diminished, showing that TRPV1 was involved in eliciting the responses. Via MAPK stimulation, activation of TRPV1 was observed to increase IL-6 expression in HCFs, which leads to corneal fibrosis [61]. Presence of a p38 MAPK inhibitor resulted in an inhibition of IL-6 rise, illustrating that TRPV1-mediated IL-6 can be elicited by p38 MAPK stimulation. Hence, suppression of TRPV1 activation may pave a way to the restoration of corneal homeostasis.



## 4. Lipid

C6 ceramide was found to be a lipid involved in the epithelial-stromal layer of the cornea.

### *4.1 C6 ceramide*

Cell death is considered an essential step in maintaining corneal homeostasis. Ceramide, a second messenger in regulating cell proliferation, differentiation and growth, was proven to play a significant role in stromal cell death after epithelial injury [63]. A study showed that a larger dose of short chain ceramide causes a greater proportion of cell death of HCF and a drastic reduction in cell viability [64]. Ceramide was further revealed to cause mitochondrial dysfunction of HCF with an increased amount of cytochrome C released from mitochondria. HCFs with ceramide exposure as well resulted in enhanced production of mitochondial reactive oxygen species and a lowered mitochondrial membrane potential.

Increased JNK phosphorylation was observed in ceramide-treated HCF samples, suggesting that ceramide may regulate harikari gene (HRK) expression, which has been found to initiate

cell death under physiological or pathological circumstances. HCFs with ceramide treatment had a prominent upregulation of HRK expression, which initiated cell death under physiological condition. An association of HRK and mitochondrial protein p32 was established by co-immunoprecipitation, reflecting that HRK translocated to mitochondria and interacted with p32, which most likely contributed to cell death. Thus, the results suggested that in corneal homeostasis, cell death of HCFs after ceramide secretion is due to mitochondrial dysfunction mediated by HRK expression [64].

**Discussion**

Cornea stromal scarring is the fourth common cause of worldwide blindness, which contributes to 5.1% of the cases according the World Health Organization [67]. Patients with corneal opacity generally result in a lowered vision-related quality of life and experience social problems such as fearing to lose residual vision and going out during night [68]. In spite of the current treatments on corneal disorders with medical advancement, consequences of corneal injury such as opacification and chronic inflammation remain to be poorly treated [69]. To identify therapeutic targets for corneal scarring, the understanding on the response of cornea to external disturbance and therefore the cornea epithelial-stromal homeostasis is of utmost importance. Previous studies aforementioned suggested that a particular gene or cell type involved in corneal homeostasis can be targeted in the course of developing novel approaches to treat stromal scarring. Hence, an in-depth understanding of the molecular pathways enables the development of new targeted therapies to minimize scarring or prolonged inflammation.

To tackle the problem of stromal fibrosis, proposed effective managements have been targeted on TGF-β1 because studies have shown the upregulation of TGF-β1 could result in differentiation of keratocytes to myofibroblasts [4]. Many pharmaceutics target to block the action of TGF-β1 secreted from the epithelium to improve corneal clarity [14]. Nonetheless,

blockage of other relevant molecules could as well be considered to minimize fibrosis. For example, CTGF production was found to be mediated by the JNK pathway, thus inhibitors of JNK pathway may be a means to hinder CTGF action [70]. Similarly, TRPV1 can be blocked by specific inhibitors such as capsazepine to reduce dysregulated ECM deposition. In contrast, proteins that inhibit excessive fibrosis may promote wound healing outcome. For example, HGF can suppress TGF-promoted myofibroblast phenotype [71], while PPM1A can terminate TGF response and reduce angiogenesis [38]. Lumican can as well maintain a proper collagen fibril architecture, contributing to transparency and stability [49]. These evidences may suggest that proteins can be administered to the cornea exogenously depending on the specific aim to be achieved, thus promotes a better prognosis upon the healing process with minimal scarring. Furthermore, understanding the roles played by each protein favours more specific healing outcome. For instance, to elicit protective inflammation and epithelial cell migration, MMP12 should be upregulated while PPM1A action ought to be suppressed since their mechanisms of action are antagonistic [40, 51]. Tear drops or ointment can be formulated in clinical settings based on the findings in *in vitro* and *in vivo* studies. For instance, recombinant NGF, which was found to accelerate cornea healing in both human cell culturing and rat *in vivo* research, has been translated to clinical patients in dry eye disease [72, 73]. It was tested to be safe and effective in improving signs and symptoms in patients at different dosage. Hence, laboratory understanding of corneal pathophysiology may prompt to novel topical drops, suspensions or solutions in clinical settings.

Different genes are being identified to be responsible for maintaining epithelial integrity and homeostasis. Gene Klf4 influences the differentiation and barrier functions of the epithelial cells, which prevents Meesmann's dystrophy and dry eye diseases [19]. Pax6 heterozygotes result in ARK and LESC deficiency, and was later discovered that the addition of transgene PAX77 helps compensate such corneal defects [23]. These genes are essential in formulating

treatments such as gene therapy towards congenital ocular diseases, particular to infants who show epithelial fragility, and such understanding of the related genetic pathophysiology may bring up novel molecular treatments for these diseases. For instance, in the treatment of Leber congenital amaurosis, *RPE65* gene therapy has been put in to phase 1 human clinical trials provided that its success in experimental models [74]. Similarly, with the discovery of the contribution of embryonic stem cells, adult stem cells and induced pluripotent stem cells in differentiating into corneal epithelial cells, these stem cells have been adopted as candidates for regenerative medicine and curation of ocular surface diseases [75]. Therefore, given the superficial and easily accessible location of the cornea, gene and cell therapy could be ways to treat corneal disorders.

Most clinicians usually prescribe topical corticosteroids which downregulate TGF-β1 to minimize corneal scarring after injury or surgery despite their significant side effects [76]. To solve this problem, in the long-term, novel topical drugs, gene or cell therapies can be developed by targeting different proteins, genes or cells that can reduce fibrosis or repair epithelial damage. A particular study highlighted that HGF was shown to have the potential ability to reverse the differentiation of human myofibroblasts back into keratocytes [35], providing hope for patients suffering from scarring to regain visual clarity through pharmaceutical means. The effects of other proteins aforementioned on the reversed differentiation of myofibroblasts could also be investigated and further studies on post-treatment of keratocytes after injury are to be warranted. Nonetheless, one of the limitations of the current research on this area is that the relevant evidence is remained at the laboratory scale, where clinical trials are required. Also, the effects of exogenous administration of a molecule on other cells types apart from cornea such as conjunctival epithelium and goblet cells were not investigated, which may potentially disrupt the homeostasis of other neighbouring tissues of cornea such as conjunctiva, lacrimal glands and sclera. Another limitation would be the lack

of further *in vitro* or *in vivo* studies to prove that administration of exogenous molecules, which were found to play an important role in the cornea physiology, can improve the healing outcome. This suggests that randomized controlled clinical trials with human subjects in the future can be conducted when *in vitro* and *in vivo* studies showed repeated positive results such as those with MMP12 and PPM1A. With more comprehensive knowledge regarding corneal homeostasis, improved and specific clinical outcomes can be achieved with fewer adverse effects using targeted pharmaceutical agents.

## Conclusion

To promote a healthy cornea epithelium and stroma, dynamics of various cells, genes, proteins and lipid are ought to be tightly regulated and maintained in a stable manner **(see figure 2)**, as they help to maintain epithelial integrity, reduce corneal fibrosis and promote protective inflammation. A thorough understanding of the roles of each relevant molecule will be essential to facilitate the development of therapeutic strategies in minimizing specific corneal conditions, such as stromal scarring and disrupted epithelial basement membrane. Hence, we believe that the investigations on the molecular pathways highlighted in this review will hint at more future therapeutic tools to treat corneal diseases.

## Statements

### Statement of Ethics

Ethics approval is not required since there is no involvement of human and animals.

### Disclosure Statement

The authors have no conflicts of interest to declare.


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### Author Contributions

All authors attest that they meet the current ICMJE criteria for authorship. HLW and KCS were involved in study design, data collection, data analysis, manuscript writing and editing. SHLP, YSB, ACYL, VJ, YKC were involved in data collection, data analysis, manuscript writing and editing. All authors have approved the final version of the manuscript.

The authors agree to make all materials, data and associated protocols promptly available to readers without undue qualifications in material transfer agreements.

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**Figure legends**

Figure 1. PRISMA flowchart of search strategy.

Figure 2. Schematic diagram of cells, genes, proteins and lipid involved in the cornea epithelial-stromal homeostasis.

## Tables

**Table 1:** Cells involved in cornea epithelial-stromal homeostasis

| Source | Cells | Mechanism of action | Effects on corneal homeostasis |
|---|---|---|---|
| **Kobayashi, Shiraishi et al., 2015** | Epithelial cells, Fibroblasts | Along with epithelial cells, fibroblasts upregulate the expression of TGF-α but downregulate TGF-β expression in fibroblasts | Interaction between cells promotes epithelial cells proliferation, migration, and stratification |
| **Marini, Mencucci et al., 2017** | Telocytes | Partake in intercellular signalling by releasing extracellular vesicles to communicate with other cell types | Participate in corneal regeneration and repair; maintain correct stromal assembly for corneal transparency |

**Table 2:** Genes involved in cornea epithelial-stromal homeostasis

| Source | Genes | Mechanism of action | Effects on corneal homeostasis |
|---|---|---|---|
| **Delp, Swamynathan et al., 2015** | Klf4 | Prevents degradation of epithelial membrane by suppressing MMP9 secretion; maintains epithelial layer integrity by expressing tight junctions and laminin-332 | Maintains the integrity of corneal epithelium by regulating the formation of basement membrane and expression cell cycle regulators |
| **Mort, Bentley et al., 2011** | Pax6 | Regulates the normal morphology of epithelial, stromal and endothelial cells | Maintains cell adhesion, movement, normal mosaic patterns, promoting corneal integrity |
| **Mohan et al., 2016** | Id | Differentially regulated by TGF-β1 and BMP7 in a time dependent manner | Minimizes corneal fibrosis and differentiation during wound healing |

**Table 3:** Proteins involved in cornea epithelial-stromal homeostasis

| Source | Protein(s) | Mechanism of action | Effects on corneal homeostasis |
|---|---|---|---|
| **Reneker, Bloch et al., 2009** | TGF-β1 | Downregulates E- and N-cadherin and promotes cadherin-11 and α-SMA expression | Leads to myofibroblasts formation and stromal thickening for the wound closure, and loss of endothelial cell contact |
| **van Setten, Trost et al., 2016** | CTGF | Acts as a downstream mediator of TGF-β via the JNK pathway; induces mesenchymal cells formation from epithelium | Leads to corneal fibrosis upon injury and plays a role in tissue regulation or ECM deposition |
| **Miyagi, Jalilian et al., 2018** | HGF | Reduces the expression of TGF-β and thus α-SMA; reduces the stiffness of the stroma with fewer keratocyte differentiation | Improves wound healing by reducing the over-expression of α-SMA; reverses myofibroblasts back to keratocytes |
| **Dvashi, Sar Shalom et al., 2014** | PPM1A | Lowers the number of monocytes and proliferating cell nuclear antigen, VEGF, TGF-β related genes through p38 phosphorylation | Reduces corneal inflammation, fibrosis and angiogenesis upon damage |
| **Swamynathan and Swamynathan, 2014** | SLURP-1 | Inhibits leukocyte infiltration and suppresses epithelial cell proliferation; binds to uPA to hinder cell migration | Downregulates upon injury to elicit protective inflammation and ECM degradation |
| **Jiang, McClellan et al., 2011** | VIP | Upregulates EGF, HGF and VEGF-A upon infection; increases β-defensins 2 expression with reduced bacterial count | Facilitates infectious injury and suppresses bacterial growth |
| **Shao, Chaerkady et al., 2011** | Lumican | Regulates ECM deposition such as collagen I and IV, fibromodulin; and stromal proteoglycans such as decorin, biglycan | Maintains stromal thickness, transparency and light scatter via proper collagen fibril architecture |

| | | | |
|---|---|---|---|
| **Wolf, Maltseva et al., 2017** | MMP12 | Recruits more neutrophils and promotes epithelial cells migration | Promotes epithelial early repair and protective inflammation |
| **Gallego-Munoz, Ibares-Frias et al., 2018** | PDGF-BB, bFGF | PDGF-BB increases collagen III, α5 and β1 integrins subunits expression; both PDGF-BB and bFGF increase SDC4 and perlecan expression in fibroblasts | Increases wound closing rate and promotes a non-fibrotic wound |
| **Berthaut, Mirshahi et al., 2009** | VEGFR-1 | Increases interconnections or reticulum formations between fibroblasts; promotes cell motility | Decrease in receptor numbers leads to diminution of autocrine function and cell renewal, leading to poor vision |
| **Kumari, Varadaraj et al., 2012** | AQP-5 | cAMP regulates AQP5 localization via PKA pathway; participates in water conductance across membrane barriers | Promotes wound healing and maintains transparency; enhances water permeability so as to prevent oedema |
| **Yang, Yang et al., 2013** | TRPV1 | Activation leads to increased $Ca^{2+}$ concentration and p38 MAPK phosphorylation, followed by IL-6 release | Leads to opacification and dysregulated inflammation of cornea during wound healing |

**Table 4:** Lipid involved in cornea epithelial-stromal homeostasis

| Source | Lipid | Mechanism of action | Effects on corneal homeostasis |
|---|---|---|---|
| **Rizvi, Heimann et al., 2011** | Ceramide | Upregulates HRK expression and JNK phosphorylation, leading to mitochondrial dysfunctions in fibroblasts | Leads to corneal fibroblasts cell death and promotes wound healing |