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The role of hepatocyte growth factor in corneal wound healing

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Abstract

Hepatocyte growth factor (HGF) is a glycoprotein produced by mesenchymal cells and operates as a key molecule for tissue generation and renewal. During corneal injury, HGF is primarily secreted by stromal fibroblasts and promotes epithelial wound healing in a paracrine manner. While this mesenchymal-epithelial interaction is well characterized in various organs and the cornea, the role of HGF in corneal stromal and endothelial wound healing is understudied. In addition, HGF has been shown to play an anti-fibrotic role by inhibiting myofibroblast generation and subsequent production of a disorganized extracellular matrix and tissue fibrosis. Therefore, HGF represents a potential therapeutic tool in numerous organs in which myofibroblasts are responsible for tissue scarring. Corneal fibrosis can be a devastating sequella of injury and can result in corneal opacification and retrocorneal membrane formation leading to severe vision loss. In this article, we concisely review the available literature regarding the role of HGF in corneal wound healing. We highlight the influence of HGF on cellular behaviors in each corneal layer. Additionally, we suggest the possibility that HGF may represent a therapeutic tool for interrupting dysregulated corneal repair processes to improve patient outcomes.

Keywords

HGF; wound healing; myofibroblast; fibrosis; TGF-β

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1. Introduction

The cornea is a protective barrier and the primary refractive element of the visual system. The cornea generally consists of four transparent and avascular layers: epithelium, stroma, Descemet's membrane and endothelium. Additionally, in some species (e.g. humans, lizards, birds), the cornea can possess a Bowman's layer, a thickened acellular collagenous zone that lies between the epithelium and stroma. The cornea is continuously subjected to physical, chemical and biological insults from the external environment that can result in wounding. Corneal injury by trauma, infection, or surgery initiates multiple complex cellular processes including cell migration, proliferation, re-stratification, as well as deposition of extracellular matrix (ECM) and tissue remodeling which are coordinated to restore a healthy and functional cornea. These wound healing processes are regulated by numerous soluble cytoactive factors, the intrinsic chemistry of ECM elements as well as by biophysical attributes of the microenvironment of corneal cells. Soluble cytoactive factors include growth factors, cytokines, proteases, and neuropeptides. These factors work through autocrine and paracrine mechanisms and are derived from epithelial cells, stromal fibroblasts, corneal nerves, lacrimal glands, and cells of the immune system (Ljubimov and Saghizadeh, 2015).

Hepatocyte growth factor (HGF) is one of the growth factors which mediate tissue regeneration in numerous organs (Nakamura and Mizuno, 2010). The liver is a potently regenerative organ, which can renew even after removal of two-thirds of its volume. The factors controlling this process have been heavily studied (Nakamura and Mizuno, 2010; Nakamura *et al.*, 2011). In 1984, HGF was identified in rat platelets as a potent mitogenic factor for hepatocytes *in vitro* (Nakamura *et al.*, 1984; Russell *et al.*, 1984). A few years later, scatter factor, originally identified as a protein which modulates cell motility of renal tubular cells (Stoker *et al.*, 1987; Weidner *et al.*, 1991), was shown to be structurally identical to HGF. Tumor cytotoxic factor, a fibroblast-derived factor that induces cell death for some kinds of cancer cells, was also shown to be indistinguishable from HGF (Shima *et al.*, 1991). In aggregate, these various functions demonstrate the diverse biologic roles that HGF can assume depending on the target tissue of interest.

Hepatocyte growth factor is primarily secreted by mesenchymal cells and stimulates morphogenesis, migration, proliferation and survival of epithelial cells that express its specific receptor, c-Met (Montesano *et al.*, 1991; Sonnenberg *et al.*, 1993; Matsumoto and Nakamura, 1996; Birchmeier and Gherardi, 1998). Hepatocyte growth factor is known as a key mediator for organ generation and maturation at defined stages of development (Schmidt *et al.*, 1995; Uehara *et al.*, 1995; Bladt *et al.*, 1995). In addition to organ development, proliferative activities of epithelial cells are critical for wound healing (Yoshida *et al.*, 2003; Chmielowiec *et al.*, 2007). Though there are numerous reports regarding the importance of HGF in wound healing processes in an array of organs (Conway *et al.*, 2006; Nakamura and Mizuno, 2010), its role in corneal biology and repair has been understudied. Here, we concisely summarize the available literature regarding the role of HGF in corneal homeostasis and wound healing and discuss its potential as a therapeutic tool in the management of corneal fibrosis.

2. Structure of HGF

The primary structure of HGF was determined in 1989 (Nakamura *et al.*, 1989; Miyazawa *et al.*, 1989; Tashiro *et al.*, 1990), and multiple splice variants encoding different isoforms have been subsequently reported (Schultz *et al.*, 2009). Hepatocyte growth factor is synthesized in an inactive pre-pro form, consisting of 728 amino acids, and secreted by mesenchymal cells such as fibroblasts and macrophages. Inactive HGF becomes activated through two-cleavage processes. First, the signal peptide comprised of the first 31 amino acids is degraded, generating the pro-HGF. The single-chain pro-HGF is subsequently cleaved between Arg 494 and Val 495 by serine proteases. Numerous serum and cell-membrane proteases are involved in this cleaving process, including HGF activator (HGFA), urokinase- and tissue-type plasminogen activator (u-PA and t-PA), plasma kallikrein, coagulation factors XII and XI, matriptase and hepsin (Miyazawa *et al.*, 1993; Tamagnone and Comoglio, 1997; Lee *et al.*, 2000; Herter *et al.*, 2005). Matriptase and hepsin are transmembrane proteases involved in pericellular activation of HGF, whereas the other proteases are resident in serum. In vascularized tissues, HGFA is the primary HGF protease and is also regulated by proteolytic cleavage in response to injury (Conway *et al.*, 2006; Miyazawa, 2010; Kataoka and Kawaguchi, 2010; Rodgers *et al.*, 2017). Inactive pro-HGFA is produced by hepatocytes in the liver and circulates in serum (Shimomura *et al.*, 1993; Okajima *et al.*, 1997). Upon tissue injury, activated thrombin, which plays to prevent further hemorrhage in blood coagulation system, concomitantly activates HGFA (Fig. 1). Therefore, HGFA represents the link between tissue injury and activation of HGF (Miyazawa, 2010). The activation process in avascular tissues such as the cornea is less well understood and remains understudied. Amongst these proteases, u-PA and t-PA are known to be present in the cornea and t-PA is found in tears (Geanon *et al.*, 1987; Stevens *et al.*, 1992; Watanabe *et al.*, 2003; Warejcka *et al.*, 2011). These proteases represent the most likely candidates to activate HGF through enzymatic processing (Mars *et al.*, 1993). While the cleaving activities of u-PA and t-PA are weak *in vitro* in comparison to other proteases such as HGFA, matriptase, and hepsin, their activity may be amplified by the *in vivo* microenvironment following wounding (Naldini *et al.*, 1995; Grierson *et al.*, 2000). Additional studies are required to better define the activation process of HGF in the cornea in health and disease.

Mature HGF is a heterodimeric molecule consisting of a 69 kDa α-chain and a 34 kDa β-chain linked by a disulfide bond. The C-terminus of the α-chain is followed directly by the N-terminus of β-chain. The α-chain has a high affinity for c-Met, but the activation of the HGF/c-Met signaling is dependent on the subsequent binding of the β-chain (Hartmann *et al.*, 1992; Matsumoto *et al.*, 1998; Gherardi *et al.*, 2006; Merchant *et al.*, 2013). The binding of HGF to c-Met induces phosphorylation of tyrosine residues of intracellular tyrosine kinase domain of c-Met, which results in biological activities including mitogenic, motogenic and morphogenic activities via downstream signaling pathways (Birchmeier and Gherardi, 1998; Nakamura *et al.*, 2011).

3. Corneal epithelial wound healing and HGF

The anterior corneal epithelium is a stratified, squamous, non-keratinized epithelium. Surface cells make tight junctional complexes between their neighbors, which create the first

barrier against the external environment. Like other epithelial barriers in the human body, the corneal epithelium is a self-renewing tissue with a distinct stem cell niche residing in the limbal basal region to provide an unlimited supply of proliferating cells for epithelial regeneration (Schermer *et al.*, 1986; Cotsarelis *et al.*, 1989; Li *et al.*, 2007a; Xie *et al.*, 2011). Proper healing of the corneal epithelium is important for maintenance of transparency and thus for preserving vision. The corneal epithelium is subjected continuously to physical, chemical, and biological insults that can result in frank defects and loss of its barrier function (Lu *et al.*, 2001). Corneal epithelial cells respond rapidly to injury, proliferating and migrating to cover the defect and to re-establish its barrier function. This process requires the coordinated interaction of numerous growth factors and cytokines, including transforming growth factor (TGF-β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1) and epidermal growth factor (EGF), secreted by epithelial cells (Pancholi *et al.*, 1998; Andresen and Ehlers, 1998; Yu *et al.*, 2010). Furthermore, HGF and keratinocyte growth factor (KGF) are secreted by fibroblasts following epithelial injury, and contribute to re-epithelialization *via* their individual receptors expressed in epithelial cells (Wilson *et al.*, 1994; Wilson *et al.*, 1999a). While HGF receptor c-Met is highly expressed in central corneal cells, the KGF receptor is more abundant in limbal cells (Li and Tseng, 1995; Li *et al.*, 1996).

Hepatocyte growth factor and c-Met are expressed in the corneal epithelium, stromal cells, endothelium, as well as in the lacrimal grand, although the amount of HGF in the epithelium appears to be extremely low (Wilson *et al.*, 1993; Wilson *et al.*, 1994; Li *et al.*, 1996; Wilson *et al.*, 1999b). Human tears also contain about 500 pg/ml of HGF, which is derived from corneal stromal cells and the lacrimal grand (Li *et al.*, 1996; Tervo *et al.*, 1997). The sources of HGF in the cornea and its surroundings are depicted in Fig. 2.

In corneal epithelial healing, HGF acts as a paracrine growth factor mediating mesenchymal-epithelial interactions. The binding of HGF to c-Met activates mitogen activated protein kinase (MAPK) pathways in human corneal epithelial cells *via* the receptor-Grb2/Sos complex to the Ras pathway or through protein kinase C (PKC) (Liang *et al.*, 1998). Phosphatidylinositol-3 kinase (PI3K) and p70 S6 kinase (S6K), which are regulated by PKC or Akt (also known as protein kinase B), are also critical for epithelial cell survival (Chandrasekher *et al.*, 2001; Kakazu *et al.*, 2004). The scheme of HGF signaling in corneal epithelial cells during wound healing is depicted in Fig. 3. Additionally, HGF is also known to induce cell motility through transactivation of the EGF receptor (Spix *et al.*, 2007).

Hepatocyte growth factor also facilitates corneal epithelial cell migration (Daniels *et al.*, 2003; McBain *et al.*, 2003), proliferation (Wilson *et al.*, 1993; Yanai *et al.*, 2006), and inhibits apoptosis (Kakazu *et al.*, 2004). These activities suggest that HGF is capable of enhancing epithelial wound healing (Chandrasekher *et al.*, 2001). However, in one study employing an *ex vivo* bovine corneal model, the retardation of re-epithelialization by HGF was reported (Carrington and Boulton, 2005). Thus, *in vivo* studies are required to further elucidate the effect of HGF on corneal epithelial wound healing.

4. Corneal stromal wound healing and HGF

The stroma of the cornea is a highly organized ECM comprised of a network of a heterodimeric complex of Type I and Type V collagen fibers, containing water, inorganic salts, proteoglycans, and glycoproteins (Birk *et al.*, 1986; Birk, 2001). Keratocytes are the primary cells of the corneal stroma and serve to maintain the extracellular environment by synthesizing collagen molecules and glycosaminoglycans, and remodeling the stroma with matrix metalloproteinases (MMPs) that are crucial to stromal homeostasis and ECM renewal (DelMonte and Kim, 2011).

Corneal stromal wounding typically results in direct damage to both stromal and epithelial elements. Wounding triggers a release of inflammatory cytokines from epithelial cells, mainly interleukin-1 (IL-1) which induces apoptosis of anterior keratocytes expressing the IL-1 receptor (Wilson *et al.*, 1996; Wilson *et al.*, 2001; Wilson *et al.*, 2007). Upon stromal injury, keratocytes differentiate into spindle-shaped fibroblasts which acquire a migratory phenotype through the increased expression of actin, to generate traction forces enabling them to proliferate and migrate towards the region of injury, repopulating the region that had been depleted of keratocytes through apoptosis (Moller-Pedersen *et al.*, 1998; Zieske *et al.*, 2001; Hinz *et al.*, 2001b). As described above, corneal wounding leads to epithelial cells secretion of growth factors and cytokines, including TGF-β, PDGF, FGF-2, IGF-1 and EGF, which have all been implicated in this differentiation (Funderburgh *et al.*, 2001; Maltseva *et al.*, 2001; Jester and Ho-Chang, 2003; Musselmann *et al.*, 2005; He and Bazan, 2008). In the process of transdifferentiation to activated fibroblasts, there is downregulation of keratocyte proteins, such as corneal crystallins and keratan sulfate proteoglycans, and the simultaneous initiation of increased proteinase activity (mostly MMPs) necessary to remodel the wounded ECM (Fini, 1999; Jester *et al.*, 1999; Carlson *et al.*, 2003; West-Mays and Dwivedi, 2006).

Upon arrival at the corneal wound bed, fibroblasts differentiate into myofibroblasts that elaborate ECM and generate contractile forces engaged in corneal wound closure (Ishizaki *et al.*, 1993; Petroll *et al.*, 1993; Jester *et al.*,1995; Kurosaka *et al.*, 1998). Myofibroblasts are characterized by the expression of α-smooth muscle actin (α-SMA) whose expression directly correlates with corneal wound contraction (Jester *et al.*, 1995). Keratocyte-fibroblast-myofibroblast (KFM) transformation is triggered by TGF-β1 and PDGF (Jester *et al.*, 1999; Carrington *et al.*, 2006; Kaur *et al.*, 2009; Singh *et al.*, 2014).

Upon proper healing, the corneal stroma is remodeled so that its arrays of collagen lamellae are orderly to ensure transparency. However, multiple reports document long-term corneal opacity from excessive numbers and/or prolonged persistence of myofibroblasts after healing (Wilson *et al.*, 2001; Ljubimov and Saghizadeh, 2015). Myofibroblasts are themselves opaque and produce a disorganized ECM, leading to the development of corneal stromal opacity and fibrosis. If the epithelial basement membrane was ablated upon initial wounding, fibrosis is often more severe than if the basement membrane was left intact (Fini and Stramer, 2005; West-Mays and Dwivedi, 2006; Torricelli *et al.*, 2016). Hepatocyte growth factor is a well-known antifibrotic molecule that counteracts TGF-β1 to reduce fibrosis in various organs (Dai and Liu, 2004; Mizuno and Nakamura, 2004; Okayama *et al.*, 2012). Specifically, HGF activates Smad7 which prevents Smad2 phosphorylation resulting

in inhibition of the TGF-β signaling pathway (Shukla *et al.*, 2009; Yong *et al.*, 2016). Additionally, HGF promotes apoptosis of myofibroblasts by inducing MMPs to degrade the ECM in general including fibronectin, specifically, which is an essential anchor for myofibroblasts (Pepper *et al.*, 1992; Mizuno *et al.*, 2005). A recent study documented that the administration of HGF can restore corneal transparency after wounding in a murine model (Mittal *et al.*, 2016). Therefore, exogenous HGF represents a potential therapeutic tool in promoting improved corneal stromal wound healing and patient outcomes (Fig. 4).

In recent years, our lab has focused its investigation on the impact of the biophysical attributes of the microenvironment of corneal cells on wound healing, and shown that biophysical cues represent potent modulators of KFM transformation (Myrna *et al.*, 2009; Pot *et al.*, 2010; Myrna *et al.*, 2012; Dreier *et al.*, 2013). Thus, HGF may be capable of affecting corneal stiffness by inhibiting the myofibroblast phenotype or degrading ECM through the induction of MMPs and u-PA (Ueki *et al.*, 1999; Ono *et al.*, 2004; Mizuno *et al.*, 2005; Kim *et al.*, 2005). The effect of HGF on the biophysical attributes of the cornea and KFM transformation represents a promising avenue to explore with this molecule to increase our understanding of compounds that may reverse unwanted fibrotic scars.

In addition to its classical paracrine mechanism, HGF may act on corneal fibroblasts in an autocrine manner. The c-Met receptor is expressed in not only corneal epithelium but also in corneal stromal and endothelial cells (Wilson *et al.*, 1993) (Fig. 2). While HGF does not induce proliferation of corneal fibroblasts, an autocrine HGF/c-Met loop is known to operate in other cell types (Sheehan *et al.*, 2000; Warn *et al.*, 2001; Xie *et al.*, 2001; Kawase *et al.*, 2006). Thus, it remains poorly understood how endogenous HGF may exert its effects in corneal stromal wound repair.

5. Corneal endothelial wound healing and HGF

The intact human corneal endothelium is a layer of simple cuboidal cells, which appears as a honeycomb-like mosaic when viewed from the posterior aspect. Corneal endothelial cells are essential to maintain corneal transparency through preservation of stromal deturgescence. Damage to the corneal endothelium can be inflicted both directly by trauma, corneal endotheliitis and surgical removal of dysfunctional endothelium or indirectly by cataract surgery. Corneal endothelial cells exhibit certain peculiarities in their healing processes. Specifically, *in vivo*, with few exceptions, corneal endothelial cells have a very low regenerative capacity, and typically fill any areas devoid of cells by migration and increased cell spreading (Joyce *et al.*, 1990; Ichijima *et al.*, 1993; Mimura *et al.*, 2013). Recently, corneal endothelial cells have been found to be capable of proliferative capacity under certain conditions *in vitro* (Nayak and Binder, 1984; Blake *et al.*, 1997; Senoo *et al.*, 2000; Li *et al.*, 2007b; Okumura *et al.*, 2009), and inhibition of Rho kinase has been reported to be able to stimulate the proliferation of corneal endothelial cells *in vivo* (Koizumi *et al.*, 2014; Okumura *et al.*, 2016). Similar to other corneal cells, corneal endothelial cells express mRNAs for HGF and c-Met, and the addition of HGF to culture medium stimulates endothelial cell proliferation (Wilson *et al.*, 1993). One recent study supports the possibility that HGF acts on c-Met of corneal endothelial cells and promote their growth in an autocrine manner (Kimoto *et al.*, 2012), as described above. Also, HGF is found in the aqueous humor,

and its concentration is correlated with corneal endothelial cell density (Araki-Sasaki *et al.*, 1997; Grierson *et al.*, 2000), suggesting that corneal endothelial cells may contribute to aqueous HGF. Therefore, HGF is thought to be capable of maintaining corneal endothelial cells not only *in vitro* but also *in vivo*.

In cases of severe corneal endothelial injury such as alkaline burns and syphilitic interstitial keratitis, corneal endothelial cells can undergo epithelial-mesenchymal transformation (EMT) (Ishizaki *et al.*, 1993; Saika *et al.*, 1993; Kawaguchi *et al.*, 2001). Additionally, cultured corneal endothelial cells can result in a phenotypic switch that changes their morphology from polygonal to spindle-shaped *in vitro* (Peh *et al.*, 2013; Okumura *et al.*, 2013; Roy *et al.*, 2015). In a model of freeze injury, EMT occurs along the migrating front, whereby cells lose the tight junction protein ZO-1 and begin expressing α-SMA (Petroll *et al.*, 1997). These findings suggest that corneal endothelial cells, like KFM transformation in corneal stroma or EMT in epithelial cells, require a transient acquisition of a fibroblast-like morphology and actin stress fibers for migration to close the wound gap (Hinz *et al.*, 2001a). A potent inducer of EMT is TGF-β which leads to abnormal ECM accumulation and production of a fibrous retrocorneal membrane on the posterior surface of the Descemet's membrane (Sumioka *et al.*, 2008; Miyamoto *et al.*, 2010). The overexpression of Smad7, an inhibitor of TGF-β signaling, can suppress corneal endothelial fibrosis without compromising endothelial wound healing (Sumioka *et al.*, 2008). Therefore, exogenous HGF holds promise as a therapeutic agent to prevent fibrogenic EMT and the formation of retrocorneal membranes *via* Smad7 activation (Shukla *et al.*, 2009; Yong *et al.*, 2016) (Fig. 4).

6. Conclusion

In this review, we have highlighted the roles of HGF in the normal cornea as well as during corneal wound healing. Hepatocyte growth factor is mainly secreted by fibroblasts, and accelerates proliferative activities of epithelial and endothelial cells. Besides operating as a key molecule in corneal wound healing state, the ability of HGF to modulate the transdifferentiation of cells implicated in the development of fibrosis motivates its investigation as a potential therapeutic tool to minimize corneal fibrosis and improve wound healing outcomes. While corneal cells in each layer respectively have nuances to their engagement in wound healing processes, corneal stromal and endothelial cells share the involvement of the myofibroblast phenotype to close a wound gap. Since TGF-β is one of the strongest profibrotic factors inducing differentiation to myofibroblasts, the inhibition of TGF-β activation by HGF presents a promising tool to ameliorate fibrosis.

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Abbreviations

ECM	extracellular matrix
HGF	hepatocyte growth factor
HGFA	hepatocyte growth factor activator
u-PA	urokinase-type plasminogen activator
t-PA	tissue-type plasminogen activator
TGF-β	transforming growth factor
PDGF	platelet derived growth factor
FGF	fibroblast growth factor
IGF-1	insulin-like growth factor-1
EGF	epidermal growth factor
KGF	keratinocyte growth factor
MAPK	Ras-mitogen activated protein kinase
PKC	protein kinase C
PI3K	phosphatidylinositol-3 kinase
S6K	p70 S6 kinase
MMP	matrix metalloproteinase
IL-1	interleukin-1
α-SMA	α-smooth muscle actin
KFM	keratocyte-fibroblast-myofibroblast
EMT	epithelial-mesenchymal transformation

Highlights

- HGF directs mesenchymal-epithelial interaction in corneal wound healing.
- The persistence of the myofibroblast phenotype results in corneal fibrosis.
- HGF can improve corneal fibrosis by inhibiting the myofibroblast phenotype.
- HGF is also involved in maintaining corneal endothelial cells *in vivo*.
- Corneal cells need the myofibroblast phenotype to close a wound gap.

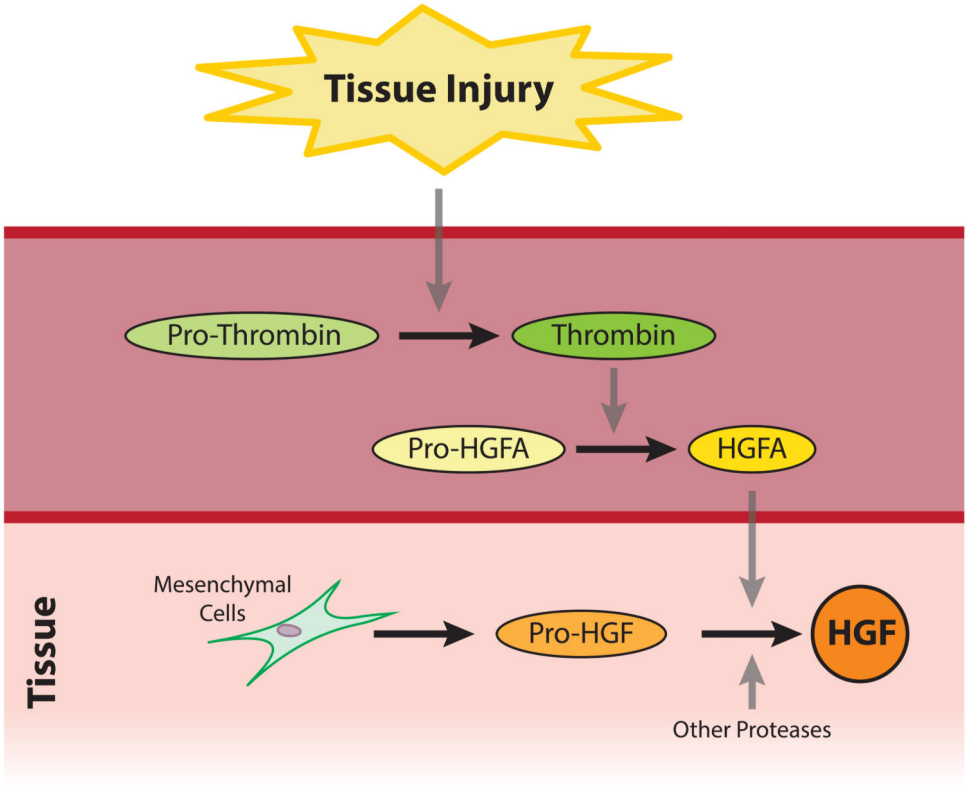

Fig. 1. The Activating Process of HGF upon the Tissue Injury

Tissue injury activates the blood coagulation system leading to conversion of pro-thrombin to thrombin to form blood clots and prevent further hemorrhage. Concomitantly, thrombin activates HGFA by processing an enzymatically inactive pro-HGFA produced by hepatocytes in the liver into an active HGFA that possesses HGF-processing enzymatic activity. Therefore, HGFA represents the link between tissue injury and activation of HGF. This diagram was modified after Conway *et al.*, 2006.

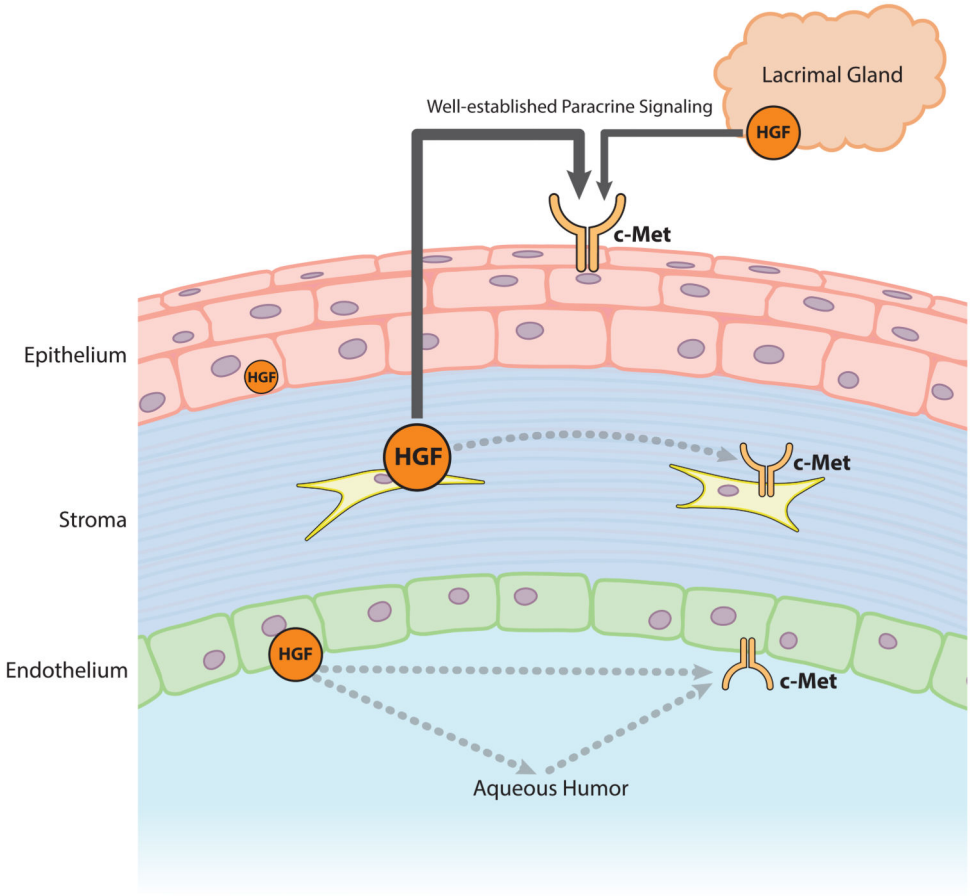

Fig. 2. The Sources of HGF in the Cornea

Hepatocyte growth factor and c-Met are expressed in the corneal epithelium, stromal cells, endothelium, as well as in the lacrimal grand. The size of the HGF and c-Met icons and width of arrows represent relative contributions. In addition to its classical paracrine mechanism, this expression pattern shows that HGF has the potential to act in an autocrine manner.

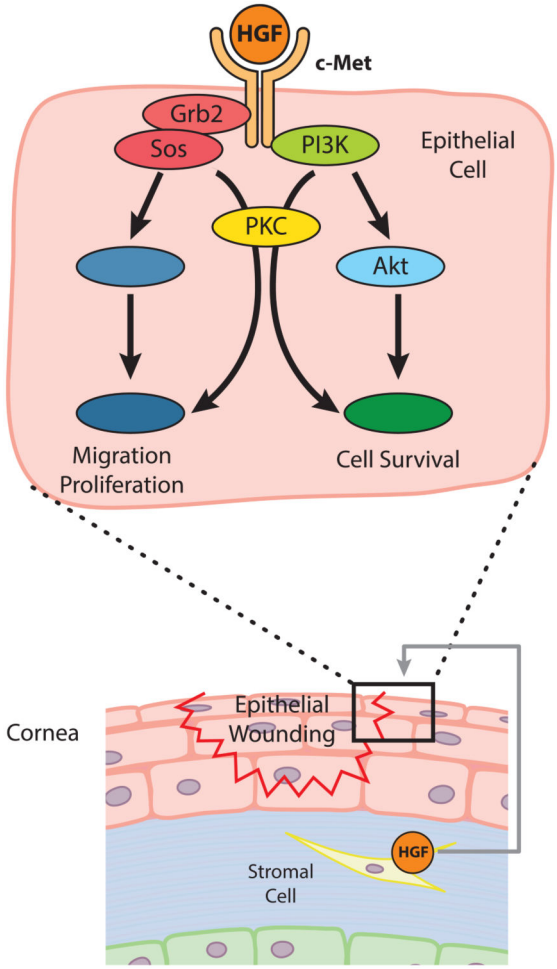

Fig. 3. The Role of HGF in Epithelial-Mesenchymal Crosstalk in Corneal Epithelial Wound Healing

Upon epithelial wounding, HGF mRNA is highly induced in stromal fibroblasts, while the expression of c-Met is upregulated in epithelial cells. The binding of HGF to c-Met activates the MAPK pathway *via* Grb2/Sos complex to the Ras or through PKC to promote epithelial wound healing. PI3K-S6K pathway mediated by PKC or Akt is another route influenced by HGF that promotes epithelial cell survival.

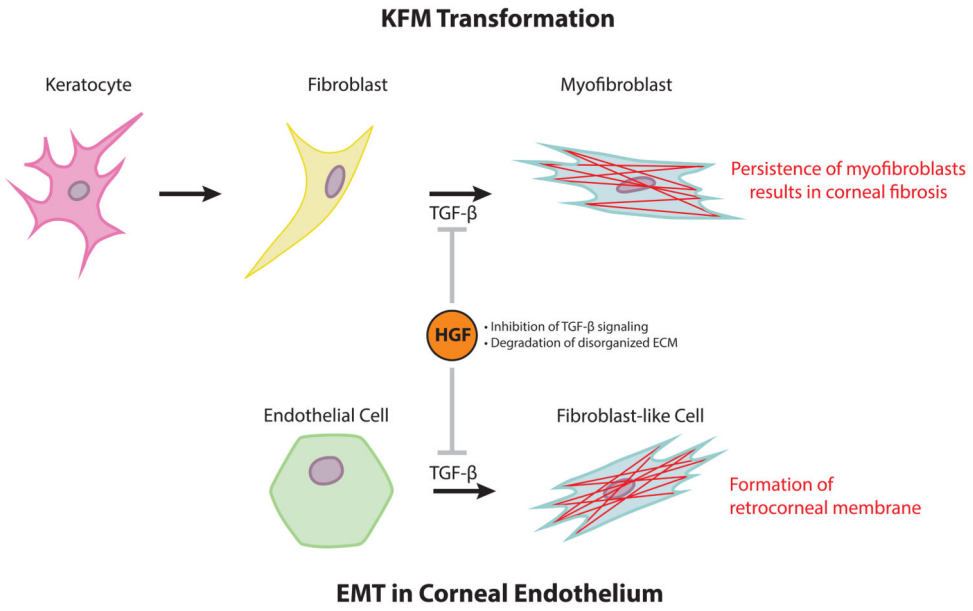

Fig. 4. HGF represents a potential therapeutic tool to minimize corneal fibrosis

In the cornea, TGF-β induces a myofibroblast-phenotype following KFM transformation in the stroma and EMT in the endothelium, which can result in corneal fibrosis and retrocorneal membrane formation, respectively. Hepatocyte growth factor could play an antifibrotic role to counteract TGF-β promotion of myofibroblast generation by activating Smad7, an inhibitory Smad. Additionally, HGF promotes apoptosis of myofibroblasts by inducing MMPs which degrade the ECM including fibronectin, which is an essential anchor for myofibroblasts.