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Authors

Miyagi, Hidetaka
Jalilian, Iman
Murphy, Christopher J
et al.

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Modulation of human corneal stromal cell differentiation by hepatocyte growth factor and substratum compliance

Hidetaka Miyagi[a,b], **Iman Jalilian**[a], **Christopher J. Murphy**[a,c,*], and **Sara M. Thomasy**[a,c,*]

[a]Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, California, USA

[b]Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

[c]Department of Ophthalmology & Vision Science, School of Medicine, UC Davis Medical Center, Sacramento, CA, USA

Abstract

Corneal wound healing is a complex process that consists of cellular integration of multiple soluble biochemical cues and cellular responses to biophysical attributes associated with the matrix of the wound space. Upon corneal stromal wounding, the transformation of corneal fibroblasts to myofibroblasts is promoted by transforming growth factor-β (TGFβ). This process is critical for wound healing; however, excessive persistence of myofibroblasts in the wound space has been associated with corneal fibrosis resulting in severe vision loss. The objective of this study was to determine the effect of hepatocyte growth factor (HGF), which can modulate TGFβ signaling, on corneal myofibroblast transformation by analyzing the expression of α-smooth muscle actin (αSMA) as a marker of myofibroblast phenotype particularly as it relates to biomechanical cues. Human corneal fibroblasts were cultured on tissue culture plastic (> 1 GPa) or hydrogel substrates mimicking human normal or wounded corneal stiffness (25 and 75 kPa) in media containing TGFβ1 ± HGF. The expression of αSMA was analyzed by quantitative PCR, Western blot and immunocytochemistry. Cellular stiffness, which is correlated with cellular phenotype, was measured by atomic force microscopy (AFM). In primary human corneal fibroblasts, the mRNA expression of αSMA showed a clear dose response to TGFβ1. The expression was significantly suppressed when cells were incubated with 20 ng/ml HGF in the presence of 2 ng/ml of TGFβ1. The protein expression of αSMA induced by 5 ng/ml TGFβ1 was also decreased by 20 ng/ml of HGF. Cells cultured on hydrogels mimicking human normal (25 kPa) and fibrotic (75 kPa) cornea also showed an inhibitory effect of HGF on αSMA expression in the presence or absence of TGFβ1. Cellular stiffness was decreased by HGF in the presence of TGFβ1 as measured by AFM. In this study, we have demonstrated that HGF can suppress the

[*]**Correspondence:** Sara M. Thomasy, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA Tel: +1530 752 0926; fax +1 530 752 3708; smthomasy@ucdavis.edu.
Christopher J Murphy, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616, USA Tel: +1 530 752 0926; fax +1530 752 3708; cjmurphy@ucdavis.edu.

myofibroblast phenotype promoted by TGFβ1 in human corneal stromal cells. These data suggest that HGF holds the potential as a therapeutic agent to improve wound healing outcomes by minimizing corneal fibrosis.

Keywords

Hepatocyte growth factor; Corneal wound healing; Myofibroblast transformation; α-smooth muscle actin; Transforming growth factor-β; Substratum compliance

1. Introduction

Upon corneal stromal injury, quiescent keratocytes differentiate into spindle-shaped fibroblasts which acquire a migratory phenotype through the increased expression of actin. This expression generates traction forces enabling fibroblasts to proliferate and migrate towards the wound, repopulating the region that had been depleted of keratocytes through apoptosis (Moller-Pedersen *et al.*, 1998; Zieske *et al.*, 2001; Hinz *et al.*, 2001). Upon arrival to the corneal wound bed, fibroblasts differentiate into myofibroblasts that elaborate extracellular matrix (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 (αSMA) whose expression directly correlates with corneal wound contraction (Jester *et al.*, 1995). After proper corneal healing, myofibroblasts disappear from the wound space through apoptosis (Wilson *et al.*, 2007). 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 less transparent than keratocytes and produce a disorganized ECM, leading to the development of corneal stromal opacity and fibrosis. The transformation from fibroblasts to myofibroblasts is mainly triggered by transforming growth factor-β (TGFβ) (Jester *et al.*, 1999; Carrington *et al.*, 2006; Singh *et al.*, 2014) which is a critical cytoactive factor that participates in the signaling cascades that promote corneal scarring. Additionally, we have shown that biophysical cues represent potent modulators of this corneal stromal cell transformation (Myrna *et al.*, 2009; Pot *et al.*, 2010; Myrna *et al.*, 2012; Dreier *et al.*, 2013) as well as the response of these cells to therapeutic agents (Thomasy *et al.*, 2018), and cellular elastic modulus was strongly correlated to myofibroblast phenotype, corneal fibrosis and haze formation (Raghunathan *et al.*, 2017).

Hepatocyte growth factor (HGF) is primarily secreted by mesenchymal cells and stimulates morphogenesis, migration, proliferation and survival of epithelial cells that express its specific receptor (Montesano *et al.*, 1991; Sonnenberg *et al.*, 1993; Matsumoto and Nakamura, 1996; Birchmeier and Gherardi, 1998; Miyagi *et al.*, 2018). Additionally, HGF is a well-known antifibrotic molecule that counteracts TGFβ to reduce fibrosis in various organs (Dai and Liu, 2004; Mizuno and Nakamura, 2004; Okayama *et al.*, 2012). HGF inhibits TGFβ production in cultures of myofibroblasts (Mizuno and Nakamura, 2004; Nakamura *et al.*, 2005) and intercepts the TGFβ signaling pathway by inhibiting nuclear Smad2 and Smad3 activation (Dai and Liu, 2004). Moreover, HGF reduces the TGFβ-

receptor (Yang and Liu, 2002) and induces decorin, an inhibitor of TGFβ1 (Kobayashi *et al.*, 2003), all of which can lead to antifibrotic outcomes *in vivo*. Additionally, HGF induces urokinase-type plasminogen activator (Pepper *et al.*, 1992), and matrix metalloprotease in myofibroblasts (Mizuno *et al.*, 2005). These proteases are critical for degradation of ECM and fibronectin, an essential anchor maintaining cell survival. As a result, HGF indirectly promotes myofibroblast apoptosis by decreasing the number of ECM anchors. A growing body of evidence supports the concept that HGF elicits the regression of fibrosis in numerous organs which TGFβ-induced myofibroblasts are implicated as central players in promoting tissue scarring. However, the role of HGF in corneal biology and repair has been understudied (Miyagi *et al.*, 2018). A recent study documented that the administration of HGF can restore corneal transparency after wounding in a murine model (Mittal *et al.*, 2016). Therefore, we hypothesized that HGF would modulate fibroblast to myofibroblast transformation promoted by TGFβ1 as well as substrate stiffness.

2. Materials and methods

2. 1. Isolation, culture and treatment of primary human corneal fibroblasts

Primary human corneal fibroblasts (HCFs) were isolated from donated human corneoscleral rims unsuitable for transplantation (Heartland Lions Eye Bank, Kansas City, MO). All experiments complied with the Declaration of Helsinki. The cell isolation was performed as previously described (Dreier *et al.*, 2013) with slight modifications. Briefly, corneal epithelial and endothelial cells were removed by gentle debridement of the anterior and posterior surface. Then, a central corneal button was obtained using an 8 mm-diameter biopsy punch (Integra Miltex, York, PA) followed which was then hemisected with a scalpel. The remaining corneal stroma containing keratocytes was incubated in a four-well plate (Nunc, Penfield, NY) filled with Dulbecco's modified Eagle's medium (DMEM) high glucose (HyClone; GE Healthcare) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin-streptomycin-amphotericin B (P/S/F; Life Technologies). In this study, all cell culture media contained serum because the presence of serum is known to cause a transition of quiescent keratocytes to active fibroblasts (Masur *et al.*, 1996; Jester and Ho-Chang, 2003). Stromal cells were isolated and were expanded up to five passages.. Primary HCFs from up to six different donors were isolated and cultured in DMEM high glucose with 10% FBS and 1% P/S/F at 37℃ under 5% CO_2 and were utilized between passages two and five. Primary HCFs were plated on six-well plates at a density of 7.5×10^4 per well for Quantitative real-time PCR (qPCR), on four-well plates at a density of 2.0×10^5 for Western blotting and on coverslips in 24-well plate at a density of 1.0×10^4 for immunocytochemistry in culture medium and were left to adhere overnight. To confirm the biological activity of TGFβ and HGF, we treated HCFs grown on tissue culture plastic (TCP) with human recombinant TGFβ1 (T7039; Sigma-Aldrich, St. Louis, MO) and/or human recombinant HGF (R&D Systems, Minneapolis, MN) for 24 hours in serum-containing medium.

2. 2. Fabrication of compliant polyacrylamide substrates

Polyacrylamide hydrogels were prepared to approximate the stiffness of human normal (25 kPa) and fibrotic cornea (75 kPa) as previously described (Wood *et al.*, 2011; McKee *et al.*,

2011). To remove unreacted reagents, the gels were rinsed three times in PBS (HyClone; GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England) and were sterilized in a hydrated state by exposure to short-wavelength UV light (280 nm) for 30 minutes. Following another PBS rinse, the gels were stored in a 5% CO_2 incubator at 37°C for 24 hours incubation to attain full hydration. Then, the gels were cut into pieces and rinsed six times over at least 72 hours. The gels were then allowed to equilibrate in the growth media for 24 hours before utilized. Atomic force microscopy (AFM) was used to determine the elastic modulus of hydrogels (Radmacher *et al.*, 1992).

2. 3. Cell culture on compliant polyacrylamide substrates

The gels were placed in the culture medium for 24 hours to attain equilibrium. Before cell plating, gels were treated for 5 minutes with a mixture of 97% collagen I and 3% collagen III (PureCol; Advanced Biomatrix, Fremont, CA) diluted in an equal volume of 12 mM HCl (Acros Organics, Geel, Belgium) to achieve a molecular coating of collagen and were subsequently rinsed three times with PBS. The stiffness of the gels was not impacted by the collagen coating as measured by AFM (data not shown). Primary HCFs were plated on gels in four-well plates at a density of 1.5×10^5 per well for PCR and 2.0×10^5 per well for Western blotting in culture medium and were left to adhere overnight. The following day, the medium was changed with the appropriate medium containing TGFβ1 or HGF. After 24-hour incubation, cells were harvested for RNA or proteins.

2. 4. RNA extraction and quantitative real-time PCR

RNA was extracted 24 hours after treatment with TGFβ1 or HGF using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA) following the manufacturer's protocol. Quantitative real-time PCR was performed using the SensiFAST Probe Hi-ROX One-Step kit (Bioline, Taunton, MA) and TaqMan aptamers specific to human glyceraldehyde 3-phospate dehydrogenase (GAPDH, Hs99999905; Applied Biosystems, Carlsbad, CA) or αSMA (ACTA2, Hs00426835; Applied Biosystems) in total volume 10 μl per reaction as previously described (Dreier *et al.*, 2013); GAPDH expression served as a reference. Each experiment was performed with samples from at least three individuals. Gene expression data were calculated as previously reported (Thomasy *et al.*, 2013), and normalized relative to the expression of mRNA from cells grown on TCP in the absence of TGFβ1 or HGF.

2. 5. Western blot analysis

Protein was extracted 24 hours after the TGFβ1 or HGF treatment using a RIPA buffer and Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). Equivalent amounts of protein (15 μg) were loaded onto a 10% NuPAGE Bis-Tris gel (Life Technologies, Carlsbad, CA). Gel electrophoresis was performed at 100 milliampere for 35 minutes per one gel, followed by transfer to a nitrocellulose membrane (Life Technologies, Carlsbad, CA) at 1.3 ampere for 10 minutes. The membrane was blocked at 37°C for 1 hour with a blocking buffer containing 80% PBS, 10% FBS, and 10% Superblock (Thermo Scientific). The membrane was incubated with a primary antibody specific to anti-αSMA (A5228; Sigma-Aldrich) diluted 1:100 in a blocking buffer or anti-Transketolase (sc-390179; Santa Cruz Biotechnology) diluted 1:150 in a blocking buffer at 37°C for 1 hour. The blot was washed three times in TBS with 0.1% Tween-20 (TBS-T) before incubating with peroxidase-

conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD) diluted 1:20,000 in a blocking buffer at 37°C for 1 hour. After washing twice with TBS-T and once with TBS, protein bands of interest were detected using Western blotting detection kit (WesternBright Quantum, Advansta, Menlo Park, CA) and ChemiDoc-It2 Imaging system (UVP, Upland, CA). The same blots were used for detection of GAPDH (sc-32233; Santa Cruz Biotechnology) diluted 1:150 in blocking buffer as a reference protein. Densitometry analyses were done with ImageJ software (National Institutes of Health, Bethesda, MD). The band densities of αSMA were normalized by the values of GAPDH. Western blot analyses were performed on samples from at least three independent experiments.

2. 6. Immunocytochemistry and fluorescent microscopy

Cells were fixed with 4% formaldehyde (Polysciences Inc., Warrington, PA) in PBS for 20 minutes at room temperature and subsequently incubated with 0.3% H_2O_2 (Sigma-Aldrich) in ice-cold PBS for 30 minutes at 4℃. Nonspecific binding sites were blocked with blocking buffer (10% FBS/10% Superblock in PBS) for 30 minutes at 37℃. Then, cells were incubated with primary antibody (mouse anti-αSMA, A5228; Sigma-Aldrich) 1:100 in a blocking buffer for 1 hour at 37℃ and washed three times in PBS. Incubation with secondary antibody (goat anti-mouse IgG, AlexaFluor 488; Life Technologies) 1:250 in a blocking buffer for 30 minutes at 37℃ was followed by three washes in PBS. Nuclei were stained for 10 minutes at 37℃ with 4’,6-diamidino-2-phenylindole (DAPI; BioGenex, San Ramon, CA) 1:10,000 in PBS. Cells were imaged in Mowiol (Sigma-Aldrich) with ×20 objective using the Axiovert 200 M microscope (Carl Zeiss, Jena, Germany).

2. 7. Atomic force microscopy on cells in vitro

Cells (2.5×10^4) were seeded on 50 mm polystyrene cell culture dishes (Falcon, Corning Inc., NY). Before the measurements, medium was replaced with new pre-warmed medium to remove any debris and dead cells. Force versus indentation curves were obtained using the MFP-3D Bio AFM (Asylum Research, Santa Barbara, CA) mounted on a Zeiss Axio Observer inverted microscope (Carl Zeiss, Thornwood, NY). A V-shaped silicon nitride PNP-TR-50 cantilevers with conical indenter, actual spring constant (κ) of 55–246 pN nm^{-1} and length of 100 μm (Nano World, Switzerland) were used to measure the stiffness of cells. Deflection sensitivity of the probes was measured by taking the average of five force curves on a cell culture dish with the culture medium. The spring constant of each cantilever probe was determined using a thermal tuning method. To minimize thermal drift, cantilever was equilibrated in the culture medium for at least 45 minutes prior to obtaining the measurements. For determination of the elastic modulus, indentation at a scan rate of 2 μm s^{-1} was performed at a region just above the nucleus and an average of 5–10 force curves were recorded out of a maximum of 15 indentations per cell. For each treatment, up to 12 cells with similar morphologies were chosen for measurements. To minimize the effect of successive indentations on membrane deformation, a 3–5 second rest between each indentation point was applied. The elastic modulus (E) was extracted from force curves using the Sneddon fit (Sneddon, 1965) for a conical indenter, as 229 shown in Equation 1,

$$F = \frac{2}{\pi}\tan\alpha\frac{E}{\left(1-\nu^2\right)}\delta^2$$

where F is the force applied by the indenter, E is Young's modulus, ν is Poisson's ratio (assumed to be 0.5 for biological samples (Herrmann, 1965), δ is the indentation depth and α is half angle of the indenter.

2. 8. Statistical analysis

In qPCR experiments, three reactions were run for each sample, and the standard deviation (SD) was calculated. A one-way analysis of variance (ANOVA) was used to calculate significant differences among values of αSMA mRNA expression to clarify the effect of treatment, and a two-way ANOVA was used to clarify the effect of treatment and substratum stiffness. A Holm-Sidak pairwise comparison test was used for post-hoc analysis. For analysis of protein expression from the Western blotting, a one-way ANOVA was used to calculate significant differences between band densities. A one-way ANOVA was used to compare the effect of treatment on cellular stiffness as measured by AFM. Significance was defined as $P < 0.05$ for all analyses. Statistically significant differences are indicated in figures as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ unless stated otherwise.

3. Results

We initially tested a range of TGFβ1 concentrations to determine the optimal dose to induce transformation of HCFs to myofibroblasts under our conditions. The addition of TGFβ1 to the culture medium for 24 hours stimulated the expression of the myofibroblast marker αSMA in HCFs as previously reported (Jester and Ho-Chang, 2003). The mRNA expression of αSMA showed a clear dose response to TGFβ1 at concentrations from 0 to 5 ng/ml, while concentrations 5 to 10 ng/ml did not cause a further increase (Figure 1). A degree of variability in the response of primary cells from different human donors is common (Oh *et al.*, 2006; Russell *et al.*, 2008). To prevent data trends from being masked by averaging, we presented the result from three different donors separately in Supplementary Tables, while we showed representative results in Figures. The pattern of expression observed in all experiments was similar between donors. The dose response curves of αSMA expression in cells from other donors showed similar trends to Figure 1 (Supplementary Table 1). By contrast, the addition of HGF to the culture medium for 24 hours did not significantly alter the expression of αSMA (Figure 2 and Supplementary Table 2), although high HGF concentrations sporadically inhibited αSMA expression in some donors.

To investigate the effect of HGF on the induction of αSMA mRNA by TGFβ1, we treated HCFs with TGFβ1 (2 ng/ml) and a range of HGF concentrations for 24 hours. The concentration of 2 ng/ml TGFβ1 was selected to allow for detection of both down regulation and upregulation of αSMA in response to the addition of HGF. Concentrations 5 to 50 ng/ml of HGF suppressed the induction of αSMA by TGFβ1, with maximal inhibition observed at 20 ng/ml HGF (Figure 3A and Supplementary Table 3). Western blot analyses were also performed using cell lysates of HCFs incubated with medium ± 5 ng/ml TGFβ1 or 20 ng/ml

HGF for 24 hours. We detected significant reduction of αSMA protein in HCFs incubated with TGFβ1 and HGF compared to cells cultured with TGFβ1 alone (Figure 3B, C).

Next, we tested the effect of HGF on the myofibroblast phenotype. We incubated HCFs with the medium containing 5 ng/ml TGFβ1 for 24 hours to induce the differentiation into myofibroblasts, subsequently cells were incubated with fresh medium ± 5 ng/ml TGFβ1 or 20 ng/ml HGF for another 24 hours. Cells incubated with TGFβ1 for 24 hours had equivalent amounts of αSMA mRNA expression versus cells incubated with TGFβ1 for 48 hours even after 24-hour recovery (Figure 4A and Supplementary Table 4A). The protein expression was consistent with the qPCR results, which indicated that 24-hour incubation of TGFβ1 promoted transformation of HCFs to myofibroblasts (Supplementary Figure 1A). The expression of αSMA mRNA and protein was significantly decreased by HGF (Figure 4B, Supplementary Table 4B and Supplementary Figure 1B), showing that HGF has the potential to revert the myofibroblast phenotype to that of fibroblasts. Transketolase, one of the corneal crystallins, was not dramatically altered in this time period. The ability of HGF to dramatically decrease the expression of αSMA was confirmed by immunocytochemistry (Figure 4C).

In other tissues, fibrosis increases the elastic modulus (Hinz, 2009; Liu *et al.*, 2010; Hinz, 2012; van Putten *et al.*, 2016). Similarly, we have reported that corneal tissue undergoes alterations in compliance throughout the stromal wound healing process with strong correlations between increased elastic modulus, corneal haze formation, fibrosis and incidence of myofibroblasts observed (Raghunathan *et al.*, 2017). We also investigated the cellular stiffness of HCFs treated with medium ± 5 ng/ml TGFβ1 ± 20 ng/ml HGF for 24 hours. Our data showed the elastic modulus of human corneal stromal cells was increased by TGFβ1, and the increase was significantly suppressed with HGF (Figure 5 and Supplementary Table 5).

We previously reported the elastic modulus of different layers of the normal human cornea (Last *et al.*, 2012). These studies found the stiffness of normal corneal stroma to be approximately 25 kPa. We also documented that the stromal stiffness changes throughout wound healing in rabbits with wounded cornea becoming 3-fold stiffer (Raghunathan *et al.*, 2017). Using the rabbit data as a rational basis for projecting biomechanical changes in the human corneal stroma during wound healing, we estimated that the stiffness of wounded human cornea to achieve a value of approximately 75 kPa. We seeded HCFs on the substrates mimicking the stiffness values of normal and (projected) wounded human cornea (25 and 75 kPa) as well as TCP (> 1 GPa), and incubated cells with medium ± 5 ng/ml TGFβ1 or 20 ng/ml HGF for 24 hours. The expression of αSMA was dramatically increased by TGFβ1 on all substrates (***$P < 0.001$ and **$P < 0.01$, Figure 6 and Supplementary Table 6). Consistent with a previous report (Dreier *et al.*, 2013), substratum compliance altered the expression of αSMA with or without TGFβ1 ($^{###}P < 0.001$ and $^{##}P < 0.01$, Figure 6 and Supplementary Table 6). Specifically, softer substrates decreased the induction of αSMA mRNA in comparison to TCP. On softer substrates, treatment with HGF significantly suppressed the expression of αSMA induced by TGFβ1 as well as on TCP ($^{†††}P < 0.001$, $^{††}P < 0.01$ and $^{†}P < 0.05$, Figure 6 and Supplementary Table 6). HGF inhibited αSMA expression even in absence of TGFβ1 on stiffer substrates. This inhibitory

effect was accentuated in the presence of TGFβ1. The protein expression of αSMA on hydrogels was consistent with mRNA expression levels (Supplementary Figure 2).

4. Discussion

Keratocytes have been shown to differentiate to fibroblasts by serum using an *in vitro* bovine culture system (Beales *et al.*, 1999), and fibroblasts to myofibroblasts transformation (KFM transformation) triggered by TGFβ has been reported in rabbit, bovine and murine cells (Jester *et al.*, 1999; Carrington *et al.*, 2006; Singh *et al.*, 2014). Jester et al. described the TGFβ1-induced transformation of primary human corneal keratocytes and immortalized corneal fibroblasts to myofibroblasts *in vitro* (Jester *et al.*, 2003). We previously reported substratum compliance modulates the expression of αSMA in human corneal stromal cells *in vitro*, with more compliant (softer) substrates inhibiting the transition of fibroblasts to myofibroblasts (Dreier *et al.*, 2013). In the current study, we tested a range of TGFβ1 and HGF concentrations in primary HCFs from donors to determine the optimal concentrations of TGFβ1 and HGF required to modulate KFM transformation. All data showed some variability in the response because primary cells from human tissues have individual characteristics and responses (Oh *et al.*, 2006; Russell *et al.*, 2008). However, αSMA mRNA expression was directly proportional to the concentration of TGFβ1, and the response curve of αSMA expression showed little variation among three donors. In the present study, 5 ng/ml of TGFβ1 was considered to be sufficient to induce maximal expression of αSMA in HCFs grown on TCP. While HGF by itself did not show dramatic effect on αSMA expression in HCFs, mRNA and protein expression induced by TGFβ1 was significantly suppressed by exposure to HGF. This result is consistent with previous reports (Dai and Liu, 2004; Shukla *et al.*, 2009; Yong *et al.*, 2016).

Additionally, we investigated how HGF affected the myofibroblast phenotype. HGF reduced the expression of αSMA mRNA and protein in myofibroblasts promoted by 24-hour incubation of TGFβ1. We also evaluated the protein expression of transketolase, one of the corneal crystallins (Sax *et al.*, 1996; Sax *et al.*, 2000) and a marker of the keratocyte phenotype. The expression was not dramatically altered by treatment with TGFβ1 or HGF. Our results indicate that HGF has the potential to induce reversal of the myofibroblast phenotype back to fibroblast phenotype likely via a TGFβ dependent mechanism, but not to the keratocyte phenotype in the time period evaluated. We note that the presence of serum in the culture media used in the reported experiments may have prevented reversion to the keratocyte phenotype. Myofibroblasts have been reported to be reversible to fibroblast phenotype under certain conditions (Ramos *et al.*, 2010; Hecker *et al.*, 2011; Artaud-Macari *et al.*, 2013; Garrison *et al.*, 2013). Corneal myofibroblasts have also been shown to revert to the fibroblast phenotype under the influence of fibroblast growth factor with heparin in an *in vitro* rabbit culture system (Maltseva *et al.*, 2001), and corneal myofibroblasts were decreased by the bone morphogenetic proteins-7 via antagonizing TGFβ in an *in vivo* murine alkali injury model (Saika *et al.*, 2005). Recently, HGF was reported to have the potential to decrease the number of myofibroblasts in a murine corneal injury model (Mittal *et al.*, 2016). In this study, we showed the possibility of reversal of human corneal myofibroblast differentiation by HGF *in vitro*. This result suggests that HGF should be investigated as a potential therapeutic agent for the prevention/treatment of corneal fibrosis.

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). The effect of HGF on the biophysical attributes of the cornea and KFM transformation represents a promising avenue to explore to increase our understanding of compounds that may reverse unwanted fibrotic scars. TGFβ1 is known to induce myofibroblast transformation with subsequent expression of αSMA and increased intracellular actin stress fiber formation (Jester *et al.*, 2005). In this study, we demonstrated that the increase of cellular elastic modulus by exposure to TGFβ1 was significantly suppressed by HGF. In aggregate, these data suggest that cellular elastic modulus correlated with the expression of αSMA in our cultures and can be used as an additional phenotypic marker for myofibroblasts. In the present study, HCFs cultured on TCP showed greater expression of αSMA than cells cultured on softer substrates, which is consistent with previous reports (Dreier *et al.*, 2013; Thomasy *et al.*, 2018). We also identified the inhibitory effect of HGF on αSMA expression in HCFs cultured on hydrogels mimicking normal and fibrotic human corneas. This inhibitory effect was accentuated in the presence of TGFβ1 and in cells cultured on stiffer substrates. Cellular stiffness, which is correlated with the number of actin stress fibers, parallels the stiffness of underlying substrates (Pelham and Wang, 1997; McKee *et al.*, 2011; Thomasy *et al.*, 2014). Additionally, we recently documented that substratum compliance modulated elastic modulus of corneal stromal cells (Raghunathan *et al.*, 2017). Thus, it appears that HGF modulates the corneal stromal cell phenotype via inhibition of TGFβ and prevention of myofibroblast transformation. The use of biologically relevant substratum stiffness in the conduction of *in vitro* experiments gives important insights into the cellular response to drugs that may more accurately predict responses observed *in vivo* (Thomasy *et al.*, 2012; Dreier *et al.*, 2013; Thomasy *et al.*, 2013; Thomasy *et al.*, 2018).

5. Conclusions

In this study, HGF and substratum compliance modulated the differentiation of human corneal stromal cells *in vitro*. Corneal stromal cell differentiation plays critical role in normal wound healing as well as the development of fibrosis. Our data suggest the possibility that HGF may represent a therapeutic tool for interrupting dysregulated corneal repair processes to improve patient outcomes. Further investigations are needed to determine if HGF can reduce corneal stromal fibrosis *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ECM	extracellular matrix
αSMA	α-smooth muscle actin
TGFβ	transforming growth factor-β
HGF	hepatocyte growth factor
HCFs	human corneal fibroblasts
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
P/S/F	penicillin-streptomycin-amphotericin B
TCP	tissue culture plastic
AFM	atomic force microscopy
qPCR	quantitative real-time PCR
GAPDH	glyceraldehyde 3-phospate dehydrogenase
TBS-T	TBS with 0.1% Tween-20
DAPI	4',6-diamidino-2-phenylindole
SD	standard deviation
ANOVA	analysis of variance
KFM transformation	keratocyte and fibroblast to myofibroblast transformation

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Highlights

- HGF suppresses the induction of αSMA mRNA and protein by TGFβ1.
- HGF suppresses αSMA expression on substrates mimicking normal and fibrotic cornea.
- HGF can promote reversion of the myofibroblast phenotype to that of fibroblasts.

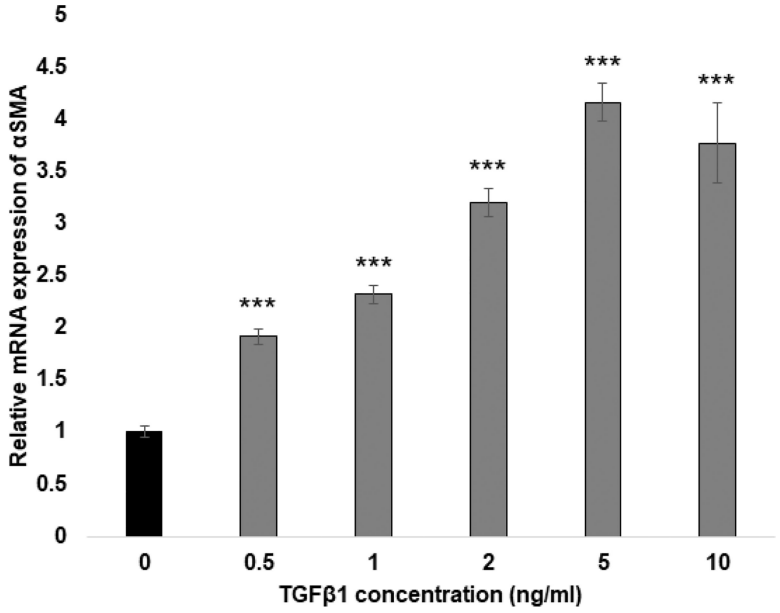

Figure 1. Transforming growth factor-β1 increases the mRNA expression of αSMA in HCFs.

Shown is a representative graph from one of at least three experiments from three donors demonstrating the relative mRNA expression of αSMA in HCFs cultured on TCP for 24 hours with various concentrations of TGFβ1. A degree of variability in the response of primary cells from different human donors is common (Oh *et al.*, 2006; Russell *et al.*, 2008). Results from three different donors are presented in Supplementary Tables. The pattern observed in all cases was similar. Maximal expression of αSMA was achieved at 5 ng/ml of TGFβ1. Data are mean ± SD for donor 14.

* Statistically significant differences compared with the expression in cells cultured without TGFβ1 or HGF (***$P < 0.001$, one-way ANOVA, followed by Holm-Sidak pairwise comparison test)

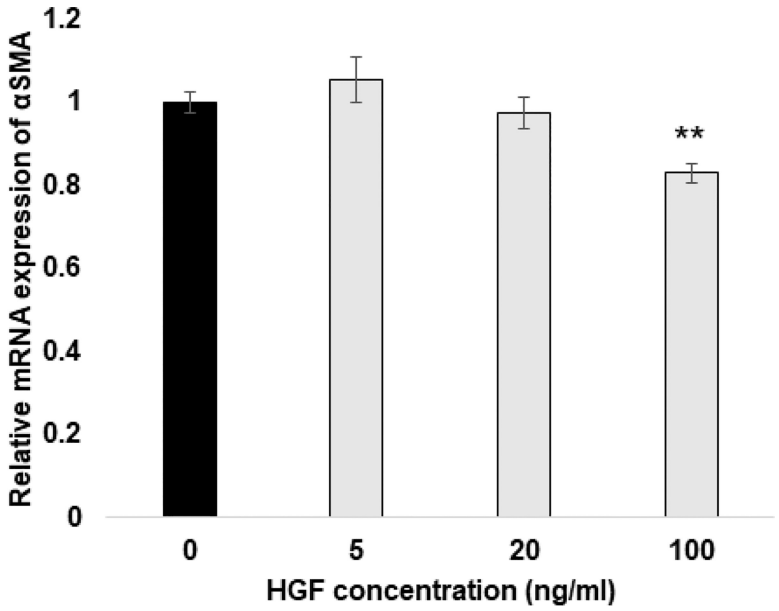

Figure 2. Hepatocyte growth factor had little effect on the mRNA expression of αSMA in HCFs.

Shown is a representative graph from one of at least three experiments from three donors demonstrating the relative mRNA expression of αSMA in HCFs cultured on TCP for 24 hours with various concentrations of HGF. In one donor, inhibition of αSMA was observed at 20 and 100 ng/ml, while no inhibitory effect was observed in another donor (See Supplementary Table 2). Data are mean ± SD for donor 14. $^{**}P < 0.01$ compared with the expression in cells cultured without HGF (one-way ANOVA, followed by Holm-Sidak pairwise comparison test)

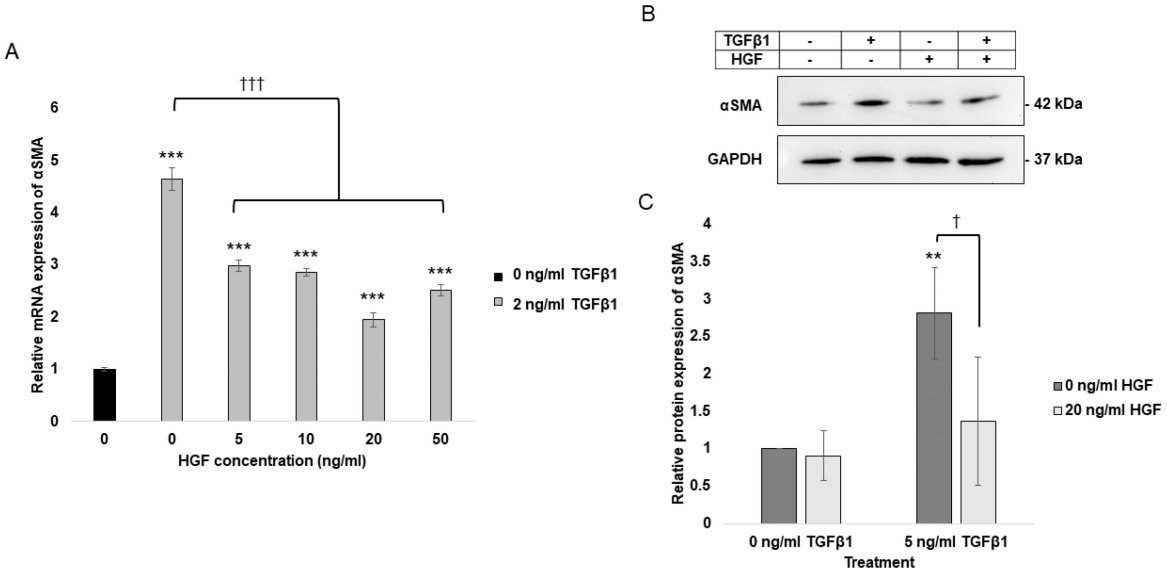

Figure 3. Hepatocyte growth factor suppresses the induction of αSMA mRNA and protein by TGFβ1 in HCFs.

(**A**) Shown is a representative graph from one of at least three experiments from three donors demonstrating the relative mRNA expression of αSMA in HCFs cultured on TCP for 24 hours with medium ± 2 ng/ml TGFβ1 and various concentrations of HGF. HGF significantly suppressed the induction of αSMA mRNA by TGFβ1 at all concentrations tested, with maximal inhibition observed at 20 ng/ml HGF. Data are mean ± SD for donor 38. (**B**) A representative Western blot from one experiment demonstrates the reduced expression of αSMA protein in HCFs cultured on TCP for 24 hours with medium ± 5 ng/ml TGFβ1 ± 20 ng/ml HGF. HGF significantly suppressed the induction of αSMA protein by TGFβ1. (**C**) A densitometry analysis was performed to account for differences in the loaded protein amounts. The graph shows mean ± SD obtained from four independent experiments for donor 20. ***$P < 0.001$ and **$P < 0.01$ compared with the expression in cells cultured without TGFβ1 or HGF; $^{\dagger\dagger\dagger}P < 0.001$ and $^{\dagger}P < 0.05$ for cells cultured without versus with HGF (one-way ANOVA, followed by Holm-Sidak pairwise comparison test)

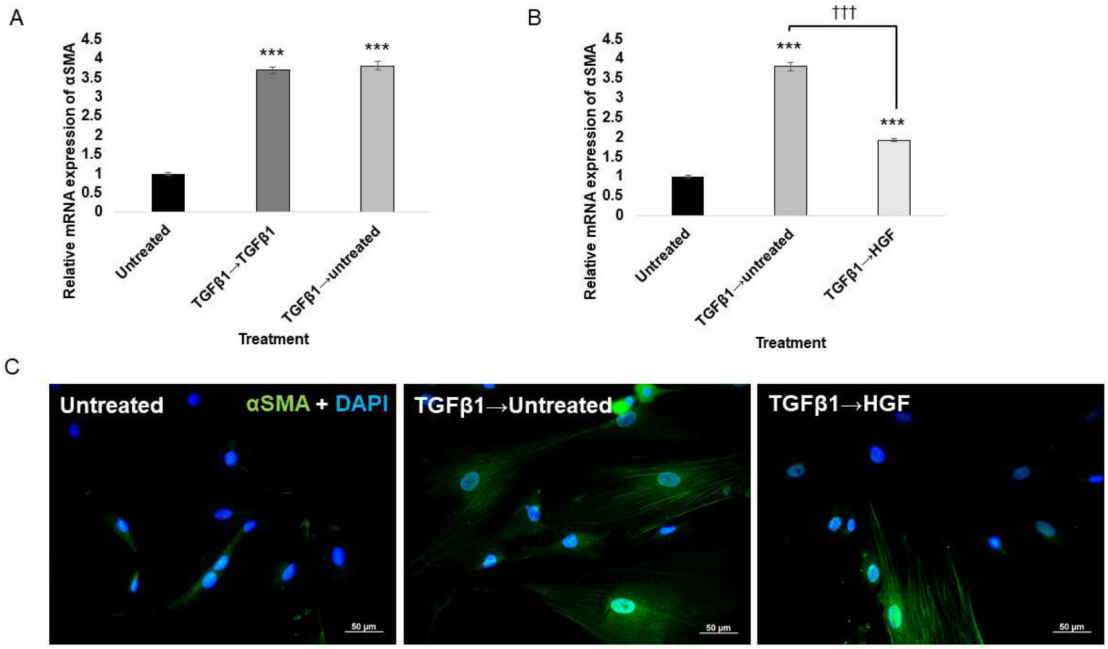

Figure 4. Hepatocyte growth factor decreases the expression of αSMA after the induction by TGFβ1 in HCFs.

Cells were cultured on TCP for 24 hours with medium ± 5 ng/ml TGFβ1 to promote fibroblast to myofibroblast transformation, and subsequently cells were incubated with medium ± 20 ng/ml HGF for another 24 hours. (**A, B**) Shown are representative graphs from at least three experiments from three individuals demonstrating the relative mRNA expression of αSMA. (**A**) The mRNA expression of αSMA induced by 24-hour incubation of TGFβ1 was maintained after 24-hour recovery. (**B**) Addition of HGF decreased αSMA mRNA expression even after 24-hour incubation with TGFβ1. Data are mean ± SD for donor 50. ***$P < 0.001$ compared with the expression in cells cultured without TGFβ1 or HGF; †††$P < 0.001$ for cells cultured with TGFβ1 for 24 hours followed by 24-hour incubation without versus with HGF (one-way ANOVA, followed by Holm-Sidak pairwise comparison test) (**C**) Shown are representative images of immunocytochemistry for donor 82. These images show the fluorescent staining of αSMA (*green*) and DAPI (*blue*). The data show the possibility that the presence of HGF promotes reversion of the myofibroblast phenotype back towards that of the fibroblast.

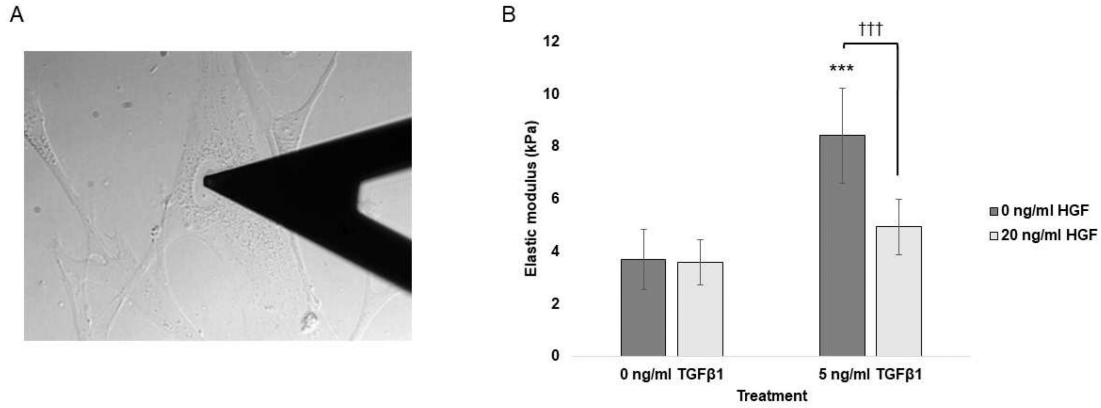

Figure 5. Corneal stromal cells treated with TGFβ1 are stiffer than cells co-treated with TGFβ1 and HGF.

Cellular elastic modulus (stiffness) was measured by AFM. (**A**) The image shows the cantilever probe of the AFM touching a region just above the nucleus of a cell treated with 5 ng/ml TGFβ1 for 24 hours. (**B**) Shown is a representative graph from one of at least three experiments from three donors demonstrating the elastic modulus of HCFs cultured on TCP for 24 hours with medium ± 5 ng/ml TGFβ1 ± 20 ng/ml HGF. HGF suppressed the increase of cellular elastic modulus induced by TGFβ1. Data are mean ± SD for donor 20. $^{***}P < 0.001$ compared with cells cultured without TGFβ1 or HGF; $^{\dagger\dagger\dagger}P < 0.01$ for cells cultured without versus with HGF (one-way ANOVA, followed by Holm-Sidak pairwise comparison test)

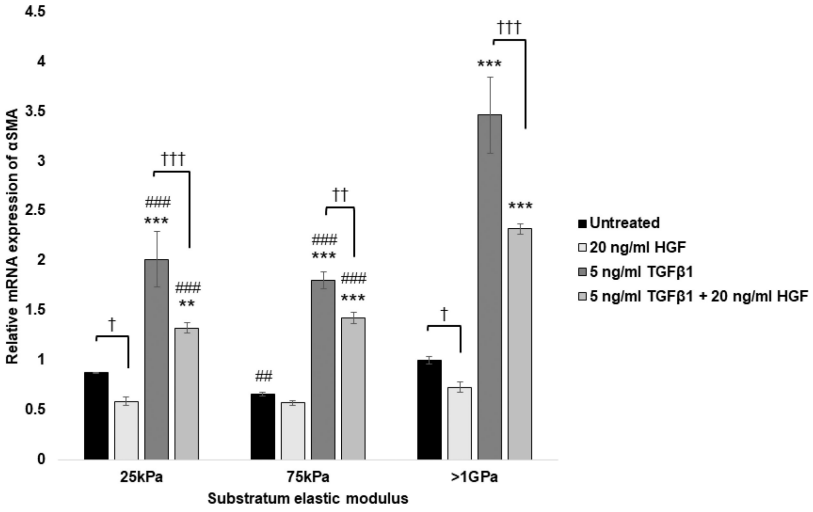

Figure 6. Hepatocyte growth factor suppresses αSMA mRNA induction by TGFβ1 on tissue culture plastic (TCP; > 1 GPa) as well as softer substrates mimicking the stiffness of normal and fibrotic corneas.

Shown is a representative graph from one of at least three experiments from three donors demonstrating the relative mRNA expression of αSMA in HCFs cultured on hydrogels mimicking substratum compliance in human corneas and TCP for 24 hours with medium ± 5 ng/ml TGFβ1 ± 20 ng/ml HGF. Except for cells incubated with HGF alone, HCFs cultured on TCP showed greater expression of αSMA than cells cultured on softer substrates. HGF showed the inhibitory effect on the αSMA expression in the presence or absence of TGFβ1 on a range of substratum compliances. Results from other donors showed a similar trend with slight differences among them. (See supplementary Table 5). The expression of αSMA in HCFs cultured on TCP without TGFβ1 or HGF (untreated) was arbitrarily set as 1.0. Data are mean ± SD for donor 82. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared with the expression in cells cultured without TGFβ1 or HGF on each substrate; $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger}P < 0.05$ for cells cultured without versus with HGF; $^{\#\#\#}P < 0.001$ and $^{\#\#}P < 0.01$ for hydrogels versus TCP (two-way ANOVA, followed by Holm-Sidak pairwise comparison test)