

# Keratoconus in vitro and the key players of the TGF- $\beta$ pathway

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**Purpose:** Keratoconus (KC) is a corneal thinning disease of unknown etiology whose pathophysiology is correlated with the presence of a thin corneal stroma and altered extracellular matrix (ECM). Transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling is a key regulator of ECM secretion and assembly in multiple tissues, including the anterior segment of the eye, and it has been linked to KC. We have previously shown that human keratoconus cells (HKCs) have a myofibroblast phenotype and altered ECM assembly compared to normal human corneal fibroblasts (HCFs). Moreover, TGF- $\beta$ 3 treatment promotes assembly of a more normal stromal ECM and modulates the fibrotic phenotype in HKCs. Herein, we identify alterations in TGF- $\beta$  signaling that contribute to the observed fibrotic phenotype in HKCs.

**Methods:** HCFs and HKCs were stimulated with TGF- $\beta$ 1, TGF- $\beta$ 2, or TGF- $\beta$ 3 isoforms (0.1 ng/mL) in the presence of a stable vitamin C derivative (0.5 mM) for 4 weeks. All samples were examined using RT-PCR and western blotting to quantify changes in the expressions of key TGF- $\beta$  signaling molecules between HCFs and HKCs.

**Results:** We found a significant downregulation in the SMAD6 and SMAD7 expressions by HKCs when compared to HCFs ( $p \leq 0.05$ ). Moreover, stimulation of HKCs with any of the three TGF- $\beta$  isoforms did not significantly alter the expressions of SMAD6 or SMAD7. HCFs also showed an upregulation in TGF- $\beta$ RI, TGF- $\beta$ RII, and TGF- $\beta$ RIII following TGF- $\beta$ 3 treatment, whereas HKCs showed a significant two-fold downregulation.

**Conclusions:** Overall, our data shows the decreased expressions of the regulatory SMADs SMAD6 and SMAD7 by HKCs contribute to the pathological ECM structure observed in KC, and TGF- $\beta$ 3 may attenuate this mechanism by downregulating the expression of the key profibrotic receptor, TGF- $\beta$ RII. Our study suggests a significant role of altered regulation of TGF- $\beta$  signaling in KC progression and that it may enable novel therapeutic developments targeting TGF- $\beta$  receptor regulation.

Keratoconus (KC) is a corneal thinning disease characterized by the formation of a cone-like cornea that affects 1:2,000 people around the world [1-5]. KC is characterized by the loss of corneal integrity and decreased visual acuity due to altered extracellular matrix (ECM) assembly and cornea structure, which can lead to increased scarring and fibrosis [6,7]. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a known regulator of ECM formation and has been linked to the development of various diseases, including cancer [8,9], rheumatoid arthritis [10], and corneal dystrophies [11]. Previous reports have suggested a role of aberrant TGF- $\beta$  signaling in KC disease in conventional 2D in vitro models [12-14]. The primary goal of our study is to dissect the role of key TGF- $\beta$  molecules in the fibrotic phenotype characteristic of KC using our 3D in vitro model.

TGF- $\beta$  signaling has been well studied and is known to occur with initial binding of the TGF- $\beta$  ligand to TGF- $\beta$ RI, which then dimerizes with TGF- $\beta$ RII and stimulates phosphorylation of SMAD2/3 followed by translocation to the

nucleus and activation of the transcription of TGF- $\beta$ -target genes [15-17]. TGF- $\beta$  signaling is tightly regulated by SMAD6 and SMAD7, which serve to negatively regulate TGF- $\beta$  signaling by 1) competing for the binding of receptor-regulated SMAD3 to co-mediators [18], 2) promoting recruitment of ubiquitin E3 ligases that result in degradation of the TGF- $\beta$  receptor [17], and 3) functioning as nuclear transcription repressors by binding histone deacetylases and inhibiting the transcription of TGF- $\beta$ -responsive genes [19].

The three TGF- $\beta$  isoforms, TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3, whose secretion and binding initiate signaling, are known to be important modulators of ECM remodeling [16], the matrix metalloproteinase expression [20,21], and terminal differentiation to the myofibroblast [22,23]. Of the three TGF- $\beta$  isoforms, TGF- $\beta$ 1 and TGF- $\beta$ 2 have been identified as stimulating a profibrotic response following injury [22,24], whereas TGF- $\beta$ 3 is known for its antifibrotic effects [25,26]. We have previously reported that TGF- $\beta$ 3 stimulates human keratoconus cells (HKCs) to secrete and assemble a normal stroma-like ECM, mimicking what is seen in vivo with high expressions of Collagen I and V, and low expressions of Collagen III and  $\alpha$ -smooth muscle actin [25]. Our current study was designed to identify key players of the TGF- $\beta$  signaling pathway involved in KC-derived cells.

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In this study, we identified significant variations in the TGF- $\beta$  pathway in HKCs, suggesting alterations in TGF- $\beta$  signaling contribute to developments in the fibrotic phenotype observed in KC. We show that HKCs have down-regulated the SMAD6 and SMAD7 expressions compared to HCFs. Furthermore, we show that TGF- $\beta$ 3 significantly downregulates the expression of the key receptor TGF- $\beta$ RII, which may lead to the attenuation of the profibrotic signaling characteristic of HKCs. Our primary goal is to understand the underlying molecular mechanism of KC progression to develop therapeutic methods to promote normal, stromal ECM assembly by HKCs. Our current study suggests that understanding the role of the TGF- $\beta$  pathway in KC ECM assembly may prove fundamental to revealing the basis of this corneal thinning disease.

## METHODS

**Ethics:** All procedures used in these studies adhered to the tenets of the Declaration of Helsinki. Healthy human corneas were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Keratoconus donor corneas were obtained from Jesper Hjortdal (Aarhus University Hospital, Aarhus, Denmark).

**Cell isolation:** Human corneas of healthy patients without any ocular disease were used for HCF isolation and age-matched HKCs were isolated from corneas of patients with Keratoconus defects, as previously described [27,28]. Briefly, the corneal epithelium and endothelium were removed from the stroma by scraping it with a razor blade. Stromal tissues were cut into small pieces of size 2×2 mm and placed into T25 culture flasks. The explants were allowed to adhere to the bottom of the flask at 37 °C for about 30 min and Eagle's Minimum Essential Medium (EMEM: ATCC: Manassas, VA) containing 10% fetal bovine serum (FBS: Atlantic Biologic's; Lawrenceville, CA) and 1% Antibiotic (Gibco® Antibiotic-Antimycotic, Life technologies) was added carefully without disturbing the ex-plants. Cells were further passaged into T75 culture flasks upon 100% confluence after 1–2 weeks of cultivation at 37 °C, 5% CO<sub>2</sub>.

**Cell growth and ECM assembly of HCFs and HKCs:** In addition, 1×10<sup>6</sup> cells of HCF and HKC cells/well was seeded and cultured in an EMEM 10% FBS medium stimulated with 0.5 mM 2-O- $\alpha$ -D-Glucopyranosyl-L-Ascorbic Acid (Vit C, American Custom Chemicals Corporation, San Diego, CA) [27,29]. The adherent cells were further stimulated with one of the three TGF- $\beta$  isoforms: TGF- $\beta$ 1, TGF- $\beta$ 2, or TGF- $\beta$ 3. According to the previous optimized concentration of TGF- $\beta$  isoforms, a 0.1 ng/mL concentration was used [27,29,30]. The experimental culture was grown for 4 weeks before further

processing. Cultures without any growth factors served as the controls (C), and fresh media were supplied every other day for the duration of the experiment.

**Real time PCR:** RNA was extracted using the Ambion RNA mini extraction kit (Ambion TRIzol® Plus RNA Purification Kit: Life technologies, Carlsbad, CA) followed by cDNA synthesis using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. The TaqMan gene expression assays (Applied Biosystems, Foster City) GAPDH (Hs99999905\_m1) and 18S (Hs99999901\_s1) were used as the control and SMAD3 (Hs00969210\_m1), SMAD4 (Hs00929647\_m1), SMAD6 (Hs00178579\_m1), SMAD7 (Hs00998193\_m1), TGFBR1 (Hs00610320\_m1), TGFBR2 (Hs00234253\_m1), TGFBR3 (Hs01114253\_m1), TGFB1 (Hs00998133\_m1), TGFB2 (Hs00234244\_m1), TGFB3 (Hs01086000\_m1) as the study probes (Table 1). Furthermore, 10 ng of cDNA was used for initiating the PCR reaction for a 20- $\mu$ l reaction mixture containing our desired probes and the Taqman FaSt Advanced Master Mix (Applied Biosystems, Life technologies, Foster city, CA). Amplification of samples was performed using the StepOnePlus™ real-time PCR system (Life Technologies) in accordance with the manufacturer's protocol. Results were confirmed in three separate analyses and Graph Pad Prism 6 and MS-Excel were used for data analysis.

**Western blot:** Western blot analyses of HCF and HKC cells were performed with lysis of cells, as previously described [27,31,32]. Protein concentration and purity were assessed by Bradford assay (Thermo Scientific, IL). As well, 4%–20% Tris-Glycine gels (Novex, Life technologies, Carlsbad, CA) was used for gel electrophoresis, to which equal amounts of proteins were loaded and a protein transfer was done using Nitrocellulose membrane (Novex, Nitrocellulose membrane filter par sandwich, Life Technologies). After incubation in a 5% BSA blocking solution (Thermo Scientific), the membranes were incubated with primary rabbit antibodies (Table 2): anti-Smad3 (Invitrogen, Camarillo, CA), anti-SMAD7 (Sigma-Aldrich, Saint Louis, MO), anti-SMAD4 (Abcam, Cambridge, MA), anti-SMAD6 (Abcam), Anti-TGF- $\beta$ R1 (Abcam), anti-TGF- $\beta$ R2 (Abcam), anti-TGF- $\beta$ R3 (Abcam), anti-TGF- $\beta$ 1 (Abcam), anti-TGF- $\beta$ 2, and anti-TGF- $\beta$ 3 (Abcam) at 1:1,000 dilution overnight at 4 °C separately. This was followed by washing of the membranes and incubation with a secondary antibody (Alexa Flour® 568 Donkey anti-Rabbit, IgG [H<sup>+</sup>L], Abcam) at 1:2,000 dilutions for 1 h. The Kodak imaging system was used for detecting the antibody binding to the membrane. GAPDH (Abcam) was used as the loading control and results were analyzed by normalizing

TABLE 1. PCR PROBES AND THEIR CONCENTRATIONS.

Probes	Catalogue #	Final concentration	Company
GAPDH	Hs99999905_m1	1X	Life technologies
18S	Hs99999901_s1	1X	Life technologies
TGFB I	Hs00998133_m1	1X	Life technologies
TGFB II	Hs00234244_m1	1X	Life technologies
TGFB III	Hs01086000_m1	1X	Life technologies
TGFBR I	Hs00610320_m1	1X	Life technologies
TGFBR II	Hs00234253_m1	1X	Life technologies
TGFBR III	Hs01114253_m1	1X	Life technologies
SMAD 3	Hs00969210_m1	1X	Life technologies
SMAD 4	Hs00929647_m1	1X	Life technologies
SMAD 6	Hs00178579_m1	1X	Life technologies
SMAD 7	Hs00998193_m1	1X	Life technologies

List of PCR probes used in this study. Final concentration, company, and catalog number information are listed.

the value to that of the loading control expression and plotting the fold expression.

*Statistical analysis:* All experiments were independently performed at least in triplicate and the statistical significance was evaluated using a non-parametric *t* test analysis, where a *p* value of  $p < 0.05$  was considered to be statistically significant. The Graph Pad Prism 6 software was used for statistical analysis.

## RESULTS

*Altered TGF- $\beta$  isoform expression in HKCs with TGF- $\beta$ 3 treatment:* Our previous study looking into the effect of TGF- $\beta$  isoforms on ECM assembly by HCFs and HKCs showed that TGF- $\beta$ 3 stimulated HKCs to generate a more

normal stromal ECM, consistent with low Collagen III and high Collagen I and V assembly [25]. In our current study, we investigated the role of key TGF- $\beta$  regulator molecules in HKCs compared to normal HCFs in an effort to further define the role of TGF- $\beta$  signaling in KC. We have compared expression changes in the HKCs to the control HCFs in an effort to define differences that contribute to the disease phenotype and the role of the TGF- $\beta$  isoforms in regulating ECM assembly. Figure 1A-C shows RT-PCR data of the TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3 transcripts following stimulation of HCFs and HKCs with the three TGF- $\beta$  isoforms. The gene expression of TGF $\beta$ 1 was similar for all conditions in normal HCFs. However, the TGF- $\beta$ 1 expression was approximately two-fold higher ( $p \leq 0.05$ ) in HCFs compared to HKCs with TGF- $\beta$ 3 treatment (Figure 1A). The TGF- $\beta$ 2 gene expression

TABLE 2. WB ANTIBODIES AND THEIR DILUTIONS.

Antibody	Catalogue #	Dilution	Company
Anti-TGF beta I	ab53169	1/1000	Abcam
Anti-TGF beta II	ab66045	1/1000	Abcam
Anti-TGFbeta III	ab15537	1/1000	Abcam
Anti-TGF beta R I	ab31013	1/500	Abcam
Anti-TGF beta R II	ab61213	1/500	Abcam
Anti-TGF beta R III	ab97459	1/500	Abcam
pSMAD3	ab52903	1/1000	Abcam
Anti-SMAD3	ab55479	1/1000	Abcam
Anti-SMAD4	ab137861	1/1000	Abcam
Anti-SMAD6	ab13727	1/1000	Abcam
Anti-SMAD7	AV32008	1/1000	Sigma Aldrich
Anti-GAPDH	ab9485	1/1000	Abcam

showed a significant expression reduction (3.5-fold, 1.8-fold, and 2.2-fold,  $p \leq 0.05$ ) in HKCs compared to HCFs with TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 treatment, respectively (Figure 1B). Furthermore, the TGF- $\beta$ 3 expression was significantly downregulated (2.2-fold, eight-fold, and 5.6-fold,  $p \leq 0.05$ ) in HKCs in the control, TGF- $\beta$ 1-, TGF- $\beta$ 2-, and TGF- $\beta$ 3-treated samples, respectively (Figure 1C). These data suggest TGF- $\beta$ 3 reduces the expression of the profibrotic ligand TGF- $\beta$ 1 in HKCs, and this reduction contributes to attenuating the ECM generated by HKCs.

We compared the gene expressions with protein expressions using a western blot analysis following TGF- $\beta$  isoform treatments (Figure 1D-G). Our western blot results showed the TGF- $\beta$ 1 protein expression was comparable in both cell types at basal levels and following stimulation with TGF- $\beta$ 1 and TGF- $\beta$ 2 (Figure 1D). In addition, TGF- $\beta$ 3 treatment in HKCs increased the TGF- $\beta$ 1 protein expression significantly (1.5-fold,  $p \leq 0.05$ ) compared to control HCFs (Figure 1D). The

TGF- $\beta$ 2 protein expression was lower (Figure 1E, two-fold,  $p \leq 0.05$ ) in HKCs with TGF- $\beta$  isoform treatments compared to HCFs, supporting our gene analysis results that the TGF- $\beta$ 1 expression is the more abundant isoform expressed in HKCs following stimulation with the three isoforms. Furthermore, we found a reduction in the TGF- $\beta$ 3 protein expression (two-fold,  $p \leq 0.35$ ) in HKCs following TGF- $\beta$ 3 treatment (Figure 1F).

*Altered TGF- $\beta$ IIR expression in HKCs induced by TGF- $\beta$ 3 stimulation:* The TGF $\beta$  receptors, TGF- $\beta$ R1, TGF- $\beta$ R2, and TGF- $\beta$ R3, showed substantial variations in expressions in the diseased HKCs versus the normal HCFs. We used RT-PCR to measure changes in receptor expressions in both cell types (Figure 2A-C). Our results show the TGF- $\beta$ 2 stimulation significantly upregulates the expression of TGF- $\beta$ R1 in HKCs (2.5-fold,  $p \leq 0.05$ ) compared to control HKC levels (Figure 2A). In contrast, HCFs did not significantly upregulate the expressions of the TGF- $\beta$  receptors with TGF- $\beta$ 1 and

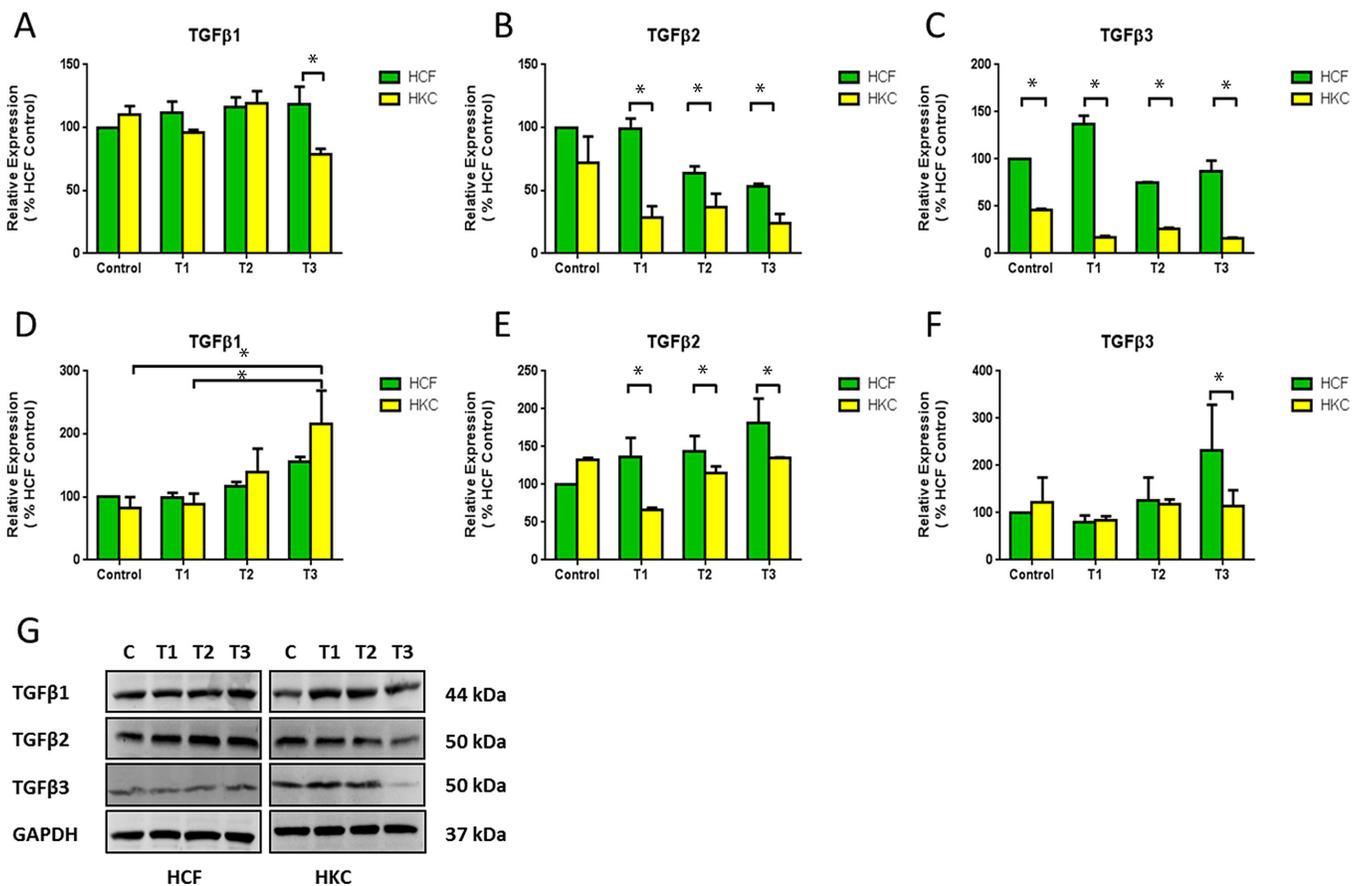


Figure 1. Quantification of TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 expression in HCFs and HKCs following stimulation with all three TGF- $\beta$  isoforms. RT-PCR analysis shows gene expression for (A) TGF- $\beta$ 1, (B) TGF- $\beta$ 2, and (C) TGF- $\beta$ 3. Western blot analysis shows protein expression for (D) TGF- $\beta$ 1, (E) TGF- $\beta$ 2, and (F) TGF- $\beta$ 3. G: Representative Western blots from three independent experiments. All samples were repeated at least three times.  $p < 0.05$  was considered to be statistically significant (\* $p < 0.05$ ).

TGF- $\beta$ 2 treatment, suggesting HKCs are more responsive to TGF- $\beta$ 3 isoform stimulation, as indicated by the increased expressions of the profibrotic receptors. The reduction in TGF- $\beta$ R1 in HKCs following TGF- $\beta$ 3 treatment correlates with the reduction in the profibrotic TGF- $\beta$ 1 ligand under the same conditions, suggesting TGF- $\beta$ 3 favors an antifibrotic mechanism by downregulating the expressions of both the profibrotic receptor and ligand in HKCs. In addition, the TGF- $\beta$ R2 and TGF- $\beta$ R3 expressions are also significantly downregulated ( $p \leq 0.05$ ) in HKCs following TGF- $\beta$ 3 treatment (Figure 2B-C), suggesting TGF- $\beta$ 3 regulates the expressions of TGF- $\beta$  receptors in KC.

We also analyzed the protein expressions of the TGF- $\beta$  receptors by western blot (Figure 2D-G), showing TGF- $\beta$ 3 stimulation significantly downregulated TGF- $\beta$ R1 (1.6-fold,  $p \leq 0.0005$ ), TGF- $\beta$ R2 (three-fold,  $p \leq 0.001$ ), and TGF- $\beta$ R3 (2.6-fold,  $p \leq 0.001$ ) in HKCs compared to HCFs (Figure 2D-F). The TGF- $\beta$ R2 expression (Figure 2E) was significantly reduced in HKCs in the control, TGF- $\beta$ 1-, TGF- $\beta$ 2-,

and TGF- $\beta$ 3-treated cells (two-fold, three-fold, three-fold, and three-fold, respectively,  $p \leq 0.05$ ). We also saw a significant reduction in TGF- $\beta$ R3 in HKCs in the control and TGF- $\beta$ 1-stimulated samples (2.7-fold and 1.5-fold, respectively,  $p \leq 0.05$ ) compared to HCFs (Figure 2F). Moreover, TGF- $\beta$ 3 stimulation ameliorates the fibrotic response evident in HKCs by significantly downregulating the expressions of TGF- $\beta$ R1 and TGF- $\beta$ R2 without altering the expression of TGF- $\beta$ R3, suggesting a differential response to TGF- $\beta$ 3 between diseased versus normal corneal fibroblasts, with TGF- $\beta$ R1 and TGF- $\beta$ R2 being the dominant receptors for TGF- $\beta$ 3.

*Regulatory SMAD6 and SMAD7 expressions decreased in HKCs:* We measured the gene expressions of the inhibitory SMADs (SMAD6 and SMAD7) to identify whether alterations in the basal levels following isoform treatment contribute to the fibrotic phenotype observed in HKCs (Figure 3A-B). Our RT-PCR data showed HCFs have a significantly upregulated expression of SMAD6 in the control, TGF- $\beta$ 1-, TGF- $\beta$ 2-, and TGF- $\beta$ 3-stimulated cells compared to HKCs (1.7-fold,

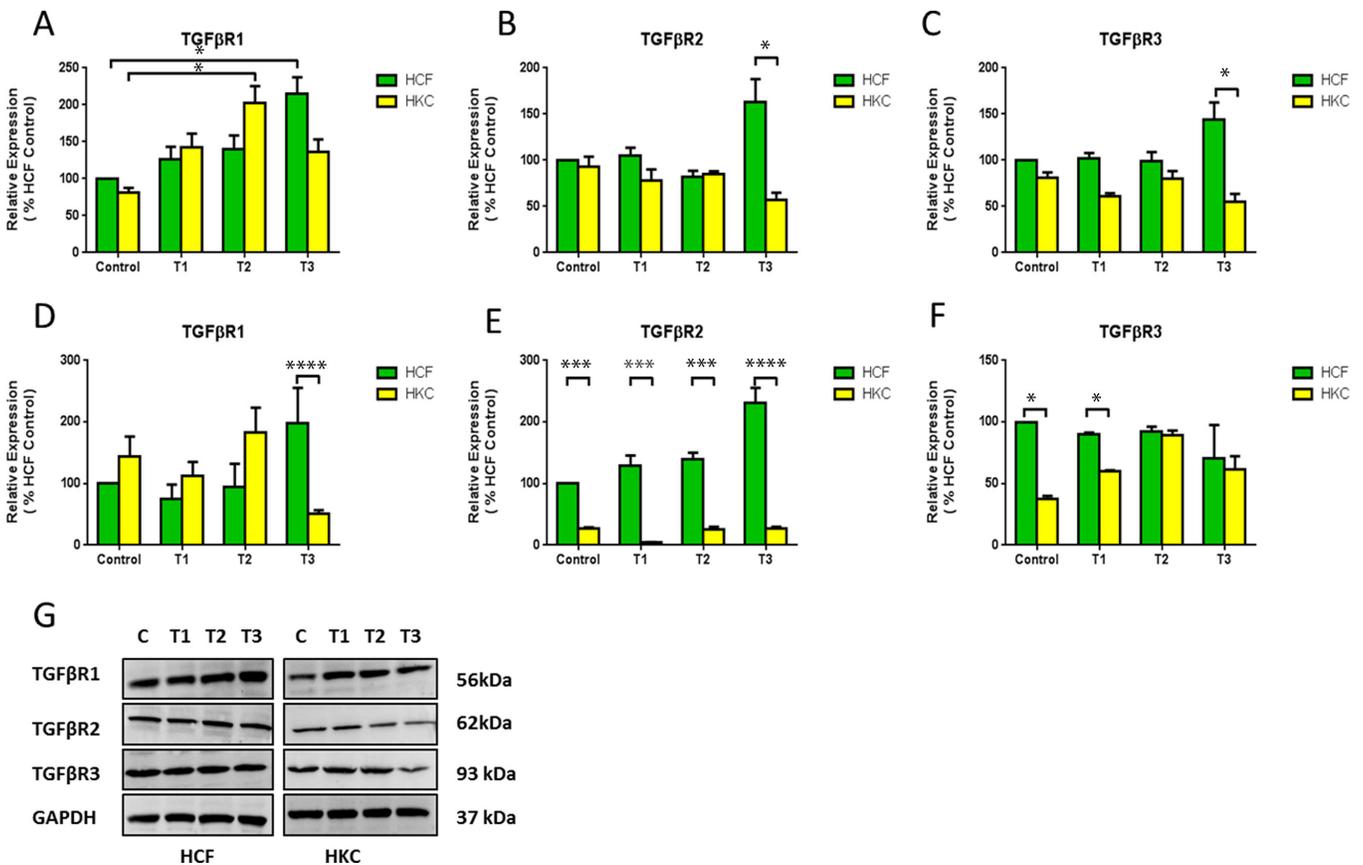


Figure 2. Quantification of TGF- $\beta$ R1, TGF- $\beta$ R2, and TGF- $\beta$ R3 expression in HCFs and HKCs following stimulation with all three TGF- $\beta$  isoforms. RT-PCR analysis shows gene expression for (A) TGF- $\beta$ R1, (B) TGF- $\beta$ R2, and (C) TGF- $\beta$ R3. Western blot analysis shows protein expression for (D) TGF- $\beta$ R1, (E) TGF- $\beta$ R2, and (F) TGF- $\beta$ R3. G: Representative Western blots from three independent experiments. All samples were repeated at least three times.  $p < 0.05$  was considered to be statistically significant (\*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.001$ , \* $p < 0.05$ ).

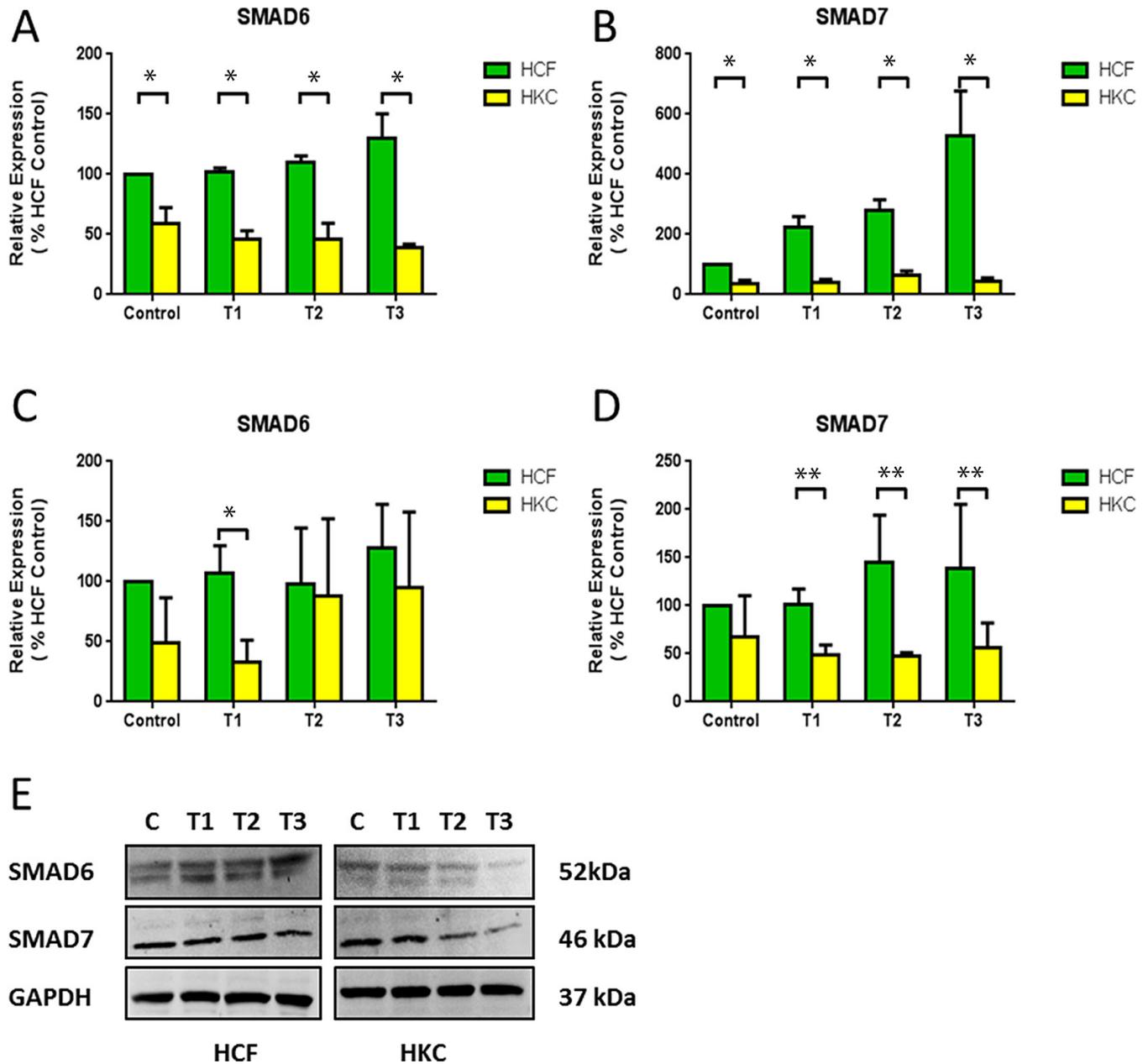


Figure 3. Quantification of SMAD6 and SMAD7 expression in HCFs and HKCs following stimulation with all three TGF- $\beta$  isoforms. RT-PCR analysis shows gene expression for (A) SMAD6 and (B) SMAD7. Western blot analysis shows protein expression for (C) SMAD6 and (D) SMAD7. E: Representative Western blots from three independent experiments. All samples were repeated at least three times.  $p < 0.05$  was considered to be statistically significant (\*\* $p < 0.01$ , \* $p < 0.05$ ).

2.2-fold, 2.4-fold, and 3.3-fold, respectively,  $p \leq 0.05$ , Figure 3A). A similar pattern was seen in the SMAD7 expression, where we found a significant downregulation in the control, TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3 treatments (5.4-fold, 4.4-fold, and 12-fold, respectively,  $p \leq 0.05$ ) in HKCs compared to HCFs (Figure 3B). This data shows a significant reduction in the regulatory SMADs—SMAD6 and SMAD7—in HKCs, providing the first evidence of the author’s knowledge,

associating a malfunction in TGF- $\beta$  signaling regulation with KC.

We analyzed the protein expressions of regulatory SMAD6 and SMAD7 by western blot (Figure 3C-E). The SMAD6 expression was reduced in HKCs with TGF- $\beta$ 1 treatment (1.1-fold,  $p \leq 0.05$ , Figure 3C). The SMAD7 expression was reduced in HKCs following TGF- $\beta$ 1, TGF- $\beta$ 2,

and TGF- $\beta$ 3 stimulation (two-fold, three-fold and 2.5-fold, respectively,  $p \leq 0.03$ ) compared to HCFs (Figure 3D). Our results show that HKCs fail to increase the expressions of the regulatory SMAD6 or SMAD7 with TGF- $\beta$  treatment, suggesting a significant malfunction in the activation of the TGF- $\beta$  responsive genes associated with regulating TGF- $\beta$  signaling.

**Altered SMAD3/4 expressions in HKCs:** To determine whether the altered expressions of TGF- $\beta$  isoforms, receptors, and regulatory SMAD6 and SMAD7 contribute to TGF- $\beta$  signaling modulation, we measured the expressions of SMAD3 and SMAD4 using RT-PCR (Figure 4A-B). TGF- $\beta$ 3 stimulation caused a significant reduction in SMAD3 and SMAD4 in HKCs (1.7-fold and 1.6-fold, respectively,  $p \leq 0.05$ ) compared to HCFs (Figure 4A-B). HKCs showed a one-fold increase in the pSMAD3 expression, as measured by western blot, compared to HCFs following TGF- $\beta$ 3 treatment (Figure 4F). This data correlates with the observed increase in the TGF- $\beta$ 1 isoform expression in HKCs with TGF- $\beta$ 3 treatment and suggests the elevated expression of TGF- $\beta$ 1 is directly linked to the significant increase in pSMAD3. The lack of TGF- $\beta$ RII with TGF- $\beta$ 3 stimulation in HKCs suggests this receptor is required for the activation of profibrotic genes, and its absence fails to promote differentiation to the fibrotic phenotype.

We measured differential expressions of SMAD3 and SMAD4 by western blot in both cell types (Figure 4C-D). HKCs showed a 3.2-fold ( $p \leq 0.007$ ) increase in SMAD3 at basal levels compared to HCFs (Figure 4C). TGF- $\beta$ 1 treatment also showed an elevated SMAD3 expression in HKCs (2.4-fold,  $p \leq 0.006$ ) compared to HCFs (Figure 4C). Moreover, TGF- $\beta$ 3 stimulation caused an increase in the SMAD3 expression by both cell types, suggesting TGF- $\beta$ 3 may also activate gene expressions via a SMAD-dependent mechanism similar to the other isoforms. SMAD4, which is an adaptor protein that colocalizes with pSMAD3 following TGF- $\beta$  receptor activation, also showed an increase (1.6-fold,  $p \leq 0.02$ ) in expression in HKCs compared to HCFs (Figure 4D). The SMAD4 expression was upregulated (2.8-fold,  $p \leq 0.01$ ) in HKCs compared to HCFs following TGF- $\beta$ 2 stimulation (Figure 4D). Collectively, our data show TGF- $\beta$ 3 significantly downregulates TGF- $\beta$ 1, TGF- $\beta$ RI, SMAD3, and SMAD4 expressions, suggesting a plausible mechanism by which TGF- $\beta$ 3 favors antifibrotic ECM assembly.

## DISCUSSION

KC is a corneal thinning disease with a pathophysiology directly linked to altered ECM assembly, myofibroblast differentiation, and distorted corneal structure [27]. KC

is known to be associated with scarring and altered ECM assembly that leads to structural thinning of the cornea in vivo [33,34]. As TGF- $\beta$  signaling is an important modulator of ECM assembly, we focused our current study on identifying the role of key TGF- $\beta$  signaling molecules in a 3D in vitro model of KC.

HKCs are isolated from severe cases of KC, in which the patients have undergone corneal transplantation due to the progressive nature of the disease. It has been shown that myofibroblasts are not expressed in the early stages of KC [35], but are present only in severe cases associated with scarring or disruptions to the Bowman's membrane [36]. In this study, we used fibroblasts isolated from KC patients and normal human controls isolated in the presence of serum. Our approach ensures we focus on the defects between the healthy and diseased corneal stromal cells. Isolation of keratocytes from KC patients is ideal; however, preliminary observations from our laboratory have found this to be difficult without additives included in the media. There are differences between keratocytes isolated from healthy corneal stroma and those that are KC derived. We did not utilize altered conditioned media, as our primary goal was to identify the native expressions of key TGF- $\beta$  mediators without altering pathways that may skew the data.

The TGF- $\beta$  pathway is a known regulator of ECM secretion and assembly. The three TGF- $\beta$  isoforms, TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3, are the primary ligands that bind to TGF $\beta$ RII, which has been found to recruit and phosphorylate TGF $\beta$ RI, promoting phosphorylation of cytoplasmic SMAD2 and SMAD3 [37]. The TGF- $\beta$ 1 and TGF- $\beta$ 2 isoforms are ligands associated with promoting a profibrotic response characterized by the secretion of excess Collagen secretion and  $\alpha$ -SMA expression [16,22]. The three TGF- $\beta$  isoforms have differential roles in wound healing, with TGF- $\beta$ 3 having an antifibrotic function [26,29,38,39]. We have previously shown that TGF- $\beta$ 3 promotes the assembly of a normal ECM by HKCs in vitro by decreasing Collagen III secretion and the expressions of profibrotic markers characteristic of the diseased phenotype [25,29]. Several previous clinical studies have shown that TGF $\beta$ 3 is a key modulator during the wound healing process [29,40,41]. Briefly, in the skin, TGF $\beta$ 3 was found to be a key component in improving the quality of healing and it has made it to the clinical trials stage [42]. TGF $\beta$ 3 has also been proposed as an antiscarring factor in buccal mucosa [43]. Furthermore, in an embryonic wound microenvironment, high levels of the TGF $\beta$ 3 expression have been reported to contribute to a scar-less healing mechanism [42].

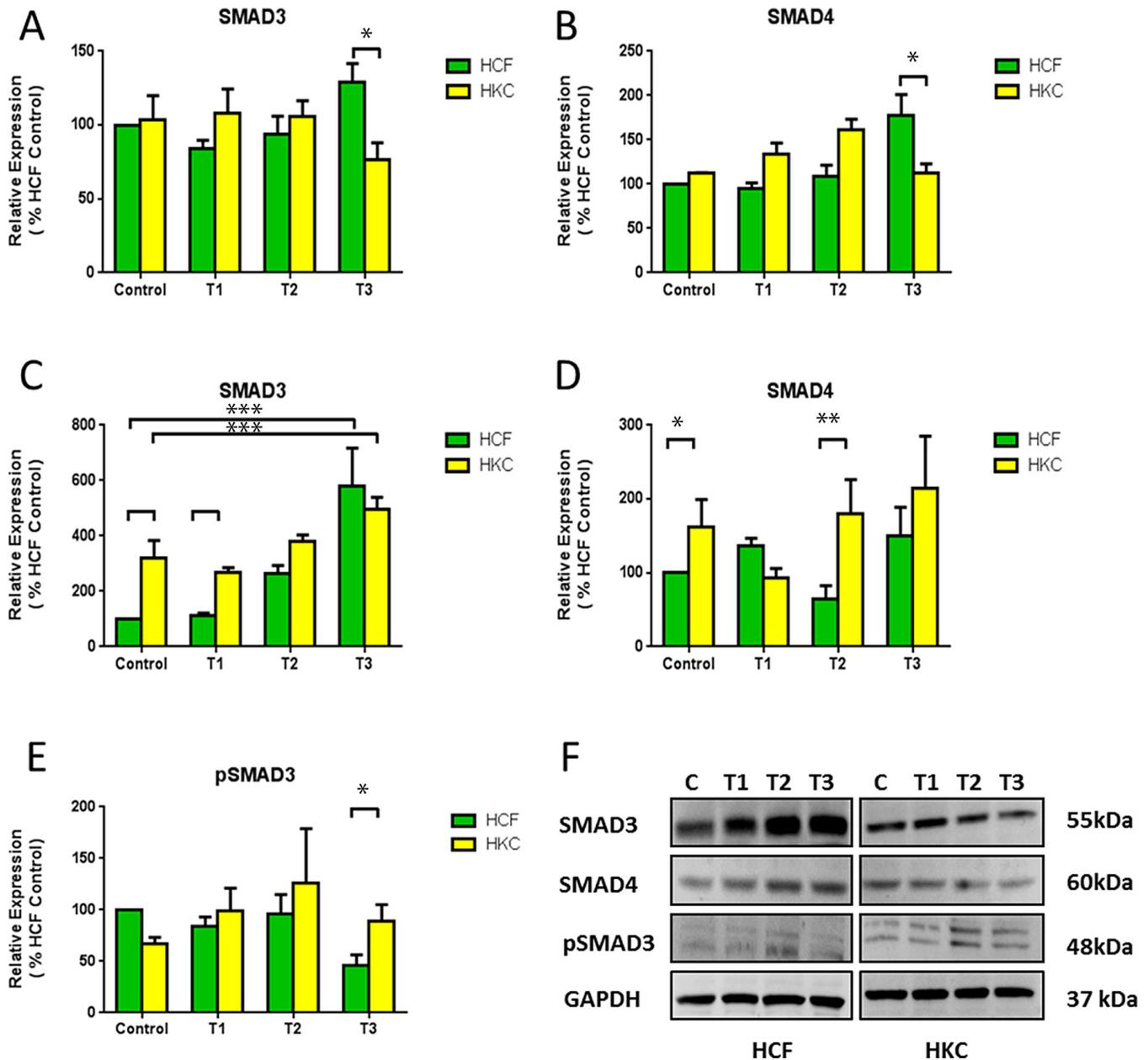


Figure 4. RT-PCR analysis shows gene expression for (A) SMAD3 and (B) SMAD4. Representative western blots show protein expression for (C) SMAD3, (D) SMAD4 and (E) pSMAD3, following stimulation with all three TGF- $\beta$  isoforms. F: Representative Western blots. All samples were repeated at least three times.  $p < 0.05$  was considered to be statistically significant (\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ ).

Due to the direct relationship associated with excessive TGF- $\beta$  signaling and the development of fibrosis, inhibitors have been developed, targeting TGF- $\beta$  signaling in vivo [44,45]. However, no treatments have been developed that directly promote ECM secretion and assembly to treat corneal diseases. Moreover, studies have associated KC with variations in TGF- $\beta$  signaling [12,13,37].

Due to this direct correlation of KC with ECM assembly, we sought to identify the role of TGF- $\beta$  signaling in contributing to the fibrotic phenotype observed in HKCs and to explore the potential of TGF- $\beta$ 3 in promoting normal ECM assembly by HKCs. In our experimental set-up, HCFs and HKCs were treated with TGF- $\beta$  isoforms in a 4-week study, where gene and protein expressions of TGF- $\beta$  signaling molecules were measured for both cell types. TGF- $\beta$ 1 was found to be elevated in HKCs with TGF- $\beta$ 3 treatment in comparison

to HCFs. Both TGF $\beta$ 2 and TGF $\beta$ 3 were reduced in HKCs, suggesting that ECM assembly in KC is directly modulated primarily by TGF- $\beta$ 1, rather than by TGF- $\beta$ 2 and TGF- $\beta$ 3. This data supports previous studies suggesting the importance of TGF- $\beta$ 1 in KC disease [12,46,47]. Mechanistically, studies have shown that TGF- $\beta$  pathway activation is dependent on receptor expression, receptor kinase activity, ligand concentration, and adaptor protein expression [18,22,48,49].

We measured changes in both gene expressions and protein expressions in key TGF- $\beta$  signaling mediators in HKCs compared to HCFs to characterize fully the role of the TGF- $\beta$  pathway in the pathobiology of KC. In addition, the expression at the mRNA level is known to deviate from that at the protein level due to the regulation of mRNA stability, transport, and translation [50-52]. We found differences in gene expressions compared to protein expressions in both cell types, which suggests the TGF- $\beta$  pathway is highly regulated. The mechanism of KC pathogenesis is still unknown; however, our work suggests TGF- $\beta$  signaling may play a significant role in the altered ECM assembled by HKCs.

Our results showed substantial variations in the expressions of the TGF $\beta$  receptors TGF- $\beta$ R1, TGF- $\beta$ R2, and TGF- $\beta$ R3 in HKCs compared to HCFs, where TGF- $\beta$ 3 stimulation caused a significant downregulation of the TGF- $\beta$ R1, TGF- $\beta$ R2, and TGF- $\beta$ R3 expressions in HKCs when compared to HCFs. We speculate TGF- $\beta$ 3 favors an antifibrotic mechanism in HKCs by downregulating the expression of the profibrotic receptor, TGF- $\beta$ R2, thereby antagonizing profibrotic signaling in the abundance of the TGF- $\beta$ 1 ligand.

The SMAD proteins, which are considered to be key regulators in the propagation of the TGF- $\beta$  pathway, also showed significant regulation in HKCs compared to HCFs. Here, we measured the expressions of the regulatory SMAD6 and SMAD7 at basal levels, as well as following stimulation with the three TGF- $\beta$  isoforms. TGF- $\beta$  signaling is known to regulate directly the regulatory SMAD expressions by activating SMAD6 and SMAD7 gene transcriptions upon ligand binding, which provides a negative feedback loop to curb aberrant TGF- $\beta$  signaling [18]. In our study, we found that stimulation with the TGF- $\beta$  isoforms caused a significant increase in the SMAD7 expression in HCFs compared to HKCs, whereas HKCs lacked any increase in the expressions of SMAD6 or SMAD7 following TGF- $\beta$  isoform treatments. These results suggest a significant malfunction in the activation of TGF- $\beta$  responsive genes associated with the negative regulation of TGF- $\beta$  signaling in HKCs that may contribute to the altered ECM generated in KC.

To evaluate whether the observed altered expressions of TGF $\beta$  receptors and regulatory SMAD6 and SMAD7 proteins by HKCs resulted in increased TGF $\beta$  signaling, we measured the pSMAD3 expression. We show a significant increase in pSMAD3 with TGF- $\beta$ 3 in HKCs. The lack of the TGF- $\beta$ R2 expression with TGF- $\beta$ 3 stimulation in HKCs suggests this receptor is required for the activation of profibrotic genes, and its absence fails to promote differentiation to the fibrotic phenotype. Mechanistically, our data also suggest TGF- $\beta$ 3 activates the TGF- $\beta$  pathway in a SMAD-dependent manner following the SMAD2/3/4 axis that leads to the transcription of genes associated with ECM deposition without terminal differentiation to the myofibroblast, which may prove useful in targeting the TGF- $\beta$  signaling pathway in the treatment of KC.

We would like to propose a new model/mechanism for the relationship between the altered expressions of key TGF- $\beta$  signaling molecules and the HKC fibrotic phenotype. HKCs have reduced SMAD6/7 expressions following stimulation with the three TGF- $\beta$  isoforms compared to HCFs. Normal corneal fibroblasts increase the expressions of SMAD6/7 upon TGF- $\beta$  isoform treatment, which serves to curb successfully aberrant activation of the TGF- $\beta$  pathway by decreasing receptor availability and recruitment of SMAD2/3. Phosphorylation of SMAD2/3 promotes translocation to the nucleus and activation of the genes associated with myofibroblast differentiation. The lack of inhibition of TGF- $\beta$  signaling by SMAD6/7 results in increased TGF- $\beta$  signaling that promotes the formation of a fibrotic ECM characteristic of HKCs.

Our data directly link the altered expressions of the regulatory SMAD6/7 with the observed aberrant TGF- $\beta$  signaling and increased fibrotic phenotype observed in HKCs. In addition, we show that TGF- $\beta$ 3 decreases the expression of TGF- $\beta$ R2 in HKCs. This result gives a mechanistic role to the antifibrotic phenotype stimulated by TGF- $\beta$ 3 by suggesting TGF- $\beta$ 3 regulates directly profibrotic signaling in HKCs by downregulating the expression of the key TGF- $\beta$  receptor responsible for promoting activation of the pathway. In this study, we have identified a significant role for the regulatory SMADs, SMAD6 and SMAD7, in regulating TGF- $\beta$  signaling, as well as highlight how alterations in expressions can contribute to the HKC phenotype. We also propose a plausible mechanistic role of TGF- $\beta$ 3 in downregulating the receptor expression, thereby stimulating an antifibrotic response. Clearly, the TGF- $\beta$  pathway plays an important role in the development and progression of KC and may provide clues to the development of therapeutics to treat KC.

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