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Role of 5′TG3′-interacting factors (TGIFs) in Vorinostat (HDAC inhibitor)-mediated corneal fibrosis inhibition

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Purpose: We have previously reported that vorinostat, an FDA-approved, clinically used histone deacetylase (HDAC) inhibitor, attenuates corneal fibrosis in vivo in rabbits by blocking transforming growth factor β (TGFβ). The 5′TG3′-interacting factors (TGIFs) are transcriptional repressors of TGFβ1 signaling via the Smad pathway. The present study was designed to explore the expression of TGIFs in human corneal fibroblasts and to investigate their role in mediating the antifibrotic effect of vorinostat.

Methods: Human corneal fibroblast cultures were generated from donor corneas. RNA isolation, cDNA preparation, and PCR were performed to detect the presence of TGIF1 and TGIF2 transcripts. The cultures were exposed to vorinostat (2.5 µM) to test its effect on TGIF mRNA and protein levels using qPCR and immunoblotting. Myofibroblast formation was induced with TGFβ1 (5 ng/ml) treatment under serum-free conditions. The changes in fibrosis parameters were quantified by measuring fibrosis marker α-smooth muscle actin (αSMA) mRNA and protein levels with qPCR, immunostaining, and immunoblotting. Smad2/3/4 and TGIF knockdowns were performed using pre-validated RNAi/siRNAs and a commercially available transfection reagent.

Results: Human corneal fibroblasts showed the expression of TGIF1 and TGIF2. Vorinostat (2.5 µM) caused a 2.8–3.3-fold increase in TGIF1 and TGIF2 mRNA levels and a 1.4–1.8-fold increase in TGIF1 and TGIF2 protein levels. Vorinostat treatment also caused a significant increase in acetylhistone H3 and acetylhistone H4. Vorinostat-induced increases in TGIF1 and TGIF2 were accompanied by a concurrent decrease in corneal fibrosis, as indicated by a decrease in αSMA mRNA by 83±7.7% and protein levels by 97±5%. The RNAi-mediated knockdown of Smad2, Smad3, and Smad4 markedly attenuated TGFβ1-evoked transdifferentiation of fibroblasts to myofibroblasts. The siRNA-mediated knockdown of TGIF1 and TGIF2 neutralized vorinostat-evoked decreases in αSMA mRNA by 31%–45% and protein levels by 12%–23%.

Conclusions: Human corneal fibroblasts demonstrate the expression of TGIF1 and TGIF2 transcription factors. These transcriptional repressors are critical, at least partially, in mediating the antifibrotic effect of vorinostat in the cornea.

Corneal scarring is a third leading cause of global blindness, often caused due to injury, trauma, and/or infection to the eye [1]. No potent and safe therapeutic agents are currently available to treat corneal fibrosis, and corneal transplantation remains the only option to restore vision. The epithelial-stromal interaction in the cornea plays an important role in corneal wound healing following injury. To counter damage caused by trauma or injury, corneal epithelial and stromal cells release a wide variety of cytokines and growth factors [2-4]. Out of many cytokines, transforming growth factor β (TGFβ) has been identified as a key inducer of corneal fibrosis [5,6]. TGFβ has been shown to activate both Smad-dependent and Smad-independent pathways, such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling [5-8]. However, very limited studies have shown the direct evidence of Smad signaling in mediated TGFβ-induced transdifferentiation of human fibroblasts to myofibroblasts [8,9].

Numerous literature reports suggest that HDAC inhibitors can cause repression of TGFβ-induced profibrotic genes, including collagen (Col) type 1 and smooth muscle actin (αSMA) [10,11]. Additionally, it is also reported that HDAC inhibitors attenuate TGFβ-driven epithelial-to-mesenchymal transition (EMT) [12,13]. Recently, our laboratory demonstrated that epigenetic modulation of corneal wound healing by histone deacetylase (HDAC) inhibitors is an innovative approach to effectively treat corneal fibrosis without significant complications or side effects [14,15]. We found that HDAC inhibitors are selective and potent inhibitors of corneal fibrosis in rabbits in vivo, and that they attenuate TGFβ-induced profibrotic expression of genes in canine, equine, and human corneal fibroblasts in vitro [14-17]. Although several HDAC inhibitors are being investigated for their antifibrotic effects, vorinostat is the only HDAC inhibitor that is already FDA-approved for humans, and is currently in clinical use to treat cutaneous T-cell lymphoma.

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Previous studies from our laboratory have demonstrated that vorinostat can inhibit TGFβ-mediated transdifferentiation of cultured corneal fibroblasts to myofibroblasts and laser surgery-induced corneal scarring in rabbits [15-17]. Unlike other antifibrotic agents, vorinostat is relatively non-toxic and does not affect the viability or proliferation of corneal fibroblasts [15-17]. However, the mechanism mediating the antifibrotic effect of vorinostat in the cornea is not yet known.

The TGFβ–Smad pathway is modulated by a wide variety of Smad co-repressors, including c-Ski, SnoN, Smads6, Smad7, and 5′TG3′-interacting factors (TGIFs) [18-20]. Recently, TGIFs have gained more attention because of their role in cell proliferation and cell differentiation [21,22]. TGIFs are members of the three amino acid loop extension (TALE) class of homeodomain proteins and consist of two closely related transcription factors, TGIF1 and TGIF2. Both TGIF1 and TGIF2 can cause repression of TGFβ1-activated genes by direct competition with the coactivator p300/CBP for Smad2 interactions [23]. TGIF1 expression has been detected in few adult tissues, whereas TGIF2 was ubiquitously expressed in several adult human tissues [24-27]. The expression and role of TGIFs in the adult cornea is still unknown. In the present study, we tested the hypothesis that TGIF1 and TGIF2 are expressed in human corneal fibroblasts, and that these proteins are involved in mediating the anti-fibrotic effects of vorinostat in the cornea using an in vitro model. Our results demonstrate that human corneal fibroblasts express TGIF1 and TGIF2 transcription factors, and that upregulation of these transcriptional repressors contributes, at least partially, to the antifibrotic effect of vorinostat in the cornea.

METHODS

Human corneal fibroblast and myofibroblast cultures: Primary corneal fibroblast cultures were generated from donor human corneas procured from an eye bank (Heartland Eye Bank, Kansas City, MO). Based on the slit lamp, specular microscopy, physical examination, and donor medical record data provided by the eye bank, it was ensured that only healthy and disease-free corneas were used in the present study. Corneal tissues were washed with a sterile cell culture medium, and the epithelium and endothelium were removed by gentle scraping with a #15 scalpel blade. The corneal stroma was cut into small sections, placed on a culture dish, and incubated in a humidified 5% CO_2 incubator at 37 °C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum for two to three weeks. The primary human corneal fibroblasts harvested from these corneal buttons were seeded in six-well plates in MEM supplemented with 10% fetal bovine serum and allowed to reach 60%–70%

confluence. To generate myofibroblast cultures, the fibroblasts were seeded using MEM containing 10% fetal bovine serum, switched to serum-free medium containing TGFβ1 (5 ng/ml) after 12 h, and incubated for 5 days. The cultures were fed with fresh serum-free TGFβ1-containing medium every 24 h.

Vorinostat, siRNA and RNAi treatment: A 10 mM stock solution of vorinostat (Cayman Chemical Company, Ann Arbor, MI) was prepared in dimethylsulfoxide (DMSO) and diluted with MEM to achieve a final concentration of 2.5 µM in the culture medium. The equivalent amount of DMSO (0.5 µl in 2 ml culture medium) served as controls. For the knockdown of TGIF1 and TGIF2 gene expression, prevalidated siRNA (Ambion Life technologies, Grand Island, NY) were used at a 30 nM concentration (Table 1). For the knockdown of Smad2/3/4, the prevalidated RNA interference (RNAi) oiligos (Table 1) were cloned into a pcDNA 6.2 miR RNAi expression vector under the control of Pol II promoter (Life Technologies, Grand Island, NY). The siRNA and RNAi plasmid transfections were performed using the commercially available transfection reagent lipofectamine3000 (Life technologies corporation, Grand Island, NY) as per the manufacturer's instructions. Briefly, a transfection solution was prepared by adding 10 µl lipofectamine3000 in 200 µl of optiMEM medium to the appropriate amounts of siRNA/RNAi plasmid in 200 µl of optiMEM. The mixture was incubated for 5 min at room temperature. The above transfection mixture was added to the human corneal fibroblasts in 1 ml of a serum-free culture medium.

RNA extraction and cDNA synthesis: Total RNA from cells was extracted with an RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA following the vendor's instructions (Promega, Madison, WI). PCR was performed for the detection of TGIF1 and TGIF2. A 50 µl reaction mixture containing 2 µl cDNA, 2 µl forward (200 nM) and 2 µl reverse (200 nM) primer, 0.2 mM of each deoxynucleotide triphosphates (dNTP), and 1.25 U of Taq polymerase was run one cycle at 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, followed by 55 °C for 30 s and 72 °C for 60 s, using a thermocycler (Bio-Rad Laboratories, Hercules, CA) and primers listed in Table 2. The cDNA samples were prepared from fibroblasts originating from two different human donor corneas. Each PCR was repeated at least three times.

Gene expression quantification by qPCR: The relative quantification of TGIFs mRNA was performed using the StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA). A 20 µl reaction mixture containing 2 µl of cDNA, 2 µl of forward primer (200 nM), 2 µl of reverse primer (200 nM), and 10 µl of 2X SYBR green super mix

TABLE 1. NUCLEOTIDE SEQUENCES OF TWO VALIDATED RNAi AND siRNA OLIGOS USED IN THE STUDY.	
Smad2 RNAi-1 validated (5′ to 3′)	GAGCAGAACCTCTCTGAATTTGGTTTTGGCCACTGACTGAC-CAAATTCAGAGGTTCTGCT
Smad2 RNAi-2 validated (5′ to 3′)	GTGTAAAGGCCTGTTGTATCCCGTTTTGGCCACTGACTGAC-GGGATACAAGGCCTTTACA
Smad3 RNAi-1 validated (5′ to 3′)	GCACTGAGGCACTCTGCGAAGAGTTTTGGCCACTGACT-GACTCTTCGCAGTGCCTCAGTG
Smad3 RNAi-2 validated (5′ to 3′)	GTTCATCTGGTGGTCACTGGTTGTTTTGGCCACTGACTGACAAC-CAGTGCACCAGATGAA
Smad4 RNAi-1 validated (5′ to 3′)	GTATGATGGTAAGTAGCTGGCTGTTTTGGCCACTGACTGACAGC-CAGCTTTACCATCATA
Smad4 RNAi-2 validated (5′ to 3′)	GTTTCCGACCAGCCACCTGAAGGTTTTGGCCACTGACT-GACCTTCAGGTCTGGTCGGAAA
TGIF1 siRNA-1 validated (5′ to 3′)	GCAUGGACAUUCCCUUGGATT
TGIF1 siRNA-2 validated (5′ to 3′)	GCACCGUUACAAUGCCUAUTT
TGIF2 siRNA-1 validated (5′ to 3′)	GCCUCCUAGCUGGGAUUGUTT
TGIF2 siRNA-2 validated (5′ to 3′)	GGACGACAGCCUUUAGUUUTT

(Bio-Rad Laboratories, Hercules, CA) was run at a universal cycle (95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s) in accordance with the manufacturer's instructions, using primers listed in Table 2.

Immunoblotting: Both the treated and untreated HCF cultures were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA) followed by centrifugation at 10,000 g for 10 min. Samples were suspended in a NuPAGE LDS buffer containing a reducing agent (Life Technologies Corporation, Grand Island, NY) and heated at 70 °C for 10 min. Protein samples were resolved by NuPAGE Novex Bis-Tris mini gels (Invitrogen) and transferred onto the polyvinylidene difluoride membranes using wet transfer at 25 V. The transferred proteins were detected by incubating the membrane with primary antibodies: TGIF1, TGIF2 (Santa Cruz Biotechnology, Santa Cruz, CA), acetylhistone H3,

acetyl histone H4 (EMD Millipore, Billerica, MA), alpha smooth muscle actin (αSMA Dako, Carpinteria, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology), followed by alkaline phosphatase conjugated anti-mouse, anti-goat, or anti-rabbit secondary antibody. After washing three times in 0.05% Tween-20 in Tris-buffered saline of pH 8.0 for 5 min each, the blot was developed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate method. Three separate western blots were performed for each experiment. The digital quantification of western blots was performed using NIH Image J software.

Immunofluorescence: Cells were fixed with 4% paraformaldehyde, and immunofluorescence staining for α–smooth muscle actin (1:200 dilution) and acetyl histone H3 (1:100 dilution EMD Millipore, Billerica, MA) was performed. Samples were incubated with 5% BSA for 30 min at room

TABLE 2. SEQUENCES OF PRIMERS USED IN THE STUDY.			
Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Accession Number
For Real Time PCR			
Smooth muscle actin	TGGGTGACGAAGCACAGAGC	CTTCAGGGGCAACACGAAGC	NM_001613
TGIF1	CTGTGTTTCGAGGATGGTTC	CATGGAAAGGAATCAGAGCC	NM_170695
TGIF2	CCTGTCAGTGCTGCAAAT	GGCGGGAAATGGTAAACT	NM_021809.5
β-actin	CGGCTACAGCTTCACCACCA	CGGGCAGCTCGTAGCTCTTC	NM_007393.4
For PCR			
TGIF1	GACATTCCCTTGGACCTTTCT	GACCACTCTGTGTATTCGTACTT	NM_170695
TGIF2	GGCACCTAGCAGAGAAA-GATAAG	GGGAGGCTGAGGCAAATAAT	NM_021809.5

temperature, followed by primary antibody incubation for 90 min. The secondary antibodies Alexa 488 donkey anti-rabbit and Alexa 594 donkey anti-mouse IgG secondary antibody (1:500 dilution) were incubated for 1 h. The cells were washed three times in PBS buffer, mounted in Vectashield containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and viewed and photographed with a Leica fluorescent microscope (Leica DM 4000B) equipped with a digital camera (SpotCam RT KE).

Statistical analyses: Results were expressed as a mean ± standard error. Statistical analysis was performed using one-way ANOVA and Tukey's test. A value of $p < 0.05$ was considered significant.

RESULTS

Detection of TGIF1 and TGIF2 in human corneal fibroblasts: To investigate whether human corneal fibroblasts express TGIFs, PCR was performed to detect the presence from mRNA transcripts for TGIF1 and TGIF2. Figure 1 shows the agarose gel electrophoresis of the PCR demonstrating the presence of amplified bands for TGIF1 and TGIF2 in the cDNA obtained from human corneal fibroblasts. An appropriately sized amplification product of 608 bp was detected for TGIF1 in two cDNA samples of human corneal fibroblasts originating from two separate donor human corneas. Amplification products for TGIF2 corresponding to the product size of 646 bp were also present in the human corneal fibroblast cDNA samples. No PCR product was detected in the negative controls that contained primers for TGIFs but no cDNA (Figure 1).

Effect of vorinostat on TGIF1 and TGIF2 expression and histone acetylation: After confirming that human corneal fibroblasts express TGIF1 and TGIF2, we next tested if

vorinostat treatment of human corneal fibroblasts increased TGIF expression. The mRNA and protein lysates were obtained from the human corneal fibroblasts +/− vorinostat. As is evident from Figure 2A, treatment of human corneal fibroblasts with vorinostat (2.5 µM) caused a 2.8±0.81-fold increase ($p < 0.05$) in TGIF1 mRNA and 3.3±0.25-fold increase ($p < 0.01$) in TGIF2 mRNA levels as compared to DMSO-treated vehicle control groups (−vorinostat). Figure 2B shows the western blot detection of TGIF protein levels in DMSO-treated vehicle control and vorinostat (2.5 µM)-exposed human corneal fibroblasts. The immunoblotting data demonstrated that vorinostat caused a 1.4-fold increase ($p < 0.05$) in TGIF1 and 1.8-fold increase ($p < 0.05$) in TGIF2 protein in comparison to vehicle-treated control human corneal fibroblasts. Further, we also tested the effect of vorinostat on TGIF1 and TGIF2 expression in corneal keratocytes. The corneal keratocytes were isolated using dispase and collagenase enzymatic digestions and grown in serum-free medium in the presence or absence of vorinostat. Our results demonstrate that vorinostat increased the TGIF1 and TGIF2 mRNA in human corneal keratocytes as observed in human corneal fibroblasts (data not shown).

We further postulated that the observed increase in TGIFs was likely mediated by enhanced transcription due to histone acetylation and DNA unfolding caused by vorinostat. To test whether the 2.5 µM dose of vorinostat used in the present study was sufficient to cause histone acetylation, we performed immunoblotting for acetylated histone H3 and acetylated histone H4. As can be seen in Figure 3A, vorinostat treatment of human corneal fibroblasts caused an 8-fold increase ($p < 0.001$) in acetylhistone H3 and a 50-fold increase ($p < 0.001$) in acetylhistone H4. Figure 3B demonstrated that only 36±4% of nuclei stained +ve for acetylhistone H3 in DMSO-treated vehicle control corneal
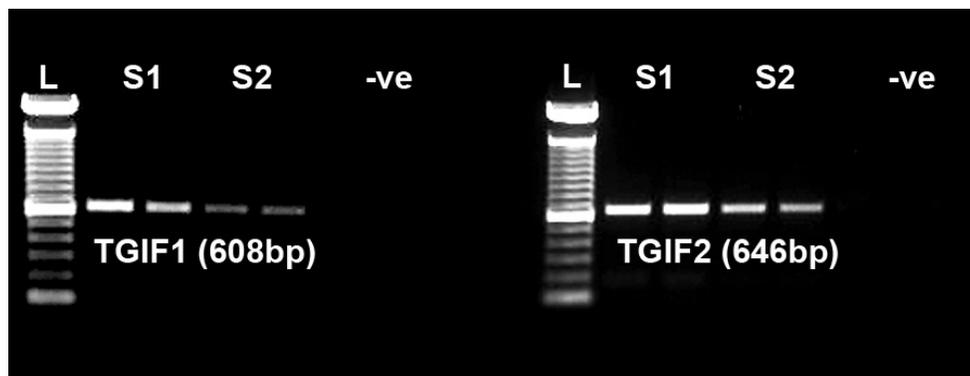

Figure 1. Agarose gel electrophoresis of PCR showing the detection of TGIF1 and TGIF2 mRNA expression in human corneal fibroblasts. Appropriately sized PCR amplification products for TGIF1 and TGIF2 were detected in two independent cDNA samples (S1 and S2) of corneal fibroblasts prepared from two separate human donor corneas. The lower level of TGIF detected in sample S2 is due to a smaller amount of starting cDNA in the PCR reaction. -ve denotes negative controls that contained TGIF1 and TGIF2 primers but water instead of cDNA. L denotes a 100-base-pair DNA ladder.
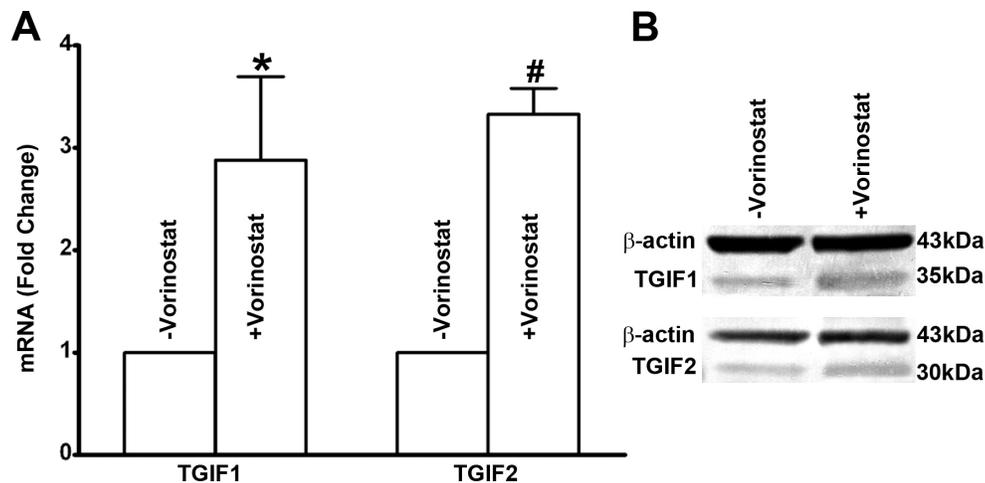
Figure 2. Quantitative real-time PCR and immunoblotting demonstrating the effect of vorinostat (2.5 µM) on TGIF1 and TGIF2 mRNA and protein levels. A significant increase in TGIF1 and TGIF2 mRNA (**A**) and protein (**B**) levels is evident after vorinostat treatment (+vorinostat) as compared to DMSO-treated vehicle controls (−vorinostat). * $p < 0.05$, # $p < 0.01$ compared to vorinostat.

fibroblasts (−vorinostat). On the other hand, vorinostat treatment of human corneal fibroblasts significantly increased the acetylhistone H3 to 75±3.4% ($p < 0.001$) nuclei showing +ve staining. A similar trend demonstrating the increase in acetylhistone H4-stained nuclei was also observed with vorinostat (data not shown). No statistically significant difference in the total number of DAPI-stained nuclei (blue) was observed in the control and vorinostat-exposed human corneal fibroblasts (Figure 3B).

Effect of Smad knockdown on TGFβ1-induced myofibroblasts formation: TGIFs are negative regulators of TGFβ1-mediated Smad signaling. Therefore, we next tested the hypothesis that TGFβ1-mediated transdifferentiation of human corneal fibroblasts to myofibroblasts is largely mediated through Smad signaling. To test this hypothesis, we transfected the human corneal fibroblasts with plasmids expressing Smad2/Smad3 or Smad4 RNAi. Figure 4 shows the effect of RNAi-mediated knockdown of Smad2/Smad3 or Smad4 on TGFβ1-evoked SMA formation. As is evident from Figure 4, the knockdown of Smad2/Smad3 or Smad4 markedly attenuated TGFβ1-mediated increase in αSMA in cultured human corneal fibroblasts tested using immunofluorescence (Figure 4A-E) and immunoblotting (Figure 4F). This suggests that TGFβ1-mediated transdifferentiation of human corneal fibroblasts to myofibroblasts is largely mediated through Smad signaling.

Effect of vorinostat on myofibroblast formation: Next, we tested the hypothesis that vorinostat-induced increases in TGIF1 and TGIF2 were accompanied by a concurrent decrease in corneal fibrosis. An in vitro model of corneal fibrosis in which the application of TGFβ1 induces myofibroblasts formation was used to test this hypothesis. The transformation of corneal fibroblasts to myofibroblasts is a key pathological feature in corneal fibrosis. Myofibroblasts are

contractile, metabolically active, and opaque cells containing intracellular microfilament bundles of αSMA, which serves as a maker for fibrosis. As expected, TGFβ1 treatment of human corneal fibroblasts caused a 3.8±0.2-fold increase ($p < 0.001$) in αSMA mRNA (Figure 5A) and a 4.3-fold increase ($p < 0.01$) in SMA protein (Figure 5B). Vorinostat treatment significantly attenuated TGFβ 1-induced increases in αSMA mRNA by 83±7.7% ($p < 0.001$, Figure 5A) and protein levels by 97±5% ($p < 0.001$, Figure 5B).

The antifibrotic effects of vorinostat were further investigated by αSMA immuno-cytochemistry (Figure 6). As anticipated, human corneal fibroblasts cultured in the absence of TGFβ1 (−TGFβ) under serum-free conditions showed minimal αSMA expression (Figure 6). TGFβ1 (5 ng/ml) treatment caused a robust myofibroblast formation, as indicated by an intense αSMA immunostaining in 82%–94% of cells ($p < 0.001$). Following vorinostat treatment, only 6%–8% of cells ($p < 0.001$) showed αSMA immunostaining, confirming that vorinostat remarkably attenuates TGFβ1-evoked myofibroblast formation (Figure 6).

Effect of TGIFs knockdown on the antifibrotic effect of vorinostat: Finally, we tested whether the increase in TGIF expression is an essential step for the antifibrotic effect of vorinostat. If so, then the TGIF knockdown should impede the antifibrotic effect of vorinostat. To test this hypothesis, we designed a reverse experiment wherein we tested the antifibrotic effect of vorinostat along with siRNA-mediated knockdown of TGIFs. Figures 5A and B show that TGIF1 and TGIF2 knockdown prevented vorinostat-mediated decreases in αSMA mRNA by 31%–45% ($p < 0.05$) and protein levels by 12%–23% ($p < 0.05$), respectively. Similarly, immunostaining data for αSMA shows that TGIF1 or TGIF2 siRNA knockdown significantly prevented vorinostat-induced decreases

in myofibroblast formation. Only 6%–8% of cells (p<0.05) showed αSMA immunostaining when exposed to vorinostat alone, whereas 12%–18% of cells (p<0.05) stained for αSMA when cells were exposed to vorinostat after either TGIF1 or TGIF2 knockdown (Figure 6). Overall, our data suggest that the knockdown of either TGIF1 or TGIF2 partially blocks the antifibrotic effect of vorinostat.

To test whether the simultaneous knockdown of both TGIF1 and TGIF2 had any additive or synergistic effect on blocking the antifibrotic effect of vorinostat, we treated the cells with siRNA of both TGIF1 and TGIF2. However, the simultaneous knockdown of TGIF1 and TGIF2 did not cause any additive neutralization of vorinostat on αSMA mRNA (Figure 5A) or protein (Figure 5B and Figure 6) as compared to the knockdown of either the TGIF1 or TGIF2 alone.

DISCUSSION

TGIFs belong to the three-amino acid loop extension (TALE) family of homeodomain proteins consisting of two transcription factors, TGIF1 and its closely related paralog TGIF2. TGIFs are highly expressed during mammalian embryonic development and have been shown to play a critical role in the development of many organs, including the eyes [28-30]. However, after birth, TGIF expression sharply declines, with only a handful of tissues showing detectable levels of TGIFs in adult animals [25,27]. In the present study, we demonstrate that human corneal fibroblasts express both TGIF1 and TGIF2. A literature survey reveals that very limited information is available concerning the biologic functions of TGIFs in fully differentiated adult tissues. Therefore, our data open up
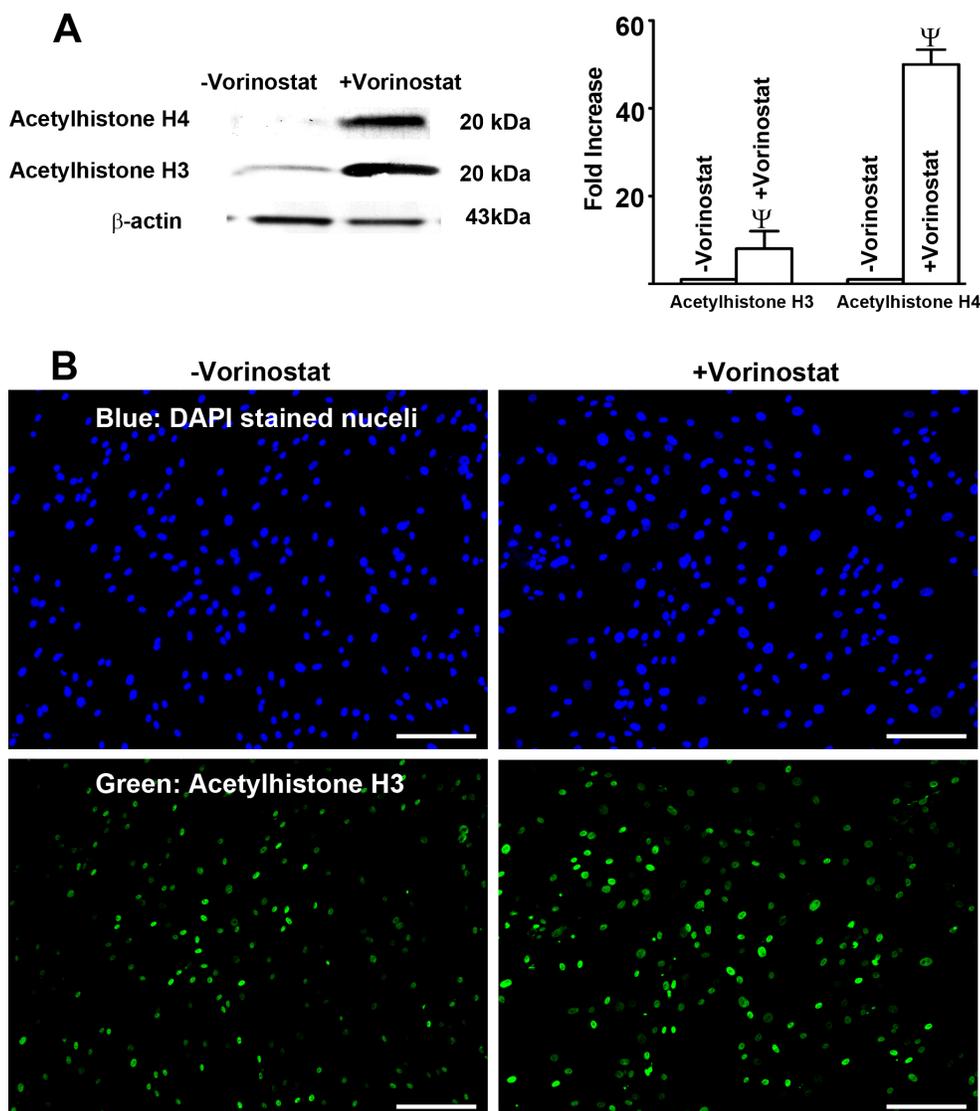

Figure 3. Representative immunoblotting and immunofluorescence images showing the effect of vorinostat (2.5 µM) on histone acetylation. Immunoblotting image and the quantification graph (**A**) showing that vorinostat (+vorinostat) caused eight fold and 50 fold (Ψ p<0.001) increase in acetylhistone H3 and acetylhistone H4 protein levels in comparison to DMSO-treated vehicle controls (–vorinostat). Immunofluorescence images (**B**) show significantly more nuclei (p<0.001) stained +ve for acetylhistone H3 (Green) in vorinostat-exposed human corneal fibroblast cultures (+vorinostat) as compared to non-treated controls (–vorinostat). The DAPI-staining (blue) depicts that there was no significant difference in the total number of nuclei between control and vorinostat-treated cultures. Scale bar=100 µm.
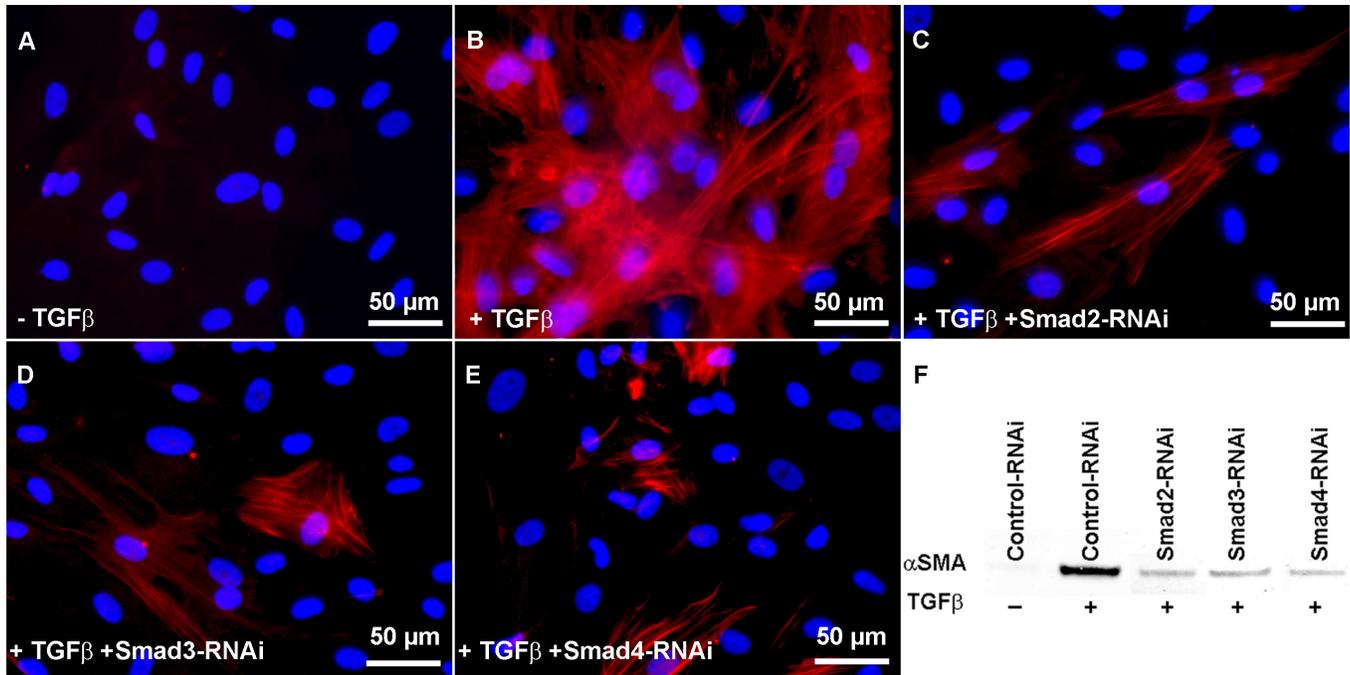
Figure 4. Representative immunofluorescence images and immunoblotting showing the effect of RNAi-mediated Smad2/3/4 knockdown on αSMA. Control human corneal fibroblast cultures (–TGFβ) showed no αSMA staining (**A**) or protein levels (**F**). Cultures treated with TGFβ1 (5 ng/ml) showed robust αSMA staining (**B**) and protein levels (**F**). The knockdown of Smad2 (**C**, **F**)/Smad3 (**D**, **F**)/Smad4 (**E**, **F**) markedly blocked TGFβ-evoked increase in αSMA.

the exciting possibility of investigating the biologic functions of these transcription factors in the corneal tissue.

HDACs are a group of enzymes that deacetylate histone and non-histone proteins and are critically involved in the epigenetic regulation of gene transcription, cellular proliferation, survival, and differentiation [31-33]. Given their critical role in modulating cell cycles, HDACs have been extensively studied in cancer biology. In addition, the role of HDACs and

epigenetic modulation has been implicated in many other diseases, including fibrosis, autoimmune, inflammatory, and metabolic diseases processes [34-40]. This has led to the testing of a variety of HDAC inhibitors to treat fibrotic diseases, inflammatory bowel diseases, multiple sclerosis, and systemic lupus erythematosus [34-40]. Our laboratory has recently demonstrated that the HDAC inhibitor trichostatin A and its FDA-approved derivative, vorinostat, effectively
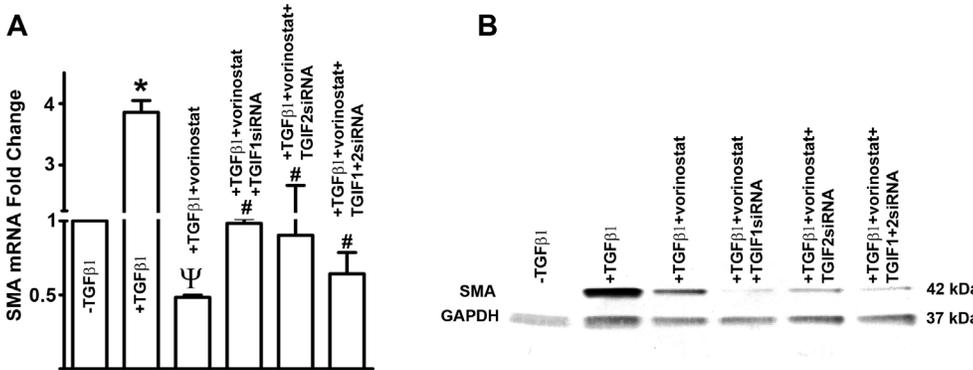

Figure 5. Quantitative real-time PCR and immunoblotting showing the effect of vorinostat (2.5 μM), TGIF1, and TGIF2 siRNA knockdown on αSMA protein levels. Control human corneal fibroblast cultures (–TGFβ) showed no αSMA expression. Cultures treated with TGFβ1 (5 ng/ml) showed robust αSMA increase. Vorinostat (+TGFβ+vorinostat) treatment markedly attenuated TGFβ-evoked αSMA mRNA (**A**) and protein (**B**) expression. The knockdown of TGIF1 (+TGFβ+vorinostat+TGIF1siRNA) and TGIF2 alone (+TGFβ+vorinostat+TGIF2siRNA) or in combination (+TGFβ+vorinostat+TGIF1+2siRNA) significantly reversed the effect of vorinostat on αSMA. * $p<0.001$ compared to –TGFβ, Ψ $p<0.001$ compared to +TGFβ, # $p<0.05$ compared to +TGFβ+vorinostat.

reduce corneal fibrosis primarily through the inhibition of TGFβ effects [14-17]. Precisely how HDAC inhibitors block the biologic effects of TGFβ is not known. TGIFs are known transcriptional repressors of TGFβ-mediated Smad signaling, and our RNAi data suggest that TGFβ-driven transdifferentiation of human corneal fibroblasts to myofibroblasts is largely mediated through Smad signaling. TGIFs have been shown to directly associate with Smad2/3 and inhibit TGFβ-driven profibrotic gene expression [23]. Therefore, we postulated that HDAC inhibitors attenuate corneal fibrosis by increasing TGIFs. Indeed, the detection of significantly increased levels of TGIF mRNA and protein in vorinostat-treated human corneal fibroblasts not only supports our hypothesis but also provides the evidence that TGIF levels can be potentially altered in the adult human cornea through pharmacological manipulation. Furthermore, vorinostat-induced increases in TGIFs were concurrently accompanied by a remarkable increase in the acetylation of histones H3 and H4, which is a well-known effect of HDAC inhibitors. Histone acetylation increases DNA transcriptional activity, and may explain the

increased transcriptional levels of TGIFs in human corneal fibroblasts following vorinostat treatment. Next, we reasoned that, if increased TGIF expression is an essential step for the antifibrotic effect of vorinostat, then the drug should lose its antifibrotic effect without TGIFs. To test this hypothesis, we used siRNAs to knockdown TGIFs. We observed that vorinostat showed a significantly diminished antifibrotic effect, as indicated by increased αSMA when either TGIF1 or TGIF2 was knocked down. These results suggest that TGIFs are vital for the antifibrotic effect of vorinostat in the cornea. However, it is worthwhile to point out that only a partial reversal of the antifibrotic effect of vorinostat was achieved by knocking down the TGIFs. Multiple factors can account for this observed partial reversal. TGIFs are short-lived proteins and undergo rapid synthesis and degradation [41]. Therefore, the noted partial reversal of vorinostat effects could be due to the quick de novo synthesis of TGIFs that can partially counterbalance the siRNA knockdown. Alternatively, vorinostat may have caused a concurrent increase in the transcription levels of other know TGFβ co-repressors,
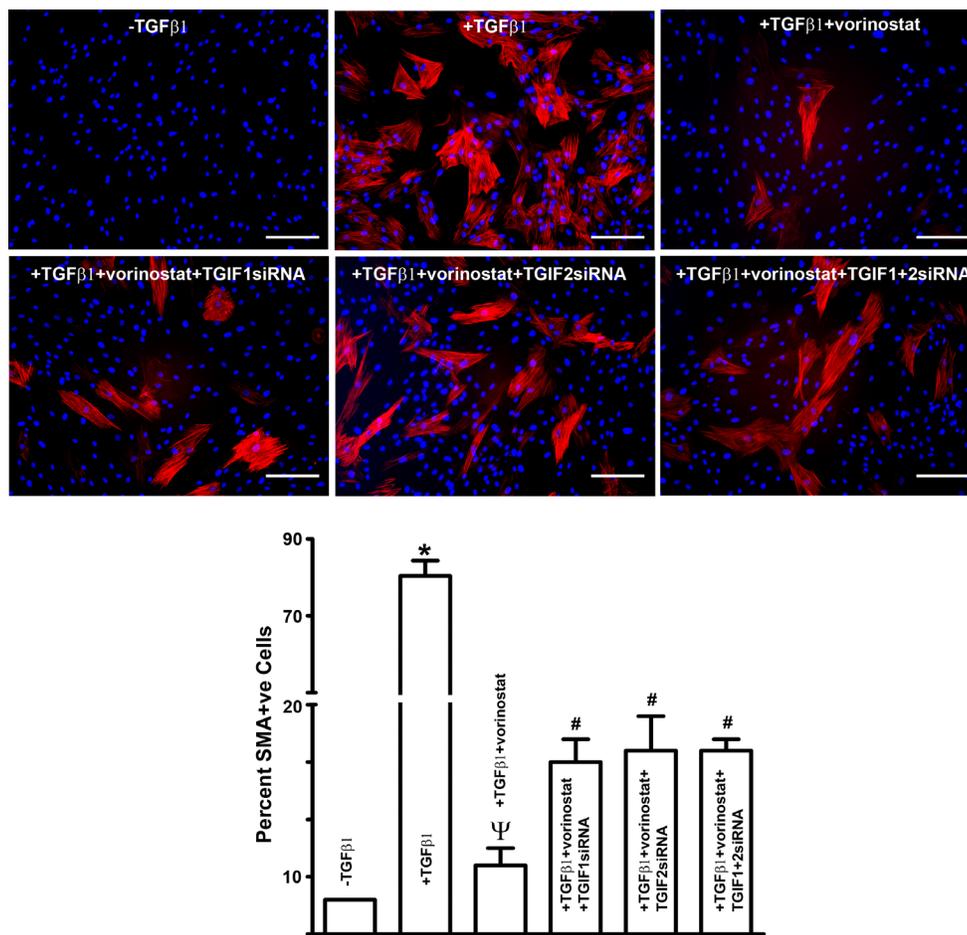

Figure 6. Representative immuno-fluorescence images and the quantification graph showing the effect of vorinostat (2.5 µM), TGIF1, and TGIF2 siRNA knockdown on αSMA. Control human corneal fibroblast cultures (-TGFβ) showed no αSMA staining. Cultures treated with TGFβ1 (5 ng/ml) showed a robust αSMA staining. Vorinostat treatment markedly attenuated TGFβ-evoked αSMA staining. The knockdown of TGIF1 (+TGFβ+vorinostat+TGIF1siRNA) and TGIF2 alone (+TGFβ+vorinostat+TGIF2siRNA) or in combination (+TGFβ +vorinostat +TGIF1 +2siRNA) markedly blocked the effect of vorinostat on αSMA. Scale bar=100 µm. * p<0.001 compared to −TGFβ, Ψ p<0.001 compared to +TGFβ, # p<0.05 compared to +TGFβ+vorinostat.
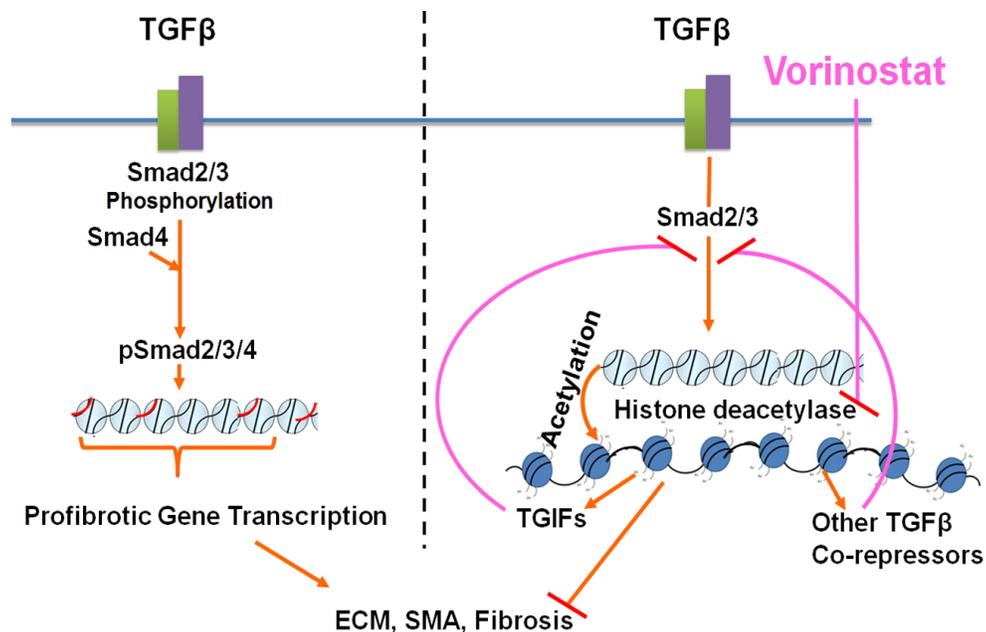
Figure 7. Schematic representation of the role of TGIFs in the antifibrotic effect of vorinostat in the cornea.

such as c-Ski, SnoN, Smad6, and Smad7 [18-20], and in that case, the selective knockdown of only TGIFs will merely partially reverse the antifibrotic effects of vorinostat. Future studies will test the role of other TGFβ co-repressors in modulating the antifibrotic effect of vorinostat.

In conclusion, we demonstrate that human corneal fibroblasts express TGIF1 and TGIF2 transcription factors. These transcriptional repressors are critical, at least partially, in mediating the antifibrotic effect of vorinostat in the cornea (Figure 7).

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