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YAP and TAZ are distinct effectors of corneal myofibroblast transformation

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Abstract

Purpose: Transforming growth factor β1 (TGFβ1) is elevated in wounds after injury and promotes the transdifferentiation of quiescent cells in the stroma (keratocytes, to activated fibroblasts and subsequently myofibroblasts-KFM transformation). Coactivators of transcription, YAP (Yes-associated protein) and TAZ (Transcriptional coactivator with PDZ-binding motif), are mechanotransducers that intersect with the TGFβ pathway via interactions with Smad proteins. Here, we examined the distinct role of YAP and TAZ on TGFβ1 induced myofibroblast transformation of primary human corneal fibroblasts (HCFs).

Methods: A knockdown approach was used to silence YAP and TAZ individually in HCFs. Forty-eight hours post siRNA transfection, cells were cultured in the presence or absence of 2 ng/ml TGFβ1 for 24h. The cells were subjected to nuclear and cytoplasmic fractionation. The expression of α-smooth muscle actin (αSMA), Smad 2, 3 and 4, CTGF and phospho-Smad2, 3, and 4 were assessed by qPCR and Western blotting.

Results: TGFβ1 stimulation resulted in the decreased phosphorylation of YAP in the cytosol, and increased levels of phosphorylated TAZ and Smad2/3/4 in the nucleus. Knockdown of TAZ resulted in elevated YAP expression but not vice versa. Additionally, knockdown of TAZ but not YAP resulted in upregulation of αSMA expression in the presence and absence of TGFβ1. In the presence of TGFβ1 YAP knockdown increased Smad2/3/4 expression and Smad4 phosphorylation, while TAZ knockdown had no effect on Smad2/3/4 expression and

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Appendix A. Supplementary data

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phosphorylation. YAP knockdown inhibited CTGF expression while TAZ knockdown resulted in its increased expression. Finally, simultaneous knockdown of YAP and TAZ resulted in cell death.

Conclusion: Our findings suggest that YAP and TAZ function as distinct modulators of TGFβ1 induced myofibroblast transformation and have different roles in signalling. Specifically, TAZ limits YAP's ability to mediate KFM transformation via Smad proteins. The data also suggest that while having distinct effects, YAP and TAZ have redundant or combinatorial functions critical to cell survival. These results suggest that a loss of TAZ may help drive corneal haze and fibrosis and that the balance between YAP/TAZ is essential in controlling myofibroblast differentiation.

1. Introduction

A healthy transparent cornea is essential for vision. This is dependent on its structural integrity which can be damaged upon exposure to various biological, chemical and/or physical insults. Keratoablative surgical procedures such as laser-assisted in situ keratomileusis (LASIK) and photorefractive and phototherapeutic keratectomies (PRK and PTK) create substantial wounds in the corneal stroma which can lead to corneal haze and vision impairment if the repair process is dysregulated. Healing of the wounded cornea is a complex process that involves cell proliferation, migration, differentiation, and extracellular matrix (ECM) remodelling. During this process, integration of biochemical and biomechanical factors tightly control the transformation of quiescent keratocytes to fibroblasts and subsequently to contractile myofibroblasts, a process known as KFM transformation (Jester JV et al., 1995; Netto MV et al., 2005; Snyder MC and Doughty, 1998).

The transforming growth factor β (TGFβ) superfamily of proteins are potent regulators of extracellular matrix (ECM) turnover. Of particular interest, TGFβ1 is a potent pro-fibrotic cytokine that plays a central role in the transition of fibroblasts into myofibroblasts during corneal wound repair (K, 2003; Nakamura et al., 2001). We have recently demonstrated that the mechanical properites of the corneal stroma are dynamically altered throughout wound repair (Raghunathan et al., 2017). Our lab and others have consistently shown that mechanical properties of the cellular microenvironment have a profound influence on cellular phenotype, differentiation, and response to drugs (Lee et al., 2016; Petroll and Lakshman, 2015; Shin and Mooney, 2016; Thomasy et al., 2018; Thomasy SM et al., 2012; Vining and Mooney, 2017; Wei et al., 2015). With specific relevance to corneal wound healing, we showed that a stiffer substrate promoted KFM transformation and this was enhanced in the presence of TGFβ1 (Dreier et al., 2013). Studies have shown that activation of TGFβ signalling leads to the translocation of *Yorkie* homologues YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ binding motif) to the nucleus (Fujii et al., 2012a, b; Grannas et al., 2015; Mauviel et al., 2012). YAP and TAZ were identified as nuclear relays of mechanical signals exerted by alterations in the extracellular environment (Dupont et al., 2011; Wada et al., 2011). The TAZ protein is encoded by the gene *WWTR1.* In this study, both the mRNA and the protein encoded by the *WWTR1* gene are termed TAZ.

The function of YAP and TAZ are distinct and are highly specific to sub-cellular localization. For instance, YAP in the cytoplasm synergizes with Smad7 to co-repress Smad

signalling (Ferrigno et al., 2002), but in the nucleus, it can elicit transcriptional regulation through association with Smad2/3/4. Canonical Smad signalling is initiated with the binding of TGFβ to its receptor at the cell surface resulting in the phosphorylation of Smad2 and Smad3 proteins (pSmad2/3) in the cytoplasm (Varelas et al, 2010a, b). Phosphorylated Smad2/3 complex then interacts with Smad4 to form a trimeric Smad2/3/4 complex that subsequently binds to cytoplasmic YAP/TAZ leading to nuclear translocation and activation of the transcription cascade by binding to the transcriptional enhanced associate domain/transcription enhancing factor (TEAD/TEF) family of transcriptional factors (Fujii et al., 2012a, b; Varelas et al, 2010a, b; Varelas et al., 2008). Transcriptional activation by YAP/TAZ can induce upregulation of multiple genes known to be important in corneal wound healing including growth factors (TGFβ and CTGF (Sudol, 2010; Vassilev et al., 2001; Yagi et al., 1999; Zhao et al., 2008)) and integrins (α3β1, αvβ3)(Dupont et al., 2011; Halder et al., 2012). Both YAP and TAZ can interact with TEAD independently and recent reports suggest that YAP/TAZ co-activators have non-redundant functions in regulating gene expression. In this study we specifically investigated the effect of individual knockdown of YAP or TAZ on corneal stromal cell response in the presence or absence of TGFβ1.

2. Methods

2.1. Cell isolation and culture

Primary human corneal fibroblasts (HCFs) were isolated from donor corneal buttons (Saving Sight, Springfield, MO) as described previously (Thomasy SM et al., 2012; Wood JA et al., 2011). Isolated cells were maintained in DMEM with 2.5 mM L-glutamine (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrence, GA) and 1% penicillin/streptomycin/amphotericin B (Lonza, Walkerville, MD). Cells in passages 2 to 6 were used for all experiments.

2.2. Gene knockdown by siRNA transfection

Using siRNA knockdown approach, we investigated the distinct role of YAP or TAZ on TGFβ1 mediated myofibroblast transformation. Once the HCFs reached 70–80% confluency, siRNA transfections were performed using a transfection reagent (Hi Perfect transfection reagent; 301705; Qiagen) following the manufacturer's instructions with final concentrations of 30 nM of YAP (S102662954; Qiagen), WWTR1 (TAZ) (S100111216; Qiagen) and siControl (1022076; Qiagen). For all knockdown experiments, cells were transfected with 30 nM control siRNA or siRNAs to YAP or TAZ, or siRNAs to YAP and TAZ (for double knockdown) for 48h and followed by 24h TGFβ1 (2 ng/ml) or vehicle control treatments. Subsequently, the cells were rinsed with phosphate buffered saline and RNA or protein was extracted. A knockdown efficiency of over 70% was achieved as determined by qPCR analyses. All experiments were performed at least three times.

2.3. RNA isolation and quantitative real-time PCR

RNA was isolated using an RNA purification kit (RNeasy Mini Kit; Qiagen, Valencia, CA) following the manufacturer's instructions and equal amounts were used for the quantitative real time PCR (qPCR) reactions. qPCR was performed using a reagent kit (TaqMan One-Step RT-PCR Master Mix Reagents Kit; Applied Biosystems, Carlsbad, CA) and

commercially available aptamers for GAPDH (Hs99999905_m1); 18S (Hs99999901_s1); YAP (Hs00371735_m1); CTGF (Hs00170014_m1); WWTR1 (Hs00210007_m1), Smad2 (Hs00183425_m1); Smad3 (Hs00969210_m1); and Smad4 (Hs00929647_m1) in total volumes of 10 μL per reaction (Applied Biosystems). The reverse transcription reaction was performed in a qPCR machine (StepOne; Applied Biosystems) with the following parameters: Thirty minutes at 50 °C followed by 10 min at 95 °C, and the product was amplified with the following parameters: 40 cycles of 60 °C for 1 min followed by 95 °C for 15 s. Relative expression of the mRNAs of interest were normalized to the expression of the ribosomal RNA 18S or GAPDH. The experiments were performed at least three times. Gene expression was normalized relative to the expression of mRNA from cells without TGFβ1 which was given a value of 1.0. In brief, equal amounts of RNA (10 ng) were loaded for all PCR reactions to account for variations in cell density. The C_t values obtained represent logarithmic changes in gene expression. The difference in C_t (i.e., ΔC_t) between the gene of interest (e.g., YAP, TAZ) and the calibrator gene (18S or GAPDH) were calculated. Next, the $\Delta\Delta C_t$ values of TGFβ treated samples were normalized with the control sample data.

2.4. Protein isolation and western blotting

Cells were washed once in PBS, lysed and scraped into RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Fisher Scientific, Hampton, NH) on ice. Cell lysate was homogenized and centrifuged at 1000g for 1 min to remove any cell debris. Protein was quantified using a modified Lowry assay (DC assay; Bio-Rad Laboratories) with bovine serum albumin as the standard. Protein homogenate was then denatured in Laemmli buffer (Sigma-Aldrich) by boiling for 20 min. Approximately 20 μg protein was loaded per well for each sample. Protein was separated on precast gels (NuPAGE 10% Bis-Tris; Invitrogen) and transferred onto nitrocellulose membranes. Immunoblotting was done against anti-human YAP-H9 (sc-271134, Santa Cruz Biotechnologies, Santa Cruz, CA); TAZ-H70 (sc-48805, Santa Cruz Biotechnologies, Santa Cruz, CA); and pan-actin (MAB1501, Millipore), pTAZ (ser 89, sc-17610; Santa Cruz Biotechnologies); pYAP (S127, D9W21; Cell Signalling); pSmad4 (T277, PA5–12695; Thermo Scientific); Smad4 (B-8, sc-7966; Santa Cruz); pSmad3 (S423 + S425, ab52903; Abcam); Smad3 (C67H9, Cell Signalling); pSmad2 (S467, ab53100; Abcam); Smad2 (D43B4, Cell Signalling), αSMA (A5228; Sigma), β-Tubulin (ab15568, abcam), GAPDH (CS207795, Millipore), CTGF (ab6992; Abcam) and Lamin B1 (sc-20682, Santa Cruz Biotechnologies) overnight at 4 °C. This was followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP; SeraCare) for 1 h at 37 °C. The bands were then amplified and detected calorimetrically following protocols detailed using a substrate kit (Amplified Opti-4CN kit; Bio-Rad Laboratories). Blots were then imaged using an imaging system (UVP ChemiDoc-It™2, UVP LLC). The optical densities of the protein bands were quantified using a Java-based image processing program (ImageJ; National Institutes of Health) (Schneider et al., 2012).

2.5. Cytoplasmic and nuclear fractionation

To determine whether TGFβ1 stimulation modulated phosphorylation and sub-cellular localization of YAP/TAZ and Smad2/3/4 in primary HCFs, cells were washed with PBS, trypsinized and subcellular franctionation was performed following manufacturer's protocol

using the NE-PER™ kit (78833, Thermo Fisher Scientific). All samples were kept on ice throughout the procedure. The lysates were stored in −80 °C for 24h. Protein quantification and western blot analysis were performed as described above. For quantitative analyses, all cytoplasmic protein expression were normalized to GAPDH in control cells (no TGFβ1 stimulus) while nuclear protein expression were normalized to Lamin B1 in control cells (no TGFβ1 stimulus). Where whole cell lysates were used to perform Western blots (i.e. in the siRNA experiments) all protein expression were compared with siCtrl treated cells without TGFβ1 stimulus.

2.6. Statistics

Statistical significance was calculated using one-way ANOVA followed by Dunnett's multiple comparison test or Student's t-test as appropriate. Specifically, in sub-cellular fractionation experiments, cytoplasmic or nuclear protein expression in TGFβ1 stimulated cells were compared with control cells (no TGFβ1 stimulus) using Student's t-test. In siRNA experiments, using one-way ANOVA and Dunnett's post-hoc comparison, protein levels were evaluated comparing with siCtrl treated cells without TGFβ1 stimulus. For experiments comparing protein amounts within each subcellular fraction (i.e. cytoplasmic or nuclear), comparisons were performed by Student's t-test. For all gene expression experiments, comparisons were performed by one-way ANOVA followed by Dunnett's multiple comparison test with control siRNA cells in the absence of TGFβ1 stimulus as the control. For all other experiments where the effect of YAP/TAZ knockdown on protein levels was determined, comparisons were performed by one-way ANOVA followed by Dunnett's multiple comparison test with control siRNA cells in the absence of TGFβ1 stimulus as the control. For all analyses, a P value of < 0.05 was considered significant.

3. Results

3.1. YAP phosphorylation is decreased and TAZ phosphorylation is enhanced by TGFβ1

In the absence of TGFβ1 stimulus, YAP was observed to be predominantly in the cytoplasm in its phosphorylated form (S127) with minimal protein amounts detected in the nuclear fraction (Fig. 1). By contrast, total TAZ was significantly overexpressed in the nucleus with a minimal amount of its phosphorylated form (S89) in the cytoplasm. In addition, the overall amount of TAZ is greater than that of YAP in HCFs. With TGFβ1 treatment, we observed a decrease in pYAP (*$p < 0.05$) amounts in the cytoplasmic fraction; concurrently a subtle but significant increase in nuclear YAP was observed. We note that quantitation was hampered by the absence of nuclear pYAP in unstimulated cells. Further, in the nucleus, total TAZ levels were reduced (*$p < 0.05$) while pTAZ levels increased (*$p < 0.05$) with TGFβ1 stimulus. This was accompanied by significant increases in pSmad2/3/4 amounts in the nuclear fraction (*$p < 0.05$ for pSmad 3 and ***$p < 0.001$ for pSmad 2/4) with TGFβ1 stimulus suggesting that pTAZ forms a complex with pSmad2/3/4 to translocate to the nucleus.

3.2. YAP and TAZ act distinctly to induce fibroblast transformation

We observed that knockdown of TAZ resulted in 2-fold elevation of YAP mRNA expression (Fig. 2a; $p < 0.05$), while YAP knockdown did not affect TAZ expression (Fig. 2b). When

these cells were stimulated with TGFβ1, we observed a potent overexpression of αSMA in the siTAZ group greater than that observed in siCtrl or siYAP groups (Fig. 2c and d; *p < 0.05, ***p < 0.001). A double knockdown of YAP and TAZ resulted in cell death (data not shown) suggesting that YAP and TAZ are have redundant or combinatorial effects essential for corneal stromal cell viability.

3.3. YAP and TAZ knockdown differentially modulates the expression and phosphorylation of Smad 2, 3, and 4 proteins

Knockdown of YAP and/or TAZ was confirmed by western blotting (Fig. 3a and b; ***p < 0.001). With YAP knockdown and in the absence of TGFβ1, Smad4 expression and phosphorylation was increased by ~2–~3 fold (**p < 0.01) respectively, Smad 2 expression was increased by ~1.5 fold (*p < 0.05), while Smad3 expression and phosphorylation and Smad 2 phosphorylation remained unaltered. With YAP knockdown, TGFβ1 stimulation resulted in a significant upregulation of Smad4 expression and phosphorylation (~2.5–3.5 fold respectively) compared with its untreated siControl counterpart (Fig. 3a). By contrast, TAZ knockdown did not alter Smad2/3 expression (Fig. 3b).

3.4. YAP/TAZ mediated CTGF expression

CTGF is a downstream target of YAP/TAZ signalling (Dupont et al., 2011), a target of TEAD transcription, and a critical cytokine in corneal wound healing (Gibson et al., 2014; Lim et al., 2003; Tall et al., 2010). In the absence of TGFβ1 stimulus, knockdown of YAP did not significantly alter mRNA expression of CTGF (Fig. 4a) although the protein amounts tended to be slightly inhibited (non-significant statistically; Fig. 4b). Stimulation of YAP deficient cells with TGFβ1 restored CTGF (mRNA and protein) expression to those observed in control cells without TGFβ1 stimulus. By contrast, knockdown of TAZ significantly increased CTGF protein amounts, in the presence of TGFβ1, compared to siControl (***p < 0.001, *p < 0.05; Fig. 4a and b) consistent with what was observed with αSMA expression.

4. Discussion

Fibrosis in the corneal stroma is characterized by the accumulation of contractile myofibroblasts, which can be identified by expression of α-smooth muscle actin (αSMA). During routine corneal wound healing, quiescent keratocytes are initially activated to become fibroblasts then subsequently transform to form myofibroblasts, a process known as KFM transformation (Myrna et al., 2009). Potent fibrogenic cytokines such as TGFβ play a significant role in this process and are regulated via a positive feedback mechanism mediated by the Smad pathway. The potency of TGFβ in modulating KFM transformation in corneal stromal cells is largely governed by substratum stiffness (Dreier et al., 2013). Recently we demonstrated that corneal stroma stiffens upon wounding leading to the sequential activation and incidence of αSMA positive myofibroblasts (Raghunathan et al., 2017) suggesting that changes in biophysical properties of tissues are translated to cell differentiation *in vivo*.

Components of the Hippo pathway, a central pathway that effects mechanotransduction, YAP and TAZ, have been shown to form complexes with Smad 2/3 (Varelas et al., 2010a, b; Wrighton et al., 2008) and thus intersect with the TGFβ pathway. The specific functions of YAP/TAZ differ depending on their spatial location (within the nucleus or cytoplasm). When localized to the nucleus, YAP/TAZ interact with transcriptional enhancer associate domain (TEAD) transcription factors and activate the expression of target genes critical to corneal wound healing such as TGFβ2, connective tissue growth factor (CTGF), thrombospondin, transglutaminase-2 (TGM-2), and fibroblast growth factor 2 (Dupont et al., 2011). The interaction of YAP/TAZ with TEAD mediated by Smad2/3 is documented (Hiemer et al., 2014; Liu et al., 2014). Since several studies have irrefutably documented the formation of Smad/YAP/TAZ complexes in the nucleus (Grannas et al., 2015; Piersma et al., 2015; Varelas et al., 2008; Wrighton et al., 2008), we did not attempt to repeat the same findings here. We observed elevated amounts of phosphorylated Smads2/3/4 in nuclei of corneal stromal cells treated with TGFβ1 with concurrent loss of phosphorylation of YAP at Ser 127 in the cytoplasm accompanied by an increase in nuclear YAP as expected. Curiously, an increase in TAZ phosphorylated at Ser 89 was observed in the nuclear fraction and not sequestered in the cytoplasm. LATS-dependent phosphorylation of YAP at Ser 127 or TAZ at Ser 89 has previously been shown to result in cytoplasmic sequestration to limit YAP/TAZ accumulation in the nucleus (Hao et al., 2008; Kanai et al., 2000; Lei et al., 2008) thereby inhibiting their transcriptional activators.

To determine the distinct role that YAP and TAZ have in KFM transformation, and to understand the implication of nuclear Ser 89 TAZ, we performed individual knockdown experiments. A double knockdown of YAP and TAZ in these cells was lethal, strongly suggesting that YAP and TAZ may either have redundant roles in supporting functions critical to corneal stromal cell survival or that they have combinatorial effects whereby the inhibition of distinct downstream effects is lethal *in vitro*. Further studies are required to delineate the specific role of YAP and TAZ in corneal stromal cell survival and is beyond the scope of this manuscript. This is in apparent contrast to the observation in primary dermal fibroblasts where double knockdown of YAP and TAZ induced Smad 7 to impair TGFβ signaling via suppression of Smad 3 phosphorylation (Qin et al., 2018). It is likely that the discrepancy between our findings may be attributed to the difference in cell types – corneal stromal cell vs dermal fibroblasts.

In our study, no change in αSMA mRNA or protein expression was observed when YAP was knocked down, and these cells responded to TGFβ1 with upregulation of αSMA. This was accompanied with elevated Smad2/4 amounts in the presence/absence of TGFβ1 stimulus suggesting that, in corneal stromal cells, TAZ associates with Smad proteins and is sufficient to elicit a response to TGFβ1 stimulus. Confirming this hypothesis, knockdown of TAZ resulted in overexpression of αSMA protein amount in the absence of any stimulus, and this was further enhanced with TGFβ1 treatment. Interestingly, no alterations in phosphorylation of Smad2/3/4 occurred even with TGFβ1 treatment when compared with the control group. This is in apparent contradiction with at least two contrasting studies. In one study upregulation of pSmads was observed in human embryonic stem cells treated with TGFβ1 with TAZ knockdown (Varelas et al., 2008), while in the other individual knockdown of YAP or TAZ did not alter Smad 3 phosphorylation or TGFβ target genes

compared with control siRNA in dermal cell culture (Qin et al., 2018). This further emphasizes the importance of studying signaling mechanisms in appropriate cell type/disease models.

In the present study, we also observed that knockdown of TAZ increased YAP mRNA expression suggesting that in the absence of TAZ, YAP occupies all the transcriptional sites and regulates the expression of αSMA independent of Smad proteins. We next hypothesized that YAP associates with TEAD transcription factor to regulate transcription in the presence of TGFβ1 stimulus. Thus we determined the effect of YAP/TAZ knockdown on CTGF expression, a known TEAD target (Shimomura et al., 2014). Indeed, TAZ knockdown resulted in a significant increase in CTGF while YAP knockdown suppressed its expression. Previous reports document that knockdown of CTGF results in inhibition of accumulation of αSMA positive myofibroblasts and limits fibrosis (Sakai et al., 2017). Although we did not establish the causal relationship between CTGF and αSMA in this study, it is likely that in the absence of TAZ, YAP interacts with TEAD to overexpress CTGF and subsequently αSMA expression. By contrast, in the absence of YAP, TAZ interacts with Smad proteins to mediate αSMA expression. Lending credence to the non-redundant regulatory function of YAP and TAZ in corneal cells, is a recent report that shows that transcriptional co-activator functions of YAP and TAZ are inversely regulated by the tyrosine phosphorylation of parafibromin in HEK cells (Tang et al., 2018). Whether such a system is active in the cornea or implicity partakes in KFM transformation and wound healing remains to be shown.

Collectively, our data suggests that YAP and TAZ have distinct roles in KFM transformation and may interact with the TGFβ pathway partially via Smad proteins (Fig. 5). Specifically, TAZ knockdown resulted in elevated YAP, CTGF and αSMA expression even in the absence of TGFβ stimulus, with no changes in phosphorylation of SMAD proteins suggesting (i) a YAP-TEAD axis for transcriptional regulation, and (ii) that TAZ may function to limit YAP's activity. Conversely, YAP knockdown resulted in elevated phosphorylation of SMAD proteins in the presence of TGFβ1 to effect αSMA expression suggesting a TAZ-SMAD axis for transcriptional regulation. The clinical implications of this diversity in KFM transformation warrant further investigation. However, considering corneal stromal stiffening early on during wound healing (Raghunathan et al., 2017), and that YAP/TAZ are effectors of mechanotransduction, it is feasible to infer that the differentiation of quiescent keratocytes to contractile myofibroblasts may be mediated by YAP/TAZ. During normal wound repair, resolution of corneal haze with time suggests that a balance in YAP and TAZ activities exist. Based on our *in vitro* findings we speculate that TAZ may be absent or functionally impaired when fibrosis develops or when myofibroblasts persist. We therefore posit that restoring TAZ and/or inhibiting YAP specifically when there is sustained presence of myofibroblasts may be a viable therapeutic option.

5. Summary

This study suggests YAP and TAZ have distinct functions in corneal stromal cells and thus regulate KFM transformation differently. The key findings are listed below:

- TGFβ1 stimulation results in a decrease in cytosolic pYAP and an increase in nuclear pTAZ, and increases the accumulation of YAP but decreases TAZ in the nuclear fraction.
- Phosphorylation of Smad2/3/4 is increased in the nuclear fraction with TGFβ1.
- Knockdown of TAZ increased mRNA expression of YAP but knockdown of YAP does not modulate TAZ expression, suggesting TAZ limits YAP in corneal stromal cells.
- Knockdown of TAZ but not YAP upregulates αSMA expression and promotes KFM transformation.
- Knockdown of YAP stimulates expression of Smad 2/4, while TAZ knockdown does not alter Smad expression.
- Knockdown of YAP or TAZ inhibits versus induces CTGF expression, respectively.
- Simultaneous knockdown of YAP and TAZ is lethal.

The data presented demonstrate that in the absence of YAP, the TAZ-Smad axis may play a dominant role in upregulation of pSmad2/3/4 concurrent with αSMA expression A schematic summarizing our finding is presented in Fig. 5. This is the first study, to the best of our knowledge, demonstrating the role of YAP and/or TAZ in corneal stromal cell transformation. This is particularly important when corneal stromal mechanics during wound healing is taken into account. A stiffer stroma would promote nuclear translocation of YAP and TAZ and transcriptional activation. Our data suggests that under normal wound healing process TAZ helps restrict the extent on myofibroblast differentiation. Whether YAP/TAZ expression and localization change during the course of wound healing is a subject of investigation in our laboratory and is currently out of the scope of this manuscript. We infer from these *in vitro* findings that a loss of TAZ may help drive corneal haze and fibrosis, and thus shifting the balance of intracellular YAP/TAZ amounts to reflect substratum changes may lead to the identification of novel therapeutic targets.

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Abbreviations

KFM	keratocytes, to activated fibroblasts to myofibroblasts
YAP	Yes-associated protein
TAZ	Transcriptional coactivator with PDZ-binding motif
FBS	Fetal Bovine Serum
HCFs	Primary Human Corneal Fibroblasts

ECM	Extracellular Matrix
αSMA	Alpha-Smooth Muscle Actin
TGF β1	Transforming Growth Factor β1
PRK	photorefractive keratectomies
PTK	phototherapeutic keratectomies
LASIK	laser-assisted in situ keratomileusis

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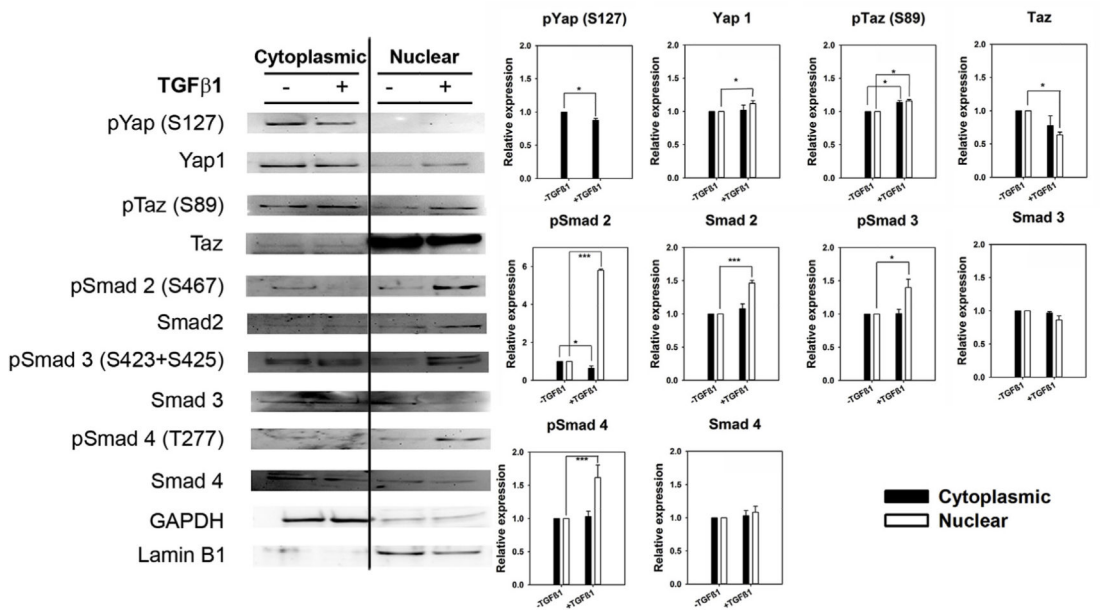

Fig. 1. TGFβ1 stimulation differentially alters phosphorylation states of YapYAP, TAZ and pSmads.

Representative Western blots of HCFs cytoplasmic and nuclear lysates after treatment with either control or TGFβ1 (2 ng/ml) for 24h. Western Blot analysis analyses of proteins in cytosolic fraction were normalized with GAPDH while those in the nuclear fraction were normalized with Lamin B1 respectively and the intensities of TGFβ1 treated samples were compared with control samples. Purity of cytoplasmic and nuclear fractions respectively were determined by immunoblotting for GAPDH and Lamin B1. Graphs demonstrate quantitative results from n = 3 independent experiments (mean ± standard deviation). *$p < 0.05$, ***$p < 0.001$ Student's t-test compared with untreated samples (-TGFβ1) within each group (cytoplasmic or nuclear fraction).

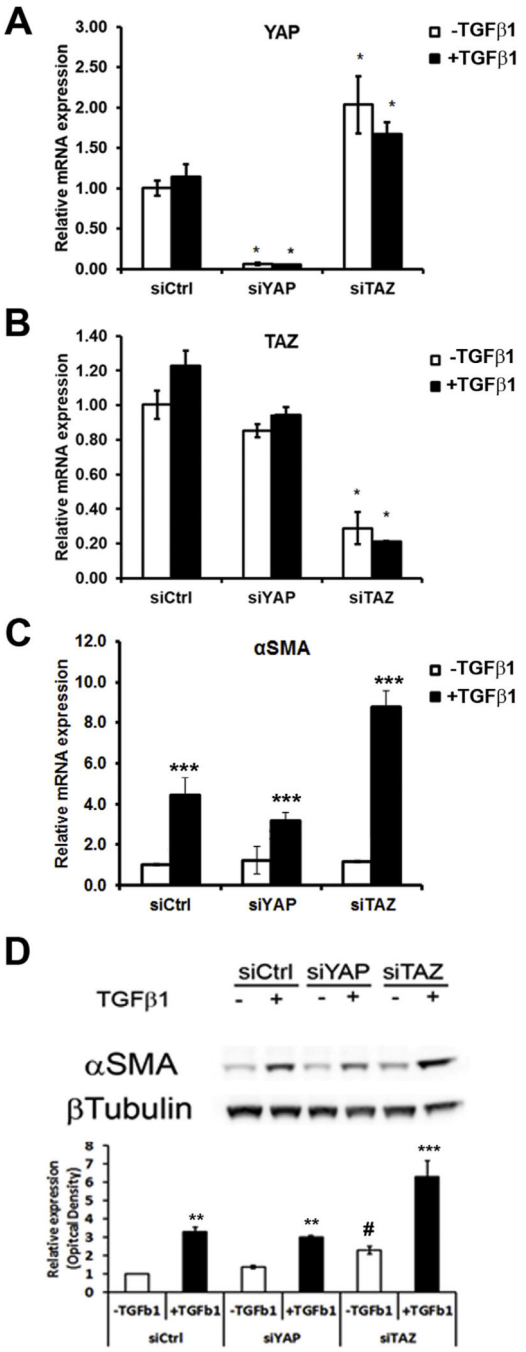

Fig. 2. Knockdown of YAP and TAZ differentially modulates αSMA expression.

(2a, 2b) Representative hisotgrams of gene expression confirming YAP/TAZ knockdown in HCFs treated with siRNA to YAP, WWTR1 (TAZ) and siControl for 48h, followed by control or TGFβ1 (2 ng/ml, T7039, Sigma Aldrich) treatments for 24h. Knock-down efficiency of YAP and TAZ were determined by normalizing to GAPDH and/or 18S. **(2c):** Representative histogram of αSMA expression after YAP/TAZ knockdown. **(2d):** Western Blot analysis of αSMA is determined and the bands were normalized with β-Tubulin. Results are mean ± standard deviation; *p < 0.05, **p < 0.01, ***p < 0.001 one-way

ANOVA followed by Dunnett's multiple comparison test compared with untreated (–TGFβ1) group; #$p < 0.05$ compared with siCtrl untreated samples.

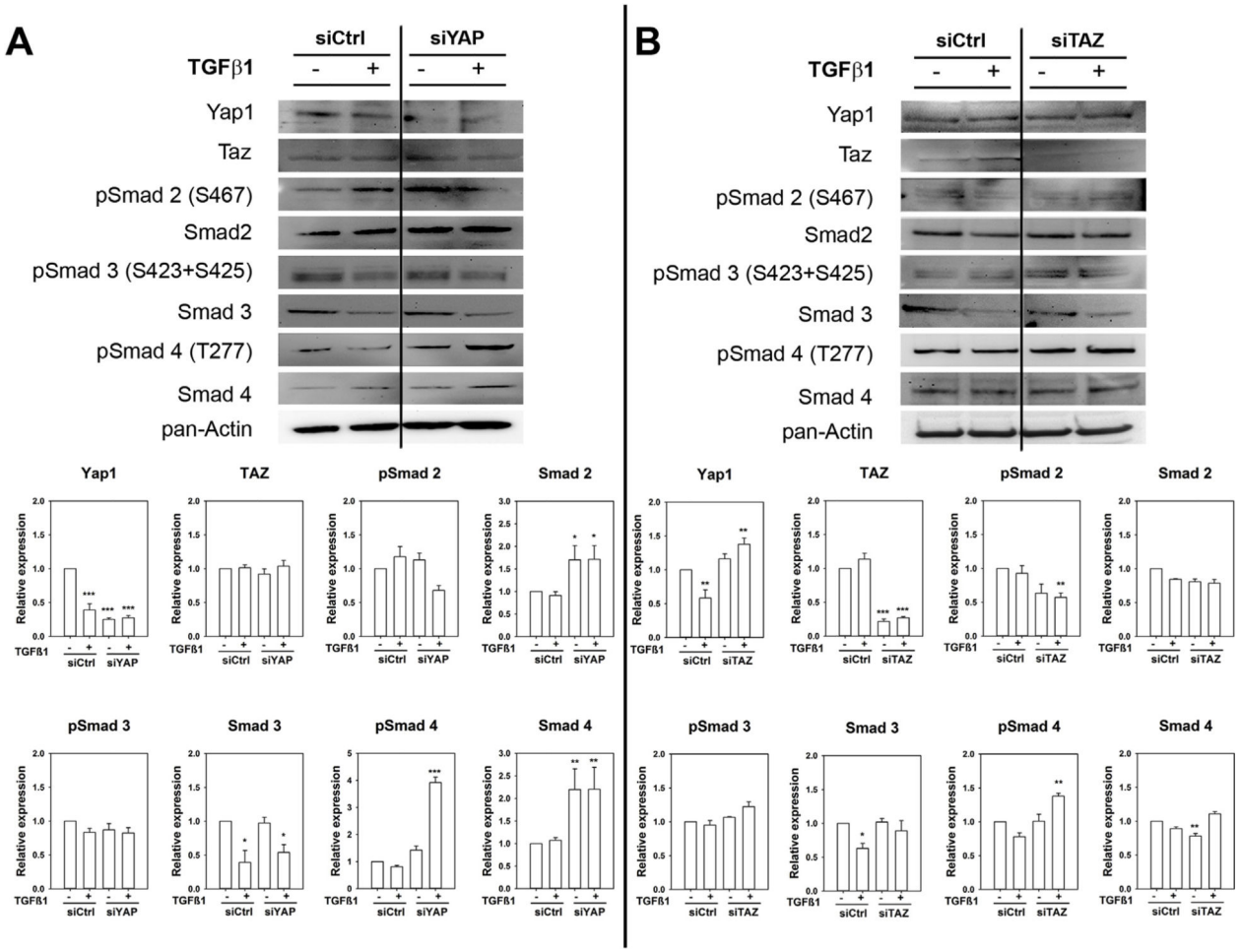

Fig. 3. YAP and TAZ knockdown differentially modulates Smad2/3/4 expression and phosphorylation.

Representative Western blots in HCFs after siRNA knockdown of YAP, WWTR1 (TAZ) and siControl for 48h followed by control or TGFβ1 (2 ng/ml) treatments for 24h. Western blot analyses performed with bands normalized to pan actin and the intensities of siTAZ treated samples were compared with control siRNA samples with no TGFβ1. Graphs demonstrate quantitative results from n = 3 independent experiments (mean ± standard deviation). *p < 0.05, **p < 0.01, ***p < 0.001, one way ANOVA followed by Dunnett's multiple comparison test compared with siCtrl untreated (–TGFβ1) cells.

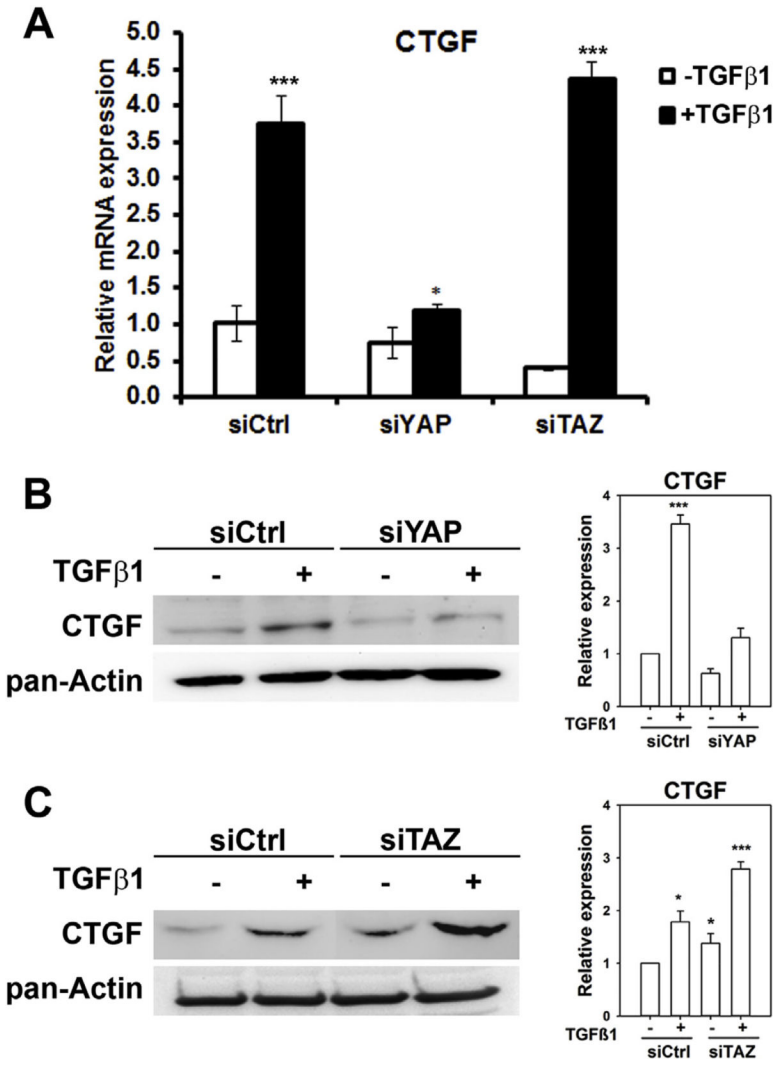

Fig. 4. Knockdown of YAP and TAZ differentially regulates CTGF expression.

(**A**) Histogram depicting CTGF expression after YAP/TAZ knockdown in primary HCFs. (**B–C**): Western blot analysis of CTGF was normalized to pan actin and the intensities of siYAP or siTAZ treated samples were compared with control siRNA sample with noTGFβ1. Graphs demonstrate quantitative results from n = 3 independent experiments (mean ± standard deviation). *p < 0.05, **p < 0.01, ***p < 0.001, one way ANOVA followed by Dunnett's multiple comparison test compared with siCtrl untreated (–TGFβ1) cells.

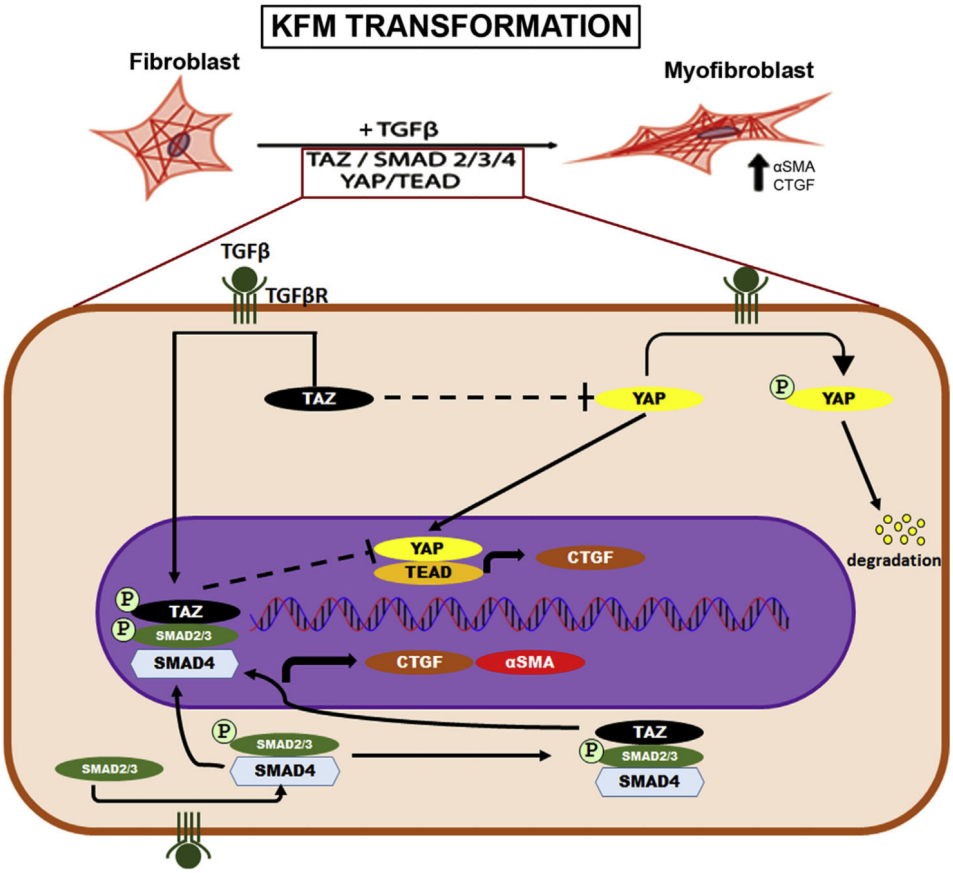

Fig. 5. YAP and TAZ differentially regulate KFM transformation via interactions with Smads and TEAD.

TGFβ stimulus results in a decrease in cytosolic pYAP and increased nuclear accumulation of pTAZ and pSmad2/3/4.TAZ predominantly regulates αSMA and CTGF expression via Smad2/3/4 pathway, whereas YAP modulates their expression through TEAD in HCFs. Further, our data suggests that TAZ limits YAP activity to prevent myofibroblast differentiation.