Critical Role of mTORC2-Akt Signaling in TGF-β1-Induced Myofibroblast Differentiation of Human Pterygium Fibroblasts

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Pterygium is a common ocular surface disease characterized by a triangular-shaped growth of fibrotic subconjunctival connective tissue and hypertrophy of overlying conjunctival epithelium.1,2 UV radiation damage, chronic irritation, inflammation, and genetic factors appear to be associated with its pathogenesis. Pathologic changes in pterygial tissues include profibrotic activation of stromal fibroblasts, neovascularization, oxidative stress, chronic inflammation, and extracellular matrix (ECM) remodeling.3–7 Surgical excision is the current standard treatment for pterygium, however, excisions in other organs, pterygium excision often leads to recurrent fibrosis due to dysfunctional wound healing. Because fibrovascular growth in recurrent pterygia is more extensive than that in the primary occurrence, recurrence is a major concern.6,8 Therefore, medical treatments targeting pterygium fibroblasts that avoid facilitating recurrences after surgery are highly desired.

The profibrotic growth factor TGF-β plays a pivotal role in myofibroblast formation, and the presence of myofibroblasts in pterygia is suggestive of TGF-β1 involvement in the pathogenesis and progression of this disorder.1,5 It has been reported that TGF-β1 expression levels are higher in recurrent pterygium fibroblast cultures than those in primary pterygium fibroblast cultures.9,10 Therefore, TGF-β signaling has been identified as a promising antifibrotic target, and numerous studies have demonstrated that inhibition of TGF-β1 successfully reduces organ fibrosis, including in the eye.11–13 Despite these promising results, TGF-β1 inhibitors currently remain unused in the clinic, partly because TGF-β1 also regulates many processes essential for maintaining normal homeostasis.

In addition to canonical TGF-β signaling, phosphoinositide 3-kinase (PI3K)-initiated pathways leading to activation of the mechanistic target of rapamycin (mTOR) have been implicated in the activation and differentiation of fibroblasts.14,15 This evolutionarily conserved serine/threonine kinase, mTOR, links a variety of environmental cues to a wide spectrum of cellular processes.16–18 There are two distinct enzyme complexes engaged by mTOR: mTOR complex 1 and 2 (mTORC1 and 2, respectively). The substrate specificities of mTORC1 and mTORC2 are defined by their unique structural components raptor and rictor, respectively. In response to growth factors, mTORC1 promotes growth and proliferation by upregulating biosynthesis of cellular components, such as...
proteins, lipids, and nucleic acids. Conversely, mTORC2 phosphorylates the hydrophobic motifs of some AGC family members, including Akt, serum- and glucocorticoid-induced kinase 1 (SGK1), and protein kinase C isoforms, and controls proliferation and actin cytoskeleton remodeling.16,17 A potential role for mTOR signaling in the promotion of fibrosis has been reported in studies that have predominantly used the immunosuppressive drug rapamycin.14,15 However, mTORC1 activity is partially inhibited by the allosteric inhibitor rapamycin in many cells, and mTORC2 is largely insensitive to mTORC1 activity.16,17-20 In this regard, it is still unclear how mTOR signaling mediates TGF-β-induced profibrotic activation and to what extent mTORC1 and mTORC2 contribute to fibrosis. Recently, adenosine triphosphate (ATP)-competitive mTOR catalytic inhibitors have been developed and many are currently being assessed in clinical trials for cancer treatment.21 The mTOR active-site inhibitors are more effective than rapamycin but may exhibit greater toxicity due to completely shutting down both the mTORC1 and mTORC2 pathways.22 In the present study, we compared the effects of mTOR inhibitors on human pterygium fibroblasts (HPFs) and investigated the role of mTOR signaling in the regulation of profibrotic activity by using a combination of mTOR inhibitors and knockdown approaches.

**Materials and Methods**

**Isolation and Culturing of Primary HPFs**

Pterygium tissues were obtained from 18 patients with a mean age, in years, of 49 ± 5.2 (range, 38–66) who underwent pterygium excision surgery. This study protocol was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine (YWMR-15-0-053) and was conducted according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each patient prior to their participation in this research.

Pterygium tissue samples were placed in 60-mm culture dishes in Dulbecco’s modified Eagle medium (DMEM; Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Thermofisher Scientific, Seoul, Korea), and 1× penicillin/streptomycin (ThermoFisher Scientific). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 during which cells migrated from the explant. Migrated cells were dissociated using 0.25% trypsin (Welgene) and subcultured in DMEM containing 5% FBS. Cells passaged 2 to 5 times were used for all experiments. The passaged HPFs were seeded into a 6-well plate in 5% FBS-containing medium. After attachment, serum-free medium was added 8 to 12 hours prior to treatment to promote cellular quiescence. These cells were then pretreated with mTOR inhibitors for 30 minutes and cultured for 2 days in the presence or absence of TGF-β1 (2.5 ng/ml; PeproTech, Seoul, Korea).

**Western Blot Analysis**

Cells were lysed in buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and protease and phosphatase inhibitors (GenDEPOT, Barker, TX, USA), and protein concentrations were determined using a BCA protein assay kit (ThermoFisher Scientific, Seoul, Korea). Western blotting was performed as previously described.22 Briefly, 20 μg of each protein lysate was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). This membrane was blocked for 1 hour in PBS containing 5% skim milk and 0.05% Tween-20 and then incubated for 3 hours with primary antibodies. The primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA), except for those against z-smooth muscle actin (z-SMA; Sigma-Aldrich Corp., St. Louis, MO, USA) and fibronectin (FN; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody-reactive proteins were detected using the appropriate HRP-conjugated secondary antibodies and an enhanced chemiluminescence reagent (Tech & Innovation, Chuncheon, Korea).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

HPFs were cultured for 48 hours in the presence or absence of TGF-β1 as described above. After 48 hours, total RNA was extracted using an RNA extraction kit (Qiagen, Venlo, Netherlands) and reverse-transcribed into complementary DNA with a LaboPass cDNA synthesis kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer’s instructions. qRT-PCRs were performed using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR qPCR mix (Toyobo, Osaka, Japan). Primer pairs used to amplify target genes are listed in the Table. All mRNA levels were normalized to 18S ribosomal RNA levels.

**Immunofluorescence Staining**

HPFs were plated onto coverslips in 24-well plates and cultured in the presence or absence of TGF-β1 as described above. After 48 hours, the cells were fixed with 4% paraformaldehyde, permeabilized, and blocked with PBS containing 2% FBS and 0.1% Tween-20. The slides were incubated with primary antibodies against z-SMA and FN for 2 hours and then incubated with Alexa Fluor 488-conjugated secondary antibodies (ThermoFisher Scientific). The coverslips were rinsed with blocking buffer and mounted onto slide glasses using 4’,6-diamidino-2-phenylindole (DAPI)-containing ProLong gold antifade mountant (ThermoFisher). Fluorescence images were taken with a fluorescence microscope (Nikon Eclipse 80i; Nikon, Minato, Japan).

**Viability Assay and Flow Cytometry**

HPFs were seeded in a 96-well culture plate at a density of 1,000 cells per well, pretreated with mTOR inhibitors as described above, and then cultured in the presence of TGF-β1. After 48 hours, cells were incubated with 10 μl EZ-Cytox (DoGEN, Seoul, Korea) according to the manufacturer’s instructions, and cell viability was assessed by measuring the absorbance at 450 nm by using a microplate reader (Bio-Rad).
Apoptosis and proliferation were assessed by flow cytometry. HPFs were incubated with mTOR inhibitors for 48 hours and stained with PE-conjugated annexin-V (BD Biosciences, San Jose, CA, USA) in 10 mM HEPES (pH 7.4) containing 140 mM NaCl and 2.5 mM CaCl2 at room temperature for 20 min. Data were acquired using a BD FACScalibur and analyzed with FlowJo software (TreeStar, Ashland, OR, USA). To measure cell cycling rates, HPFs were cultured as described and then labeled with the modified thymidine analogue EdU using a Click-IT EdU flow cytometry kit (Life Technologies, Carlsbad, CA, USA). After 12 hours, the cells were fixed, permeabilized, and stained with Alexa Fluor 647-conjugated anti-EdU according to the manufacturer’s instructions. The EdU+ cell population (i.e., proliferating cell population) was measured by flow cytometry.

Migration Assay
The migration of HPFs was assessed using scratch-wound assays as described previously. HPFs were seeded (3 × 103 cells per well) in a 6-well culture plate and maintained in serum-free medium. After a 24-hour incubation, a straight line was made across the cells with a P200 pipette tip to create a scratch wound. The plates were then rinsed with serum-free medium to remove the cells in suspension. Samples were treated as previously described. Identical sites were observed at 40× magnification 0, 2, and 4 days postscratching by using a light microscope (Olympus, Tokyo, Japan) equipped with a camera (Canon, Tokyo, Japan).

Collagen Gel Contraction Assay
Fibroblast-embedded collagen gels were prepared using a modified version of a previously described method. Briefly, HPFs were resuspended in DMEM on ice and then immediately mixed with neutralized collagen solution (type 1 collagen from rat tail tendon, Sigma-Aldrich Corp.), yielding a final concentration of 1.5 × 105 cells/mL in 1 mg/mL collagen. Aliquots of the cold gel solution were added to 24-well plates at 600 µL per well and allowed to polymerize for 1 hour at 37°C. The gels were then gently detached from the bottom of the wells, DMEM containing 2% FBS was added, and then the gels were pretreated with mTOR inhibitors for 30 minutes and then incubated with TGF-β1 for the indicated time. Subsequently, the gels were photographed and the degree of contraction was quantified by measuring the surface area by using ImageJ software.

RNA Interference
Short interfering RNAs (siRNAs) targeting genes encoding human raptor and rictor were purchased from Ambion (ThermoFisher Scientific). HPFs were plated in 6-well plates and transfected with 50 nM raptor- or rictor-targeting or control siRNA duplexes by using Lipofectamine-2000. Two days posttransfection, the cells were activated for an additional 2 days with 2.5 ng/mL TGF-β and then analyzed by Western blot.

Statistical Analysis
At least three independent experiments were performed in duplicate or triplicate for each assay, and data are presented as a mean ± standard error of the mean. Differences between groups were analyzed using Kruskal-Wallis 1-way analysis of variance on ranks and Bonferroni’s multiple comparisons. A P value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad InStat (GraphPad software, La Jolla, CA, USA).

RESULTS
Activation of mTOR Signaling Pathway in Human Pterygia
The growth and activation of fibroblasts depend on multiple trophic signaling pathways that include mTOR. To determine whether mTOR signaling has a role in human pterygia, we first compared mTOR activity in HPFs derived from clinically severe pterygium and subconjunctival fibroblasts. Activation of mTORC1 enhances protein synthesis by suppressing eukaryotic translation initiation factor 4E; TSCs, tuberous sclerosis complexes; Rheb, Ras homolog enriched in brain. Human pterygial and subconjunctival cells were cultured, as described in the Materials and Methods section and analyzed by Western blotting with the indicated antibodies. The figures show representative results from three independent experiments. α-tubulin and unphosphorylated proteins served as the loading control. Numbers above the panels indicate relative signals for each phosphorylated protein, normalized to that of unphosphorylated protein in each sample and expressed as arbitrary units with conjunctiva samples set as 1.0.

FIGURE 1. Increased mTORC1 and mTORC2 activity in human pterygium. (A) Schematic of mTOR signaling pathway. Lines ending with arrowheads and bars indicate activating or inhibitory phosphorylation, respectively. elf-4E, eukaryotic translation initiation factor 4E; TSCs, tuberous sclerosis complexes; Rheb, Ras homolog enriched in brain. (B) Human pterygial and subconjunctival cells were cultured, as described in the Materials and Methods section and analyzed by Western blotting with the indicated antibodies. The figures show representative results from three independent experiments. α-tubulin and unphosphorylated proteins served as the loading control. Numbers above the panels indicate relative signals for each phosphorylated protein, normalized to that of unphosphorylated protein in each sample and expressed as arbitrary units with conjunctiva samples set as 1.0.

TORin2 Suppressed TGF-β1-Induced Profibrotic Activation of Pterygium Fibroblasts
We next tested the effect of mTOR inhibition on profibrotic activation of pterygia by using the mTOR inhibitors Torin2 and
Figure 2. Inhibition of mTOR suppresses TGF-β1-induced expression of myofibroblastic markers and ECM proteins in pterygium fibroblasts. Human pterygium fibroblasts (HPFs) were pretreated with Torin2 and rapamycin at the indicated concentrations and then cultured in the presence or absence of TGF-β1. After 48 hours, these cells were analyzed by (A) Western blotting and (B) real-time reverse transcription–PCR. (A) Shown are the representative immunoblots from four or five independent experiments and the relative mean (± SEM) intensities of α-SMA and FN, normalized to the α-tubulin in each sample. (B) The concentration of mRNA was normalized to 18S ribosomal RNA concentration, and the mean (± SEM) of the
rapamycin. When HPFs were activated using TGF-β1, ECM proteins, such as FN and FN-containing extra domain A (EDA-FN), and α-SMA expression were induced, the latter of which is indicative of myofibroblastic differentiation (Fig. 2). Intriguingly, Torin2, an ATP-competitive mTOR inhibitor was sufficient to suppress the induction of α-SMA, FN, and EDA-FN in TGF-β1-activated HPFs (Fig. 2A). Expression of vimentin, a mesenchymal fibroblast marker, was not affected by mTOR inhibition. Induction of mRNAs encoding α-SMA (ACTA2), FN (FN1), and type 1 collagen (COL1A1) was also significantly attenuated by Torin2, suggesting transcriptional control of these fibrotic markers by the mTOR signaling pathway (Fig. 2B). Conversely, the allosteric mTOR inhibitor rapamycin marginally inhibited the induction of α-SMA and ECM proteins (Figs. 2A, 2B). These results were further confirmed by immunofluorescent staining that indicated that Torin2 suppressed TGF-β1-induced expression of α-SMA and FN more potently than rapamycin (Figs. 2C, 2D). Thus, mTOR signaling promoted the induction of α-SMA and ECM proteins in HPFs, suggesting that mTOR complexes differentially contribute to myofibroblastic differentiation of pterygium cells.

HPFs express different amounts of α-SMA, FN, and EDA-FN, which depend on the tissue of origin (i.e., disease severity) and culture condition. For example, HPFs derived from severe pterygium showed higher expression of α-SMA than mild pterygium, and TGF-β1 treatment did not further enhance α-SMA expression in the spontaneously activated HPFs with high basal α-SMA (Fig. 1A). In addition to our focus on the role of mTOR in TGF-β1-induced myofibroblast differentiation, we assessed the effect of mTOR inhibition on the activated HPFs, which showed high basal α-SMA expression without TGF-β1 treatment. The protein levels of α-SMA and FN were decreased by Torin2 in a dose-dependent manner, which coincided with reduced intrinsic expression of TGF-β1 (Figs. 3B, 3C). On the other hand, rapamycin did not modulate the expression of α-SMA and FN in the activated myofibrotic HPFs. These results imply that mTOR inhibition by Torin2 might suppress myofibroblast function of the activated HPFs, partially through blocking of the autocrine regulation of TGF-β1. However, it remains to be determined whether TGF-β is required for the activated pterygia in regulating the sustained expression of α-SMA and FN.

**Inhibitors of mTOR Attenuated Pterygium Fibroblast Proliferation**

The mTOR inhibitor Torin2 suppressed myofibroblastic activation of HPFs. However, this reduced induction of profibrotic proteins may be due to the cytotoxic and/or cytostatic effects of mTOR inhibition. When we assessed the...
viability of the HPFs, we observed fewer viable cells following treatment with Torin2 and, to a lesser extent, rapamycin (Fig. 4A). Annexin-V staining indicates Torin2 and rapamycin treatment did not induce apoptosis in HPFs at the concentrations tested in this study (Fig. 4B). To determine the effect of mTOR inhibition on rates of proliferation, we pulsed HPFs with EdU, a thymidine analogue, and measured EdU incorporation into genomic DNA by using flow cytometry. As shown in Figures 4C and 4D, mTOR inhibitors significantly attenuated EdU uptake, with rapamycin being relatively less potent than Torin2. Overall, these data suggest mTOR inhibitors exert cytostatic, but not cytotoxic, effects on HPFs.

**Inhibition of mTOR Signaling Impaired Migration and Contractile Phenotype of Pterygium Fibroblast**

Many fibrotic diseases are associated with severe or repetitive wound-healing processes. In the scratch-wound assays
with HPF cells, we observed that TGF-β1 accelerated healing of wound gaps through cell migration and growth toward the center of the gap. However, inhibition of mTOR signaling in HPFs with Torin2 or rapamycin treatment significantly attenuated healing of wounds (Fig. 5). This impairment of HPF wound healing with mTOR inhibitor treatment implies mTOR signaling regulates TGF-β1 mediated migration, as well as proliferation, of pterygium fibroblasts.

To further validate these observations with respect to the functional characteristics of myofibroblasts, we performed collagen gel contraction assays and examined the degree of contraction of a three-dimensional collagen lattice, an accepted model of myofibroblastic contractility. As shown in Figure 6, treatment of HPF-embedded collagen gels with TGF-β1 gradually reduced the gel area of the collagen disk. When we compared the effects of mTOR inhibitors on the contractile phenotype of the HPFs, Torin2 was found to be sufficient to prevent TGF-β1-induced contraction of the collagen disk. In accordance with the moderate effect of rapamycin on induction of α-SMA and FN, the gel area of the rapamycin-treated collagen disk was slightly larger than that in the vehicle-treated samples (Fig. 6). Combined with biochemical data

**FIGURE 5.** Effect of mTOR inhibition on migration of pterygium fibroblasts. HPFs were grown until confluent, subjected to a scratch-wound assay, and then imaged using phase-contrast microscopy. (A) Representative images of cells migrating into the center of the wound are shown for the indicated timepoints. Red lines define the boundary of migrating cells. (B) The rate of wound healing was measured by quantifying the wound gap area using ImageJ software. The relative scratch gap compared to that of control sample (media alone) at day 0 is shown as a mean (± SEM) obtained from three independent experiments.

**FIGURE 6.** Effect of mTOR inhibition on the contractile phenotype of pterygium fibroblasts. Collagen gel-embedded HPFs were prepared and placed in medium with or without TGF-β1. Photographs were taken at the indicated times, and HPF-mediated gel contraction was measured by quantifying the area of the collagen disk in the gel using ImageJ software. The relative gel area compared to that of the original gel (day 0) is shown as a mean (± SEM) from three independent experiments performed in duplicate is shown. *P < 0.05, **P < 0.01.
assessing the induction of fibrotic markers and ECM proteins, these results reveal an important role for mTOR in the myofibroblastic differentiation and fibrotic functions of pterygium fibroblasts.

Abrogation of the mTORC2-Akt Signaling Axis Impeded Myofibroblastic Differentiation of Pterygium Fibroblasts

Biochemical analyses were performed to investigate mechanisms by which mTOR signaling regulates profibrotic activation of HPFs. In accordance with other studies,20,32 the allosteric inhibitor of mTOR rapamycin did not suppress mTORC2 activity (Fig. 7A). Rapamycin also failed to inhibit 4E-BP1 phosphorylation in HPFs but blocked phosphorylation of S6K1 and S6 proteins (Fig. 7A). Conversely, the ATP-competitive mTOR catalytic inhibitor Torin2 efficiently suppressed both mTORC1 and mTORC2 activity in HPFs, as shown by reduced phosphorylation of S6K1 and 4E-BP1 and inhibition of phosphorylation of S473 in Akt, respectively (Fig. 7A). When we tested whether mTOR inhibitors affect canonical signaling triggered by TGF-β receptors in HPFs, we observed that mTOR inhibition did not interfere with TGF-β-induced phosphorylation of Smad2/3, which occurs downstream of the TGF-β receptor (Fig. 7B). This indicates the observed suppression of profibrotic activation of HPFs was not likely from Torin2 inhibition of canonical TGF-β signaling. Moreover, our data suggest distinct contributions by mTOR signaling components to profibrotic activation of pterygium fibroblasts, where mTORC2 might be vital for TGF-β-induced myofibroblastic differentiation.

We were unable to distinguish between the impact of mTORC1 and mTORC2 on myofibroblastic differentiation of HPFs by using mTOR inhibitors alone. Therefore, to better assess the impact of each mTOR complex on pterygium fibroblasts, we used RNA interference-based approaches using siRNAs. HPF transfection with siRNAs targeting raptor- and rictor-encoding mRNAs displayed notable decreases in raptor and rictor protein levels, respectively. Although target mRNAs were not completely removed, the reduced expression of rictor substantially decreased the induction of α-SMA and FN in TGF-β1-treated HPFs (Figs. 8A, 8B). The depletion of raptor from HPFs also attenuated the induction of α-SMA and FN but was less potent than that observed when rictor was depleted (Figs. 8A, 8B). The hydrophobic motifs of Akt, SGK1, and PKC isoforms are phosphorylated by mTORC2, which regulates differentiation of and cytoskeletal changes in many cells.32–34 We examined the requirement for Akt in myofibroblastic differentiation of pterygium fibroblasts by using the Akt inhibitor MK2206. MK2206 potently inhibited phosphorylation of Akt S473 and attenuated TGF-β-induced expression of α-SMA in a dose-dependent manner (Figs. 8C, 8D). In contrast to Torin2 (Fig. 3), however, MK2206 did not reduce the basal expression of α-SMA in the spontaneously activated HPFs (Fig. 8E). These results raise a possibility that unlike the induction of α-SMA in TGF-β-stimulate HPFs, the basal expression of α-SMA in the activated myofibrotic pterygia might be regulated through an Akt-independent mTOR signaling pathway.55–57 Overall, our experimental data suggest the mTORC2-Akt signaling axis is crucial for the TGF-β-induced profibrotic programming of pterygium fibroblasts.

**DISCUSSION**

In the present study, we dissected the role of mTOR complexes in the growth and profibrotic activation of HPFs. By comparing the effects of mTOR inhibitors, we found that mTOR signaling promoted proliferation and myofibroblastic differentiation of HPFs, which was highly sensitive to mTOR active-site inhibitor Torin2 but largely insensitive to the allosteric inhibitor.
rapamycin. To further evaluate the relative contribution of different mTOR complexes, we used RNA interference-based approaches to target and inhibit raptor and rictor, which revealed that mTORC2 plays a critical role in myofibroblastic differentiation of HPFs. Intriguingly, Akt, which is regulated by mTORC2,38 was indispensable for profibrotic activation of pterygium cells, suggesting a key role for the mTORC2-Akt signaling axis. Inhibition of mTOR signaling was not cytotoxic to HPFs but did have a cytostatic effect, as determined by flow cytometry. These data indicate the advantage of the therapeu-

**FIGURE 8.** Abrogation of mTORC2-Akt signaling axis potently inhibited myofibroblastic activation of pterygium fibroblasts. (A) HPFs were transfected with control and raptor- or rictor-targeting small interfering RNA duplexes, and immunoblotting was performed 48 hours poststimulation with TGF-β1. Representative data from three independent experiments are shown. (B) Shown are means (± SEM) of the relative intensities of α-SMA and FN normalized to α-tubulin in each sample. *P < 0.05, **P < 0.01. (C) HPFs were pretreated with the Akt inhibitor MK2206 at the indicated concentration for 30 minutes and then cultured in the presence of TGF-β1. After 48 hours, the cells were analyzed by Western blotting. Representative immunoblots from three independent experiments. (D) Bar graph showing the mean (± SEM) of the relative α-SMA intensity normalized to the α-tubulin in each sample. (E) Spontaneously activated HPFs with high basal α-SMA were treated with MK2206 in the absence of TGF-β1 for 48 hours and then analyzed by Western blotting as above.
Effect of mTOR Inhibition on Pterygium

Many studies have defined complex intercellular signaling networks dictating myofibroblast differentiation. Although TGF-β-triggered cellular transduction is a key event in many aspects of fibrotic pathogenesis, it is unclear to what extent other signaling pathways contribute to myofibroblastic differentiation. Some studies have identified an association of the PI3K/Akt pathway with α-SMA expression during myofibroblast differentiation in several tissues.\(^9,10\) It has also been reported that the Akt/mTOR pathway regulates FN translation and ECM network secretion and assembly in pulmonary fibrosis.\(^53\) Accordingly, our biochemical analysis revealed a profibrotic role for Akt in human pterygium by comparing alterations in signaling downstream from mTORC1 and mTORC2 after treatment with inhibitors or interfering RNAs. Moreover, the Akt inhibitor MK2206 markedly suppressed α-SMA induction in pterygium fibroblasts. Therefore, we demonstrated that mTORC2-dependent activation of Akt is a vital step in myofibrotic differentiation of pterygium, although it remains to be determined how Akt regulates profibrotic gene expression with regard to canonical TGF-β signaling.

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