

# Inhibition by a retinoic acid receptor $\gamma$ agonist of extracellular matrix remodeling mediated by human Tenon fibroblasts

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**Purpose:** Scar formation is most frequently responsible for the failure of glaucoma filtration surgery. Retinoic acids are vitamin A derivatives that play diverse roles in development, immunity, and tissue repair. The effects of the retinoic acid receptor (RAR)  $\gamma$  agonist R667 on the contractility of human Tenon fibroblasts (HTFs) cultured in a three-dimensional collagen gel as well as on intraocular pressure (IOP) in a rat model of glaucoma filtration surgery were investigated.

**Methods:** HTFs were cultured in a type I collagen gel, the contraction of which was evaluated by measurement of the gel diameter. The release of matrix metalloproteinases (MMPs) into culture supernatants was assessed with immunoblot analysis and gelatin zymography. Phosphorylation of focal adhesion kinase (FAK) was examined with immunoblot analysis, and production of fibronectin and type I collagen was measured with immunoassays.

**Results:** R667 inhibited transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-induced collagen gel contraction mediated by HTFs in a concentration- and time-dependent manner, whereas an RAR $\alpha$  agonist inhibited this process to a lesser extent and an RAR $\beta$  agonist had no effect. TGF- $\beta$ 1-induced MMP-1 and MMP-3 release, FAK phosphorylation, and fibronectin and type I collagen production in HTFs were also attenuated by R667. Furthermore, R667 lowered IOP in rats after glaucoma filtration surgery.

**Conclusions:** R667 inhibited TGF- $\beta$ 1-induced contraction and extracellular matrix synthesis in HTFs. Such effects might have contributed to the lowering of IOP by R667 in a rat model of glaucoma filtration surgery. RAR $\gamma$  agonists might thus prove effective for inhibition of scar formation after such surgery.

Glaucoma is the major cause of irreversible blindness worldwide. Filtration surgery remains the gold standard for the treatment of glaucoma in individuals whose intraocular pressure (IOP) is not well controlled by medication or laser surgery. The long-term success of filtration surgery depends on the postoperative wound healing response at the subconjunctival filtering bleb site [1,2]. Scar formation due to excessive synthesis of new extracellular matrix (ECM) and contraction of subconjunctival tissue can obstruct the aqueous flow and thus result in an increase in IOP and surgery failure [3]. Antimitotic agents such as mitomycin C and 5-fluorouracil are administered to reduce the extent of postoperative scarring and improve the outcome of filtration surgery, but the severe side effects of these agents limit their application [4]. Alternative strategies that are safe and modulate the wound healing response more effectively to prevent scar formation are thus needed.

The wound healing response at the subconjunctival filtering bleb site is mediated in part by matrix metalloproteinases (MMPs) and is regulated by various molecules, including growth factors and inflammatory mediators. The cytokine transforming growth factor (TGF)- $\beta$  is a pivotal regulator of wound healing and fibrosis and a major driving force of conjunctival scarring [5,6]. TGF- $\beta$ 1 and TGF- $\beta$ 2 have been detected at subconjunctival wounds after filtering surgery [7]; these factors, thus, have become major targets of evolving antifibrotic strategies. Antisense oligonucleotides that target TGF- $\beta$  for downregulation were found to prolong bleb survival in vivo and to improve surgical outcome [8]. Neutralizing antibodies to TGF- $\beta$ 2 were also shown to reduce scarring in animal models of glaucoma [9] as well as to maintain good bleb morphology in a pilot clinical study [10], but the antibodies did not have a significant effect on bleb survival in a randomized clinical trial [11].

Retinoic acids are derivatives of vitamin A that play complex roles in eye development and physiology [12]. They also possess anti-inflammatory properties and antifibrotic potential as a result of their attenuation of TGF- $\beta$  actions [13]. Such effects of retinoic acids are mediated by nuclear receptors— $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms of retinoic acid receptors

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(RARs)—that function as ligand-inducible transcriptional regulators and are expressed in most parts of the eye [14]. We recently showed that the RAR $\gamma$  agonist R667 attenuated the epithelial-mesenchymal transition (EMT) in RPE cells as well as subretinal fibrosis [15]. In the present study, we examine the effects of R667 on collagen gel contraction mediated by human Tenon's capsule fibroblasts (HTFs) as well as on fibronectin and collagen production by these cells exposed to TGF- $\beta$ 1. We also assess the effect of R667 on IOP in a rat model of glaucoma filtration surgery.

## METHODS

**Materials:** Fetal bovine serum, Eagle's minimum essential medium (MEM), and 10X MEM were obtained from Invitrogen-Gibco (Rockville, MD). Reconstitution buffer and acid-solubilized native porcine type I collagen were from Nitta Gelatin (Osaka, Japan). Antibodies to phospho-FAK (phospho-Tyr<sup>576/577</sup>) and to FAK were obtained from Cell Signaling (Beverly, MA). The RAR $\alpha$  agonist Am580, the RAR $\beta$  agonist BmS453, collagenase, and antibodies to  $\beta$ -actin and to alpha-smooth muscle actin ( $\alpha$ -SMA) were from Sigma-Aldrich (St. Louis, MO), and the RAR $\gamma$  agonist R667 (palovarotene) was from NARD Institute (Hyogo, Japan). Recombinant human TGF- $\beta$ 1 and antibodies to MMPs and to tissue inhibitors of metalloproteinases (TIMPs) were obtained from R&D Systems (Minneapolis, MN). Enzyme immunoassay (EIA) kits for fibronectin and the C peptide of procollagen type I were from Takara Shuzo (Shiga, Japan). Viscoelastic material (Healon) was obtained from Abbott Medical Optics (Santa Ana, CA), 30G needles were from NIPRO (Osaka, Japan), and silicon microtubes with a diameter of 0.3 mm were from ARAM (Osaka, Japan).

**Isolation and culture of HTFs:** This study adhered to the tenets of the ARVO statement on human subjects. Human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki and with approval of the human ethics committee of the Yamaguchi University Graduate School of Medicine. Tenon's capsule fibroblasts were isolated with informed consent from subconjunctival tissue of three individuals undergoing eyelid or strabismus surgery. The subconjunctival tissue was digested with collagenase (2 mg/ml) for 1 h at 37 °C to provide a suspension of Tenon fibroblasts. The cells derived from each patient were maintained separately at 37 °C under a humidified atmosphere containing 5% CO<sub>2</sub> and in MEM supplemented with 10% fetal bovine serum. Cells were used for experiments before the sixth passage.

**Assay of collagen gel contraction:** Free-floating collagen gels containing relaxed HTFs were prepared as previously

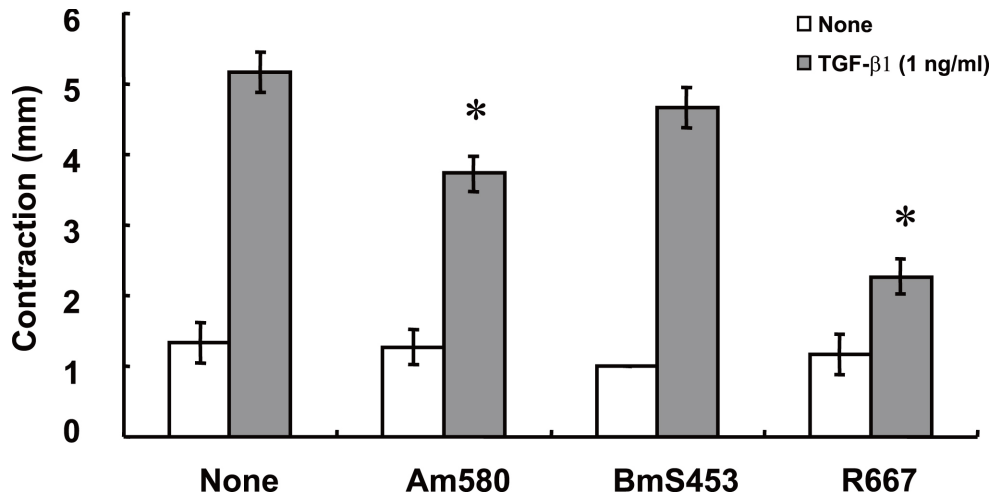
described [16]. Briefly, type I collagen, reconstitution buffer, 10X MEM, and HTF suspension were mixed on ice. Portions (0.5 ml) of the mixture containing HTFs at a density of  $2 \times 10^5$ /ml and collagen at 1.9 mg/ml were transferred to the wells of a 24-well culture plate that had been coated with 1% bovine serum albumin (BSA). After gelation of the mixture, the collagen gels were detached from the wells of the culture plate, and serum-free MEM (0.5 ml) containing vehicle (4 mM HCl containing 0.1% BSA), TGF- $\beta$ 1, or RAR agonists was then added on top of each gel. The diameter of the gels was determined daily with a ruler for calculation of the extent of gel contraction.

**Immunoblot analysis:** Immunoblot analysis was performed as described [16]. For immunoblot analysis of FAK and  $\alpha$ -SMA, HTFs incubated in collagen gels were lysed in an ice-cold lysis buffer, and portions (10  $\mu$ g of protein) of the lysates were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel. Immunoblot analysis of MMP-1, MMP-3, TIMP-1, and TIMP-2 was performed as described [17], with culture supernatants subjected to SDS–PAGE on a 10% gel. After electrophoresis, the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was exposed to 5% dried skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 h at room temperature before incubation overnight at 4 °C with primary antibodies (each at a 1:1,000 dilution). The membrane was then washed extensively with TBST before incubation for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies and detection of immune complexes with enhanced chemiluminescence reagents.

**Gelatin zymography:** Gelatin zymography of culture supernatants was performed as described previously [18]. Briefly, 8  $\mu$ l of culture supernatants mixed with 4  $\mu$ l of non-reducing SDS sample buffer were subjected to SDS–PAGE at 4 °C on a 10% gel containing 0.1% gelatin. The gel was then incubated first for 1 h at room temperature in 2.5% Triton X-100 and then for 18 h at 37 °C in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, and 5 mM CaCl<sub>2</sub>. The gel was then stained with 0.5% Coomassie brilliant blue.

**EIAs for fibronectin and the C peptide of procollagen type I:** Fibronectin and the C peptide of procollagen type I, which reflects the amount of newly synthesized type I collagen, were measured with EIAs [19]. Conditioned medium from collagen gel incubations was collected and stored at –80 °C until analysis. Standards were included with each assay to ensure accuracy.

**Glaucoma filtering surgery:** Animal experiments were approved by the animal ethics committee of the Yamaguchi



or presence of Am580, BmS453, or R667, respectively. Data are means  $\pm$  standard deviation (SD) from three independent experiments. \* $p < 0.01$  versus the corresponding value for cells cultured with TGF- $\beta$ 1 alone (the Tukey-Kramer test).

Figure 1. Effects of RAR agonists on TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs. Cells were incubated in collagen gels first for 12 h in the absence or presence of 1  $\mu$ M Am580, BmS453, or R667 and then for 3 days in the additional absence or presence of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; 1 ng/ml), after which the change in gel diameter was measured. The extent of TGF- $\beta$ 1-induced gel contraction was 5.17 $\pm$ 0.29, 3.73 $\pm$ 0.25, 4.67 $\pm$ 0.29, and 2.27 $\pm$ 0.25 mm in the absence

University Graduate School of Medicine. Surgery was performed on the left eye of 6-week-old male Wistar rats (five animals per treatment group) by a clinical glaucoma specialist according to a modified version of a previously described procedure [20]. Briefly, a fornix-based conjunctival flap was created at a position 3 to 4 mm behind the limbus with a conjunctival incision and dissection of the underlying Tenon's capsule from the sclera. No antimetabolite was applied. A full-thickness scleral tunnel into the intraocular space (either anterior or posterior chamber) was generated by insertion of a 30G needle with care so as to avoid iridal hemorrhage. Viscoelastic material was injected through the needle to maintain the anterior chamber, and the needle was then withdrawn. A silicon microtube with a bevel at the distal end and a slit at the proximal end was inserted through the scleral tunnel. R667 (50  $\mu$ g/ml) or PBS (1X; 155 mM NaCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, pH 7.4; 0.2  $\mu$ l) was injected into Tenon's capsule, and the conjunctival flap was closed with a 10–0 nylon suture. IOP was measured with a portable applanation tonometer (Tono-Pen AVIA Vet; Reichert Technologies, Depew, NY) before and for 2 weeks after surgery with the animals under inhalation anesthesia with diethyl ether.

**Statistical analysis:** Data are presented as means  $\pm$  standard deviation (SD). All in vitro experiments were performed in triplicate and repeated three times with three different cell isolates, and the data were analyzed with the Tukey-Kramer test. Nonparametric statistical analysis of IOP was performed with the Holm pairwise *t* test and the Mann–Whitney U test. A *p* value of  $< 0.05$  was considered statistically significant.

## RESULTS

**Effects of RAR agonists on TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs:** RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$  have been found to be expressed at the human ocular surface [14]. We first examined whether RAR agonists affect TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs. The cells were incubated with 1  $\mu$ M Am580 (RAR $\alpha$  agonist), BmS453 (RAR $\beta$  agonist), or R667 (RAR $\gamma$  agonist) for 12 h before exposure to TGF- $\beta$ 1 (1 ng/ml) for 3 days. The stimulatory effect of TGF- $\beta$ 1 on collagen gel contraction was inhibited by 36%, 12%, or 73% in the presence of Am580, BmS453, or R667, respectively (Figure 1). None of the RAR agonists affected HTF-mediated collagen gel contraction in the absence of TGF- $\beta$ 1.

We further characterized the inhibitory effect of R667 on TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs. Incubation of the cells for 3 days with various concentrations of R667 (0.01 to 1  $\mu$ M) revealed that the drug attenuated TGF- $\beta$ 1-induced gel contraction in a concentration-dependent manner (Figure 2A). The stimulatory effect of TGF- $\beta$ 1 on collagen gel contraction was thus inhibited by 41%, 57%, or 66% by R667 at concentrations of 0.01, 0.1, or 1  $\mu$ M, respectively. The inhibitory effect of R667 (1  $\mu$ M) on TGF- $\beta$ 1-induced gel contraction was also time dependent (Figure 2B), becoming statistically significant at day 2. Vehicle (4 mM HCl containing 0.1% BSA) for TGF- $\beta$ 1 had no effect on collagen gel contraction mediated by HTFs (Figure 2B).

**Effects of R667 on the release of MMPs and TIMPs by HTFs in collagen gel cultures:** MMPs play an important role in cell-mediated collagen contraction [21]. Therefore, we next examined the effects of R667 on the release of MMPs and

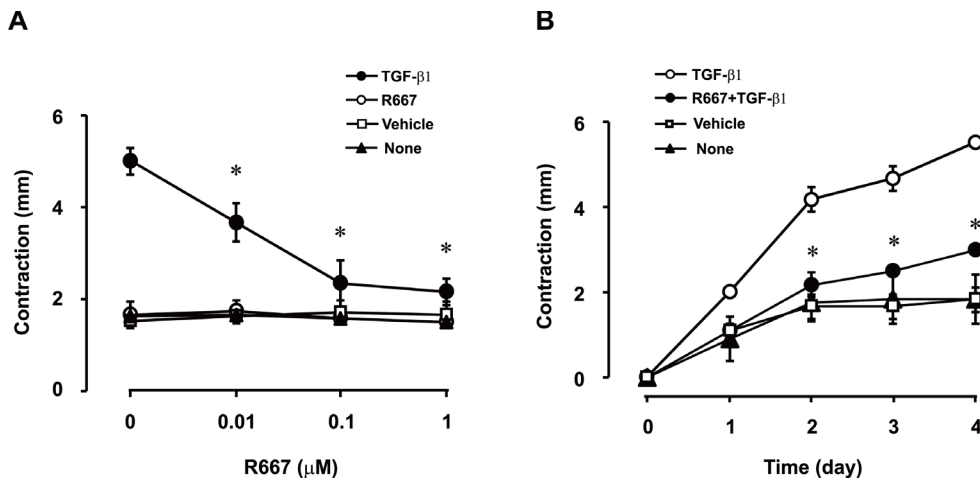


Figure 2. Concentration dependence and time course for the inhibitory effect of R667 on TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs. Cells were cultured in collagen gels either for 3 days in the absence or presence of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; 1 ng/ml) or vehicle (4 mM HCl containing 0.1% bovine serum albumin, BSA) and in the presence of the (A) indicated concentrations of R667 or for the indicated times with TGF- $\beta$ 1 (1 ng/ml) or vehicle in the absence or presence of R667 (1  $\mu$ M; B). The extent of gel contraction was then determined. The extent of TGF- $\beta$ 1-induced gel contraction was  $5.50 \pm 0.17$  mm,  $3.67 \pm 0.29$ ,  $2.83 \pm 0.29$ , and  $2.43 \pm 0.12$  mm in the presence of R667 at 0, 0.01, 0.1, or 1  $\mu$ M, respectively (A). Data are means  $\pm$  standard deviation (SD) of triplicates from experiments that were repeated a total of three times with similar results. \* $p < 0.05$  (the Tukey-Kramer test) versus the corresponding value for cells cultured with TGF- $\beta$ 1 alone.

TIMPs by HTFs with the use of immunoblot analysis and gelatin zymography. HTFs were cultured in collagen gels for 3 days in the presence of TGF- $\beta$ 1 (1 ng/ml) and various concentrations of R667. Analysis with antibodies to MMP-1 revealed that the culture supernatants of cells maintained in the absence of TGF- $\beta$ 1 and R667 contained small amounts of 49- and 45-kDa immunoreactive proteins corresponding to active forms of MMP-1 (Figure 3A). Culture of the cells in the presence of TGF- $\beta$ 1 resulted in an increase in the intensity of these bands. The additional presence of R667 in the cultures induced a concentration-dependent decrease in the abundance of active MMP-1 in the culture supernatants. Immunoblot analysis with antibodies to MMP-3 did not detect either pro or active forms of MMP-3 in the culture supernatants of cells incubated in the absence of TGF- $\beta$ 1 and R667 (Figure 3A). In the presence of TGF- $\beta$ 1, such analysis revealed 49- and 45-kDa immunoreactive proteins corresponding to active forms of MMP-3. The further addition of R667 induced a concentration-dependent decrease in the abundance of active MMP-3 in the culture supernatants.

Gelatin zymography of culture supernatants revealed that culture of the cells with TGF- $\beta$ 1 resulted in an increase in the intensity of the bands corresponding to pro-MMP-2 and active MMP-2, but that this effect of TGF- $\beta$ 1 was not influenced by R667 (Figure 3A). Immunoblot analysis with antibodies to TIMP-1 or to TIMP-2 revealed that neither TGF- $\beta$ 1 nor R667 affected the abundance of these proteins in culture supernatants (Figure 3B).

*Effects of R667 on phosphorylation of FAK and  $\alpha$ -SMA expression in HTFs cultured in collagen gels:* We next examined whether R667 might affect the phosphorylation of FAK in HTFs cultured in collagen gels. Immunoblot analysis revealed that TGF- $\beta$ 1 (1 ng/ml) induced the phosphorylation of FAK in HTFs and that this effect was inhibited by R667 in a concentration-dependent manner (Figure 4). Immunoblot analysis also revealed that culture of the cells for 3 days in the presence of TGF- $\beta$ 1 (1 ng/ml) and various concentrations of R667 had no effect on the abundance of  $\alpha$ -SMA (Figure 5).

*Effects of R667 on fibronectin and type I collagen release from HTFs:* With the use of specific EIAs, we examined whether R667 might affect the release of fibronectin and collagen type I induced by TGF- $\beta$ 1 from HTFs. The release of fibronectin (Figure 6A) and the C peptide of procollagen type I (Figure 6B) induced by TGF- $\beta$ 1 (1 ng/ml) was inhibited by R667 in a concentration-dependent manner.

*Effect of R667 on IOP in rats after glaucoma filtration surgery:* Finally, we examined the effect of R667 on the change in IOP in rats after glaucoma filtration surgery (Figure 7A). IOP was significantly reduced at 7 days after the surgery in rats treated with R667 during the procedure compared with those that received PBS vehicle (Figure 7B).

## DISCUSSION

We have shown that the RAR $\gamma$  agonist R667 inhibited TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs as well as MMP-1 and MMP-3 release, FAK phosphorylation, and the production of fibronectin and type I collagen induced



in these cells by TGF-β1. Furthermore, we found that R667 lowered IOP in a rat model of glaucoma filtration surgery.

Retinoic acids have diverse effects on development, immunity, and tissue repair and regulate the differentiation and function of many cell types [22]. RARs, including RARα, RARβ, and RARγ, belong to the steroid and thyroid hormone receptor superfamily of ligand-dependent transcription factors [23] and bind all-*trans* retinoic acid (ATRA) and its isomer 9-*cis* retinoic acid [24]. We have previously shown that ATRA attenuates TGF-β signaling in HTFs [16]. Failure of filtration surgery due to excessive postoperative scarring remains a major problem, and recent studies into alternative methods for the prevention of such tissue fibrosis have focused on the regulation of HTFs by various growth factors [9,25]. TGF-β is a key mediator of scarring after glaucoma surgery and has become a major target for modulation of the wound healing response. We have now shown that the

RARγ agonist R667 inhibited TGF-β1-induced collagen gel contraction mediated by HTFs in a concentration- and time-dependent manner, whereas the RARα agonist Am580 inhibited this process to a lesser extent and the RARβ agonist BmS453 had no such effect. Our results thus suggest that R667 may prove effective as a drug to reduce scar formation after glaucoma filtration surgery.

Matrix contraction and remodeling during wound healing are mediated in part by the release of MMPs and the formation of focal adhesions by fibroblasts. The regulation of MMP activity is a complex and finely tuned process in which TIMPs as well as MMP synthesis and activation play an important role and to which TGF-β is thought to contribute [26]. Increased expression of MMPs and TIMPs is associated with the formation of filtering blebs [27]. An MMP inhibitor was found to have a pronounced antiscarring effect in an experimental model of glaucoma filtration surgery [2]. We

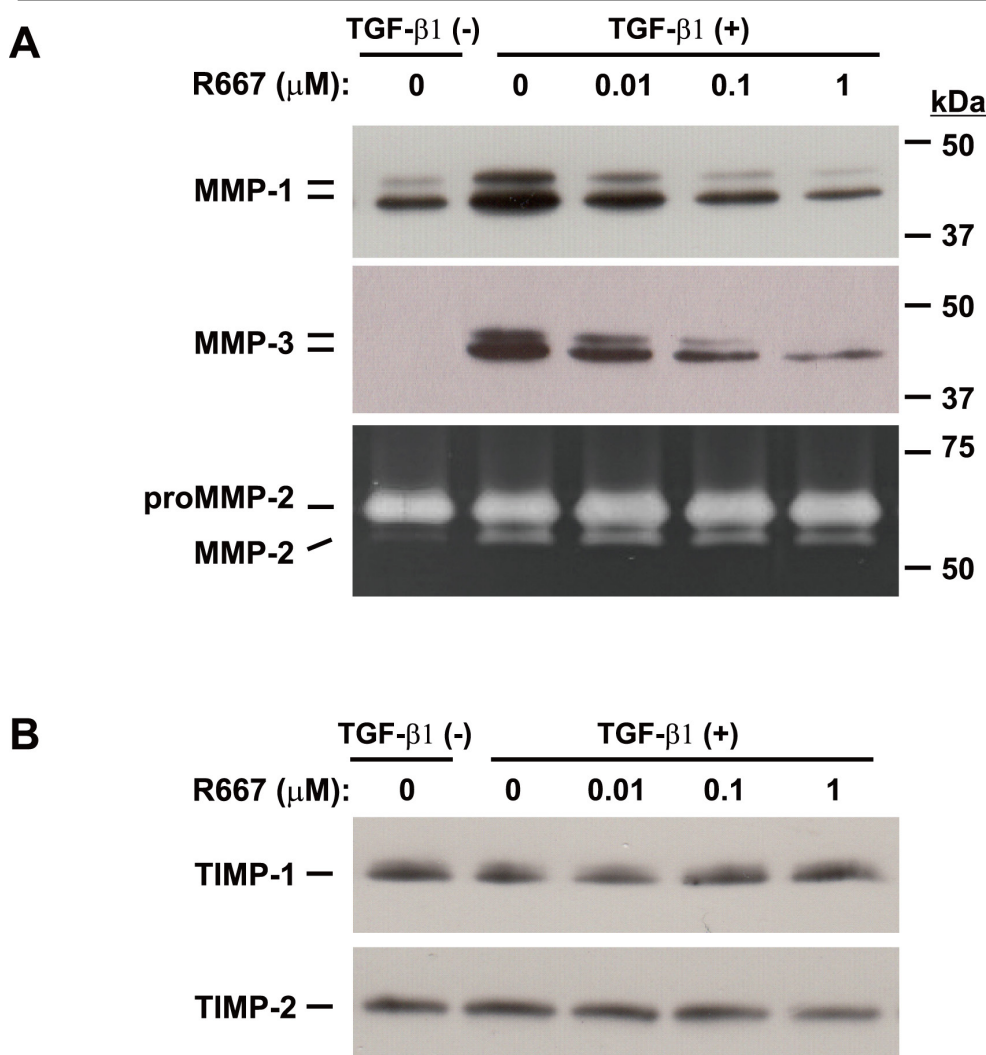


Figure 3. Effects of R667 on the expression of MMPs and TIMPs by HTFs. Cells were cultured in collagen gels for 3 days in the absence or presence of transforming growth factor-β1 (TGF-β1; 1 ng/ml) and in the presence of the indicated concentrations of R667. **A:** The culture supernatants were then either subjected to immunoblot analysis with antibodies to matrix metalloproteinase-1 (MMP-1) and to MMP-3 or analyzed with gelatin zymography for detection of pro and active forms of MMP-2. **B:** The culture supernatants were also subjected to immunoblot analysis with antibodies to tissue inhibitor of metalloproteinase-1 (TIMP-1) and to TIMP-2. All data are representative of three independent experiments.

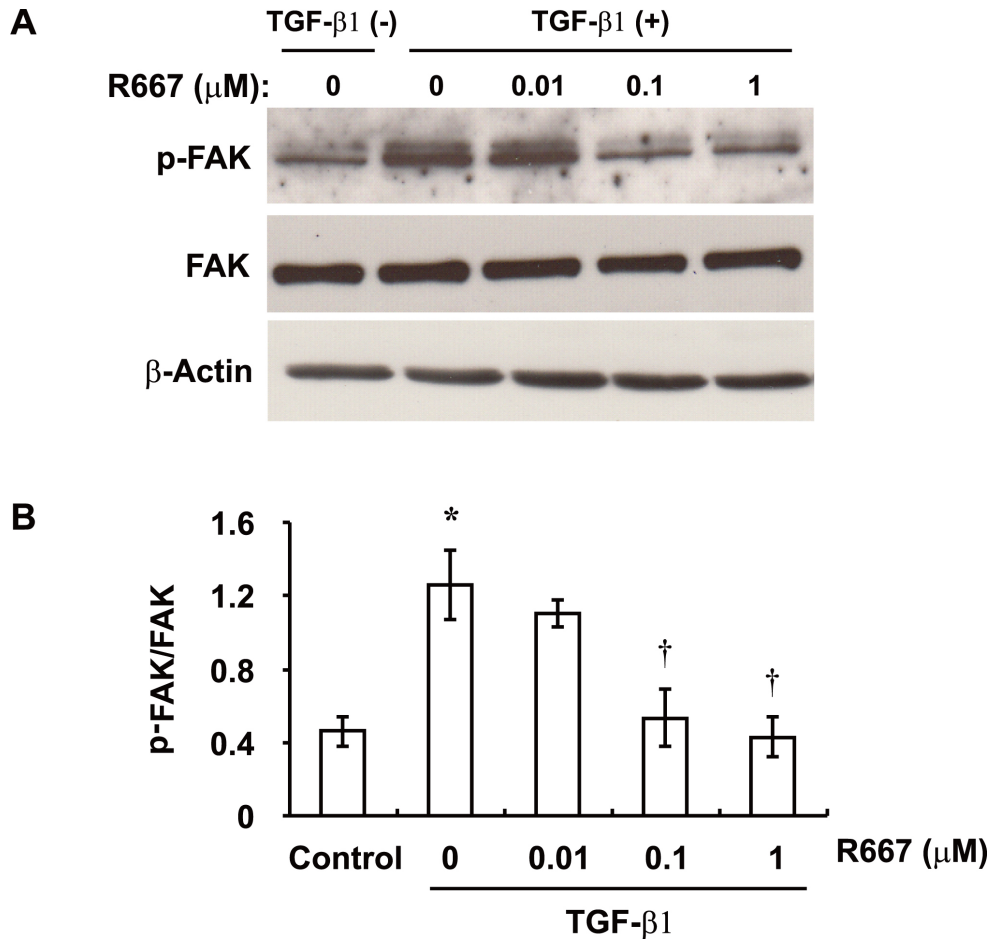


Figure 4. Inhibitory effect of R667 on the TGF- $\beta$ 1-induced phosphorylation of FAK in HTFs cultured in collagen gels. **A:** Cells embedded in collagen gels were incubated first for 12 h with various concentrations of R667 and then for 3 days in the additional absence or presence of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; 1 ng/ml). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of focal adhesion kinase (FAK). The abundance of  $\beta$ -actin was examined as an internal control. **B:** Immunoblots similar to those in (A) were subjected to densitometric analysis to determine the intensity of each band for phosphorylated FAK relative to that of the corresponding band for FAK. Data are means  $\pm$  standard deviation (SD) from three independent experiments. \* $p$ <0.01 versus the corresponding value for cells cultured without TGF- $\beta$ 1; † $p$ <0.05 versus the corresponding value for cells cultured with TGF- $\beta$ 1 alone (the Tukey-Kramer test).

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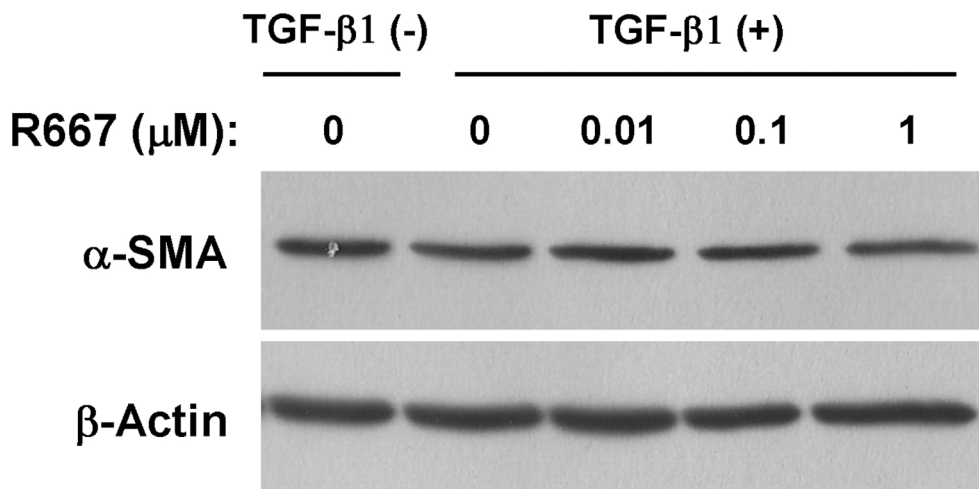


Figure 5. Effect of R667 on  $\alpha$ -SMA expression in HTFs cultured in collagen gels. Cells embedded in collagen gels were incubated first for 12 h with various concentrations of R667 and then for 3 days in the additional absence or presence of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; 1 ng/ml). Cell lysates were then prepared and subjected to immunoblot analysis with

antibodies to alpha-smooth muscle actin ( $\alpha$ -SMA). The abundance of  $\beta$ -actin was examined as an internal control. Data are representative of three independent experiments.

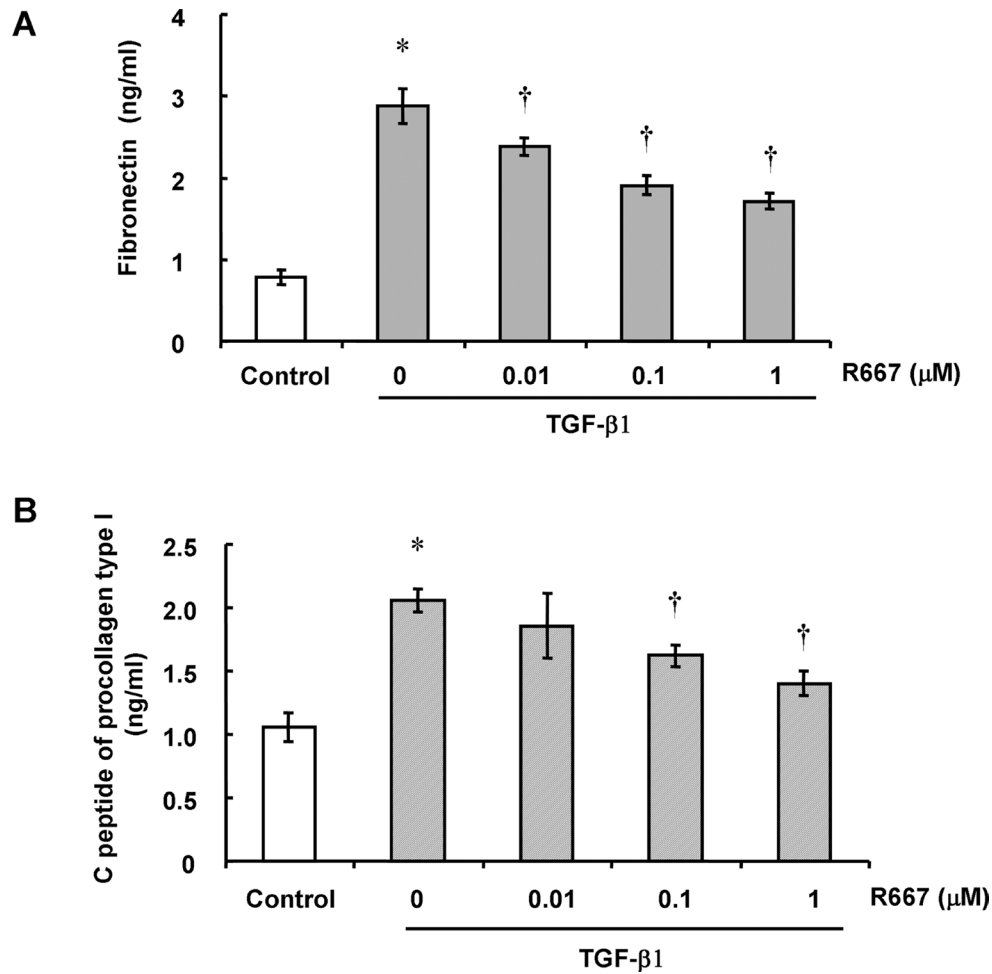


Figure 6. Inhibitory effects of R667 on TGF-β1-induced fibronectin and type I collagen release by HTFs. Cells were cultured in collagen gels with or without transforming growth factor-β1 (TGF-β1; 1 ng/ml) and in the presence of various concentrations of R667 for 3 days, after which the culture supernatants were assayed for (A) fibronectin and the (B) C peptide of procollagen type I. Data are means ± standard deviation (SD) from three independent experiments. \*p<0.01 versus the corresponding value for cells cultured without TGF-β1; †p<0.05 versus the corresponding value for cells cultured with TGF-β1 alone (the Tukey-Kramer test).

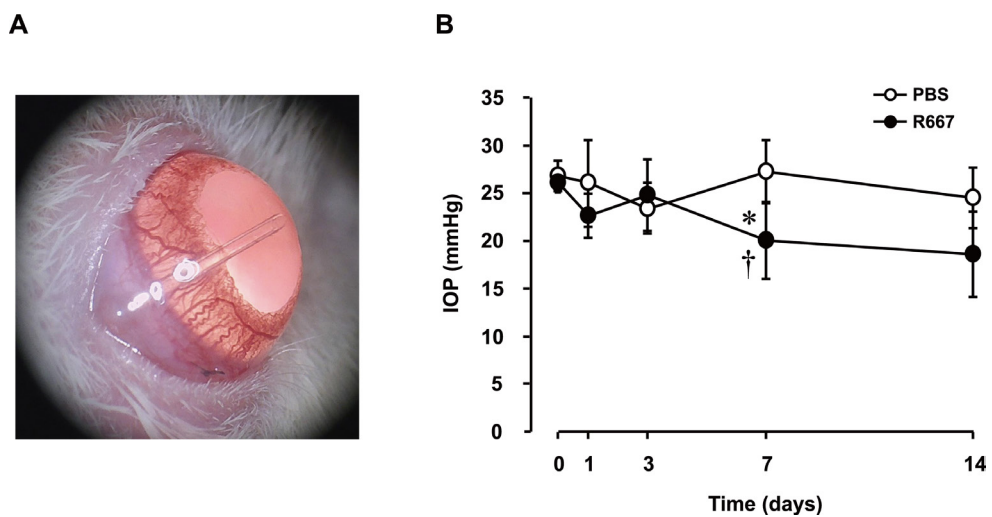


Figure 7. Effect of R667 on IOP in rats after glaucoma filtration surgery. A: Representative photograph of the left eye at 14 days after surgery. B: Intraocular pressure (IOP) of the left eye at the indicated times after filtration surgery for rats treated with R667 or vehicle (PBS) during the procedure. Data are means ± standard deviation (SD) for five animals in each group. \*p<0.05 versus the corresponding

preoperative (time 0) value (Holm's pairwise *t* test); †p<0.05 versus the corresponding value for rats treated with PBS (the Mann-Whitney U test).

have previously shown that MMPs contribute to TGF- $\beta$ 1-induced collagen gel contraction mediated by HTFs [17]. In the present study, R667 inhibited the TGF- $\beta$ 1-induced release of MMP-1 and MMP-3 by HTFs in a concentration-dependent manner, without affecting the release of MMP-2, TIMP-1, or TIMP-2, suggesting that this inhibition of MMP-1 and MMP-3 production contributes to the attenuation of TGF- $\beta$ 1-induced collagen gel contraction by R667. The formation of actin stress fibers as well as the assembly of focal adhesions that connect cells to the ECM contribute to TGF- $\beta$ -induced cell contractility and tissue contraction [16]. FAK is a protein tyrosine kinase that plays an important role in the assembly of focal adhesions and cell contractility [28]. We have now shown that the inhibition of TGF- $\beta$ 1-induced HTF contraction by R667 was associated with attenuation of the TGF- $\beta$ 1-induced phosphorylation of FAK at Tyr<sup>576/577</sup> in these cells. TGF- $\beta$  also induces FAK phosphorylation at other tyrosine residues, including Tyr<sup>397</sup> and Tyr<sup>925</sup>, in some cell types [29,30]. The effect of R667 on FAK phosphorylation at other tyrosine sites remains to be determined.

Transdifferentiation of fibroblasts into myofibroblasts, which is characterized by the onset of expression of the contractile protein  $\alpha$ -SMA, also plays a key role during wound contraction [31]. Consistent with our previous results [16], we found that  $\alpha$ -SMA expression was not affected by TGF- $\beta$ 1 or R667 in HTFs cultured in a floating collagen gel, likely because tension is required for the induction of  $\alpha$ -SMA expression and is not generated to a sufficient extent in the floating matrix [32]. We found that  $\alpha$ -SMA was expressed by HTFs without TGF- $\beta$ 1 stimulation (Figure 5), suggesting that prior exposure of the cells to serum induced myofibroblast differentiation to some extent, consistent with previous observations showing a low level of  $\alpha$ -SMA expression in cultured HTFs [33]. Further examination of the effects of R667 on native Tenon fibroblasts is warranted.

Tissue scarring is associated with changes in the composition of the ECM, which acts as a primary scaffold for cell attachment and migration. Excessive accumulation of ECM proteins such as collagen and fibronectin in the subconjunctival space contributes to bleb failure from wound obstruction in glaucoma filtration surgery [34]. The expression of fibronectin is strongly associated with hypertrophic scar formation and enhanced TGF- $\beta$ -driven scarring responses after glaucoma revision surgery [35]. We have previously shown that fibronectin contributes to the contractility of fibroblasts cultured in a three-dimensional collagen gel [36]. In the present study, the TGF- $\beta$ 1-induced production of type I collagen and fibronectin by HTFs was attenuated by R667 in association with the inhibitory effect of this drug on

TGF- $\beta$ 1-induced collagen gel contraction mediated by these cells. We found that R667 also effectively reduced IOP after experimental glaucoma filtration surgery in rats. Inhibition of the contractility of Tenon fibroblasts and ECM synthesis by R667 may contribute to this *in vivo* action. Comparison of surgical outcomes for animals treated with R667 and mitomycin C is now warranted for further evaluation of the potential of RAR $\gamma$  agonists as alternative, more physiologic agents to protect against postoperative scarring.

Several studies have shown that ATRA possesses antifibrotic potential as a result of its attenuation of TGF- $\beta$  actions [13]. TGF- $\beta$  signaling via Smad and other pathways and retinoid signaling via nuclear receptors intersect in complex ways that are frequently cell dependent [37]. A derivative of ATRA was previously shown to inhibit TGF- $\beta$ -induced collagen expression by blocking Smad2/3 phosphorylation in hepatic stellate cells [38]. We recently showed that the RAR $\gamma$  agonist R667 attenuated the EMT in RPE cells by suppressing mitogen-activated protein kinase, Smad2, and Akt signaling [15]. Modulation of such signaling pathways may also contribute to the inhibitory effects of R667 on ECM remodeling mediated by HTFs.

Humans express three isoforms of TGF- $\beta$  (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3), all of which were detected in association with the conjunctival scarring response [7]. All three isoforms of TGF- $\beta$  also stimulate the proliferation, migration, and contractility of fibroblasts *in vitro*, and they show similar stimulatory effects on conjunctival scarring *in vivo* [7]. Inflammatory processes as well as other growth factors such as vascular endothelial growth factor play a role in conjunctival scarring [39]. Neutralizing antibodies to TGF- $\beta$ 2 did not have a significant effect on bleb survival after glaucoma filtration surgery in a randomized clinical trial [11], suggesting that an agent that targets all three isoforms of TGF- $\beta$  or a combination of treatments may be necessary for such an effect. In the present study, the RAR $\gamma$  agonist R667 lowered IOP in a rat model of glaucoma filtration surgery *in vivo*. We also previously showed that R667 attenuated TGF- $\beta$ 2-induced EMT in RPE cells [15]. Attenuation of TGF- $\beta$ 2 activity might thus also contribute to the lowering of IOP by R667.

Retinoic acids play complex roles in eye development and physiology [12]. The availability of retinoic acids in the anterior chamber has been implicated as a determinant of the pathological changes associated with primary congenital glaucoma [40]. Whether RAR $\gamma$  agonists affect other aspects of ocular physiology, such as aqueous flow or the metabolism of aqueous humor, remains to be determined. Whereas the pharmacokinetics and toxicity of R667 in the eye have not



been examined to date, those after oral administration of the drug have been studied in human subjects [41,42]. The half-life of R667 in plasma was found to be about 10 h, and a single dose of 1 mg was well tolerated, with no unexpected safety concerns [41]. Moreover, we have previously shown that injection of R667 into the eye ameliorated subretinal fibrosis in mice [15]. Further studies are warranted to assess the pharmacokinetics and possible side effects of ocular administration of this drug.

In conclusion, our results show that the RAR $\gamma$  agonist R667 inhibited the contractility of HTFs as well as ECM production by the cells induced by TGF- $\beta$ 1 in vitro. Moreover, this agent lowered IOP in a rat model of glaucoma filtration surgery in vivo. RAR $\gamma$  agonists might thus prove effective for inhibition of scar formation during subconjunctival wound healing after such surgery.

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### REFERENCES

- Addicks EM, Quigley H, Green W, Robin A. Histologic characteristics of filtering blebs in glaucomatous eyes. *Arch Ophthalmol* 1983; 101:795-8. [PMID: 6847472].
- Wong TT, Mead A, Khaw P. Matrix metalloproteinase inhibition modulates postoperative scarring after experimental glaucoma filtration surgery. *Invest Ophthalmol Vis Sci* 2003; 44:1097-103. [PMID: 12601035].
- Fuller JR, Bevin T, Moltano A, Vote B, Herbison P. Anti-inflammatory fibrosis suppression in threatened trabeculectomy bleb failure produces good long term control of intraocular pressure without risk of sight threatening complications. *Br J Ophthalmol* 2002; 86:1352-4. [PMID: 12446362].
- Lama PJ, Fechtner R. Antifibrotics and wound healing in glaucoma surgery. *Surv Ophthalmol* 2003; 48:314-46. [PMID: 12745005].
- Cordeiro MF, Reichel M, Gay J, D'Esposito F, Alexander R, Khaw P. Transforming growth factor- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 in vivo: effects on normal and mitomycin C-modulated conjunctival scarring. *Invest Ophthalmol Vis Sci* 1999; 40:1975-82. [PMID: 10440251].
- Horbelt D, Denkis A, Knaus P. A portrait of Transforming Growth Factor  $\beta$  superfamily signalling: Background matters. *Int J Biochem Cell Biol* 2012; 44:469-74. [PMID: 22226817].
- MF C. Role of transforming growth factor  $\beta$  in conjunctival scarring. *Clin Sci (Lond)* 2003; 104:181-7. [PMID: 12546640].
- Cordeiro MF, Mead A, Ali RR, Alexander RA, Murray S, Chen C, York-Defalco C, Dean NM, Schultz GS, Khaw PT. Novel antisense oligonucleotides targeting TGF- $\beta$  inhibit in vivo scarring and improve surgical outcome. *Gene Ther* 2003; 10:59-71. [PMID: 12525838].
- Cordeiro MF, Gay J, Khaw P. Human anti-transforming growth factor- $\beta$ 2 antibody: a new glaucoma anti-scarring agent. *Invest Ophthalmol Vis Sci* 1999; 40:2225-34. [PMID: 10476787].
- Siriwardena D, Khaw P, King A, Donaldson ML, Overton BM, Migdal C, Cordeiro MF. Human antitransforming growth factor  $\beta$ 2 monoclonal antibody—a new modulator of wound healing in trabeculectomy: a randomized placebo controlled clinical study. *Ophthalmology* 2002; 109:427-31. [PMID: 11874742].
- Khaw P, Grehn F, Hollo G, Overton B, Wilson R, Vogel R, Smith Z. A phase III study of subconjunctival human anti-transforming growth factor  $\beta$ 2 monoclonal antibody (CAT-152) to prevent scarring after first-time trabeculectomy. *Ophthalmology* 2007; 114:1822-30. [PMID: 17908591].
- Duester G. Keeping an eye on retinoic acid signaling during eye development. *Chem Biol Interact* 2009; 178:178-81. [PMID: 18831967].
- Wen X, Li Y, Hu K, Dai C, Liu Y. Hepatocyte growth factor receptor signaling mediates the anti-fibrotic action of 9-cis-retinoic acid in glomerular mesangial cells. *Am J Pathol* 2005; 167:947-57. [PMID: 16192631].
- Nezzar H, Chiambaretta F, Marceau G, Blanchon L, Faye B, Dechelotte P, Rigal D, Sapin V. Molecular and metabolic retinoid pathways in the human ocular surface. *Mol Vis* 2007; 13:1641-50. [PMID: 17893666].
- Kimura K, Orita T, Liu Y, Yang Y, Tokuda K, Kurakazu T, Noda T, Yanai R, Morishige N, Takeda A, Ishibashi T, Sonoda KH. Attenuation of EMT in RPE cells and subretinal fibrosis by an RAR- $\gamma$  agonist. *J Mol Med (Berl)* 2015; 93:749-58. [PMID: 25947075].
- Liu Y, Kimura K, Orita T, Teranishi S, Suzuki K, Sonoda K. Inhibition by all-trans-retinoic acid of transforming growth factor- $\beta$ -induced collagen gel contraction mediated by human tenon fibroblasts. *Invest Ophthalmol Vis Sci* 2014; 55:4199-205. [PMID: 24894398].
- Liu Y, Kimura K, Orita T, Teranishi S, Suzuki K, Sonoda K. All-trans-retinoic acid inhibition of transforming growth factor- $\beta$ -induced collagen gel contraction mediated by human Tenon fibroblasts: role of matrix metalloproteinases. *Br J Ophthalmol* 2015; 99:561-5. [PMID: 25614514].
- Kimura K, Orita T, Fujitsu Y, Liu Y, Wakuta M, Morishige N, Suzuki K, Sonoda KH. Inhibition by female sex hormones of collagen gel contraction mediated by retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2014; 55:2621-30. [PMID: 24609629].

19. Fujitsu Y, Fukuda K, Kumagai N, Nishida T. IL-4-induced cell proliferation and production of extracellular matrix proteins in human conjunctival fibroblasts. *Exp Eye Res* 2003; 76:107-14. [PMID: 12589780].
20. Sherwood MB, Esson D, Neelakantan A, Samuelson D. A new model of glaucoma filtering surgery in the rat. *J Glaucoma* 2004; 13:407-12. [PMID: 15354080].
21. Daniels JT, Cambrey A, Ocleston N, Garrett Q, Tarnuzzer RW, Schultz GS, Khaw PT. Matrix metalloproteinase inhibition modulates fibroblast-mediated matrix contraction and collagen production in vitro. *Invest Ophthalmol Vis Sci* 2003; 44:1104-10. [PMID: 12601036].
22. Chytil F. Rough and rocky road to the retinoid revolution. *Annu Rev Nutr* 2003; 23:1-16. [PMID: 12524430].
23. Mark M, Ghyselinck N, Chambon P. Function of retinoic acid receptors during embryonic development. *Nucl Recept Signal* 2009; 7:e002-[PMID: 19381305].
24. Rochette-Egly C, Germain P. Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). *Nucl Recept Signal* 2009; 7:e005-[PMID: 19471584].
25. Li Z, Van Bergen T, Van de Veire S, Van de Vel I, Moreau H, Dewerchin M, Maudgal PC, Zeyen T, Spileers W, Moons L, Stalmans I. Inhibition of vascular endothelial growth factor reduces scar formation after glaucoma filtration surgery. *Invest Ophthalmol Vis Sci* 2009; 50:5217-25. [PMID: 19474408].
26. Genevay S, Finckh A, Mezin F, Tessitore E, Guerne P. Influence of cytokine inhibitors on concentration and activity of MMP-1 and MMP-3 in disc herniation. *Arthritis Res Ther* 2009; 11:R169-[PMID: 19906289].
27. Mathalone N, Marmor S, Rahat M, Lahat N, Oron Y, Geyer O. MMP expression in leaking filtering blebs and tears after glaucoma filtering surgery. *Graefes Arch Clin Exp Ophthalmol* 2011; 249:1047-55. [PMID: 21452038].
28. Novak IL, Slepchenko B, Mogilner A, Loew L. Cooperativity between cell contractility and adhesion. *Phys Rev Lett* 2004; 93:268109-[PMID: 15698031].
29. Nakamura K, Yano H, Schaefer E, Sabe H. Different modes and qualities of tyrosine phosphorylation of Fak and Pyk2 during epithelial-mesenchymal transdifferentiation and cell migration: analysis of specific phosphorylation events using site-directed antibodies. *Oncogene* 2001; 20:2626-35. [PMID: 11420674].
30. Tian H, Mythreye K, Golzio C, Katsanis N, Blobel G. Endoglin mediates fibronectin/ $\alpha 5\beta 1$  integrin and TGF- $\beta$  pathway crosstalk in endothelial cells. *EMBO J* 2012; 31:3885-900. [PMID: 22940691].
31. Darby I, Skalli O, Gabbiani G.  $\alpha$ -smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 1990; 63:21-9. [PMID: 2197503].
32. Arora PD, Narani N, McCulloch C. The compliance of collagen gels regulates transforming growth factor- $\beta$  induction of  $\alpha$ -smooth muscle actin in fibroblasts. *Am J Pathol* 1999; 154:871-82. [PMID: 10079265].
33. Meyer-Ter-Vehn T, Katzenberger B, Han H, Grehn F, Schlunck G. Lovastatin inhibits TGF- $\beta$ -induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* 2008; 49:3955-60. [PMID: 18421080].
34. Seet LF, Su R, Barathi V, Lee WS, Poh R, Heng YM, Manser E, Vithana EN, Aung T, Weaver M, Sage EH, Wong TT. SPARC deficiency results in improved surgical survival in a novel mouse model of glaucoma filtration surgery. *PLoS One* 2010; 5:e9415-[PMID: 20195533].
35. Meyer-Ter-Vehn T, Grehn F, Schlunck G. Localization of TGF- $\beta$  type II receptor and ED-A fibronectin in normal conjunctiva and failed filtering blebs. *Mol Vis* 2008; 14:136-41. [PMID: 18334936].
36. Liu Y, Yanai R, Lu Y, Kimura K, Nishida T. Promotion by fibronectin of collagen gel contraction mediated by human corneal fibroblasts. *Exp Eye Res* 2006; 83:196-204. .
37. Xu Q, Kopp J. Retinoid and TGF- $\beta$  families: crosstalk in development, neoplasia, immunity, and tissue repair. *Semin Nephrol* 2012; 32:287-94. [PMID: 22835460].
38. Yang KL, Chang W, Hung K, Li E, Chuang C. Inhibition of transforming growth factor- $\beta$ -induced liver fibrosis by a retinoic acid derivative via the suppression of Col 1A2 promoter activity. *Biochem Biophys Res Commun* 2008; 373:219-23. [PMID: 18558083].
39. Lockwood A, Brocchini S, Khaw P. New developments in the pharmacological modulation of wound healing after glaucoma filtration surgery. *Curr Opin Pharmacol* 2013; 13:65-71. [PMID: 23153547].
40. Bouhenni RA, Al Shahwan S, Morales J, Wakim BT, Chomyk AM, Alkuraya FS, Edward DP. Identification of differentially expressed proteins in the aqueous humor of primary congenital glaucoma. *Exp Eye Res* 2011; 92:67-75. [PMID: 21078314].
41. Brennan B, Chiu Y, Berthelon L, Kolis S, Davies B. Effect of age and gender on the pharmacokinetics of R667, a novel agent for the treatment of emphysema, in healthy volunteers. *J Pharm Pharm Sci* 2007; 10:9-16. [PMID: 17498389].
42. Chiu YY, Roth M, Kolis S, Rogovitz D, Davies B. Pharmacokinetics of a novel agent, R667, in patients with emphysema. *Br J Clin Pharmacol* 2007; 63:527-33. [PMID: 17324249].

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