• Basic Research •

Anti-scarring effects of butaprost on human subconjunctival Tenon's fibroblasts

Jong Hoon Shin[1], Je Hyun Seo[2,3], Jae Ho Jung[2,3], Tae Woo Kim[4]

[1]Department of Ophthalmology, Pusan National University Hospital, Busan 49241, Korea
[2]Department of Ophthalmology, Pusan National University Yangsan Hospital, Yangsan 50612, Korea
[3]Research Institute for Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital, Yangsan 50612, Korea
[4]Department of Ophthalmology, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam 13620, Korea
Correspondence to: Je Hyun Seo. Department of Ophthalmology, Pusan National University Yangsan Hospital, 20 Geuo-ro, Mulgum, Yangsan 50612, Korea. jazmin2@naver.com
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Abstract

• AIM: To investigate the toxicity of the E-prostanoid 2 (EP2) receptor agonist, butaprost against human subconjunctival (Tenon's capsule) fibroblasts, and to determine the underlying mechanism.

• METHODS: We isolated Tenon's fibroblasts from the subconjunctival area of healthy subjects and evaluated the types of EP receptors expressed using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The toxicity of butaprost against the fibroblasts was evaluated using methyl thiazolyl tetrazolium and lactic dehydrogenase assays. The inhibition of conjunctival fibroblast proliferation by butaprost was assessed by measuring α-actin levels. The underlying mechanism was assessed by measuring intracellular cyclic adenosine monophosphate (cAMP) levels. Intergroup differences were statistically analyzed using an independent *t*-test. Densitometry of the Western blot bands was performed using the Image J software.

• RESULTS: Quantitative real-time RT-PCR revealed that the fibroblast EP2 receptor levels were higher than those of the other EP receptors. Butaprost did not show toxicity against Tenon's tissue, but inhibited conjunctival fibroblast proliferation by reducing collagen synthesis. EP2 receptor activation enhanced the cAMP cascade, which might be an important mechanism underlying this effect.

• CONCLUSION: Butaprost effectively reduces the subconjunctival scarring response. Given the significance of wound healing modulation in blebs, butaprost's inhibitory effect on subconjunctival Tenon's fibroblasts may be beneficial in managing postoperative scarring in glaucoma surgery.

• **KEYWORDS:** butaprost; Tenon's capsule; trabeculectomy; fibroblasts

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INTRODUCTION

Glaucoma is a progressive optic neuropathy characterized by structural changes in the optic disc and retinal nerve fiber layers, as well as by specific patterns of functional abnormality within the visual field that may eventually lead to severe visual impairment and blindness[1]. Although glaucoma treatment options have improved, intraocular pressure (IOP) remains the major modifiable risk factor in the development and aggravation of glaucoma[2-3]. Glaucoma filtration surgery has been mandatory for achieving target IOP and preventing visual function loss, despite the administration of maximal tolerated medical therapy or laser treatment[3]. Several studies have shown that failure of glaucoma filtration surgery is associated with subconjunctival fibrosis, which leads to conjunctival scarring *via* a postoperative wound-healing response[3-6]. Over the past decade, the success rate of glaucoma filtration surgery has significantly increased because of treatment with intraoperative or postoperative anti-metabolic agents such as mitomycin-C (MMC) and 5 fluorouracil (5-FU)[2]. However, previous reports showed that the toxicity of anti-metabolic agents increased the incidences of severe complications such as corneal melting, conjunctival toxicity, hypotony maculopathy, bleb leaks, and endophthalmitis. Therefore, alternative agents that are less toxic and have higher target specificity should be investigated[7-8].

Prostaglandins are a group of hormone-like substances that are involved in various processes such as smooth muscle contraction and relaxation, blood vessel dilatation and constriction, blood pressure control, and modulation of inflammation[9-10]. Prostaglandin E2 (PGE2) signaling occurs *via* four identified G-protein-coupled receptors, termed

E-prostanoid (EP) receptors 1-4 (EP1-EP4)[11-12]. PGE2 inhibits myofibroblast differentiation *via* activation of EP2 receptors in the lungs, and contributes to the regulation of the pathogenesis of fibrotic disorders of the lungs and other organs[11,13-15]. Nilsson *et al*[16] found that the selective EP2 receptor agonist, butaprost had ocular hypotensive effects in the monkey eye by increasing uveoscleral outflow. These findings suggest that butaprost may lower IOP *via* both inhibiting conjunctival fibrosis after glaucoma filtration surgery and increasing uveoscleral outflow.

The purpose of the present study was to evaluate the anti-scarring effect of the selective EP2 agonist butaprost on subconjunctival (Tenon's tissue) fibroblasts. We expect that our findings may suggest a new therapeutic strategy for the inhibition of conjunctival fibrosis associated with glaucoma filtration surgery.

SUBJECTS AND METHODS

Collection of Human Conjunctival Tissue (Tenon's Tissue) The study design and protocols were approved by the Pusan National University Yangsan Hospital Institutional Review Board (No.04-2015-004), tissue derived from humans in the Declaration of Helsinki. Human subconjunctival fibroblasts were derived from Tenon's capsule after scheduled strabismus surgery by a skillful pediatric eye doctor (Jung JH) at Pusan National University Yangsan Hospital in January-March, 2015. Fourteen subjects were eligible for inclusion in the study. Except for the presence of strabismus, we included only subjects with healthy eyes. Subjects with a history of ocular surgery, intraocular disease, glaucoma, and chronic use of topical eye drops were excluded. Finally, Tenon's fibroblasts derived from 10 subjects were used in this study. Written informed consent was obtained before the strabismus correction surgery, and approval from the institutional human experimentation committee was also granted. Briefly, small pieces of non-functional episcleral tissue were removed during surgery for purification of the subconjunctival Tenon's fibroblasts.

Cell Culture The subconjunctival Tenon's fibroblasts were cultured at 37℃ under a 5% humidified CO_2 atmosphere and in a culture medium containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 50 μg/mL penicillin, and 50 μg/mL streptomycin. The medium was changed every three days. The collected fibroblasts were expanded and cultured in 10-mm dishes. Once they had reached 80% confluency, they were serum-starved for 48h before the addition of medium alone or medium containing 10 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MO, USA) and butaprost (Sigma-Aldrich, St Louis, MO, USA) or LPS alone. We used LPS to induce inflammation followed by fibrosis in the fibroblast cultures. Butaprost was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA).

Assessment of E-prostanoid Receptor Expression Using Quantitative Real-time Reverse Transcription Polymerase Chain Reaction and Southern Blotting RNA was extracted from the fibroblasts according to the manufacturer's instructions (Life Technologies, Gaithersberg, MD, USA) to assess EP receptor expression in the cultured fibroblasts. EP receptor expression was normalized against that of α-actin. The sequences of the polymerase chain reaction (PCR) primers (sense and antisense, respectively) were 5'-CGCAGGGTTCACGCACACGA-3' and 5'-CACTGTGCCGGGAACTACGC-3' for EP1, 5'-AGGACTTCGATGGCAGAGGAGAC-3' and 5'-CAGCCCCTTACACTTCTCCAATG-3' for EP2, 5'-CCGGGCACGTGGTGCTTCAT-3' and 5'-TAGCAGCAGATAAACCCAGG-3' for EP3, and 5'-TTCCGCTCGTGGTGCGAGTGTTC-3' and 5'-GAGGTGGTGTCTGCTTGGGTCAG-3' for EP4. The primers and probes were designed using a commercial software (Primer Express 10; Applied Biosystems, Foster City, CA, USA). Total RNA prepared from subconjunctival fibroblasts was analysed by reverse transcription polymerase chain reaction (RT-PCR) and Southern blotting for expression of EP1 to EP4, and α-actin.

Lactic Dehydrogenase and Methyl Thiazolyl Tetrazolium Assays In the lactate dehydrogenase (LDH) assay, leakage of cytoplasmic LDH into the extracellular cell culture medium was measured as a marker of cell membrane damage. Twenty-four hours after cell seeding, the cells were exposed to butaprost-free saline as control, 10, 100, 500 and 1000 nmol/L concentrations of butaprost. LDH activity was measured in both the supernatants and the cell lysate fraction using CytoTox 96®, a non-radioactive cytotoxicity assay kit (Promega, WI, USA). The viability of human Tenon's fibroblasts was evaluated using the methyl thiazolyl tetrazolium (MTT) assay. Butaprost at each of the concentrations described above was added to the tissue culture plates, and the plates were incubated for 30min; thereafter, the plates were washed with phosphate buffered saline (PBS) to remove butaprost. To measure cell viability after 24h of incubation, 100 μL of ten-fold diluted PBS containing MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; thiazoyl blue, Sigma Aldrich, St. Louis, MO, USA] (5 mg/mL) were added to each well. The samples were incubated for 4h at 37℃ in the dark, and the medium was removed. The precipitates were resuspended in 100 μL of DMSO. The absorbance was measured at a wavelength of 570 nm using a plate reader.

Cyclic Adenosine Monophosphate Assay To assess the intracellular production of cyclic adenosine monophosphate (cAMP), the subconjunctival Tenon's fibroblasts were cultured until they were subconfluent. They were subsequently serum-starved for 24h, followed by treatment with butaprost for 15min. The media were aspirated and the fibroblasts were

washed. The intracellular cAMP levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

Western Blot Analysis The subconjunctival Tenon's fibroblasts were washed twice with ice-cold PBS and were subsequently lysed using cold lysis buffer (1% Triton X-100, 1 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.4, and protease inhibitors). Cell debris was removed *via* centrifugation at 10 000×g for 10min at 4℃. The resultant supernatants were resolved using SDS-PAGE and were transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30min, and were incubated with an anti-α-actin (Sigma-Aldrich, St Louis, MO, USA) and anti-GAPDH antibody (Sigma-Aldrich, St Louis, MO, USA). The membranes were subsequently washed and were incubated with a horseradish peroxidase-conjugated secondary antibody. Western blot bands were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Statistical Analysis All statistical analyses were performed with SPSS for Windows 21.0 (SPSS Inc, Chicago, Illinois, USA), and the sample size were calculated with MedCalcversion 10.0 (MedCalc Software; Ostend, Mariakerke, Belgium). Then, total 10 samples obtained from human conjunctiva were analyzed by the present experiment in each group. Differences between two groups were evaluated using an independent *t*-test, and analysis of variance (ANOVA) was used to determine the differences among the different groups. Bonferroni's post-hoc test was used, if there is a significance of ANOVA test. Densitometry of the Western blot bands was performed using the Image J software (version 1.31; National Institutes of Health, Bethesda, ML, USA). *P* values <0.05 were considered statistically significant. All data are represented as mean±SD.

RESULTS

Total 10 samples obtained from human conjunctiva were analyzed by the present experiment in each group. Sample size was determined by using Medcalc statistical program with establishing significant level as 0.05, and power as 0.2.

RNA Analysis of E-prostanoid Receptor Expression in Subconjunctival Tenon's Fibroblasts The biological effects of PGE2 are mediated *via* EP1, EP2, EP3 and EP4. Both EP2 and EP4 are activated by adenyl cyclase[15]. Quantitative real-time RT-PCR of human Tenon's fibroblasts revealed that the EP2 (123.2, relative expression $\times 10^3$) and EP4 receptors (87.83, relative expression $\times 10^3$) were the significantly dominant receptors in human cultured subconjunctival Tenon's fibroblasts than EP1 (10.42, relative expression $\times 10^3$) and EP3 receptor (33.52, relative expression $\times 10^3$), respectively (*P*<0.001).

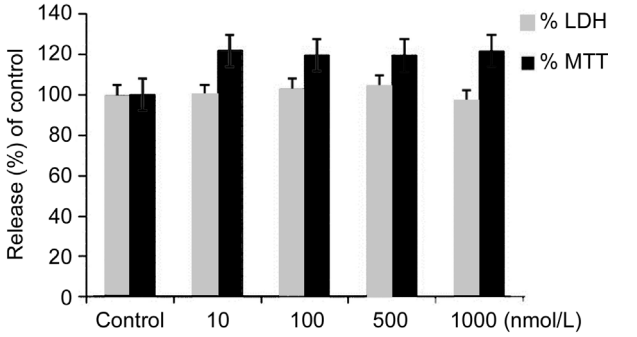

Figure 1 Cytotoxicity against subconjunctival Tenon's fibroblasts was assessed by the release of MTT and LDH into the medium Compared to that observed for the control, butaprost does not show significant cytotoxicity up to a concentration of 1000 nmol/L.

Lipopolysaccharide and Butaprost Toxicity The levels of LDH and MTT in the culture media were analyzed as indicators of the cytotoxicity of LPS and butaprost. Butaprost was non-toxic at all concentrations tested up to 1000 nmol/L compared to that observed for the butaprost-free saline as control (*P*=0.987 and 0.281, respectively) (Figure 1). In addition, LPS did not have a negative effect on cell survival at any of the tested concentrations. Thus, both butaprost and LPS could be safely used at these concentrations in the present study.

Effect of Butaprost on the Inhibition of Lipopolysaccharide-induced α-actin Expression We assessed whether PGE2 could inhibit the proliferation of subconjunctival Tenon's fibroblasts by measuring the protein expression levels of α-actin in the fibroblasts in a time- and dose-dependent manner.

Dose-dependent α-actin expression Dose-dependent response experiments were performed to examine the effect of butaprost on migration of subconjunctival Tenon's fibroblasts. In these experiments, subconjunctival Tenon's fibroblasts were serum-starved for 48h before incubation with LPS±butaprost for 48h. Butaprost at all concentrations measured, *i.e.* 100-1000 nmol/L, inhibited the proliferation of subconjunctival Tenon's fibroblasts. The expression of α-actin protein showed in a butaprost concentration-dependent manner (Figure 2A). A concentration-dependent decrease in LPS-induced α-actin protein expression was also observed in subconjunctival Tenon's fibroblasts treated with butaprost as compared to that of the subconjunctival Tenon's fibroblasts that were treated with LPS alone. The inhibition of LPS-induced α-actin expression was 62.6%±8.43%, 45.7%±6.68% and 36.1%±5.12% at 100, 500 and 1000 nmol/L butaprost, respectively.

Time-dependent α-actin expression To determine the time-dependent effect of butaprost on the proliferation of subconjunctival Tenon's fibroblasts, we treated human subconjunctival Tenon's fibroblasts with 500 nmol/L butaprost for different amounts of time. We observed a time-dependent increase in the proliferation inhibition after treatment with

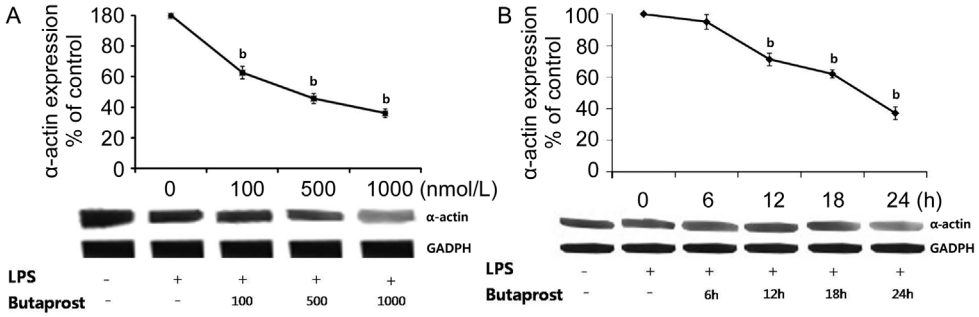

Figure 2 The cytotoxic effect of butaprost against human subconjunctival Tenon's fibroblasts as assessed by the α-actin protein expression levels using Western blotting A: LPS-induced α-actin protein expression decreases in a butaprost dose-dependent manner; the expression of α-actin protein was decreased significantly in 100, 500 and 1000 nmol/L compared with control condition; B: As the duration of the treatment with butaprost (500 nmol/L) was increased, α-actin protein expression was also significantly inhibited throughout the time course; α-actin protein was inhibited significantly after 12h of treatment as compared to the treatment without butaprost. $^{b}P<0.001$ by Bonferroni's post-hoc test.

butaprost, which was significant after 6h of treatment as compared to that observed for LPS-induced α-actin proliferation in the absence of butaprost. Maximal inhibition was observed at 24h after treatment. Western blotting showed a 38.9%±8.17% decrease in α-actin levels after 24h of treatment relative to that observed for butaprost-free saline as the control (Figure 2B). Thus, butaprost inhibited the proliferation of human subconjunctival Tenon's fibroblasts.

Increased Cyclic Adenosine Monophosphate Production Through E-prostanoid 2 Receptor Stimulation The inhibition of the proliferation of human subconjunctival Tenon's fibroblasts by butaprost we observed (Figure 2) prompted us to assess the potential underlying mechanism. It has been postulated that such inhibition might be mediated by elevated cAMP levels[15]. To assess this in our study, human subconjunctival Tenon's fibroblasts were treated with butaprost for 48h followed by measurement of the intracellular cAMP levels. As expected, the cAMP levels in the fibroblasts increased in a butaprost dose-dependent manner, and were significantly increased at butaprost concentrations ≥100 nmol/L compared to that observed for the butaprost-free saline as control ($P<0.05$) (Figure 3). Therefore, we speculated that the ability of butaprost to decrease the scarring effect on subconjunctival fibroblasts might be related to its ability to stimulate cAMP production in subconjunctival Tenon's fibroblasts.

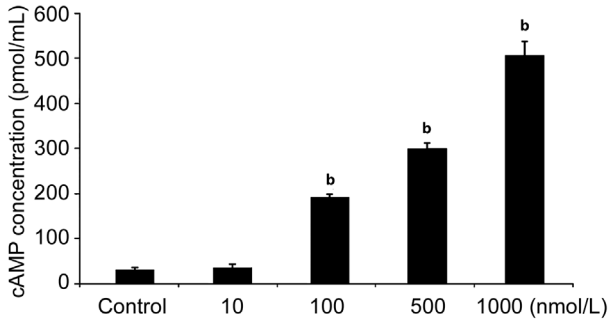

Figure 3 Butaprost induces a dose-dependent increase in cAMP levels Concentrations of butaprost above 100 nmol/L significantly increase the production of cAMP relative to baseline levels ($^{b}P<0.001$ by Bonferroni's post-hoc test).

DISCUSSION

Excess scarring of the conjunctiva after glaucoma filtration surgery is a major cause of bleb failure[17]. The introduction of anti-fibrotic drugs such as MMC and 5-FU has revolutionized filtration surgery and improved the success rate of glaucoma surgery, even in patients known to be at a high risk of scarring. However, these drugs have serious adverse effects such as bleb leakage, hypotony, and bleb-related infections. Further, they cause varied responses, and prediction of the surgical outcome during the intraoperative soaking time is difficult[2,7-8]. When signs of bleb failure are found, needle bleb revision with or without 5-FU injections are needed. This procedure increases the severe complications caused by anti-fibrotic agents. Thus, some investigators have attempted to identify alternative drugs that show lesser toxicity and cause fewer complications[8,18-20]. These potential drugs included steroids, non-steroidal anti-inflammatory agents, fibrinolytic agents such as tissue plasminogen, cytokines such as interferon-α, growth factors such as transforming growth factor-β, and angiogenesis inhibitors such as anti-vascular endothelial growth factor (anti-VEGF). However, to date, none of these have replaced the intraoperative application of MMC and 5-FU.

Recent studies have shown that the selective EP2 receptor agonist butaprost inhibited myofibroblast differentiation *via* activation of EP2 receptors in the lungs, and contributed to the regulation of the pathogenesis of fibrotic disorders of the lungs and other organs[9-10,13,15]. Considering the relationship between conjunctiva fibrosis and failure of glaucoma filtration surgery, EP2 receptor agonists may inhibit fibroblasts in the conjunctiva. The chief aim of our study was to determine whether the EP2 receptor was present in human subconjunctival Tenon's fibroblasts and could inhibit fibrosis in the conjunctiva. Quantitative real-time RT-PCR of human subconjunctival

Tenon's fibroblasts showed that the EP2 and EP4 expression levels were higher than those of EP1 and EP3. Hence, targeting of EP2 and EP4 in subconjunctival Tenon's fibroblasts may be therapeutically effective in glaucoma surgery patients. We also showed that both butaprost and LPS could be safely used at all the concentrations tested in this study. To our knowledge, this is the first study that demonstrated that the EP2 receptor is present in the conjunctiva. Further, we showed that the EP2 receptor agonist butaprost inhibited the proliferation of human subconjunctival Tenon's fibroblasts as assessed by the α-actin protein expression levels. Moreover, butaprost inhibited α-actin in a time- and dose-dependent manner, suggesting that collagen synthesis might have been reduced in butaprost-treated subconjunctival Tenon's fibroblasts. In addition, we have shown that butaprost increased cAMP levels in the subconjunctival Tenon's fibroblasts, which might be the mechanism underlying the inhibition of the proliferation of these fibroblasts by butaprost.

A previous study reported that butaprost decreased the IOP in the monkey eye by increasing uveoscleral outflow[16]. The authors showed that butaprost induced changes in the trabecular meshwork as well as in cilliary muscle tissue. In contrast to what we observed in our study, they postulated that butaprost caused up-regulation of Cyr61 expression to induce remodeling of ciliary muscle cells, and up-regulation of Nur 77 expression in human ciliary cells *via* a protein kinase C-dependent pathway[21-22]. These findings suggested that butaprost had protective effects by lowering the IOP. In our study, we found an additional benefit of butaprost, *i.e.* the inhibition of human subconjunctival Tenon's fibroblast migration and proliferation. The results of our present study and those of the previous studies indicate that the application of butaprost in bleb failure may be clinically relevant because of its IOP-lowering effect by inhibiting conjunctival fibrosis in the bleb and restoration of bleb function, and by increasing uveoscleral outflow of the aqueous humor as is observer for other prostagladin analogues.

There are known risk factors of excess wound healing, *e.g.* young age, aphakia, uveitic glaucoma, and anterior segment neovascularization[23] for which postoperative management, including needling combined with the injection of an anti-metabolite, is required. Some patients are reluctant to receive anti-metabolite injections because of pain sensations. In this case, topical application of the drug can be useful for maintaining bleb survival. Another study has shown that the combination of tacrolimus and octreotide in the form of topical eye drops administered after glaucoma surgery effectively inhibited the proliferation of fibroblasts in a rabbit model[24]. In addition to this finding, in the present study, butaprost lowered the IOP by increasing uveoscleral outflow *via* increasing cAMP levels in the subconjunctival Tenon's fibroblasts. We further showed that butaprost could be safely used *in vitro*.

Our study has several limitations. First, all subconjunctival Tenon's fibroblasts were obtained from healthy subjects. In another study, subconjunctival fibroblasts of glaucoma patients showed higher levels of inflammation than those of healthy subjects after benzalkonium chloride (BAK) use[25-26]. However, the purpose of our study was to determine EP2 receptor toxicity, the effective concentration levels in the conjunctiva, and the underlying mechanism of action of butaprost; therefore, we first studied normal conjunctiva tissue. Future studies assessing the conjunctiva obtained from glaucoma patients during trabeculectomy or glaucoma drainage implants are needed. Second, this study assessed the effects of butaprost only *in vitro*, and, therefore, its clinical effects might be different. To address this issue, the application of topical butaprost in an animal glaucoma surgery model is necessary. Third, this study did not fully answer whether butaprost could be used instead of the anti-fibrotic drugs MMC and 5-FU; further comparison and non-inferiority studies should be performed. In addition, the potential long-term implications of butaprost treatments should be investigated. Fourth, we concluded that butaprost inhibited the proliferation of subconjunctival Tenon's fibroblasts on the basis of the decreased α-actin protein expression. However, a decrease in α-actin expression does not necessarily imply that butaprost inhibits proliferation. To address this issue, future studies should quantify the number of fibroblasts or investigate cell cycle markers.

In conclusion, our study showed that EP2 receptors are present in the human conjunctiva and that butaprost effectively reduced the subconjuctival scarring response. Given the significance of wound-healing modulation in blebs, butaprost's inhibitory effect on subconjunctival Tenon's fibroblasts may be a potential therapeutic strategy in the postoperative management of glaucoma filtration surgery, especially if signs of bleb failure are observed.

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Conflicts of Interest: Shin JH, None; **Seo JH,** None; **Jung JH,** None; **Kim TW,** None.

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