• Basic Research •

Effects of exogenous recombinant human bone morphogenic protein-7 on the corneal epithelial mesenchymal transition and fibrosis

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Abstract

• AIM: To evaluate the effect of exogenous recombinant human bone morphogenic protein-7 (rhBMP-7) on transforming growth factor-β (TGF-β)-induced epithelial mesenchymal cell transition (EMT) and assessed its antifibrotic effect *via* topical application.

• METHODS: The cytotoxic effect of rhBMP-7 was evaluated and the EMT of human corneal epithelial cells (HECEs) was induced by TGF-β. HECEs were then cultured in the presence of rhBMP-7 and/or hyaluronic acid (HA). EMT markers, fibronectin, E-cadherin, α-smooth muscle actin (α-SMA), and matrix metaloproteinase-9 (MMP-9), were evaluated. The level of corneal fibrosis and the reepithelization rate were evaluated using a rabbit keratectomy model. Expression of α-SMA in keratocytes were quantified following treatment with different concentrations of rhBMP-7.

• RESULTS: Treatment with rhBMP-7 attenuated TGF-β-induced EMT in HECEs. It significantly attenuated fibronectin secretion (31.6%; *P*<0.05), the α-SMA protein level (72.2%; *P*<0.01), and MMP-9 expression (23.6%, *P*<0.05) in HECEs compared with cells grown in the presence of TGF-β alone. E-cadherin expression was significantly enhanced (289.7%; *P*<0.01) in the presence of rhBMP-7. Topical application of rhBMP-7 combined with 0.1% HA significantly reduced the amount of α-SMA$^+$ cells by 43.18% (*P*<0.05) at a concentration of 2.5 μg/mL and by 47.73% (*P*<0.05) at 25 μg/mL, compared with the control group, without disturbing corneal reepithelization.

• CONCLUSION: rhBMP-7 attenuates TGF-β-induced EMT *in vitro*, and topical application of rhBMP-7 reduces keratocyte myodifferentiation during the early wound healing stages *in vivo* without hindering reepithelization. Topical rhBMP-7 application as biological eye drops seems to be feasible in diseases involving TGF-β-related corneal fibrosis with corneal reepithelization disorders.

• KEYWORDS: bone morphogenic protein-7; corneal fibrosis; epithelial mesenchymal transition; myodifferentiation; transforming growth factor-β
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INTRODUCTION

Scarring of the cornea can occur for many reasons. Scarring changes the optical properties of the cornea, thus altering eye sight. It is caused *via* fibrotic cellular responses; it heals the tissue, but fails to restore transparency. Debris and/or chemicals entering the eye, infection, inflammation, and diseases of the cornea can all lead to permanent scarring[1]. In laser refractive surgeries including photorefractive keratectomy (PRK), laser subepithelial keratomileusis (LASEK), and laser *in situ* keratomileusis (LASIK), certain degrees of corneal haze develop during the wound healing process. However, if severe corneal haze remains, it may induce glare disability and result in decreased visual acuity. Previous studies indicated that LASIK causes less corneal haze than does PRK or LASEK[2-4]. One deciding difference between LASIK and the other procedures during the wound healing process after surgery is whether epithelial regeneration takes place.

Interactions between corneal epithelial cells and stromal cells during the wound healing process have been suggested. Nakamura[5] demonstrated that the intact corneal epithelium curbs the differentiation of myofibroblasts during corneal wound healing. However, injured epithelial cells stimulated fibroblast myodifferentiation in a rabbit model. Another pos-

sible contribution is the epithelial mesenchymal transition (EMT). Epithelial cells have the potential to undergo phenotypic changes and differentiation into fibroblastic cells in response to morphogenic pressure from injured tissue. The EMT is characterized by a transition from a polarized epithelial phenotype to a mesenchymal phenotype or highly motile fibroblatoid by promoting the downregulation of epithelial markers such as E-cadherin, β-catenin, and zonular occludens-1, as well as upregulation of mesenchymal markers such as fibronectin, vimentin, and α-smooth muscle actin (α-SMA)[6-7]. It has been reported that the EMT is involved in mouse corneal wound healing after alkali burn, human pterygium, and subepithelial corneal fibrosis in patients with limbal stem cell deficiency[8-10].

Corneal wound healing involves various cellular systems including cell migration and proliferation. It is generally accepted that transforming growth factor-β1 (TGF-β1) regulates the diverse events surrounding wound healing[11]. TGF-β signaling components are also involved in the EMT of various fibrosis models and diseases[11-14]. Kaji *et al*[2] reported that PRK increased the expression of TGF-β1 and induced corneal haze, and Lee *et al*[15] demonstrated a release of tear fluid TGF-β1 during the early postoperative days following LASEK and PRK and its association with corneal haze. Several studies have shown the expression of TGF-βs and their receptors during the wound healing process after laser ablation in animal models[16-17].

Bone morphogenic proten-7 (BMP-7) is a 35-kDa homodimeric protein and a member of the TGF-β superfamily[18]. It antagonizes TGF-β activity in tissue fibrosis. BMP-7 in the cornea is mediated through the activation of its receptor signaling, that is Smad 1/5/8 signaling, which counteracts TGF-β/Smad signaling.

Moreover, inhibitors of differentiation, Id2 and Id3, reportedly control the EMT response to TGF-β1 and BMP-7[19-20]. Zeisberg *et al*[12] reported that recombinant human BMP-7 (rhBMP-7) reversed TGF-β1-induced EMT in a mouse chronic renal injury model. In the cornea, the BMP-7 gene, introduced *via* gold nanoparticles or adenoviral gene transfer, showed inhibition of corneal fibrosis in rabbit cornea or suppression of myofibroblast generation in a mouse corneal alkali injury model[21-22]. The primary aims of the present study were to evaluate the effect of exogenous rhBMP-7 on TGF-β-induced EMT in human corneal epithelial cells and corneal fibrosis during wound healing after keratectomy in a rabbit model.

MATERIALS AND METHODS

We used human corneal epithelial cells (HCECs) and New Zealand white rabbits for *in vitro* and *in vivo* studies, respectively. The Institutional Animal Care and Use Committee of the Korea Animal Medical Science Institute approved all animal experiments. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Hyaluronic acid (HA) is a naturally occurring viscoelastic hydrophilic polymer that reduces ocular surface damage, possibly by improving corneal hydration and decreasing ocular friction[23]. Eye drops containing HA are commonly used in patients after undergoing laser refractive surgery and those with dry eye syndrome. Treatment with 0.1% HA alone was used as a control, and rhBMP-7 was used at various concentrations with 0.1% HA in the treatment groups.

Cell Culture and Reagents HCECs were obtained from the American Type Culture Collection (PCS-700-010, ATCC; VA, USA) and cultured in corneal epithelial cell basal medium containing growth supplements according to the manufacturer's instructions (ATCC). Cells from passage 3 were used in all experiments. To evaluate the effect of BMP-7 on cell viability and proliferation, cells were seeded into 96-well plates at a density of 2×10^5/mL. Cells were serum-starved for 4h and cultured in one of the following media for 24h: 1) fresh medium; 2) fresh medium containing 500 ng/mL BMP-7 (Cellumed, Seoul, Korea); 3) fresh medium containing 500 ng/mL BMP-7 and 0.1% HA (Bioland, Chunan, Korea); or 4) fresh medium containing 0.1% HA.

To evaluate the effect of rhBMP-7 on the EMT, cells were cultured in 6-well plates at a density of 1×10^5/mL at 37℃ for 48h. Cells were serum-starved for 4h and washed with phosphate buffered saline (PBS) and switched to medium containing TGF-β1 (Sigma. St. Louis, MO, USA; 5 ng/mL). After 1h, rhBMP-7 and/or HA were added to the cells as described above and cultured for 24h.

Cell Viability and Proliferation Assay Cytotoxicity was assessed by measuring dehydrogenase activity in cells using the Ez-cytox Kit (Daeillab service, Seoul, Korea) according to the manufacturer's instructions. Cellular proliferation was assessed using a bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Enzyme-linked Immunosorbent Assay To evaluate the effect of rhBMP-7 on the EMT, secreted levels of fibronectin, an extracellular matrix and upregulated mesenchymal marker in culture media, was measured. Cells were cultured in 6-well plates at a density of 1×10^5/mL at 37℃ for 48h. Cells were serum-starved for 4h and switched to medium containing TGF-β1 (5 ng/mL). After 1h, rhBMP7 and/or HA were added to cells and cultured for 24h. Cell-free culture media were harvested and stored at -70℃ before use. Secreted fibronectin was assessed using a fibronectin ELISA kit (Abcam).

Western Blotting To evaluate the effect of rhBMP-7 on the EMT, Western blot analyses were performed. The effect of TGF-β on the EMT was determined by the appearance of α-SMA and loss of E-cadherin[10]. Matrix metaloproteinase-9

(MMP-9) has been shown to be important in both normal corneal wound healing and in EMT-like changes[24]. Cultures in four different media were washed with PBS, and trypsinized cells were harvested. Cell lysates were prepared in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail (Pierce Biotechnology, IL, USA). Lysate samples were quantified, and 60 μg lysates were boiled for 10min. Samples were transferred onto a nitrocellulose membrane (Bio-Rad, CA, USA) and detected using the following primary antibodies: α-SMA (1:500 dilution, 1A4; Sigma-Aldrich, MO, USA), MMP-9 (1:1000 dilution; Cell Signaling Technology, MA, USA), E-cadherin (1:1000 dilution, 24E10; Cell Signaling Technology), and GAPDH (1:1000 dilution, 1D4, Santa Cruz), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (AbDSerotec; Oxford, UK) for α-SMA or a HRP-conjugated goat anti-rabbit secondary antibody (Pierce biotechnology) for MMP-9 and E-cadherin. Membranes were washed with PBS containing 0.05% Tween-20 and developed using enhanced chemiluminescence (ECL) solution (Pierce Biotechnology).

***In Vivo* Rabbit Corneal Injury Model of Keratectomy** Fifteen male New Zealand white rabbits (DreamBio, Seoul, Korea) were anesthetized by intravenous injection of a mixture of tiletamine hypochloride and zolazepam hydrochloride (VIRBAC, Carros, France) and xylazine (Bayer AG, Leverkusen, Germany). Several drops of 0.5% proparacaine hydrochloride (Alcon, TX, USA) were instilled on the cornea for local anesthesia. Keratectomy was performed to create a free flap 6 mm in diameter and 150 μm in depth using a microkeratome (MK-2000, Nidek, Gamagori, Japan), which has been show to create a flap of predictable thickness[25]. Rabbits were divided into four groups (three rabbits for each group). Only the left eye of each rabbit was used for subsequent experiments. Animals in Group 1 were treated with 0.1% HA, those in Group 2 with 0.1% HA and rhBMP-7 (0.25 μg/mL), those in Group 3 with 0.1% HA and rhBMP-7 (2.5 μg/mL), and those in Group 4 with 0.1% HA and rhBMP-7 (25 μg/mL).

Corneal Epithelization Evaluation To evaluate corneal epithelial healing *in vivo* using a rabbit keratectomy model, photographs of fluorescein-stained cornea were obtained to measure the area of the epithelial defect immediately or 4 or 7d after keratectomy. The area was quantified from the photographs using an image analyzer (Image J; National Institute of Health, Bethesda, MD, USA). The extent of healing was determined by the ratio of the difference between the initial wound and the remaining epithelial defect area at 4 and 7d after keratectomy.

Immunofluorescence Staining Immunofluorescence staining for α-SMA was performed by incubating corneal sections with 1% bovine serum albumin for 10min at room temperature followed by incubation with a mouse monoclonal primary α-SMA antibody (1:200 dilution, 0N5; Abcam, Cambridge, UK) overnight. For detection, sections were exposed to an Alexa 488 goat anti-mouse IgG secondary antibody (1:200 dilution; Abcam, Cambridge, UK) for 60min. After completion of immunostaining, tissue sections were mounted in medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA, USA) and photographed under a fluorescence microscope (Axio Imager 2, Zeiss, Oberkochen, Germany) equipped with a digital camera system. $\alpha\text{-SMA}^+$ cells in six randomly selected non-overlapping full-thickness central corneal columns were counted manually for quantitative analysis. The diameter of each column was that of a 400× magnification field.

Statistical Analysis Data are presented as the mean±standard deviation (SD) or standard error of the mean (SEM). Statistical analyses were performed using SPSS ver. 12.0 (IBM SPSS, Chicago, IL, USA). The Mann-Whitney *U* test following Kruskal-Wallis test was used to compare results between groups. A P value <0.05 was considered statistically significant.

RESULTS

Recombinant Human Bone Morphogenic Protein-7 was Non-toxic and Increased Human Corneal Epithelial Cell Proliferation To evaluate the feasibility of ocular surface application of rhBMP-7, cytotoxicity and the proliferation rate were assessed initially. The number of metabolically active cells was significantly higher in cells cultured with rhBMP-7 alone or rhBMP-7 with 0.1% HA compared with those cultured in fresh medium or 0.1% HA-containing medium, as determined by water-soluble tetrazolium assay (P<0.05; Figure 1A). The proliferation rate of cultivated HCECs cultured with rhBMP-7 alone or rhBMP-7 with 0.1% HA was also significantly higher compared with those cultured in fresh medium or 0.1% HA-containing medium, as determined by BrdU uptake (P<0.01; Figure 1B).

Recombinant Human Bone Morphogenic Protein-7 Attenuated TGF-β-induced Epithelial Mesenchymal Cell Transition *in Vitro* Increased fibronectin levels in HCECs induced by TGF-β stimulation were attenuated in the presence of rhBMP-7 (31.6%; P<0.05) or rhBMP-7 with HA (35.7%; P<0.01) (Figure 2A). α-SMA protein levels were significantly decreased in the presence of rhBMP-7 (72.2%; P<0.01) or rhBMP-7 with HA (69.3%; P<0.01) compared with TGF-β alone (Figure 2B). MMP-9 expression stimulated by TGF-β was attenuated by rhBMP-7 (23.6%; P<0.05) or rhBMP-7 with HA (27.4%; P<0.05) (Figure 2C). E-cadherin expression was enhanced in the presence of rhBMP-7 (289.7%; P<0.01) or rhBMP-7 with HA (290.2%; P<0.01) compared with TGF-β alone (Figure 2D).

Effect of Recombinant Human Bone Morphogenic Protein-7 on Cornel Epithelization and Fibrosis *in Vivo* Corneal epithelization rates were not significantly different at 4d (P=0.053) or 7d (P=0.593) after keratectomy among the four groups (Figure 3).

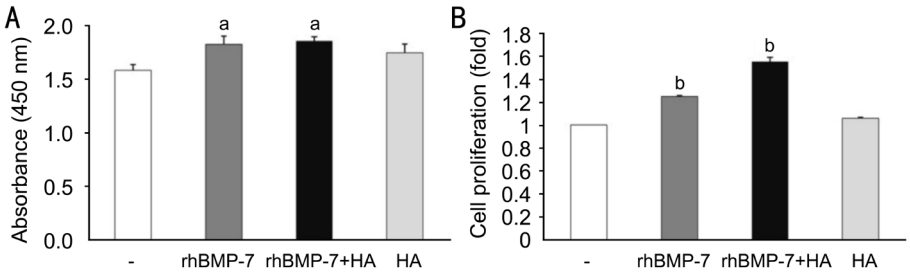

Figure 1 Effect of rhBMP-7 and HA on survival and proliferation of HCECs A: Water soluble tetrazolium assay showing significant increases in metabolically active cells in medium containing rhBMP-7 with or without HA; B: BrdU uptake assay showing increased cell proliferation rates in medium containing rhBMP-7 with or without HA. Data represent the mean±SEM or the ratio relative to control cells of three independent experiments (^{a}P<0.05, ^{b}P <0.01).

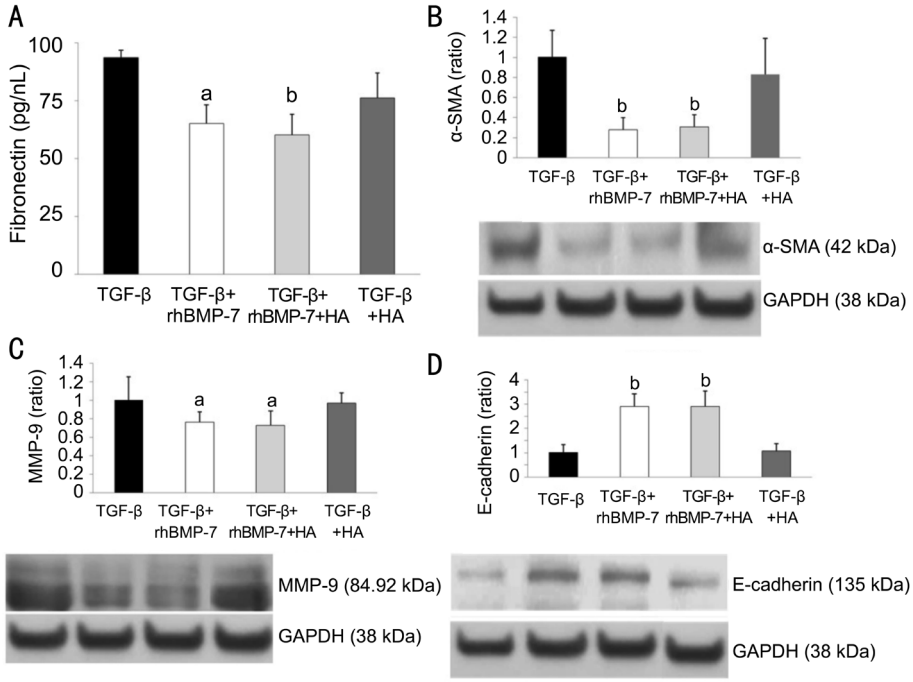

Figure 2 Effect of rhBMP-7 with HA treatment on the expression of EMT markers in cultivated HCECs A: Fibronectin secretion was attenuated 31.6% in the presence of rhBMP-7 (P<0.05) and 35.7% in the presence of rhBMP-7 with HA (P<0.01). The effect of HA alone was not significant; B, C: Both rhBMP-7 and rhBMP with HA showed a significant attenuation of α-SMA (72.2%, P<0.01 and 69.3%, P<0.01, respectively) and MMP-9 (23.6%, P<0.05 and 27.4%, P<0.05, respectively) expression compared with TGF-β stimulation; D: E-cadherin expression was enhanced 289.7% by rhBMP-7 (P<0.01) and 290.2% by rhBMP-7 with HA (P<0.01) compared with TGF-β alone. The graph summarizes the densitometric analysis from three independent experiments. Representative immunoblots for α-SMA, E-cadherin, or MMP-9 with GAPDH are displayed below. All data represent the mean±SEM of three independent experiments (^{a}P<0.05, ^{b}P <0.01).

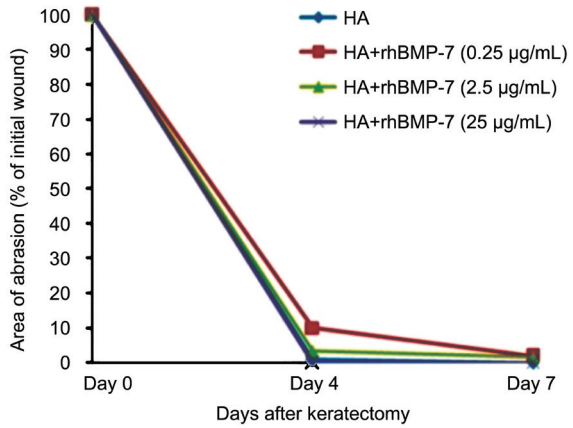

Figure 3 Rate of *in vivo* epithelial healing in the four groups Residual epithelial defect areas evaluated by fluorescein staining showed no significant differences among the four groups at 4d (P=0.053) or 7d (P=0.593) after keratectomy. The graph summarizes the ratio of residual epithelial defects to the initial wound area from three independent experiments. All data represent the mean±SD.

Corneal sections obtained from the rabbits 7d after keratectomy showed α-SMA^{+} cells in the anterior stroma. Specifically, the presence of myofibroblasts was confirmed (Figure 4). rhBMP-7 (2.5 and 25 μg/mL)-treated rabbit corneal tissue sections showed significantly fewer α-SMA^{+} cells. Topical application of 2.5 and 25 μg/mL rhBMP-7 caused a significant (43.18%, P<0.05 and 47.73%, P<0.05, respectively) decrease in α-SMA^{+} cells compared with untreated corneas (Figure 5).

DISCUSSION

In the present study, we confirmed that rhBMP-7 has no cytotoxic effects, and that exogenous rhBMP-7 suppresses TGF-β-induced EMT in cultivated HCECs. Moreover, we showed that topical application of rhBMP-7 suppresses the expression of α-SMA expression, a well-known fibrosis marker, without disturbing reepithelization *in vivo*.

TGF-β is one of the most important growth factors regulating tissue fibrosis and inflammation. TGF-β modulates the

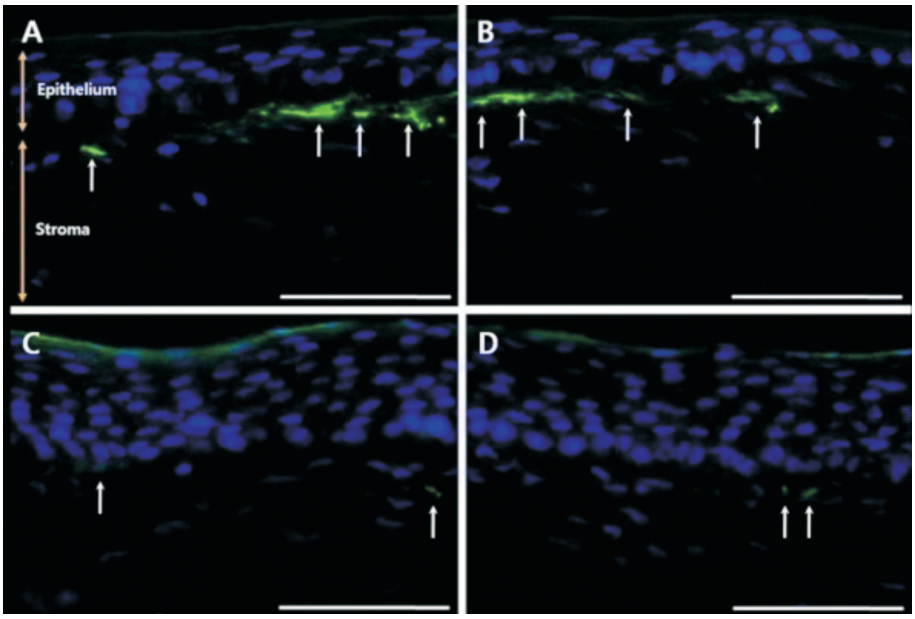

Figure 4 Representative images showing immunofluorescence staining for α-SMA (green), a myofibroblast marker, in the stroma of keratectomized corneal tissue sections obtained from rabbit eyes treated with topical rhBMP-7 and/or 0.1% HA A: Corneal tissues treated with 0.1% HA showing abundant α-SMA$^+$ cells; B, C, D: Corneal tissues treated with 0.25, 2.5, or 25 μg/mL rhBMP-7 with 0.1% HA. Scanty α-SMA$^+$ cells were identified in the 2.5 (C) and 25 μg/mL (D) rhBMP-7-treated groups. Scale bar denotes 100 μm.

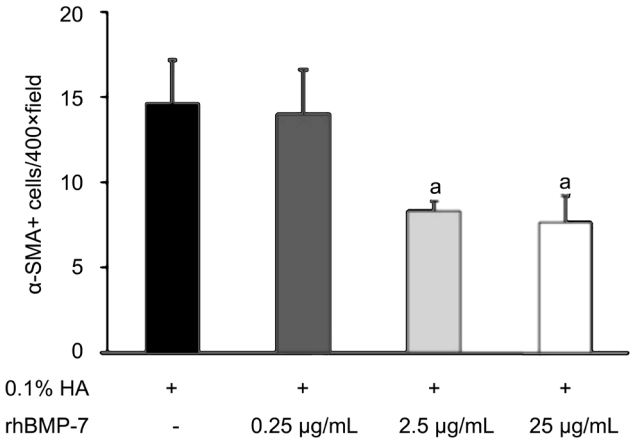

Figure 5 Quantification of α-SMA$^+$ cells in corneal tissues 7d after keratectomy Topical application of 2.5 or 25 μg/mL rhBMP-7 with 0.1% HA significantly decreased α-SMA$^+$ cells compared with application of 0.1% HA and/or 0.25 μg/mL rhBMP-7. A concentration of 0.25 μg/mL rhBMP-7 was not sufficient to attenuate corneal fibrosis. All data represent the mean±SD (^aP<0.05).

behaviors of several corneal cell types (*e.g.* epithelial cell migration and proliferation and transdifferentiation of keratocytes into myofibroblasts). TGF-β is also chemotactic to monocytes/macrophages during wound healing in the cornea as in other tissues[26-28]. It is detected in tears and corneal tissue, and its receptors are expressed in component cells of the cornea[15,29]. Several studies have demonstrated the role of TGF-β in corneal wound healing processes, especially fibrosis after PRK. Mita *et al*[16] reported that an abnormal fibrotic layer contains fibroblast-like keratocytes and expresses fibrotic markers, TGF-β isoforms, and TGF-β receptors in the subepithelial layer. Kaji *et al*[2] also reported a causal relationship between corneal haze and TGF-β1 expression, which is not detected in normal keratocytes. Tear fluid analysis demonstrated significantly increased TGF-β1 levels during the early healing stages after corneal ablation and its association with corneal haze[15,30].

In the present study, TGF-β1 induced loss of the epithelial phenotype marker E-cadherin and induced expression of mesenchymal markers such as α-SMA and fibronectin in cultivated HCECs. Contrary to TGF-β1, rhBMP-7 significantly inhibited the EMT by enhancing E-cadherin expression (289.7%) and attenuating that of α-SMA (72.2%), MMP-9 (23.6%), and fibronectin (31.6%). Our findings suggest that rhBMP-7 has an antagonistic effect in the process of TGF-β-induced EMT in HCECs.

TGF-β is a well-known inducer of the EMT, and BMP-7 counteracts TGF-β-induced EMT[12]. BMP-7 is a multifunctional cytokine with a wide range of effects on cell growth, differentiation, and apoptosis[31-32]. BMP receptors and several BMPs have also been described in human corneal epithelium and stroma[33]. BMP-7 counteracts the EMT and is involved in the induction of the mesenchymal to epithelial transition, alongside its role in sustaining the epithelial phenotype and wound healing[34-35]. The mechanism of action of BMP-7 involves Smad1/5/8 activation. Using a corneal fibrosis model, Tandon *et al*[21] reported significantly increased phospho-Smad1/5/8 levels and inhibition of Smad6, suggesting that the anti-fibrotic effects of BMP-7 in the cornea are mediated by the suppression of TGF-β-driven profibrotic Smad signaling by increasing the expression of inhibitory Smad6 *in vivo*. A study of adenoviral BMP-7 gene transfer in mice cornea also showed

activation of Smad1/5/8 signaling and partial suppression of the phosphor-Smad2 signal[22]. Although the effect of BMP-7 against TGF-β is organ-dependent, our findings and previous studies in the cornea support the hypothesis that BMP-7 counteracts TGF-β-induced EMT[36].

The presence of myofibroblasts is a characteristic feature of laser-induced corneal haze. Typically, these myofibroblasts express bundles of filamentous proteins and therefore can be detected readily by staining for α-SMA[21]. Significant decreases in α-SMA$^+$ cells (43.18%, P<0.05 and 47.73%, P<0.05) at concentrations of 2.5 and 25 μg/mL, respectively, compared with untreated cells, suggest that topical application of rhBMP-7 could be an effective treatment for corneal fibrosis. Keratocyte activation results in the generation of myofibroblasts and increases the contractile characteristics and extracellular matrix production of these cells. This process is also regulated by TGF-β, which modulates transdifferentiation of keratocytes into myofibroblasts[28]. BMP-7 has a specific binding affinity to ActR-I, which activates Smad1/5[37]. A previous study of BMP-7 adenoviral gene transfer in corneal tissue demonstrated an attenuation of Smad2/3 signaling along with upregulation of Smad1/5/8 signaling[22].

Epithelial healing is also involved in the process of stromal healing. Cell proliferation in regenerated epithelium is modulated by growth factors and cytokines *via* complex autocrine and paracrine systems[22]. Our evaluation of the effect of rhBMP-7 on HCEC cytotoxicity *in vitro* and reepithelization *in vivo* did not show any negative effects. That is, topical application of rhBMP-7 is feasible in diseases involving wound healing processes in both the stromal and epithelial cell layers. Injured epithelial cells stimulate fibroblast myodifferentiation through one or more soluble factors, including TGF-β[5]. The HA used in the present study is a naturally occurring glycosaminoglycan of the extracellular matrix and is widely used for the treatment of dry eye syndrome and many epithelial disorders by promoting the migration of HCECs[38-39]. The viscoelastic properties of HA prolong its residence on the ocular surface[40]. When used with rhBMP-7, this mixture rendered a prolonged ocular surface residence time. In contrast to previous reports that used BMP-7 gene transfer *via* adenoviral transfection or gold nanoparticles[21-22], we demonstrated an antifibrotic effect on cornea stromal cells *via* topical application of recombinant protein with 0.1% HA. The direct application of eye drops seems not to be affected by viral vector-induced inflammatory response, poor transfection efficiency of non-viral vectors, or weak transgene delivery. Moreover, its preparation is more convenient.

The major limitation of this study was the relatively short follow-up period of the *in vivo* study. Although corneal opacity after laser ablation reaches its peak at 4wk clinically, TGF-β1 was released in the tears after LASEK and PRK during the first and second postoperative days[15]. The wound healing process started initially at the epithelium, followed by a stromal reaction at 2-5d, and spindle-shaped fibroblast-like keratocytes developed 10d after laser ablation[16]. Despite this limitation, the present study clearly showed that topical application of rhBMP-7 attenuated α-SMA$^+$ cells during the early wound healing stages. A study with a longer follow-up period designed to explain the interaction between corneal epithelial cells and stromal cells under the influence of BMP-7 should be performed.

In conclusion, this study demonstrated that rhBMP-7 attenuated TGF-β-induced EMT *in vitro*, and topical application of rhBMP-7 reduced keratocyte myodifferentiation during the early wound healing stages *in vivo* without cytotoxicity or disturbance of reepithelization.

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