Extracellular Matrix Production Regulation by TGF-β in Corneal Endothelial Cells

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PURPOSE. Production of extracellular matrix (ECM) by corneal endothelial cells is related to physiologic functions and pathologic conditions and is regulated by many cytokines, including transforming growth factor-β (TGF-β). In this study, the molecular mechanism of ECM production regulation by TGF-β was investigated in cultured corneal endothelial cells.

METHODS. The production of ECM components (laminin and fibronectin) was detected in cultured corneal endothelial cells by western blot analysis. To determine the signal transduction pathways, mutant TGF-β type I receptor (TβR-I) and/or Smad protein family members (intracellular signal transducers in TGF-β signaling) were overexpressed by transfecting their cDNA into the cultured cells, and the effects on ECM production were observed.

RESULTS. The production of laminin and fibronectin was stimulated by treatment with TGF-β, or TGF-β2. After transient transfection of cDNA of the constitutively active (CA) mutant of TβR-I, the production of laminin and fibronectin was stimulated even in the absence of TGF-β. The transfection of the dominant negative mutant of TβR-I counteracted the effects of TGF-β. These results confirm that TGF-β directly stimulates ECM production from corneal endothelial cells through TβR-I. The ECM production stimulation by TGF-β or CA TβR-I was accelerated by the overexpression of Smad2, Smad3, and/or Smad4 and inhibited by that of Smad7. These results show that TGF-β signals connected to ECM production are regulated by Smad family members, located downstream of TβR-I.

CONCLUSIONS. The results of this study show that TGF-β stimulates ECM production from corneal endothelial cells through TβR-I and Smad family transducers. (Invest Ophthalmol Vis Sci. 1998;39:1981-1989)

Corneal endothelial cells are essential for corneal transparency. When these cells are injured by trauma, surgery, or intraocular inflammatory diseases, endothelial cells surrounding the defects extend, migrate, and cover denuded areas in human eyes.1-3 In other species, including rabbits, corneal endothelial cells also proliferate in situ.4 Regulation of cell migration and proliferation is closely related to the production of extracellular matrix (ECM). These processes are regulated by many growth factors and cytokines, including transforming growth factor-β (TGF-β).5,6

TGF-β is a multifunctional cytokine that controls cell proliferation, differentiation, migration, ECM production, and apoptosis in various types of cells.7 It regulates formation of granulation tissue with regulation of angiogenesis and ECM deposition, which is important in the wound healing process in many organs.8-10 In mammals, three isoforms of the TGF-β family, which contain TGF-β1, TGF-β2, and TGF-β3, are expressed. Many structurally related factors, including activins and bone morphogenetic proteins, form the TGF-β superfamily.11

The TGF-β superfamily exerts its effects through type I and type II receptors with serine-threonine kinase activity. TGF-β binds to TGF-β type II receptors (TβR-II) first; thereafter, complexes of TGF-β and TβR-II recruit TGF-β type I receptors (TβR-I).12 In these complexes, TβR-I is phosphorylated by TβR-II and is activated to phosphorylate intracellular signal transducers, including those in the Smad family.13,14 According to studies in which nonocular cell lines were used, Smad2 and/or Smad3 are phosphorylated by the TβR-I receptor or the activin type I receptor (activin type I, IB receptors), form complexes with Smad4, and translocate from cytoplasm into nuclei.14-16 The translocated Smad complexes recruit factors to form a transcription-factor complex and initiate transcription of genes. Smad1, Smad5, and/or Smad8 are involved in the signal transduction of the bone morphogenetic protein family.15,17 Smad6 and Smad7 have been recently identified as inhibitors of TGF-β superfamily signaling.18-20

In the present study, we determined the molecular mechanism of the regulation of ECM production by TGF-β in cultured bovine corneal endothelial cells, which has been reported to express TβR-I and TβR-II at the protein level.21 This

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Supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture, the Ministry of Health and Welfare of Japan, and the Sankyo Foundation of Life Science, Tokyo, Japan (HY).

Submitted for publication January 26, 1998; revised May 28, 1998; accepted June 14, 1998.

Proprietary interest category: N.

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is the first report that has clarified the signal transduction mechanism of TGF-β in cells derived from ocular tissue.

MATERIALS AND METHODS

Cell Culture

Bovine corneal endothelial cells (BCECs) were used in this study. The cultured cells were prepared according to methods reported previously. Briefly, eyes of 3-year-old cattle were obtained from an abattoir, and corneal buttons were prepared within 3 hours after enucleation. BCECs with Descemet’s membrane were obtained under microscopy and separated in the presence of trypsin-EDTA (Gibco, Grand Island, NY). Cells were cultured in 60-mm culture dishes (Falcon, Lincoln Park, NJ) in Eagle’s minimum essential medium (EMEM; Gibco) with 15% fetal bovine serum (FBS) and 20 μg/ml gentamicin (Gibco) in humidified atmosphere with 5% CO₂ at 37°C. When cells grew to confluence, they were subcultured into a 6-well culture plate (Falcon) treated with trypsin-EDTA. Second-passage cells were used in the study.

ECM Production after TGF-β₁ and TGF-β₂ Treatment

Recombinant human TGF-β₁ and TGF-β₂ (R&D, Minneapolis, MN) were applied to BCECs in EMEM with 1% FBS after starvation in EMEM with 1% FBS for 24 hours. After BCECs were incubated for 2 days in 5% CO₂ at 37°C, the culture medium was collected as the sample for determination of ECM content. The same volume of the sample buffer was added to the collected medium, then the sample was boiled for 3 minutes. The sample buffer contained 100 mM Tris-HCl (pH 6.8), 0.001% bromphenol blue, 20% glycerol, 4% sodium dodecyl sulfate (SDS), and 12% mercaptoethanol. The samples were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis, Coomassie brilliant blue (CBB) stain, and western blot analysis. For analysis with CBB stain, the polyacrylamide gel after electrophoresis was stained by CBB for 1 hour, then washed in destaining solution (25% methanol, 7% acetic acid) overnight. For western blot analysis, proteins were blotted onto polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK). Antilaminin and antifibronectin polyclonal antibodies (Cosmo-Bio, Tokyo, Japan) were used. According to the manufacturer’s protocol and information, this antilaminin antibody detects all the subtypes of laminin as a single band. Binding was detected by the peroxidase-antiperoxidase method using an immunoblotting kit (ABC-POD; Wako, Osaka, Japan).

ECM Production after Transient Transfection of Plasmid DNA in BCECs

To analyze the signal transduction mechanism, cDNA of mutant forms of TßRI and/or Smad family members were transiently transfected into BCECs (procedures described later) to overexpress mutant TßRI and/or the Smad family at the protein level. In a 6-well culture plate, 70% confluent cells were used for transient transfection. Transient transfection was performed using reagent (DMRIE-C; Gibco) according to the manufacturer’s protocol. Briefly, two kinds of solution were initially prepared: a diluted solution containing a few micrograms of plasmid DNA in 500 μl serum-free medium (Opti-MEM; Gibco) with 20 μg/ml gentamicin and a solution containing 2 to 12 μl reagent in 500 μl serum-free medium with 20 μg/ml gentamicin. The solutions were mixed gently and incubated for 30 minutes at room temperature. After the cells were washed in serum-free EMEM, the mixed solution for transfection was applied to the cells.

The cells were incubated 8 to 10 hours for the preparation of cDNA transfection. Thereafter, the medium was changed to EMEM with 1% FBS, and the cells were incubated in humidified atmosphere with 5% CO₂ at 37°C for 24 or 48 hours. The medium was collected as the sample for western blot analysis.

Transient Transfection of cDNA of TGF-β Type 1 Receptors and the Smad Family

To confirm the direct involvement of TGF-β in the regulation of ECM production, two types of mutant TßRI cDNA were subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA) and used for transient transfection: the CA form T204D and the kinase-deficient form K232R, generous gifts of Masao Saito, Hidenori Ichijo, and Kohji Miyazono of The Cancer Institute, Tokyo. The T204D constitutively active form transduces the signals of TGF-β in the absence of TGF-β, in which the threonine residue 204 has been changed into aspartic residue. The K232R kinase-deficient form blocks the TGF-β signal transduction, in which the lysine residue 232 has been changed into arginine residue.

To investigate the involvement of the Smad family in signal transduction to ECM production, transient transfection of the cDNA of the Smad family was performed. Smad family cDNA, which was subcloned in pcDNA3.1 (Invitrogen) and fused with FLAG tag protein, was generously provided by K. Miyazono and Takeshi Imamura of The Cancer Institute. In these experiments, total DNA in each well was adjusted to the same amount using vector DNA.

Immunocytochemistry

The localization of Smad2, Smad3, or Smad4 in the absence or presence of TGF-β was observed immunocytochemically. Endogenous Smad family members were not detected; therefore, the overexpressed Smad2, Smad3, or Smad4 was observed. cDNA of Smad2, Smad3, or Smad4 was transiently transfected into BCECs, as previously described. Transfected BCECs were exposed to 10 ng/ml TGF-β for 2 hours. Thereafter, the cells were washed twice with phosphate-buffered saline and fixed with 70% ethanol for 15 minutes. After washing with phosphate-buffered saline, the cells were incubated with 10 μg/ml anti-FLAG M2 monoclonal antibody (Product no. F3165; Sigma, St. Louis, MO) as primary antibody at room temperature for 1 hour. Then the cells were incubated at room temperature for 30 minutes with fluorescein-conjugated anti-mouse IgG (Cappel, Malvern, PA) as the second antibody. After washing with phosphate-buffered saline, the cells were dehydrated using 100% ethanol and xylene, then observed with a fluorescence microscope (Nikon, Tokyo, Japan).

RESULTS

TGF-β₁ or TGF-β₂ Stimulation of ECM Production in BCECs

The effect of TGF-β₁ and TGF-β₂ on ECM production was observed by western blot analysis using antilaminin polyclonal antibody. TGF-β₁ or TGF-β₂ stimulated the production of laminin from BCECs in a bell-shaped manner: TGF-β₁ stimulated
laminin production from 1 ng/ml and reached a peak at 3 ng/ml, and the production decreased at 10 ng/ml and 100 ng/ml (Fig. 1A). TGF-β2 was less effective than TGF-β1, with a peak at 10 ng/ml (Fig. 1B). Staining of the gel with CBB confirmed that loaded protein was not different among the lanes, with or without TGF-β1 and TGF-β2, which shows that the same amount of sample was loaded in each lane (Figs. 1A, 1B). This evidence also suggests that total production of protein from the culture cells did not change, even with TGF-β treatment. TGF-β stimulated fibronectin production in a dose-dependent manner; TGF-β1 stimulated fibronectin production from a concentration of 1 ng/ml and reached a peak at 10 ng/ml; production decreased at 100 ng/ml (data not shown). Staining of the gel with CBB confirmed that loaded protein was not different among the lanes (data not shown). The representative data are shown in Figure 1; similar results were obtained in three repetitions of the experiments.

**Direct Involvement of TGF-β in ECM Production in BCECs**

To investigate whether TGF-β stimulates ECM production directly, the effects of the overexpression of mutant forms of TβR-I cDNA were observed. As shown in the introduction, TGF-β exerts its effects through binding to TβR-II and thereafter to TβR-I. The T204D mutant TβR-I initiates the TGF-β signals without the stimulation of TGF-β and is designated a constitutively active (CA) form. When cDNA of CA TβR-I (T204D) was transiently transfected into BCEC, laminin production increased significantly compared with the nontransfected BCEC or BCEC transfected with only the pCDNA3 vector (the mock transfection) in the absence of TGF-β1 (Fig. 2A). Fibronectin production was also stimulated by CA transfection (Fig. 2A). K232R mutant TβR-I loses kinase activity, blocks the signal transduction of TGF-β, and is designated a dominant negative (DN) form. When cDNA of DN TβR-I (K232R) was transiently transfected into BCEC, laminin production stimulation by TGF-β1 and TGF-β2 was blocked in a dose-dependent manner according to the amount of DN cDNA (Fig. 2B). The representative data are shown in Figure 2; similar results were obtained in three repetitions of the experiments.

In the following experiments of signal transduction pathways containing the Smad family, only laminin production was observed, because TGF-β and CA affected the laminin and fibronectin production similarly, and the laminin band in western blot analysis was sharper than fibronectin, which made the analysis reliable. The sharpness of the laminin band in western blot analysis may be because of lower glycosylation in our experimental conditions than that of fibronectin.

**Acceleration of Laminin Production Caused by Overexpression of Smad2, Smad3, and/or Smad4**

After transient transfection of 1.0 μg/ml Smad2, Smad3, or Smad4 cDNA into BCECs, the cells were incubated for 24 hours in 1% EMEM in the presence or absence of 10 ng/ml TGF-β1, and medium was collected as samples. Laminin production was not accelerated by the overexpression of Smad2, Smad3, or Smad4 in the absence of TGF-β1 (Fig. 3A, lower column). However, laminin production stimulation by TGF-β1 was significantly accelerated by the overexpression of Smad2, Smad3, or Smad4 (Fig. 3A, upper column). The effects of the Smad family members and their synergistic effects were investigated in the presence of CA TβR-I. We chose the amount of cDNA of Smad family members and CA TβR-I to avoid the saturation of response by only one cDNA (Fig. 3B). The transfection effect of 1.0 μg/ml CA TβR-I cDNA was significantly accelerated by the
FIGURE 2. Laminin production regulation in bovine corneal endothelial cells (BCECs) with overexpression of mutant transforming growth factor (TGF-β) type I receptor (TβRI). (A) After transfection of cDNA of constitutively active (CA) mutant TβRI (T204D), cells were incubated in Eagle’s minimum essential medium (EMEM) with 1% fetal bovine serum (FBS) for 2 days. Medium was analyzed by western blot analysis. Cells transfected with CA produced laminin and fibronectin more than nontransfected or mock-transfected cells in a manner dependent on the concentration of cDNA of CA. In each lane, total DNA concentration of CA and/or vector (in micrograms per milliliter) was adjusted to the same level except in the left lane (CA, 0; vector, 0). (B) After transfection of cDNA of dominant negative (DN) mutant TβRI (K232R), cells were incubated in EMEM with 1% FBS in the presence of 10 ng/ml of TGF-β1 or TGF-β2 for 2 days. Laminin production was inhibited relative to the concentration of cDNA of dominant negative (DN). In each lane, total cDNA concentration of DN and/or vector (in micrograms per milliliter) was adjusted to the same level. These results show that TGF-β directly regulates extracellular matrix production in BCEC.

transfection of 0.5 μg/ml Smad2, Smad3, or Smad4 cDNA alone (Fig. 3B). Cotransfection of Smad2 + Smad4, Smad3 + Smad4, or Smad2 + Smad3 + Smad4 accelerated the laminin production significantly compared with that induced in cells transfected with Smad2, Smad3, or Smad4 alone in the presence of CA (Fig. 3B). The representative data are shown in Figure 3; similar results were obtained in four repetitions of the experiments.

Specificity of the Smad Family
The ECM production stimulated by overexpression of CA TβRI was not accelerated by the overexpression of Smad1 or Smad5 (concentrations of CA TβRI and Smad cDNA were each 1.0 μg/ml; Fig. 4). The overexpression of Smad1 or Smad5 did not accelerate laminin production by the stimulation of TGF-β (data not shown). The representative data are shown in Figure:

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ECM Production Regulation by TGF-β

Smad6 and Smad7 have been reported to inhibit the signal transduction for TGF-β, activin, or bone morphogenetic proteins. In this study, the function of Smad6 and Smad7 in TGF-β signal transduction was investigated in BCEC (concentrations of CA TβR-1 and Smad cDNA were each 1.0 μg/ml). Expression of Smad6 did not significantly inhibit laminin production stimulation by the CA overexpression, otherwise the overexpression of Smad7 abrogated the laminin production increase by the overexpression of CA (Fig. 5). Laminin production decreased below the baseline level with the overexpression of Smad7, which suggests that Smad7 counteracted the laminin production from BCECs in 1% FBS, which probably contained TGF-β. The representative data are shown in Figure 5; similar results were obtained in three repetitions of the experiments.

In the experiments shown in Figures 2A, 3A, and 4, laminin bands appeared in double bands, whereas they appeared in single bands in experiments shown in Figures 1A, 1B, 2B, 3B, and 5. The lower bands of the double bands corresponded to the single bands and were confirmed to be laminin. These data were consistent with the manufacturer’s information. The identity of the agent in the upper bands is unknown.
Nuclear Accumulation of Smad Family Members Induced by TGF-β1

The localization change of Smad2, Smad3, or Smad4 by TGF-β stimulation was investigated immunocytochemically. The endogenous Smad family protein could not be detected immunocytochemically (data not shown), so BCECs with the overexpressed Smad protein were used. cDNA of Smad2, Smad3, or Smad4 with FLAG tag was transiently transfected into BCECs, and the effect of TGF-β1 treatment was observed immunocytochemically using the anti-FLAG M2 monoclonal antibody.

Nuclear accumulation was not detected in the transfected cells without TGF-β1 treatment (Fig. 6, middle). Overexpressed Smad2, Smad3, or Smad4 translocated into the nuclei after stimulation by 10 ng/ml TGF-β1 for 2 hours (Fig. 6, right). The accumulation of Smad protein in the nuclei was detected in approximately 10% of the BCECs transfected with Smad2, Smad3, or Smad4 alone in the presence of TGF-β1 (Fig. 6). Figure 6 shows the representative data of the nuclear accumulation of the overexpressed Smad4 by the treatment of TGF-β1. Similar results were obtained in the experiments with Smad2, Smad3, and Smad4 repeated three times each.

DISCUSSION

The present study shows for the first time that corneal endothelial cells produce ECM components (laminin and
ECM Production Regulation by TGF-β

Figure 6. Nuclear accumulation of Smad4 by treatment with transforming growth factor (TGF-β). Bovine corneal endothelial cells (BCECs) were transfected with FLAG-tagged Smad4 and incubated in the absence or presence of 10 ng/ml TGF-β, for 2 hours. FLAG-Smad4 was localized in the cells by immunofluorescence, using an anti-FLAG M2 antibody. Smad4 was distributed diffusely in the absence of TGF-β (middle). However, Smad4 accumulated in nuclei in the presence of TGF-β, (right, arrows).

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fibronectin) under the direct regulation of TGF-β, which has been reported to transduce signals through TβR-II and TβR-I. The results of this study suggest that ECM component production (laminin and fibronectin) was located downstream of TβR-I, because laminin and fibronectin production was stimulated by the overexpression of constitutively active mutant TβR-I (T204D) (Fig. 7), and the acceleration by addition of TGF-β was abrogated by the overexpression of mutant DN TβR-I (K232R).

In Figure 1, TGF-β, accelerated laminin production at a lower concentration than TGF-β2. Our group has reported that the cultured BCECs used in the present study expressed endoglin in addition to TβR-I and TβR-II, but not the TGF-β type III receptor (TβR-III). Endoglin is an accessory receptor expressed mainly on vascular endothelial cells and blood cells. It forms a heteromeric complex with the signaling receptors for TGF-β. It binds TGF-β2 and TGF-β3 with higher affinity than TGF-β2, which is thought to have contributed to the result in our experiments that TGF-β, accelerated laminin production in BCECs more effectively than TGF-β2. Joyce and Zieske reported that human corneal endothelial cells in situ express TβR-III. The reasons for this discrepancy are unknown at present; however, the different experimental conditions (in situ versus culture) and species differences (human versus bovine) may contribute to the difference in the expression profiles. In the experiments conducted to investigate the signal transduction pathway, CA mutant TβR-I was used to overcome the difference between TGF-β and TGF-β2, and the difference in the accessory receptors (TβR-II and endoglin).

Expression of Smad2, Smad3, and/or Smad4 accelerated the stimulation of laminin production by addition of T204D mutant CA TβR-I or TGF-β. Therefore, Smad2, Smad3, and/or Smad4 are the candidates for the downstream component(s) of TβR-I. The synergistic effects of Smad2, Smad3, and/or Smad4 were confirmed compared with the overexpression of Smad2, Smad3, and/or Smad4 by treatment with TGF-β, which has been reported to transduce signals through TβR-II and TβR-I. The results of this study suggest that ECM component production (laminin and fibronectin) was located downstream of TβR-I, because laminin and fibronectin production was stimulated by the overexpression of constitutively active mutant TβR-I (T204D) (Fig. 7), and the acceleration by addition of TGF-β was abrogated by the overexpression of mutant DN TβR-I (K232R).

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Figure 7. Schematic illustration of transforming growth factor (TGF-β) signal transduction pathway for TGF-β in bovine corneal endothelial cells. TGF-β accelerates the assembly of heteromeric complex of mutant TGF-β type II (TβR-II) and type I (TβR-I) receptors in which TβR-I is downstream of TβR-II. TβR-II phosphorylates and activates kinase activity of TβR-I. Among Smad family members, Smad2, Smad3, and/or Smad4 are transducers downstream of TβR-I and translocate into nuclei by treatment with TGF-β. This evidence is confirmed by observation that constitutively active (CA) TβR-I (T204D) stimulated laminin production in the absence of TGF-β. In nuclei, complexes containing Smad2, Smad3, and/or Smad4 enhanced gene transcription, including that of laminin.
pression of Smad2, Smad3, or Smad4 alone, which is consistent with the results of the assay of the reporter gene expression systems using plasminogen activator inhibitor-1 promoter in previous reports.\textsuperscript{11,15,18,19} Our study showed that Smad1 and Smad5 were not involved in the TGF-\( \beta \) signaling. The downstream components of TGF-\( \beta \) (Smad2, Smad3, or Smad4) translocated from cytoplasm into nuclei during stimulation by TGF-\( \beta \), which is supposed to correspond to the signal transduction from cytoplasm into nuclei (Fig. 6). This evidence is summarized in the schematic illustration (Fig. 7).

The inhibitory signal transduction pathways have been shown in various cytokines (interleukin-4 and interleukin-\( \gamma \)),\textsuperscript{25,26} and the TGF-\( \beta \) superfamily,\textsuperscript{14,18,20} which may be important in the negative feedback mechanisms in the signal transduction of cytokines.\textsuperscript{14} In corneal endothelial cells, TGF-\( \beta \) signals were abrogated in the presence of Smad7, but not of Smad6, at least in the laminin production regulation pathways. This means that Smad7 blocks the stimulatory effect of TGF-\( \beta \) on laminin production.

Taken together, in our experimental systems using cultured BCECs, the evidence suggests that production of ECM components is regulated directly by TGF-\( \beta \) through the stimulatory pathway(s) initiated by TGF-\( \beta \) receptors and involving Smad2, Smad3, and Smad4 and the inhibitory pathways containing Smad7. These results do not mean that TGF-\( \beta \) receptors and Smad family members are functioning the overexpression systems of mutant receptors and/or Smad family members. It is possible that the bona fide signal transduction pathways in situ without the overexpression are different from those in this study. We used the overexpression systems for the following reasons: Used cultured bovine corneal endothelial cells express their own TGF-\( \beta \) receptors,\textsuperscript{21} and it was therefore necessary to transfect the mutant receptor cDNA to overexpress the mutant receptor and to modulate the functions of the endogenous receptors. The expression level of Smad family members was too low to be detected by immunocytochemistry or western blot analysis (data not shown), and we could not trace the localization of endogenous Smad family members or functions. With the completion of this study, it becomes mandatory to investigate the signal transduction pathways in which endogenous TGF-\( \beta \) receptors and Smad family members are functioning in corneal endothelial cells, using much more sensitive methods without cDNA transfection, which should be developed in the future.

The physiological and pathologic significance of ECM production by corneal endothelial cells is in the formation of the basement membrane\textsuperscript{27} and the posterior collagenous layer\textsuperscript{28-30} and in the regulation of cell migration and cell proliferation.\textsuperscript{5,6} Descemet's membrane, which is the natural basement membrane of corneal endothelium, is formed by corneal endothelium during development.\textsuperscript{31} Defects of corneal endothelial cells were repaired by migration and proliferation of adjacent endothelial cells and subsequent remodeling of the re-established cell layer.\textsuperscript{32} TGF-\( \beta \) is related to ECM synthesis and remodeling in various cells including corneal endothelial cells.\textsuperscript{33} Therefore, from the clinical point of view, the regulation of TGF-\( \beta \) functions can be used for regulating corneal endothelial cell functions, accelerating corneal endothelial wound healing, and diminishing abnormal fibrosis tissue formation. Our study is the first that has shown the molecular mechanisms of TGF-\( \beta \) signal transduction.

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**ERRATUM**


Grant support should have been acknowledged as follows: Supported by National Institutes of Health, Bethesda, Maryland, Grants EY07065, EY01894, and EY11906, and by an unrestricted grant from Research to Prevent Blindness, New York, New York. TB is a Jules and Doris Stein Research to Prevent Blindness Professor awardee. The Journal regrets the error.