



Anterior segment dysgenesis after overexpression of transforming growth factor- β -induced gene, β igh3, in the mouse eye

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Purpose: β igh3 is a transforming growth factor- β -inducible cell adhesion molecule and its mutations are responsible for human autosomal dominant corneal dystrophies. Previously, we have studied the molecular properties of β igh3 in vitro and reported that β igh3 polymerizes to form a fibrillar structure and interacts with several extracellular matrix proteins including type I collagen. This study aimed to understand the role of elevated circulating levels of normal β igh3 in eye development and corneal diseases.

Methods: We generated Alb-h β igh3 transgenic mice that have liver-specific expression of human β igh3 (h β igh3) under the control of the albumin (Alb) enhancer/promoter and investigated the influence of β igh3 overexpression in mouse eye. Polymerase chain reaction (PCR) genotyping, western blotting, and ELISA were performed to generate Alb-h β igh3 transgenic mouse lines. To identify the ocular pathology, electron microscopy and histological staining were employed in Alb-h β igh3 transgenic mice and wild-type mice.

Results: Normal h β igh3 was ectopically overexpressed in the liver, secreted into blood stream, and reached the cornea of Alb-h β igh3 transgenic mice. Among transgenic mice, some mice had anterior segment defects including corneal opacity, disorganization of the collagen layers in the corneal stroma, and corneolenticular adhesion.

Conclusions: These results suggest that β igh3 may be involved in anterior segment morphogenesis and eye development in mice. In addition, this indicates that the level of normal β igh3 expression must be properly maintained during ocular development. The phenotype observed in Alb-h β igh3 transgenic mice is similar to human eye disorders such as anterior segment dysgenesis and Peters' anomaly. Thus, this model provides a very useful tool to study human eye diseases and the control of proliferation and differentiation of neural crest-originated cells.

β igh3 (keratoepithelin), also known as TGF β 1, is a transforming growth factor- β (TGF- β)-induced extracellular matrix (ECM) protein that was first identified in human adenocarcinoma cells [1]. β igh3 is strongly induced by TGF- β in several cell lines including human epithelial cells, keratinocytes, and fibroblasts [2,3]. β igh3 is ubiquitously expressed in many normal human tissues such as the heart, liver, pancreas, and skin, suggesting that it may have an important function throughout the body [1]. It has been reported that β igh3 is not only expressed in the cornea of the normal human eye but also in healing corneal wounds [4-6]. During mouse development, expression of β igh3 in the cornea begins around embryonic day 15.5 (E15.5) and is sustained until E18.5 with localization in the corneal epithelium and stroma [7].

Mutations of β igh3 are responsible for 5q31-linked human autosomal dominant corneal dystrophies such as granular corneal dystrophy (GCD), Reis-Bückler corneal dystrophy (RBCD), lattice corneal dystrophy (LCD) type I and IIIA, and Avellino corneal dystrophy (ACD) [8,9]. These diseases are most often characterized by progressive accumulation of deposits in the cornea, resulting in a loss of transparency and severe visual impairment. Although mutations of β igh3 are well described in corneal dystrophy, the function of normal β igh3 in the eye is not well known. We recently reported that normal β igh3 mediates human corneal epithelial cell adhesion through α 3 β 1 integrin [10] and that β igh3 and its mutants polymerize to form a fibrillar structure and interact with type I collagen, laminin, and fibronectin [11].

TGF- β is a multifunctional cytokine that regulates cell growth and differentiation as TGF- β inhibits epithelial cell proliferation and stimulates the proliferation of smooth muscle cells and skin fibroblasts [12,13]. TGF- β and its receptors are localized in the human anterior segment of the eye, including the cornea, and may regulate various pathophysiological re-

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sponses in the anterior segment by controlling cell proliferation, differentiation, and ECM composition [14,15]. Moreover, TGF- β highly induces β h3, which associates with ECM molecules. Based on the expression of TGF- β and β h3 in the eye and their correlation, β h3 as well as TGF- β may be key molecules in the pathogenesis of ocular disorders or in the eye development.

In this study, to characterize the role of β h3 responses in ocular development, we generated transgenic mice that have liver-specific expression of normal β h3 under the control of the albumin (Alb) enhancer/promoter [16] and we investigated in the eyes of these mice the influence of overexpressed normal β h3 secreted from the liver. The data from this study showed that the elevated levels of normal β h3 caused corneal opacity and anterior segment dysgenesis. Therefore, these results had particular relevance for human fetal conditions characterized by ocular abnormalities such as anterior segment mesenchymal dysgenesis. The results established an experimental model in which overexpression of normal β h3 resulted in the gross ocular pathophysiological characteristics of these conditions.

METHODS

Animals: All procedures concerning animal experiments in the present study were conducted according to the guidelines of Kyungpook National University. All mice were maintained on 12 h light/dark cycles in specific pathogen free (SPF) conditions and fed a sterilized standard diet.

Construction of the Alb-h β h3 transgene and generation of transgenic mice: To generate an Alb-h β h3 transgene, a *SalI/XhoI* fragment of a full-length human β h3 (h β h3, amino acids 1-683), which has high identity with mouse β h3, was first cloned into the *SalI* site of a plasmid that has a 4.3 kb fragment of IRES-LacZ-mp1 intron/polyA. After insertion of h β h3, a 2.3 kb fragment of the albumin (Alb) enhancer/promoter (kindly provided by Dr. R. Palmiter, Seattle, WA) [16], which is essential for expression in the liver, was cloned into the *NorI/SalI* site at the 5'-end of h β h3-IRES-LacZ-mp1 intron/polyA. The entire construct was cut with *NorI* and *NruI* and the 8.8 kb transgene named Alb-h β h3 was purified. Purified Alb-h β h3 transgene was injected into the pronucleus of fertilized eggs of C57BL/6 embryos by MacroGen Inc. (Seoul, Korea). Two hundred animals were screened and seven founders were identified that exhibited integration of the Alb-h β h3 transgene by polymerase chain reaction (PCR) genotyping. Transgenic founders were backcrossed to C57BL/6 or intercrossed. The transgenic mice and their wild-type control littermates were maintained under standard temperature and lighting.

Identification of transgenic mice by genotype analysis: The transgene in Alb-h β h3 founders and offspring was identified by PCR analysis of genomic DNA obtained from tail biopsies. PCR was performed in 30 μ l reaction mixtures, each containing 100 ng genomic DNA, 0.2 μ M each primer set, 1 mM dNTP mixture, 3 μ l of 10X Taq buffer, and 1 unit of Taq polymerase. The primers were specific for h β h3 cDNA (forward: 5'-TCA TCG ATA AGG TCA TCT CC-3', reverse: 5'-

CGG TTC AAA GTC TCA CTA GG-3') and LacZ cDNA (forward: 5'-TAA TCA CGA CGC GCT GTA TC-3', reverse: 5'-CGG ATA AAC GGA ACT GGA AA-3') to amplify a 202 bp and 500 bp fragment, respectively. Amplification was performed for 35 cycles in the following PCR conditions: one min at 95 °C, one min at 48 °C for h β h3 and at 58 °C for LacZ, and one min at 72 °C using a GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, CA). PCR products were then separated electrophoretically on 1% agarose gels and visualized after ethidium bromide staining. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward: 5'-TGA AGG TCG GTG TGA ACG ATT TGG C-3', reverse: 5'-CAT GTA GGC C-AT GAG GTC CAC CAC-3') were used as a PCR control.

Western blotting for human β h3 in the liver: Liver and eye from wild-type and transgenic mice was prepared in radioimmunoprecipitation assay (RIPA) lysis buffer including 150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, and 5 mM EDTA. Each sample was mixed with 2X sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 10 min. Then, the samples were separated by 10% sodium dodecyl sulfate-PAGE and transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Blocking was performed with 5% nonfat milk in phosphate-buffered saline (PBS) for 1 h at room temperature (RT). The membrane was incubated for 2 h at RT with anti-human β h3 antibody (diluted 1:1000 in PBS), and then reacted for 1 h at RT with peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:3000 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA). The blot was developed with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti- β -actin antibody was used as a control on the same filter after deprobing.

Immunohistochemistry of human β h3 in the liver and eye: To investigate transgenic expression, immunohistochemical staining of the mouse liver and eye was performed as described previously [17]. Briefly, sections were deparaffinized, rehydrated, and blocked by incubation in 10% H₂O₂ before blotting. The sections were then put in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and heated in a microwave for 10 min to reveal the antigens. After blocking in PBS, supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin, sections were incubated overnight at 4 °C with anti-human β h3 antiserum in a humidified chamber. Sections were then washed three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) for 90 min at RT. The signal was visualized by incubating the sections with liquid diaminobenzidine tetrahydrochloride (DAB) Chromogen (DAKO). Hematoxylin staining was used to counterstain sections.

Enzyme-linked immunoSorbent assay of human β h3 in blood: The level of human β h3 in mouse plasma was measured by enzyme-linked immunosorbent assay (ELISA; Regen Biotech, Seoul, Korea). Blood was collected through the saphenous vein into potassium EDTA-coated Microvette tubes

(Sarstedt, Nümbrecht, Germany). Recombinant human β igh3 proteins and the anti-human β igh3 antibody for ELISA were prepared as described previously [10]. For ELISA, 96 well plastic flat microtiter plates (Corning, Lowell, MA) were coated overnight at 4 °C with wild-type human β igh3 protein in 20 mM carbonate-bicarbonate buffer (pH 9.6) with 0.02% sodium azide. The coated plates were then washed with PBS with 0.05% Tween-20 (PBS-T). Mouse plasma samples were diluted in PBS-T and preincubated with anti-human β igh3 antibodies in 96 well plastic round microtiter plates for 90 min at 37 °C. The preincubated samples were then transferred to the precoated plates and incubated for 30 min at RT. Thereafter, the samples were incubated with the peroxidase-conjugated anti-rabbit IgG antibodies (Santa Cruz Biotechnology) for 90 min at RT, and the plates were washed as before. The assay was developed with a substrate solution of 0.1 mg/ml o-phenylenediamine and 0.003% H_2O_2 for 60 min at RT in the dark. After stopping the reaction with 8 N H_2SO_4 , the absorbance was read at 492 nm in a Bio-Rad model 550 microplate reader. The paired t-test was used to determine statistical significance with $p < 0.05$ considered to denote statistical significance. Values were expressed as mean \pm SD.

Gross pathology and ocular stereology: To analyze the gross appearance of the eye, enucleated mouse eyeball samples were examined under a slit lamp biomicroscope (Haag-Streit, Bern-Koeniz, Switzerland) equipped with a digital camera; images were captured with the eye image capture system (eMedio@Inc., Seoul, Korea). To capture cataracts, pupils were dilated with a drop of 1% Mydracyl (Alcon Laboratories, Hemel Hempstead, UK) for 20-30 min while the animals were under anesthesia. The eyeball sizes were observed under a stereomicroscope with a ruler.

Light microscopy: For the histological analysis of the cornea, enucleated mouse eyeballs were fixed in 4% paraformaldehyde (PFA) in PBS for 16 h at 4 °C, dehydrated in a graded series of ethanol, and then embedded in paraffin. Serial sections, including anterior segment defects in the eyes of Alb- β igh3 transgenic mice, were cut at 4 μ m thickness and stained with hematoxylin and eosin (H&E) or with Masson's trichrome. Masson's trichrome stained collagen fibers blue and most other intracellular and extracellular proteins red.

Electron microscopy: Corneas were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2-4 h at 4 °C. The central portion of the cornea, including the opaque region, in Alb- β igh3 transgenic mice was removed, washed three times in 0.1 M PB, and post-fixed in 1% osmium tetroxide in 0.1 M PB for 2 h at 4 °C. After rinsing with 0.1 M PB, the samples were dehydrated in a graded series of ethanol, immersed in propylene oxide, infiltrated with an Epon mixture, embedded in a Beem capsule, and polymerized for three days. Serial ultrathin sections were cut with an ultramicrotome, collected on Formvar film-coated single slot nickel grids, and counterstained with uranyl acetate and lead citrate. The grids were examined on a Hitachi H6000 electron microscope at 80 kV accelerating voltage.

RESULTS

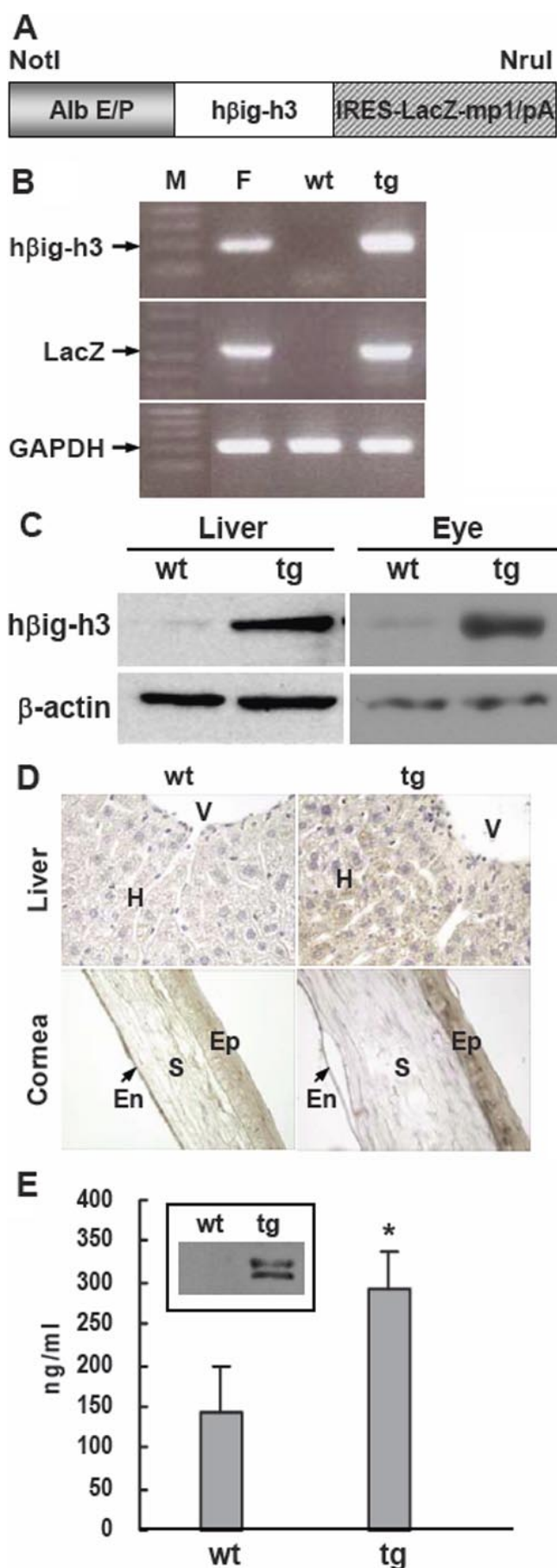
Transgenic mice overexpressing normal β igh3 in the liver and blood: The mouse albumin (Alb) enhancer/promoter was used to generate transgenic mice expressing human β igh3 (β igh3) in the liver with increased levels of β igh3 in the blood. The full-length β igh3 was inserted into the plasmid with Alb enhancer/promoter, followed by IRES-LacZ-mp1 intron/polyA to detect β -galactosidase activity in the liver (Figure 1A). This *NotI/NruI* transgene fragment was injected into fertilized eggs of C57BL/6 and then 200 animals from injected eggs were screened to select transgenic founders. Seven founder mice were identified and three of these lines showed high expression of the transgene. To maintain the lines, they were crossed with C57BL/6 and the offspring were identified by PCR genotyping of β igh3 and LacZ gene 10 days after birth (Figure 1B). PCR results showed 202 bp and 500 bp PCR products for β igh3 and LacZ, respectively. To investigate ectopic expression of the β igh3 transgene, western blotting and immunohistochemistry were performed in the liver and eye of two-month-old mice using anti-human β igh3 antibody. High expression of β igh3 was detected by western blot analysis in the liver and in eye extracts of transgenic mice (Figure 1C). Transgenic mice also showed strong expression of the β igh3 transgene in liver hepatocytes and in corneal epithelium by immunostaining (Figure 1D). Figure 1B-D show representative data for β igh3 transgene expression in 28 transgenic mice. ELISA was completed for β igh3 in plasma and the level of β igh3 in 28 transgenic mice was 1.5-2 fold higher than in 19 wild-type mice (Figure 1E). A high level of β igh3 expression was also observed by western blot analysis in the plasma of transgenic mice (Figure 1E). As the human β igh3 antibody partially cross-reacted with both mouse and human β igh3, the amount in wild-type mice likely represented the level of endogenous mouse β igh3 in tissue and blood. Finally, we generated Alb- β igh3 transgenic mice that overexpress β igh3 under the control of the Alb enhancer/promoter. Stable transgenic mice were continuously propagated in the C57BL/6 background to maintain the lines and to analyze the phenotype.

Gross phenotype and electron microscopic examination of the mice eyes with β igh3 overexpression: Among the Alb- β igh3 transgenic mice identified by PCR genotyping 10 days after birth, eight transgenic mice displayed a central corneal opacity, a characteristic phenotype that was visible when the eyelids opened around two weeks after birth. At the age of two months, when plasma could safely be collected, the level of β igh3 was measured in all wild-type and transgenic mice to establish β igh3 overexpression in plasma. The average bodyweight and eye size were identical for wild-type (22 ± 0.6 g, 3.5 ± 0.05 mm) and transgenic mice (22 ± 0.4 g, 3.5 ± 0.08 mm) at two months of age. Internal organs such as the liver and kidney were phenotypically normal (data not shown) and there was no specific phenotype, even in the liver where ectopic normal β igh3 was produced under control of the albumin promoter (Figure 1D). This was performed through systematic

sampling and measurements in all wild-type and transgenic mice. Even though the bodyweight and the size of eyes were no different between wild-type and transgenic mice, various phenotypes with diverse defect sizes were investigated in the defective eye of transgenic mice that had a corneal opacity (Figure 2). The gross ocular phenotypes of Alb-h β igh3 transgenic mice showed corneal opacification (Figure 3B). The opacity sometimes accompanied a cataract, which was investigated in the eye of transgenic mice after pupil dilatation (Figure 3C). These anomalies were never seen in normal eyes of wild-type mice (Figure 3A). Corneal opacity was observed bilaterally with cataracts in Alb-h β igh3 transgenic mice with eye defects. Among eight Alb-h β igh3 transgenic mice with corneal opacity, bilateral defects with cataracts were observed in five mice and unilateral defect was shown in the remaining three mice. Electron microscopy showed that the collagen fibers and fibrils were disorganized with an irregular arrangement in the corneal stroma of the defected eye in transgenic mice compared to the stroma in wild-type mice, which were compact and well-organized (Figure 4A). Additionally, tissue debris was frequently observed in the spaces among the disorganized collagen fibers (Figure 4B).

Histological analysis of the anterior segment: Ocular histology was analyzed in the eyes of wild-type and Alb-h β igh3 transgenic mice. Though the extent of the defect varied in each transgenic mouse, all mutant eyes had abnormalities at layers of the cornea, anterior chamber, and lens. In H&E stained sections, normal cornea was separated from the lens by a distinct

Figure 1. Generation of Alb-h β igh3 transgene. **A:** The diagram of Alb-h β igh3 transgene shows the albumin enhancer/promoter (Alb E/P), the human β igh3 (h β igh3), and IRES-LacZ-mp1 intron/polyA (IRES-LacZ-mp1/pA). *NotI* and *NruI* indicate enzyme sites in the transgene for pronuclear injection. **B:** PCR genotyping for h β igh3 and LacZ gene in wild-type (wt) and transgenic (tg) mice. The 202-bp h β igh3 and 500 bp LacZ amplification products were produced by PCR in Alb-h β igh3 transgenic mice. PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as a control. M and F indicate a DNA size marker and a founder mouse as a positive control for PCR, respectively. **C:** Western blot analysis of h β igh3 expression in liver and eye extracts from wild-type (wt) and transgenic (tg) mice. The h β igh3 protein was highly expressed in the transgenic mouse compared to the wild-type mouse. β -Actin was used as an internal protein control. **D:** Immunohistochemical study of wild-type (wt) and transgenic (tg) liver and cornea at two months of age using anti-human β igh3 antibody. Expression of h β igh3 transgene was strongly detected in liver hepatocytes and corneal epithelium of Alb-h β igh3 transgenic mouse. Abbreviations: H, hepatocyte; V, terminal hepatic venule; Ep, corneal epithelium; S, corneal stroma; En, corneal endothelium. **E:** Human β igh3 levels in plasma of wild-type (wt) and transgenic (tg) mice. ELISA showed that h β igh3 levels in plasma were elevated two-fold in transgenic mice. The plasma levels show the average for 19 wild-type mice and 28 transgenic mice. High expression of h β igh3 protein was detected in plasma of transgenic mice by western blotting. The asterisk indicates a statistically significant difference ($p < 0.001$) calculated by paired t-test. The western blotting and ELISA data are representative of three independent experiments.



endothelial layer and the corneal epithelium showed well-defined, stratified squamous epithelium with a smooth surface (Figure 5A, Figure 6A). In comparison, transgenic mice with corneal opacity displayed an obvious abnormal cornea with an irregular corneal epithelium and an uneven corneal stroma, including disorganization of collagen layers in the defective area (Figure 6B,C). The defective eye also had a narrow anterior chamber and showed either partially formed or discontinued corneal endothelium (Figure 5B,C). Unlike the thin monolayer of lens epithelial cells in the lens' anterior surface of wild-type mice, the anterior region of the lens had abnormal multilayer cells in transgenic mice (Figure 5).

In another transgenic mouse that showed more serious deformity of the eye (Figure 7), the separation between lens and cornea was not complete. The anterior portion of the lens attached to the posterior surface of the cornea and the anterior chamber was obliterated with the iris adherent to the posterior surface of the cornea. Proliferated lens epithelial cells under the lens capsule were observed in the attached portion between the protruded lens and cornea. Thin corneal epithelium and disordered corneal stroma were also observed in the defective eye of the transgenic mouse. As shown in the wild-type mouse of Figure 5, the cornea was separated from the lens by a distinct endothelial layer; this was also the case in the seven-month-old wild-type mouse (Figure 7E).

DISCUSSION

A transforming growth factor- β (TGF- β)-induced extracellular matrix (ECM) protein, β igh3, has been considered a major component of abnormal extracellular deposits in the cornea and its mutations and responsible for 5q31-linked human autosomal dominant corneal dystrophies (CDs) [8]. Immunohistochemical studies show that β igh3 is strongly stained in pathologic deposits in all mutant β igh3-related corneal dystrophies and that, above all, high levels of normal β igh3 are detected in scarring cornea [18] as if TGF- β is present on the edge of wound healing in the cornea [19]. These data suggest that β igh3 as well as TGF- β would be key molecules in the pathogenesis of corneal opacification. In this study, we investigated whether β igh3 is essential to ocular development in vivo and whether it is pathologically important to corneal disorganization.

First, to study the function of β igh3 in eye, it is necessary to discover suitable methods or animal models that express ectopic or mutant β igh3. Many researchers have tried direct injections or eye drops of plasmid DNA or infection with an adenovirus vector in the eye. However, the difficulty of gene transfer into the cornea or the eye without inflammation prevents the study of certain protein's roles in these tissues [20,21]. Therefore, diverse methods to overcome this difficulty have been studied and reported. Several mouse model systems have

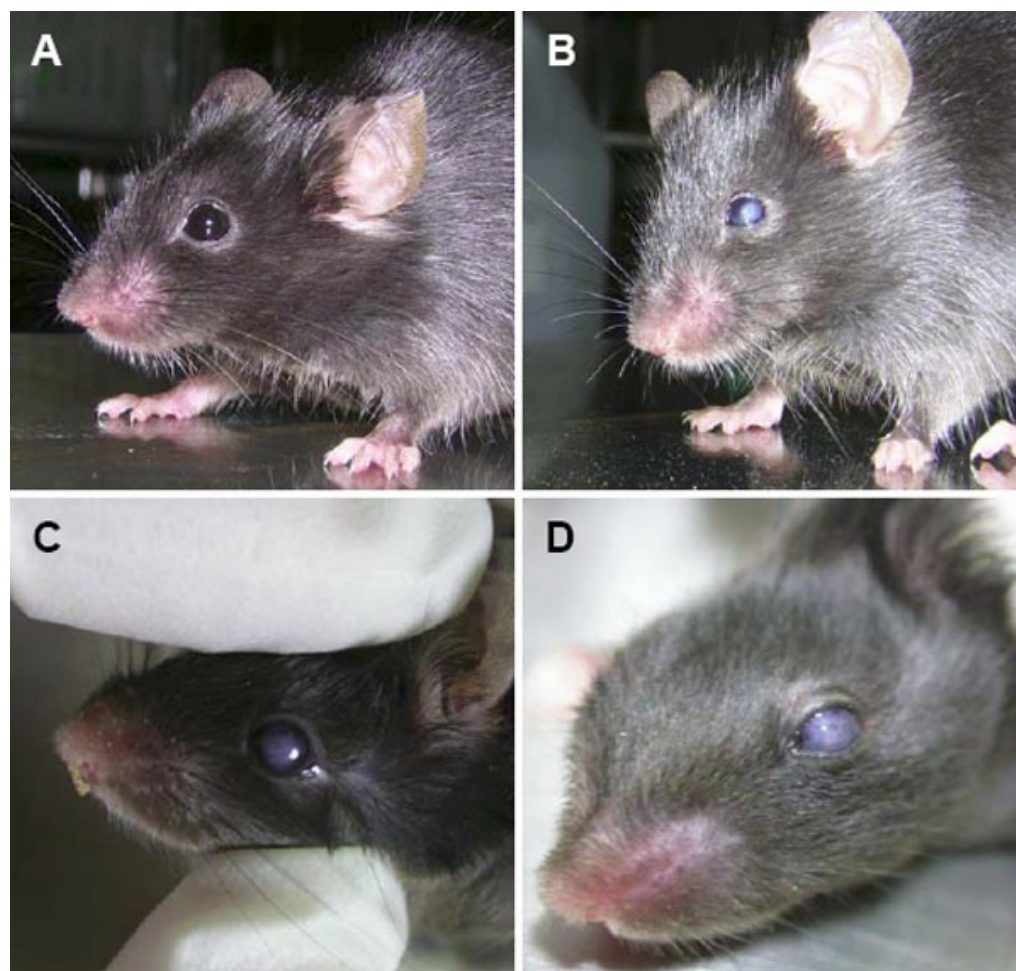


Figure 2. Gross appearance of wild-type and Alb-h β igh3 transgenic mouse. The eye of a two-month-old transgenic mouse (B) had a corneal opacity with a characteristic phenotype compared to wild-type (A). C and D show the left eyes with corneal opacity in one-year-old Alb-h β igh3 transgenic mice.

been developed to observe the function of TGF- β , which is an inducer of β hgh3 and has a critical role in the development of normal cornea [22,23]. Even though overexpression of TGF- β 1 driven by the lens-specific α A-crystallin promoter does not cause a corneal phenotype in the embryonic mouse eye, cataract formation is observed in adult mice with ocular defects including corneal opacity and structural changes in the iris and ciliary body [24]. Transgenic overexpression of TGF- β 1 by lens-specific chicken β B1-crystallin promoter also revealed severe disruption of corneal and anterior chamber development from mouse embryonic day 13.5 [25]. These results suggest not only the importance of precise quantitative control of TGF- β but also of good transgenic model systems to study the role of TGF- β in eye development. However, TGF- β overexpression regulated by these specific promoters may cause an artificial and excessive phenotype that is restricted to a specific time and tissue in lens development.

Sakamoto et al. [26] constructed an adenoviral vector expressing a soluble TGF- β receptor fused to the Fc portion of human IgG and injected this construct into the skeletal muscle of mice. The soluble TGF- β receptor produced in the muscle

reached the cornea by means of normal blood circulation, sequestered local TGF- β , and acted in the cornea but not in the lens. Based on this idea, we generated transgenic mice that overexpressed human β hgh3 (h β hgh3) in blood through the liver-specific albumin (Alb) enhancer/promoter. Albumin, which is a major secretory protein of the liver, is induced in fetal liver and its expression is maintained in the adult liver [27]. Since serum albumin is synthesized particularly by hepatocytes, the albumin promoter/enhancer is activated during liver development. Previously, it had also been reported that the most abundant water-soluble protein in the human cornea, serum albumin, can diffuse from peripheral blood vessels around the cornea [28,29]. β hgh3 is a secretory protein and h β hgh3 ectopically expressed in liver under the control of Alb promoter was able to secrete into blood. Eventually, this secreted protein reached the cornea via blood vessels surrounding the limbal area of the cornea where it affected the anterior segment. In this model system, we can exclude possible artificial outcomes in the lens caused by using α A- or β B1-crystallin lens-specific promoters and explain the role of β hgh3, which causes corneal diseases in human.

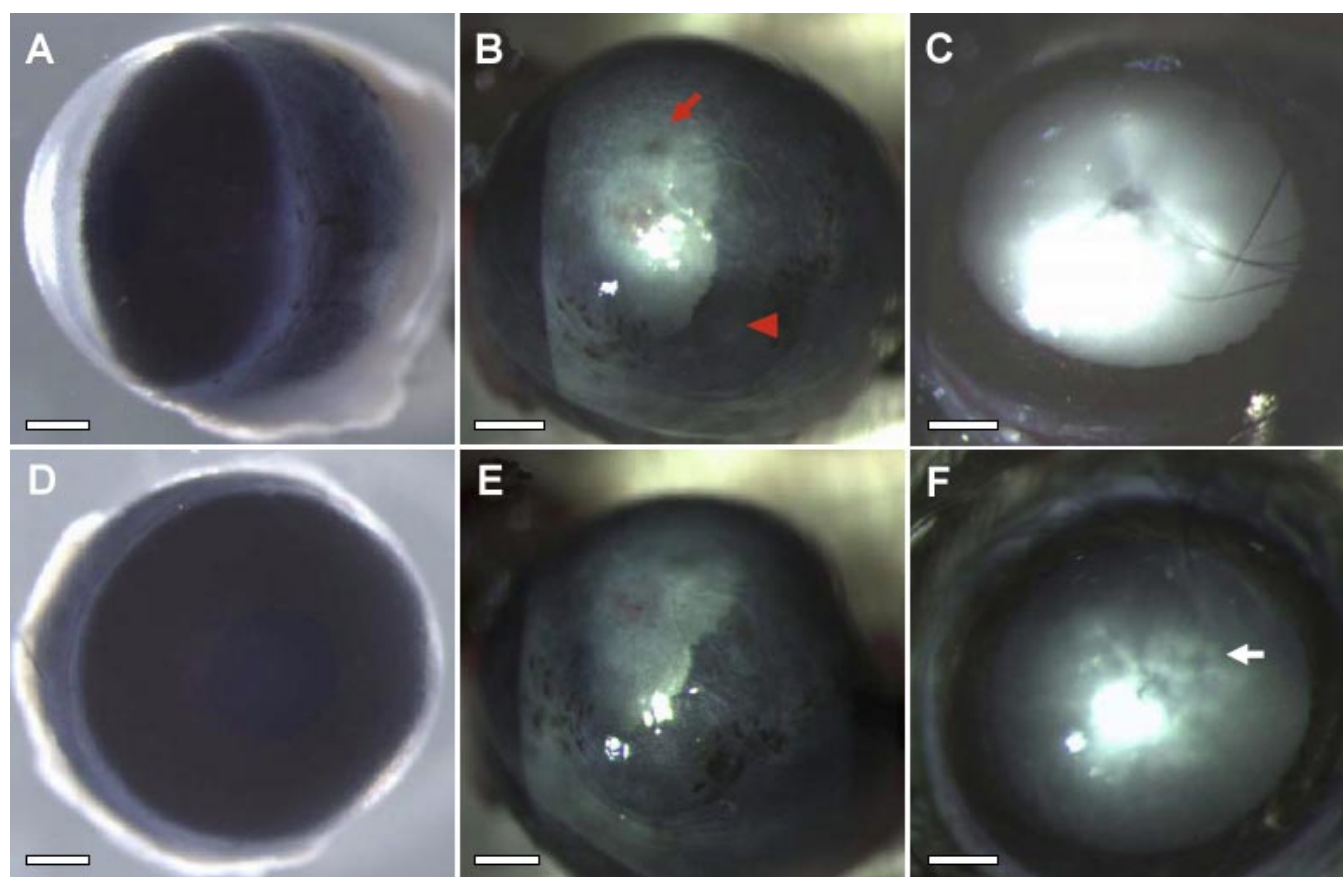


Figure 3. Phenotypes in two-month-old Alb-h β hgh3 transgenic mouse eye. **A** and **D**: Normal eye of a wild-type mouse. **A** and **D** are a lateral view and a frontal view of the eye with transparent cornea, respectively. **B** and **E**: Mutant eye with corneal opacity in Alb-h β hgh3 transgenic mouse. **B** and **E** show different angle views of the defective eye in the transgenic mouse. The **Red** arrow indicates the corneal opacification and the **red** arrowhead indicates the relatively clear zone in the transgenic mouse. **C** and **F**: The mutant eye with cataract in Alb-h β hgh3 transgenic mouse. **C** and **F** show different angle views of the eye with cataract in the transgenic mouse. The white arrow indicates cortical opacity of the lens. The scale bars are equal to 0.5 mm.

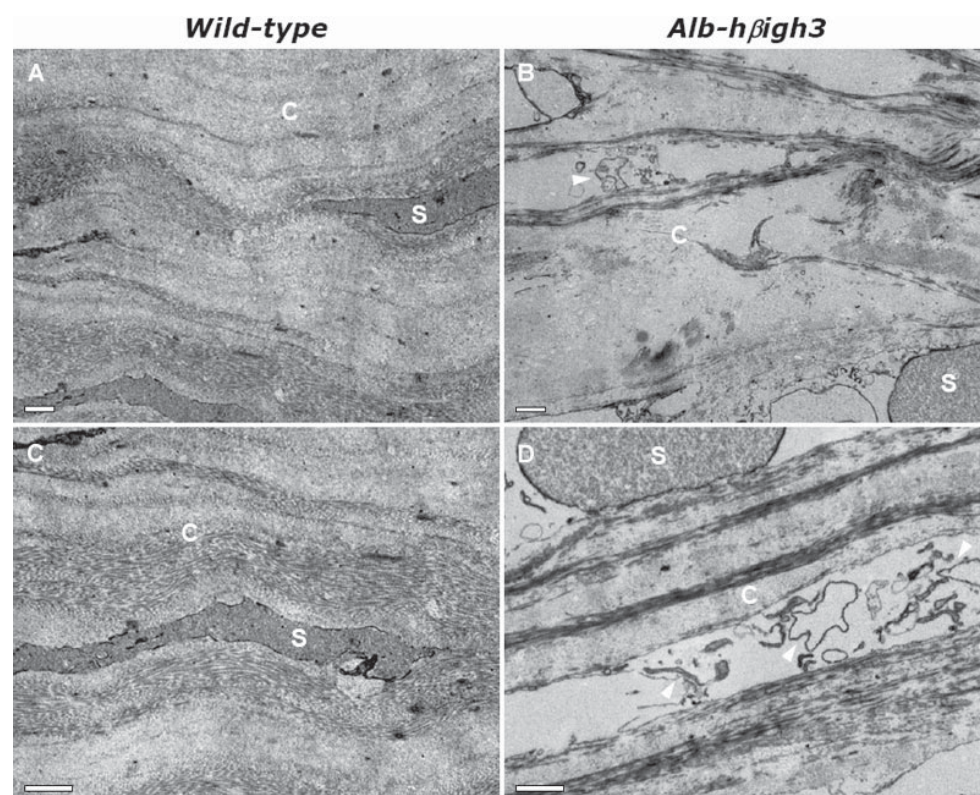


Figure 4. Electron micrographs showing stroma of central portion of cornea in wild-type and Alb-hβigh3 transgenic mice. Corneas from mice were fixed in 2.5% glutaraldehyde and subjected to electron microscopy. **A** and **C**: In wild-type mice, collagen fibers and fibrils were regularly and compactly arranged parallel to the epithelial surface. However, in Alb-hβigh3 transgenic mice (**B** and **D**), the collagen fibers and fibrils were disorganized. Tissue debris was occasionally observed between the collagen fibers (arrowhead). **A** and **B** are images corresponding to 5K and **C** and **D** are images corresponding to 8K. The scale bars are equal to 2 μm. Abbreviations: C, collagen layers; S, stromal cells.

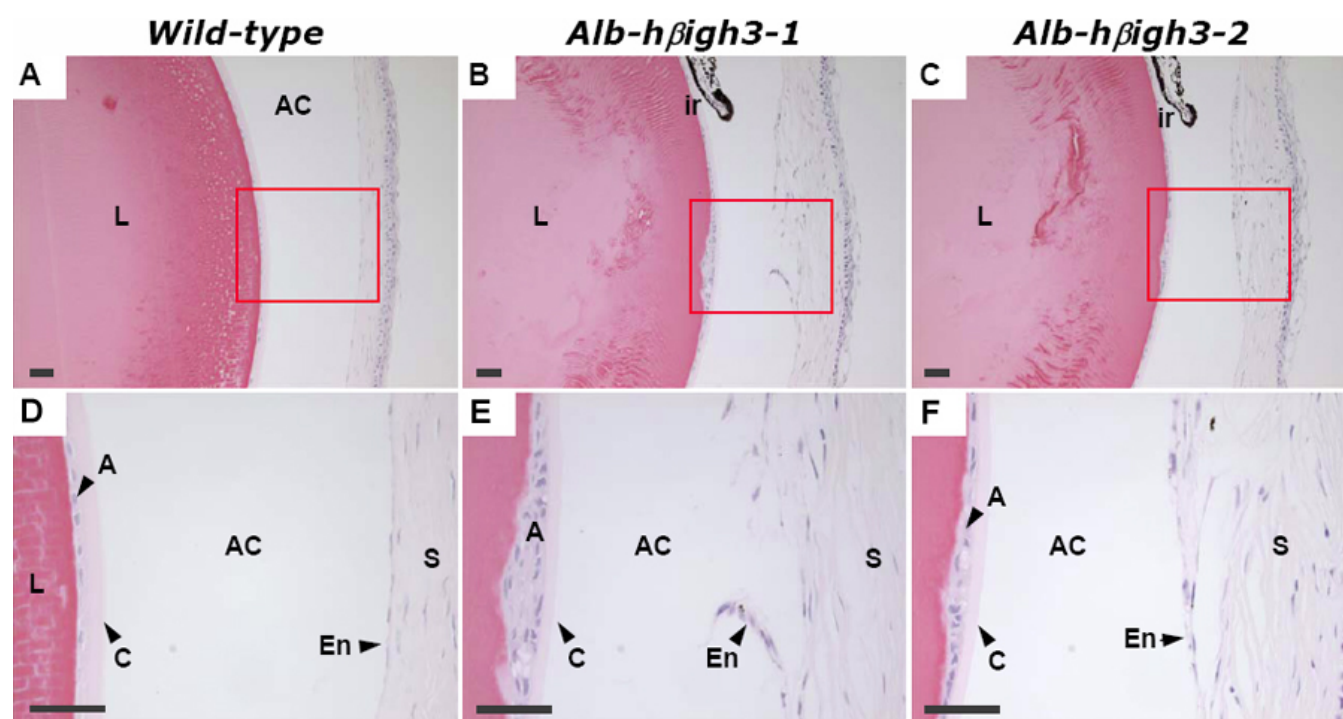


Figure 5. Histopathologic findings of normal eye in wild-type mouse and the defective eye in transgenic mouse at two months of age. Staining was performed with H&E in each 4 μm thick paraffin section. **C** is the serial section of **B**. Images in row 2 are higher-magnification images corresponding to small red rectangles in row 1. The abnormal cornea of transgenic mice (**B**, **C**) had disorganized corneal stroma, disconnected corneal endothelium, and a narrow anterior chamber compared to wild-type (**A**). The anterior surface of the lens had multilayer epithelial cells in transgenic mice. The scale bars are equal to 50 μm. Abbreviations: L, lens; C, lens capsule; A, anterior epithelium of lens; AC, anterior chamber; ir, iris; S, corneal stroma; En, corneal endothelium.

An exogenous transgene in the transgenic mouse model should be clearly distinct from an endogenous gene. For this, many researchers use specific marker genes or the same gene from different species. By using the human β igh3 transgene, exogenous transgene is easily distinguished from endogenous mouse β igh3. Since the nucleotide sequence of human β igh3 has high identity with mouse β igh3 and the amino acid sequence also has more than 91% identity with mouse, there were no undesirable phenotypes caused by using a transgene from a different species.

Here, we demonstrated that the Alb-h β igh3 transgenic mouse had a serious failure in anterior segment development including an irregularity of the corneal epithelium, disorganization in collagen layer of the corneal stroma, and discontinuity of corneal endothelium following corneal opacity. Thickened corneal abnormality due to endothelial disruption was

observed in the cornea of Alb-h β igh3 transgenic mice with corneal opacity. Moreover, the attachment of the iris or lens to the cornea in some transgenic mice indicated that overexpression of β igh3 resulted in incomplete central migration of neural crest cells during ocular development. Cataract formation was also examined in defective eyes of Alb-h β igh3 transgenic mice. In a previous paper, we showed that β igh3 expression greatly increased in lens epithelial cells from patients with anterior polar cataracts and in human lens epithelial cells treated with TGF- β [30]. Thus, cataract formation in transgenic mice was due to overexpression of β igh3 in their affected eyes. The bilateral phenotype with cataract in Alb-h β igh3 transgenic mice was very similar to the clinicopathologic findings of Peters' anomaly that is a kind of anterior mesenchymal dysgenesis in human developmental anomalies. Most cases of Peters' anomaly are bilateral with lens abnor-

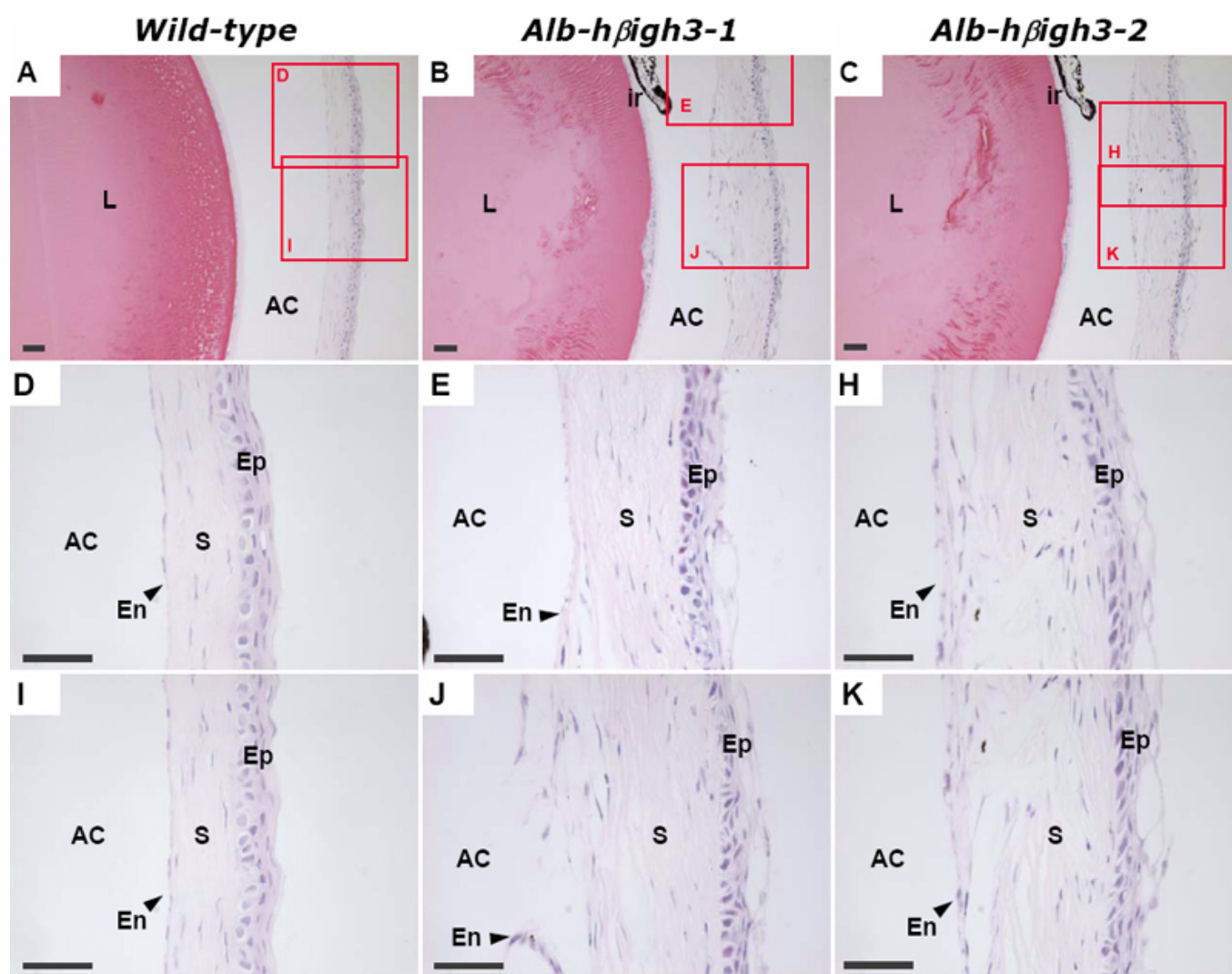


Figure 6. Histological examination of the defective eye in transgenic mouse compared to normal eye in wild-type mouse at two months of age. Staining was performed with H&E in each 4 μ m thick paraffin sections. **C** is the serial section of **B**. Images in rows 2 and 3 are higher-magnification images of the cornea corresponding to the two red rectangles in column 1. The abnormal cornea of transgenic mice (**B**, **C**) had disorganized corneal stroma and disconnected Descemet's membrane and endothelial cell layer compared to wild-type (**A**). The scale bars are equal to 50 μ m. Abbreviations: L, Lens; ir, iris; AC, anterior chamber; Ep, corneal epithelium; S, corneal stroma; En, corneal endothelium.

malinity and variable severity [31,32]. However, mild unilateral cases are often observed in patients who do not have cataract. Additionally, even though $h\beta gh3$ was overexpressed in the entire mouse body through the blood stream, the effect was observed only in the eye. This result corresponds exactly with the observation that mutant $\beta gh3$ in humans causes only corneal dystrophy in the eye and no other deformities in other organs [33].

The anterior segment of the vertebrate eye is structurally defined by the cornea, iris, ciliary body, and lens [34]. Various anterior segment mesenchymal dysgenesis have been reported in humans and animals. In particular, anterior segment disorders in humans include autosomal dominant iridogoniodysgenesis anomaly, family glaucoma with goniodysgenesis, congenital endothelial dystrophy, and aniridia [35-38]. Although coordinated interactions between dif-

ferent cell types in these disorders are considered essential for proper spatial positioning and differentiation, the requisite intercellular signals and the proper animal model to verify these signals have not been well defined. One human anterior segment disorder, Peters' anomaly, is characterized by congenital corneal opacity with defects in the cornea [39]. By histological examination, Peters' anomaly in humans shows dense corneal opacity, iridocorneal adhesions, and occasional lens abnormality with direct adhesion to the posterior corneal surface. In addition, human Peters' anomaly accounts for a thickened cornea due to endothelial layer disruption. The histological views of Alb- $h\beta gh3$ transgenic mice with corneal opacity are comparable to phenotypes observed in human Peters' anomaly. Likewise, a mouse model of fetal alcohol syndrome (FAS) has been reported to exhibit similar defects to Peters' anomaly with malformations in Descemet's membrane and

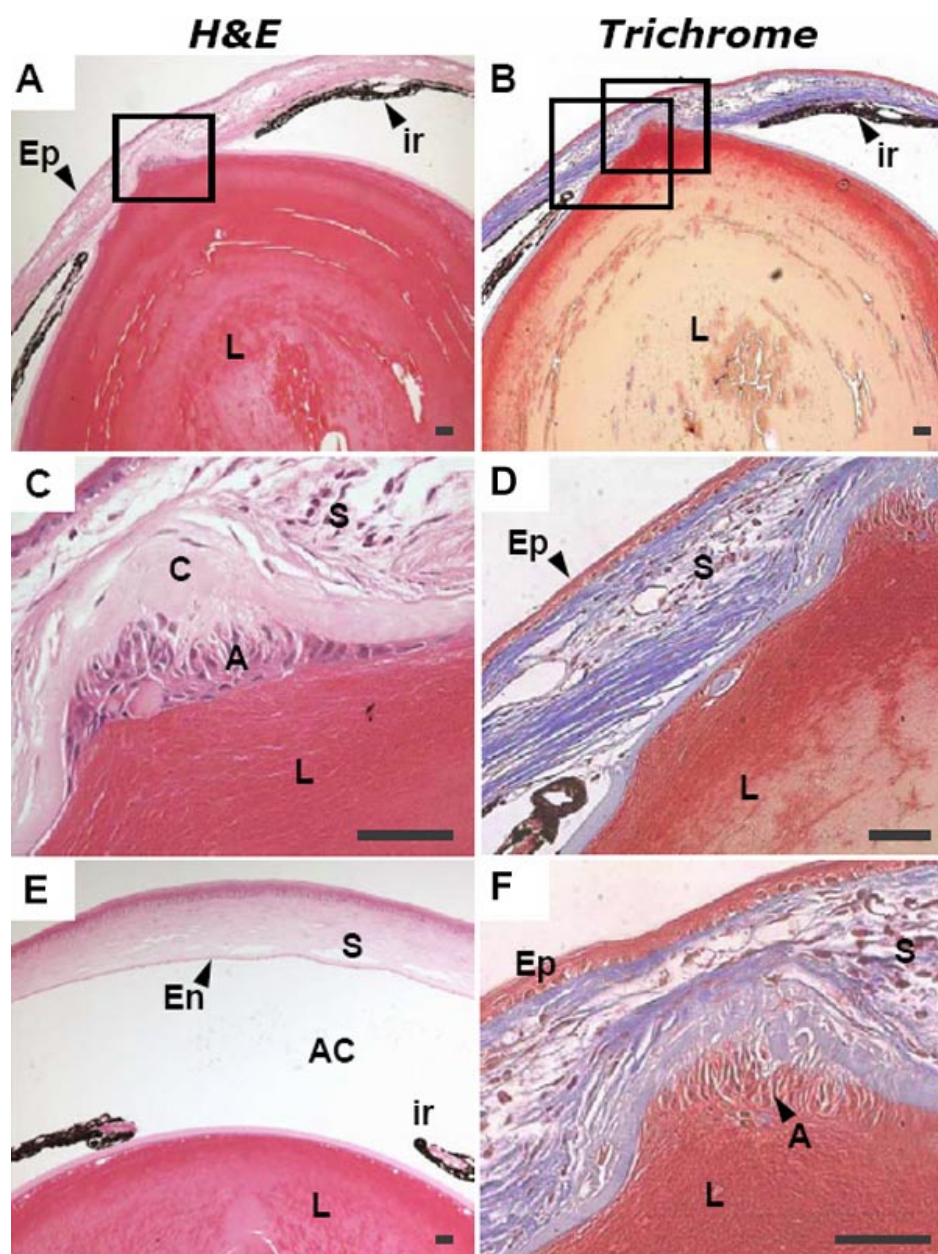


Figure 7. Histologic analysis of the anterior segment in a seven-month-old Alb- $h\beta gh3$ transgenic mouse. Staining was performed with H&E (A, C, and E) and Trichrome (B, D, and F) in each 4 μ m thick paraffin section. Images in rows 2 and 3 are higher-magnification images corresponding to the small rectangles in column 1. Separation between lens and cornea was not complete in the defective eye of the Alb- $h\beta gh3$ transgenic mouse. The attached portion between the protruded lens and cornea showed proliferated lens epithelial cell under the lens capsule. The terminal part of the iris was attached to the posterior surface of the cornea and there was no space for the anterior chamber. E shows the normal eye of a seven-month-old wild-type mouse. The scale bars are equal to 50 μ m. Abbreviations: L, Lens; C, lens capsule; A, anterior epithelium of lens; ir, iris; Ep, corneal epithelium; S, corneal stroma; En, corneal endothelium.

corneal endothelium as well as delayed or failed separation of the lens [40]. Accordingly, Alb-h β igh3 transgenic mice will be a practical model to study these eye disorders.

Though we have not examined the signaling pathway that is altered by overexpression of β igh3, these results sufficiently explain that β igh3 is involved in anterior segment morphogenesis and must be properly expressed for normal anterior segment development. This suggests that β igh3 may play an important role in the corneolenticular adhesion and the normal development of the cornea during ocular morphogenesis. Therefore, β igh3 may play a role in the normal formation of the anterior segment. Taken together, this study shows that β igh3 expression must be critically maintained during ocular development to avoid severe deformity, especially in the cornea. The Alb-h β igh3 transgenic mice described in this study also provide a useful animal model for the study of anterior segment dysgenesis and of the control of proliferation and differentiation of neural crest-originated cells.

ACKNOWLEDGEMENTS

We thank Dr. R. Palmiter of University of Washington (Seattle, WA) and Dr. M. Schuchmann of I. Med. Klinik (Germany) for providing the albumin enhancer/promoter plasmid. This work was supported by the grant No. RTI04-01-01 from the Regional Technology Innovation Program of the MOCIE, Advanced Medical Technology Cluster for Diagnosis and Prediction at Kyungpook National University, a faculty research grant of Yonsei University College of Medicine for 2006, and the Brain Korea 21 Project in 2007.

REFERENCES

- Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD, Purchio AF. cDNA cloning and sequence analysis of beta igh3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. *DNA Cell Biol* 1992; 11:511-22.
- LeBaron RG, Bezverkov KI, Zimmer MP, Pavelec R, Skonier J, Purchio AF. Beta IG-H3, a novel secretory protein inducible by transforming growth factor-beta, is present in normal skin and promotes the adhesion and spreading of dermal fibroblasts in vitro. *J Invest Dermatol* 1995; 104:844-9.
- Skonier J, Bennett K, Rothwell V, Kosowski S, Plowman G, Wallace P, Edelhoff S, Distecche C, Neubauer M, Marquardt H, Rodgers J, Purchio AF. beta igh3: a transforming growth factor-beta-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice. *DNA Cell Biol* 1994; 13:571-84.
- Escribano J, Hernando N, Ghosh S, Crabb J, Coca-Prados M. cDNA from human ocular ciliary epithelium homologous to beta igh3 is preferentially expressed as an extracellular protein in the corneal epithelium. *J Cell Physiol* 1994; 160:511-21.
- Hirano K, Klintworth GK, Zhan Q, Bennett K, Cintron C. Beta igh3 is synthesized by corneal epithelium and perhaps endothelium in Fuchs' dystrophic corneas. *Curr Eye Res* 1996; 15:965-72.
- Rawe IM, Zhan Q, Burrows R, Bennett K, Cintron C. Beta-ig. Molecular cloning and in situ hybridization in corneal tissues. *Invest Ophthalmol Vis Sci* 1997; 38:893-900.
- Ferguson JW, Mikesch MF, Wheeler EF, LeBaron RG. Developmental expression patterns of Beta-ig (betaIG-H3) and its function as a cell adhesion protein. *Mech Dev* 2003; 120:851-64.
- Munier FL, Korvatska E, Djemai A, Le Paslier D, Zografos L, Pescia G, Schorderet DF. Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. *Nat Genet* 1997; 15:247-51.
- Fujiki K, Hotta Y, Nakayasu K, Yokoyama T, Takano T, Yamaguchi T, Kanai A. A new L527R mutation of the betaIGH3 gene in patients with lattice corneal dystrophy with deep stromal opacities. *Hum Genet* 1998; 103:286-9.
- Kim JE, Kim SJ, Lee BH, Park RW, Kim KS, Kim IS. Identification of motifs for cell adhesion within the repeated domains of transforming growth factor-beta-induced gene, betaig-h3. *J Biol Chem* 2000; 275:30907-15.
- Kim JE, Park RW, Choi JY, Bae YC, Kim KS, Joo CK, Kim IS. Molecular properties of wild-type and mutant betaIG-H3 proteins. *Invest Ophthalmol Vis Sci* 2002; 43:656-61.
- Massague J. The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990; 6:597-641.
- Barnard JA, Lyons RM, Moses HL. The cell biology of transforming growth factor beta. *Biochim Biophys Acta* 1990; 1032:79-87.
- Pasquale LR, Dorman-Pease ME, Luttly GA, Quigley HA, Jampel HD. Immunolocalization of TGF-beta 1, TGF-beta 2, and TGF-beta 3 in the anterior segment of the human eye. *Invest Ophthalmol Vis Sci* 1993; 34:23-30.
- Song QH, Singh RP, Richardson TP, Nugent MA, Trinkaus-Randall V. Transforming growth factor-beta1 expression in cultured corneal fibroblasts in response to injury. *J Cell Biochem* 2000; 77:186-99.
- Pinkert CA, Ornitz DM, Brinster RL, Palmiter RD. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. *Genes Dev* 1987; 1:268-76.
- Ha SW, Bae JS, Yeo HJ, Lee SH, Choi JY, Sohn YK, Kim JG, Kim IS, Kim BW. TGF-beta-induced protein beta igh3 is upregulated by high glucose in vascular smooth muscle cells. *J Cell Biochem* 2003; 88:774-82.
- Takacs L, Csutak A, Balazs E, Berta A. Immunohistochemical detection of betaIG-H3 in scarring human corneas. *Graefes Arch Clin Exp Ophthalmol* 1999; 237:529-34.
- Saika S. TGF-beta signal transduction in corneal wound healing as a therapeutic target. *Cornea* 2004; 23:S25-30.
- Noisakran S, Campbell IL, Carr DJ. Ectopic expression of DNA encoding IFN-alpha 1 in the cornea protects mice from herpes simplex virus type 1-induced encephalitis. *J Immunol* 1999; 162:4184-90.
- Tsubota K, Inoue H, Ando K, Ono M, Yoshino K, Saito I. Adenovirus-mediated gene transfer to the ocular surface epithelium. *Exp Eye Res* 1998; 67:531-8.
- Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997; 124:2659-70.
- Saika S, Saika S, Liu CY, Azhar M, Sanford LP, Doetschman T, Gendron RL, Kao CW, Kao WW. TGFbeta2 in corneal morphogenesis during mouse embryonic development. *Dev Biol* 2001; 240:419-32.
- Srinivasan Y, Lovicu FJ, Overbeek PA. Lens-specific expression of transforming growth factor beta1 in transgenic mice causes anterior subcapsular cataracts. *J Clin Invest* 1998; 101:625-34.
- Flugel-Koch C, Ohlmann A, Piatigorsky J, Tamm ER. Disruption of anterior segment development by TGF-beta1

- overexpression in the eyes of transgenic mice. *Dev Dyn* 2002; 225:111-25.
26. Sakamoto T, Ueno H, Sonoda K, Hisatomi T, Shimizu K, Ohashi H, Inomata H. Blockade of TGF-beta by in vivo gene transfer of a soluble TGF-beta type II receptor in the muscle inhibits corneal opacification, edema and angiogenesis. *Gene Ther* 2000; 7:1915-24.
27. Rothschild MA, Oratz M, Schreiber SS. Serum albumin. *Hepatology* 1988; 8:385-401.
28. Maurice DM, Watson PG. The distribution and movement of serum albumin in the cornea. *Exp Eye Res* 1965; 4:355-63.
29. Nees DW, Fariss RN, Piatigorsky J. Serum albumin in mammalian cornea: implications for clinical application. *Invest Ophthalmol Vis Sci* 2003; 44:3339-45.
30. Lee EH, Seomun Y, Hwang KH, Kim JE, Kim IS, Kim JH, Joo CK. Overexpression of the transforming growth factor-beta-inducible gene betaig-h3 in anterior polar cataracts. *Invest Ophthalmol Vis Sci* 2000; 41:1840-5.
31. Townsend WM. Congenital anomalies of the cornea. In: Kaufman HE, Baron BA, McDonald MB, editors. *The Cornea*. 2nd ed, CD-ROM. London: Butterworth-Heinemann; 1999.
32. Townsend WM. Congenital corneal leukomas. 1. Central defect in Descemet's membrane. *Am J Ophthalmol* 1974; 77:80-6.
33. El Kochairi I, Letovanec I, Uffer S, Munier FL, Chaubert P, Schorderet DF. Systemic investigation of keratoepithelin deposits in TGFBI/BIGH3-related corneal dystrophy. *Mol Vis* 2006; 12:461-6.
34. Pei YF, Rhodin JA. The prenatal development of the mouse eye. *Anat Rec* 1970; 168:105-25.
35. Williams DL. A comparative approach to anterior segment dysgenesis. *Eye* 1993; 7:607-16.
36. Walter MA, Mirzayans F, Mears AJ, Hickey K, Pearce WG. Autosomal-dominant iridogoniodysgenesis and Axenfeld-Rieger syndrome are genetically distinct. *Ophthalmology* 1996; 103:1907-15.
37. Mullaney PB, Risco JM, Teichmann K, Millar L. Congenital hereditary endothelial dystrophy associated with glaucoma. *Ophthalmology* 1995; 102:186-92.
38. Churchill A, Booth A. Genetics of aniridia and anterior segment dysgenesis. *Br J Ophthalmol* 1996; 80:669-73.
39. Kivlin JD, Fineman RM, Crandall AS, Olson RJ. Peters' anomaly as a consequence of genetic and nongenetic syndromes. *Arch Ophthalmol* 1986; 104:61-4.
40. Cook CS, Nowotny AZ, Sulik KK. Fetal alcohol syndrome. Eye malformations in a mouse model. *Arch Ophthalmol* 1987; 105:1576-81.