Establishment of a transgenic mouse model of corneal dystrophy overexpressing human *BIGH3*

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Abstract. This study aimed to establish a transgenic mouse model of corneal dystrophy (CD) overexpressing the human transforming growth factor, β-induced, 68 kDa (TGFBI, also known as *BIGH3*) gene. A purified and linearized recombinant plasmid carrying the expression cassette *BIGH3*-IRES-EGFP was microinjected into the pronuclei of C57BL/6J mouse fertilized eggs under the control of the phosphoglycerate kinase (PGK) promoter. The expression of human *BIGH3* in the transgenic mice was confirmed by PCR using DNA extracted from tail tissue. Four founder transgenic mice were identified by PCR and the increased expression of BIGH3 was observed in the corneas of the transgenic mice by RT-PCR and western blot analysis. The abnormal corneas with central opacity were observed in the transgenic mice by corneal photography. We concluded that the exogenous gene, *BIGH3*, was integrated successfully into the mouse genome through microinjection. In addition, the phenotype observed in this *BIGH3* transgenic mouse model was similar to CD. Therefore, this transgenic model may prove useful in the investigation of the pathogenesis of CD.

Introduction

Human corneal dystrophy (HCD) is a group of genetic disorders characterized by a non-inflammatory, bilateral opacity of the cornea (1). Many types of HCD are associated with transforming growth factor, β-induced, 68 kDa (TGFBI, also known as BIGH3) gene mutations. The symptoms may appear before the age of 20, developing during adolescence and gradually progressing throughout life. HCD often affects only one layer of the cornea at first and then the disease progresses to the remainder of the cornea. Histopathological studies have also proven that the BIGH3 protein can be found in these corneal deposits. Penetrating keratoplasty has commonly been performed for extensive CD. However, recurrence and deterioration of the disease has often been observed even after surgery (2).

Although studies of *BIGH3*-related HCD are of great interest (3,4), the role of *BIGH3* mutations in HCD remains to be fully understood due to the lack of animal models (5). Since there are many limiting factors in the study of HCD, relevant animal models would greatly contribute to the study of this pathogenesis. Transgenic mice produced by microinjection are the most widely used animal models. Therefore, in this study, a transgenic mouse model expressing the human *BIGH3* gene was established. Our data also indicated that the human *BIGH3* gene was overexpressed in the mouse corneas and induced corneal opacity in the eyes of the mice.

Materials and methods

Animals. C57BL/6J mice were purchased from the Shanghai Biomodel Research Center (Shangai, China). All animals were fed with sterile mouse food and water at pH 2.8-3.2 under specific pathogen-free (SPF) conditions and were kept in an isolated room with an automatic light control (14 h day light and 10 h dark cycle). The animal experiments were approved by the Institutional Animal Care and Use Commitee (IACUC) of the Shangai Biomodel Research Center for Experimental Animal Management (permit no. IACUC 2010-0001). All animal experiments were conducted in compliance with the relevant provisions of the Association for Research in Vision and Ophthalmology (ARVO; American Association of Ophthalmology) for animal research.

Construction of the pPGK-BIGH3-IRES-EGFP transgene vector. A 0.5 kb phosphoglycerate kinase (PGK) promoter fragment was amplified by PCR using DNA polymerase Ex Taq (Takara Bio, Inc., Shiga, Japan) and pPL451 as a template (Shanghai Biomodel Research Center). The forward and reverse primers were as follows: 5'-CGACTCGAGACC GGGTAGGGGAGGCGCTTT-3' and 5'-GGCGTCGACTCG AAAGGCCCGGAGATGAGG-3', respectively. The amplicon flanked with *Xho*I and *Sal*I was recovered from the agarose gel

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and ligated into the similarly double digested plasmid, pCAG-IRES-EGFP, which was obtained from the same supplier. The ligation was transformed into *E. coli* in order to isolate the recombinant plasmid, pPGK-CAG-IRES-EGFP. The plasmid DNA was digested with *PhB*I and purified to remove the CAG promoter fragment by agarose gel electrophoresis. The recovered DNA was self-ligated to generate the recombinant plasmid, pPGK-IRES-EGFP.

The cDNA fragment of *BIGH3* (Source BioScience, Nottingham, UK) was digested with *Sal*I and *Sac*II and ligated to the similarly digested pPGK-IRES-EGFP. The ligation mixture was transformed into *E. coli* to obtain the recombinant plasmid, pPGK-*BIGH3*-IRES-EGFP. The plasmid was sequenced for confirmation before being linearized with *Xba*I, purified with phenol-chloroform extraction and used in microinjection buffer (pH 7.4) at a concentration of 4 ng/μl to produce the transgenic mice.

Generation of the transgenic animals. The purified pPGK-*BIGH3*-IRES-EGFP plasmid DNA was microinjected into the fertilized eggs of C57BL/6J mice. After the injection, the fertilized eggs that were in a good condition were transplanted into the fallopian tubes of pseudo-pregnant female mice of the same strain, to produce the F_0 generation animals. All animals were kept in an isolated room at 22˚C, with an automatic light control (14 h day and 10 h dark cycle). The F_0 animals were mated with the wild-type mice to generate F_1 animals.

Genomic DNA extraction and PCR genotyping. The transgenic founders and offsprings were identified by PCR of the genomic DNA extracted from tail biopsies. The tissues were digested overnight in a water bath at 55˚C in 500 μl of a lysis buffer, containing 20-100 mg/ml proteinase K, 50 mmol/l Tris, pH 8.0, 100 mmol/l EDTA, 100 mmo1/l NaCl and 1% SDS (Bio-Rad, USA). The digested tissues were extracted twice with a double volume of phenol-chloroform and the supernatants were diluted in a double volume of absolute ethanol to precipitate the DNA. After having been washed twice with 70% ethanol, the pellets were air-dried and dissolved in 100 μ1 TE buffer. The DNA concentration was determined using a UV spectrophotometer. PCR was conducted to confirm the presence of the exogenous gene in pPGK-*BIGH3*-IRES-EGFP using the forward primer, 5'-GACTAGCCCCTGTCTATCAAAAGTT-3' and the reverse primer, 5'-AACCTCGACTAAACACATGTAAAGC-3', which produced a product of 578 bp. PCR was carried out under the following conditions: 95˚C for 3 min, followed by 25 cycles of 95˚C for 15 sec, 65˚C for 30 sec, 72˚C for 1 min and with an extension at 72˚C for 10 min at the end of the reaction. The results of the reaction were visualized by gel electrophoresis. Wild-type DNA was run as a negative control on a 1% agarose gel electrophoresis.

RT-PCR of human BIGH3 expression in the cornea. Several corneas were removed from two randomly selected wild-type and PCR-positive F_1 transgenic mice, which were euthanized, irrespective of their gender. Euthanasia was performed by cervical dislocation. Total RNA was extracted using TRIzol reagent and digested with DNase. Phenol-chloroform was used to extract the residual genomic DNA before being subjected to reverse transcription. cDNA was obtained using an RT-PCR kit and Gapdh as the control. The following PCR primers were used for RT-PCR: Gapdh forward, 5'-TGGGAAGCTGGTCATCAAC-3' and reverse, 5'-GCATCACCCCATTTGATGTT-3'; and BIGH3 forward, 5'-CAGGCGTCAGCGTATTCC-3' and reverse, 5'-CCTTCCCTACCCGTCCAA-3'. PCR was conducted under the following conditions: 90˚C for 30 sec, 61˚C for 20 min followed by pre-denaturation at 94˚C for 30 sec and 35 cycles of amplification at 55-61˚C for 30 sec, 72˚C for 30 sec and then extension at 72˚C for 5 min. The products were analyzed on a 1% agarose gel. All experiments were repeated three times independently.

Western blot analysis of human BIGH3 expression in the cornea. Three wild-type mice served as the controls. Corneas were collected under a stereo microscope from the eye balls of euthanized mice following cervical dislocation, each randomly selected from wild-type and PCR-positive F_1 transgenic mice. The corneal tissues were separated using micro-tweezers, weighed and cut into small sections using micro scissors. The tissues were added with equal volumes of lysis buffer, homogenized on ice and incubated for 30 min at 4˚C in a refrigerator. The digested tissues were transferred to 1.5 ml centrifugation tubes and spun for 1 min at 12,000 rpm at 4˚C. The supernatants were heated for 5 min in a water bath at 100˚C before being used for SDS-PAGE. The proteins were blotted onto nitrocellulose membranes following electrophoresis and reacted with 1:500 diluted antibodies against BIGH3 (Abcam, Cambridge, UK) to detect the expression product. The images were scanned using the LI-COR Odyssey® Infrared Laser Imaging System (LI-COR Biotechnology; Lincoln, NE, USA). The results were expressed as a percentage of the control optical density. All experiments were repeated three times.

Corneal photography. The corneas of these transgenic mice were photographed using a digital camera following anesthesia to determine the gross appearance of the corneas.

Statistical analysis. Data were statistically analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Quantitative data were tested using the F-test for homogeneity of variance. Data with homogeneous variance were tested using the independent sample t-test, while non-homogeneous data were analyzed by the Mann-Whitney U test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Construction of PGK-BIGH3-IRES-EGFP vector. The PGK promoter fragment and the *Xho*I and *Sal*I digested plasmid, pCAG-IRES-EGFP, were ligated to produce pPGK-CAG-IRES-EGFP. The plasmid was digested and gel purified to remove the CAG fragment and then self-ligated to obtain pPGK-IRES-EGFP. Human *BIGH3* cDNA was cloned into the *Sal*I/*Sac* II site of pPGK-IRES-EGFP to generate a new 7416 bp recombinant vector, pPGK-*BIGH3*-IRES-EGFP.

Identification of founder transgenic mice. Following pre-nuclei microinjection of the linearized plasmid pPGK-*BIGH3*-IRES-EGFP, 36 founder mice were generated. PCR indicated

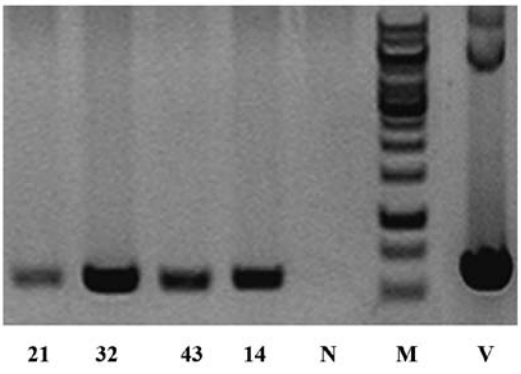

Figure 1. Agarose gel electrophoresis of PCR products on genomic DNA from mouse tails. The PCR products from transgenic mice, numbered 21, 32, 43 and 14, are shown in lanes 1-4, respectively; the PCR products using wild-type mouse DNA as templates are shown as negative (N); the 1 kb DNA ladder as (M) and the PCR product with the transgenic vector (V) as a template is shown as positive.

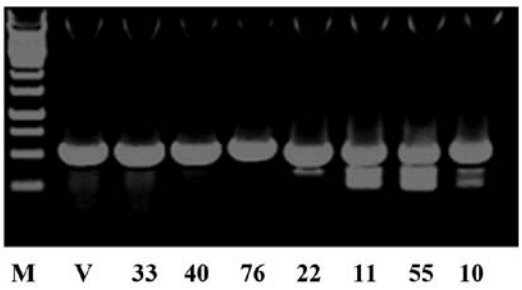

Figure 2. Identification of F_1 transgenic mice by PCR. The 1 kb DNA ladder is shown as a marker (M); the PCR product with the transgenic vector (V) as positive; the PCR products from transgenic mice, numbered 33, 40, 76, 22, 11, 55 and 10, are shown in lanes 3-9, respectively.

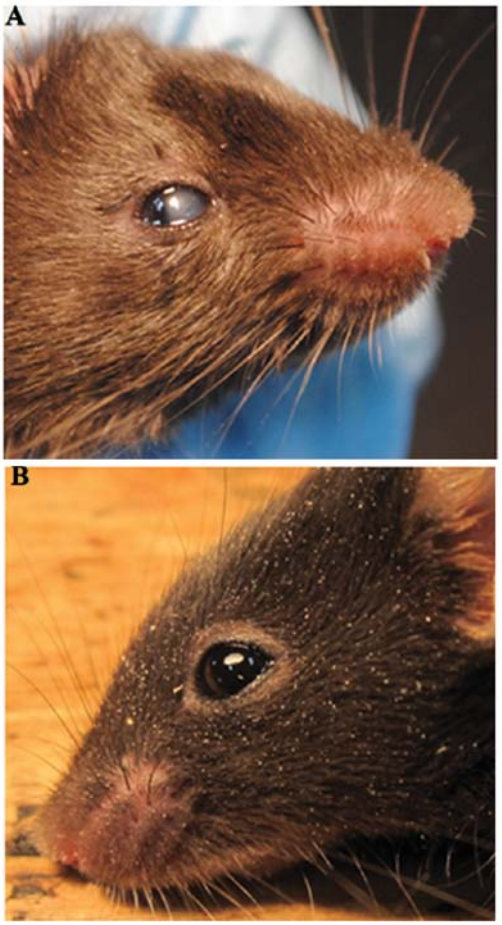

Figure 3. Gross phenotype of the wild-type and *BIGH3* transgenic mice. The (A) transgenic mice showed corneal opacity compared with the (B) transparent cornea of the wild-type animals.

that four of them (M21, male; M32, female; M43, female; M14, male) were transgenic founders. The PCR-positive animals were confirmed twice along with three wild-type animals using DNA prepared from the sampled tail tips with an amplified product of 578 bp in the transgenic but not in the wild-type mouse samples (Fig. 1).

Generation of F_1 transgenic mice. The four F_0 transgenic mice were mated with wild-type C57BL/6J mice to produce F_1 animals and the offsprings were identified by PCR genotyping ten days after birth. A total of 30 mice were born. PCR analysis indicated that seven of them [M33, female; M40, female; M76, male; M22, male; M11, female; M55, female; M10, male] were transgenic (Fig. 2).

Gross phenotype of transgenic mice. The corneas of these transgenic mice were photographed using a digital camera following anesthesia to determine the gross appearance of the corneas. There were five transgenic mice among the seven F_1 transgenic mice, that displayed a centrally reduced corneal transparency, which was visible when the eyelids opened at approximately two weeks after birth (Fig. 3).

RT-PCR of corneal tissues from F_1 transgenic mice. The corneas were removed from the dissected F_1 transgenic mouse eyes. RT-PCR was performed to examine the corneas of two F_1 transgenic mice in order to investigate the relative expression of human *BIGH3* mRNA in the corneas from wild-type and transgenic mice. As illustrated in Fig. 4, compared with the wild-type mice, the expression of BIGH3 in the transgenic mice was significantly upregulated in their corneas ($p<0.01$).

Western blot analysis of the corneas of BIGH3 transgenic mice. Western blot analysis was performed to examine the corneas of three F_1 transgenic mice in order to investigate the expression of the human *BIGH3* gene in the corneas from wild-type and transgenic mice. As the anti-human BIGH3 antibody partially cross-reacted with both the mouse and human proteins, the amount of BIGH3 in the wild-type mice likely represented the level of endogenous mouse BIGH3 in the corneas. As illustrated in Fig. 5, in comparison with the wild-type mice, the transgenic mice had higher levels of BIGH3 in their corneas ($p<0.01$).

Discussion

BIGH3 (68 kDa), also known as TGFBI, is located on chromosome 5q31, spanning a region of 30 kb (6). A mutation was initially discovered when TGF was used to treat adenocarcinoma cell lines (7-9). Research to date on BIGH3 has focused on two point mutations of arginine residues 124 and 555 (10,11).

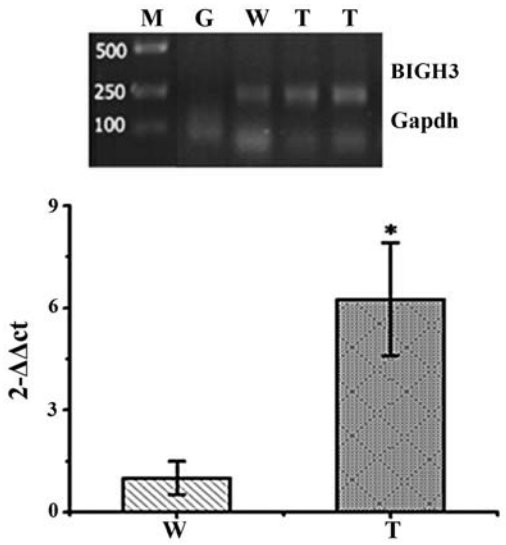

Figure 4. RT-PCR of the human *BIGH3* gene in corneas from wild-type (W) and transgenic (T) mice. A higher expression of the human *BIGH3* gene (242 bp) was detected by RT-PCR in the corneas of transgenic mice compared with the wild-type mice. Gapdh (78 bp) was used as an internal control. M, DNA size marker; G, Gapdh; *p<0.01.

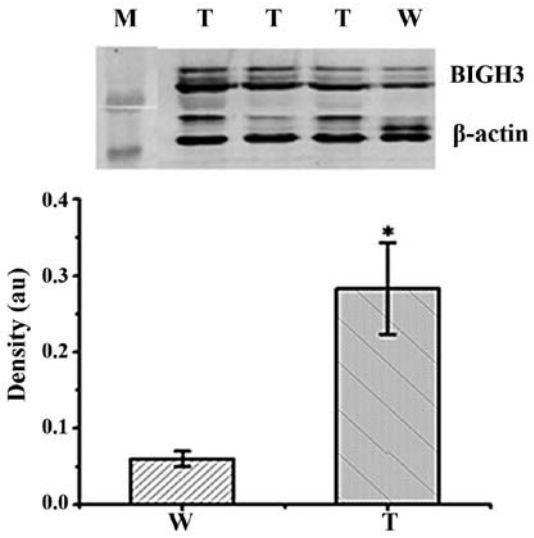

Figure 5. Western blot analysis of the human *BIGH3* protein in the corneas from wild-type (W) and transgenic (T) mice. β-actin protein (42 kDa) was used as an internal protein control. The *BIGH3* protein (68 kDa) was significantly increased in the corneas of the transgenic mice compared with those of the wild-type mice. The results are expressed as the means ± SD. M, marker; *p<0.01.

They are the most common mutations in the human population (12). Few reports are available regarding the establishment of animal models for CD. Kim *et al* (13) reported the use of the Alb promoter to establish a transgenic mouse model of CD overexpressing *BIGH3*. However, the mice were subsequently found to have anterior segment disease, including corneal opacity, cataract and iris abnormalities. Bustamante *et al* (14) also described the development of the model with the mutated BIGH3 gene R555W using lentiviral vectors. However, they found that the animals tended to age more rapidly, with retinal degeneration, although their corneas were normal.

To achieve a high level of expression of exogenous genes and efficient secretion in mammalian cells, it is necessary to use suitable vectors and recipient systems that enable the effective induction of expression. The key element is a strong promoter to drive the expression cassette. In this study, we used the PGK promoter to drive the high level expression of the *BIGH3* gene. The PGK promoter has been widely used in yeast expression systems (15,16) and is recognized as a strong constitutive promoter with a wide range of hosts. It has three transcriptional start sites with a high GC content. The GC content upstream of the transcriptional start sites can reach 70%. It does not contain conserved sequences, such as TATA or CAAT boxes as often observed in other promoters. There is a repeat sequence GGGGCGG upstream from the transcriptional start sites (17,18). All these features describe the high efficiency of the PGK promoter and the reasons we selected it.

The pCAG-IRES-EGFP vector, also used in this study, has an enhanced GFP gene with an internal ribosomal entry site (IRES). GFP is non-toxic, stable and easy to visualize (19). It has been widely used to monitor transfection efficiency, expression levels and to sort the transfected cells. IRES is a conserved *cis*-acting element existing in the mRNA of eukaryotes or viral genomes. It binds to ribosomes to mediate the 5' cap-dependent translation of downstream genes. The sequence has been widely used in various binary expression vectors (20-22). In our study, we intended to use EGFP as a reporter to measure the expression of the *BIGH3* transgene. However, the GFP fluorescence observed *in vivo* under a fluorescence microscope was too weak to fulfill this purpose. We speculate that this may be due to the dark fur color of the C57BL/6J mice, leading to the absorption of most of the GFP fluorescence.

Male pronuclear microinjection was applied to generate transgenic mice in our study, which is currently the most commonly used method for transgenic mouse production. The recombinant plasmid, PGK-BIGH3-IRES-EGFP, was injected into the pronuclei of 85 fertilized eggs of C57BL/6J mice to produce *BIGH3* transgenic mice. Four transgenic founder mice (M21, male; M32, female; M43, female; M14, male) of 36 born mice were successfully generated, as confirmed by PCR analysis. After mating with wild-type C57BL/6J mice, a total of 30 F_1 offspring mice were produced and seven of them were PCR-positive (M33, female; M40, female; M76, male; M22; male, M11; female, M55; female, M10; male), indicating that the human *BIGH3* gene had integrated into the mouse genome and was able to be transmitted to the next generation. However, we also noted that only 6-25% of the transgenic mice were able to transmit the *BIGH3* gene to the next generation, which is a low integration rate. We speculate that this is due to the multiple integration and chimerical structure of the foreign genes.

Subsequently, we observed that the *BIGH3* transgenic mice did not present apparent macroscopic abnormalities compared with the wild-type mice, suggesting that *BIGH3* overexpression did not severely impair mouse development. A digital camera was also applied to determine the gross appearance of the corneas of these transgenic mice following anesthesia. There were five among the seven F_1 transgenic mice that displayed centrally reduced corneal transparency, as illustrated in Fig. 3, which was similar to the symptoms of HCD.

Furthermore, RT-PCR and western blot analysis were also performed with the corneas of F_1 transgenic mice to investigate the expression of the human *BIGH3* gene in the corneas from wild-type and transgenic mice. As the anti-human BIGH3 antibody partially cross-reacted with both mouse and human proteins, the amount of BIGH3 in the wild-type mice likely represented the level of the endogenous mouse BIGH3 in the corneas. We demonstrated that, in comparison with the wild-type mice, the transgenic mice had a significantly increased expression of BIGH3 in their corneas (Fig. 5).

Taken together, the results from our study suggest that we successfully generated a transgenic mouse model overexpressing the human *BIGH3* gene under the control of the PGK promoter, which may serve as a practical model for studying HCD. In addition, we believe that the *BIGH3* gene is essential for ocular development *in vivo* and pathologically important to corneal disorganization. Therefore, this transgenic mouse model is likely to contribute to further studies concerning the detailed pathogenesis of the overexpression of BIGH3 protein in corneal dystrophy.

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