·Basic Research·

Construction of eukaryotic plasmid expressing human TGFBI and its influence on human corneal epithelial cells

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Abstract

• AIM: To detect the expression of transforming growth factor beta-induced gene (TGFBI) protein in human corneal tissue and overexpress it in the human corneal epithelial cells in order to discuss the function of TGFBI in the pathogenesis of corneal dystrophy.

• METHODS: Immunohistochemistry (IHC) was used to detect the expression of TGFBI in the human cornea tissue. TGFBI cDNA was obtained by reverse transcription-PCR from human corneal total RNA extracted from cornea transplant donor and cloned into pCMV-N-HA vector. The recombinant pCMV-N-HA-TGFBI plasmid transfected human corneal epithelial cells. Forty-eight hours later, mRNA and proteins were harvested from cells for real-time PCR analysis and western blot assay respectively.

• RESULTS: IHC indicated TGFBI mainly exist below the human corneal epithelium layer. Transfection of recombinant pCMV-N-HA-TGFBI into human corneal epithelial cells resulted in effective expression of TGFBI, as shown by increased mRNA level detected by real-time PCR as well as increased protein level detected by Western blot. Meanwhile the result of real-time PCR and Western blot shown the expression of MMP1,MMP3(matrix metalloproteinases MMP) increased while the expressin of TIMP1 (tissue inhibitors of matrix metalloproteinases TIMP) decreased.

• CONCLUSION: TGFBI mainly exists below the corneal epithelial layer, recombinant eukaryotic expression vector harboring human TGFBI cDNA was obtained and efficiently overexpressed in human corneal epithelial cells. Meanwhile the TGFBI overexpression in human corneal epithelial cells result in MMP1, MMP3 increasing and TIMP1 decreasing. The result might be helpful for studying the function and role of TGFBI in pathogenesis of corneal dystrophy.

• KEYWORDS: TGFBI; human corneal epithelial cells; matrix metalloproteinases

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INTRODUCTION

The transforming growth factor beta induced protein (TGFBIp; also known as kerato-epithelin[1], beta ig-h3, βig-h3, RGD-containing collagen-associated protein [RGD-CAP][2], and MP78/70 [3,4]) is an extracellular matrix protein encoded by the transforming growth factor beta induced gene (TGFBI; formerly designated BIGH3), which was first discovered in a lung adenocarcinoma cell line exposed to transforming growth factor beta [5]. TGFBI is strongly induced by TGF-β in several cell lines including human epithelial cells, keratinocytes, and fibroblasts[6,7]. The TGFBIp is composed of 683 amino acids containing short amino acid regions homologous to similar motifs in Drosophila fasciclin-I and four homologous internal domains. TGFBIp has been established in a wide variety of tissues including developing nuchal ligament, aorta, lung, and kidney and mature cornea, skin, bladder, and bone[8-12]. It is involved in many cell processes such as cell growth, differentiation, wound healing, angiogenesis and apoptosis[13,14], although the underlying mechanisms for these effects are still unclear.

Mutations of TGFBI are responsible for 5q31-linked human autosomal dominant corneal dystrophies [15]. These diseases are most often characterized by progressive accumulation of deposits in the cornea, resulting in a loss of transparency and severe visual impairment. Corneal dystrophy results in a reduction of visual power and in eventual blindness owing to the accumulation of protein deposits in the cornea. Development of a clinical therapy for corneal dystrophy has been slow because it is not known how the protein deposit accumulates progressively in the cornea. Although the immunohistochemical studies [16,17] demonstrated that TGFBI is strongly stained in the pathologic deposits in all TGFBI-related corneal dystrophies, the role of TGFBI in the formation of deposits is largely unknown, even though its exact expressing parts in cornea is unclear. In an attempt to better understand the function of TGFBI, in this study we detected the TGFBI expressing parts in human cornea and constructed eukaryotic expression vector expressing human TGFBI gene and provide new information about the physical and biochemical mechanisms of βigh3 protein aggregation.

MATERIALS AND METHODS

Materials

Instruments and chemicals The following instruments and chemicals were used in the study: centrifugal machine and mastercycler PCR system (Eppendorf Co., Germany); electrophoresis apparatus, and gel imaging system and 450 microplate reader (BIO-RAD Co., USA); paraffin slicing machine (Leica Co., Germany); polarization microscope (Chongqing Kanghua Medical Equipment Co., Ltd); 7500 Real-Time PCR System (Applied Biosystems Co., USA); DNA Ligation Kit (Mighty Mix), BamH Ⅰ and Xho Ⅰ (Takara Co., Japan); TGFBI polyclonal antibody (Proteintech Group Inc Co., USA), goat anti-rabbit immunoglobulins (DAKO Co., Denmark), NucleoSpin RNA Ⅱ (10 preps) (MachereyCanada-Cronkhite syndrome Co., Germeny); AMV First Strand cDNA Synthesis Kit (Bio Basic Inc Co., Canada); DMEM medium and fetal bovine serum (Gibco Co., USA); Effectence Transfection Reagent (1ml) (Qiagen Co., Germeny); SYBR Green Master Mix (Tiangen biotech Co., China); Super Signal West Femto Trial Kit (Pierce Co., USA); CCK-8 (Beyotime Institute of Biotechnology, China).

Cells and corneal tissue The human corneal epithelial cells were obtained from Zhongshan Eye Institute (Guangzhou, China), the human corneal tissue what was not suitable for the patients was from the the corneal transplant donors in the first affiliated hospital of Jinan University. Research related to this work was supported by a grant from Guangzhou Science and Technology Commission, Guangzhou, China.

Methods

Immunohistochemistry (IHC) detect the expression of TGFBI in the cornea tissue To investigate TGFBI expression, immunohistochemical staining of the human corneal tissue was performed as described previously. Briefly, sections were deparaffinized, rehydrated, and blocked by incubation in 10% H_2O_2 before blotting. The sections were then put in 1mmol/L Tris solution (pH 9.0) supplemented with 0.5mmol/L EGTA (ethylene glycol tetraacetic acid) and heated in a microwave for 10min to reveal the antigens. After blocking in PBS (phosphate buffered saline), supplemented with 1% BSA(bovine serum albumin), 0.05% saponin, and 0.2% gelatin, sections were incubated overnight at 4℃ with TGFBI polyclonal antibody in a humidified chamber. Sections were then washed three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins for 90 minutes at RT (room temperature). The signal was visualized by incubating the sections with liquid diaminobenzidine tetrahydrochloride (DAB) Chromogen. Hematoxylin staining was used to counterstain sections.

The total RNA isolation and reverse transcription PCR The total RNA was isolated from the corneal tissue from cornea transplant donors with Macherey-Nagel NucleoSpin RNA Ⅱ (10 preps). 2μg total RNA was reverse transcribed using a reverse transcription kit (Bio Basic Inc AMV First Strand cDNA Synthesis Kit) to synthesize a single-stranded cDNA (ss-cDNA) product. The dsDNA was identified by PCR analysis. PCR was performed in 20μL reaction mixtures, containing 0.8μL cDNA, 0.5μL (10 μmol/L)each primer set (forward primer and reverse primer), 8.2μL ddH_2O, 10μL 2×Pfu PCR MasterMix. The primers were specific for TGFBIcDNA (Forward:5'-ccgatatcATGGCGCT CCTCATGCGACTGC-3', Reverse: 5'- ttctcgagAATTGGC GGAGAGCTGCCGTGG-3') to amplify a 2098 bp fragment. Amplification was performed for 30 cycles in the following PCR conditions: 30 seconds at 95℃, 30 seconds at 55℃ and 3 minutes at 72℃ using a Mastercycler PCR System. PCR products were then separated electrophoretically on 2% agarose gels and visualized after ethidium bromide staining.

Construction of recombinant eukaryotic expression vector The eukaryotic expression vector pCMV-N-HA and the TGFBI purifed products were digested with BamH Ⅰ and Xho Ⅰ and the resulting products were ligated with DNA Ligation Kit (Mighty Mix), transformed DH5α strains, extracted plasmids and recombinant vectors were identified and analyzed by the restrictive endonuclease digestion map and sequence analysis. The new recombinant plasmid vectors were designated as pCMV-N-HA-TGFBI.

Human corneal epithelial cells culture and transfection The human corneal epithelial cells were obtained from Zhongshan Eye Institute and cultured as the method

mentioned in *Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells* [18], the cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in 5%CO_2 at 37 ℃. Before transfection, the cells were digested by 0.25% trypsin and 0.05% EDTA (1:1) into 6-well plates. When the cells reached 80%, transfected the plasmids following the manufacturer's instructions. The plasmids diluted in buffer EC, add enhancer and vortex. Incubate at RT for 5 minutes then add effectene transfection reagent to the DNA-Enhancer mixture. Vortex for 10 seconds. Incubate the samples for 10 minutes at RT to allow transfection-complex formation. While complex formation took place, gently aspirate the growth medium from the 6-well plates and wash cells once with PBS. Add fresh growth medium to the cells. Add fresh growth medium to the tube containing the transfection complexes, mix and add the transfection complexes into the cells in 6-well plates. Gently swirl the plates to ensure uniform distribution of the transfection complexes. Then incubate the cells with transfection complexes under their normal growth conditions for 48 hours.

Real-time PCR analysis Forty-eight after transfection, collected the cells and extracted the total RNA. 2μg total RNA were respectively reverse transcribed to synthesize single-stranded cDNA (ss-cDNA) products. A standard 20μL reaction contained 0.5μL of the cDNA mixture, 0.25μL 100μm of the forward and reverse primers and the SYBR Green Master Mix，amplification was performed for 40 cycles in the following PCR conditions: 1 minute at 95℃, 1 minute at 60℃ and 1 minute at 72℃. Samples were run in triplicate in a 96-well plate format in a 7500 Real-Time PCR System. Primers were: F: 5'- GCTGTATA CAGACCG CACAGAA-3', R: 5'-GCAGGCAAGGAAGA CCAGG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were also tested cDNA samples from cells transfected recombinant vectors for control. F: 5'-CTGCCCAGAACATCATCCCT-3', R: 5'-GGTCCTCAG TGTAGCCCAAGA-3'.The MMP1, MMP3 and TIMP1 primers were:

MMP1 F: 5'-AAGTGTGACCCAGCCCTATC-3'
R: 5'-CACATGGTTGGGAAGTTCTG-3'
TIMP1 F: 5'-TACCAGCGTTATGAGATCAAG-3'
R: 5'-CCATGAGGATCTGATCTGTCC-3'
MMP3 F: 5'-CTATTCCTGGTTGCTGCTCA-3'
R: 5'-GAGATGGAAACGGGACAAG-3'

Western blot assay Forty-eight hours after transfection, the cells were lysed with EBC buffer (50mmol/L Tris pH8, 170mmol/L NaCl, 0.5% NP40, 50mmol/L NaF) supplemented with protease inhibitors. Protein lysate (30g) was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane in 25mmol/L Tris, 200mmol/L glycine, 20% methanol, at 80 V for 3 hours at 4℃. After blocking nonspecific binding sites with 5% nonfat milk in TBS-T (20mM Tris-HCl, pH7.6, 137mmol/L NaCl, and 0.1%Tween), the membrane was incubated for 2 hours at RT with TGFBI polyclonal antibody (diluted 1:100 in PBS) and washed 3×15 minutes with TBS-T. Then the membrane was incubated for 1 hour with the goat anti-rabbit immunoglobulins (diluted 1:3 000 in PBS) and washed 3× 15 minutes with TBS-T. The blot was developed with Super Signal West Femto Trial Kit. Anti-GAPDH antibody was used as a control on the same menbrane. MMP1, MMP3 and TIMP1 were analysised as the same method.

CCK-8 assay Proliferation and viability of HCECs after transfection in 96-well culture plate were assessed by CCK-8 assay. Briefly, cells were seeded in the concentration of 1.5×10^3 cells per well in 100μL of fresh medium with serum 96-well culture plate. Then, the recombinant plasmid transfected (the method as above) the cells in 96-well culture plate (the cells diverse three groups: transfected with pCMV-N-HA-TGFBI; transfected with pCMV-N-HA; human corneal epithelial cells control). After 48 hours, 10μL of CCK-8 solution was added in the culture plate and incubated for 4 hours at 37℃. The colored product was quantified at 450nm in a Bio-Rad model 450 microplate reader. The test was performed after 0.5, 1, 2 and 4 hours.

Statistical Analysis Statistical analysis was performed using SPSS11.0 (SPSS Inc. Chicago, Illinois, USA) and one-way single factor ANOVA. The Fisher's exact test was used to perform categorical data. The Student-Newman-Keuls (SNK) test was used to conduct pairwise comparisons among the three groups. P value less than 0.05 indicates that total comparison differences are statistically significant, and a P value less than 0.01 indicates that pair-wise comparisons among groups are statistically significant.

RESULTS

Expression of TGFBI in Human Corneal Tissue βIG-H3 has been known to be synthesized in the corneal epithelium and has been suggested to play a role in maintaining the integrity of the corneal epithelium[13]. While in our findings IHC indicated TGFBI exist mainly in the corneal stormal layer not the epithelial layers. This may suggest that the βIG-H3 is secreting protein. It was synthesized in the corneal epithelium and transported to the corneal stromal layer (Figure 1).

Amplification of TGFBI and Construction and Identification of Plasmids pCMV-N-HA-TGFBI The PCR products were electrophoresed on a 2% agarose gel. Results showed that the lengths of the TGFBI were 2098bp, which were identical to the expected lengths respectively (Figure 2).The recombinant vector was identified by Restriction endonuclease analysis and DNA sequence analysis (Figure 3).The results showed that synthesised TGFBI gene was identical to the TGBFI sequence recently

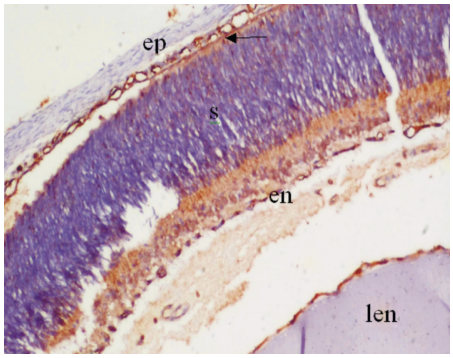

Figure 1 Positive expression of TGFBI in human corneal tissue (SP ×100) ep: corneal epithelium; S: corneal stroma; en: corneal endothelium.

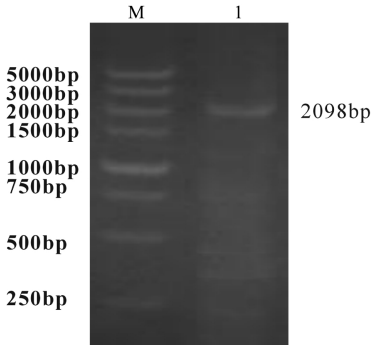

Figure 2 PCR product M: DNA marker DL5000; Lane 1: PCR product of human TGFBI.

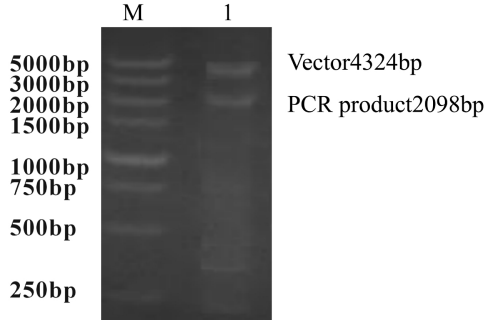

Figure 3 Restrictive endonuclease digestion of recombinant plamid M: DNA marker DL5000; Lane 1: Product of recombinant plasmid by restriction enzyme.

reported on pubmed. We concluded that the recombinant plasmid vectors pCMV-N-HA-TGFBI were constructed successfully.

Human Corneal Epithelial Cells Culture and Transfected Forty-eight hours after transfection, the cells were collected and the expression of TGFBI was detected by real time PCR and Western blot. Real-time PCR demonstrated the plasmids transfecting cells expressing TGFBI gene enhanced (Figure 4) and the Western blot results showed the expression of TGFBI expressed stronger in pCMV-N-HA-TGFBI transfected cells than in pCMV-N-HA transfected cells and untransfected cells(Figure 5).

CCK-8 Assay Result CCK-8 indicate the human corneal epithelial cell proliferation that tansfected with pCMV-N-HA-TGFBI and tansfected with pCMV-N-HA and cells

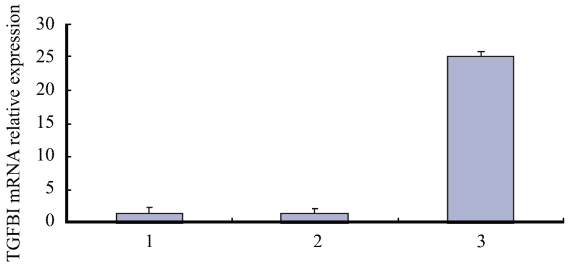

Figure 4 Decetion of TGFBI mRNA by SYBR PCR and calculate the relative expression amount according to $2^{-\triangle\triangle\alpha}$ 1: human corneal epithlial cells control; 2: transfected with pCMV-N-HA; 3: transfected with pCMV-N-HA-TGFBI. F=4.45, P< 0.05, 3 control to 1:# t=3.434, P<0.05; 3 control to 2: * t=3.233, P<0.05.

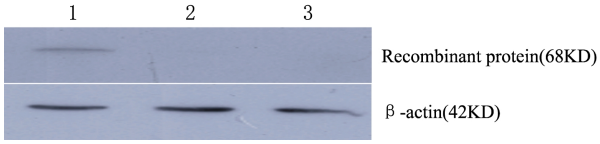

Figure 5 Western blotting of TGFBI proteins in human corneal epithelial cells 1: Transfected with pCMV-N-HA-TGFBI; 2: Transfected with pCMV-N-HA; 3: human corneal epithelial cells control.Upper: 68KD recomniant protein; Lower:β-actin protein.

Table 1 Detection of the transfected corneal epithlial cells proliferation by CCK-8 (mean ±standard deviation, n=12)

Group	CCK-8(A450)
1: Transfected with pCMV-N-HA-TGFBI;	3.2147±0.045
2: Transfected with pCMV-N-HA;	3.1898±0.032
3: Human corneal epithlial cells control	3.2563±0.035

Notes:Control to 3:1) $t_{1,3}$=2.35,P>0.05; 2) $t_{2,3}$=2.43,P>0.05.

control has no statistical significance. That means the recombinant pCMV-N-HA-TGFBI plamid has no obvious influence to the human corneal epithelial cell proliferation (Table 1).

Expression of MMP1, MMP3 and TIMP1 in Human Corneal Epithelial Cells 48 hours after transfection, the cells were collected and the expression of MMP1, MMP3 and TIMP1 were detected by real time PCR and western blot.Real-time PCR demonstrated the recombinant plasmids transfecting cells expressing MMP1, MMP3 gene enhanced and TIMP1 decreased (Figure 6), the Western blot results showed the expression of MMP1, MMP3 expressed stronger while TIMP1 expressed less in pCMV-N-HA-TGFBI transfected cells than in pCMV-N-HA transfected cells and untransfected cells(Figure 7).

DISCUSSION

A transforming growth factor-β (TGF-β)-induced extracellular matrix (ECM) protein (βIG-H3) has been considered a major component of abnormal extracellular deposits in the cornea and its mutations were responsible for 5q31-linked human autosomal dominant corneal dystrophies (CDs). Although the immunohistochemical studies [16,17]

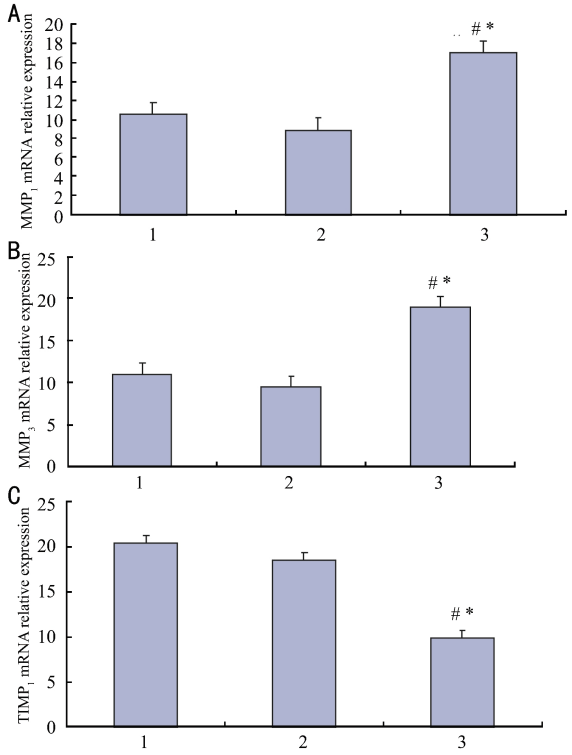

Figure 6 Decetion of TGFBI mRNA by SYBR PCR and calculate the relative expression amount according to $2^{-\triangle\triangle\alpha}$ 1:human corneal epithelial cells control; 2:transfected with pCMV-N-HA; 3: transfected with pCMV-N-HA-TGFBI.Upper:the relative expression amount of MMP1 mRNA; Middler: the relative expression amount of MMP3 mRNA; Lower: the relative expression amount of TIMP1 mRNA. A: F=5.32, P<0.05.3 control to 1: # t=4.619, P<0.05; 3 control to 2: * t=3.548, P< 0.05; B: F=5.79, P<0.05. 3 control to 1: # t=4.619, P<0.05; 3 control to 2: * t=3.548, P<0.05; C: F=4.93, P<0.05. 3 control to 1: # t=4.619, P<0.05; 3 control to 2: * t=3.548, P<0.05.

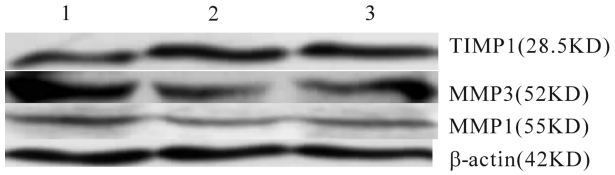

Figure 7 Western blotting of TGFBI proteins in human corneal epithelial cells 1: Transfected with pCMV-N-HA-TGFBI; 2: Transfected with pCMV-N-HA; 3: Human corneal epithelial cells control. From up to down: TIMP1 protein; MMP3 protein; MMP1 protein; β-actin protein.

demonstrated that βIG-H3 is strongly stained in the pathologic deposits in all βIG-H3-related corneal dystrophies, the role of the different mutations in the formation of different types of deposits is largely unknown. Even, the structure of wild-type βIG-H3 and its interaction with other extracellular matrix (ECM) proteins are not known. βIG-H3 protein is keratoepithelin, while our IHC result indicated the βIG-H3 protein mainly existed below the corneal epithelial layer and in the corneal stromal layer. Mutations of βIG-H3 were demonstrated to be responsible for granular (GCD), Reis-Buckler (RBCD), lattice type I (LCD-I), and Avellino (ACD) corneal dystrophies [1] and so on. The pathological changes of there CDs were in the corneal stromal layer. And that was consistent with our finding. As keratoepithelin, less βIG-H3 protein expression in the corneal epithelial layer in our experiment, but it may be synthetized in corneal epithelium and transported to the stromal layer. It may play an important role in corneal epithelial cells. Based on that, we constructed the pCMV-N-HA-TGFBI recombinant vector to transfect the human corneal epithelial cells in order to detect the influence of overexpression TGFBI to the human corneal epithelial cells *in vitro*.

After the pCMV-N-HA-TGFBI recombinant vector was successfully constrcured, it transfected the human corneal epithelial cells. We found first the cells successfully overexpressed the TGFBI gene in mRNA level and protein level. Second the plasmid has less influences on the transfected cells growth. This suggested TGFBI play its role in human corneal epithelial cells mainly not through regulating the cell proliferation. In other words, the recombinant plasmid did not effect the transfected cells normal growth. Then, the expression of MMP1, MMP3 and TIMP1 changed in the transfected cells according to the untransfected cells.

The MMPs (matrix metalloproteinases) constitute a large family of proteolytic enzymes that degrade the extracellular matrix and facilitate remodeling under normal and pathological conditions[19]. MMPs can be strictly regulated at multiple levels through the control of gene transcription, posttranslational activation of zymogens, and the interactions of secreted MMPs with TIMPs(tissue inhibitors of matrix metalloproteinases), which are small proteins that inhibit MMPs by noncovalently binding them with a 1:1 stoichiometry. MMPs are classified into several groups, such as interstitial collagenases (MMP-1, -8, -13), gelatinases (MMP-2,-9), stromelysins (MMP-3, -7, -10, -11), and membrane type-MMPs (MMP-1, -2, -3, -4). The MMP-1 causes degradation of collagen fibers and other extracellular matrix components. MMP3 (Stromelysine-1) is capable of cleaving the triple helical fibrillar of collagen (IV, V, IX, X), elastin, gelatin, laminin, aggrecan and E-cadherin. MMP3 is activated by MMP1. TIMP-1 not only inhibits MMPs [20,21] but also affects cellular proliferation [22,23], apoptosis [24-26], and angiogenesis [27], both dependent on and independent of its MMP-inhibiting function. In our experiment, after 48 hours the recombinant plasmid transfected the cells, the MMP1,MMP3 expression increased both in mRNA level and in protein level while the TIMP1 expression is opposite. These show the function of TGFBI overexpression in human corneal epithelial cells

may have important relationship with the MMP and TIMP.

Based on the above findings, we hypothesized that TGFBI in the βIG-H3-related cornea dystrophies may play a role through this way: TGFBI overexpression in corneal epithelial cells may activate the MMP1, then MMP1 activated the MMP3 and at the same time the TIMP1 was inhibited. At last it broke the banlance of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases lead to the matrix metalloproteinases degradate more normal protein. The enhanced abnormal protein deposit in the corneal stromal and epithelium induce cornea optication decreased. All these process, lots of deeper research were needed.

MMPs, MMP inducer and tissue inhibitors may play an important role in the pathogenesis of βIG-H3-related corneal dystrophies. The expression change of MMPs through the TGFBI overexpession may provide an opportunity to deeperly study the mechanisms[28-32] and novel therapies in corneal dystrophies. By advancing knowledge of the expression of MMPs and TIMPs in corneal epithelium layer and in corneal stromal layer this information may contribute to the understanding of the biology of corneal dystrophies and foster development of new therapies.

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