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Induction of Corneal Myofibroblasts by Lens-derived Transforming Growth Factor $\beta 1$ (TGF $\beta 1$): A Transgenic Mouse Model

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Abstract

Purpose—Transforming growth factor β (TGF β) is an important cytokine in corneal development and wound healing. Transgenic mice that express an active form of human TGF $\beta 1$ driven by a lens-specific promoter were used in the current study to determine the biological effects of lens-derived TGF $\beta 1$ on postnatal corneal development and homeostasis.

Methods—The postnatal corneal changes in the TGF $\beta 1$ transgenic mice were examined by fluorescein labeling and histology. Epithelial/endothelial-to-mesenchymal transition (E/EnMT) in the transgenic mouse cornea was demonstrated by immunostaining for α -smooth muscle actin (α -SMA) and cadherin-11. Expression of E- and N-cadherin in the corneal epithelial and endothelial cells, respectively, was analyzed by *in situ* hybridization.

Results—Among the established TGF $\beta 1$ transgenic lines, mice from line OVE853 and OVE917 had normal-sized eyeballs but developed a corneal haze after eyelid opening. Histological examination showed that prenatal corneal development appeared to be normal. However, after postnatal day 7 (P7), the corneal endothelial cells in transgenic line OVE853 began to lose normal cell-cell contact and basement membrane structure. The endothelial layer was eventually absent in the inner surface of the transgenic mouse cornea. The morphological changes in the cornea correlated with abnormal expression of α -SMA, a molecular marker of EMT, and stress fiber formation in myofibroblast-like cells, which initially appeared in the corneal endothelial layer and subsequently in the corneal epithelial and stromal layers. The E/EnMT in the transgenic mouse cornea was further demonstrated by loss of E- and N-cadherin expression in the corneal epithelial and endothelial cells respectively, and meanwhile increasing expression of cadherin-11 in both corneal epithelium and stroma.

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Conclusions—Elevated levels of active TGF β 1 in the anterior chamber can lead to myofibroblast formation in the corneal endothelial layer and subsequently in the corneal epithelial and stromal layers. Our data suggest that the levels of biologically active TGF β in the aqueous humor must be under tight control to maintain corneal homeostasis. TGF β 1 is the major cytokine during wound healing. Therefore, our findings also suggest a potential mechanism to explain the loss of corneal endothelial barrier and corneal opacification after intraocular surgery or trauma.

1. Introduction

Transforming growth factor β (TGF β) is a multifunctional cytokine that controls a diverse set of cell processes including production of extracellular matrix (ECM), cell proliferation, migration, differentiation, and apoptosis [1;2;3;4;5]. TGF β has emerged as one of the most important ligands involved in tissue development, homeostasis and wound healing in the eye [6;7;8;9]. Three mammalian isoforms of TGF β (TGF β 1, TGF β 2 and TGF β 3) have been identified [10]. While all are expressed in the eye, TGF β 2 is expressed a much higher level than TGF β 1 or TGF β 3 [11;12;13;14;15]. TGF β 2 knockout mice exhibit multiple defects in anterior segment structures, including thinning of the corneal stroma and an absence of the corneal endothelial layer and anterior chamber [6;16;17]. Additional ocular defects in the TGF β 2-null mouse embryos include immature retina and vitreous hypercellularity [16;18]. The abnormalities found in the eye of the TGF β 2-null mouse are mostly due to the impaired immigration of neural crest cells [6]. Deletion of TGF β 1 and β 3 did not cause any visible abnormalities in the cornea or in the eye [19;20;21;22].

TGF β also plays an important role in corneal maintenance and wound healing [23;24;25]. In contrast to the developmental role of TGF β 2 in the eye, TGF β 1 is believed to be the principle isoform during corneal wound healing [25]. In the normal cornea, TGF β 1 and TGF β 2 localize to both corneal epithelium and stroma, and both are present in the tear fluid [26;27;28]. Additionally, TGF β 3 is present at a very low level in the cornea [29]. Interestingly, after a corneal wound, the levels and spatial locations of the TGF β isoforms are altered in the cornea [30]. For instance, TGF β 1 increases dramatically in the tear fluid [31]. Previous studies suggested that increase of TGF β 1 in the cornea may induce scar formation on the ocular surface [32;33], a process that is also seen in other internal organs such as liver and kidney [34;35;36]. As a result of stromal fibrosis and scarring, the transparency of the cornea is reduced, ultimately leading to the impairment of the patients' vision.

TGF β signals are relayed through type I and II serine/threonine kinase receptors (TGF β RI and TGF β RII) [37;38]. Among the three TGF β isoforms, TGF β 1 and TGF β 3 can independently bind to TGF β RII and then recruit TGF β RI to form an RI-RII-ligand complex. TGF β RII must phosphorylate TGF β RI to transfer the signal to the cell interior. TGF β 2 binds to a type III receptor (TGF β RIII), also known as β -glycan, which then presents TGF β 2 to TGF β RII and phosphorylates TGF β RI [39]. The phosphorylated (active) TGF β RI transduces the signal by phosphorylating the carboxyl terminal serines of the receptor-regulated Smad (R-Smad) proteins, Smad2 and Smad3 [40]. The activated R-Smads form a complex with the common Smad (co-Smad), Smad4. The R-Smad/Co-Smad complex translocates into the nucleus and regulates target gene transcription in conjunction with other nuclear factors. TGF β receptor activity can be negatively regulated by two inhibitory Smads (I-Smads), Smad6 and Smad7, which compete with R-Smads, to attenuate the receptor signaling activity.

TGF β RI and TGF β RII are located in epithelial, stromal and endothelial layers of the cornea [41;42;43]. The non-signaling type III receptor (β -glycan receptor) has been located on both the epithelium and endothelium *in vivo*, but appears to be absent in keratocytes *in vivo*. The presence of all three types of receptors in the epithelial and endothelial layers suggests that these corneal cells can respond to any of the three TGF β isoforms.

Transgenic mice that express a self-activating human TGF β 1 in the ocular lens were generated several years ago to test the biological effects of elevated levels of TGF β 1 on ocular tissue morphogenesis and homeostasis [18;44]. The pathological consequences of TGF β 1 overexpression on the lens and ocular vascular development have been examined in detail and reported previously [18;44]. In some of these studies, corneal and anterior segment defects were documented but not investigated in detail [44]. Among the three established transgenic lines, OVE920A had the highest expression level for the transgene, whereas OVE853 and OVE917 had relatively lower expression levels [18]. The developmental abnormalities in the eyes of the OVE920A mice were similar to transgenic mice in which TGF β 1 overexpression was driven by the stronger lens-promoter: chicken β B1-crystallin promoter [45]. In these high expression transgenic lines, differentiation of the corneal mesenchymal cells was disrupted, leading to severe defects in the anterior segment structures. In contrast to OVE920A or β B1-crystallin-TGF β 1 transgenic mice, the development of the cornea and anterior segment in OVE853 and OVE917 transgenic lines appeared to be normal during the prenatal period, but corneal defects were found postnatally [44].

In this study, we show that active TGF β 1 released from the lens of the transgenic mouse has a negative effect on corneal integrity. Data are consistent with corneal cells undergoing classical TGF β 1-induced epithelial-to-mesenchymal transition (EMT), including 1) detachment of the corneal endothelial cells from the basement membrane, 2) induction of α -smooth muscle actin (α SMA) expression and myofibroblast formation in the corneal endothelial and epithelial layers, and later in the corneal stroma, and 3) down-regulation of E- and N-cadherin expression in the corneal epithelial and endothelial cells respectively, and increased expression of cadherin-11, a mesenchymal cell cadherin. Our results suggest that activation of TGF β 1 in the aqueous humor of the postnatal mouse eye can disrupt the maintenance of corneal tissue homeostasis. The findings from this transgenic mouse model may also have clinical implications since increased activation of TGF β 1 after injury, intraocular surgery or in diabetes may potentially lead to pathological changes in the cornea.

2. Materials and Methods

2.1. Transgenic mice

Generation of transgenic mice has been described previously [44]. Briefly, the mouse α A-crystallin promoter was used to drive expression of a secreted and constitutively active form of human TGF β 1 in the lens. All the animals were used in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all experimental procedures were approved by the Animal Care and Use Committee at the University of Missouri (MU) and University of Oklahoma Health Science Center (OUHSC).

2.2. Fluorescein labeling of the cornea surface

To evaluate the smoothness of the cornea surface, 3 mice at postnatal day 16 (P16) were anesthetized with ketamine and xylazine, and fluorescein eye drops (Fluress, Akorn Inc.) were applied to the cornea surface. The mouse eyes were examined under blue light using a fluorescent stereomicroscope (SZX12, Olympus).

2.3. Histology

Mouse eyes were fixed in 10% formalin overnight at room temperature, rinsed twice with phosphate buffered saline (PBS), dehydrated by emersion in an ethanol series, cleared with xylene, and embedded in paraffin. Sections were cut at 5 μ m and stained with hematoxylin and eosin (H&E). To label the cell nuclei, sections were incubated with DAPI for 5 min and then rinsed twice with PBS before analysis by fluorescence microscopy. Periodic Acid Schiff (PAS) reaction was used to reveal the basement membrane structures in mouse cornea.

2.4. Immunohistochemistry and immunofluorescence

For immunohistochemical staining of TGF β receptors in mouse cornea, tissue sections were pretreated with 10% methanol and 3% H₂O₂ in PBS to block endogenous peroxidase. Antigen retrieval was done by heating the slides to 100°C in 10 mM citrate buffer (pH 6.0) for 10 min using a microwave oven. After blocking with 5% normal serum in PBS, sections were incubated with primary antibody overnight at 4°C. The anti-TGF β RI (sc-398) and anti-TGF β RII (sc-220) primary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). After washing, the sections were incubated with biotinylated secondary antibody (Vector Laboratory Inc. Burlingame, CA) for 1 h at room temperature. Immune complexes were visualized by using the avidin-biotin peroxidase complex method with diaminobenzidine as the substrate [46]. Sections were counterstained with hematoxylin. For Cadherin-11 immunofluorescence, after blocking, tissue sections were labeled with anti-cadherin-11 antibody (Cat#71-7600, Invitrogen, Carlsbad, CA) overnight at 4°C, and then with Alexa Fluor 568 anti-rabbit secondary antibody for 1 hour at room temperature. Images were captured under Leica fluorescence microscope.

2.5. *In situ* hybridization

In situ hybridization was performed as described previously [47]. Tissue sections were incubated with radioactive ³⁵S-labeled riboprobes specific for E- and N-cadherin overnight at 50°C. After stringent washing, sections were coated with Kodak NTB-2 emulsion and exposed for 1 week at 4°C before developing. Sections were counterstained with hematoxylin.

2.6. Whole-mount immunofluorescence staining

For whole mount immunofluorescence staining of corneas, mice were killed by CO₂ asphyxiation and the eyes were removed and fixed overnight in 4% paraformaldehyde. The following day, corneas were dissected and rinsed three times in PBS. Corneas were then incubated in PBS containing 10% horse serum and 1% Triton for 2 hours to permeabilize tissues and block non-specific binding. Corneas were then incubated with FITC or Cy3 conjugated anti-alpha smooth muscle actin (monoclonal antibody, Sigma-Aldrich) diluted 1:200 in PBS containing 10% horse serum and 1% triton. Following a 16 hour incubation at 4°C corneas were washed three times in PBS with 1% triton, then flat mounted unto glass slides and coverslipped in PBS containing 10% glycerol. Stained corneas were imaged on an Olympus Flowview confocal microscope. Optical sections were obtained using identical detector settings. Maximum projections of image stacks were obtained to demonstrate flat perspective, or rotated in the Z-plane to produce Z-rotated images. Whole corneas were also imaged using epi-fluorescence illumination on a Nikon E800 microscope.

3. Results

3.1. Lens-specific expression of biologically active TGF β 1 causes corneal defects

Among the three previously reported TGF β 1 transgenic mouse lines [18;44], mouse from line OVE853 and OVE917 share a similar corneal phenotype, this study focused on line OVE853. When OVE853 transgenic mice opened their eyes by P14, the cornea had already lost its transparency and developed haze (Fig. 1A). The defect of the corneal surface in these transgenic mice was also demonstrated by fluorescein binding. Exposure of wild-type corneas to fluorescein dye revealed a smooth surface (Fig. 1B), whereas the TGF β 1 transgenic corneas retained strong fluorescent staining, indicating the roughness of the corneal surface and defects in the epithelial layer (Fig. 1C).

The progression of the corneal abnormalities in transgenic mice was examined by histology (Fig. 2 and Fig. 3). At P7 (Fig. 2A, B), the wild type cornea contained a single layer of

endothelial cells that formed a barrier at the inner surface of the cornea. The corneal endothelial layer in the transgenic mice began to lose its normal cell contact and to detach from the inner surface. The stroma of the transgenic mouse cornea was thicker, probably resulting from the loss of barrier function of the endothelial layer. At P14, the inner surface of the transgenic mouse cornea lacked the distinctive corneal endothelial layer (Fig. 2D, F). Additionally, the corneal epithelial cells were poorly differentiated, for example, the stratified layers and the super basal layers of the corneal epithelium were not seen in transgenic cornea. The mouse corneas were stained with PAS to identify the basement membrane structures (Fig. 3). At P21, the Descemet's membrane, synthesized by the corneal endothelial cells, can be seen in the wild type cornea (Fig. 3A, indicated by white arrows), but not in either the hemizygous (Fig. 3B) or homozygous (Fig. 3C) transgenic mouse corneas. Loss of corneal endothelial layer and Descemet's membrane caused the lens and iris to adhere to the cornea in the transgenic eye (Fig. 3C and data not shown).

To determine whether corneal cells during the postnatal stage can directly respond to TGF β 1 stimulation, we examined the expression of TGF β RI and TGF β RII in wild-type corneas from P7 mice. Figure 4 shows that both TGF β RI and TGF β RII were expressed in the corneal epithelial, stromal and endothelial cells. The downstream signaling protein Smad 4 was also expressed in all the corneal layers (data not shown). Therefore, it is likely that the corneal phenotype of the TGF β 1 transgenic mice is a direct response to the active TGF β 1 secreted from the lens.

TGF β -signaling is known to play a role in EMT [4;48]. One hallmark feature of EMT is the expression of α -smooth muscle actin (α -SMA). At P16, α -SMA-positive cells were found in both corneal endothelial and epithelial layers (Fig. 5). Although most of the cells still retain their epithelial and endothelial cell morphology, a few cells at the center of the cornea in the transgenic eye experienced morphological changes, and transformed to myofibroblast like cells (Fig. 5B, C).

To follow the progression of En/EMT in the TGF β 1 transgenic cornea, α -SMA expression was examined at different postnatal ages (Fig. 6). α -SMA expression in the corneal endothelial layer was first detected as early as P2 (Fig. 6B), and the expression level (as indicated by the number of α -SMA-positive cells) increased with age (Fig. 6C–F). At P16, the α -SMA-positive cells had a proto-myofibroblast phenotype but the stain was still prominent in the cytoplasm. Between P21 to P30, corneal endothelial cells had increased α -SMA staining and formed well-organized stress fibers, which are typical of a myofibroblast phenotype.

The distribution of α -SMA-positive cells in different corneal layers was monitored through the confocal x–z imaging stacking technique. α -SMA-expressing cells were initially identified in the corneal endothelial and epithelial layers, and subsequently in the corneal stroma (Fig. 7), suggesting that corneal endothelial and epithelial cells are more susceptible to TGF β 1-induced En/EMT than the keratocytes in the stroma.

Additional features of EMT include changes in cadherin expression [49;50]. Cadherins belong to a family of calcium-dependent adhesion molecules that have been shown to play critical roles in tissue morphogenesis and homeostasis [51;52]. Previous studies have documented that E-cadherin is expressed in corneal epithelial cells whereas N-cadherin is expressed in corneal endothelial cells [47]. Therefore, E- and N-cadherin can serve as molecular markers for cornea epithelial and endothelial cells, respectively. *In situ* hybridization showed that at P0, the expression patterns of E- and N-cadherin in the cornea of the transgenic mice appeared similar to their expression patterns in wild-type mice, suggesting that prenatal development of these cell layers were not affected in the OVE853 mice (Fig. 8 A–D). However, by P7, the expression levels of E- and N-cadherin in the transgenic cornea were downregulated significantly, a

finding consistent with the observation that En/EMT occurred in these two corneal cell layers during the postnatal development.

Cadherin-11 is a non-classical cadherin found in mesenchymal cells [53]. Its expression was detected in the corneal myofibroblasts in a cell culture model [54]. We compared the cadherin-11 expression patterns in the wild type and transgenic mouse corneas (Fig. 9). In the wild type eye, cadherin-11 was expressed at a low level in the corneal epithelial layer and in the cells associated with the vasculature systems (e.g. the blood vessel in the iris in Fig. 9A). In the transgenic eye, cadherin-11 expression was increased in both corneal epithelial and stromal layers, and also in a group of lens epithelial cells that detached from the lens capsule (arrow in Fig. 9B). Increase of cadherin-11 expression, along with α SMA expression, confirmed the myofibroblast phenotype in the cornea of TGF β 1 transgenic mouse.

4. Discussion

Earlier studies have shown that TGF β 1 overexpression can alter the development patterns of the anterior segments and the ocular vasculature in the eye [18;44;45]. TGF β 1 overexpression can also induce EMT in the lens [44;55;56]. In this study, we found that the two transgenic lines OVE853 and OVE917 that have relatively lower levels of TGF β 1 transgene expression had normal prenatal development of the anterior segment (Figure 8). The postnatal corneal defects were investigated in detail in this study using line OVE853. Histological examination revealed that the corneal endothelial layer started to deteriorate by P7. Initially the endothelial cells lost both normal cell contact and adhesion to the basement membrane. By P14, the resulting edema suggested that the corneal endothelial barrier was lost in the eye of the transgenic mice. At P21, the distinctive basement membrane structure of the corneal endothelium, the Descemet's membrane, was absent in the transgenic mouse cornea (Fig. 3B, C). Changes in the corneal endothelial layer in transgenic mice is consistent with endothelial-to-mesenchymal transformation (EnMT), as demonstrated by the expression of myofibroblast marker α -SMA, and loss of N-cadherin expression combined with the formation of stress fibers in the inner surface of the cornea. Following EnMT, the fate of endothelial cells is not certain. It is possible that they die or detach from the basement membrane or they migrate as myofibroblast into the stroma, as suggested by the loss of Descemet's membrane (Fig. 3B, C) and the appearance of α -SMA adjacent to the endothelial layer. Following the loss of corneal endothelial barrier function, EMT occurred in the corneal epithelium and later in the stromal layer. Our data suggest that balancing the biological activity of TGF β 1 in the eye is crucial not only for normal anterior segment development, but also for maintaining the cornea integrity and homeostasis during the postnatal period.

TGF β 1 is known to induce EMT in many tissues. The transgenic mice we used in this study develop anterior subcapsular cataracts (ASC) by P21 [44]. Previous studies showed that the underlying mechanism of ASC is through TGF β 1-induced EMT in the lens epithelial cells [57;58]. Despite the lens defects in the OVE853 transgenic mice, we conclude that EnMT and EMT in the corneal endothelial and epithelial layers respectively are a direct response to the TGF β 1 released from the lens, not a secondary effect resulting from EMT in the lens epithelial cells. In our study, α -SMA expression in the cornea occurred earlier than that in the lens. When the eyelids open on P14, the cornea in the OVE853 transgenic mice looked hazy and the EMT marker, α -SMA, was expressed in the corneal endothelial and epithelial layers before P14. In contrast, the lens of OVE853 transgenic mice looked normal and no ASC was found at P14 [44]. The expression of α -SMA in the lens epithelial cells was detected at P21. During mouse eye development, both type I and the type II TGF β receptors are highly expressed in the anterior corneal mesenchymal cells, from which the corneal endothelial and stromal keratocytes differentiate. The type II TGF β receptor was not expressed at a detectable level in the lens epithelial cells before P3, but significant expression of both type I and type II receptors occurred

by age P21 [59]. These results together suggest that the corneal cells are susceptible to TGF β 1 stimulation at an earlier age than the lens.

Our data indicate that active TGF β 1 from the lens can directly induce epithelial/endothelial-to-myofibroblast transformation in the cornea. However, we can not rule out the possibility that other factors, such as the growth factors in the aqueous humor and tears, also play a role in the progression of EnMT and EMT in the cornea [60;61;62]. A recent report showed that epidermal growth factor (EGF), which is normally present in tears, synergizes with TGF β 1 in the induction of differentiation of keratocytes to myofibroblasts [63]. Growth factors, including PDGF, IGF-1 and FGFs, are present in the anterior chamber. Some of these, such as PDGF, were shown to synergistically induce keratocyte-to-myofibroblast differentiation with TGF β in a cell culture system [64;65]. The initial loss of the barrier function of the corneal endothelial layer in the transgenic eye can result in the exposure of stromal keratocytes and the corneal epithelial cells to various growth factors in the aqueous humor. Therefore, it is likely that the increase of α -SMA expression and stress fiber formation in the cornea of the transgenic mice are the combined effects of TGF β 1 and other growth factors.

It is noteworthy to mention that while our data demonstrate that TGF β 1 can induce EnMT and EMT in the cornea, they do not demonstrate that TGF β 1 is responsible for this activity in injured eyes. While TGF β 1 levels increase during normal corneal wound healing [25;66], TGF β 2 is the predominant form of TGF in normal eyes, and TGF β 2 is present at a relatively high level in the aqueous humor [67;68]. TGF β 2 expression is found in the ciliary and iris epithelial cells as well as in the equatorial region of lens cells in mice [15]. These findings raise a question as to why a high level of endogenous TGF β 2 does not induce the same EMT response in normal eyes. One possibility is that the endogenous TGF β 2 in the anterior chamber is normally present in the inactive form [67]. TGF β proteins are secreted as inactive complexes that contain the mature TGF β homodimer tightly associated with dimers of the amino-terminal domain, which is referred as the latency-associated peptide (LAP) [69]. The complexes are termed “latent” because they do not bind to TGF β receptors. The release of the active TGF β form from the latent complex is thought to be regulated by specific proteases *in vivo*. The protease activity is tightly controlled under the normal physiological conditions to keep the levels of active TGF β 2 in the aqueous humor low. Another possibility is that the co-receptor TGF β RIII is required to present TGF β 2 to TGF β RII [69]. Expression of TGF β RIII in the corneal endothelial layer of the normal rat cornea was not detectable before P10 [23]. Therefore, the developing corneal endothelial cells would not be sensitive to TGF β 2 stimulation until after P10. Another possibility is that inhibitory molecules, which antagonize TGF β activity, are present in the anterior chamber of the normal eye [70].

In the cornea, TGF β 1 plays a critical role in wound healing [7]. After corneal injury, quiescent keratocytes at the wound periphery become activated and transform into corneal fibroblasts or myofibroblasts. The transformed cells can migrate to the damaged area, proliferate, and deposit a disorganized and fibrotic ECM to repair the wound. Abnormal wound healing can lead to corneal fibrosis as a result of deregulated production of ECM components and contractile elements by corneal fibroblasts and myofibroblasts [71;72;73;74]. In our transgenic model, we found that TGF β 1 from the lens is able to induce corneal endothelial- and epithelial-to-myofibroblast transformation. The transformation in these two cell layers occurred before myofibroblast formation in the stroma. In our transgenic mouse model, TGF β 1 is secreted from the lens, therefore, it is not surprising that the corneal endothelial cells underwent EnMT prior to the stromal cells. After the loss of endothelial barrier, the differential susceptibility to TGF β 1 stimulation in the corneal epithelium and stroma could be a result of different levels of receptor expression. Zieske et al showed that both type I and type II TGF β receptors were upregulated in the corneal epithelial cells during wound repair [43]. In cultured primary human corneal epithelial cells, EGF and TGF β 1 can induce the expression of TGF β RII. If this is also

true in our transgenic mouse model, TGF β 1 overexpression in the lens can upregulate the TGF β RII expression in the corneal epithelial cells, give the cells the potential for enhanced TGF β 1-signaling, and result in an earlier onset of EMT in these cells than in the stromal keratocytes.

In this study, we demonstrated that TGF β 1-induced EMT in corneal endothelial and epithelial layers is associated with loss of N- and E-cadherin expression respectively. Our result supports the hypothesis proposed by Masur et al based on their cell culture model that myofibroblast differentiation requires at least two factors: action of TGF β and loss of cell contact [75]. In their in vitro system, the low-density culture had more myofibroblasts and active proliferating cells than the high-density culture. Interestingly, the same change in cell proliferation was also observed in our TGF β 1 transgenic mouse and was reported in the previous study [44]. It was shown that BrdU-labeled cells in the basal corneal epithelium were markedly increased in the P21 TGF β 1 transgenic mouse. In contrast to the in vitro cell culture model, it is unclear to us whether TGF β 1-induced EMT in the transgenic mouse cornea resulted in the formation of both fibroblasts and myofibroblasts. Cadherin-11 is a non-classical cadherin which was expressed in cultured corneal myofibroblasts [54]. We found that cadherin-11 expression was upregulated in the corneal epithelium and stroma in TGF β 1 transgenic mouse. Cadherin-11 is a marker for loosely connected and migratory mesenchymal cells. In human cancers, cadherin-11 expression is associated with the most aggressive and metastatic subsets of cancer cells [76; 77]. In our TGF β 1 transgenic mouse model, loss of E- and N-cadherin and increase of cadherin-11 expression in the cornea can potentially increase cell motility and promote the migration/invasion of transformed cells into the stromal layer. Our data raise the question: during corneal fibrosis, can EMT in corneal epithelial and EnMT in endothelial layer also contribute to the myofibroblasts in the corneal stroma? This hypothesis would challenge the original theory that myofibroblast formation during corneal wound healing is derived from mesenchymal cells, i.e. subconjunctival fibroblasts or keratocytes (corneal fibroblasts).

In summary, a threshold level of TGF β 1 appears to be critical for corneal development and homeostasis. As seen in the OVE920A transgenic mice and the TGF β 1 transgenic mice with a β B1-crystallin lens promoter [18;45], TGF β 1 at high levels acts as a mitogen to the corneal mesenchymal cells and prevents these cells from differentiating into the corneal endothelial cells and keratocytes. In the OVE853 and OVE917 transgenic mice that have lower TGF β 1 expression, TGF β 1 does not interfere with the normal differentiation of the corneal mesenchymal cells. As a result, the corneal endothelial layer is formed properly, as shown by the expression of N-cadherin in the inner corneal surface (Figure 7). However, after P7 the corneal endothelium of the OVE853 strain started to lose its integrity and some of the cells expressed α -SMA, a molecular marker of EMT.

The effect of TGF β 1 on the postnatal cornea could be of clinical relevance. During intraocular injury or post-operative wound healing, the level of TGF β 1 in the ocular media may rise. Our data suggest that such an increase can result in the loss of corneal endothelial cells through the EnMT process. For patients with relatively low corneal endothelial cell density, blocking the increase of TGF β activity in the aqueous humor after insult or surgery may help to preserve corneal endothelium and prevent corneal complications.

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Figure 1.

Corneal defects in OVE853 TGF β 1 transgenic mice. (A) Eyes from P14 OVE853 transgenic (Tg) and wild-type (Wt) mice. Arrow points to the cloudy corneal surface of the transgenic eye. (B and C) Fluorescein staining of mouse corneas. The wild-type cornea (B) did not retain the fluorescent dye, whereas the transgenic cornea (C) was fluorescein positive (marked with an asterisk).

Figure 2.

Corneal histology of wild-type and TGF β 1 transgenic mice. Wild-type (WT) and transgenic (TG) corneas were stained with H&E (A–D) and DAPI (E, F). WT corneas (A, C and E) had three distinctive cell layers at P7 and P14: the corneal epithelium (Epi), the stroma (Str) and the endothelium (End). At P7, the endothelial cells of the TG corneas began to detach from the inner surface (arrows in B). By P14, the endothelial layer was absent from the TG cornea (D and indicated by arrows in F). Additionally, the epithelial layer of the TG cornea was thinner and less differentiated (indicated by arrowheads in D) than the WT cornea (C). The stroma was thicker in the TG cornea, which is indicative of corneal edema. Scale bar represents 100 μ m.

Figure 3. PAS staining. In P21 WT cornea (A), the corneal endothelial cells synthesize a distinctive basement membrane structure, Descemet's membrane, shown in red purple color (pointed by arrows). In either hemizygous (B) or homozygous (C) transgenic mouse cornea, the Descemet's membrane was not apparent.

Figure 4.

Immunohistochemical staining for TGFβRI and TGFβRII in the wild-type mouse cornea at P7. Both TGFβRI (upper panel) and TGFβRII (lower panel) were expressed in the corneal epithelial (Epi), stroma (Str) and endothelial (End) cells, suggesting that all the corneal cells can directly respond to TGFβ1 stimulation. Scale bar represents 100 μm.

Figure 5.

Abnormal expression of α -SMA in TGF β 1 transgenic mouse cornea at postnatal day 16. Whole-mount immunofluorescent staining for α -SMA and images were analyzed by using confocal microscopy. α -SMA-expressing cells were present in both the corneal endothelial (A–C) and epithelial (D) layers of the TGF β 1 transgenic mouse. Cells with a myofibroblast shape and a greater level of α -SMA were found in the corneal endothelial layer (as indicated by the arrows in B and C).

Figure 6.

Increase of α SMA expression along with the progression of myofibroblast formation in the postnatal TGF β 1 transgenic cornea. α -SMA expression was not detected in the transgenic cornea at postnatal day (P) 0 (A). A few α -SMA-positive cells were found at P2 (B) and the number of α -SMA-expressing cells increased significantly between P6 to P30 (C-F). The myofibroblast-like morphology became visible in the transgenic cornea at P16 (D). By P30 almost all of the α SMA-positive cells in the transgenic cornea were transformed into myofibroblast phenotypes including irregular shape, and the presence of stress fibers.

Figure 7. Distribution patterns of α SMA-expressing cells in the cornea of the TGF β 1 transgenic mice. Confocal x-z images of whole-mount-stained corneas shown at different postnatal ages. At P7 (A) and P16 (B), most of the α SMA-expressing cells were localized in the corneal epithelial and endothelial layers. By P30 (C), strong α -SMA staining was distributed in all corneal cell layers. Cornea edema along with the enhanced expression of α -SMA progressed in the transgenic corneas from P7–P30.

Figure 8.

Decrease in cadherin expression in the cornea of the $TGF\beta 1$ transgenic mouse. At P0 (A–D), E-cadherin was expressed in the corneal epithelial cells (arrowheads) and N-cadherin was in the corneal endothelial cells (arrows). E-cadherin expression was also detected in the epithelial cells of the eyelid and lens, and N-cadherin in the lens epithelial cells. Similar E- and N-cadherin expression patterns were found for the wild-type and the $TGF\beta 1$ transgenic mice, suggesting that prenatal development of the cornea and lens were not affected in the OVE853 transgenic mice. At P7 (E–H), E- and N-cadherin expression decreased in the corneal epithelial (arrowhead in F) and endothelial layers (arrows in H), respectively, in the $TGF\beta 1$ transgenic mouse, a feature consistent with EnMT and EMT in the cornea of the transgenic mouse. Scale bar represents 100 μm .

Figure 9.

Cadherin-11 immunofluorescence in P21 mouse cornea. Cadherin-11 staining was found in the vesicular tissue of the iris (arrow in A). A low level of cadherin-11 expression was also detected in the WT corneal epithelial layer (A). The intensity of cadherin-11 staining was increased in the transgenic mouse cornea, particularly in the corneal epithelial layer (B). On the same section, cadherin-11 was also shown in the group of lens epithelial cells (arrow in B) which were positive for α SMA and had transformed into myofibroblasts.