Histol Histopathol (2009) 24: 1405-1416

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Temporal and spatial distribution of TGF-ß isoforms and signaling intermediates in corneal regenerative wound repair

Man-IL Huh[1], Yongmin Chang[2] and Jae-Chang Jung[1]

[1]Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu, Korea and [2]Department of Diagnostic Radiology, College of Medicine, Kyungpook National University, Daegu, Korea

Summary. The present study analyzed the temporal and spatial expression of TGF-ß isoforms and activated pSmad2 and p38MAPK during epithelial debridement wound repair, using chick cornea by immunohistochemistry. Normal corneas showed low-level TGF-ßs staining. Following wounding, TGF-ß1 expression was strong in the Bowman's layer (BL). TGF-ß3 expression was confined to basal cells in the regenerating and unwounded regions, and was not detected in migrating epithelial, stromal or endothelial cells. In addition, TGF-ß3 treatment stimulated the proliferation of cultured epithelial cells. Our present findings seem to suggest that the TGF-ß3 signal may be required for epithelial cell proliferation. TGF-ß2 expression was strong in migrating and proliferating epithelial cells, many active migrating fibroblasts at the wound edge, endothelial cells and Descemet's membrane (DM). Although both nuclear pSmad2 and p38MAPK staining was observed in many basal epithelial cells, pSmad2 positive cells were co-localized with PCNA positive cells. Therefore, it seems likely that the pSmad2 signal may affect epithelial cell proliferation in healing corneas. Both pSmad2 and p38MAPK expression were also observed in endothelial cells. Interestingly, many active fibroblasts over the whole stroma in early wound healing at day 2 expressed nuclear pSmad2, but little if any cytoplasmic p38MAPK. Collectively, temporal/spatial up-regulation and distribution of the three TGF-ß isoforms, as well as concerted activation of both Smad2 and p38MAPK, appears to be a key aspect of regenerative corneal wound healing in the chick.

Key words: Corneal wound healing, TGF-ßs, pSmad2, p38 MAPK

Offprint requests to: J.C. Jung, Ph.D. Developmental Biology Laboratory, Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea. e-mail: jcjung@mail.knu.ac.kr

Introduction

The three layers of the cornea are the outer squamous epithelium, inner endothelium and the central stroma, which contains quiescent stromal cells (keratocytes) embedded in a thick collagenous matrix. Upon epithelial debridement, epithelia at the margin of undamaged areas begin to migrate and can resurface the damaged area completely within several days, depending on the wound size. At the same time, keratocytes underlying the epithelial wound regions are programmed to die within several hours after injury (Mohan et al., 2000; Wilson et al., 2001). In contrast, keratocytes located at the wound edges of the stroma change from a quiescent to an active state and undergo mitosis, transforming into active fibroblasts evoking an altered fibroblast phenotype, and then migrate into the damaged area (Weimar, 1960; Moller-Pedersen et al., 1998a,b; Jester et al., 1999b; Mohan et al., 2000; Wilson et al., 2001; Zieske et al., 2001a; Stramer et al., 2003; Fini and Stramer, 2005a). These active fibroblasts induced by epithelial debridement eventually return to normal keratocytes.

Previous studies have shown that a variety of cytokines and growth factors are involved in corneal wound healing (Schultz et al., 1992; Andresen et al., 1997; Strissel et al., 1997; Andresen and Ehlers, 1998; Moller-Pedersen et al., 1998a; Jester et al., 1999b; Mohan et al., 2000; Wilson et al., 2001; Zieske et al., 2001a; Stramer et al., 2003; Fini and Stramer, 2005a). As demonstrated first in skin (Desmouliere et al., 1993), TGF-ß is a key modulator of the quality of healing in cornea (Jester et al., 1996; Myers et al., 1997; Moller-Pedersen et al., 1998b; Jester et al., 1999a,b). The cellular source of TGF-ß appears to be different in these two organs. TGF-ß is derived primarily from platelets in skin wounds (Assoian et al., 1983), but the cornea is

Abbreviations: TGF-ß, Transforming growth factor-ß; BL, Bowman's layer; DM, Descemet's membrane.

avascular and evidence suggests that an important source of TGF-ß in cornea is the regenerating epithelium (Strissel et al., 1995; Ivarsen et al., 2003; Stramer et al., 2003; Fini and Stramer, 2005b). Once stimulated by epithelial TGF-ß corneal stromal cells can then make their own TGF-ß in an autocrine cytokine loop, amplifying the response (Song et al., 2000).

The three TGF-ß isoforms identified in mammals are TGF-ß1, -ß2 and -ß3 (Cheifetz et al., 1990). Despite structural and functional similarities, they can exert distinct biological functions in vivo depending on the cell and tissue type, and in vitro depending on culture conditions, such as plating cell density and the presence of serum or other growth factors (Cheifetz et al., 1990; Jakowlew et al., 1992; Barcellos-Hoff, 1996; Koli et al., 2001). In general, the secreted form of TGF-ß is released into the extracellular milieu in its latent form, and is activated in response to tissue injury that also stimulates the synthesis and release of TGF-ß (Barcellos-Hoff, 1996; Koli et al., 2001). Activation of TGF-ß is the key event in initiating and mediating the response to tissue damage and the tissue repair process. Active TGF-ß binds to TGF-ß receptor type II (TßR-II), which then complexes with TGF-ß receptor type I (TßR-I). The activated TßR-I receptor phosphorylates Smad2 and Smad3, which then form heteromeric complexes with Smad4 in the cytoplasm. These complexes translocate to the nucleus and activate transcription of specific genes (Hata et al., 1998; Piek et al., 2001). Furthermore, TGF-ß can also activate p38MAPK signaling pathways which also influence the transcription of specific genes (Tsukada et al., 2005).

Studies of corneal wound healing in rats and mice have shown that both Smad and p38MAPK signaling pathways are involved in early corneal wound healing, particularly in regulation of corneal epithelial migration and proliferation (Ashcroft et al., 1999; Datto et al., 1999; Mohan et al., 2002; Saika, 2004; Saika et al., 2004; Hutcheon et al., 2005). However, data generated using these models may be of limited utility in comparisons with humans, since rat and mouse corneas do not have a Bowman's layer (BL). Like humans, the chicken cornea has a BL, suggesting it may be a better model for human cornea wound repair. Using the chick cornea, the present study analyzed the temporal and spatial expression of TGF-ß isoforms and Smad2 and p38MAPK activation during regenerative corneal repair over 2 weeks.

Materials and methods

Epithelial debridement procedures and paraffin embedding

Surgical procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A previously-characterized mouse epithelial debridement wound model (Stramer et al., 2003) was adapted for the chick in order to study regenerative repair. Two-month old chicks were anesthetized with an intramuscular injection of ketamine (25 mg/kg) and xylazine (10 mg/kg) before debridement. Briefly, the central corneal epithelium demarcated by trephine was debrided using an Alger brush within a 4 mm region, leaving the basement membrane intact. Corneal wound repair was examined by removing the corneas at 12 hours, 2 days, 7 days and 14 days, with 10 corneas examined at each time point. To remove and examine corneas, chicks were sacrificed using an overdose of ketamine and xylazine, and whole corneas were excised and fixed overnight in 4% paraformaldehyde at 4°C. Corneas were then dehydrated and trimmed, leaving some tissue around the healing wound, cleared with xylene and embedded in paraffin. Six m sections were prepared and kept until use.

Antibodies and reagents

Polyclonal antibodies against TGF-ß1, TGF-ß2, TGF-ß3 and p38MAPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal pSmad2 and proliferating cell nuclear antigen (PCNA) antibodies were from Cell signaling Technology (Danvers, MA), and the Alexa-Fluor 488-conjugated goat anti-mouse and rabbit IgG were from Molecular Probes (Eugene, OR). Propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were from Vector Laboratories (Burlingame, CA). Cell counting Kit-8 (CCK-8) was purchased from Dojindo laboratories (Kumamoto, Japan) for proliferation assay of corneal epithelial cells. F-12 medium, fetal calf serum (FCS), and 0.25% trypsin-EDTA were purchased from Gibco (Grand Island, NY). TGF-ß3 was purchased from R&D system (Minneapolis, MN).

Immunohistochemistry

Slides containing paraffin sections were deparaffinized in xylene and subsequently rehydrated using an ethanol concentration series. Sections were rinsed in 0.1 M tris-buffered saline (TBS; pH 7.4) and then permeabilized by incubating in 0.3% Triton X-100 in TBS at room temperature for 10 minutes. To block non-specific binding, sections were incubated in a blocking solution (5% normal goat serum and bovine serum albumin in TBS) at room temperature for 1 hour. Sections were then processed for indirect immuno-fluorescence localization using antibodies against TGF-ß1, TGF-ß2, TGF-ß3, pSmad2 and p38MAPK. For the negative control, sections were incubated with 5% normal goat serum rather than primary antibodies. Sections were washed with TBS and then incubated with the corresponding secondary antibodies conjugated with Alexa-Fluor 488 at room temperature for 1 hour.

Double-labeling immunofluorescence was performed by using the same tissue section with two primary antibodies (PCNA and pSmad2) of different species. After blocking nonspecific binding, the sections were incubated with the anti-pSmad2 antibody, and then Alex-Fluor 488 conjugated anti-rabbit IgG for 1 hour.

After washing the slides with TBS, the sections were incubated with anti-PCNA antibody and then TRITC-conjugated anti-mouse IgG for 1 hour. Prior to mounting, slides were washed three times with TBS, and all sections were counterstained with PI (1 µg/ml) or DAPI (1 µg/ml) for nuclei staining. Digital images were captured using a laser scanning confocal image system (MRC-1000; Bio-Rad) and Axiocam MRc10 (Carl Ziess, Germany).

Epithelial cell culture

Corneal cell culture was carried out according to the previously described protocol (Cai and Linsenmayer, 2001). In brief, 10 corneas dissected from 2-month old chicks were treated with 0.5% dispase in PBS for 1 hour at 4°C, and the epithelial layers were gently scraped away with a scalpel. The epithelia were rinsed with PBS and further digested in 0.25% trypsin-EDTA at 37°C for 5 min. Epithelial cells were grown in F-12 medium supplemented with heat-inactivated 10% FCS.

Cell proliferation assay

Equal numbers ($1x10^4$ cells/well) of sub-cultured corneal epithelial cells were plated on a 96-well culture plate in F-12 medium containing 10% FCS, and allowed to attach and spread. After 24 hours of culture, the cell layer was washed twice with PBS and the medium was replaced with a serum-free medium. To determine whether epithelial cell proliferation is regulated by TGF-ß3, cells were treated with TGF-ß3 (2 ng/ml) for 24 hours. Cell proliferation analysis was performed by using Cell Counting Kit-8 (CCK-8). After cultivation with TGF-ß3, CCK-8 solution (10 µl) was added to each well plate, and continued to culture for 4hours until the medium turned yellow. The absorbance was measured at 450 nm using a microplate reader.

Statistical analysis

The PCNA-positive and total number of epithelial cells were counted in ten fields selected through each 66 µm epithelium length, starting from the migrating-edge to wound edge in the midperiphery. Quantitative data were presented as mean ± SE. Difference was analyzed with student t-test using Microsoft Excel. $P<0.05$ was considered statistically significant.

Results

Temporal and spatial expression of TGF-ß1, TGF-ß2 and TGF-ß3 in the healing cornea

Secreted and matrix-associated TGF-ß has been detected transiently after injury in vivo (Trinkaus-Randall and Nugent, 1998), suggesting that it plays a role in regulating cell proliferation and migration, matrix deposition, interaction of stromal cells with the ECM, and tissue remodeling (Song et al., 2000). Epithelial debridement causes up-regulation of TGF-ß receptor expression in migrating corneal epithelial cells (Zieske et al., 2001b). We therefore examined TGF-ß isoform expression during chick cornea wound healing using immunohistochemistry. In normal corneas, TGF-ß1 was detected at low levels only in the BL (Fig. 1A). In the healing cornea at 12 hours after wounding (Fig. 1B), TGF-ß1 was detected at high levels extracellularly along the BL in the unwounded region. The level of expression gradually decreased along the BL wound region, and was very weak in the BL at the central epithelial defect area. The BL was clearly visible using hematoxylin and eosin staining (Fig. 1B; Inset). There was weak cytoplasmic TGF-ß1 staining in stromal cells at the wound edge and at the posterior region of the wound stroma. Debridement of the corneal epithelium from its basement membrane causes underlying stromal cells to undergo apoptosis (Helena et al., 1998). Interestingly, weak TGF-ß1 staining was also observed in the cytoplasm of many endothelial cells, regardless of the wound region. After wound closure at day 2 (Fig. 1C), there was strong TGF-ß1 staining clearly present in the BL at the central wound region, and comparisons with the staining shown in Fig. 1B suggest the source of TGF-ß1 was the resurfacing epithelium. While weak cytoplasmic staining was observed in some basal cells, strong staining was observed in many endothelial cells. In the stroma, weak TGF-ß1 staining was observed in the active fibroblasts of the wound region, except in the anterior region due to lack of stromal cells. Both cytoplasmic and nuclear TGF-ß1 staining was observed in endothelial cells (Fig. 1F; higher magnification of arrowhead in Fig. 1C). By day 7 (Fig. 1D), the anterior stroma region had been covered by active fibroblasts. Although TGF-ß1 was detected in the BL, the levels had decreased dramatically, especially in the BL at the wound region. By day 14 (Fig. 1E), while only relatively weak TGF-ß1 staining was observed along the BL, this level was higher than that observed in the normal cornea. Interestingly, cytoplasmic TGF-ß1 staining was detected in many basal and endothelial cells. Negative control using the healing cornea at 12 hours after wounding failed to show TGF-ß1 staining (Fig. 1G).

In normal corneas, cytoplasmic TGF-ß2 staining was prominent in most basal cells, and was also observed in some endothelial cells (Fig. 2A). In the healing corneal wound at 12 hours (Fig. 2B), TGF-ß2 staining was strongest in the epithelial cells migrating into the wound region as well as the basal and wing cells in the unwounded region. Following wounding, most endothelial cells expressed TGF-ß2 continuously throughout the healing period up to day 14. In the stroma, a high level of TGF-ß2 staining was detected, especially in active fibroblasts at the wound edge. Active fibroblasts migrating into the central wound region also expressed TGF-ß2. By day 2 (Fig. 2C), strong TGF-2 staining was clearly present in the basal and wing epithelial cells, as well as endothelial cells. High levels of secreted TGF-ß2, presumably from endothelial cells, were observed in the Decemet's membrane (DM) up to

day 14. By day 7 (Fig. 2D), TGF-ß2 staining in endothelial cells had slightly decreased, and the strongest signal throughout the healing period was observed in both basal and wing cells. By day 14 (Fig. 2E), TGF-ß2 staining in the basal and wing cells had decreased dramatically, and was virtually absent in stromal cells. Negative control failed to show TGF-ß2 staining (Fig. 2F).

In normal corneas, TGF-ß3 staining was observed at low levels only in the cytoplasm of basal cells (Fig. 3A). At 12 hours after wounding (Fig. 3B), strong TGF-ß3 staining was observed only in the basal cells of the unwounded region, and not in epithelial cells migrating into the wound region. By days 2 and 7 (Fig. 3C,D), weak TGF-ß3 staining was observed only in the basal cells of the regenerating epithelium. In contrast, at day 14, strong TGF-ß3 staining was again observed in the cytoplasm of basal cells (Fig. 3E). Unlike TGF-ß1 and TGF-ß2 staining, TGF-ß3 staining was not observed in either stromal or endothelial cells throughout healing.

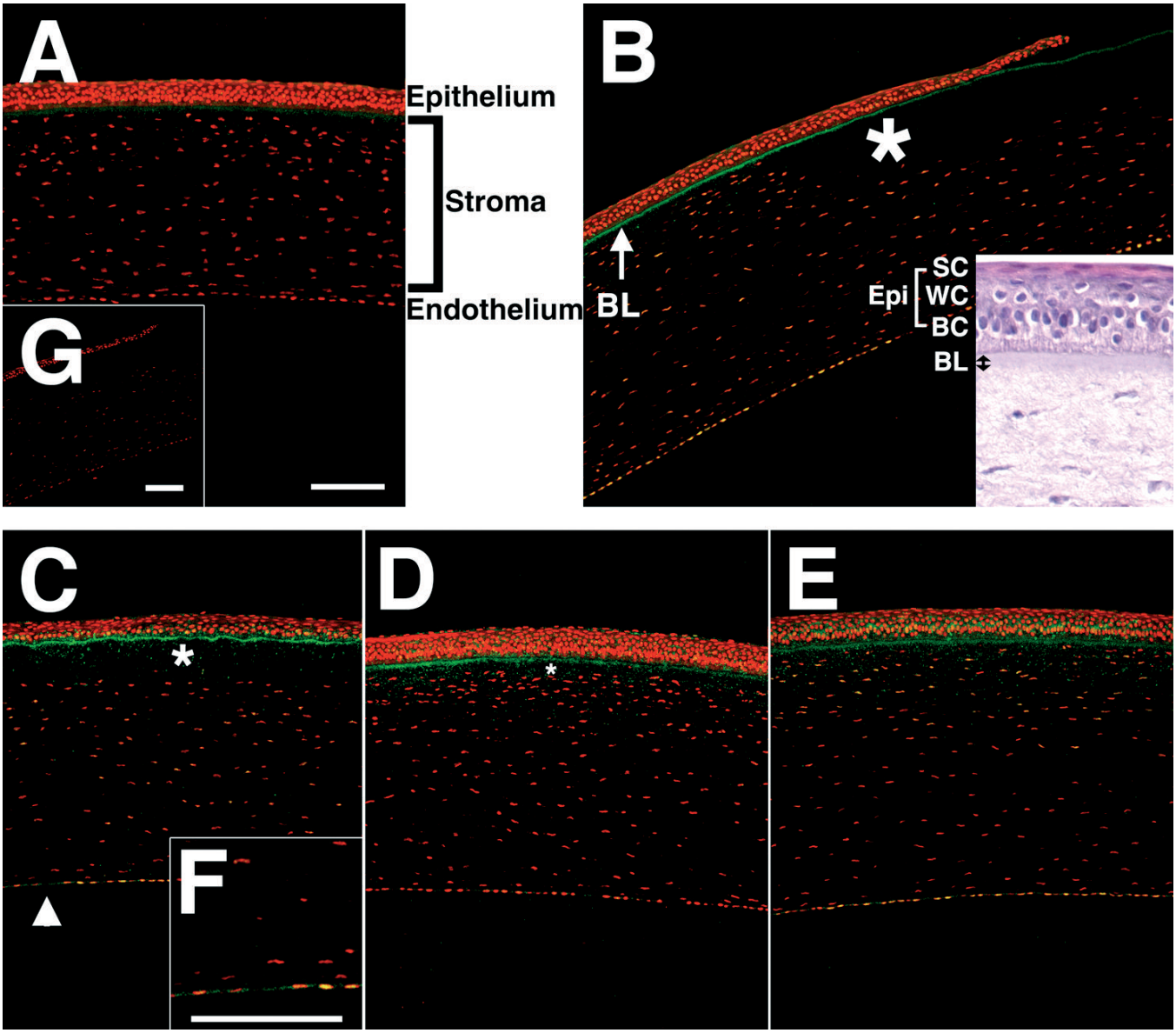

Fig. 1. Immunolocalization of TGF-ß1 in healing chick corneas after epithelial debridement wounding. **A.** TGF-ß1 expression in a normal cornea. **B.** TGF-ß1 expression at 12 hours after wounding. Note staining along the Bowman's layer (BL) of the unwounded region. Inset shows the BL using hematoxylin & eosin staining. (*) indicates the prominent acellular zone in the anterior stroma. **C.** TGF-ß1 expression at day 2 after wounding (after wound closure). **F.** Higher magnification of arrowhead in C. **D.** TGF-ß1 expression at day 7. (*) indicates the acellular anterior stroma. **G.** Negative control using the healing cornea at 12 hours after wounding. **E.** TGF-ß1 expression at day 14. Sections were counterstained for nuclei (red). Scale bars: 100 µm.

Negative control failed to show TGF-ß3 staining (Fig. 3F).

Temporal and spatial p38MAPK activation in the healing cornea

Activated p38MAPK is reported to be involved in epithelial cell migration and inhibition of corneal epithelial cell proliferation during the early phase of wound healing in corneas (Saika et al., 2004). Inhibition of p38MAPK induces phosphorylation of ERK1/2 mediating cell proliferation(Saika et al., 2004). We examined p38MAPK expression in healing chick corneas. In normal corneas, p38MAPK staining was observed in the cytoplasm of some epithelial cells, but not in either stromal or endothelial cells (Fig. 4A). Similar to a previous report (Saika et al., 2004), both strong cytoplasmic (green) and nuclear (yellow) p38MAPK staining was also observed in the many regenerating and migrating epithelial cells at 12 hours after wounding (Fig. 4B). Very few migrating epithelial cells at the wound edge showed cytoplasmic p38MAPK staining at 12 hours (Fig. 4C). There was very low p38MAPK expression in a small number of stromal cells, but strong expression in many endothelial cells regardless of their location in the wound region. Once

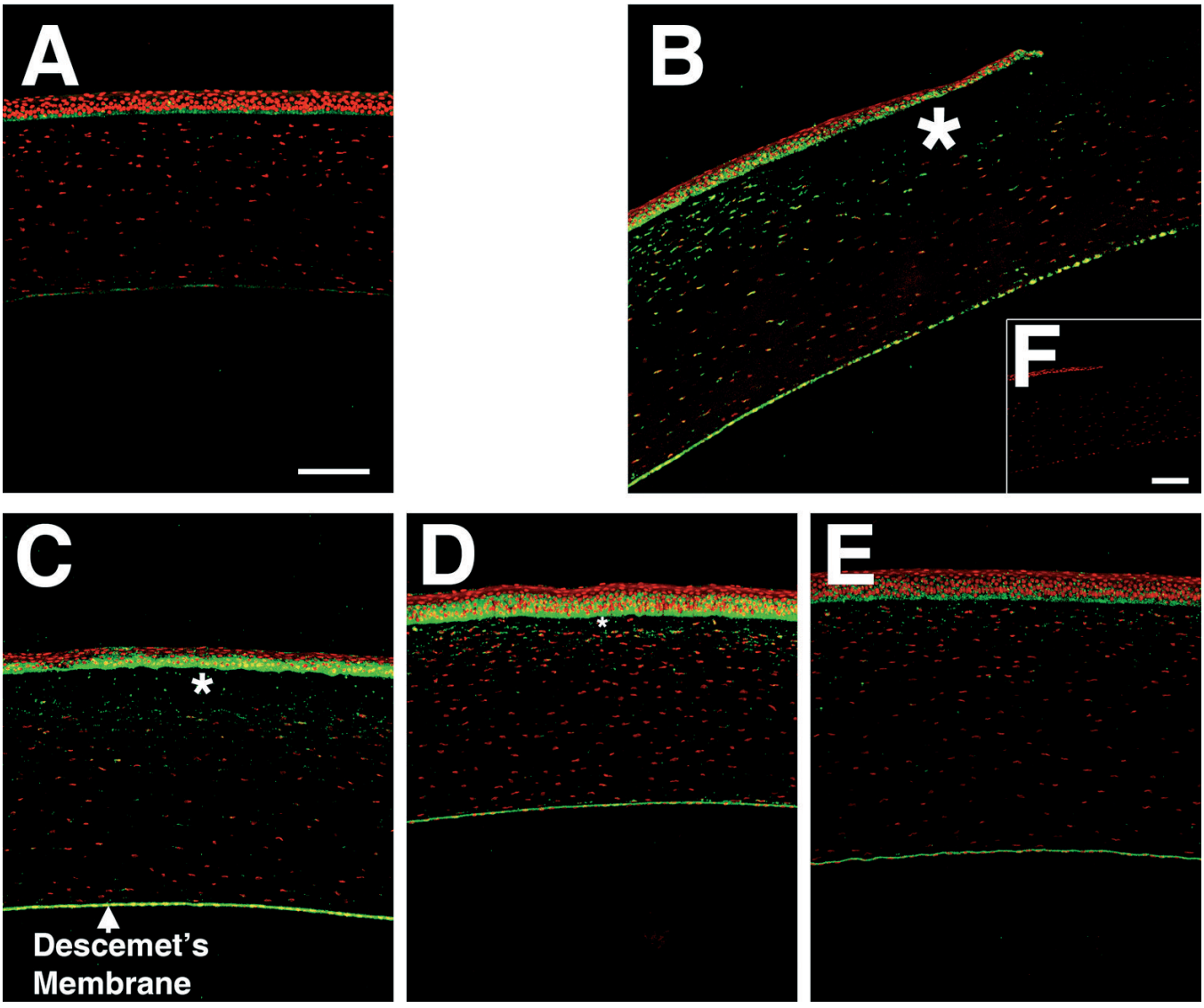

Fig. 2. Immunolocalization of TGF-ß2 in chick corneas after epithelial debridement wounding. **A.** TGF-ß2 expression in a normal cornea. **B.** TGF-ß2 expression at 12 hours after wounding. **C.** TGF-ß2 expression at day 2 after wounding. (*) indicates the acellular anterior stroma. **D.** TGF-ß2 expression at day 7 after wounding. **E.** TGF-ß2 expression at day 14 after wounding. **F.** Negative control at 12 hours after healing cornea. Sections were counterstained for nuclei (red). Scale bars: 100 µm.

the defect area had resurfaced completely by day 2 (Fig. 4D), high level nuclear and cytoplasmic p38MAPK staining was observed in many basal epithelial cells and most endothelial cells, but in few stromal cells. Both cytoplasmic and nuclear p38MAPK staining was observed in few stromal cells (Fig. 4G; higher magnification of arrowhead in Fig. 4D). By day 7 (Fig. 4E), there was no nuclear p38MAPK staining in the epithelium, and while cytoplasmic staining was detected in most basal and wing cells, the levels were far lower than those observed at day 2. Interestingly, weak cytoplasmic p38MAPK staining was detected in a small number of cells in the anterior region of stroma, but not in any endothelial cells. Negative control failed to show p38MAPK staining (Fig. 4H). Although we do not understand why, by day 14 (Fig. 4F), strong diffuse cytoplasmic p38MAPK staining was again observed in most basal and wing cells, and nuclear p38MAPK staining was observed in many basal cells. A small number of the anterior region stromal cells showed faint cytoplasmic p38MAPK staining. Both cytoplasmic and nuclear p38MAPK staining was observed to re-appear in many endothelial cells.

Temporal and spatial pSmad2 activation in the healing cornea

The temporal and spatial expression of active pSmad2 during wound repair was examined. Although both Smad2 and Smad3 are major TGF-ß signaling

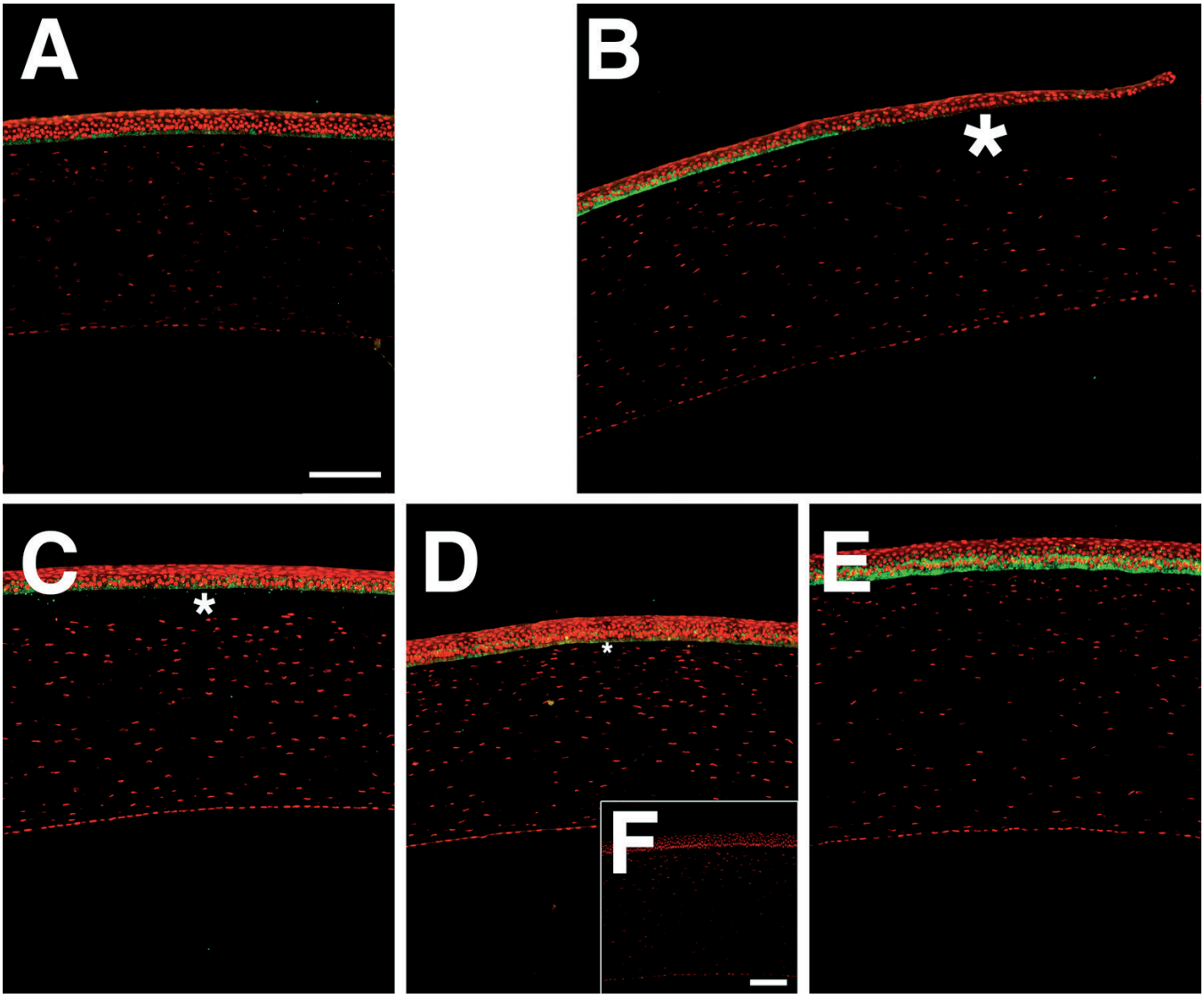

Fig. 3. Immunolocalization of TGF-ß3 in chick corneas after epithelial debridement wounding. **A.** TGF-ß3 expression in a normal cornea. **B.** TGF-ß3 expression at 12 hours after wounding. TGF-ß3 expression at day 2 **(C)**, day 7 **(D)** and day 14 **(E)** after wounding. **F.** Negative control at 7 days after healing cornea. Sections were counterstained for nuclei (red). Scale bars: 100 µm.

mediators (Derynck and Zhang, 2003), there is limited availability of antibodies against chick pSmad3. Therefore, the present study focused on the nuclear expression of activated Smad2 using an anti-phospho-Smad2 antibody (shown as a yellow color in merged images).

In normal corneas, pSmad2 staining was observed in some basal epithelial cells, stromal cells and endothelial cells (Fig. 5A). At 12 hours after wounding, strong nuclear pSmad2 staining was observed in the many regenerating and migrating epithelial cells (Fig. 5B). However, very few migrating epithelial cells at the wound edge expressed nuclear pSmad2 (Fig. 5C). Nuclear pSmad2 staining was observed in the many active fibroblasts located immediately underneath the regenerating epithelial cells, but not in the acellular region of the anterior stroma (Fig. 5B). The migrating epithelial cells adjacent to the central defect area do not proliferate during the early phase of wound healing (Saika et al., 2004). Once the defect area is resurfaced, the epithelial cells begin to proliferate and stratify by day 2 (Saika et al., 2004). Strong nuclear pSmad2

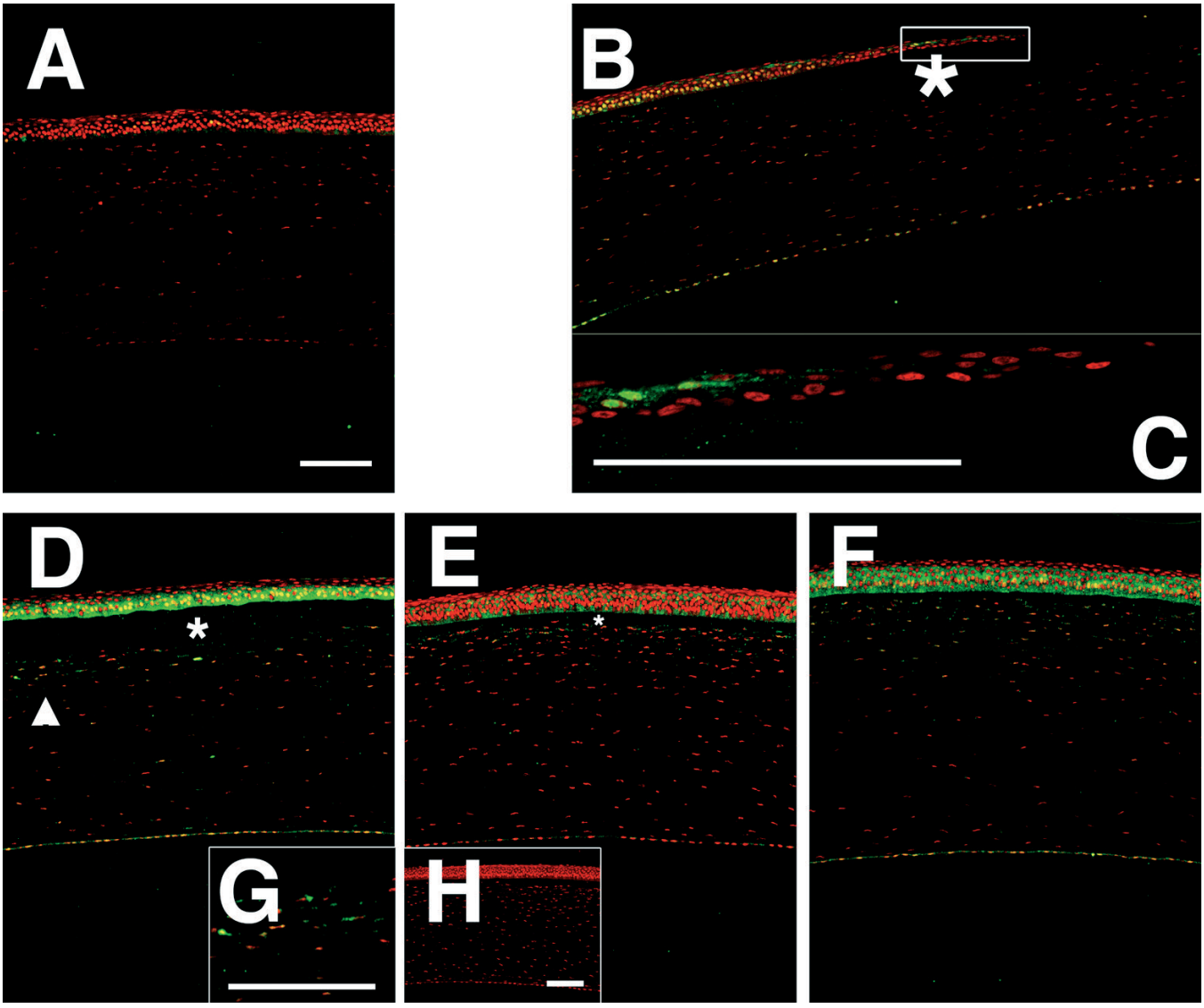

Fig. 4. Immunofluorescence detection of p38MAPK nuclear translocation in chick cornea after epithelial debridement wounding. **A.** p38MAPK expression in a normal cornea. **B.** Cytoplasmic (green) and nuclear (yellow) p38MAPK expression in the wound region at 12 hours after wounding. **C.** Cytoplasmic (green) and nuclear (yellow) p38MAPK expression at the wound edge at 12 hours after wounding. **D.** Cytoplasmic (green) and nuclear (yellow) p38MAPK expression at day 2 after wounding. (*) indicates stromal cells just below the acellular region. **G.** Higher magnification of arrowhead in D. **E.** Cytoplasmic (green) and nuclear (yellow) p38MAPK expression at day 7 after wounding. **H.** Negative control at 7 days after healing cornea. **F.** Cytoplasmic (green) and nuclear (yellow) p38MAPK expression at day 14 after wounding. Sections were counterstained for nuclei (red). Scale bars: 100 μm.

staining was observed in many basal cells by day 2 (Fig. 5D), and persisted up to day 14 (Fig. 5E,F). By day 2, nuclear pSmad2 staining was observed in many active fibroblasts over the whole stroma, except for the acellular stroma anterior region (Fig. 5D). By day 7, strong pSmad2 staining was confined to the active and migratory fibroblasts in the stroma anterior region (Fig. 5E). By day 14, the pSmad2 staining pattern was similar to that at day 7, although somewhat weaker overall (Fig. 5F). In the endothelium, nuclear pSamd2 staining was observed at low levels in only a few endothelial cells at 12 hours (Fig. 5B). In contrast, strong nuclear pSmad2 staining was detected in most endothelial cells at day 2 (Fig. 5D). By days 7 and 14, strong staining was detected in many, but not all cells.

TGF-3ß stimulates corneal epithelial cell proliferation

TGF-ßs have been implicated in inhibition of the epithelial cell proliferation in many organs and cell types (Zhu and Burgess, 2001; Derynck and Zhang, 2003; Massague, 2003). In cornea wound healing, although TGF-ß1 and 2 have been implicated in mediating inhibition of the epithelial cell proliferation induced by EGF, KGF, and HGF (Honma et al., 1997), there is no study for TGF-ß3 mediating cell proliferation. Therefore, we examined whether TGF-ß3 affects in vitro cultured corneal epithelial cell proliferation. Compared to control cultures, epithelial cell proliferation was stimulated by TGF-ß3 (2 ng/ml) treatment (Fig. 6). Such increased cell proliferation, stimulated by TGF-ß3, was correlated with the expression patterns of TGF-ß3 observed only in the basal cells of the regenerating epithelium (Fig. 2).

Co-localization of pSmad2 and PCNA in proliferating corneal epithelial cells

To examine whether Smad2 is activated in proliferating epithelial cells at 1day after wounding, we determined the nuclear distribution of pSmad2 and PCNA, known as the marker of cell proliferation, by

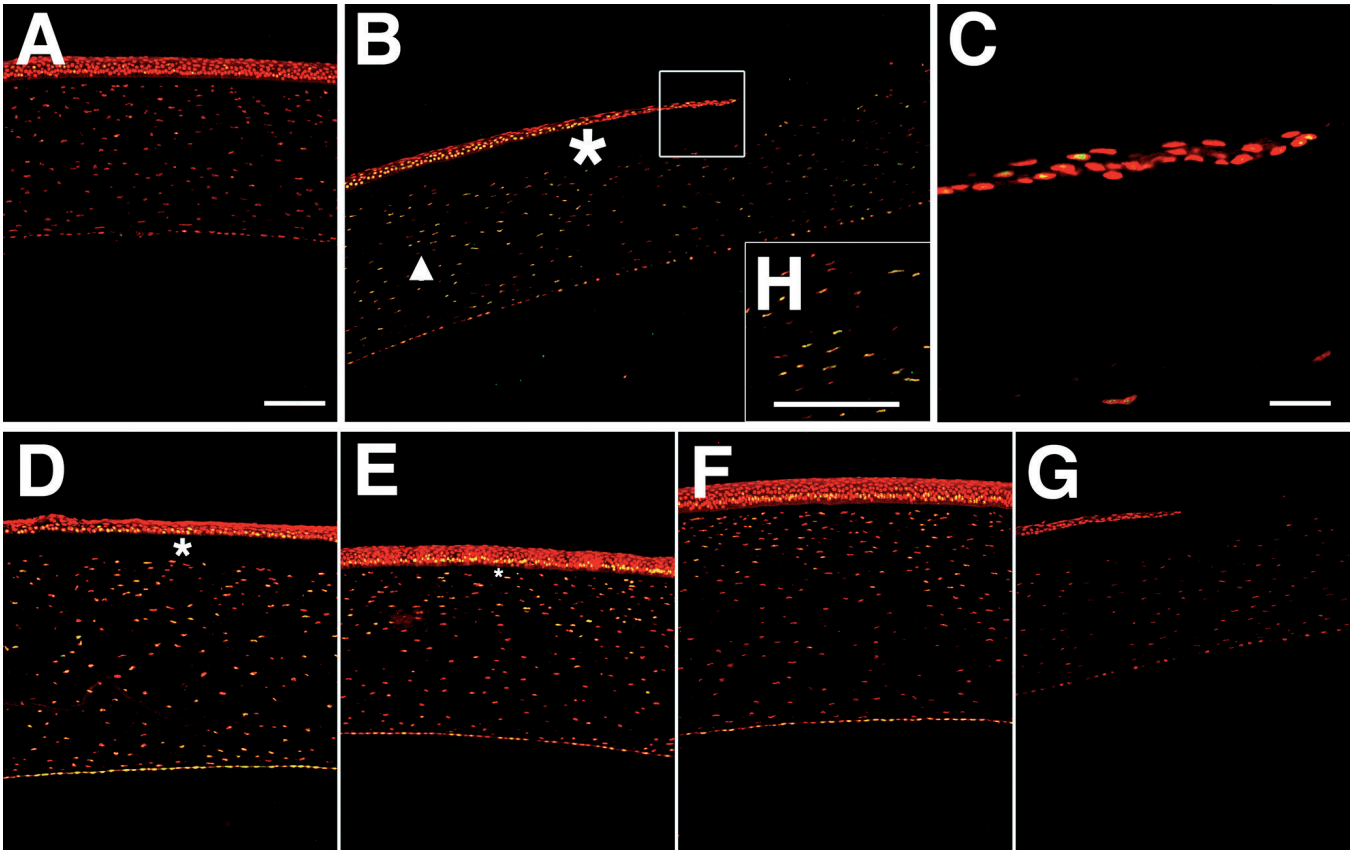

Fig. 5. Immunofluorescence detection of nuclear pSmad2 expression (shown in yellow) in chick corneas after epithelial debridement wounding. **A.** pSmad2 expression in a normal cornea. **B.** pSmad2 expression at 12 hours after wounding in epithelial and endothelial cells in the wound region. **C.** pSmad2 expression at 12 hours after wounding in active fibroblasts located just beneath regenerating epithelial cells. (*) indicates the acellular zone of the anterior stroma. **D.** pSmad2 expression at day 2 in basal and endothelial cells. (*) indicates the anterior region of the acellular stroma. **E.** pSmad2 expression at day 7. **F.** pSmad2 expression at day 14. **G.** Staining in 12 hour wound samples in which no primary antibody was used (negative control). Sections were counterstained for nuclei (red). Scale bars: 100 µm.

double-labeling immunofluorecence microscopy (Fig. 7A). DAPI staining showed total numbers of epithelial cells in the healing corneas (Fig. 7Ac,Ag). Few epithelial cells were observed at the leading edge, but many in the wound margin of the regenerated epithelium (Fig. 7A,B). Higher magnifications of the wound margin (asterisks) of Aa, Ab, and Ac were Ae, Af, and Ag, respectively. Importantly, strong PCNA-positive cells in the nucleus (Ab and Af; arrowheads) were also stained with pSmad2 (Aa and Ae; arrowheads). Although both proliferating and pSamd2 positive cells were nearly detected in the leading edge of the migrating epithelium (arrows), many proliferating and pSmad2 positive cells were co-localized in the basal epithelial cells (arrowheads) of the wound margin (Fig. 7A). Negative controls incubated without the primary antibodies failed to show staining (Fig. 7Ad,Ah).

Discussion

The basement membrane binds particular growth factors (Soubrane et al., 1990, Kim et al., 1999), suggesting that it acts as a barrier to pro-fibrotic substances from the epithelium or tear fluid (Zieske et al., 1994, 2001b, Ivarsen et al., 2003, Stramer et al., 2003). Although we observed high levels of TGF-ß1 in the BL of healing corneas, weak TGF-ß1 staining was detected in fibroblasts during early healing (Fig. 1), suggesting that the integrity of the chick BL may also have a barrier function for TGF-ß1 release into the stroma in the regenerative differentiation program. Interestingly, TGF-ß3 expression was observed in basal epithelial cells in the regenerating and unwounded regions, but not in migrating epithelial cells, stromal cells or endothelial cells (Fig. 3). In addition, TGF-ß3 stimulated epithelial cell proliferation (Fig. 6), suggesting that TGF-ß3 may play a key role in epithelial cell proliferation. Compared to TGF-ß1 and TGF-ß3, high levels of TGF-ß2 were observed in migrating and proliferating epithelial cells, active fibroblasts and endothelial cells (Fig. 2). Others report that levels of both TGF-ß receptors, TR-ßI and -ßII, are elevated in non-proliferating corneal epithelial cells migrating to cover the wound area (Zieske et al., 2001b), suggesting that high levels of TGF-ß2 in the migrating epithelial cells (Fig. 2B) bind to both elevated TGF-ß receptors. In the regenerative pathway, the anterior stroma wound region just below the epithelium is regenerated by migration of active fibroblasts from the wound edges with no obvious hypercellularity, and these fibroblasts return to being quiescent keratocytes after wound repair (Wilson et al., 1992; Fini, 1999). Thus, it seems likely in the stroma that autocrine and paracrine TGF-ß2 stimulation may transform keratocytes into fibroblasts (Fig. 2B), and that in the absence of TGF-ß2 after wound repair these fibroblasts return to being keratocytes. Overall, the observations suggest that TGF-ß2 exerts multiple effects and plays crucial roles in regenerative repair.

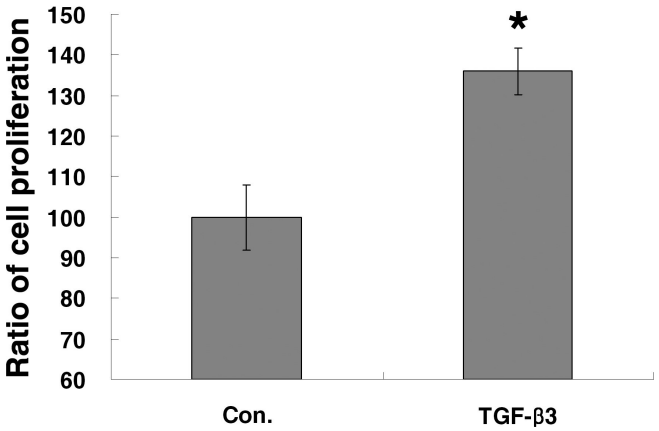

Fig. 6. Stimulation of TGF-ß3 on corneal epithelial cell proliferation. Sub-cultured corneal epithelial cells were treated with TGF-ß3 (2 ng/ml) for 24 hours, and cell proliferation analysis was performed. Compared to control cultures (Con), epithelial cell proliferation was stimulated by TGF-ß3 (2 ng/ml) treatment (136.1±5.8 %). The data are means ± SE of values from 4 independent experiments. * p<0.01 versus corresponding value for controls.

TGF-ß can activate multiple signaling cascades involving ERK, JNK, p38MAPK and Smads (Derynck and Zhang, 2003). Members of the MAPK family, including Extracellular signal-regulated kinase (ERK), c-Jun NH-terminal kinase (JNK), and p38MAPK have been implicated in a wide variety of wound healing processes, such as corneal epithelial migration and corneal epithelial cell proliferation (Saika et al., 2004; Kimura et al., 2008). JNK regulates epithelial cell migration by modulating the phosphorylation of paxillin and the consequent formation of focal adhesion (Kimura et al., 2008). Previous studies have found that TGF-ß activated the p38MAPK pathway, rather than the Smad pathway, in migrating epithelial cells in epithelial debridement wounds (Saika, 2004; Hutcheon et al., 2005). Furthermore, inhibition of the p38MAPK pathway slowed epithelial cell migration in organ-cultured cornea after debridement wounding (Saika et al., 2004). In contrast to previous reports, the present study found that both pSmad2 and p38MAPK pathways appeared to be involved in epithelial cell migration. However, it is possible that neither pathway is essential for migration, since very few migrating epithelial cells at the wound edge expressed cytoplasmic p38MAPK and nuclear pSmad2 (Figs. 4C, 5C). Although we observed nuclear p38MAPK expression in many basal epithelial cells (Fig. 4B-F), not all p38MAPK positive cells were co-localized with PCNA positive cells (data not shown). Therefore, it seems likely that the pSmad2 pathway is required for epithelial cell proliferation in healing corneas due to co-localization of pSmad2 and PCNA (Fig. 7). It was previously reported that nuclear Smad2 staining was merely observed in the healing epithelium up to 48 hours after debridement wounding in rats (Hutcheon et al., 2005). Those authors suggested that the presence or absence of a basement membrane plays a

role in Smad translocation into the nucleus (Hutcheon et al., 2005), and it appears likely that the differences in pSmad2 staining patterns between the studies relate to the different species used, although there is no clear understanding of these phenomena at present.

A previous in vitro study showed that exogenous TGF-ß3 expression increased the levels of pSmad2, but not Smad2 (Shiomi et al., 2006). In addition, although the endogenous Smad2 level was unchanged in epithelial cells isolated from TGF-ß3 homozygous null mutant mice, pSmad2 was not found (Cui et al., 2003). In the present study, there were similar staining patterns for TGF-ß3, pSmad2 and p38MAPK in healing epithelium (Figs. 3-5). Although TGF-ß2 was strongly expressed in most migrating and proliferating epithelial cells (Fig. 2), it is intriguing that very few migrating epithelial cells at the leading edge expressed cytoplasmic p38MAPK or nuclear pSmad2 (Figs. 4C, 5C). The wound healing process is highly regulated, especially in terms of growth factor release from healing epithelial cells (Wilson et al.,

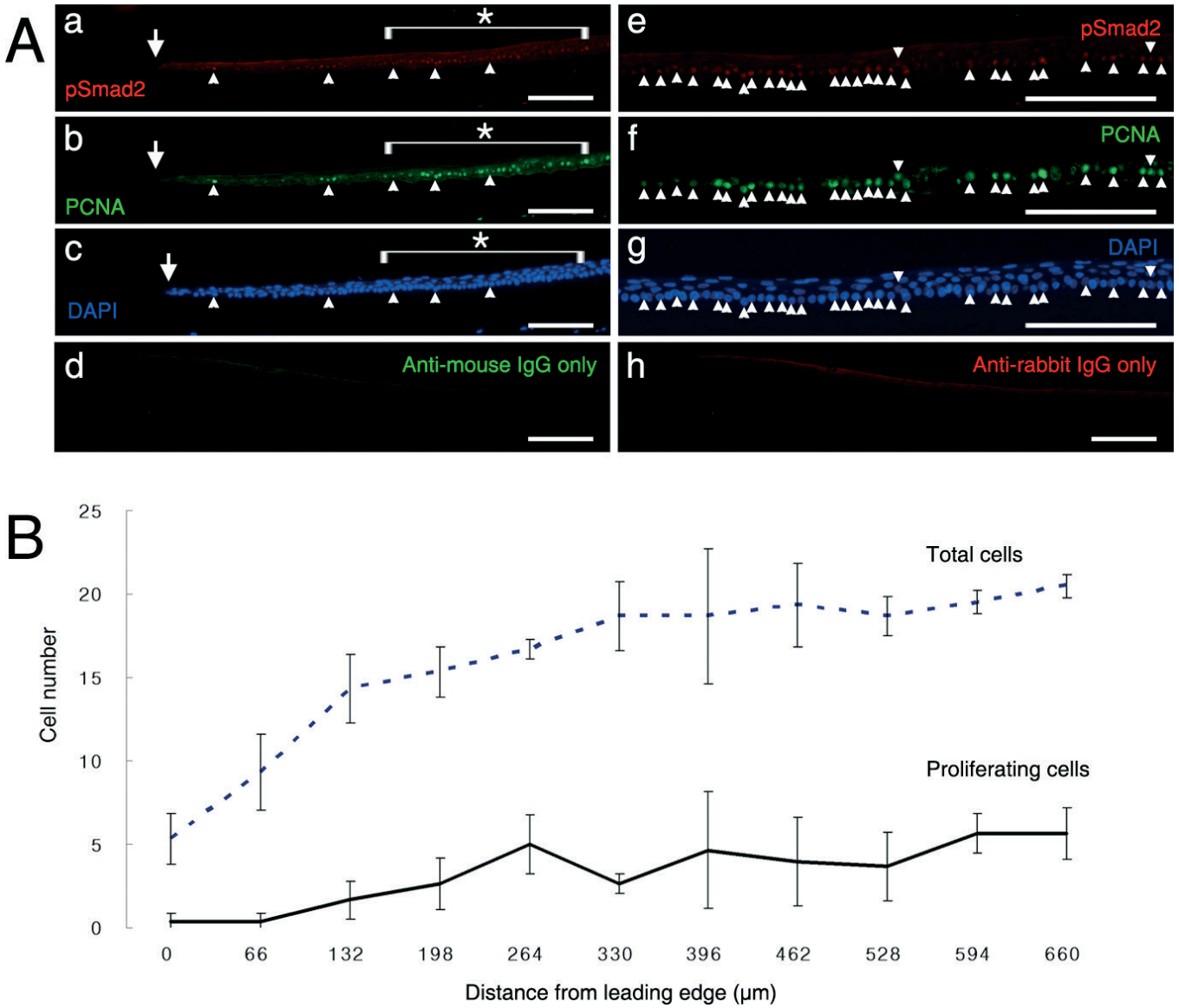

Fig. 7. Similar distribution patterns of pSmad2 with PCNA in proliferating epithelial cells at 1 day after healing corneas. **A.** Double-labeling immunofluorecence microscopy. Many PCNA positive proliferating cells **(b)** were immunostained with pSmad2 **(a)** in the wound margin of basal epithelial cells (arrowheads). DAPI staining showed total numbers of epithelial cells (**c** and **g**). Higher magnifications of the wound margin (asterisks) of **a, b,** and **c** were **e, f,** and **g**, respectively. White arrows indicate the leading edge of the migrating epithelium. Negative controls **(d** and **h). B.** Statistical analysis. Proliferating and total cell numbers in the healing corneas were counted in ten fields through each 66 μm (200 pixels) length, starting from the leading edge to the wound margin of epithelia. Data are means ± SE of values from 4 independent experiments. Scale bars: 100 μm.

1992, 1999). Thus, we suspect that migrating and proliferating epithelial cells at different locations receive multiple cross-talking signals (Massague, 2003; Sharma et al., 2003; Hutcheon et al., 2005), and that their behavior is influenced by growth factors in addition to TGF-ß2, and that different signal transduction pathways may be induced in these two different cell populations.

In the healing stroma, one of our most striking observations was that nuclear pSmad2 staining was detected in many active fibroblasts during the period of migration into the wound region between 12 hours and day 2 after wounding, but not in keratocytes (Fig. 5). Earlier immunostaining data for Mac1, a marker of both monocytes and polymorphonuclear cells, showed that infiltrating inflammatory cells into the anterior stroma shortly after wound closure (Mohan et al., 2002). Unfortunately, we could not evaluate the role of inflammatory cells. However, we assume that little if any cytoplasmic p38MAPK positive cells in the anterior stroma are inflammatory cells. By day 7 after wounding, the number of fibroblasts with nuclear pSmad2 staining, confined to the anterior stroma, gradually diminished. Taken together, these data suggest that stromal cell transformation into fibroblasts is mediated in part by activation of pSmad2. In summary, the repair of chick corneal epithelial injury involved specific temporal and spatial expression of all three TGF-, isoforms, activation of Smad2 and p38MAPK, and translocation of pSmad2 and p38MAPK. These results suggest that spatial Smad2 and p38MAPK activation play critical roles in regenerative repair, and that those roles may differ depending on the cell types.

Acknowledgements. This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea. (A080763).

References

Andresen J.L. and Ehlers N. (1998). Chemotaxis of human keratocytes is increased by platelet-derived growth factor-BB, epidermal growth factor, transforming growth factor-alpha, acidic fibroblast growth factor, insulin-like growth factor-I, and transforming growth factor-beta. Curr. Eye Res. 17, 79-87.

Andresen J.L., Ledet T. and Ehlers N. (1997). Keratocyte migration and peptide growth factors: the effect of PDGF, bFGF, EGF, IGF-I, aFGF and TGF-beta on human keratocyte migration in a collagen gel. Curr. Eye Res. 16, 605-613.

Ashcroft G.S., Yang X., Glick A.B., Weinstein M., Letterio J.L., Mizel D.E., Anzano M., Greenwell-Wild T., Wahl S.M., Deng C. and Roberts A.B. (1999). Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat. Cell Biol. 1, 260-266.

Assoian R.K., Komoriya A., Meyers C.A., Miller D.M. and Sporn M.B. (1983). Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J. Biol. Chem. 258, 7155-7160.

Barcellos-Hoff M.H. (1996). Latency and activation in the control of TGF-beta. J. Mammary Gland Biol. Neoplasia 1, 353-363.

Cai C.X. and Linsenmayer T.F. (2001). Nuclear translocation of ferritin in corneal epithelial cells. J. Cell Sci. 114, 2327-2334.

Cheifetz S., Hernandez H., Laiho M., ten Dijke P., Iwata K.K. and Massague J. (1990). Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. J. Biol. Chem. 265, 20533-20538.

Cui X.M., Chai Y., Chen J., Yamamoto T., Ito Y., Bringas P. and Shuler C.F. (2003). TGF-beta3-dependent SMAD2 phosphorylation and inhibition of MEE proliferation during palatal fusion. Dev. Dyn. 227, 387-394.

Datto M.B., Frederick J.P., Pan L., Borton A.J., Zhuang Y. and Wang X.F. (1999). Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol. Cell Biol. 19, 2495-2504.

Derynck R. and Zhang Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425, 577-584.

Desmouliere A., Geinoz A., Gabbiani F. and Gabbiani G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J. Cell Biol. 122, 103-111.

Fini M.E. (1999). Keratocyte and fibroblast phenotypes in the repairing cornea. Prog. Retin. Eye Res. 18, 529-551.

Fini M.E. and Stramer B.M. (2005a). How the cornea heals: cornea-specific repair mechanisms affecting surgical outcomes. Cornea 24, S2-S11.

Fini M.E. and Stramer B.M. (2005b). How the cornea heals: Cornea-specific repair mechanisms affecting surgical outcomes. Cornea 24, S2-S11.

Hata A., Shi Y. and Massague J. (1998). TGF-beta signaling and cancer: structural and functional consequences of mutations in Smads. Mol. Med. Today 4, 257-262.

Helena M.C., Baerveldt F., Kim W.J. and Wilson S.E. (1998). Keratocyte apoptosis after corneal surgery. Invest. Ophthalmol Vis. Sci. 39, 276-283.

Honma Y., Nishida K., Sotozono C. and Kinoshita S. (1997). Effect of transforming growth factor-beta1 and -beta2 on in vitro rabbit corneal epithelial cell proliferation promoted by epidermal growth factor, keratinocyte growth factor, or hepatocyte growth factor. Exp. Eye Res. 65, 391-396.

Hutcheon A.E., Guo X.Q., Stepp M.A., Simon K.J., Weinreb P.H., Violette S.M. and Zieske J.D. (2005). Effect of wound type on Smad 2 and 4 translocation. Invest. Ophthalmol. Vis. Sci. 46, 2362-2368.

Ivarsen A., Laurberg T. and Moller-Pedersen T. (2003). Characterisation of corneal fibrotic wound repair at the LASIK flap margin. Br. J. Ophthalmol. 87, 1272-1278.

Jakowlew S.B., Cubert J., Danielpour D., Sporn M.B. and Roberts A.B. (1992). Differential regulation of the expression of transforming growth factor-beta mRNAs by growth factors and retinoic acid in chicken embryo chondrocytes, myocytes, and fibroblasts. J. Cell Physiol. 150, 377-385.

Jester J.V., Barry-Lane P.A., Cavanagh H.D. and Petroll W.M. (1996). Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. Cornea 15, 505-516.

Jester J.V., Huang J., Barry-Lane P.A., Kao W.W., Petroll W.M. and Cavanagh H.D. (1999a). Transforming growth factor(beta)-mediated corneal myofibroblast differentiation requires actin and fibronectin assembly. Invest. Ophthalmol. Vis. Sci. 40, 1959-1967.

Jester J.V., Petroll W.M. and Cavanagh H.D. (1999b). Corneal stromal wound healing in refractive surgery: the role of myofibroblasts. Prog. Retin. Eye. Res. 18, 311-356.

Kim W.J., Mohan R.R. and Wilson S.E. (1999). Effect of PDGF, IL-1alpha, and BMP2/4 on corneal fibroblast chemotaxis: expression of the platelet-derived growth factor system in the cornea. Invest. Ophthalmol. Vis. Sci. 40, 1364-1372.

Kimura K., Teranishi S., Yamauchi J. and Nishida T. (2008). Role of JNK-dependent serine phosphorylation of paxillin in migration of corneal epithelial cells during wound closure. Invest. Ophthalmol. Vis. Sci. 49, 125-132.

Koli K., Saharinen J., Hyytiainen M., Penttinen C. and Keski-Oja J. (2001). Latency, activation, and binding proteins of TGF-beta. Microsc. Res. Tech. 52, 354-362.

Massague J. (2003). Integration of Smad and MAPK pathways: a link and a linker revisited. Genes Dev. 17, 2993-2997.

Mohan R., Chintala S.K., Jung J.C., Villar W.V., McCabe F., Russo L.A., Lee Y., McCarthy B.E., Wollenberg K.R., Jester J.V., Wang M., Welgus H.G., Shipley J.M., Senior R.M. and Fini M.E. (2002). Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. J. Biol. Chem. 277, 2065-2072.

Mohan R.R., Mohan R.R., Kim W.J., Stark G.R. and Wilson S.E. (2000). Defective keratocyte apoptosis in response to epithelial injury in stat 1 null mice. Exp. Eye Res. 70, 485-491.

Moller-Pedersen T., Cavanagh H.D., Petroll W.M. and Jester J.V. (1998a). Corneal haze development after PRK is regulated by volume of stromal tissue removal. Cornea 17, 627-639.

Moller-Pedersen T., Cavanagh H.D., Petroll W.M. and Jester J.V. (1998b). Neutralizing antibody to TGFbeta modulates stromal fibrosis but not regression of photoablative effect following PRK. Curr. Eye Res. 17, 736-747.

Myers J.S., Gomes J.A., Siepser S.B., Rapuano C.J., Eagle R.C. Jr and Thom S.B. (1997). Effect of transforming growth factor beta 1 on stromal haze following excimer laser photorefractive keratectomy in rabbits. J. Refract. Surg. 13, 356-361.

Piek E., Ju W.J., Heyer J., Escalante-Alcalde D., Stewart C.L., Weinstein M., Deng C., Kucherlapati R., Bottinger E.P. and Roberts A.B. (2001). Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. J. Biol. Chem. 276, 19945-19953.

Saika S. (2004). TGF-beta signal transduction in corneal wound healing as a therapeutic target. Cornea 23, S25-30.

Saika S., Okada Y., Miyamoto T., Yamanaka O., Ohnishi Y., Ooshima A., Liu C.Y., Weng D. and Kao W.W. (2004). Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. Invest. Ophthalmol. Vis. Sci. 45, 100-109.

Schultz G., Chegini N., Grant M., Khaw P. and MacKay S. (1992). Effects of growth factors on corneal wound healing. Acta. Ophthalmol. Suppl. 60-66.

Sharma G.D., He J. and Bazan H.E. (2003). p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. J. Biol. Chem. 278, 21989-21997.

Shiomi N., Cui X.M., Yamamoto T., Saito T. and Shuler C.F. (2006). Inhibition of SMAD2 expression prevents murine palatal fusion. Dev. Dyn. 235, 1785-1793.

Song Q.H., Singh R.P., Richardson T.P., Nugent M.A. and Trinkaus-Randall V. (2000). Transforming growth factor-beta1 expression in cultured corneal fibroblasts in response to injury. J. Cell. Biochem. 77, 186-199.

Soubrane G., Jerdan J., Karpouzas I., Fayein N.A., Glaser B., Coscas G., Courtois Y. and Jeanny J.C. (1990). Binding of basic fibroblast growth factor to normal and neovascularized rabbit cornea. Invest. Ophthalmol. Vis. Sci. 31, 323-333.

Stramer B.M., Zieske J.D., Jung J.C., Austin J.S. and Fini M.E. (2003). Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes. Invest. Ophthalmol. Vis. Sci. 44, 4237-4246.

Strissel K.J., Rinehart W.B. and Fini M.E. (1995). A corneal epithelial inhibitor of stromal cell collagenase synthesis identified as TGF-beta 2. Invest. Ophthalmol. Vis. Sci. 36, 151-162.

Strissel K.J., Rinehart W.B. and Fini M.E. (1997). Regulation of paracrine cytokine balance controlling collagenase synthesis by corneal cells. Invest. Ophthalmol. Vis. Sci. 38, 546-552.

Trinkaus-Randall V. and Nugent M.A. (1998). Biological response to a synthetic cornea. J. Control Release 53, 205-214.

Tsukada S., Westwick J.K., Ikejima K., Sato N. and Rippe R.A. (2005). SMAD and p38 MAPK signaling pathways independently regulate alpha1(I) collagen gene expression in unstimulated and transforming growth factor-beta-stimulated hepatic stellate cells. J. Biol. Chem. 280, 10055-10064.

Weimar V. (1960). Healing processes in the cornea. In: The transparacy of the cornea, S.a.P. Duke-Elder E.S. (ed). Blackwell Scientific Publications. Oxford. pp 111-124.

Wilson S.E., Chen L., Mohan R.R., Liang Q. and Liu J. (1999). Expression of HGF, KGF, EGF and receptor messenger RNAs following corneal epithelial wounding. Exp. Eye Res. 68, 377-397.

Wilson S.E., He Y.G. and Lloyd S.A. (1992). EGF, EGF receptor, basic FGF, TGF beta-1, and IL-1 alpha mRNA in human corneal epithelial cells and stromal fibroblasts. Invest. Ophthalmol. Vis. Sci. 33, 1756-1765.

Wilson S.E., Mohan R.R., Ambrosio R. Jr, Hong J. and Lee J. (2001). The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. Prog. Retin. Eye Res. 20, 625-637.

Zhu H.J. and Burgess A.W. (2001). Regulation of transforming growth factor-beta signaling. Mol. Cell Biol. Res. Commun. 4, 321-330.

Zieske J.D., Guimaraes S.R. and Hutcheon A.E. (2001a). Kinetics of keratocyte proliferation in response to epithelial debridement. Exp. Eye Res. 72, 33-39.

Zieske J.D., Hutcheon A.E., Guo X., Chung E.H. and Joyce N.C. (2001b). TGF-beta receptor types I and II are differentially expressed during corneal epithelial wound repair. Invest. Ophthalmol. Vis. Sci. 42, 1465-1471.

Zieske J.D., Mason V.S., Wasson M.E., Meunier S.F., Nolte C.J., Fukai N., Olsen B.R. and Parenteau N.L. (1994). Basement membrane assembly and differentiation of cultured corneal cells: importance of culture environment and endothelial cell interaction. Exp. Cell Res. 214, 621-633.

Accepted May 25, 2009