Conditional Knockout of CTGF Affects Corneal Wound Healing

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Purpose. This study aimed to elucidate the role of connective tissue growth factor (CTGF) in healthy eyes and wounded corneas of mice and rabbits. Conditional knockout mice were used to determine the role of CTGF in corneal healing.

Methods. *CTGF* expression was determined using transgenic mice carrying *CTGF promoter driven–eGFP,* quantitative RT-PCR, and immunofluorescent staining. Mice that carried two floxed *CTGF* alleles and a *Cre/ERT2* transgene under the control of human *ubiquitin C* (*ubc*) promoter were used to conditionally delete *CTGF* gene in a tamoxifen-inducible manner. Phototherapeutic keratectomy (PTK) was used to generate an acute corneal wound and corneal re-epithelialization was assessed by fluorescein staining.

Results. Connective tissue growth factor expression was found in multiple ocular tissues with relatively high levels in the corneal endothelium, lens subcapsular epithelium, and in the vasculature of the iris and retina. Wounded corneas responded with an immediate upregulation of CTGF in the epithelium at the wound margin and a sustained CTGF induction during re-epithelialization. At the onset of haze formation, CTGF protein becomes more focused in the basal epithelium. Deletion of the *CTGF* gene caused a 40% reduction ($P < 0.01$) in the cornea re-epithelialization rate in knockout mice compared with wild-type mice.

Conclusions. Connective tissue growth factor is expressed in the naïve cornea, lens, iris, and retina, and is expressed immediately after epithelial injury. Loss of *CTGF* impairs efficient re-epithelialization of corneal wounds.

Keywords: CTGF, animal models, pre-fibrosis

Corneal scarring following acute injury or vision correction surgery can lead to vision degrading opacification of the cornea through the generation of light reflecting myofibroblasts in the anterior stroma.[1] Currently, mitomycin C is considered the most potent therapy, and it effectively devitalizes the stromal, thereby, precluding the arrival of the light reflecting myofibroblasts.[2] This approach has proven effective, but carries with it the risk of substantial side effects, including endothelial toxicity, punctual stenosis, and worst of all, it adds to the risk for later corneal melting, all of which have caused concern over its continued use.[3–10]

Research is currently ongoing to find more targeted approaches, which seek to neutralize the effectors of the cellular phenotypic change from quiescent clear fibrocytes into light reflecting myofibroblasts. Transforming growth factor–β (TGF-β) had emerged as an early target due to its ability to force fetuses and marsupial pouch young to scar.[11–13] However, due to its pleiotropy, TGF-β has proven to be a difficult therapeutic target.[14,15] Work in seeking targets further down TGF-β's fibrotic activity pathway has led to connective tissue growth factor (CTGF) as a candidate further downstream of TGF-β with potentially fewer pleiotropic effects. Fibroblasts produce CTGF in response to TGF-β,[16] and neutralization of CTGF reduces fibrosis-related activities in cell culture.[17–19] These in vitro data have begun to be translated into an antifibrotic therapy in the skin via the use of an anti-CTGF antisense oligodeoxynucleotide.[20] The results in improving skin healing are encouraging, but the cornea will require a higher degree of antifibrotic effect than the skin since an equivalent scar in the cornea will be accompanied by a higher morbidity than it would in the skin.

With regards to CTGF in the cornea, the literature concerning the expression timing and localization is contradictory for models like the rat and nonexistent for mice. The most extensive data on the time course of CTGF is only published for the rat[18,21]; though these data came from two different wounding models. In the first report, CTGF was found in all corneal cell layers in healing corneas, but had an

apparent higher concentration in the epithelium.[18] These first data indicated a constant rise in both CTGF mRNA and protein through day 28 post wounding,[18] though later reports now indicate a peak at day 3 followed by a continual recession toward baseline values.[21] The later profile is also supported by an immunofluorescent study of healing rabbit corneas, which peaked at day 2 or 3 post wounding and returned to baseline levels by day 21 post wounding.[22] The disparity found in these publications, and the lack of knowledge of CTGF's disposition in the mouse cornea, currently precludes confidence in the use of mouse genetic models to test the hypothetical role of CTGF in corneal wound healing. Additionally, the lack of clear knowledge of the timing and precise cellular sources of synthesis of CTGF in the early stages of wound healing currently impedes the study of CTGF-targeted RNA interference (RNAi) approaches.

In the continued pursuit of gene neutralization-based approaches to improve wound healing outcomes, one clear hurdle remains apparent: a lack of good, positive controls. Two impediments currently exist for validating CTGF's role in corneal scaring. The first is that no data exist on the molecular or physiologic equivalence of corneal scaring in mice compared with more accepted models like rabbits. Second is that constitutive, *CTGF* knockout mice die shortly after birth.[23] In order to address these two short comings, we compared the expression profile and protein localization of CTGF in naïve and wounded mouse and rabbit corneas to determine equivalence. Then, we generated a drug-inducible, postnatal, conditional *CTGF* knockout mouse ($CTGF^{k/k}$) to enable normal development in the presence of CTGF and the testing of wound healing in its absence. The results of these experiments are expected to both improve the spatial and temporal profile of CTGF synthesis in the healing cornea, and to test the outcome of corneal healing in the absence of CTGF.

Materials and Methods

Transgenic Mice

CTGF Promoter–Driven Enhanced Green Fluorescent Protein Mice. These mice possess a transgene composed of the *CTGF* promoter upstream from an enhanced green fluorescent protein (CTGFp-eGFP).[24,25] The *CTGF* promoter driven eGFP mice (*Tg*[*Ctgf-EGFP*]*FX156Gsat/Mmucd*) were originally from Mutant Mouse Regional Resource Centers (in the public domain, http://www.mmrrc.org/catalog/sds.php?mmrrc_id=11899). This strain was developed using a modified bacterial artificial chromosome (BAC) containing *eGFP* immediately upstream of the coding sequence of the targeted *CTGF* gene, but downstream of *cis* acting regulatory elements. The genetic background of the mice used in this study was FVB/N-Swiss Webster and crossed to FVB/N mice for at least for two generations to facilitate the eGFP visualization in the absence of melanin. This construct results in the accumulation of eGFP in cells that possess activities capable of stimulating transcription from the *CTGF* promoter. The mouse pups were chosen by phenotypic observation of the green fluorescence in the lens in place of standard genotyping.

Postnatal *CTGF* Conditional Knockout Mice. Mating occurred between $CTGF^{floxed/floxed}$ mice and hemizygotes carrying human ubc promoter driven *Cre/ERT2* (*ubiquitin C [ubc]-Cre/ERT2*; Jackson Laboratory, Sacramento, CA, USA) for at least 3 generations before the study. Adult littermate mice (3-weeks old) that were hemizygous for *ubc-Cre/ERT2* and homozygous for the floxed *CTGF* allele were given intraperitoneal injections of the tamoxifen suspension (75 mg/kg body weight) once per day for 5 days. One month after the final dose, the eyes of these mice were confirmed to be free of opacities occasionally observed with continuous tamoxifen treatment.[26] Deletion of *CTGF* exon 4 was determined by PCR genotyping of mouse tails according to Liu et al.[27]

Whole-Mount Confocal Micrography

Euthanasia occurred for CTGFp-eGFP mice and their eyes were immediately enucleated and placed in 10% neutral buffered formalin. Following overnight fixation at 4°C, the eyes were grossly dissected and the tissues were cut into small pieces and placed into a custom-made slide with a reservoir. The tissues were immersed in mounting medium containing DAPI counterstain (Vector Labs, Burlingame, CA, USA) and a coverslip was affixed in place with nail hardener. The pieces of tissue were then imaged with either an epifluorescent or a fluorescent confocal microscope.

CTGF ELISA

Briefly, frozen, whole, young rabbit globes were purchased from Pel-Freez Biologicals, Inc. (Rogers, AR, USA). The tissues were removed from deep freeze storage and thawed on ice. Each tissue was then grossly dissected from the globe, homogenized, and cleared by centrifugation. Total extractable CTGF was measured via a sandwich ELISA in a 96-well plate using polyclonal antibodies (C7978-25C and C7978-25D; US Biological, Swampscott, MA, USA). A standard curve was plated in duplicate using recombinant human CTGF at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, and 0.0 ng/mL.

Excimer Laser Surgery

All animals used in experiments reported herein were treated in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and in protocols that were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

Rabbits and mice were both topically and generally anesthetized, then wounded as previously described.[28,29] with one modification to the mouse protocol. The mice used herein were wounded with a 1.0- (whole mount) or 2.0-mm (all others) diameter wound by 40-μm deep. All rabbits received a daily dose of oral meloxicam (0.2 mg/kg; Boehringer Ingelheim, St. Joseph, MO, USA) until wound closure or euthanasia.

Wound Closure Analysis

The $CTGF^{+/+}$ and $CTGF^{k/k}$ mice were anesthetized at 30 minutes, 6, 12, 24, and 30 hours, and the wound size was imaged by fluorescein staining and digital photography with a fixed focal length macro lens set to a 1:1 reproduction ratio and a pair of macro flashes with blue light filters for all images taken. The images were checked for good focus using the image review function of the camera prior to imaging the next eye. The size of the fluorescein stained area was determined using Photoshop CS3 Extended (Adobe Systems, Inc., San Jose, CA, USA) and the data were exported for analysis using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). Briefly, the area measured at each time point was normalized to the area at 30 minutes post wounding. The wound closure percentages for each group were averaged for each time point and were compared using Student's *t*-test to determine if the observed differences were statistically significant ($P \leq 0.05$).

Gross Corneal Dissection and CTGF Transcript Quantification

Corneas were obtained from six rabbits without observable corneal wounds, and six rabbits wounded as described above and euthanized approximately 30 minutes later. A fresh scalpel was soaked in lysis buffer and then used to immediately scrape the epithelium off with care taken to ensure that the scraped mass was retained on the blade. The scraped epithelial mass was then transferred to 350 μL of tissue lysis buffer (buffer RLT; Qiagen, Inc., Valencia, CA, USA) and the blade was rinsed with 250 μL of additional lysis buffer. The cornea was then excised from the globe by cutting with a fresh scalpel and scissors at the limbus. The cornea was placed face down and yet another fresh scalpel was used to scrape and peel off the endothelium; which consistently delaminated from Descemet's layer. The endothelial sheet was transferred to 350 μL of lysis buffer and the blade rinsed with an additional 250 μL of lysis buffer. The residual stroma was cut into approximately 1 mm × 1 mm pieces, which were all submerged in 600 μL of lysis buffer. Each grossly isolated cellular layer was then subjected dounce homogenization and subsequent ultrasonication on iced saline for further tissue disruption. Fresh dounces were used and the ultrasonication probe was rigorously washed, rinsed, and dried in between each sample. The homogenates were then immediately loaded onto Qiagen genomic DNA removal columns and the RNA was purified in accordance with the manufacturer's provided protocol (Qiagen, Inc., Valencia, CA, USA).

The purified RNA was quantified and equal masses of RNA were loaded for each sample with 2-fold concentrated TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in a 96-well PCR reaction plate with the primers listed in Table 1. The relative gene expression of *CTGF* versus *GAPDH* was calculated for each tissue and these normalized values (ΔCt) were used in the subsequent statistical analyses. First, to determine whether there were differences in *CTGF* transcription among the corneal cell layers in either the unwounded or wounded corneas, a one-way ANOVA was performed with Tukey's post hoc test to determine which cell layers had significant differences. To determine whether a given cell layer's CTGF production changed following wounding, the ΔCt values of the unwounded and wounded cell layers were compared using an unpaired, two-tailed, Student's *t*-test. A threshold of P less than or equal to 0.05 was chosen to determine statistical significance for all tests. Relative quantities were determined using the $2^{-\Delta\Delta Ct}$ method.[30]

Tissue Harvesting, Processing, and Sectioning

At the terminal time point, the rabbits and mice were euthanized and each globe was immediately enucleated and placed in fresh 10% neutral buffered formalin on ice. Rabbit corneas were removed after 1 hour of prefixation and then resubmerged in fixative. The mouse globes were punctured with a 25 gauge needle after 1 hour to improve fixative penetration. Reporter mouse tissues were grossly dissected and flat mounted on poly-l-lysine slides. Rabbit and mouse corneas were either paraffin embedded and cut (4–5 μm) or cryoprotected, embedded, and cut (12–15 μm).

Immunohistochemical Staining

In order to visualize the localization of CTGF protein, rabbit corneas were stained as previously described[28] using a mouse anti-CTGF monoclonal antibody (50 μg/mL; University of Florida Interdisciplinary Center for Biotechnology Hybridoma Core, Gainesville, FL, USA), while a rabbit anti-CTGF polyclonal antibody was used as previously described[31] for the mouse corneas. All slides were kept refrigerated in the dark until imaged via confocal fluorescent micrography.

Table 1. TaqMan RT-PCR and End-Point RT-PCR Primers and Probe Sequences

Growth Factor	Species	Direction	Accession Number
CTGF	Rabbit	Forward	AGGAGTGGGTGTGTGATGAG
		Reverse	CCAAATGTGTCTTCCAGTCG
		Probe	ACCACACCGTGGTTGGCCCT
	Mouse	Forward	CAAAGCAGCTGCAAATACCA
		Reverse	AGTGGAGCGCCTGTTCTAAG
GAPDH	Rabbit	Forward	GAGACACGATGGTGAAGGTC
		Reverse	ACAACATCCACTTTGCCAGA
		Probe	CCAATGCGGCCAAATCCGTT
Actin	Mouse	Forward	TCCTGCTTGCTGATCCACAT
		Reverse	TCCTCCCTGGAGAAGAGCTA

Results

The most immediate finding is that the lenses of these mice are extremely green with eGFP. The green fluorescence was confirmed to be limited to the lens subcapsular epithelium (Fig. 1A), and these findings have been confirmed to be present in rabbits as well by ELISA (Figs. 1A–D, labels) and Western blot.[32] Unwounded CTGFp-eGFP reporter mice also had observable *CTGF* promoter activity in the corneal endothelium (Fig. 1B), the iris (Fig. 1C), and in at least two cell types in the retina (Fig. 1D). An ELISA of grossly dissected rabbit globes confirmed both the presence of CTGF protein in these tissues and the dominance of the CTGF quantity in the lens (Figs. 1A–D, subset). Interestingly, the distribution of promoter activity in the lens, corneal endothelium, and portions of the retina all share a similar mosaic pattern. Further investigation of the apparently elevated *CTGF* promoter activity in the corneal endothelium (Fig. 1E) was supported by our findings in grossly dissected naïve rabbit corneas (Fig. 1F), where the relative expression level of CTGF mRNA is highest in the endothelium ($P < 0.05$, Fig. 1G). As would be expected, the quantitative PCR (qPCR) method is more sensitive than the reporter mouse since no signal was observed in the epithelium and stroma, while CTGF mRNA was detected in these cell layers. These data indicate that the relative CTGF distribution in the naïve mouse eye is similar to that of the naïve rabbit eye, with the endothelium being the most abundant source of CTGF in the normal cornea.

In addition to investigating CTGF in normal eyes, we sought to determine how corneal CTGF expression changes in response to wounding, and how the response in rabbits compares with that of mice. The excimer wounding was well tolerated by both the rabbits and mice with no complications. The wounds were consistently circular and the rough appearance of the ablated surface evidences that the wound penetrated into the stroma (Fig. 2A). Within 30 minutes after wounding, *CTGF* promoter activity was observed as an annular ring around the wounded reporter mouse cornea (Fig. 2B). The presence of CTGF protein was observed in this same pattern in similarly wounded rabbit corneas at the 30-minute time point as well (Fig. 2C). The staining of CTGF protein appears to be associated with a fibrous material, which appears to be being deposited on the basal surface at the epithelium/stroma interface. The staining was demonstrated as specific by the lack of staining in sections with the primary antibody withheld (Fig. 2C, inset). The presence of 7.86-fold more CTGF

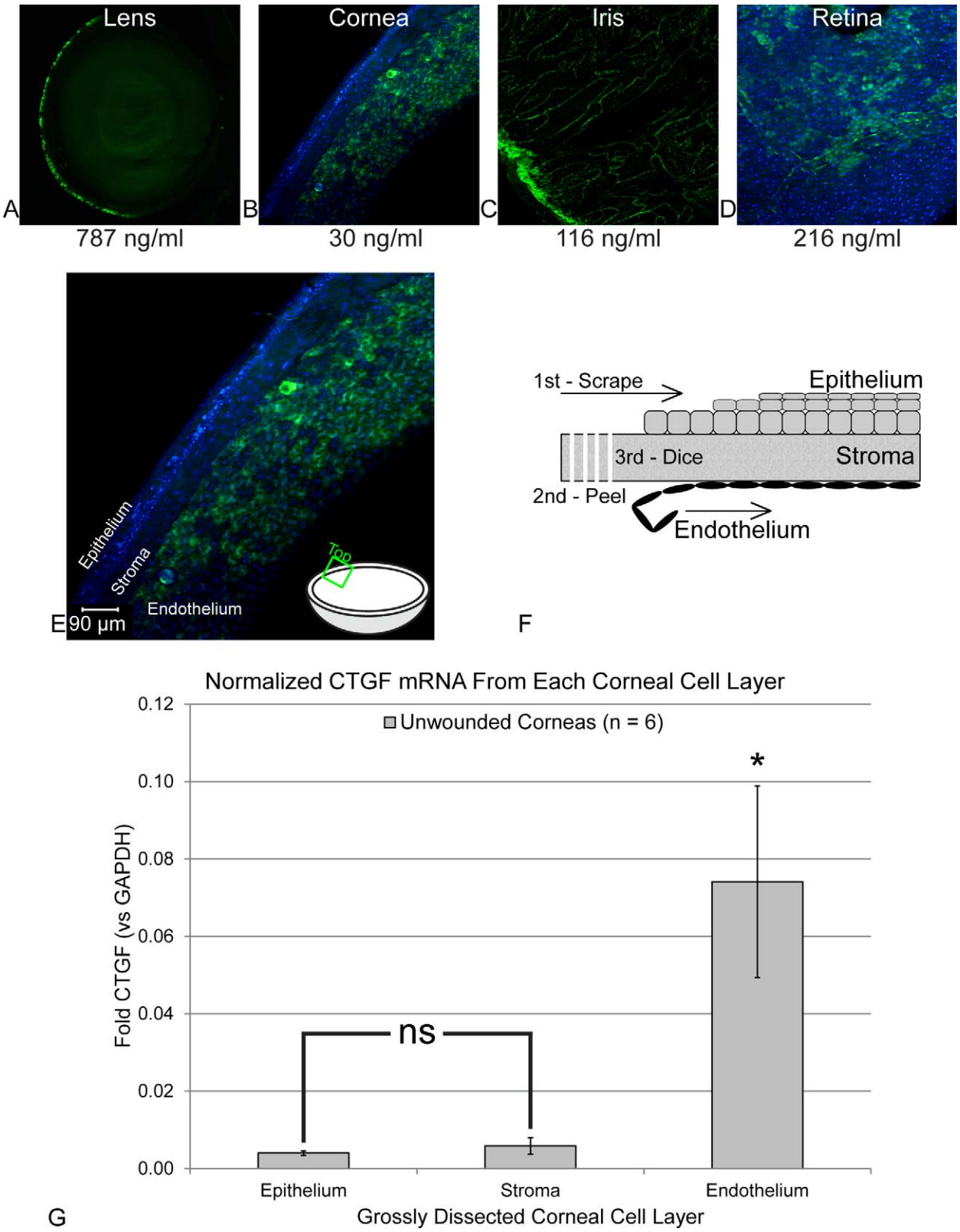

Figure 1. Connective tissue growth factor in the naïve eye. (**A–D**) *CTGF* promoter activity and protein in the naïve eye (*green*, DAPI in *blue*) and CTGF protein from grossly dissected rabbit globes (ELISA, subset number). *CTGF* promoter activity and protein are present in all of the tissues investigated including (**A**) the lens subcapsular epithelium, (**B**) the corneal endothelium (viewed en face), (**C**) various cells in the iris (en face), and (**D**) in the retinal vasculature and in foci within the retinal pigmented epithelium (en face). Quantitative assessment of CTGF content via ELISA indicated that the naïve lens has the most and the cornea the least. (**E**) Within the cornea, *CTGF* is constitutively expressed in the corneal endothelium. The inset graphic depicts the tissue and image orientation. The epithelium and stroma are visible at the cut face, while the posterior surface of the cornea (endothelium) is visible as viewed en face. Note the mosaic pattern. (**F**) Unwounded rabbit corneas were grossly dissected prior to RT-qPCR to quantify the amount of CTGF mRNA. (**G**) The endothelium has more CTGF mRNA than the epithelium (18.53-fold, $P < 0.05$) and stroma (11.37-fold, $P < 0.05$).

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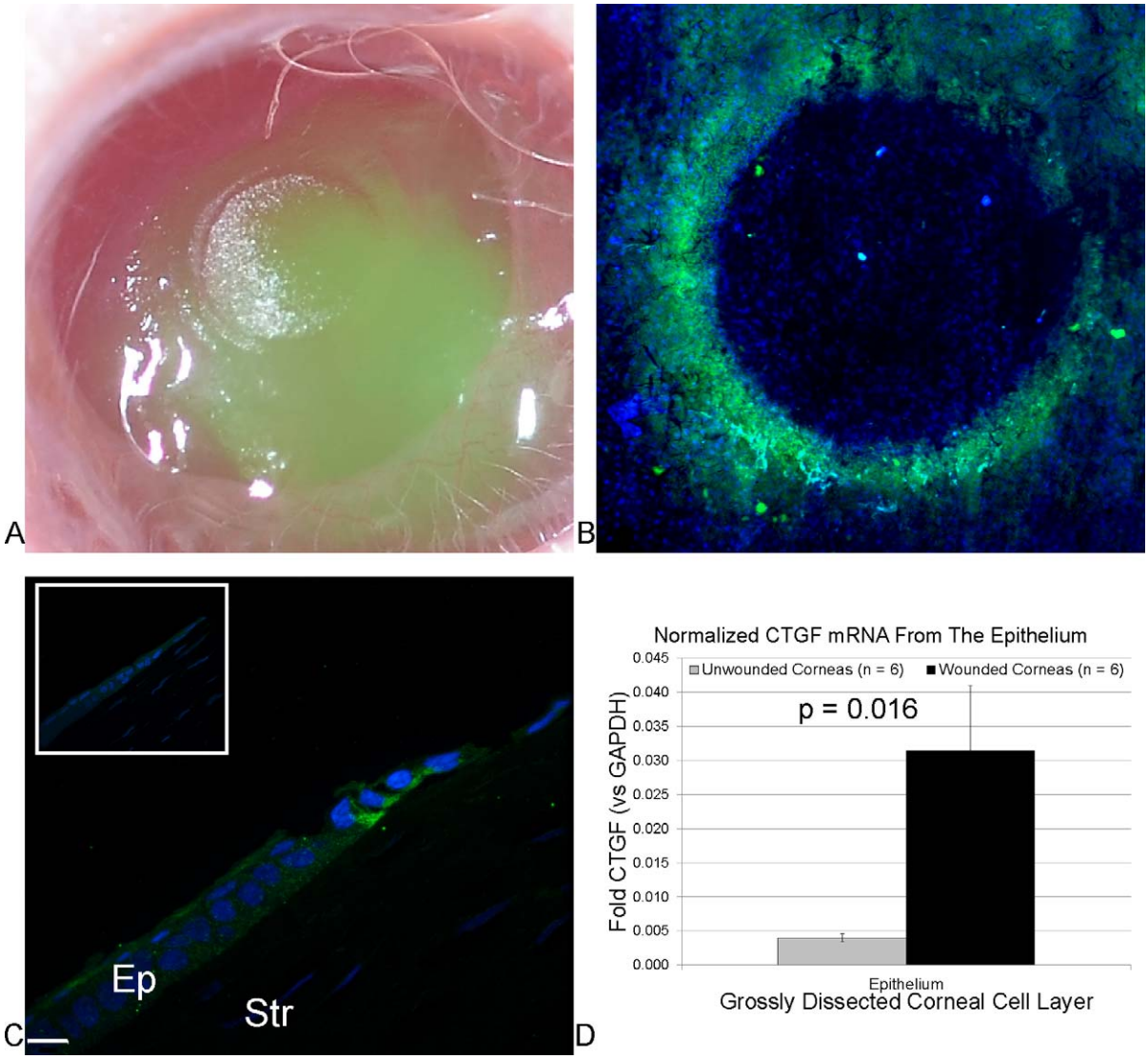

Figure 2. Connective tissue growth factor is immediately upregulated in the epithelium at the wound margin. (**A**) A representative 1.0-mm phototherapeutic keratectomy (PTK) wound on a CTGFp-eGFP mouse. (**B**) An en face mosaic of the entire 1.0 mm-PTK wound 30 minutes after wounding with CTGF promoter activity in *green* and DAPI in *blue*. (**C**) An immunofluorescent micrograph of the wound margin of a rabbit cornea 30 minutes after receiving a PTK wound (CTGF monoclonal antibody, *green*; DAPI, *blue*). The inset image is a serial section which didn't receive the CTGF mAb. (**D**) Grossly dissected epithelia from rabbits 30 minutes after wounding do have an increase in CTGF mRNA (7.86-fold, $P = 0.016$).

transcripts in grossly dissected rabbit epithelia also supports the promoter activity and immunofluorescent data (Fig. 2D). The immediate increase in CTGF mRNA was also found to be significant for the stroma and endothelium (Table 2). The epithelium in both rabbit and mouse corneas immediately increases CTGF synthesis in response to wounding.

Immunofluorescence staining for CTGF at later time points indicates that CTGF staining is highly associated with cells from all three layers in the rabbit (Figs. 3A–C). The epithelium has the highest apparent density of staining (Fig. 3A), and the staining appears to peak between day 1 and 2 post wounding (not shown). Interestingly, the staining for CTGF in the stroma reveals a novel, highly polarized, distribution on the apical surface of the stromal fibroblasts. Mouse corneas stained at the same time point demonstrate some similarities, but also a divergence from what was seen in the rabbit. First, the epithelium was consistently positive for CTGF protein in the mouse cornea (Fig. 3D), while the stroma exhibited one of two patterns. First was a paucity of both cells and CTGF staining (Fig. 3D), and the other was an abundance of cells and the highest levels of CTGF staining seen in the mouse (Fig. 4E). The divergent observation appears to hinge on the presence or absence of polymorphonuclear leukocytes (neutrophils) based on the crescent-like shape of the nuclei with the highest CTGF antigenicity (Figs. 3E, 3F, asterisks). At the expected onset of haze 5 days after wounding,[29] the mouse (Fig. 3G), and rabbit (Fig. 3H) CTGF patterns were comparable with the distribution of staining in the epithelium transitioning from homogenous to being more concentrated in the basal

Table 2. Connective Tissue Growth Factor Is Immediately Upregulated in All Three Cell Layers in the Rabbit

Wounded vs. Unwounded	Fold Difference	*P* Value
Epithelium	6.51	0.0003
Stroma	44.01	0.0004
Endothelium	7.53	0.0088

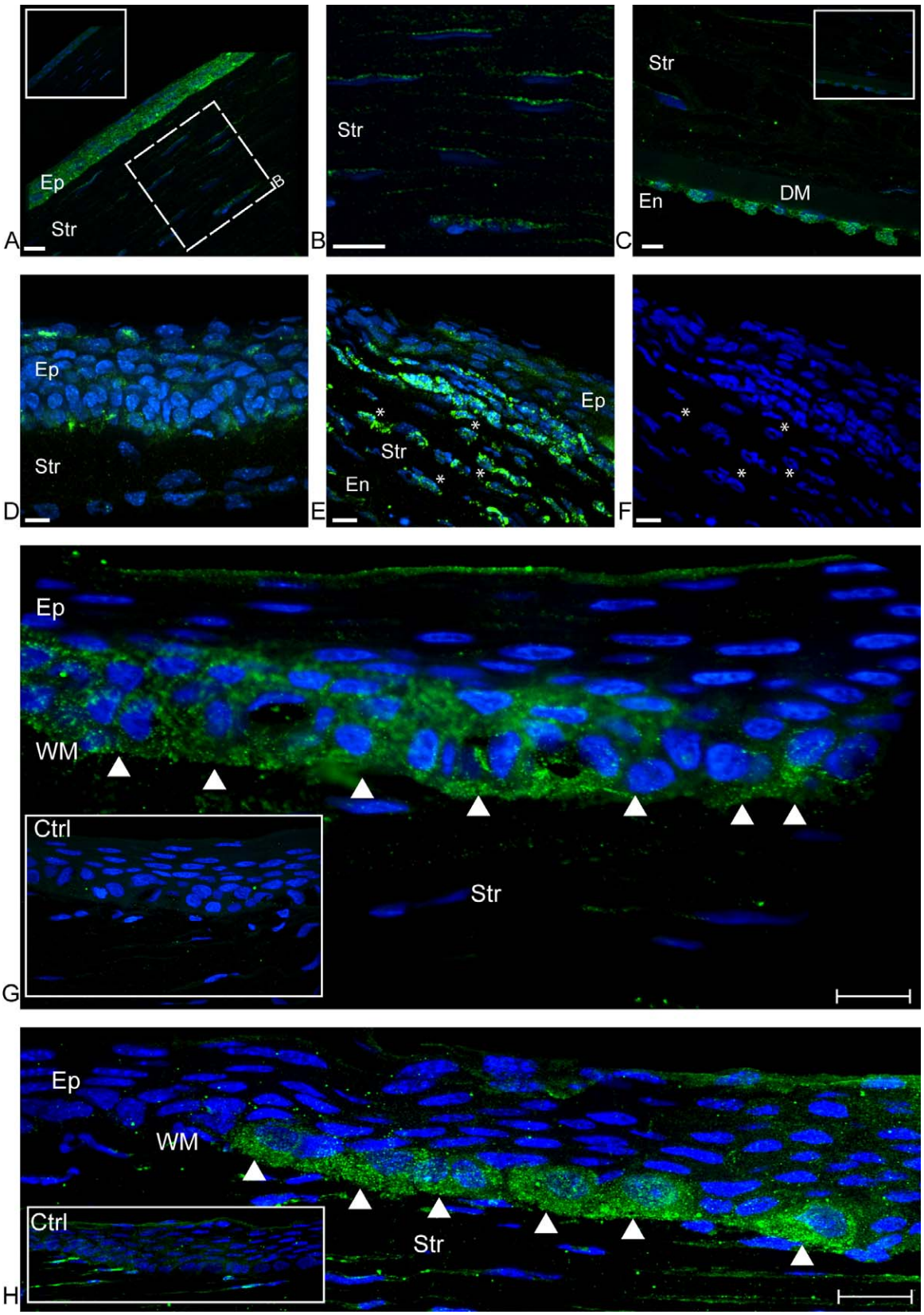

FIGURE 3. Immunofluorescent staining of CTGF protein in wounded rabbit (**A–C**) and mouse (**D–F**) corneas 1 day post wounding. All three layers have positive CTGF staining, with the epithelium having the most consistently dense staining. For the first time, (**B**) CTGF has been observed to be distributed on the stromal fibroblasts in a highly polar manner, focused only on the apical surface. (**D**, **E**) The epithelium of the mouse consistently stained for CTGF, but the stroma was highly variable, with (**E**) some having amounts of stromal staining which exceeded the epithelial staining. (**E**, **F**) The cells with the highest staining have the crescent-like nuclear shape of neutrophils (*). At 5 days post wounding, the onset of haze formation, CTGF protein becomes more focused in the basal epithelium in both (**G**) the rabbit cornea and (**H**) the mouse cornea. While the staining in the (**H**)

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mouse cornea has more off-target staining, comparison with the inset control demonstrates that the basal epithelium staining is specific. For all panels, DAPI is in *blue* and CTGF is in *green*. The *white bars* represent 10 μm.

epithelium. This similarity at the onset of expected haze formation further demonstrates another commonality of the CTGF-related response to wounding in these two model animals.

Since the *CTGF* expression data between the rabbit and mouse epithelia were comparable, we considered the use of a *CTGF* knockout mouse as a feasible model to test CTGF's role in wound healing. Dosing *ubc-Cre/CTGF$^{floxed/floxed}$* mice (Figs. 4A, 4B) with tamoxifen is sufficient to cause recombination-mediated removal of exon 4 (Fig. 4C) and to remove CTGF mRNA from the cornea, lens, and retina (Fig. 4D). Due to the consistent similarity in the response and distribution of CTGF in the epithelium, we focused solely on the function of CTGF in epithelial healing. *CTGF$^{k/k}$* mice have delayed epithelial closure rates (Fig. 5), with wild type corneas healing within 30 hours, while the *CTGF$^{k/k}$* corneas had only healed approximately 60% of the wounded area within this same time period.

Discussion

Contrary to our initial working hypothesis, which cast CTGF as primarily a profibrotic factor, the presence of CTGF in the naïve tissues of the eye indicates that it has a much broader biological role. Recent findings have begun identifying the role of CTGF as a factor in neovascularization of the retina.[31,33] Our findings of the presence of CTGF in the naïve lens, RPE, iris, and corneal endothelium all suggest that CTGF may also have a role in normal tissue homeostasis in addition to regulating the transient responses of fibrosis and neovascularization.

Our demonstration of a constitutive mosaic expression of *CTGF* in the endothelium was unexpected and contradicts previous reports of an absence of CTGF mRNA in the naïve cornea.[22] While the role of CTGF in the endothelium is not yet known, the heterogeneous pattern of expression may indicate a role for CTGF in a periodic and transient process, which occurs during normal tissue homeostasis. The emerging evidence of an ion transport role for CTGF may implicate it in the endothelium's key homeostatic role of cornea dehydration. At present, we have yet to test the CTGF$^{k/k}$ cornea's hydration content or relative edema to determine the verity of this hypothetical role.

The immediate upregulation of CTGF subsequent to wounding was demonstrated by increased promoter activity, mRNA, and protein in the cornea within 30 minutes of wounding for both model animals. This immediate response places new constraints on future RNAi-based approaches, in that the agent must be immediately bioavailable for CTGF mRNA ablation. Antifibrotic approaches using viral-vectored RNAi will require pretreatment of the cornea 1 or 2 days prior to wounding in order to allow the viral episome time to begin synthesizing its transgene.

We found that the peak of CTGF staining intensity comported with the reports of both Yang et al.[22] and Shi et al.[21] in contradiction to our previous findings in a photorefractive keratectomy (PRK) rat model.[18] The fact that the peak occurs prior to the initiation of scarring, which begins at approximately day 4 or 5 post wounding[29] doesn't contraindicate CTGF's role in fibrosis, though it might suggest a less direct role than previously hypothesized. At the time that the scar is beginning to form, the pattern of the CTGF protein staining is primarily focused on the basal epithelium (Figs. 5G, 5H). At even later time points, the distribution remains basally focused, but becomes more sporadically distributed (data not shown). These data current support two possible alternative hypotheses: (1) that CTGF is necessary to initiate the fibrotic response, but not to maintain it, or (2) that CTGF's activity on the basal epithelial cells gives rise to secondary factors, which serve to draw fibrotic myofibroblasts into the subepithelial space. This hypothesis will be tested using the conditional knockout mice developed and explained herein, and another, epithelial-specific knockout mouse currently being bred. Prior to testing these mice, the cellular kinetics of haze formation in mice must be better characterized, much as we have recently done for rabbits.[29] We do know that the mouse scar can be nearly mature as early as 7 days after wounding,[28] though we don't know if the formation pattern and mechanism differs from that of the rabbit; precluding any confidence in the relevance of any haze-related findings.

Our key phenotypic finding of CTGF's role in re-epithelialization was anticipated by in vitro experiments using antisense oligonucleotides to reduce CTGF in TGF-β–stimulated human corneal epithelial cultures.[34] While re-epithelialization still occurred in the conditional *CTGF$^{k/k}$* mice, it was impaired; demonstrating in vivo, that CTGF protein is necessary for quick and efficient epithelial wound closure. The key consequence of this finding, and the continually emerging findings in other

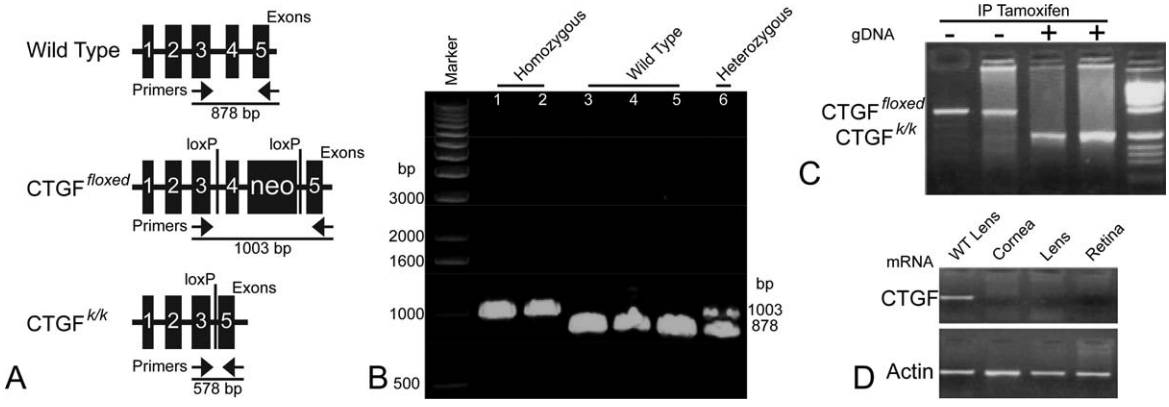

Figure 4. Establishing the conditional *CTGF* knockout mouse. (**A**) A schematic of the *CTGF^{floxed}* transgene. (**B**) A typical gel of *CTGF$^{floxed/floxed}$*, wild type, and *CTGF$^{floxed/WT}$* mice. (**C**) Systemic tamoxifen (IP) is sufficient to stimulate *ubc-Cre*–mediated recombination and (**D**), thereby, removal of *CTGF* mRNA from the cornea, lens, and retina.

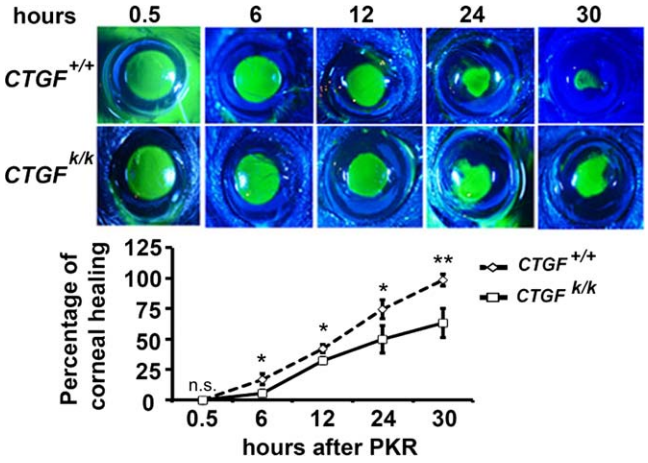

FIGURE 5. Deletion of CTGF in conditional knockout mice affected corneal epithelial wound healing. *Top*: The fluorescein staining on wounded corneas. *Bottom*: Percentage of epithelial healing coverage was expressed as percentage of fluorescein stained areas at 6 to 30 hours related to the areas at 0.5 hours ($n = 3$–4/group). Data represent mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$. Statistical analysis was performed with the unpaired Student's *t*-test for comparisons between two groups at the same time points.

tissues, is that CTGF is not specifically a profibrotic factor, though it may still be necessary for fibrosis. Within the context of CTGF-targeted antifibrotic therapies, these alternate roles raise concerns about side effects of anti-CTGF therapeutic approaches. It remains to be demonstrated if there is a level of CTGF inhibition which can both minimize the effects on wound closure and reduce stromal scarring, but at least now, we have a list of side effects to anticipate and monitor in the development and testing of CTGF-centric therapies.

In the work we have reported herein, we have observed and compared the timing and localization of CTGF synthesis and binding in two key animal models. The similarities that were observed justified the use of a conditional CTGF knockout mouse model to observe one component of corneal wound healing. At least one role for CTGF in the healing corneas was demonstrated, and evidence supporting possible later roles in the basal epithelium in the time frame of haze formation was also provided. Our goal is, and will remain to be, to understand the role of CTGF in the formation of corneal haze, and its suitability as a therapeutic target. In the work reported herein, we have demonstrated a conditional CTGF knockout corneal wound healing model, which will be instrumental in achieving this goal. Additional experiments with this model, and other models with more cell layer–specific ablation of CTGF, are currently ongoing to validate and clarify CTGF's role in opacifying subepithelial haze formation in order to finally settle whether targeted reduction of this growth factor is suitable as a viable clinical approach to improving vision though improved maintenance of corneal clarity subsequent to wounding.

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References

1. Moller-Pedersen T. Keratocyte reflectivity and corneal haze. *Exp Eye Res*. 2004;78:553–560.
2. Maldonado MJ. Intraoperative MMC after excimer laser surgery for myopia. *Ophthalmology*. 2002;109:826, author reply 826–828.
3. Coppens G, Maudgal P. Corneal complications of intraoperative Mitomycin C in glaucoma surgery. *Bull Soc Belge Ophtalmol*. 2010;19–23.
4. Kopp ED, Seregard S. Epiphora as a side effect of topical mitomycin C. *Br J Ophthalmol*. 2004;88:1422–1424.
5. Lane HA, Swale JA, Majmudar PA. Prophylactic use of mitomycin-C in the management of a buttonholed LASIK flap. *J Cataract Refract Surg*. 2003;29:390–392.
6. Netto MV, Mohan RR, Ambrosio R Jr, Hutcheon AE, Zieske JD, Wilson SE. Wound healing in the cornea: a review of refractive surgery complications and new prospects for therapy. *Cornea*. 2005;24:509–522.
7. Netto MV, Mohan RR, Sinha S, Sharma A, Gupta PC, Wilson SE. Effect of prophylactic and therapeutic mitomycin C on corneal apoptosis, cellular proliferation, haze, and long-term keratocyte density in rabbits. *J Refract Surg*. 2006;22:562–574.
8. Roh DS, Funderburgh JL. Impact on the corneal endothelium of mitomycin C during photorefractive keratectomy. *J Refract Surg*. 2009;25:894–897.
9. Santhiago MR, Netto MV, Wilson SE. Mitomycin C: biological effects and use in refractive surgery. *Cornea*. 2012;31:311–321.
10. Zhivov A, Beck R, Guthoff RF. Corneal and conjunctival findings after mitomycin C application in pterygium surgery: an in-vivo confocal microscopy study. *Acta Ophthalmol*. 2009;87:166–172.
11. Ferguson MW, Whitby DJ, Shah M, Armstrong J, Siebert JW, Longaker MT. Scar formation: the spectral nature of fetal and adult wound repair. *Plast Reconstruct Surg*. 1996;97:854–860.
12. Longaker MT, Whitby DJ, Ferguson MW, Lorenz HP, Harrison MR, Adzick NS. Adult skin wounds in the fetal environment heal with scar formation. *Ann Surg*. 1994;219:65–72.
13. Sullivan KM, Lorenz HP, Meuli M, Lin RY, Adzick NS. A model of scarless human fetal wound repair is deficient in transforming growth factor beta. *J Pediatr Surg*. 1995;30:198–202, discussion 202–203.
14. Franch HA, Shay JW, Alpern RJ, Preisig PA. Involvement of pRB family in TGF beta-dependent epithelial cell hypertrophy. *J Cell Biol*. 1995;129:245–254.
15. Laping NJ, Everitt JI, Frazier KS, et al. Tumor-specific efficacy of transforming growth factor-beta RI inhibition in Eker rats. *Clin Cancer Res*. 2007;13:3087–3099.
16. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev*. 1997;8:171–179.
17. Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-beta(1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol*. 2003;163:2043–2052.
18. Blalock TD, Duncan MR, Varela JC, et al. Connective tissue growth factor expression and action in human corneal fibroblast cultures and rat corneas after photorefractive keratectomy. *Invest Ophthalmol Vis Sci*. 2003;44:1879–1887.
19. Blalock TD, Yuan R, Lewin AS, Schultz GS. Hammerhead ribozyme targeting connective tissue growth factor mRNA blocks transforming growth factor-beta mediated cell proliferation. *Exp Eye Res*. 2004;78:1127–1136.
20. Excaliard Announces Positive Data From Phase 2 Clinical Trial of Its Anti-Scarring Drug, EXC 001 [press release]. Carlsbad: Excaliard Pharmaceuticals I; August 3, 2010.

21. Shi L, Chang Y, Yang Y, Zhang Y, Yu FS, Wu X. Activation of JNK signaling mediates connective tissue growth factor expression and scar formation in corneal wound healing. *PLoS One*. 2012;7:e32128.
22. Yang YM, Wu XY, Du LQ. The role of connective tissue growth factor, transforming growth factor and Smad signaling pathway during corneal wound healing [in Chinese]. *Zhonghua Yan Ke Za Zhi*. 2006;42:918–924.
23. Ivkovic S, Yoon BS, Popoff SN, et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development*. 2003;130:2779–2791.
24. Blake J, Bult C, Kadin J, Richardson J, Eppig J, Group Mouse Genome Database Group. The Mouse Genome Database (MGD): premier model organism resource for mammalian genomics and genetics. *Nucleic Acids Res*. 2011;39:D842–D848.
25. Kapoor M, Liu S, Huh K, Parapuram S, Kennedy L, Leask A. Connective tissue growth factor promoter activity in normal and wounded skin. *Fibrogenesis Tissue Repair*. 2008;1:3.
26. Nayfield SG, Gorin MB. Tamoxifen-associated eye disease. A review. *J Clin Oncol*. 1996;14:1018–1026.
27. Liu S, Shi-wen X, Abraham DJ, Leask A. CCN2 is required for bleomycin-induced skin fibrosis in mice. *Arthritis Rheum*. 2011;63:239–246.
28. Gibson DJ, Schultz GS. Ectopic epithelial implants following surface ablation of the cornea. *Invest Ophthalmol Vis Sci*. 2012;53:7760–7765.
29. Gibson DJ, Tuli SS, Schultz GS. The progression of haze formation in rabbit corneas following phototherapeutic keratectomy. *Invest Ophthalmol Vis Sci*. 2013;16:4776–4781.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
31. Pi L, Xia H, Liu J, Shenoy AK, Hauswirth WW, Scott EW. Role of connective tissue growth factor in the retinal vasculature during development and ischemia. *Invest Ophthalmol Vis Sci*. 2011;52:8701–8710.
32. Robinson PM, Smith TS, Patel D, et al. Proteolytic processing of connective tissue growth factor in normal ocular tissues and during corneal wound healing. *Invest Ophthalmol Vis Sci*. 2012;53:8093–8103.
33. Pi L, Shenoy AK, Liu J, et al. CCN2/CTGF regulates neovessel formation via targeting structurally conserved cystine knot motifs in multiple angiogenic regulators. *FASEB J*. 2012;26:3365–3379.
34. Secker GA, Shortt AJ, Sampson E, Schwarz QP, Schultz GS, Daniels JT. TGFbeta stimulated re-epithelialisation is regulated by CTGF and Ras/MEK/ERK signalling. *Exp Cell Res*. 2008;314:131–142.

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