

# Injury induces endothelial to mesenchymal transition in the mouse corneal endothelium in vivo via FGF2

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**Purpose:** To determine whether the mouse corneal endothelium enters endothelial to mesenchymal transition (EndoMT) following surgical injury in vivo.

**Methods:** The corneal endothelium in anesthetized mice was surgically injured in vivo under direct visualization. The secretion of interleukin-1 beta (IL-1 $\beta$ ) and fibroblast growth factor 2 (FGF2) into the aqueous humor was analyzed with western blotting. The expression of *FGF2*, *Snail*, *Zeb1*, *Colla1*, *Colla2*, *Fnl*, *Vim*, *Cdk2*, *Ccne1*, and *Cdh1* was analyzed with semiquantitative RT-PCR in the mouse corneal endothelium ex vivo and in vivo. Knockdown of FGF2 was done using siRNA. *Col8a2* was used as a corneal endothelial marker, and Keratocan (*Ktcn*) was used as a stromal marker.  $\beta$ -actin was used as a loading control.

**Results:** Sequential expression of IL-1 $\beta$  and FGF2 was detected in the aqueous humor after surgical injury. FGF2 treatment induced expression of endothelial to mesenchymal transition-related genes including *Snail*, and *Zeb1* in the mouse ex vivo corneal endothelium. This led to increased expression of *Colla1*, *Colla2*, *Fnl*, and *Vim* and suppression of *Cdh1* in a time-dependent manner. Expression of *FGF2*, *Snail*, *Zeb1*, *Colla1*, *Colla2*, *Fnl*, *Vim*, *Cdk2*, and *Ccne1* was completely abolished by FGF2 siRNA knockdown in the mouse corneal endothelium ex vivo. Surgical injury induced *FGF2* expression in the in vivo mouse corneal endothelium. The injury-dependent expression of *FGF2*, *Snail*, *Zeb1*, *Colla1*, *Colla2*, *Fnl*, *Vim*, *Cdk2*, and *Ccne1* and the suppression of *Cdh1* were inhibited by siRNA knockdown of FGF2 in the mouse corneal endothelium in vivo. Moreover, siRNA knockdown of FGF2 inhibited the formation of the injury-dependent retrocorneal membrane in the in vivo mouse corneal endothelium.

**Conclusions:** These findings suggest that after surgical injury, FGF2 induces the expression of EndoMT-related genes *Snail*, *Zeb1*, *Colla1*, *Colla2*, *Fnl*, *Vim*, *Cdk2*, and *Ccne1* in the mouse corneal endothelium in vivo, similar to the human corneal endothelium ex vivo.

The cornea endothelium is composed of a hexagonal monolayer of cells and plays a critical role in maintaining corneal transparency by regulating hydration through its pump function. Adult human corneal endothelial cells (CECs) are arrested at the G1 phase of the cell cycle throughout their life span [1,2]. Because of the cell cycle arrest, the loss of corneal endothelial cells leads to enlargement and spreading of existing endothelial cells. This, in turn, leads to a progressive decline in the cell density, and a decline below approximately 500 cells/mm<sup>2</sup> leads to decreased corneal transparency and a resultant loss in vision. Vision loss from endothelial dysfunction is a common indication for corneal transplantation. Although adult human CECs are arrested in the cell cycle, severe injury to the corneal endothelium can force the CECs to enter endothelial to mesenchymal transition (EndoMT). Endothelial to mesenchymal transition is a process in which epithelial or endothelial cells undergo a change in cell morphology to a spindle shape and show increased migratory and proliferative capacity. They secrete

fibrillar extracellular matrix leading to the formation of a retrocorneal membrane (RCM) [3-5]. Experimental models of RCM formation showed that activated polymorphonuclear leucocytes (PMNs) induce morphological alteration of CECs to spindle-shaped cells [6] and induce expression of type I collagen leading to anterior segment fibrosis [7,8].

Several soluble factors, such as interleukin-1 beta (IL-1 $\beta$ ), have been reported to have important roles in cell proliferation and wound healing [6,9-11]. IL-1 $\beta$  modulates inflammation through induction of secondary cytokines such as fibroblast growth factor 2 (FGF2) [12-16]. Corneal endothelial cells produce all isoforms of FGF2 in response to IL-1 $\beta$  stimulation [17-19]. Among the FGF2 isoforms induced by IL-1 $\beta$ , the secreted 18-KDa isoform regulates many components of endothelial to mesenchymal transition in CECs [20-22]. In CECs, FGF2 promotes cell proliferation through degradation of p27 [23,24], enhanced migration via coordinated regulation of CDC42 and RHOA [25], and upregulation of collagen type I expression [26]. Moreover, FGF2 induces a change in CEC morphology to a spindle shape along with loss of contact inhibition through regulation of RhoGTPases [25,27].

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The endothelial to mesenchymal transition consists of a change in the cell phenotype characterized by loss of cell polarity and adhesion, and reorganization of the cytoskeleton [28,29]. Downregulation of the homotypic junctional protein E-cadherin has also been shown to occur as cells enter endothelial to mesenchymal transition [30]. E-cadherin downregulation is mediated through transcriptional inhibition by SNAI (SNAI1 and SNAI2) and ZEB (ZEB1 and ZEB2) families of transcription factors [31-33]. Moreover, SNAI and ZEB transcription factors also upregulate expression of vimentin, fibronectin, and  $\alpha 1$  (COL1A1) and  $\alpha 2$  (COL1A2) chains of collagen type I, a major component of RCM, as CECs enter endothelial to mesenchymal transition [28,31,34-38]. We have also reported previously that FGF2 upregulates expression of SNAI1, which, in turn, leads to increased expression of Cdk2 and ZEB1, followed by induction of proliferation and type I collagen expression in human CECs [36]. Moreover, ZEB1 plays a central role in mediating fibrosis in endothelial to mesenchymal transition in human corneal endothelium *ex vivo* [36]. Although the downstream effects of IL-1 $\beta$  and FGF2 have been studied extensively in the human corneal endothelium *in vitro* and *ex vivo*, regulation of endothelial to mesenchymal transition in the corneal endothelium *in vivo* has not been investigated. Investigating CECs in the laboratory is difficult from a technical standpoint because their behavior is dependent on their environment; that is, they behave differently *in vitro* versus *ex vivo* versus *in vivo*, and the lack of a suitable *in vivo* model. In the present study, we present evidence that following surgical injury of the corneal endothelium, IL-1 $\beta$  and FGF2 are secreted into the aqueous humor in the mouse eye. FGF2 induces expression of endothelial to mesenchymal transition markers *Snail* and *Zeb1*, which, in turn, leads to upregulation of fibrosis- and proliferation-related genes in the mouse corneal endothelium *in vivo*, much like in the human corneal endothelium *ex vivo*. The results validate siRNA knockdown *in vivo* in the mouse and indicate that the mouse can serve as an *in vivo* model to investigate regulation of EndoMT.

## METHODS

**Animal husbandry and anesthesia:** All mouse experiments were performed in accordance with the protocol approved by the University of Southern California Institutional Animal Care and Use Committee and adhered to the ARVO Statement for Use of Animals in Research. The mice were housed in clear, air-filtered cages with a 12 h:12 h light-dark cycle and *ad libitum* feeding. Nonobese diabetic (NOD) and C57BL/6 mouse breeding pairs were purchased from Taconic (Oxnard, CA) and Jackson Laboratories (Sacramento, CA),

respectively, and the colonies used in this study were bred in-house. Mice between ages of 12 and 14 weeks were used for all experiments. Contralateral uninjured eyes were used as controls in *in vivo* studies. Mice were anesthetized with an intraperitoneal injection of ketamine (60–70 mg/kg) and xylazine (5–10 mg/kg), and they were euthanized with cervical dislocation.

**Reagents:** Mouse FGF2 was purchased from Cell Signaling Technology (Danvers, MA). IL-1 $\beta$  and rhodamine-conjugated secondary antibody were obtained from Sigma-Aldrich (St. Louis, MO). Anti-ZO1 antibody (61–7300) was purchased from Invitrogen (Carlsbad, CA). Anti-FGF2 antibody (610,072) was purchased from BD Transduction Laboratories (San Jose, CA). Anti-IL-1 $\beta$  antibody (AF-401-NA) was obtained from R&D systems (Minneapolis, MN). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA).

**Ex vivo manipulation of mouse corneas:** Mouse corneas were excised from enucleated eyes, and the whole corneas were placed endothelial side up in individual wells of a 96-well tissue culture plate. Opti-MEM (Invitrogen-Gibco, Grand Island, NY) was used for organ cultures. The corneas were treated with IL-1 $\beta$  or FGF2 for 7 days during culturing. Following organ culture, the endothelium-Descemet's membrane complex was carefully stripped and processed for semiquantitative reverse transcription PCR (RT-PCR).

**Semiquantitative RT-PCR:** Total RNA extraction from the mouse corneal endothelium was performed as previously described [36] with a slight modification. Briefly, cDNA was synthesized with 1  $\mu$ g of RNA by using iScript reverse transcriptase (Bio-Rad, Hercules, CA) and oligo(dT) primers. Reverse transcription was performed at 42 °C for 90 min. Then, PCR was performed using the first strand cDNA equivalent to 0.05  $\mu$ g of starting RNA from each sample as the template. The specific primers used are shown in Table 1. The standard PCR conditions were as follows: 5 min at 94 °C, followed by 30 s at 94 °C, 45 s at 53 °C, 30 s at 72 °C, and a final extension for 4 min at 72 °C. The PCR cycles were optimized to ensure that the product intensity fell within the linear phase of amplification, and the annealing temperature was optimized for each primer.  $\beta$ -actin transcript was used as the internal loading control. The amplified products were separated on a 1.5% agarose gel with electrophoresis and visualized with Gel-Red staining, and the band intensity was analyzed using the Image Lab program from Bio-Rad. All PCR products were verified with DNA sequencing.

**Gene knockdown with siRNA:** The Accell SMARTpool system (Dharmacon, Pittsburgh, PA) was used for siRNA knockdown as previously reported [36,39]. The mouse

TABLE 1. DNA SEQUENCE OF THE FORWARD AND REVERSE PRIMERS.

Gene	Primers (5'-3')
$\beta$ -actin	F: GCAGGAGTACGATGAGTCCGG R: CTTGGGGGATGTTTGCTCCA
Col8a2	F: TGAGGGCCTAGTCTCCTTCCC R: ACAGCTCCAATCCACAGACGT
Col1a1	F: GGAAGCTTGGTCTCTTGCTT R: CCCCATGTCCCAGCAGGATTT
Col1a2	F: CCGTTCCTTGACATTGCACCT R: ACAACAGGTGTCAGGGTGTTA
Snai1	F: CAGGACTCCTTCCAGCCTTGG R: CCCTGCTGAGGCATGGTTACA
Zeb1	F: AGAGAAGCTGAAGCACTGGGG R: ACTGCCAGGCTTAAAGACATA
Cdh1	F: TGTTCCGGCTATGTGTCTGGGG R: GGGATAGGTCTCACCGCCTGT
Fibronectin	F: TAATCTTTCCAGCCCCACCTT R: CAGAGGTGTCTGGGTGACTTT
Vimentin	F: CCTCTGGTTGACACCCACTCA R: CGCTTTTGGGGTGTGAGTTGT
FGF2	F: TGCTGGCTTCTGTGAGTAGTG R: GCCCAGTTCGTTTCAGTGCCA
Keratocan	F: AACTGAGCTACCTGCGTCTGG R: AACTAATACACGTGGCCCCTG
Ccne1	F: CCTGCAGATGCTGTGCTCTAT R: CATCCACATTTGCTCACAAAC
Cdk2 (NOD)	F: GTGGTCTGACTTGACCCTGGG R: TGAGGCCGAACGCTAAACTA
Cdk2 (C57)	F: GTGGTCTGACTTGACCCTGGG R: CCAGCCAGTTCTGGGGATTC

corneal endothelium was transfected on a 96-well plate with 1.5  $\mu$ M Accell SMARTpool of siRNA targeting FGF2 in Accell delivery medium according to the manufacturer's instructions. Seventy-two hours after transfection, based on our experience with human ex vivo CEs [36], the medium was changed to medium containing IL-1 $\beta$ . Ten days after the addition of IL-1 $\beta$ , another transfection with 1.5  $\mu$ M Accell SMARTpool of siRNA targeting FGF2 was performed, and the corneas were cultured for 4 additional days in the presence of IL-1 $\beta$ . The efficacy of FGF2 siRNA was verified with FGF2 RT-PCR. The Accell non-targeting siRNA pool (Dharmacon) was used as a negative control, and transfection efficiency was confirmed with Accell Red Cyclophilin B Control siRNA (Dharmacon).

*Surgical injury of corneal endothelium and siRNA knock-down in vivo:* Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. A paracentesis was made using a 30-gauge needle at the edge of the right

cornea, and a bent-30-gauge needle was introduced into the anterior chamber through the paracentesis to scrape the endothelium. All procedures were performed under direct visualization using an operating microscope, and care was taken not to injure the lens. For siRNA knockdown in vivo, 3  $\mu$ l of aqueous humor was aspirated, and then 3  $\mu$ l of 1.5  $\mu$ M Accell SMARTpool (Dharmacon, Pittsburgh, PA) of FGF2 siRNA or non-targeting siRNA in Accell delivery medium was injected into the anterior chamber 24 h before surgical injury. The uninjected and uninjured contralateral eye was used as the control. Mice were euthanized by intraperitoneal injection of ketamine (60–70 mg/kg) – xylazine (5–10 mg/kg) mixture followed by cervical dislocation, and their eyes were enucleated. Control and injured corneas were excised from the enucleated eyes and processed for RT-PCR and immunofluorescence imaging.

*Detection of aqueous proteins and histopathology:* Mice were anesthetized by intraperitoneal injection of ketamine

(60–70 mg/kg) – xylazine (5–10 mg/kg) mixture 24 and 30 h following surgical injury, and aqueous humor was collected using a 36-gauge needle affixed to a 10  $\mu$ l Hamilton syringe through the paracentesis under direct visualization using an operating microscope. Two to three microliters of 3  $\mu$ l aqueous humor was aspirated from the uninjured and injured eyes. A protease inhibitor cocktail (Cell Signaling Technology) was added immediately to prevent degradation of the proteins. The total protein concentration in the aqueous humor samples was measured with the Bradford protein assay kit (Bio-Rad) on the day of sample collection. The samples were placed on ice until testing and were analyzed with immunoblotting. Fifteen percent polyacrylamide gel was used for detection of FGF2 [17] and IL-1 $\beta$  [40] in aqueous humor.

**Histology:** Whole eyeballs were fixed in 10% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) at room temperature overnight. The eyes were dehydrated in a series of ethanol baths, treated with xylol, and then embedded in paraffin with the axis of the wound perpendicular to the bottom of the mold. Corneal cross sections were cut at 5  $\mu$ m thickness, and then deparaffinized with a series of xylol and alcohol rinses. The sections were rinsed in PBS three times for 5 min each, followed by a final rinse with distilled water. The sections were then stained with hematoxylin and eosin (H&E).

**Statistical analysis:** Means and standard error of the means (SEM) were calculated for each data set. One-way ANOVA (ANOVA) was performed to compare the means within the groups, and post hoc Tukey's honestly significant difference intervals at 0.05 and 0.01 levels of confidence were calculated for pairwise comparisons among the means in each group.

## RESULTS

**Injury induces IL-1 $\beta$  and FGF2 production in the mouse corneal endothelium in vivo:** We previously reported that transcorneal freezing induces sequential expression of IL-1 $\beta$  and FGF2 in the rabbit corneal endothelium in vivo [6]. To test the roles of IL-1 $\beta$  and FGF2 in corneal wound healing in the NOD mice, the corneal endothelium was surgically injured in the mouse in vivo. Hematoxylin and eosin staining showed a well-organized monolayer of normal corneal endothelial cells in the uninjured control cornea (Figure 1A). Thickened and disorganized Descemet's membrane with abnormal corneal endothelial cells was observed in the cornea 4 weeks after the surgical injury (Figure 1A). IL-1 $\beta$  and FGF2 could be detected by immunoblotting in the aqueous humor of the mouse following the surgical injury. The 17-KDa active form of IL-1 $\beta$  was detected in the aqueous humor 24 h after the surgical injury, but the amount was dramatically reduced

at 30 h after injury. No IL-1 $\beta$  was detected in the aqueous humor of the control uninjured eyes (Figure 1B). The 18-KDa isoform of FGF2 was detected in the aqueous humor 30 h after the surgical injury but not in the aqueous humor of the uninjured control eyes (Figure 1C). Interestingly, the amount of FGF2 was statistically significantly lower at 24 h than at 30 h after injury. These results are consistent with our previous findings that only the 18-KDa FGF2 is secreted into the extracellular space among the isoforms of FGF2 [41] and the secretion of IL-1 $\beta$  precedes expression of FGF2 [6,7,17].

**FGF2 induces endothelial to mesenchymal transition in the mouse corneal endothelium ex vivo:** The role of FGF2 in endothelial to mesenchymal transition of the human corneal endothelium ex vivo [36] led us to investigate whether FGF2 plays a similar role in the mouse corneal endothelium. Similar to the human corneal endothelium, FGF2 stimulation led to overexpression of *Snail* and *Zeb1*, starting at 2 days and rising steadily until 7 days post FGF2 treatment, in the mouse corneal endothelium ex vivo (Figure 2A). Time-dependent activation of *Colla1*, *Colla2*, fibronectin (*Fn1*), and vimentin (*Vim*) and suppression of E-cadherin (*Cdh1*) expression were also observed in response to FGF2 in the mouse corneal endothelium ex vivo (Figure 2B). Next, we investigated the role of the FGF2 in CEC proliferation in the mouse corneal endothelium ex vivo. FGF2 has been shown to induce proliferation following endothelial injury in the human corneal endothelium ex vivo [36]. FGF2 induced expression of *Cdk2* and Cyclin E1 (*Ccne1*) in a time-dependent manner in the mouse corneal endothelium ex vivo (Figure 2C). The expression of  $\alpha$ 2 chain of collagen type VIII (*Col8a2*), a corneal endothelial marker, and  $\beta$ -actin (*Actb*), the loading control, were not altered by FGF2 (Figure 2D).

To determine whether the strain of the mouse has an impact, we repeated the RT-PCR experiments in the C57BL/6 strain of mice. Similar to the NOD mice, FGF2 increased the expression of *Snail* and *Zeb1* in the C57BL/6 mouse corneal endothelium ex vivo (Figure 2E). Moreover, expression of *Colla1*, *Colla2*, *Fn1*, and *Vim* was also increased, while expression of *Cdh1* was decreased by in the C57BL/6 mouse corneal endothelium ex vivo (Figure 2F). FGF2 also induced *Cdk2* and *Ccne1* (Figure 2G), while there were no changes in the *Col8a2* and *Actb* expression in the C57BL/6 mouse corneal endothelium ex vivo. We used NOD mice for all the subsequent experiments because of the easier visualization of the anterior segment anatomy in the NOD mice.

**IL-1 $\beta$  induces endothelial to mesenchymal transition through FGF2 in the mouse corneal endothelium ex vivo:** It has been previously reported that IL-1 $\beta$  activates endothelial to mesenchymal transition through FGF2 in the rabbit [6,40] and human

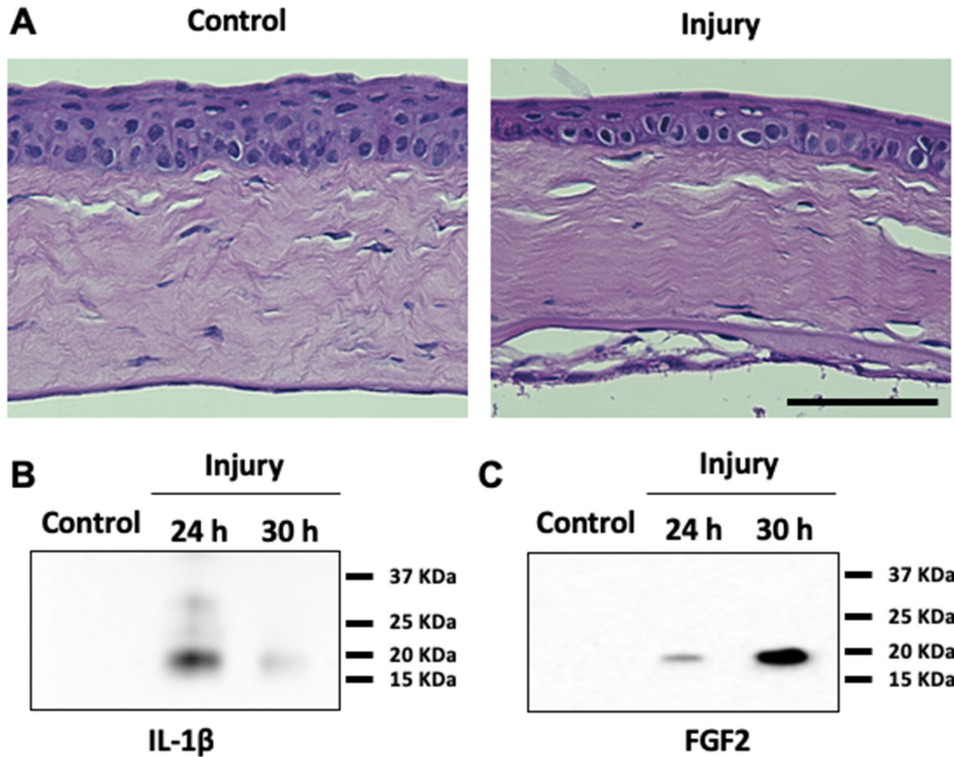


Figure 1. Surgical injury of the corneal endothelium induces PMN infiltration into the corneal endothelium and secretion of IL-1 $\beta$  and FGF2 into aqueous humor. **A:** At 4 weeks after surgical injury, hematoxylin and eosin (H&E) staining showed thickening and disorganization of Descemet's membrane with altered endothelial cells in the injured cornea. The uninjured control cornea showed a well-organized monolayer of corneal endothelial cells. Scale bar, 50  $\mu$ m. **B:** At 24 and 30 h after surgical injury, aqueous humor from ten anesthetized mice was collected, and immunoblotting was performed with an anti-interleukin 1 beta (anti-IL-1 $\beta$ ) antibody. IL-1 $\beta$  was not detected in the aqueous humor of the uninjured eyes, whereas IL-1 $\beta$  could be detected at 24 h but not 30 h after surgical injury. **C:** At

24 and 30 h after the surgical injury, the aqueous humor from ten anesthetized mice was collected, and immunoblotting was performed with anti-FGF2 antibody. FGF2 was not detected in the aqueous humor of the uninjured eyes, while FGF2 could be detected at 24 h and increased through 30 h after injury. Equal amounts of protein (2.5  $\mu$ g) of aqueous humor from uninjured and injured mice were loaded for the immunoblotting. The data shown are representative of the results in three independent experiments.

[17] corneal endothelium. To investigate the role of IL-1 $\beta$  in endothelial to mesenchymal transition in the mouse ex vivo corneal endothelium, we used siRNA-mediated knockdown of FGF2. IL-1 $\beta$  stimulation in the mouse corneal endothelium ex vivo dramatically induced FGF2 mRNA. FGF2 siRNA transfection in the mouse corneal endothelium ex vivo led to knockdown of the IL-1 $\beta$ -dependent expression of FGF2 mRNA (Figure 3A). FGF2 knockdown was not observed with non-targeting control siRNA transfection (Figure 3A). The endothelial to mesenchymal transition-related transcription factors *Snail* and *Zeb1* were highly induced by the IL-1 $\beta$  treatment, and these IL-1 $\beta$ -dependent inductions were inhibited with FGF2 siRNA knockdown in the mouse corneal endothelium ex vivo (Figure 3A). FGF2 knockdown also led to inhibition of the IL-1 $\beta$ -dependent expression of *Colla1*, *Colla2*, fibronectin (*Fnl*), and vimentin (*Vim*) and inhibited the IL-1 $\beta$ -dependent suppression of E-cadherin (*Cdh1*) expression in the mouse corneal endothelium ex vivo (Figure 3B). IL-1 $\beta$  also activated expression of *Cdk2* and cyclin E1 (*Ccn1*; Figure 3C), but this was abrogated by FGF2 knockdown. Non-targeting control siRNA transfection did not affect the expression of *Col8a2* and *Actb* (Figure 3D).

These results suggest that FGF2 acts downstream of IL-1 $\beta$  during endothelial to mesenchymal transition in the mouse corneal endothelium ex vivo.

*Injury induces endothelial to mesenchymal transition through FGF2 in the mouse corneal endothelium in vivo:* We next investigated the role of FGF2 in regulation of endothelial to mesenchymal transition in the mouse corneal endothelium in vivo by performing a surgical injury with FGF2 siRNA knockdown. FGF2 siRNA transfection before the surgical injury inhibited activation of FGF2 and the injury-dependent expression of *Snail* and *Zeb1* in the mouse corneal endothelium in vivo (Figure 4A). Moreover, FGF2 knockdown inhibited injury-dependent activation of *Colla1*, *Colla2*, *Fnl*, and *Vim*, and reversed the injury-dependent inhibition of *Cdh1* (Figure 4B). Interestingly, the surgical injury also induced expression of *Cdk2* and *Ccn1*, and this was inhibited by FGF2 knockdown in the mouse corneal endothelium in vivo (Figure 4C). These effects were not observed with non-targeting siRNA transfection, and keratocan (*Ktn*), a corneal epithelial and stromal marker [42], was used to control for stromal and epithelial cell contamination (Figure 4D). There

were no changes in the expression of *Col8a2* and *Actb* in response to the injury or siRNA transfection. These results suggest that injury-induced endothelial to mesenchymal transition proceeds through FGF2 in the mouse corneal endothelium in vivo.

*Inhibition of FGF2 signaling blocks the RCM formation induced by surgical injury in the mouse corneal endothelium in vivo:* We then investigated the role of FGF2 in

RCM formation in the mouse corneal endothelium in vivo by performing surgical injury with or without FGF2 siRNA knockdown. Hematoxylin and eosin staining of the cornea 14 weeks after the surgical injury showed a multilayered and disorganized Descemet’s membrane in the absence of FGF2 siRNA knockdown (Figure 5B). Blocking of FGF2 signaling with siRNA knockdown inhibited the RCM formation induced by the surgical injury (Figure 5C). Interestingly, a

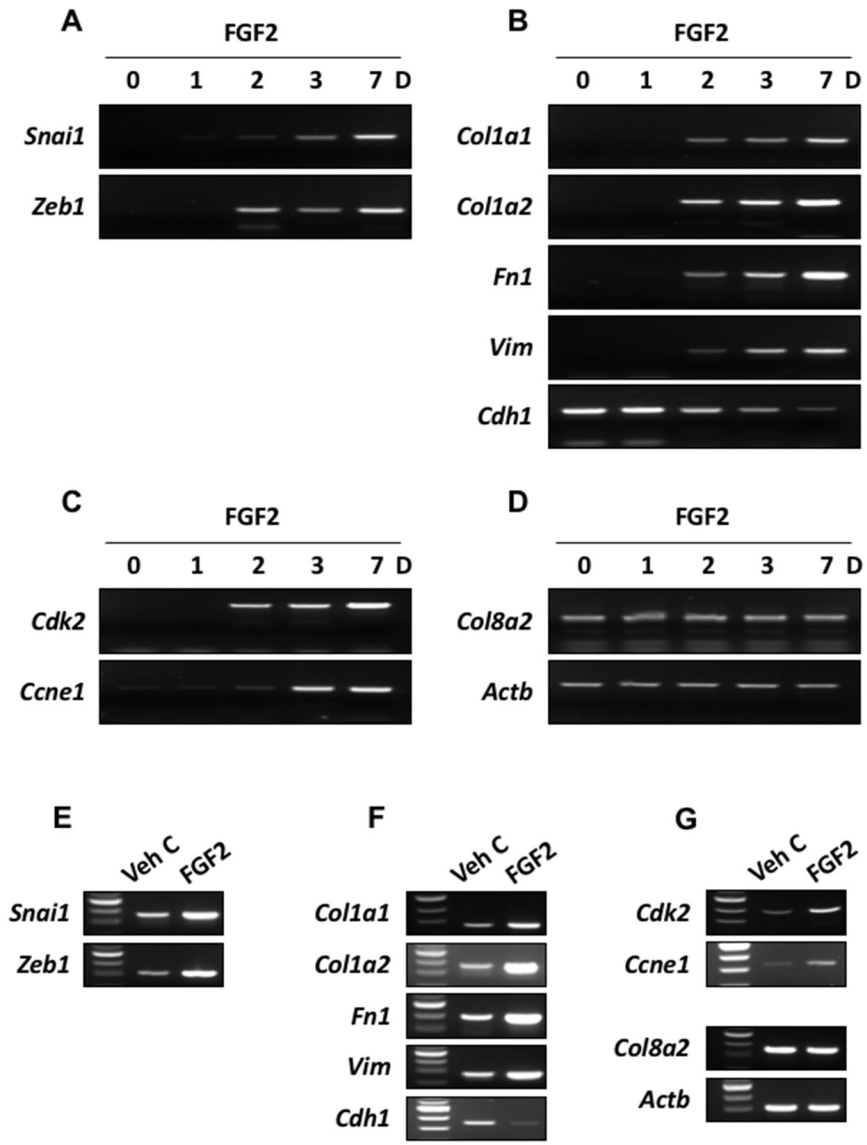


Figure 2. FGF2 regulates endothelial to mesenchymal transition-related gene expression in a time-dependent manner in mouse corneal endothelium ex vivo. Fifteen ex vivo corneas from nonobese diabetic (NOD; A, B, C, and D) and C57BL/6 (E, F, and G) mice were cultured for the indicated times (0, 1, 2, 3, or 7 days) with fibroblast growth factor 2 (FGF2). The endothelium–Descemet’s membrane complex was isolated, and total RNA was purified for reverse transcriptase PCR (RT-PCR). A: Increased expression of *Snai1* and *Zeb1* was noted in the FGF2-treated mouse corneal endothelium ex vivo as early as 2 days post-treatment. B: The FGF2 treatment led to increased expression of *Colla1*, *Colla2*, fibronectin (*Fn1*), and vimentin (*Vim*), and suppression of E-cadherin (*Cdh1*) in a time-dependent manner in the mouse corneal endothelium ex vivo. C: Increased expression of *Cdk2* and *Ccne1* were also noted in FGF2-treated corneas in a time-dependent manner. D: The expression of *Col8a2*, a corneal endothelial marker, and  $\beta$ -actin (*Actb*), loading control, was not affected by FGF2. The ex vivo corneal endothelium from the C57BL/6 mouse strain was used to

determine whether there were any strain differences in the FGF2 response. After being cultured for 7 days with FGF2, total RNA from mouse corneal endothelium ex vivo was purified, and RT-PCR was performed. E: Marked induction of the endothelial to mesenchymal transition markers *Snai1* and *Zeb1* was observed in the FGF2-treated but not in the vehicle control (Veh C) mouse corneal endothelium ex vivo. F: FGF2 treatment led to increased expression of *Colla1*, *Colla2*, fibronectin (*Fn1*), and vimentin (*Vim*), and suppression of E-cadherin (*Cdh1*) in the mouse ex vivo corneal endothelium. G: Increased expression of *Cdk2* and Cyclin E1 (*Ccne1*) was noted in the FGF2-treated but not in the control mouse corneal endothelium ex vivo. *Col8a2* and  $\beta$ -actin (*Actb*) expression was not affected by FGF2. The data shown are representative of the results in three independent experiments. Veh C, vehicle control.

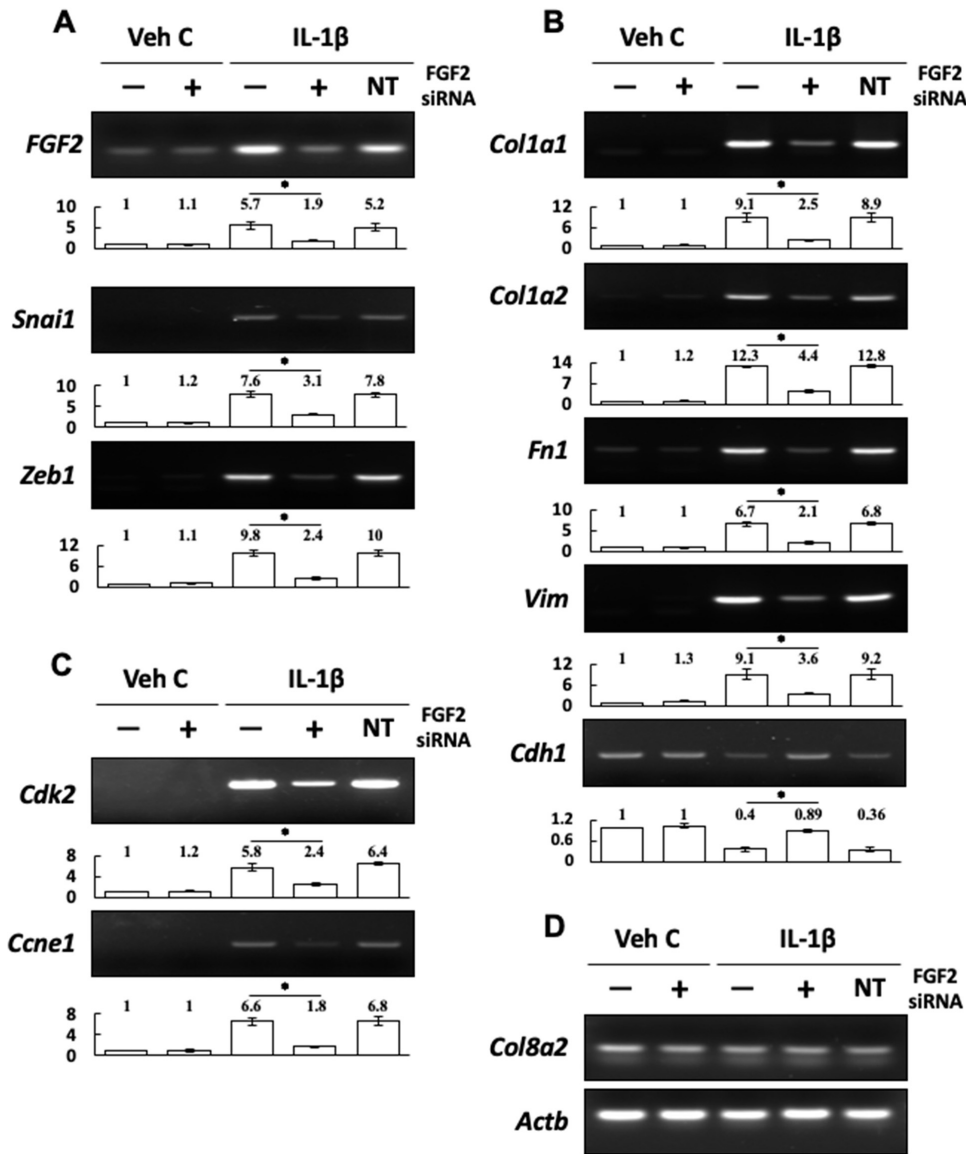


Figure 3. IL-1 $\beta$  regulates endothelial to mesenchymal transition-related gene expression through FGF2 in the mouse corneal endothelium ex vivo. The mouse corneal endothelium ex vivo was transfected with either fibroblast growth factor 2 (FGF2) siRNA or non-targeting control siRNA (NT) and then maintained in organ culture with interleukin-1 beta (IL-1 $\beta$ ) or vehicle control (Veh C). Total RNA was isolated at 14 days post-transfection. **A**: Induction of FGF2 with IL-1 $\beta$  stimulation was knocked down by FGF2 siRNA at up to 14 days post transfection. RT-PCR showed inhibition of IL-1 $\beta$ -dependent expression of Snai1 and Zeb1 by FGF2 siRNA but not with non-targeting (NT) control siRNA. FGF2 one-way ANOVA,  $F(4,10)=43.4$ ,  $p=0.002$ ,  $n=3$  per sample. Tukey's post-hoc test, honestly significant difference (HSD)[0.05]=1.6 and HSD[0.01]=2.1. Snai1 one-way ANOVA,  $F(4,10)=208.8$ ,  $p<0.0001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.1 and HSD[0.01]=1.5. Zeb1 one-way ANOVA,  $F(4,10)=182.8$ ,  $p<0.0001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.6 and HSD[0.01]=2.1. **B**: IL-1 $\beta$ -dependent expression of Col1a1, Col1a2, fibronectin (Fn1), and vimentin (Vim) was also inhibited by FGF2 siRNA but not non-targeting (NT) control

siRNA in the mouse corneal endothelium ex vivo. Suppression of E-cadherin (Cdh1) with the IL-1 $\beta$  treatment was also reversed with FGF2 siRNA knockdown. Col1a1 one-way ANOVA,  $F(4,10)=68.0$ ,  $p=0.001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=2.3 and HSD[0.01]=3.1. Col1a2 one-way ANOVA,  $F(4,10)=759.0$ ,  $p<0.0001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.0 and HSD[0.01]=1.3. Fn1 one-way ANOVA,  $F(4,10)=186.7$ ,  $p<0.0001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=0.1 and HSD[0.01]=1.3. Vim one-way ANOVA,  $F(4,10)=53.9$ ,  $p=0.001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=2.6 and HSD[0.01]=3.4. Cdh1 one-way ANOVA,  $F(4,10)=122.0$ ,  $p=0.0002$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=0.14 and HSD[0.01]=0.19. **C**: FGF2 siRNA also knocked down IL-1 $\beta$ -dependent expression of Cdk2 and Ccne1. Cdk2 one-way ANOVA,  $F(4,10)=152.9$ ,  $p=0.001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.0 and HSD[0.01]=1.3. Ccne1 one-way ANOVA,  $F(4,10)=104.8$ ,  $p=0.0003$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.4 and HSD[0.01]=1.8. **D**) Expression of Col8a2 and  $\beta$ -actin (Actb) was not affected by siRNA. The data are representative of the results in three experiments. NT, non-targeting control; Veh C, vehicle control. \*  $p<0.05$ .

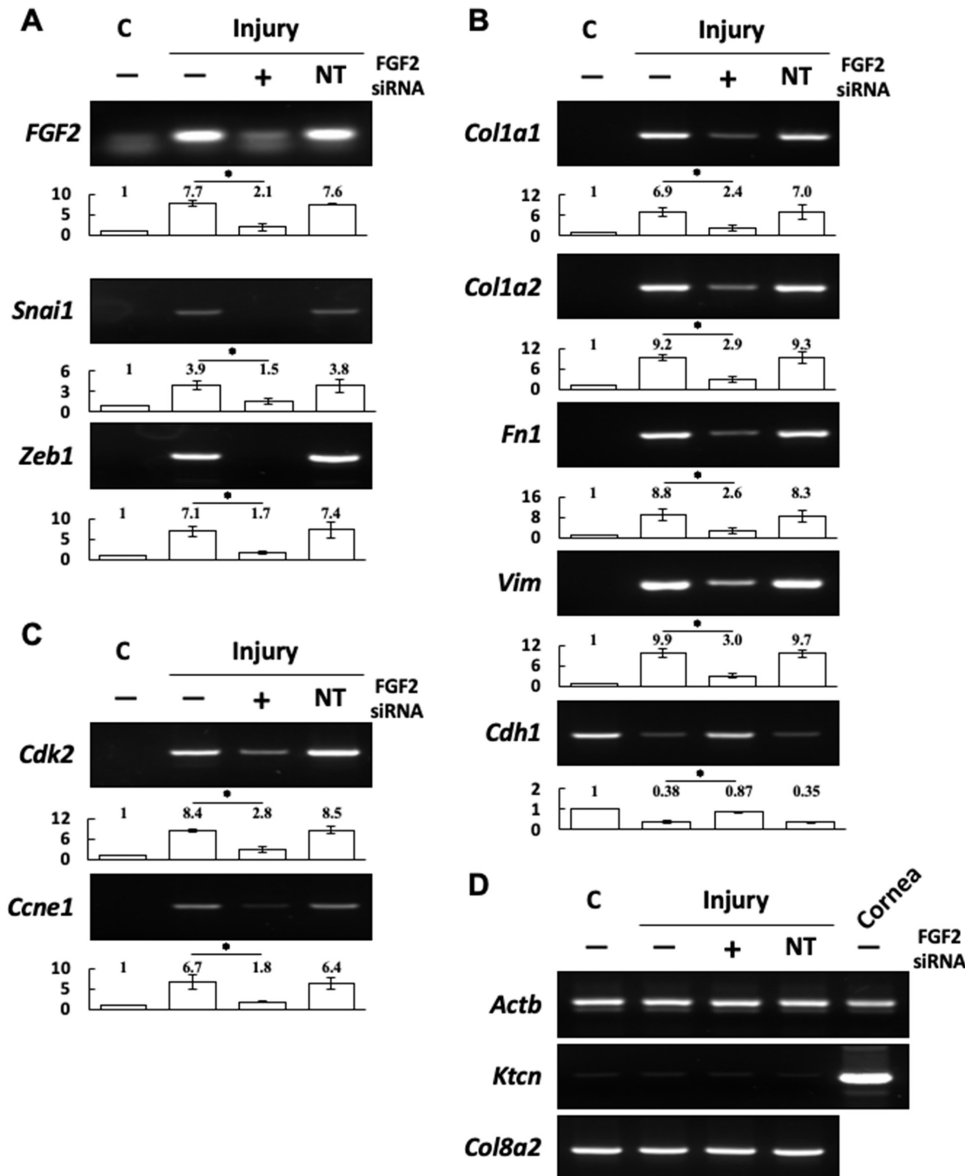


Figure 4. Surgical injury induces fibrosis- and proliferation-related genes through FGF2 in the mouse corneal endothelium in vivo. **A:** Surgical injury induced *FGF2* and endothelial to mesenchymal transition markers *Snai1* and *Zeb1* in the mouse corneal endothelium in vivo. This could be inhibited by fibroblast growth factor 2 (FGF2) knockdown using FGF2 siRNA. Non-targeting siRNA (NT) had no effect on injury-induced gene expression. The uninjured control corneal endothelium showed no expression of *Snai1* and *Zeb1*. FGF2 one-way ANOVA,  $F(3,8)=127.7$ ,  $p=0.001$ ,  $n=3$  per sample. Tukey's post-hoc test, honestly significant difference (HSD)[0.05]=1.4 and HSD[0.01]=2.0. *Snai1* one-way ANOVA,  $F(3,8)=18.0$ ,  $p=0.01$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.6 and HSD[0.01]=2.2. *Zeb1* one-way ANOVA,  $F(3,8)=25.4$ ,  $p=0.01$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=3.0 and HSD[0.01]=4.2. **B:** Surgical injury induced expression of *Colla1*, *Colla2*, *Fn1*, and *Vim*, and decreased expression of *Cdh1* in the mouse corneal endothelium in vivo. This could be inhibited by FGF2 knockdown using FGF2 siRNA. Non-targeting siRNA (NT) had no effect on injury-induced gene expression. The uninjured control corneal

endothelium showed no expression of *Colla1*, *Colla2*, *Fn1*, and *Vim*, and showed robust expression of *Cdh1*. *Colla1* one-way ANOVA,  $F(3,8)=16.3$ ,  $p=0.02$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=3.5 and HSD[0.01]=4.8. *Colla2* one-way ANOVA,  $F(3,8)=54.9$ ,  $p=0.005$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=2.6 and HSD[0.01]=3.6. *Fn1* one-way ANOVA,  $F(3,8)=14.9$ ,  $p=0.03$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=4.6 and HSD[0.01]=6.4. *Vim* one-way ANOVA,  $F(3,8)=77.8$ ,  $p=0.003$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=2.3 and HSD[0.01]=3.2. *Cdh1* one-way ANOVA,  $F(3,8)=359.2$ ,  $p=0.0001$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=0.080 and HSD[0.01]=0.12. **C:** Surgical injury-induced expression of *Cdk2* and *Ccne1* in the mouse corneal endothelium in vivo. This could be inhibited by FGF2 knockdown using FGF2 siRNA. Non-targeting siRNA (NT) had no effect on injury-induced gene expression. The uninjured control corneal endothelium showed no expression of *Cdk2* and *Ccne1*. *Cdk2* one-way ANOVA,  $F(3,8)=102.3$ ,  $p=0.002$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=1.7 and HSD[0.01]=2.4. *Ccne1* one-way ANOVA,  $F(3,8)=21.7$ ,  $p=0.02$ ,  $n=3$  per sample. Tukey's post-hoc test, HSD[0.05]=2.9 and HSD[0.01]=4.0. **D)** Keratocan (*Ktcn*) was used as the control for contamination of the stromal keratocytes. *Col8a2* and  $\beta$ -actin (*Actb*) were used as the corneal endothelial cell (CEC) marker and the loading control, respectively. The data shown are representative of the results in three independent experiments. C, Non-injured normal corneal endothelium; NT, non-targeting control. \*  $p<0.05$ .



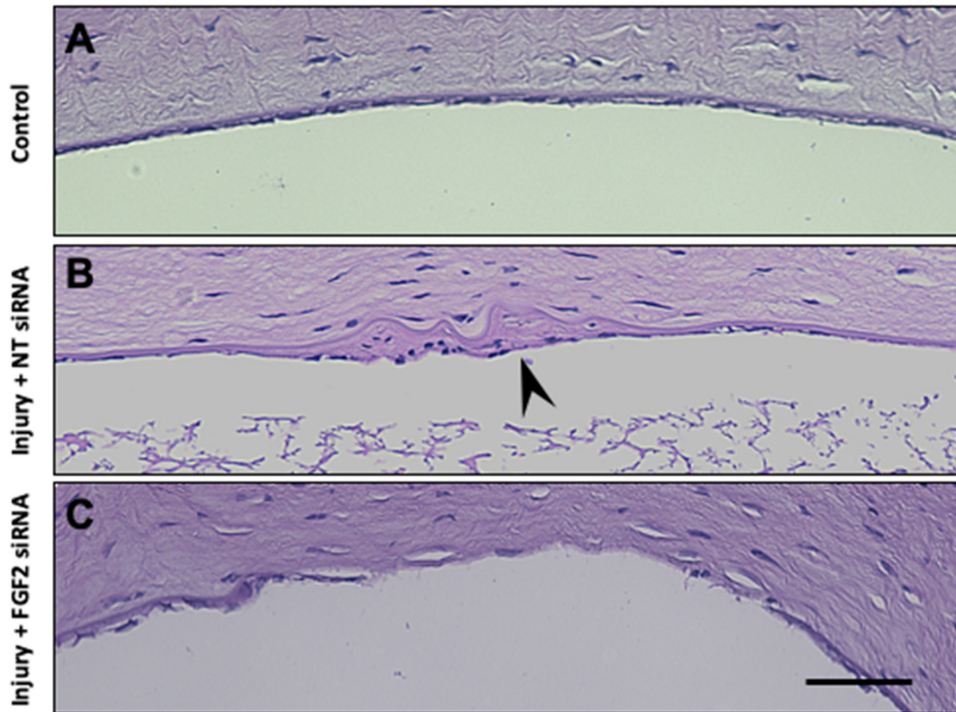


Figure 5. Inhibition of FGF2 signaling blocks the RCM formation induced by surgical injury in the mouse corneal endothelium *in vivo*. **A:** The uninjured control cornea showed a well-organized monolayer of corneal endothelial cells. **B:** At 14 weeks after surgical injury, hematoxylin and eosin (H&E) staining showed an extensive retrocorneal membrane (RCM) with thickening and disorganization of Descemet's membrane in the injured cornea (arrowhead). **C:** fibroblast growth factor 2 (FGF2) siRNA knockdown completely blocked RCM formation induced by surgical injury. FGF2 siRNA knockdown also left a persistent defect in Descemet's membrane and the endothelium 14 weeks after injury. Scale bar, 50  $\mu\text{m}$ .

defect in Descemet's membrane and the endothelium was still present at 14 weeks post injury. In the uninjured control, the cornea showed a well-organized monolayer of normal corneal endothelial cells (Figure 5A).

## DISCUSSION

Endothelial to mesenchymal transition in the corneal endothelium can lead to RCM formation that often results in irreversible vision loss due to the progressive and fibrotic nature of RCM [43,44]. Although RCM formation is undesirable, endothelial to mesenchymal transition in the corneal endothelium could be exploited therapeutically for vision loss due to endothelial dysfunction if the individual components, *i.e.*, migration, fibrosis, and proliferation, can be regulated independently of one another. Current challenges in investigating endothelial-mesenchymal transition in the human cornea include the environment-dependent behavior of CECs, *e.g.*, markers or signals critical *in vitro* are not active *in vivo*, and the lack of a suitable model for human corneal endothelial behavior *in vivo*. In this study, we attempted to address the latter problem, and present evidence that the mouse corneal endothelium *in vivo* behaves in a similar manner to the human corneal endothelium *ex vivo* in response to injury.

IL-1 $\beta$  is a major proinflammatory cytokine in the human eye [45-47]. Previous studies have reported that IL-1 $\beta$  mediates the inflammatory process by inducing the production

and release of secondary cytokines which are necessary for the wound repair process in a variety of cell types [12,13]. In human CECs *in vitro*, IL-1 $\beta$  has been shown to induce secretion of the 18-kDa isoform of FGF2 through AP-1 and nuclear factor kappa beta (NF- $\kappa$ B) [17-19]. Much like in the human corneal endothelium, we observed sequential secretion of IL-1 $\beta$  (Figure 1B) and FGF2 (Figure 1C) into the aqueous humor following surgical injury of the mouse corneal endothelium *in vivo*. Moreover, the thickening and disorganization of Descemet's membrane following surgical injury (Figure 1A) is highly suggestive of early retrocorneal membrane formation in the mouse corneal endothelium *in vivo*. This demonstrates similarities in the early phase of endothelial to mesenchymal transition among human, rabbit, and mouse corneal endothelia [37,48].

FGF2 has previously been shown to be an initiator of endothelial to mesenchymal transition in the corneal endothelium in multiple species, including humans [49-51]. Our previous report showed that FGF2 induces expression of SNAI1, an endothelial to mesenchymal transition marker, which, in turn, activates expression of ZEB1 and Cdk2/Cyclin E1 (CCNE1). ZEB1 further induces endothelial to mesenchymal transition through suppression of E-cadherin and activation of type I collagen, fibronectin, and vimentin expression in the human corneal endothelium *ex vivo* [36]. Much like in the human corneal endothelium *ex vivo*, IL-1 $\beta$

and FGF2 induced expression of *Snai1* and *Zeb1* in the mouse corneal endothelium ex vivo in a time-dependent manner (Figure 2 and Figure 3). Concomitant with the increased expression of *Snai1* and *Zeb1*, there also were increases in the expression of *Colla1*, *Colla2*, *Vim*, *Fn1*, *Cdk2*, and *Ccne1*, along with a decrease in *Cdh1* expression. Moreover, the IL-1 $\beta$ -mediated endothelial to mesenchymal transition could be abrogated with FGF2 siRNA knockdown, indicating FGF2 acts downstream of IL-1 $\beta$  in the mouse corneal endothelium ex vivo. This strongly suggests that the mouse corneal endothelium ex vivo and the human corneal endothelium ex vivo behave in a similar manner. There also does not appear to be any differences in the FGF2 response between the NOD and C57BL/6 strains of mice (Figure 2). Surgical injury induced expression of FGF2 (Figure 1), and this is followed

by increased expression of *Colla1*, *Colla2*, *Vim*, *Fn1*, *Cdk2*, and *Ccne1*, along with decreased expression of *Cdh1* in the mouse corneal endothelium in vivo (Figure 4). FGF2 siRNA knockdown attenuated surgery-induced endothelial to mesenchymal transition (Figure 4) and prevented injury-induced RCM formation (Figure 5) in the mouse corneal endothelium in vivo. Moreover, the persistent endothelial and Descemet's membrane defect 14 weeks after surgical injury (Figure 5C) indicates that FGF2 is critical for wound closure following surgical injury and plays a central role in EndoMT. The present data strongly suggest that regulation of endothelial to mesenchymal transition is very similar between the human corneal endothelium ex vivo and the mouse corneal endothelium in vivo, and support the use of the mouse as an

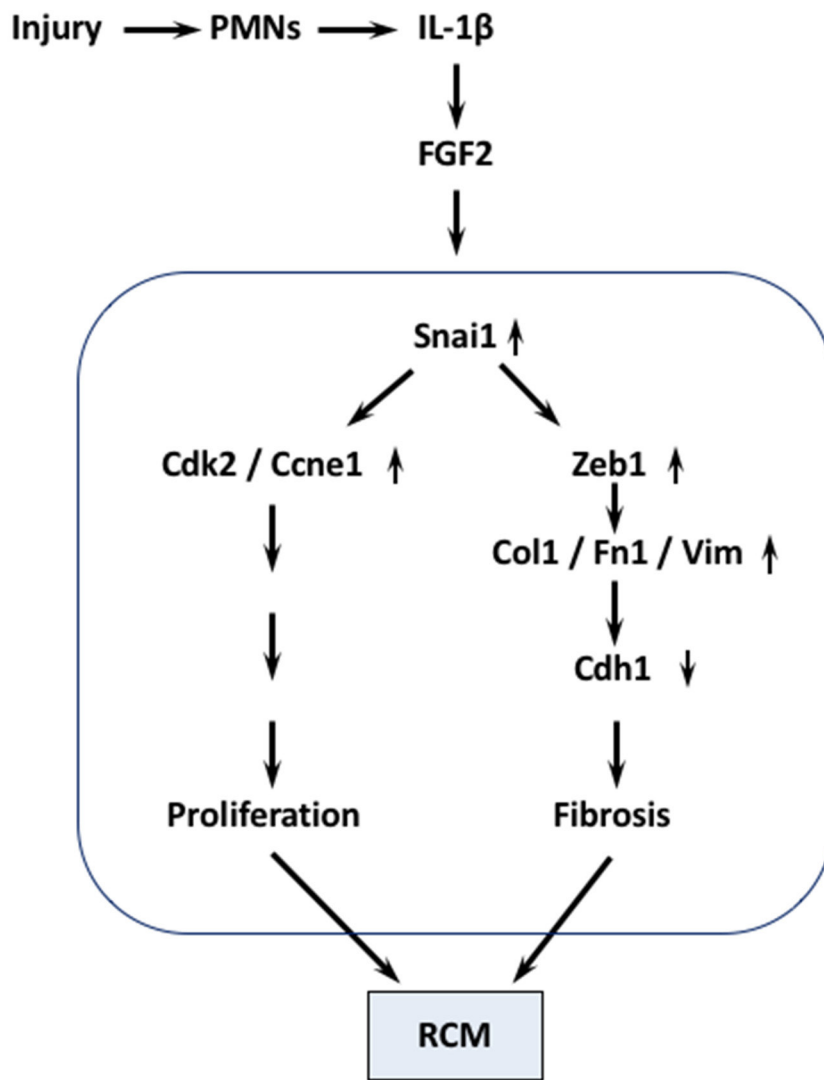


Figure 6. Regulation of the endothelial to mesenchymal transition in the mouse corneal endothelium in vivo. Interleukin-1 beta (IL-1 $\beta$ ) induced by surgical injury activates FGF2 expression. The endothelial to mesenchymal transition is mediated through Snai1, Zeb1 and Cdk2 pathways, leading to the expression of fibrosis- and proliferation-related genes resulting in the formation of the retrocorneal membrane (RCM).

in vivo model for investigating regulation of endothelial to mesenchymal transition.

Previous studies focused on the prevention of RCM due to its progressive nature. However, modulation of individual components of endothelial to mesenchymal transition, that is, cell proliferation, migration, and fibrosis, may hold the key to developing novel therapeutic strategies for management of vision loss due to endothelial dysfunction. Although endothelial to mesenchymal transition involves a progressive change in cellular phenotype, cell proliferation, migration and fibrosis represent distinct phenotypes that could be under separate regulatory control. Our data in the human corneal endothelium ex vivo support this [36], and future therapies for vision loss could focus on promoting cell proliferation while simultaneously inhibiting fibrosis. *Zeb1* is an attractive candidate for investigation because it acts downstream of FGF2, and its inhibition led to decreased expression of *Colla1* and *Colla2* in the mouse corneal endothelium in vivo. In summary, induction of FGF2 through IL-1 $\beta$  in damaged corneal endothelium leads to increased expression of *Snail* and *Zeb1*. This then leads to increased expression of *Vim* and *Fnl* along with fibrosis-related genes *Colla1* and *Colla2*, and proliferation-related genes *Cdk2* and *Cene1* ultimately resulting in formation of a retrocorneal membrane (Figure 6).

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