

ZEB1 Mediates Fibrosis in Corneal Endothelial Mesenchymal Transition Through SP1 and SP3

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PURPOSE. ZEB1 is induced during endothelial-mesenchymal transition (EnMT) in the cornea. Induction of SP1 and SP3 by ZEB1 along with identification of putative SP1 and SP3 binding sites in promoters of EnMT-associated gene lead us to investigate their roles in retrocorneal membrane formation in the corneal endothelium.

METHODS. Expressions of SP1, SP3, and EnMT associated genes were analyzed by immunoblotting and semiquantitative reverse transcription polymerase chain reaction. Accell SMARTpool siRNAs targeting ZEB1, SP1, and SP3 were used for gene knockdown. SP1 and SP3 binding to promoters of EnMT associated genes was investigated by chromatin immunoprecipitation assay. Corneal endothelium in mice was surgically injured in vivo under direct visualization.

RESULTS. Transient Fibroblast Growth Factor 2 stimulation increased the expression of both SP1 and SP3 in the human corneal endothelium ex vivo. ZEB1 siRNA knockdown inhibited FGF2-induced SP1 mRNA and protein but not the expression of SP3. FGF2-induced expression of EnMT-related genes, such as fibronectin, vimentin, and type I collagen, was reduced by both SP1 and SP3 siRNA knockdown, with inhibition of SP1 having a greater inhibitory effect than SP3. Additionally, although SP1 and SP3 proteins were found to bind together, SP1 and SP3 could bind to the same promoter binding sites of EnMT-related genes in the absence of the other. Moreover, siRNA knockdown of Zeb1 inhibited injury-dependent RCM formation in mouse corneal endothelium in vivo.

CONCLUSIONS. Zeb1, through SP1 and SP3, plays a central role in mesenchymal transition induced fibrosis in the corneal endothelium and suggests that Zeb1 could be targeted to inhibit anterior segment fibrosis.

Keywords: mesenchymal transition, corneal endothelium, Zeb1, SP1, SP3

The cornea, composed of epithelium, stroma, and endothelium, is the anterior transparent tissue of the eye. The corneal endothelium is the inner most layer of the cornea and is composed of a single-cell layer arranged on Descemet's membrane. The corneal endothelial cells (CECs) function as a pump to maintain the corneal stroma in a slightly dehydrated state, and this function is critical for corneal transparency and sharp vision.¹ Adult human CECs are arrested at the G₁ phase of the cell cycle and mitotically inactive under normal circumstances.² Because of the cell cycle arrest, there is a progressive decline in the endothelial cell density with aging that can be further accelerated by injury or disease. Once the endothelial cell density reaches below a critical threshold, approximately 500 cells/mm², corneal edema occurs and leads to loss of transparency and vision.^{3,4} Vision loss secondary to endothelial dysfunction is a common indication for corneal transplantation in developed nations.

Although CECs are mitotically inactive, they have been shown to have proliferative potential and can be expanded in vitro. Prior studies reported induction of endothelial cell proliferation and disruption of contact inhibition by treat-

ment with growth factors such as Fibroblast Growth Factor, Epidermal Growth Factor, and transforming growth factor- β (TGF- β).⁵⁻¹⁰ Moreover, severely injured or inflamed CECs can undergo mesenchymal transition where they gain some fibroblastic phenotype. In endothelial-mesenchymal transition (EnMT), CECs lose their polarity and assume a fibroblastic phenotype and exhibit enhanced proliferation, migration, and type I collagen secretion.¹¹⁻¹⁶ Our previous study showed that surgical injury induces FGF2 expression and EnMT in the mouse corneal endothelium in vivo.¹⁷ These examples illustrate that although CEC may be coaxed to proliferate through EnMT, the resulting fibrosis from type I collagen secretion manifesting clinically as a retrocorneal membrane (RCM) is undesirable.¹⁸ An RCM is a gray, opaque membrane that can obscure the visual axis and cause loss of vision. Moreover, RCM can also involve the iridocorneal angle leading to glaucoma. Although fibrosis is undesirable, CEC proliferation could be exploited therapeutically.

Previous publications have reported on the signaling pathways regulating fibrosis in EnMT. Interleukin-1 β induces FGF2 expression, which in turn, activates SNAIL and stimulates both proliferation and fibrosis in the corneal

endothelium.^{11,19} Downstream of SNAI1, CDK2 regulates proliferation whereas the transcription factor ZEB1 was shown to regulate fibrosis through increased expression of type I collagen.¹⁹ The promoters of the fibrosis-related genes such as fibronectin (FN1), vimentin (VIM), and alpha 1 (COL1A1), and alpha 2 (COL1A2) chains of type I collagen do not contain putative ZEB1 binding sites. It is likely that there are other downstream mediators involved in ZEB1-induced expression of the fibrosis-related genes. Given the critical role ZEB1 plays in fibrosis, we sought to identify the downstream mediators of ZEB1 signaling in the corneal endothelium.

Specific protein (SP) 1 and 3, originally identified as transcription factors that bind to multiple Guanine-Cytosine-boxes, play critical roles in cell cycle regulation, and oncogenesis.^{20–23} Although both transcription factors bind to the same consensus binding site, they can either activate or repress transcription depending on the promoter and cellular context.^{21,24,25} Furthermore, coordinated transcription of COL1A1 and COL1A2, the main component of RCM, involves similar cis-elements interacting with SP1 and SP3 through direct binding of their promoter.^{26,27} Identification of putative SP1 and SP3 binding sites in COL1A1, FN1, and VIM promoters lead us to investigate their roles in EnMT-induced fibrosis in the corneal endothelium.

In this study, we show the transactivating roles of SP1 and SP3 in regulation of COL1A1, COL1A2, FN1, and VIM expression with SP1 having a greater role in the activation for COL1A1, FN1 and VIM expression in the human corneal endothelium *ex vivo*. We also provide evidence for SP1 and SP3 binding and show that ZEB1 siRNA knockdown inhibited injury induced RCM formation in mouse corneal endothelium *in vivo*. These results suggest that ZEB1 can be targeted to inhibit RCM formation and anterior segment fibrosis.

MATERIALS AND METHODS

Materials

FGF2 was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-COL1 (139 kDa, ab6308) and vimentin (54 kDa, ab92547) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti- β -actin (42 kDa, A5316) and peroxidase-conjugated secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against SP1 (81 kDa, GTX110593), SP3 (110 kDa, GTX129426) and fibronectin (260 kDa, GTX60570) were obtained from GeneTex (Irvine, CA, USA). Antibody against COL8A2 (67 kDa, sc-82843) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-ZEB1 (124 kDa, PA5–40350) antibody was purchased from Thermo Fisher (Waltham, MA, USA). Deidentified human *ex vivo* corneas were obtained OneLegacy Eye Bank (Los Angeles, CA, USA).

Cell Culture

Immortalized human CEC line human CEC-B4G12 (DSMZ, Braunschweig, Germany) was cultured as previously described.^{28–30} Briefly, human CEC-B4G12 was cultured in human endothelial-serum-free medium (SFM) supplemented with 10 ng/mL human recombinant FGF2 without antibiotics (SFM-F). Cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. For subculture, confluent cultures were treated with 0.05% trypsin

and 5 mmol/L ethylenediamine tetra-acetic acid (EDTA) in phosphate-buffered saline solution (PBS) for five minutes. Cells were plated in 100-mm tissue culture dishes coated with 10 mg/mL chondroitin-6-sulfate and 10 μ g/mL laminin at a concentration of 1×10^6 cells. Second passage human CEC maintained in SFM-F were used for all experiments. Culture medium was changed twice a week. All experiments included a vehicle control (2 mmol/L Dithiothreitol in PBS) sample.

Semi-Quantitative Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA was extracted from human corneal endothelium and performed RT-PCR as previously described.¹⁹ Briefly, cDNA was synthesized with 2 μ g of RNA by using iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA, USA) and oligo(dT) primer. Reverse transcription was performed at 42°C for 90 minutes. Then, the first-strand cDNA equivalent to 0.1 μ g of starting RNA from each sample was amplified by using the specific primer pairs. The specific primers used are shown in the Table. Standard PCR conditions were as follows: five minutes at 94°C, followed by 30 seconds at 94°C, 30 seconds at 53°C, 30 seconds at 72°C, and a final extension for four minutes at 72°C. PCR cycles were optimized to ensure that the product intensity fell within the linear phase of amplification, and annealing temperature were adjusted depend on the PCR primer (Table). RT-PCR amplification of β -actin transcript was used as the internal control to verify use of equal amounts of RNA from each sample. The amplified products were separated on a 1.5% agarose gel electrophoresis, visualized by Gel-Red staining, and then band intensity was analyzed with the Image Lab program from Bio-Rad. All positive target PCR bands were verified by DNA sequencing.

Gene Knockdown by siRNA

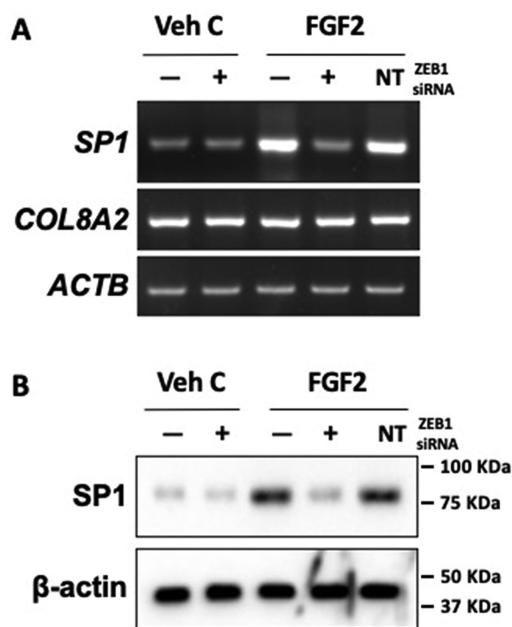
For gene knockdown by siRNA, Accell SMARTpool system was used as previously reported.²⁸ Corneal endothelial cells at 70% confluence or *ex vivo* corneal endothelium were transfected on six-well plate with 1.5 μ mol/L Accell SMARTpool of siRNA targeting ZEB1, SP1 or SP3 (Dharmacon, Pittsburgh, PA, USA) in Accell delivery medium according to the manufacturer's instructions. Seventy-two hours after transfection, the medium was changed to medium containing FGF2. Ten days after maintaining, another transfection with 1.5 μ mol/L Accell SMARTpool of siRNA targeting ZEB1, SP1 or SP3 were performed and maintained four more days with FGF2. RNA and protein levels of ZEB1, SP1, or SP3 were analyzed by RT-PCR and immunoblotting, respectively. Accell non-targeting pool siRNA (Dharmacon) was used as a negative control, and transfection efficiency was confirmed with Accell Red Cyclophilin B Control siRNA (Dharmacon).

Protein Preparation, Protein Assay, SDS PAGE, Immunoblotting Analysis and Coimmunoprecipitation

All assays were performed following previously reported protocols.^{19,30} The following gel concentrations were used to separate proteins: 10% polyacrylamide gel for COL8A2, vimentin, and β -actin, 8% polyacrylamide gel for SP1, SP3, type I collagen and ZEB1, and 6% polyacrylamide gel for

TABLE. DNA Sequence of the Forward and Reverse Primers, Annealing Temperature, and Number of Cycles Performed for the RT-PCR-Mediated Confirmation of the Different Expression of the Target Genes

Gene	Primers	Annealing Temp.	No. of Cycles	PCR Product Size
β -actin	Forward 5'-ATTGCCGACAGGATGCAGAAG-3' Reverse 5'-CTGTCACCTTCACCGTTCCAG-3'	55°C	22	371 bp
COL8A2	Forward 5'-TCCTCTCCTTCCACCCTCTGG-3' Reverse 5'-AGCAGTGCAACGAGATCACAG-3'	57°C	25	376 bp
COL1A1	Forward 5'-GAGTCACACCGGAGCCTGGGG-3' Reverse 5'-GCATGGGTCTTCAAGCAAGTG-3'	55°C	25	351 bp
COL1A2	Forward 5'-CATCCCAGCCAAGAAGTGGTA-3' Reverse 5'-GACTGGGCCAATGTCCACAAA-3'	53°C	23	460 bp
SP1	Forward 5'-TGATCTGCCTCTCAACTGCC-3' Reverse 5'-TTACCGGAAACTGGAGCACT-3'	57°C	23	222 bp
SP3	Forward 5'-CGCAGAAAGTCAGATGCCCT-3' Reverse 5'-TGGCTACCAGGCCTATGGAA-3'	57°C	23	192 bp
ZEB1	Forward 5'-TTAGTTGCTCCCTGTGCAGTTA-3' Reverse 5'-TGTGTGAGCTATAGGAGCCAGA-3'	53°C	23	261 bp
Fibronectin	Forward 5'-GCTTCTCCAAGCATCACCTG-3' Reverse 5'-CTACAGTATTGGGGCCAGAC-3'	55°C	25	262 bp
Vimentin	Forward 5'-TCACCTGTGAAGTGGATGCC-3' Reverse 5'-CAGCTTCCTGTAGGTGGCAAT-3'	55°C	25	233 bp

**FIGURE 1.** FGF2 induces SP1 expression through ZEB1 in human corneal endothelium ex vivo. Fourteen days after ZEB1 siRNA transfection of human ex vivo corneas, total RNA and protein were isolated from the endothelium. (A) RT-PCR and (B) immunoblotting showed ZEB1 siRNA knockdown inhibited FGF2-dependent expression SP1. Transfection with nontargeting control siRNA did not alter SP1 expression. Vehicle control did not alter SP1 expression. β -actin was used as a loading control and COL8A2 was used as a corneal endothelial marker. Veh C, vehicle control; NT, non-targeting control.

fibronectin. For purification of protein from human ex vivo corneal endothelium, after peeling off corneal endothelium and Descemet's membrane, cells were then lysed with 100 μ L of RIPA lysis buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Total protein was purified and concentrated with Amicon ultra

centrifugal filter devices (EMD Millipore, Burlington, MA, USA), according to the manufacturer's instructions. Briefly, cell lysates were applied to the Amicon ultra 10 K centrifugal device (molecular weight cutoff 10 K), and then spun down at 14,000g for 30 minutes. To recover the concentrated protein, the Amicon ultra filter device placed upside down in a clean tube that was then spun again in a centrifuge for 2 minutes at 1000g to transfer the concentrated protein sample from the device to the clean tube. Purified total proteins were used to for analysis of immunoblotting. For coimmunoprecipitation of SP1 and SP3, after purification of nuclear fraction, the purified nuclei were suspended with 10 volume of nucleus suspension medium (0.25 mol/L sucrose, 5 mmol/L MgCl₂, and 50 mmol/L Tris-Cl, pH 7.4), using three to four gentle pipetting and then incubated for one hour at 4°C with treatment of DNase I and RNase to a final concentration of 0.25 μ g/ μ L. Concentration of nuclear lysates were assessed with Bradford protein assay system (Bio-Rad) and then used for co-immunoprecipitation.

Chromatin Immunoprecipitation (ChIP) Assay

FGF2-starved human CEC were first transfected with 1.5 μ mol/L Accell SMARTpool of siRNA targeting SP1 or SP3 in Accell delivery medium. Seventy-two hours after transfection, the medium was changed to medium containing FGF2. Ten days after maintaining, another transfection with 1.5 μ mol/L Accell SMARTpool of siRNA targeting SP or SP3 was performed. The cells were then maintained with FGF2 for four more days before isolating protein-DNA complexes by use of anti-SP1 and SP3 antibodies. Binding of SP1 and SP3 to the *COL1A1*, *FNI*, and *VIM* promoter were evaluated by PCR using the following primer pairs. *COL1A1* (271 bp), F 5'-GTAGCCCA CGCCATTCTGAG-3' and R 5'-CCCGGGCCCCTTTTACTACTGT-3'; *FNI* (253 bp), F 5'-GCGCTGAGAAGGGAAGAAGTC-3' and R 5'-TTGAGGACATTGCGTCACTC-3'; *VIM* promoter (288 bp), F 5'-AGGGAGCGAGACAAAGGGATG-3' and R 5'-GCATCTCCTCCTGCAATCTGA-3'. Standard PCR conditions

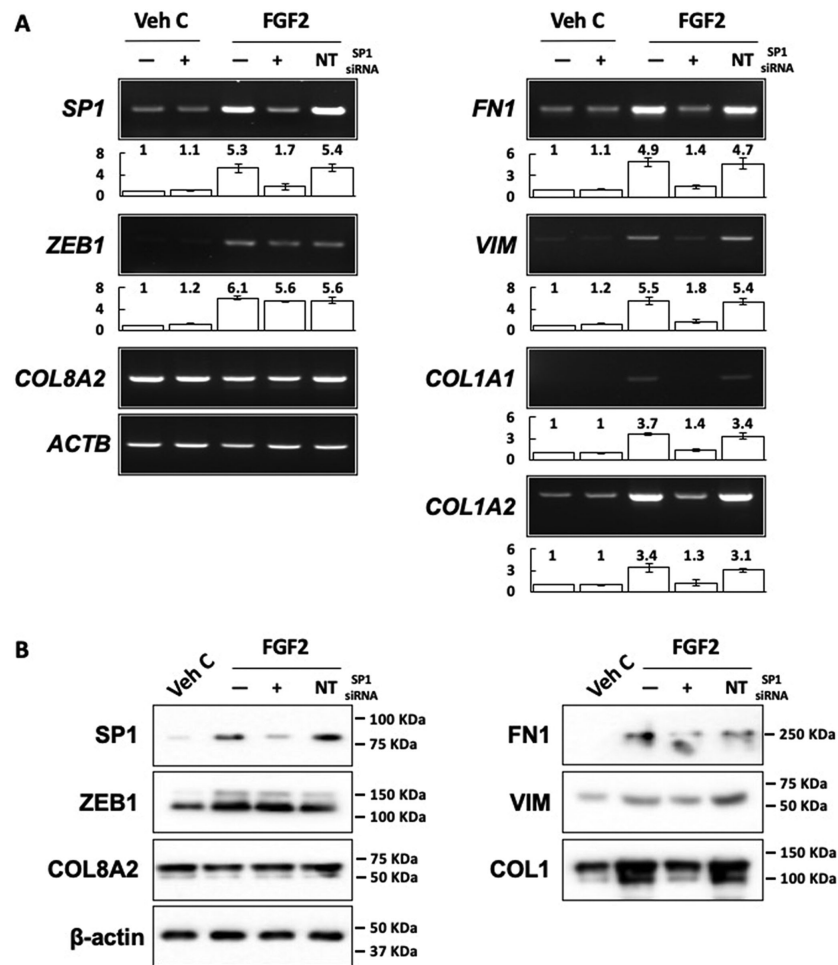


FIGURE 2. FGF2 regulates mesenchymal transition-related gene expression through SP1 in human corneal endothelium ex vivo. (A) SP1 siRNA knockdown attenuated FGF2-dependent expression of *SP1*, *FN1*, *VIM*, *COL1A1*, and *COL1A2* expression at transcriptional level in human ex vivo corneal endothelium. *ZEB1* expression level was not affected by SP1 siRNA knockdown. SP1 $F(4,10) = 52.1$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 1.4 and HSD[0.01] = 1.9. ZEB1 $F(4,10) = 213.7$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 0.8 and HSD[0.01] = 1.1. FN1 $F(4,10) = 56.8$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 1.2 and HSD[0.01] = 1.6. VIM $F(4,10) = 95.2$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 1.1 and HSD[0.01] = 1.4. COL1A1 $F(4,10) = 123.6$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 0.6 and HSD[0.01] = 0.7. COL1A2 $F(4,10) = 39.5$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, HSD[0.05] = 0.9 and HSD[0.01] = 1.1. (B) SP1 siRNA knockdown also decreased expression of SP1, FN1, VIM, COL1 proteins. SP1 siRNA transfection also did not affect FGF2-dependent expression of ZEB1 protein, indicating that SP1 functions downstream of ZEB1. Transfection with nontargeting control siRNA did not alter gene expression in all experiments. COL8A2 and β -actin were used as a corneal endothelial marker and loading control, respectively. Veh C, vehicle control; NT, nontargeting control; FN1, fibronectin; VIM, vimentin; ACTB, β -actin.

were as follows: five minutes at 94°C followed by 33 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, and a final extension for two minutes at 72°C. Annealing temperatures were adjusted depend on PCR primer. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. All positive target PCR bands were verified by DNA sequencing.

Animal Husbandry and Anesthesia

All mouse experiments were performed in accordance with the protocol approved by University of Southern California Institutional Animal Care and Use Committee and followed ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were housed in clear, air-filtered cages with 12-hour light/dark cycle and ad lib feeding. C57BL/6 mouse breeding pairs were purchased from Jackson Laboratories (Sacramento, CA, USA), and colonies used

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

Surgical Injury of Corneal Endothelium and siRNA Knockdown In Vivo

Introducing surgical injury of corneal endothelium was performed as previously described¹⁷ with slight modification. Briefly, a paracentesis was made using a 30-gauge needle at the edge of the right cornea in anesthetized mice, and a bent 30-gauge needle was introduced into the anterior chamber through the paracentesis to scrape the

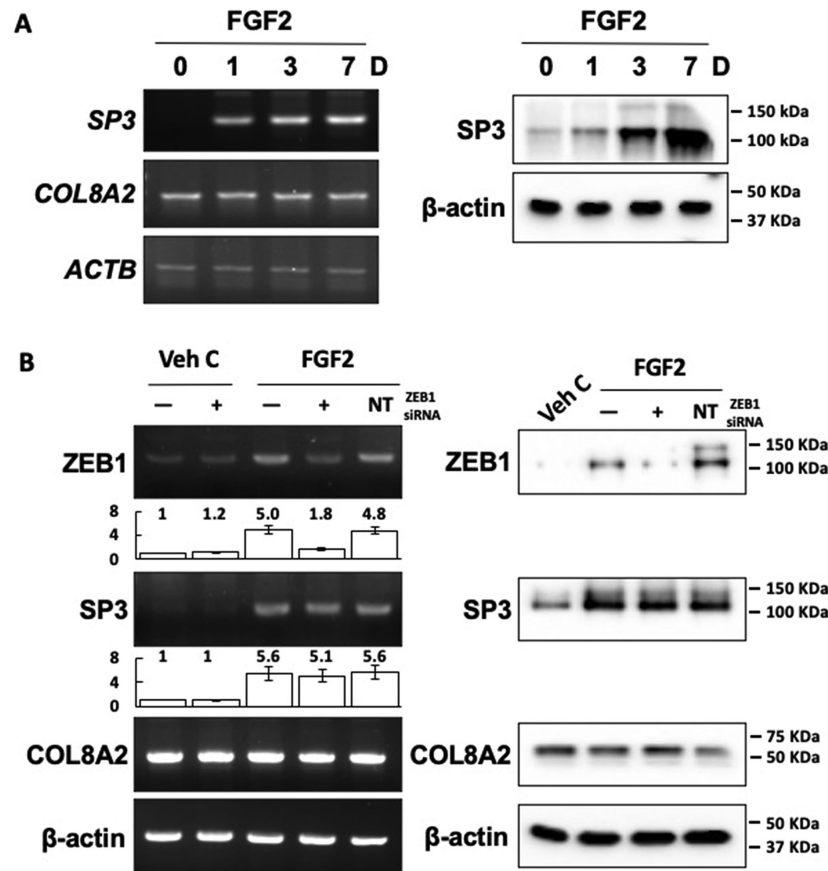


FIGURE 3. FGF2 induces SP3 expression through a ZEB1-independent pathway in human corneal endothelium ex vivo. **(A)** After FGF2 stimulation for indicated number of days times, total RNA and protein were purified from human corneal endothelium ex vivo for RT-PCR (left panel) and immunoblotting (right panel). FGF2 treatment led to an increase in SP3 expression in a time-dependent manner. D, days. **(B)** ZEB1 siRNA knockdown did not affect the FGF2-dependent expression of SP3 at either the transcriptional (*left panel*) or translational (*right panel*) level. SP3 $F(4,10) = 24.6$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 2.3$ and $HSD[0.01] = 3.0$. ZEB1 $F(4,10) = 87.6$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 1.0$ and $HSD[0.01] = 1.3$. COL8A2 was used as a corneal endothelial marker, and β -actin used as a loading control. Veh C, vehicle control; NT, non-targeting control; ACTB, β -actin.

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 μ L of aqueous humor was aspirated, and then 3 μ L of 1.5 μ mol/L Accell SMARTpool (Dharmacon) of Zeb1 siRNA or non-targeting siRNA in Accell delivery medium was injected into the anterior chamber 24 hours before surgical injury. The uninjected and uninjured contralateral eye was used as control. Mice were euthanized 14 weeks after injury, and their eyes were enucleated. Control and injured corneas were excised from the enucleated eyes and processed for hematoxylin and eosin staining. The siRNA injection experiment was performed in eight mice.

Histology

Whole eyeballs were fixed in 10% paraformaldehyde in PBS 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- μ m thickness, and then deparaffinized with a series of xylol and alcohol rinses. The sections were rinsed

in PBS three times for five minutes each, followed by a final rinse with distilled water. The sections were then stained with hematoxylin and eosin.

Statistical Analysis

One-way analysis of variance was performed to compare means within groups, and post hoc Tukey's honest significant difference tests were done to perform pairwise comparisons between means in a given group.

RESULTS

FGF2 Induces SP1 through ZEB1 in Human Corneal Endothelium Ex Vivo

We previously reported that ZEB1 mediates FGF2-dependent fibrosis in the human corneal endothelium ex vivo,¹⁹ and it has also previously reported that SP1 activates expression of type I collagen.^{31,32} To investigate the role of SP1 in ZEB1-mediated fibrosis in human corneal endothelium ex vivo, siRNA-mediated ZEB1 knockdown was performed. FGF2 stimulation increased SP1 expression at both the transcriptional and translational levels and FGF2-mediated

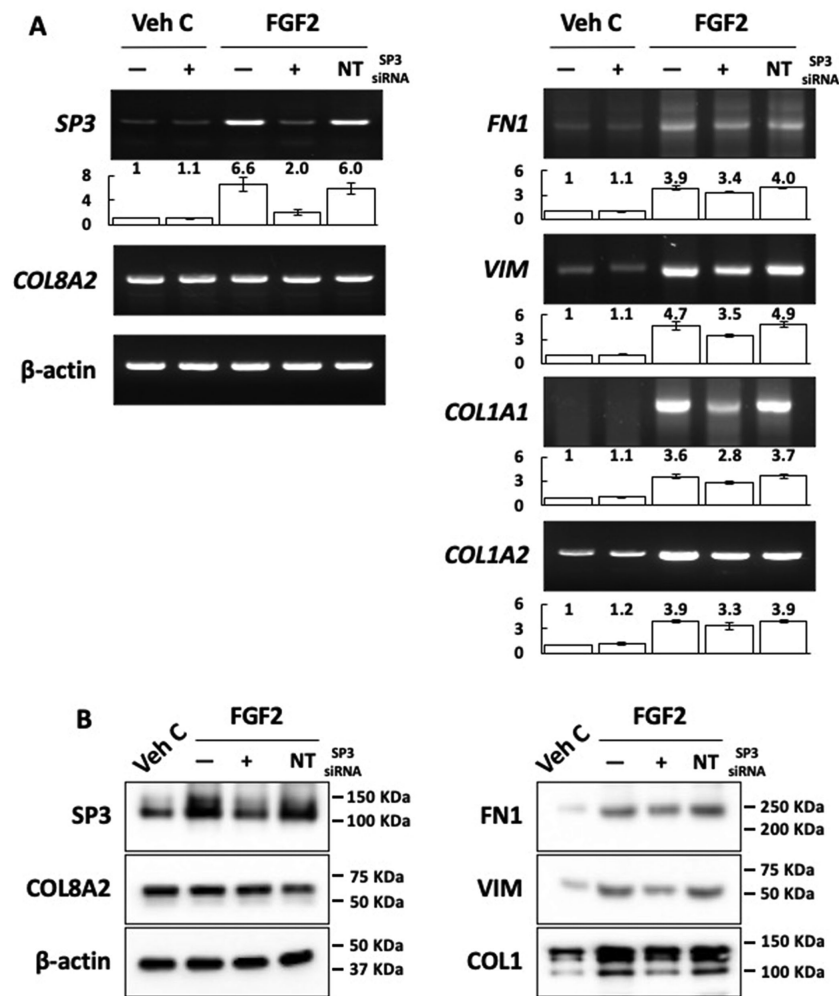


FIGURE 4. Regulation of FGF2-induced mesenchymal-related gene expression by SP3 is limited in human corneal endothelium ex vivo. **(A)** SP3 siRNA knockdown led to a decrease in FGF2-dependent *SP3* expression but only a slight inhibition of *FN1*, *VIM*, *COL1A1*, and *COL1A2* expression in human ex vivo corneal endothelium. *SP3* $F(4,10) = 48.2$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 1.8$ and $HSD[0.01] = 2.4$. *FN1* $F(4,10) = 372.7$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 0.4$ and $HSD[0.01] = 0.5$. *VIM* $F(4,10) = 125.8$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 0.8$ and $HSD[0.01] = 1.0$. *COL1A1* $F(4,10) = 130.3$, $P < 0.0001$, $n = 3$ per sample. Tukey's post hoc test, $HSD[0.05] = 0.5$ and $HSD[0.01] = 0.7$. *COL1A2* $F(4,10) = 128.5$, $P < 0.0001$, $n = 3$ per sample. Tukey's post-hoc test, $HSD[0.05] = 0.6$ and $HSD[0.01] = 0.8$. **(B)** SP3 siRNA knockdown led to a decrease in FGF2-dependent SP3 protein level but only slight decreases in FN1, VIM, COL1 proteins. Transfection with nontargeting control siRNA did not alter gene expression in all experiments. COL8A2 and β -actin were used as a corneal endothelial marker and loading control, respectively. Veh C, vehicle control; NT, nontargeting control; FN1, fibronectin; VIM, vimentin; ACTB, β -actin.

SP1 expression was attenuated by ZEB1 siRNA knockdown (Fig. 1), indicating that SP1 acts downstream of ZEB1. Nontargeting control siRNA transfection did not alter SP1 expression in the human corneal endothelium ex vivo.

Knockdown of FGF2-dependent SP1 expression led to inhibition of *FN1*, *VIM*, *COL1A1* and *COL1A2* expression in the human corneal endothelium ex vivo (Fig. 2A). SP1 knockdown had no effect on *ZEB1* expression, further confirming that SP1 signals downstream of ZEB1. The nontargeting control siRNA did not alter transcription levels, including *COL8A2*, a corneal endothelial marker, and *ACTB*, the loading control. Similarly, Western blot results also showed that siRNA-mediated SP1 knockdown led to decreased protein levels of fibronectin, vimentin, and type 1 collagen, with no change in *ZEB1*, *COL8A2*, or β -actin at the translational level (Fig. 2B).

FGF2 Induces SP3 through a ZEB1-independent Pathway in Human Corneal Endothelium Ex Vivo

After FGF2 stimulation for zero, one, three, and seven days, total RNA and protein were purified from human corneal endothelium ex vivo for RT-PCR and immunoblotting. FGF2 treatment led to an increase in SP3 expression in human corneal endothelium ex vivo in a time-dependent manner (Fig. 3A). To investigate the role of ZEB1 in FGF2-dependent SP3 expression in human corneal endothelium ex vivo, it was targeted by siRNA knockdown. ZEB1 siRNA transfection led to knockdown of ZEB1 mRNA and protein induced by FGF2 (Fig. 3B). Unlike SP1, ZEB1 siRNA knockdown did not alter the FGF2-dependent expression of SP3 at either the transcriptional or translational level (Fig. 3B). These results indicate that SP3 is induced by FGF2 stimulation

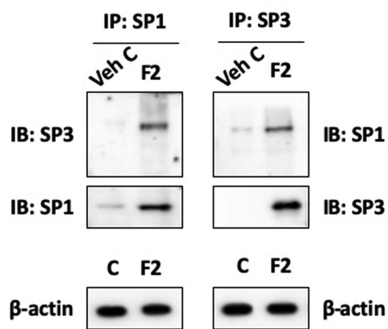


FIGURE 5. Binding between SP1 and SP3 proteins in human CECs treated with FGF2. Immunoprecipitation (IP) with anti-SP1 or anti-SP3 antibodies was performed on extracts prepared from human CECs treated with FGF2. Resulting complexes were separated on polyacrylamide gels and immunoblotted with the other anti-SP3 or anti-SP1 antibodies (IB). Binding between SP1 and SP3 in FGF2 stimulated human CECs was demonstrated by coimmunoprecipitation. SP1 and SP3 were not detected in cells treated with vehicle control. β -actin was used as a loading control. IP, immunoprecipitation; IB, immunoblotting; Veh C, vehicle control; F2, FGF2.

but independently of ZEB1. There were no changes in expression of COL8A2 and β -actin.

While SP3 is not regulated by ZEB1, we explored whether SP3 was involved in upregulation of COL1 expression in human corneal endothelium ex vivo given that SP3 has been reported to upregulate COL1 expression by binding to its promoter in other cell types.^{32,33} RT-PCR analysis showed that SP3 knockdown slightly reduced FGF2-dependent expression of *FN1*, *VIM*, *COL1A1* and *COL1A2* in human corneal endothelium ex vivo (Fig. 4A). However, magnitude of reduction was less when compared to SP1 knockdown (Fig. 2). Western blot results also showed that SP1 knockdown led to slightly decreased expression of FGF2-dependent fibronectin, vimentin, and type 1 collagen proteins (Fig. 4B). The nontargeting control siRNA did not alter mRNA levels, and there were no changes in COL8A2 and β -actin expression.

FGF2 Increases SP1-SP3 Complex Levels in Human CECs

Because binding of SP1 and SP3 transcription factors to each other has previously been shown in renal epithelial cells,³⁴ we investigated whether this was the case in human CECs. To investigate this possibility, we stimulated human CECs with FGF2 and performed coimmunoprecipitation. In cell lysates prepared from FGF2-stimulated human CECs, increased amounts of SP3 could be detected after SP1 immunoprecipitation (Fig. 5). Conversely, increased SP1 amounts could be detected after SP3 immunoprecipitation. Negligible levels SP1 and SP3, if any, could be detected after immunoprecipitation in vehicle control treated human CECs (Fig. 5). These results show that FGF2 increases SP1-SP3 complex levels in human CECs.

FGF2 Induces SP1 and SP3 to Bind to the Same Sequence in COL1A1, FN1, and VIM Promoter in Human CECs

Putative SP1 and SP3 binding sites are present in the promoters of COL1A1, FN1 and VIM (Fig. 6A). To determine whether

the putative binding sites are functional, we performed chromatin immunoprecipitation (ChIP) in human CECs. SP1 and SP3 immunoprecipitation of nuclear lysates prepared from immortalized human CECs were treated with FGF2 and resulted in pulldown of DNA fragments containing the putative binding sites in *COL1A1*, *FN1*, and *VIM* promoter, generating the expected 271, 232, and 288 bp PCR fragments, respectively (Fig. 6B). Human CECs cultured under control serum-free medium condition failed to protect the SP1/SP3 binding sites in a similar manner as FGF2 treatment.

To determine whether SP1-SP3 binding is necessary for protein-DNA binding, we carried out ChIP assays after SP1/SP3 siRNA knockdown in immortalized human CECs treated with FGF2. Both SP1 and SP3 siRNA transfection inhibited their own binding to the promoters of COL1A1, FN1 and VIM (Fig. 7). Interestingly, siRNA knockdown of one transcription factor did not alter the DNA binding of the other transcription factor (Fig. 7). The nontargeting control siRNA did not alter binding activity of SP1 and SP3.

Inhibition of Zeb1 Blocks the RCM Formation Induced by Surgical Injury in Mouse Corneal Endothelium In Vivo

We next investigated the role of Zeb1 in RCM formation in mouse corneal endothelium in vivo by performing surgical injury with or without Zeb1 siRNA knockdown. Hematoxylin and eosin staining showed a well-organized monolayer of normal corneal endothelial cells in the uninjured control cornea (Fig. 8A). Thickened, multilayered cellular architecture with disorganized Descemet's membrane, consistent with RCM formation, was observed in the cornea 14 weeks after surgical injury (Fig. 8B). Interestingly, inhibition of Zeb1 signaling by siRNA knockdown inhibited RCM formation induced by surgical injury (Fig. 8C). It should be noted that the Descemet's membrane is thicker and the endothelial cells are larger in cross section in the Zeb1 siRNA injected eye. Nontargeting siRNA yielded similar results as surgical injury alone (Fig. 8D). The in vivo Zeb1 siRNA knockdown and the control experiments were performed in eight mice, and the images representative of the group are shown in Figure 8. All Zeb1 siRNA injected eyes showed thickening of the Descemet's membrane and enlarged endothelial cells but no retrocorneal membrane.

DISCUSSION

Investigating the roles of FGF2 and its downstream mediators in regulating mesenchymal transition in the human corneal endothelium has the potential to identify new therapeutic targets for treating vision loss caused by endothelial dysfunction without relying on corneal transplantation. FGF2 induces EnMT as evidenced by changes in cell morphology and expression of mesenchymal transition markers SNAI1 and ZEB1 in human and mouse corneal endothelium.^{17,19} SNAI1 serves as a bifurcation point in the regulatory network, below which pathways for regulation of cell proliferation and fibrosis diverge. ZEB1 functions downstream of SNAI1 and promotes fibrosis by activating expression of type I collagen, a major component of retrocorneal membranes.^{12,19} However, the factors downstream of ZEB1 that regulate fibrosis have not been identified. Because severe retrocorneal membranes lead to

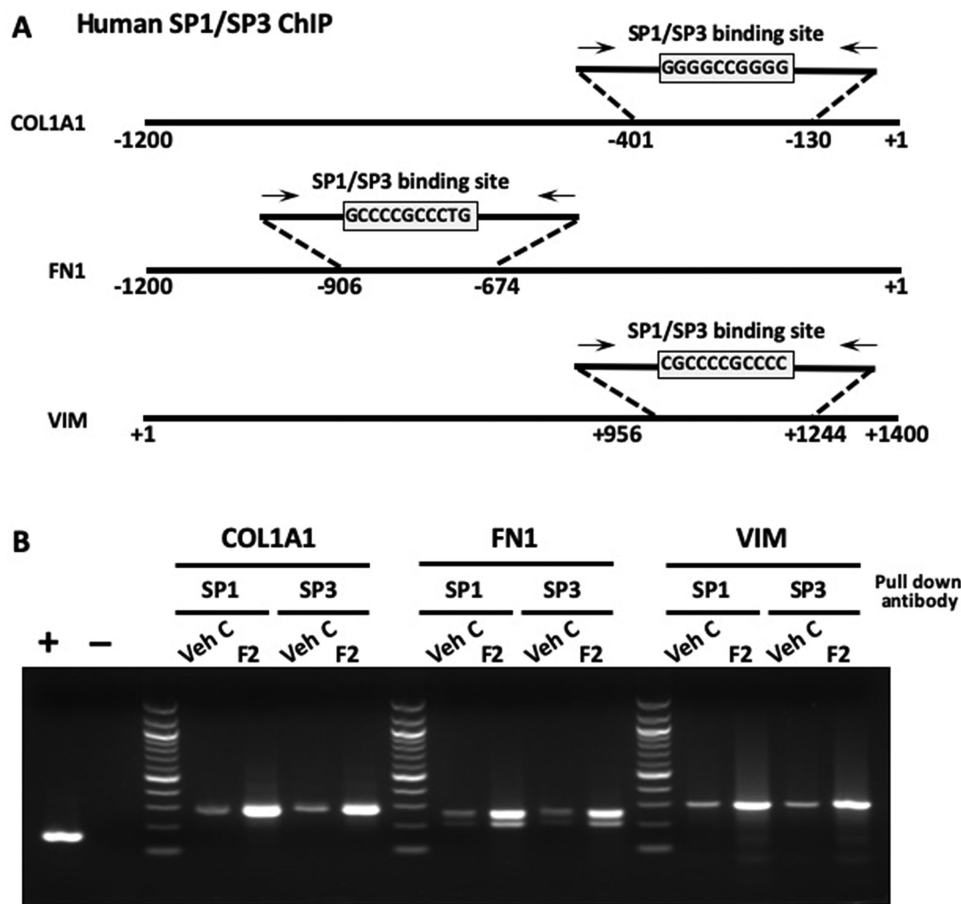


FIGURE 6. SP1 and SP3 bind to the promoters of FN1, VIM, and COL1A1 in human CECs treated with FGF2. **(A)** Illustration of the putative SP1/SP3 binding sites in the promoters of COL1A1, FN1 and VIM. The location of each primer used in the ChIP assay is indicated. **(B)** The expected PCR product sizes for the primer pairs were as follows: COL1A1, 271 bp; FN1, 253 bp; and VIM, 288 bp. Stimulation with FGF2 resulted in SP1 and SP3 binding to the *COL1A1*, *FN1* and *VIM* promoters in human CECs. Anti-histone H3 antibody was used as positive control (+) and normal rabbit IgG was used as negative control (–). Veh C, vehicle control; F2, FGF2; FN1, fibronectin; VIM, vimentin.

irreversible vision loss, inhibition of fibrosis and prevention of retrocorneal membranes have clinical utility.

SP1 and SP3 are ubiquitous transcription factors in many mammalian cells.²⁰ SP1 and SP3 are both part of the SP family of transcription factors that share many common features. Their DNA-binding domains share over 90 percent sequence homology and bind the same DNA sequence, generally GC-rich, with similar affinity.³⁵ In previous reports, the binding sites for SP1 and SP3 in the COL1A1 promoter were identified,^{26,27} and in this study, SP1 and SP3 binding sites in FN1 and VIM promoters were identified. This suggests that SP1 and SP3 play roles in regulation of fibrosis in human corneal endothelium. It has previously been reported that SP1 and SP3 are functionally distinct in vivo and can act as transcriptional activators or repressors depending on the cellular environment.^{21,36,37}

SP1 plays important roles in corneal development and homeostasis.^{38,39} It has also been implicated in keratoconus through its downregulation of alpha 1 proteinase inhibitor.⁴⁰ SP1 has also been shown to be a mediator of fibrosis in other cell types, such as skin fibroblasts through TGF- β signaling,⁴¹ and in renal tubular epithelial cells,⁴² hepatocytes,^{41,43} and alveolar epithelial cells⁴⁴ through activation of type 1 collagen expression during mesenchymal transition.

There is evidence that SP3 represses SP1-mediated transcriptional activity through competition for binding sites in the promoter and blocking SP1-mediated transcription in many cell types.^{37,45–47} However, other studies have shown that SP3 acts synergistically with SP1 to activate expression of the alpha2 chain of type 1 collagen.³³ It can also act as either an activator or repressor of SP1 function depending on the cell type³⁶ and SP3 isoform.⁴⁸

Our results indicate that SP1 and SP3 behave in a slightly different manner in the human corneal endothelium. Both are involved in fibrosis mediated by FGF2, but SP1 functions downstream of ZEB1, whereas SP3 is not regulated by ZEB1 (Figs. 2 and 3). Furthermore, SP1 seems to play a more prominent role in expression of COL1A1, COL1A2, FN1 and VIM, compared to SP3 (Fig. 4), suggesting that SP3's role in regulation of fibrosis is limited when compared to SP1. Although immunoprecipitation results showed that SP1 and SP3 bind to one another (Fig. 5), the ChIP assay results in the setting of siRNA knockdown indicate that each transcription factor's ability to bind to DNA is not dependent on the formation of SP1-SP3 complexes in the human corneal endothelium (Fig. 7).

The critical role of ZEB1 driving fibrosis in the corneal endothelium is supported by the results of Zeb1 knockdown

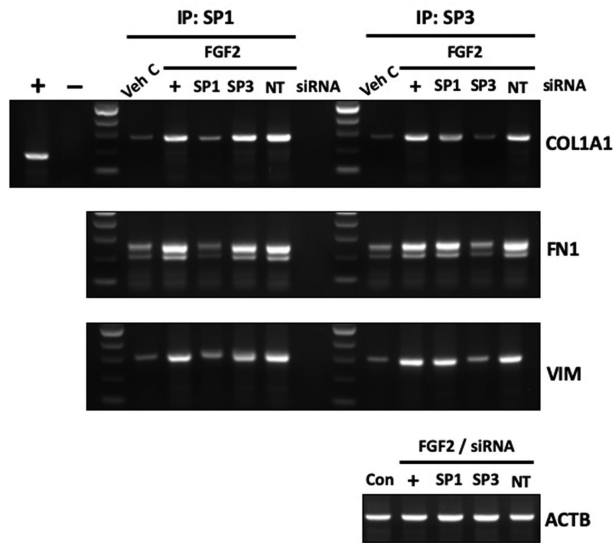


FIGURE 7. SP1 and SP3 bind independently to the promoters of FN1, VIM, and COL1A1 in human CECs treated with FGF2. After transfection with SP1 or SP3 siRNA, ChIP assays were performed with anti-SP1 or anti-SP3 antibodies. While siRNA knockdown of either SP1 or SP3 reduced their respective binding to *FN1*, *VIM*, and *COL1A1* promoters, SP1 knockdown had no effect on SP3 binding and SP3 knockdown had no effect on SP1 binding. Non-targeting control siRNA did not alter their binding activity. Anti-histone H3 antibody was used as positive control (+) and normal rabbit IgG was used as negative control (-). IP, immunoprecipitation; Veh C, vehicle control; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; ACTB, β -actin.

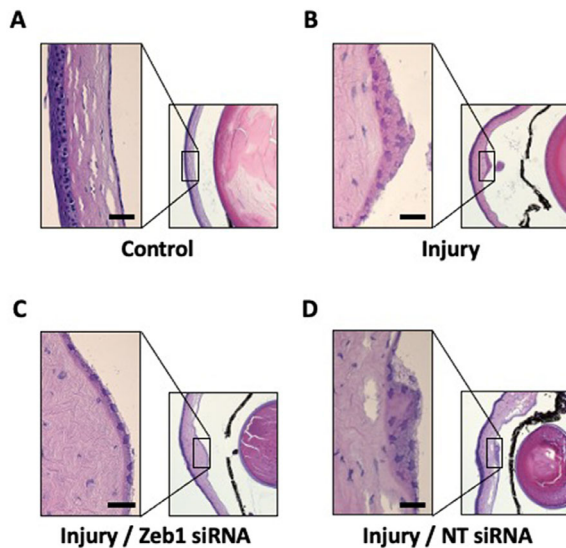


FIGURE 8. Inhibition of Zeb1 signaling blocks the RCM formation induced by surgical injury in the mouse corneal endothelium in vivo. At 14 weeks after surgical injury, corneal cross sections were stained with hematoxylin and eosin from control (A), injured (B), injured with Zeb1 siRNA transfection (C), and injured with nontargeting siRNA transfection (D) mouse eyes. Extensive RCM with thickening and disorganization of Descemet's membrane was noted in the injured cornea (B), whereas Zeb1 siRNA knockdown completely blocked RCM formation induced by surgical injury (C). Transfection with nontargeting control siRNA did not alter extensive RCM formation by injury (D). The uninjured control cornea showed a well-organized monolayer of corneal endothelial cells (A). NT, non-targeting control. Scale bar, 50 μ m.

FGF2 or Surgical Injury

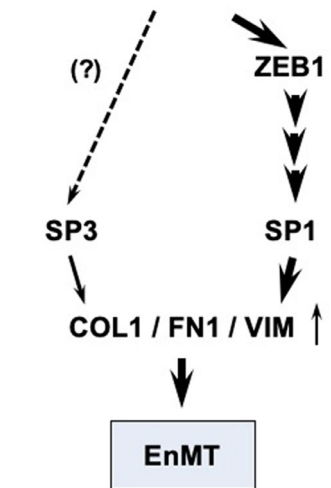


FIGURE 9. The proposed regulatory pathway for FGF2 mediated mesenchymal transition in human corneal endothelial cells. Stimulation by FGF2 leads to increased expression of SP1 and SP3. SP1 expression is regulated through ZEB1 whereas SP3 expression is not. SP1 plays a greater role in expression of COL1, FN1 and VIM than SP3.

in the mouse corneal endothelium in vivo (Fig. 8). Although the corneal endothelium showed thickening of Descemet's membrane, the disorganized multicellular architecture was not observed in Zeb1 siRNA injected eyes (Fig. 8C) compared with injured (Fig. 8B) and nontargeting siRNA injected eyes (Fig. 8D). This indicates that Zeb1 could be an attractive candidate for pharmacologic targeting to prevent RCM formation. It should be noted, however, that Zeb1 targeting did not prevent corneal edema in the injured cornea (Fig. 8C), indicating that other interventions may be necessary to prevent vision loss from corneal edema in a clinical setting. Although SP1 and SP3 act downstream of Zeb1, they are poor therapeutic targets because of their presence in many other signaling pathways. Targeting SP1 and SP3 will lead to disruption of several other signaling pathways that will have wide-ranging unintended consequences.

Having the ability to modulate EnMT in the human corneal endothelium by stimulating proliferation while inhibiting fibrosis could be potentially beneficial in several ways, treating vision loss from endothelial dysfunction without relying on corneal transplants and enhancing storage for donor corneas. These results may also have applications for managing severe organ failure due to fibrosis, such as in the kidney, lung, or pancreas, where mesenchymal transition has been shown to occur.^{49–51} Moreover, mesenchymal transition has also been shown to be involved in some forms of carcinogenesis.⁵² However, it is currently not clear whether inhibition of mesenchymal transition will prevent carcinogenesis. In conclusion, FGF2 or surgical injury induces ZEB1 expression, leading to activation of COL1A1, COL1A2, FN1, and VIM expression through SP1 and SP3 in the corneal endothelium, resulting in EnMT and RCM formation (Fig. 9).

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