Fibroblast growth factor 2 induces proliferation and fibrosis via SNAI1-mediated activation of CDK2 and ZEB1 in corneal endothelium

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*Running Title: FGF2 induces corneal endothelial mesenchymal transition

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ABSTRACT

Investigating stimulation of endogenous wound healing in corneal endothelial cells (CECs) may help address the global shortage of donor corneas by decreasing the number of transplants performed for blindness due to endothelial dysfunction. We previously reported that IL-1β stimulation leads to fibroblast growth factor (FGF2) expression, enhancing migration and proliferation of mammalian CECs. However, FGF2 also promotes the endothelial–mesenchymal transition, which can lead to retrocorneal membrane formation and blindness. This prompted us to investigate downstream FGF2 signaling targets that could be manipulated to prevent retrocorneal membrane formation. FGF2 stimulation altered cell morphology and induced expression of mesenchymal transition marker genes such as snail family transcriptional repressor 1 (SNAI1), SNAI2, zinc finger E-box–binding homeobox 1 (ZEB1), and ZEB2. This, in turn, induced expression of fibronectin, vimentin and, type I collagen and suppressed E-cadherin in CECs in vitro and ex vivo. siRNA-mediated SNAI1 knockdown revealed that SNAI1 induces ZEB1 expression, in turn inducing expression of type I collagen, the major component of retrocorneal membranes, and of cyclin-dependent kinase 2 (CDK2) and cyclin E1, promoting cell proliferation. siRNA-mediated knockdown of SNAI1 or ZEB1, but not of CDK2, inhibited FGF2-dependent expression of fibronectin, vimentin, and type I collagen and of suppression of E-cadherin expression. We conclude that SNAI1 is a key regulator of FGF2-dependent mesenchymal transition in human ex vivo corneal endothelium, with ZEB1 regulating type I collagen expression and CDK2 regulating cell proliferation. These results suggest that SNAI1 promotes fibrosis and cell proliferation in human corneal endothelium through ZEB1 and CDK2.

The cornea is the anterior, transparent tissue of the human eye and consists of epithelium, stroma and endothelium. The corneal endothelium is composed of a monolayer of cells and plays a critical role in maintaining corneal transparency that is critical for sharp vision through its pump function (1,2). Adult human corneal endothelial cells (CEC) are usually mitotically inactive and arrested at the G1 phase of the cell cycle (3,4). Because of the cell cycle arrest, there is a progressive decline in CEC density that can be further accelerated by injury, such as intraocular surgery or infection. When the density decreases below a critical threshold, corneal edema ensues, leading to loss of transparency and vision. Vision loss secondary to endothelial dysfunction is a common indication for corneal transplantation in developed nations. There have been many attempts to overcome the cell cycle arrest using various approaches including the use of growth factors such as FGF, EGF, and TGF-β, the use of endothelial cell growth supplements (5-9), and disruption of contact inhibition using EDTA (10). Moreover, severely injured CEC can undergo mesenchymal transition through which they lose their polarity and assume a fibroblastic phenotype. These CEC also exhibit enhanced migration,
proliferation and secrete type I collagen leading to retrocorneal membrane formation (11-16).

Mesenchymal transition is a process in which cells lose their polarity and adhesion, express mesenchymal makers, and reorganize their cytoskeleton, leading to morphological changes (17,18). A major hallmark of the mesenchymal transition is down-regulation of the junctional protein E-cadherin and upregulation of cytoskeletal proteins such as fibronectin and vimentin (19-21). Moreover, there is also increased expression of α1 (COL1A1) and α2 (COL1A2) chains of type I collagen (21,22), and this is also observed in CEC (12,16,23). It has been reported that overexpression of transcription factors such as members of SNAI family, SNAI1 and SNAI2, and members of ZEB family, ZEB1 and ZEB2, regulate suppression of E-cadherin expression and overexpression of fibronectin, vimentin, and α-smooth muscle actin (αSMA) (24-26). The current model suggests that SNAI1 and ZEB1 are key components in regulation of mesenchymal transition, although there is some discrepancy regarding their exact roles. There are reports that suggest that they act in a parallel manner (27-29), while other reports suggest that ZEB1 acts downstream of SNAI1 (30-33) in the regulatory network.

Another key inducer of mesenchymal transition is FGF2 (34-36), and SNAI1 has been reported to be a downstream target of FGF2, during chondrogenesis and in certain forms of achondroplasia (37). We previously reported that FGF2 signals through PI 3-kinase to induce mesenchymal transition in rabbit and human CEC. FGF2 promotes proliferation via degradation of p27 (38-40), activates migration via activation of CDC42 (41), and induces a change in cell shape from polygonal to fibroblastic morphology and the loss of contact inhibition via cross-talk of RhoGTPase (15,42). FGF2 also upregulates expression and secretion of type I collagen in CEC (12). Although the downstream effects of FGF2 signaling have been studied extensively, the mechanism through which FGF2 exerts its effects on CEC has not been reported.

In the present study, we investigated the downstream targets of FGF2 capable of driving various components of mesenchymal transition in human CEC. We show that FGF2 induces expression of SNAI1, which in turn leads to increased expression of CDK2 and ZEB1. This leads to increased proliferation and expression of type I collagen. Our results suggest that ZEB1 plays a central role in mediating fibrosis in corneal endothelial mesenchymal transition.

**EXPERIMENTAL PROCEDURES**

Reagents – FGF2 was purchased from Cell Signaling Technology (Danvers, MA). Anti-COL1 (139 KDa, cat: ab6308) and vimentin (54 KDa, cat: ab92547) antibodies were purchased from Abcam (Cambridge, MA). Anti-β-actin (42 KDa, cat: A5316) and peroxidase conjugated secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against SNAI1 (29 KDa, cat: GTX125918), SNAI2 (30 KDa, cat: GTX30813) and fibronectin (260 KDa, cat: GTX60570) were obtained from GeneTex (Irvine, CA). Antibodies against COL8A2 (67 KDa, cat: sc-82843), cytokeratin 12 (KRT12, 54 KDa, cat: sc-25722) and αSMA (43 KDa, cat: sc-53015) were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-CDK2 (33 KDa, cat: 610145), and E-cadherin (98 KDa, cat: 610404) antibody was purchased from BD Biosciences (San Jose, CA). Antibody against cyclin E (52 KDa, cat: 07-687) was obtained from EMD Millipore (Billerica, MA). Anti-ZEB1 (124 KDa, cat: PA5-40350) and ZEB2 (135 KDa, cat: PA5-40759) antibody were purchased from Thermo Fisher (Rockford, IL).

Isolation and Culture of Primary Human CEC – Isolation and culture of primary human CEC were performed according to previously published protocols (40) with minor modifications. Briefly, corneas were removed from Optisol and washed several times with OptiMEM-I (Gibco-BRL, Grand Island, NY) containing 50 µg/mL gentamicin. The Descemet’s membrane and endothelium complex was stripped from corneas and treated with 0.2% collagenase type II and 0.05% hyaluronidase (Worthington Biochemical, Lakewood, NJ) for 3 hours at 37°C. After centrifugation, the primary cells were resuspended in culture medium: OptiMEM-I supplemented with 5% fetal bovine serum, 10 µM Y-27632, 0.5 µg/mL R-spondin and 10 µM SB431542 (TGF-β RI Kinase inhibitor VI) (16) and plated on 24-well tissue culture plates precoated with FNC coating mix (Biological Research Faculty & Facility, Inc., Ijamsville, MD) and laminin (Sigma). For subculture, confluent primary cultures were treated
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with 0.05% trypsin and 5 mM EDTA in phosphate-buffered saline (PBS) for 10 minutes. Second or third passage human CEC were used for all experiments. For serum starvation, culture medium was changed to OptiMEM-I and maintained for 24 hours. Serum starved-cells were stimulated with FGF2 (10 ng/ml). Reconstitution buffer (2 mM DTT and 1% BSA in PBS) of FGF2 was used as vehicle control for some of the control samples.

Semi-quantitative Reverse Transcription-PCR (RT-PCR) Analysis – Total RNA were extracted from human primary CEC or corneal endothelium. cDNA were synthesized with 2 µg of RNA by utilizing iScript reverse transcriptase (Bio-Rad, Hercules, CA) and oligo(dT) primer. Reverse transcription was performed at 42 °C for 90 min. Then, the first strand cDNA equivalent to 0.1 µg of starting RNA from each sample was amplified by using the specific primer pairs. The specific primers used are shown in Table 1. Standard PCR conditions were as follows: 5 min at 94 °C, followed by 30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C, and a final extension for 4 min 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 1). RT-PCR amplification of β-actin transcript was used as the internal control to verify using 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 analyzed band intensity using 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 (43). Corneal endothelial cells at 70% confluence or ex vivo corneal endothelium were transfected on 6-well plate with 1.5 µM Accell SMARTpool of siRNA targeting SNAI1, ZEB1 or CDK2 (Dharmacon, Pittsburgh, PA) in Accell delivery medium according to the manufacturer’s instructions. 72 hours after transfection, the medium was changed to medium containing FGF2. 10 days after maintaining, another transfection solution with 1.5 µM Accell SMARTpool of siRNA targeting SNAI1, ZEB1 or CDK2 were added without medium change and maintained 4 more days. Samples were therefore maintained for a total of 14 days under FGF2 stimulation. RNA and protein levels of SNAI1, ZEB1 or CDK2 were analyzed by RT-PCR and immunoblotting, respectively. Accell nontargeting 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, and Immunoblotting Analysis – All assays were performed following previously reported protocols (12,40,43,44). The following gel concentrations were used to separate proteins: 15% polyacrylamide gel for SNAI1 and SNAI2, 12% polyacrylamide gel for CDK2, 10% polyacrylamide gel for COL8A2, cyclin E1, vimentin, cytokeratin 12, αSMA, and β-actin, 8% polyacrylamide gel for E-cadherin, type I collagen, ZEB1 and ZEB2, and 6% polyacrylamide gel for 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 µl of RIPA lysis buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Total protein were purified and concentrated with Amicon ultra centrifugal filter devices (EMD Millipore), 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 spin down at 14,000 x g for 30 minutes. In order to recover the concentrated protein, the Amicon ultra filter device placed upside down in a clean tube which was then centrifuged again for 2 minutes at 1000 x g to transfer the concentrated protein sample from the device to the clean tube. Purified total proteins were used to for analysis of immunoblotting.

Ex vivo Proliferation Assay – Ex vivo proliferation assays were performed according to previously published protocols (44) with minor technical modifications. Whole human corneas not suitable for transplantation were obtained from One Legacy (Los Angeles, CA) and cut into three equal sections. The endothelium of each piece was injured with a small pipette tip measuring ~0.5 mm in diameter, resulting in a linear wound ~0.5 mm wide. The corneal pieces
were then placed endothelial side up in individual wells of a 24-well tissue culture plate. The pieces were maintained with OptiMEM-I containing FGF2 for 14 days at 37 °C in a 5% carbon dioxide, humidified atmosphere. Vehicle control (2 mM DTT and 1% BSA in PBS) was used for the control samples. For siRNA knockdown in human ex vivo corneal endothelium, the Accell SMARTpool system of siRNA targeting SNAI1, ZEB1 and CDK2 (Dharmacon) was used. Accell non-targeting control siRNA (Dharmacon) was used as a negative control. For immunostaining, the corneal pieces were fixed for 20 min in 1 ml of 4% paraformaldehyde and rinsed three times with PBS. They were then permeabilized using 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at room temperature, followed by blocking with 4% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h at room temperature. The tissues were incubated for 2 h at room temperature with both mouse anti-human phospho-histone H3 IgG (EMD Millipore, cat: 05-1336) and rabbit anti-human ZO-1 IgG (Therm Fisher, cat: 61-7300) diluted 1:100 in blocking buffer. Each piece was rinsed in PBS three times for 10 min, followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich, cat: F9887) and Rhodamine-conjugated donkey anti-rabbit IgG (Genetex, cat: GTX26799) diluted 1:200 in blocking buffer for 2 h at room temperature. Incubation of primary or secondary antibody alone was used as a negative control. The corneal pieces were then washed three times for 10 min with PBS at room temperature. Excess sclera was removed to facilitate mounting (endothelial side down) with mounting solution with DAPI on a glass cover slip (MatTek Co., Ashland, MA). A glass coverslip was placed on the epithelial side of the corneal sections, and a 20-g weight was placed on the coverslip for 15 min to flatten the sections for imaging. Images were captured with a BZ-X700 fluorescence microscope (Keyence, Itasca, IL) using a ×40 objective lens with an aperture of 0.95. All images were captured at room temperature on sections mounted with Vectashield DAPI mounting medium (Vector Laboratories, Burlingame, CA) and analyzed with BZ-X analyzer software (Keyence). Phospho-histone H3-positive nuclei were counted in an 85-μm² field within the wound area. Five fields were counted per section, and three different sections were used for each culture condition. The proliferation index was calculated as the number of phospho-histone H3-positive nuclei divided by the number of all nuclei within each field, and the relative proliferation rate was calculated relative to the proliferation index of the vehicle-treated cornea.

Statistical Analysis – One-way analysis of variance (ANOVA) 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 group.

RESULTS

FGF2 induces mesenchymal transition in human primary CEC. We previously reported that FGF2 induces morphological changes in rabbit CEC (11,15). We, therefore, investigated the effect of FGF2 on morphological alternation in human CEC, as a first step towards analyzing the role of FGF2 in mesenchymal transition in human CEC. After 45 days of FGF2 exposure, primary human CEC lost their polygonal shape and became more fibroblastic in appearance (Fig. 1A). The change in cell shape was accompanied by increased expression of α1 (COL1A1) and α2 (COL1A2) chains of type I collagen, SNAI1, SNAI2 ZEB1, and ZEB2 (Fig. 1B). Expression of α2 chain of type VIII collagen (COL8A2), a CEC marker, and αSMA were not altered by prolonged FGF2 exposure (Fig. 1B).

FGF2 induces mesenchymal transition in human ex vivo corneal endothelium. The observation that FGF2 can induce mesenchymal transition in primary human CEC led us to investigate whether it also could do the same in human ex vivo corneal endothelium. The importance of this question is based on the observation that CEC behavior is dependent on its environment, i.e. in vitro versus ex vivo. Similar to the in vitro results, FGF2 stimulation, but not vehicle control, led to increased expression of SNAI1, SNAI2, ZEB1 and ZEB2, starting at 3 days and rising steadily until 14 days post treatment in human ex vivo corneal endothelium (Fig. 2A). Increased expression of COL1A1, COL1A2, vimentin (VIM) and fibronectin (FN1) and inhibition of E-cadherin (CDH1) expression in human ex vivo corneal endothelium was also noted.
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in response to FGF2 stimulation (Fig. 2B). Expression of COL8A2 and αSMA was not altered by FGF2 stimulation in human ex vivo corneal endothelium (Fig. 2B). Next, we investigated the role of the FGF2, which has been shown to induce proliferation in primary human CEC (40), on cell proliferation in human ex vivo corneal endothelium. Treatment with FGF2 increased expression of CDK2 and cyclin E1 (CCNE1) in a time-dependent manner (Fig. 2C).

Similar to the RT-PCR results, western blotting showed that treatment with FGF2 induced SNAI1, SNAI2, ZEB1 and ZEB2 protein expression (Fig. 3A). Moreover, expressions of type I collagen, vimentin and fibronectin were also increased, and expression of E-cadherin was decreased by FGF2 treatment (Fig. 3B). Expression of αSMA was not changed by FGF2 (Fig. 3B). FGF2 treatment also induced CDK2 and cyclin E proteins (Fig. 3C). Presence of keratin 12, a marker for corneal epithelium and stroma (45), was used to control for epithelial cell contamination (Fig. 3D). There were no changes in expression of COL8A2 and β-actin in response to FGF2 treatment in human ex vivo corneal endothelium.

FGF2 signaling is mediated through SNAI1 in both in human CEC and ex vivo corneal endothelium – Given that SNAI1 expression is induced by FGF2 (Fig. 1 and Fig. 2) and its role in mesenchymal transition in other tissues (25,28,31), we investigated the role of SNAI1 in the FGF2-induced mesenchymal transition in primary human CEC using siRNA knockdown. Western blotting analysis showed that SNAI1 siRNA was able to maintain knockdown of FGF2-induced expression up to 14 days post transfection (Fig. 4A). RT-PCR analysis with total RNA isolated at 14 days post transfection showed that SNAI1 siRNA knockdown also inhibited FGF2-dependent expression of SNAI2, ZEB1 and ZEB2 (Fig. 4B). Moreover, SNAI1 siRNA knockdown also inhibited FGF2-dependent expression of COL1A1, COL1A2, and FN1 and reversed FGF-2 dependent inhibition of CDH1 (Fig. 4C). Unlike ex vivo corneal endothelium, neither FGF2 nor SNAI1 siRNA had any effect on expression of VIM (Fig. 4C). Interestingly, knockdown of SNAI1 also inhibited FGF2-dependent expression of CDK2 and CCNE1 (Fig. 4D). The non-targeting control siRNA had no effect on mRNA levels.

To further investigate SNAI1’s role in FGF2-induced mesenchymal transition in the corneal endothelium, siRNA knockdown experiments were performed in human ex vivo corneal endothelium. Transfection with SNAI1 siRNA inhibited FGF2-dependent expression of SNAI2, ZEB1 and ZEB2 ex vivo (Fig. 5A). Similarly, FGF2-dependent expressions of COL1A1, COL1A2, VIM and FN1, and FGF2-dependent suppression of CDH1 were also inhibited by SNAI1 knockdown (Fig. 5B). Additionally, SNAI1 siRNA knockdown also inhibited FGF2 dependent expression of CDK2 and CCNE1 (Fig. 5C).

Western blotting results paralleled the RT-PCR results in human ex vivo corneal endothelium. SNAI1 siRNA knockdown led to decreased levels of SNAI1, ZEB1 and ZEB2 in FGF2 stimulated human ex vivo corneal endothelium (Fig. 6A). The same was observed with levels of type I collagen, fibronectin and vimentin (Fig. 6B). Inhibition of FGF2-mediated suppression of E-cadherin was also observed with SNAI1 siRNA knockdown ex vivo (Fig. 6C). The non-targeting control siRNA had no effect on protein levels, including the CEC marker COL8A2 and the loading control β-actin. These results suggest that SNAI1 acts downstream of FGF2 to induce proliferation and fibrosis during mesenchymal transition in human CEC in vitro and ex vivo corneal endothelium.

ZEB1 mediates FGF2-dependent fibrosis but not proliferation in human ex vivo corneal endothelium – It has been previously reported that ZEB1 activates expression of fibronectin and vimentin (20,46,47) and suppresses E-cadherin (20,48). To investigate the role of ZEB1 in mesenchymal transition in ex vivo corneal endothelium, we used siRNA mediated ZEB1 knockdown to investigate its role. ZEB1 siRNA transfection in human ex vivo corneal endothelium led to knockdown of ZEB1 mRNA and protein (Fig. 7A and Fig. 7B). ZEB1 knockdown also led to the inhibition of FGF2-dependent expression of type I collagen, fibronectin and vimentin, and reversed the FGF2-dependent suppression of E-cadherin expression in human ex vivo corneal endothelium (Fig. 7A and Fig. 7B). This was similar to SNAI1 knockdown, but expression of SNAI1 was not affected by ZEB1 knockdown. Additionally, unlike SNAI1 knockdown,
expression of CDK2 and CCNE1 were not affected by ZEB1 knockdown. Non-targeting control siRNA transfection did not alter gene expression in human ex vivo corneal endothelium. CDK2 knockdown did not affect expression of any of the previously mentioned genes, except CDK2 itself (Fig. 8).

**FGF2 promotes proliferation through SNAI1 but not ZEB1 in human ex vivo corneal endothelium** – To investigate the roles of SNAI1 and ZEB1 in human CEC proliferation, we used siRNA knockdown in a human ex vivo cornea wounding model. FGF2 treatment in the absence of endothelial injury was not sufficient to drive proliferation in CEC (data not shown). FGF2 stimulation, but not vehicle control, followed by mechanical injury to the corneal endothelium led to phosphorylation of histone H3 in human ex vivo corneas (Fig. 9A). siRNA knockdown of SNAI1 and CDK2 abolished phosphorylation of histone H3 in wounded ex vivo corneal endothelium treat with FGF2. ZEB1 siRNA and non-targeting siRNA control did not affect phosphorylation of histone H3 (Fig. 9A). Quantification of pH3 in human ex vivo corneal endothelium showed that there was a significant increase in the relative proliferation rate in response to FGF2 treatment (Fig. 9B). This could be inhibited significantly by knockdown of SNAI1 and CDK2, but not by ZEB1 knockdown (Fig. 9B).

**DISCUSSION**

Cell proliferation and fibrosis play important roles in a wide variety of physiologic processes such as wound healing (17). In corneal endothelial wound healing, endothelial cells that have been severely injured can undergo mesenchymal transition, where they lose some endothelial phenotype and taken on mesenchymal characteristics. These cells exhibit increased proliferation, migration, and fibrosis, which can lead to loss of corneal transparency resulting in vision loss and even blindness (49). Little is known regarding signals that regulate the individual components of mesenchymal transition in the corneal endothelium, but FGF2 has been shown to be a potent initiator of mesenchymal transition in the corneal endothelium (12,15,38,39,42). Moreover, a picture is beginning to emerge in which the individual components of EnMT are regulated by independent signaling pathways. We have previously reported that cell migration and proliferation are independently regulated by WNT5A and WNT10B respectively in human corneal endothelial cells (43,44). In this study, we investigated the downstream targets of FGF2 that regulate cell proliferation and fibrosis in primary human CEC and human ex vivo corneal endothelium. Although the use of primary CEC in vitro and ex vivo corneal endothelium may appear to be redundant, both model systems are complimentary because human corneal endothelial cell behavior is dependent on its environment. This is evidenced by differences the time it took to enter mesenchymal transition in response to FGF2 between in vitro (45 days) and ex vivo (14 days), and the difference in vimentin expression between primary human CEC in vitro and ex vivo corneal endothelium (Figs. 3 and 4).

Prolonged FGF2 exposure induced mesenchymal transition in human CEC as evidenced by changes in cell morphology and expression of mesenchymal transition markers SNAI1, SNAI2, ZEB1 and ZEB2 (Fig. 1). Moreover, these cells also showed increased expression of α1 and α2 chains of type I collagen, a major component of fibrotic retrocorneal membranes. While we did not observe any changes in vimentin mRNA expression in response to FGF2 treatment in primary human CEC in vitro (Fig. 4C), there was an increase in vimentin mRNA and protein expression in response to FGF2 treatment in human ex vivo corneal endothelium (Fig. 5 and Fig. 6). This strongly suggests that corneal endothelial cells behave differently ex vivo versus in vitro. Moreover, suppression of E-cadherin expression by FGF2 treatment in corneal endothelial cells also supports conversion to a mesenchymal phenotype and is consistent with previous reports showing similar results in ovarian cancer cells (34,36).

Induction of SNAI1 and ZEB1 by FGF2 were of particular interest because SNAI1 has previously been reported to be a key regulator of mesenchymal transition in epithelial cells, and ZEB1 mutations have been associated with two endothelial diseases; posterior polymorphous corneal dystrophy (PPMD3) and late-onset Fuchs endothelial corneal dystrophy (FECD6) (19,20,31,50,51). Both have been reported to bind
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to the same element in the E-cadherin promoter, E-box with the core 5'-CACCTG-3' sequence (20,28). This raised questions regarding the roles of SNAI1 and ZEB1 in the signaling network regulating mesenchymal transition. Previous reports have shown that SNAI1 can activate expression of ZEB1 during mesenchymal transition (30-33). Consistent with previous reports, SNAI1 siRNA knockdown led to decreased expression of ZEB1 following FGF2 stimulation in both primary human CEC and human ex vivo corneal endothelium (Fig. 4, Fig. 5, and Fig. 6). SNAI1 knockdown also led to decreased expression of vimentin and fibronectin suggesting that SNAI1 acted upstream of ZEB1 in FGF2 induced mesenchymal transition. Moreover, expression of type I collagen, CDK2, and cyclin E1 were inhibited by SNAI1 knockdown. On a functional level, SNAI1 knockdown led to decreased phosphorylation of histone H3 in FGF2-treated human ex vivo corneal endothelium (Fig. 9). Taken together, our data strongly suggest that SNAI1 occupies a nexus regulating both cell proliferation and fibrosis in FGF2-induced mesenchymal transition in the corneal endothelium.

We then turned our attention to ZEB1 to examine its role in the regulatory network. ZEB1 siRNA knockdown led to decreased expression of type I collagen, vimentin and fibronectin, but expression of SNAI1, CDK2, and cyclin E1 were not affected in human ex vivo corneal endothelium treated with FGF2, indicating that SNAI1 was acting upstream of ZEB1 (Fig. 7). Moreover, ZEB1 knockdown was also able to reverse FGF2-mediated inhibition of E-cadherin expression ex vivo. This suggested that ZEB1 plays a critical role in inducing a mesenchymal phenotype and regulates fibrosis but not proliferation in FGF2-mediated mesenchymal transition in ex vivo corneal endothelium. This is further supported by ZEB1 knockdown not having any effect in histone H3 phosphorylation in FGF2-treated ex vivo corneal endothelium (Fig. 9). Conversely, CDK2 does not play a role in regulating mesenchymal transition or fibrosis in human ex vivo corneal endothelium as CDK2 knockdown had no effect on expression of SNAI1, ZEB1, vimentin, fibronectin, and E-cadherin (Fig. 8). This implies that ZEB1 is a major regulator of fibrosis in FGF2-induced mesenchymal transition in the corneal endothelium.

The regulation of fibrosis in disease is garnering increasing attention as more pathogenic mechanisms become elucidated (52). Tissue fibrosis results from deposition of extracellular matrix from myofibroblasts, often leading to organ dysfunction and failure. In the kidney, injured renal epithelial cells stimulate the conversion of renal tubular interstitial fibroblasts into myofibroblasts to drive fibrosis (53,54). In the liver, injured hepatocytes are thought to promote the conversion of hepatic stellate cells into myofibroblasts that in turn leads to hepatic fibrosis (55). Alveolar epithelial cells from patients with idiopathic pulmonary fibrosis were found to express higher levels of type I collagen and αSMA, suggesting that their conversion to a myofibroblast phenotype was contributing to the disease etiology (56). The corneal endothelium also showed increased expression of type I collagen in response to FGF2, but unlike the previous examples, we did not see an increase in αSMA expression ex vivo (Fig. 2). This may indicate that unlike the previous examples, the corneal endothelium may be able to increase type I collagen expression without assuming a more myofibroblastic phenotype or that corneal endothelial cells ex vivo have already assumed a partial myofibroblast phenotype as evidenced by a low level expression of αSMA (Fig. 2).

Our data show that FGF2 induces expression of SNAI1 which in turn activates expression of ZEB1 and CDK2/cyclin E1. ZEB1 induces a mesenchymal phenotype through vimentin and fibronectin expression, and activates expression of type I collagen. In parallel to the ZEB1 pathway, CDK2/cyclin E1 activates cell proliferation without inducing a mesenchymal phenotype (Fig. 10). Previous studies have reported that SNAI1 is required for cell proliferation in malignant melanoma (57,58) and ZEB1 induces proliferation and tube formation through upregulation of VEGF in human umbilical vein endothelial cells (59). It has also been reported that SNAI1 and ZEB family members inhibit cell cycle progression, resulting in accumulation of cells in G1 phase of cell cycle in epidermoid A431 cells (60). These reports, along with our data, indicate that SNAI and ZEB family of proteins may have different regulatory roles in different tissues.
In conclusion, FGF2 initiates mesenchymal transition through SNAI1, which induces ZEB1 and CDK2 in parallel, leading to induction of COL1A1 and COL1A2 and proliferation (Fig. 10).

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Author contributions: JGL and MH conceived and designed the experiments. JGL and EJ performed the experiments. JGL, EJ and MH analyzed and interpreted the data, and MH performed the statistical analysis. JGL, EJ and MH wrote the manuscript and approved the final version of the manuscript.

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**FOOTNOTES**

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The abbreviations used are: CEC, corneal endothelial cells; FGF2, fibroblast growth factor 2; FN1, Fibronectin; VIM, vimentin; CDH1, E-Cadherin; CCNE1, Cyclin E1; αSMA, α-Smooth Muscle Actin; KRT12, Keratin 12; CDK2, Cyclin Dependent Kinase 2; COL1A1, α1 chain Type I Collagen; COL1A2, α2 chain Type I Collagen; COL8A2, α2 chain Type VIII Collagen; SNAI1, Snail1; SNAI2, Snail2; ZEB1, Zinc finger E-box-binding homeobox 1; ZEB2, Zinc finger E-box-binding homeobox 1; siRNA, small interfering RNA.

FIGURE LEGENDS

FIGURE 1. FGF2 induces mesenchymal transition-related gene expression in human primary corneal endothelial cells in vitro. A) After 45 days of FGF2 stimulation, the polygonal cell morphology of corneal endothelial cells (CEC) is altered to the elongated and spindle shape, while CEC treated with vehicle control showed normal morphology. B) Following treatment for designated times (h, hour; d, day) and culture conditions, total RNA from CEC were purified and RT-PCR was performed. Marked induction of COL1A1, COL1A2, SNAI1, SNAI2, ZEB1 and ZEB2 were noted in FGF2 but not in vehicle control treated human primary CEC. Expression of αSMA and TWIST was not altered by FGF2 treatment. COL8A2 and β-actin were used as corneal endothelial marker and loading control respectively. Veh C, vehicle control; F2, FGF2; M, DNA molecular weight marker.

FIGURE 2. FGF2 regulates mesenchymal transition- and proliferation-related gene expression at the transcriptional level in human ex vivo corneal endothelium. Following treatment for designated times, total RNA from human ex vivo corneal endothelium was purified and RT-PCR performed. A) Expression of SNAI1, SNAI2, ZEB1 and ZEB2, was increased by FGF2 treatment in a time dependent manner. Treatment with vehicle control had no effect. B) FGF2 treatment led to increased expression of COL1A1, COL1A2, fibronectin, and vimentin, while at the same time, it suppressed expression of E-cadherin. Similar to human CEC in vitro, FGF2 treatment had no effect on expression of αSMA. C) Increased expression of CDK2 and Cyclin E1 were also noted in FGF2 treated but not in vehicle control treated human ex vivo corneal endothelium. COL8A2 and β-actin were used as corneal endothelial marker and loading control respectively, and they were reused in panels A, B, and C. Veh C, vehicle control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1; αSMA, α-Smooth Muscle Actin.

FIGURE 3. FGF2 regulates mesenchymal transition- and proliferation-related gene expression at the translational level in human ex vivo corneal endothelium. After treatment of human ex vivo corneas for indicated times (0 or 14 days), total protein was isolated from the endothelium-Descemet’s membrane complex. A) Increased protein levels of SNAI1, SNAI2, ZEB1 and ZEB2 were noted in FGF2 treated but not in vehicle control treated human ex vivo corneal endothelium. B) Increased expression of COL1, fibronectin, and vimentin, and suppression of E-cadherin were observed in FGF2 treated but not in vehicle control treated human ex vivo corneal endothelium. FGF2 treatment also had no effect on expression of αSMA protein. C) CDK2 and cyclin E1 protein levels were also increased in FGF2 treated but not in vehicle control treated human ex vivo corneal endothelium. D) COL8A2 and keratin 12 were used as markers of corneal endothelium and epithelium respectively, and β-actin was used as loading control. Veh C, vehicle control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1; αSMA, α-Smooth Muscle Actin; KRT12, keratin 12.

FIGURE 4. FGF2 regulates mesenchymal transition- and proliferation-related gene expression at the transcriptional level through SNAI1 in human primary corneal endothelial cells in vitro. A) To determine application time point of SNAI1 siRNA, human primary corneal endothelial cells (CEC) were transfected with SNAI1 siRNA and total protein was isolated at indicated times (3, 7 or 14 days) post-transfection. siRNA knockdown of FGF2-dependent SNAI1 protein expression was observed at up to 14
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days post transfection. Keratin 12 was used as control for corneal epithelial cell contamination. B) SNAI1 siRNA knocked down FGF2 dependent SNAI1, SNAI2, ZEB1 and ZEB2 expression in human primary CEC. C) SNAI1 siRNA knockdown led to inhibition of FGF2-dependent expression of COLIA1, COLIA2, and fibronectin, however, there was no change in expression of vimentin. FGF2-dependent suppression of E-cadherin was also reversed by SNAI1 siRNA. D) SNAI1 siRNA transfection inhibited FGF2-dependent expression of CDK2 and cyclin E1 in primary human CEC. COL8A2 and β-actin were also affected on FGF2 dependent expression of COL1A1, COL1A2, fibronectin and vimentin. There was no effect on gene expression with non-targeting siRNA transfection in all experiments. Veh C, vehicle control; Epi, corneal epithelium; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1; KRT12, keratin 12.

FIGURE 5. FGF2 regulates mesenchymal transition- and proliferation-related gene expression at the transcriptional level through SNAI1 in human ex vivo corneal endothelium. A) SNAI1 siRNA knockdown attenuated FGF2-dependent expression of SNAI1, SNAI2, ZEB1 and ZEB2 in human ex vivo corneal endothelium. B) SNAI1 siRNA knockdown decreased FGF2-dependent expression of COLIA1, COLIA2, fibronectin and vimentin. There also was an inhibition of FGF2-dependent suppression of E-cadherin expression by SNAI1 siRNA knockdown. C) SNAI1 siRNA knockdown attenuated FGF2-dependent expression CDK2 and cyclin E1. There was no effect on gene expression with non-targeting siRNA transfection in all experiments. COL8A2 and β-actin were used as corneal endothelial marker and loading control respectively, and they were reused in panels B, C, and D. Transfection with non-targeting control (NT) siRNA did not have any effect on gene expression in all experiments. Veh C, vehicle control; Epi, corneal epithelium; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1.

FIGURE 6. FGF2 regulates mesenchymal transition- and proliferation-related gene expression at the translational level through SNAI1 in human ex vivo corneal endothelium. A) SNAI1 siRNA knockdown inhibited FGF2 dependent expression of SNAI1, SNAI2, ZEB1 and ZEB2 proteins. B) FGF2-dependent expression of COL1, Fibronectin and Vimentin was inhibited SNAI1 siRNA knockdown. FGF2-dependent suppression of E-cadherin was also inhibited by SNAI1 siRNA knockdown. C) SNAI1 siRNA knockdown inhibited FGF2-dependent expression of CDK2 and CCNE1. Transfection with non-targeting control (NT) siRNA did not alter gene expression in all experiments. COL8A2 and β-actin were used as corneal endothelial marker and loading control respectively. Veh C, vehicle control; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1.

FIGURE 7. ZEB1 regulates fibrosis but not proliferation in human ex vivo corneal endothelium. RT-PCR (A) and western blotting (B) showed ZEB1 siRNA knockdown inhibited FGF2-dependent expression of COL1, fibronectin, and vimentin, and inhibited FGF2-dependent suppression of E-cadherin. SNAI1, SNAI2 and ZEB2 levels were not affected by ZEB1 siRNA knockdown. ZEB1 siRNA transfection also did not affect FGF2-dependent expression of CDK2 and cyclin E1. Transfection with non-targeting control siRNA did not alter gene expression in all experiments. COL8A2 and β-actin were used as corneal endothelial marker and loading control respectively. Veh C, vehicle control; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1.

FIGURE 8. CDK2 regulates proliferation but not fibrosis in human ex vivo corneal endothelium. RT-PCR (A) and immunoblotting (B) showed CDK2 siRNA knockdown inhibited FGF2-dependent expression of CDK but not cyclin E1. Moreover, CDK2 siRNA knockdown did not alter FGF2-dependent expression of SNAI1, SNAI2, ZEB1, ZEB2, fibronectin, vimentin, and type I collagen. There also was effect on FGF2-dependent suppression of E-cadherin by CDK2 siRNA knockdown. Transfection with non-targeting control siRNA did not alter gene expression in all experiments. COL8A2 and β-actin
were used as corneal endothelial marker and loading control respectively. Veh C, vehicle control; NT, non-targeting control; FN1, fibronectin; VIM, vimentin; CDH1, E-cadherin; CCNE1, cyclin E1.

FIGURE 9. FGF2 promotes proliferation through SNAI1 but not ZEB1 in human ex vivo corneal endothelium. A) FGF2 treatment following injury of human ex vivo corneal endothelium induced phosphorylation of Histone H3 (pH3) in the nuclei (green) of endothelial cells immediately adjacent to the injury site. FGF2-induced histone H3 phosphorylation was severely attenuated by SNAI1 or CDK2 siRNA knockdown, but not by ZEB1 siRNA knockdown. Transfection with non-targeting (NT) control siRNA had no effect on FGF2-induced histone H3 phosphorylation. Corneal endothelial cell membranes were visualized using anti-ZO-1 (red) antibody and nuclei were stained with DAPI (blue). Scale bar = 50 μm. B) FGF2 treatment increased the relative proliferation rate compared to vehicle control in human ex vivo corneal endothelium, 15.3 ± 4.5 vs 1.0 ± 1.52, p < 0.05. SNAI1 and CDK2 siRNA knockdown reduced relative proliferation rates compared to FGF2 treatment alone, 3.35 ± 2.26 vs 15.3 ± 4.5, *p < 0.05 and 2.2 ± 1.24 vs 15.3 ± 4.5, **p < 0.05, respectively. There was no significant change in relative proliferation rate for ZEB1 siRNA knockdown versus FGF2 treatment alone, 14.7 ± 3.23 vs 15.3 ± 4.5. One-way ANOVA, F(5,9) = 9.0, p < 0.003, n = 9 per sample. Tukey’s post-hoc test, HSD[0.05] = 11.4 and HSD[0.01] = 15.0. Veh C, vehicle control.

FIGURE 10. FGF2 signaling enhances mesenchymal transition through SNAI1 and ZEB1 in human corneal endothelium. FGF2 induced SNAI1 activates parallel and independent ZEB1 and CDK2 pathways, leading to regulation of fibrosis and proliferation.
Figure 1

A

![Veh C](image)  ![FGF2](image)

100 µm

B

| Gene   | Veh C 0h | Veh C 48h | Veh C 45d | Veh C 45d | FGF2 0h | FGF2 48h | FGF2 45d |
|--------|----------|-----------|-----------|-----------|----------|----------|----------|
| COL1A1 | ![400bp](image) | ![200bp](image) | ![400bp](image) | ![400bp](image) | ![500bp](image) | ![300bp](image) | ![500bp](image) |
| COL1A2 | ![200bp](image) | ![500bp](image) | ![300bp](image) | ![300bp](image) | ![500bp](image) | ![500bp](image) | ![500bp](image) |
| α-SMA  | ![400bp](image) | ![200bp](image) | ![400bp](image) | ![400bp](image) | ![500bp](image) | ![300bp](image) | ![500bp](image) |
| COL8A2 | ![500bp](image) | ![300bp](image) | ![500bp](image) | ![500bp](image) | ![300bp](image) | ![500bp](image) | ![300bp](image) |
| β-actin| ![500bp](image) | ![300bp](image) | ![500bp](image) | ![500bp](image) | ![500bp](image) | ![500bp](image) | ![500bp](image) |
Figure 2
Figure 3

A

| 0 d | 14 d |
|-----|------|
| Veh C | Veh C | F2 |

- SNAI1
- SNAI2
- ZEB1
- ZEB2

B

| 0 d | 14 d |
|-----|------|
| Veh C | Veh C | F2 |

- COL1
- FN1
- VIM
- α-SMA
- CDH1

C

| 0 d | 14 d |
|-----|------|
| Veh C | Veh C | F2 |

- CDK2
- CCNE1

D

| 0 d | 14 d |
|-----|------|
| Veh C | Veh C | F2 |

- COL8A2
- β-actin
- KRT12
### Figure 4

**A**

|       | Veh C | FGF2 |
|-------|-------|------|
|       | 0 d   | 14 d |
|       | +     | −    |
|       | +     | −    |
|       | +     | −    |
|       | 14 d  |      |

|       | SNAI1 siRNA |
|-------|-------------|
|       | +           |
|       | −           |
|       | +           |
|       | −           |
|       | 14 d        |

**B**

|       | Veh C | FGF2 |
|-------|-------|------|
|       | −     | +    |
|       | −     | +    |
|       | −     | NT   |

|       | SNAI1 siRNA |
|-------|-------------|
|       | +           |
|       | −           |
|       | +           |
|       | −           |
|       | +           |

**C**

|       | Veh C | FGF2 |
|-------|-------|------|
|       | −     | +    |
|       | −     | +    |
|       | −     | NT   |

|       | COL1A1 |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | COL1A2 |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | FN1    |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | VIM    |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | CDH1   |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | COL8A2 |
|-------|--------|
|       | +      |
|       | −      |
|       | +      |
|       | −      |

|       | β-actin |
|-------|---------|
|       | +       |
|       | −       |
|       | +       |
|       | −       |

**D**

|       | Veh C | FGF2 |
|-------|-------|------|
|       | −     | +    |
|       | −     | +    |
|       | −     | NT   |

|       | CDK2  |
|-------|------|
|       | +    |
|       | −    |
|       | +    |
|       | −    |

|       | CCNE1 |
|-------|------|
|       | +    |
|       | −    |
|       | +    |
|       | −    |

|       | COL8A2 |
|-------|-------|
|       | +     |
|       | −     |
|       | +     |
|       | −     |

|       | β-actin |
|-------|---------|
|       | +       |
|       | −       |
|       | +       |
|       | −       |
Figure 5
Figure 6

A) Veh C | FGF2
---|---
— | +

SNAI1

SNAI2

ZEB1

ZEB2

SNAI1 siRNA

37 KDa
25 KDa

150 KDa
100 KDa

B) Veh C | FGF2
---|---
---|+

COL1

FN1

VIM

CDH1

SNAI1 siRNA

150 KDa
100 KDa

250 KDa

75 KDa
50 KDa

100 KDa
75 KDa

C) Veh C | FGF2
---|---
— | +

CDK2

CCNE1

SNAI1 siRNA

37 KDa
25 KDa

50 KDa
37 KDa

150 KDa
100 KDa

37 KDa
25 KDa

50 KDa
37 KDa

75 KDa
50 KDa

75 KDa
Figure 7

A  

| Veh C | FGF2 |
|-------|------|
|  —    |  +   |
| NT    |  —   |  —   |

ZEB1 siRNA

SNAI1  
SNAI2  
ZEB1  
ZEB2  

COL1A1  
COL1A2  

COL1  

FN1  
VIM  

CDH1  

CDK2  
CCNE1  

COL8A2  

β-actin

B  

| Veh C | FGF2 |
|-------|------|
|  —    |  +   |
|  —    |  +   |
|  NT   |  —   |

ZEB1 siRNA

37 KDa  
25 KDa  
150 KDa  
100 KDa  

150 KDa  
100 KDa  

250 KDa  

75 KDa  
50 KDa  

100 KDa  
75 KDa  

37 KDa  
25 KDa  
50 KDa  
37 KDa  

75 KDa  
50 KDa  
37 KDa
Figure 8

A  

|       | Veh C  | FGF2 | CDK2 siRNA |
|-------|--------|------|------------|
|       | −      | +    | −          |
|       | −      | +    | +          |
|       | −      | +    | NT         |

SNAI1
SNAI2
ZEB1
ZEB2
COL1A1
COL1A2
FN1
VIM
CDH1
CDK2
CCNE1
COL8A2
β-actin

B  

|       | Veh C  | FGF2 | CDK2 siRNA |
|-------|--------|------|------------|
|       | −      | +    | −          |
|       | −      | +    | +          |
|       | −      | +    | NT         |

COL1
FN1
VIM
CDH1
CDK2
CCNE1
COL8A2
β-actin
### A

| Treatment          | pH3 | ZO-1 | DAPI | Overlap |
|--------------------|-----|------|------|---------|
| Veh C              | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| FGF2               | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| FGF2/ SNAI1 siRNA  | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| FGF2/ ZEB1 siRNA   | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| FGF2/ CDK2 siRNA   | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |
| FGF2/ NT siRNA     | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) |

### B

| siRNA | Relative Proliferation Rate (fold of control) |
|-------|-----------------------------------------------|
| Veh C | 1.0                                           |
| SNAI1 | 15.3 **(*)**                                  |
| ZEB1  | 3.35                                          |
| CDK2  | 14.7                                          |
| NT    | 2.2                                           |
| FGF2  | 13.3 **(**)                                   |

*Significant at p < 0.05
**Significant at p < 0.01

Figure 9
Figure 10

FGF2

SNAI1/2

CDK2 / CCNE1  ZEB1 / ZEB2

COL1 / FN1 / VIM  E-cadherin

Cell Proliferation  Fibrosis
Fibroblast growth factor 2 induces proliferation and fibrosis via SNAI1-mediated activation of CDK2 and ZEB1 in corneal endothelium
JeongGoo Lee, Eric Jung and Martin Heur

J. Biol. Chem. published online January 23, 2018

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