The corneal epithelium is the outermost layer of the cornea and acts as a barrier that protects the corneal interior from infection by noxious environmental agents. The corneal epithelium accounts for 10% of corneal depth and is maintained by a relatively acellular, dense stroma of tightly packed collagen fibers in an environment that lacks blood vessels. Invasion of the corneal epithelium by vessels decreases the function of the fibers in an environment that lacks blood vessels. Invasion by a relatively acellular, dense stroma of tightly packed collagen fibers accounts for 10% of corneal depth and is sustained by proangiogenic factors, epithelial phenotypes, and wound healing in corneal epithelial cells.

### Purpose
Subconjunctival injection of antagonim-21 attenuates the progression of corneal neovascularization. We examined the underlying mechanism by investigating the regulation of microRNA (miR)-21 expression and the involvement of miR-21 in the homeostasis of corneal epithelial cells.

### Methods
Corneal epithelial cells were cultured with TGF-β1 and/or under hypoxia conditions. miR-21 expression was measured by quantitative PCR. The direct targets of miR-21 were validated by the 3’UTR luciferase reporter assay. Alterations of proangiogenic signaling and the epithelial-mesenchymal transition (EMT) phenotype after miR-21/Sprouty2 (SPRY2) knockdown were examined by Western blotting. The effect of conditioned medium on angiogenesis was assessed using the tube formation assay. Wound healing was evaluated by the migration and scratch assays.

### Results
TGF-β1 or hypoxia upregulated miR-21, and miR-21 silencing abolished TGF-β1/hypoxia-induced hypoxia inducible factor (HIF)-1α and VEGF expression. miR-21 inhibited SPRY2 by directly targeting its 3’UTR. Simultaneous silencing of miR-21 and SPRY2 significantly upregulated p-ERK, HIF-1α, and VEGF and promoted angiogenesis. Induction of miR-21 or inhibition of SPRY2 reduced the levels of cytokeratin (CK)-3 and CK-12 and promoted EMT. Transwell and wound healing assays indicated that miR-21 promoted cell migration.

### Conclusions
TGF-β1 or hypoxia induced miR-21 and inhibited SPRY2, thereby enhancing proangiogenic signaling, suppressing the epithelial phenotype, and promoting wound healing in corneal epithelial cells.

Keywords: corneal epithelial cell, miR-21, SPRY2, proangiogenic, epithelial markers, corneal wound healing.

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**The Role of the miR-21/SPRY2 Axis in Modulating Proangiogenic Factors, Epithelial Phenotypes, and Wound Healing in Corneal Epithelial Cells**

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YZ and FY contributed equally to the work presented here and should therefore be considered equivalent authors.

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calcium chloride, 0.2 ng/mL epidermal growth factor, and 25 keratinocyte serum-free medium (KSFM, Thermo Fisher, Germany), which enabled the mixing of N₂ and air to achieve an oxygen content of 1%

The culture medium was changed every 2 days. The culture was kept in a modular incubator equipped with an O₂ sensor (Eppendorf, Hamburg, Germany) at 37°C with 5% humidified CO₂ during routine passages, and the medium was incubated at 100°C for 10 minutes and separated on SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with a 5% BSA solution. The membranes were incubated overnight at 4°C with antibodies listed in the Table. Specific bands were scanned and analyzed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

Luciferase Reporter Assay

Corneal epithelial cells were seeded in 6-well plates at a density of 1 × 10⁵ cells/well and maintained in culture medium for 24 hours. The culture medium was then removed and replaced with 10 ng/ml TGF-β1 in medium. Cells were cultured in a modular incubator equipped with an O₂ sensor (Eppendorf, Hamburg, Germany), which enabled the mixing of N₂ and air to achieve hypoxic conditions (1% O₂ and 5% CO₂ balanced with N₂).

**Materials and Methods**

**Mouse Corneal Epithelial Cell Culture**

All experimental protocols were approved by the animal care and use committee of Shanghai Tenth People’s Hospital Affiliated to Tongji University in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse corneal epithelial cells were harvested from the eyeballs of BALB/c mice (SLAC Animal, Shanghai, China). Briefly, epithelium was obtained by digestion with 0.2% collagenase IV (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C for 2 hours. Single cells were obtained by digestion with 0.25% trypsin-EDTA (Sigma-Aldrich Corp.) at 37°C for 15 minutes, and cells were centrifuged and resuspended in keratinocyte serum-free medium (KSF, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.3 M calcium chloride, 0.2 ng/mL epidermal growth factor, and 25 µg/ml brain pituitary extract. The cells were maintained at 37°C in 5% humidified CO₂ during routine passages, and the medium was changed every 2 days.

**Treatment of Corneal Epithelial Cells With TGF-β1 and Hypoxia**

TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA). Corneal epithelial cells were seeded in 6-well plates at a density of 1 × 10⁵ cells/well and maintained in culture medium for 24 hours. The culture medium was then removed and replaced with 10 ng/ml TGF-β1 in medium. Cells were cultured in a modular incubator equipped with an O₂ sensor (Eppendorf, Hamburg, Germany), which enabled the mixing of N₂ and air to achieve hypoxic conditions (1% O₂ and 5% CO₂ balanced with N₂).

**Cell Transfection**

The synthesized siRNA oligonucleotides against SPRY2, the miR-21 inhibitor, and the negative control for the miRNA inhibitor were purchased from RIBOBIO (Guangzhou, China). Cells were transfected with oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM following the manufacturer’s instructions.

**Quantitative Real-Time PCR**

Total RNA was extracted from cells using the TRIzol (Invitrogen) reagent according to the manufacturer’s instructions. Aliquots of 0.5 µg RNA were reverse transcribed to cDNA using the PrimeScript RT reagent kit (Takara, China). A stem-loop primer (5'-GTCGTATCCAGTGAGGTGTCGAGCGTTAGCTTACAGACTACA-3') was included for miR-21 reverse transcription, and a specific primer (5'-AAGGCTCACGAATTGGG-3') was included for reverse transcription of U6. Quantitative PCR (QPCR) was performed using the ABI 7500 Fast System (Applied Biosystems, Foster City, CA, USA). QPCR was performed using the SYBR PrimeScript RTPCR kit (Takara), and the reaction mixtures were incubated at 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The following primers were used: miR-21 forward: 5'-CGCGGTTAGCTTATCCAAGACTGA-3', reverse 5'-CCAGTGACGGGTCCGAGGTTAGCTTATCCAAGACTGA-3'. U6 forwards: 5'-CTCGCTTGGGACACGACA-3', reverse 5'-AAGGCTCACGAATTGGG-3'. Experiments were performed at least three times.

**Western Blot Analysis**

Protein was extracted from the cells on ice for 15 minutes using RIPA lysis buffer (Beyotime, Haimen, China) supplemented with protease inhibitors and phosphatase inhibitors (Merck, Kenilworth, NJ, USA). The supernatants were collected before transfection. Cells were stained with antibodies listed in the Table. Specific bands were scanned and analyzed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

The Role of miR-21 in Corneal Epithelial Cells

**Table. Antibody Information in Western Blot**

<table>
<thead>
<tr>
<th>Name of Antibody</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
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<td>1:500</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Abcam</td>
<td>ab46154</td>
<td>1:2000</td>
</tr>
<tr>
<td>Tubulin</td>
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<td>1:2000</td>
</tr>
<tr>
<td>SPRY1</td>
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<td>#15013</td>
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</tr>
<tr>
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<td>Cell Signaling Technology</td>
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<td>Cell Signaling Technology</td>
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<td>1:1000</td>
</tr>
<tr>
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<td>Abcam</td>
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<td>1:1000</td>
</tr>
<tr>
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</tr>
<tr>
<td>vimentin</td>
<td>Abcam</td>
<td>ab20346</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

that SPRY2 and SPRY4 are downregulated in alkali-burned cornea, and their levels are restored in antagomir-21-treated corneas compared to the negative controls (antagomir-NC). However, whether the expression of HIF-1α or VEGF is affected by the SPRY family of proteins in corneal epithelial cells remains to be determined.

We showed that miR-21 was induced by TGF-β1 or hypoxia and directly targeted SPRY2 in corneal epithelial cells. We found that miR21/SPRY2 signals were involved in the regulation of p-ERK, HIF-1α, VEGF, and corneal epithelial markers including cytokeratin (CK)-3 and CK-12, as well as cell migration.

**MATERIALS AND METHODS**

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Lipofectamine 2000 (Invitrogen). At 36 hours after transfection, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). All experiments were performed in triplicate.

**Tube Formation Assay**

The 96-well plates were filled with 75 μL Matrigel (Corning Matrigel Basement Membrane Matrix; Corning, Inc., Corning, NY, USA) and allowed to solidify at 37°C. Then HUVECs (2 × 10^4 cell) were gently seeded on top of the gel in the presence of conditioned medium of corneal epithelial cells. After 16 hours of incubation, tube-forming structures were analyzed. The average number of tubules was calculated from three fields in each sample.

**Scratch Wound Closure Assay**

Corneal epithelial cells were seeded in 24-well plates at a density of 5 × 10^4 cells/cm² and incubated for 24 hours in medium to form a confluent monolayer. Two linear defects were produced by a silicone needle in monolayer cells. The scratch-wounded corneal epithelial cells were washed with 1× PBS to remove any cell fragments. Still images were acquired at two independent points in each defect under a Zeiss Observer Z1 microscope (Carl Zeiss Meditec, Jena, Germany) equipped with a CCD camera at 12 and 24 hours. The remaining wound area was measured using ImageJ software (version 1.48, NIH).

**Transwell Migration Assay**

Migration experiments were performed using a 24-well Transwell chemotaxis chamber technique. Briefly, the Transwell membrane was precoated with 30 μL of Matrigel, the lower chamber was filled with Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (ScienCell Research Laboratories, Carlsbad, CA, USA), and a total of 1 × 10^6 cells in 200 μL medium were seeded into the upper chamber. Chambers were incubated for 24 hours at 37°C. The cells remaining on the upper surface of the membrane were removed followed by PBS washes, and those on the undersurface of the membrane were fixed and stained with crystal violet. Fields on the membrane were counted under a ×20 objective. Each assay was performed in triplicate.

**Statistical Analysis**

Results were analyzed statistically using 1-way ANOVA and paired-sample t-tests. The tube formation data are expressed as the mean and each data point represents an individual sample. All other data are expressed as the average ± SD. P < 0.05 was considered statistically significant.

**RESULTS**

**TGF-β1 and Hypoxia Induce the Expression of Proangiogenic Factors in Corneal Epithelial Cells by Upregulating miR-21**

The effect of TGF-β1 or hypoxia on the expression of proangiogenic factors was assessed by exposing mouse corneal epithelial cells to TGF-β1 and/or 1% O₂ (hypoxia) for 48 hours. As shown in Figures 1A and B, TGF-β1 and 1% O₂ upregulated HIF-1α and VEGF. Although the two treatments had similar
effects on VEGF induction, HIF-1α levels increased more dramatically in response to 1% O₂ than TGF-β1 treatment. Simultaneous treatment with TGF-β1 and 1% O₂ had a stronger effect on upregulating VEGF than single agent treatment, indicating a synergistic action. We previously showed that miR-21 expression is increased in the alkali-burned cornea compared to the healthy cornea. Here, we showed that TGF-β1 or 1% O₂ for 24 and 48 hours upregulated miR-21, and the effect was stronger at 24 hours (Figs. 1C, 1D). Combination treatment resulted in a higher miR-21 induction at 24 hours (Fig. 1C).

To elucidate the biologic function of miR-21, we next inhibited the expression of miR-21 in the presence of TGF-β1 treatment and/or hypoxia conditions. Silencing of miR-21 drastically downregulated HIF-1α and VEGF protein expression, restoring VEGF expression to basal levels (Figs. 1E, 1F). These results supported that miR-21 induces the proangiogenic phenotype of corneal epithelial cells in the presence of TGF-β1 treatment and/or hypoxia.

**MiR-21 Directly Targets SPRY2 in Corneal Epithelial Cells**

Previously, we found that SPRY1/2/4, which are candidate targets of miR-21, were significantly inhibited in the alkali-burned cornea at 4 days post injury compared to their expression in the normal cornea. Based on these results, we examined whether miR-21 upregulation is required for the downregulation of the SPRY family of proteins under TGF-β1 or hypoxia treatment in corneal epithelial cells. The results showed that SPRY2, but not SPRY1 or 4, was significantly suppressed in response to TGF-β1 or hypoxia treatment, whereas p-ERK, which is negatively regulated by the SPRY family of proteins, was activated (Figs. 2A, 2B). Inhibition of miR-21 rescued SPRY2 expression and inhibited p-ERK to basal levels. The levels of SPRY1/4 remained unchanged (Figs. 2A, 2B).

Bioinformatics analyses identified a putative miR-21 binding site in the 3' UTR of Spry2 that was conserved across species (Fig. 2C). The results of the 3' UTR reporter assay showed that the luciferase activity of the wild-type Spry2 3' UTR construct was significantly inhibited by miR-21 mimic (Fig. 2D). Conversely, suppression of miR-21 increased the luciferase activity of the wild-type construct (Fig. 2E). miR-21 expression had no significant impact on the luciferase activity of the mutant Spry2 3' UTR (Fig. 2C–E). Taken together, these results suggested that miR-21 directly targeted the 3' UTR of Spry2 and inhibited its expression in corneal epithelial cells.
The miR-21/SPRY2 Axis is Vital for the Proangiogenic Phenotype of Corneal Epithelial Cells

We showed that TGF-β1 or hypoxia downregulated SPRY2 and upregulated p-ERK, HIF-1α, and VEGF, and these effects were reversed by silencing miR-21.

To examine the biologic significance of the miR-21-mediated downregulation of SPRY2, we simultaneously knocked down miR-21 and SPRY2 in corneal epithelial cells. SPRY2 siRNA specifically decreased SPRY2 level without altering SPRY1/4 levels. Knockdown of Spry2 abolished the effect of miR-21 inhibition on downregulating p-ERK, HIF-1α, and VEGF. Silencing of SPRY2 almost completely reversed the effects of miR-21 inhibition, suggesting that high levels of SPRY2 are essential for the suppression of p-ERK, HIF-1α, and VEGF (Fig. 3). These data confirmed that miR-21 promoted p-ERK activation and the proangiogenic phenotype of corneal epithelial cells by suppressing SPRY2.

Since proangiogenic factors were induced in corneal epithelial cells in response to TGF-β1 or 1% O2 treatment, we hypothesized that this treatment also may induce angiogenesis. A tube formation assay was performed to evaluate the effect of conditioned medium from corneal epithelial cells on the angiogenesis of HUVECs in Matrigel. The results showed that conditioned medium from TGF-β1 and/or hypoxia-treated cells promoted the development of tubes at a rate 2.01- to 3.19-fold higher than that in cells exposed to untreated conditioned medium. However, the effect of conditioned medium from miR-21 inhibitor-treated cells was comparable to that of medium from untreated cells. Inhibition of SPRY2 notably induced angiogenesis of HUVECs, whereas miR-21 levels were largely reduced (Fig. 4).

The miR-21/SPRY2 Axis Regulates the Expression of Corneal Epithelial Markers and Induces EMT

To evaluate whether the miR-21/SPRY2 axis regulates the expression of corneal epithelial markers as well as the epithelial-mesenchymal transition (EMT) process, the levels of CK3, CK12, and vimentin were measured in the presence of miR-21 inhibitor and/or SPRY2 siRNA. The results showed that TGF-β1 and/or 1% O2 suppressed the expression of CK3, whereas only TGF-β1 and 1% O2 suppressed CK3 and increased vimentin expression. Inhibition of miR-21 abolished the downregulation of epithelial markers and upregulation of vimentin. Coinhibition of miR-21 and SPRY2 downregulated CK3 and CK12 and upregulated vimentin level (Fig. 5). These results indicated that corneal epithelial markers, such as CK3, CK12, and EMT, were regulated by the miR-21/SPRY2 axis under TGF-β1 treatment or hypoxia conditions.
miR-21 Promotes the Migration of Corneal Epithelial Cells and Increases Wound Healing

We next set out to investigate the impact of TGF-β1 or hypoxia on corneal epithelial cell migration and proliferation. Corneal epithelial cells were exposed to TGF-β1 and/or 1% O2 in the presence of miR-21 or control inhibitor for 0, 12, or 24 hours. The scratch wound closure assay was performed to measure cell migration. The results showed that wound closure was enhanced after 24 hours in TGF-β1-treated cultures and at 12 hours in response to 1% O2 and combination treatment with TGF-β1 and 1% O2 compared to vehicle controls (Figs. 6A, 6B). However, inhibition of miR-21 attenuated the overall wound healing rate (Figs. 6A, 6B). The Transwell assay was performed to examine the effects of miR-21 on migration, and the results showed that TGF-β1 and 1% O2 increased the migration of corneal epithelial cells, whereas inhibition of miR-21 significantly decreased migration (Figs. 6C, 6D). Collectively, these results showed that miR-21 induced the migration of corneal epithelial cells.

Figure 4. Conditioned medium from corneal epithelial cells affected angiogenesis of HUVECs. (A, B) Corneal epithelial cells were treated with miR-21 inhibitor in the presence or absence of siRNA against SPRY2 and subsequently exposed to TGF-β1, 1% O2, or TGF-β1 plus 1% O2. The conditioned medium from each group was added to HUVEC culture. Tube formation was photographed (A) and counted (B). The experiment was repeated three times, and each sample was tested in triplicate. Scale bar: 500 μm.

Figure 5. EMT of corneal epithelial cells is regulated by the miR-21/SPRY2 axis under TGF-β1 treatment or hypoxia conditions. (A, B) Corneal epithelial cells were treated with miR-21 inhibitor in the presence or absence of siRNA against SPRY2 and exposed to TGF-β1 and/or 1% O2. (A, B) Cells were subjected to Western blot analyses (A) of corneal epithelial markers (CK3 and CK12) and vimentin followed by densitometry analysis using ImageJ software (B). Tubulin was used as the loading control. Experiments were repeated three times.
**DISCUSSION**

The normal cornea actively maintains avascularity, and the ingrowth of new blood vessels into the cornea reduces visual acuity. In a previous study, we showed that antagonim-21 attenuates the progression of corneal neovascularization, and this effect may be mediated by SPRY2/4 inhibition of p-ERK. Here, we investigated the role of miR-21 in corneal epithelial cells. The results showed that miR-21 was induced by TGF-β1 or hypoxia, and suggested that the miR-21/SPRY2 axis is vital for wound healing and the regulation of proangiogenic and epithelial markers in corneal epithelial cells.

We previously showed that miR-21 was significantly increased in the alkali-burned cornea compared to the healthy...
corneal epithelial wound healing is crucial for the health of the cornea. Corneal epithelial wound healing is a complex process that involves cell proliferation, migration, and differentiation. Several miRNAs have an important role in inducing corneal epithelial cell proliferation and migration. For instance, miR-184 has been shown to promote cell proliferation and migration by inhibiting the expression of Spry2, which negatively regulates FGF–ERK signaling. This regulation is critical for maintaining corneal transparency and preventing irreplaceable cell loss. Furthermore, the conditioned medium from TGF-β1 or hypoxia-treated cells induced angiogenesis. To determine whether the effects of TGF-β1 or hypoxia were mediated by miR-21, we inhibited the expression of miR-21 and found that the levels of HIF-1α and VEGF were reduced, and CK3 and CK12 levels were increased. The conditioned medium failed to induce tube formation. Taken together, these results indicated that TGF-β1 or hypoxia induced angiogenic factors and inhibited corneal epithelial cell markers in corneal epithelial cells by increasing the expression of miR-21.

SPRY proteins are downstream targets and negative feedback regulators of the FGF-Ras-ERK signaling pathway. Kuracha et al. reported that Spry1 and Spry2-mediated negative modulation of FGF-ERK signaling regulates lens vesicle separation and corneal epithelial proliferation. We previously reported that SPRY1/2/4 were significantly inhibited in the alkali-burned cornea 4 days after injury compared to normal corneas. However, inhibition of SPRYs to determine their function was not performed previously. Here, we demonstrated that SPRY2, but not SPRY1 or 4, was suppressed in response to TGF-β1 or hypoxia treatment. Klein et al. showed that different Sprouty genes are expressed in different tissue compartments (Spry2 in the epithelium and Spry4 in the mesenchyme) to prevent diastema tooth formation. We speculated that Spry2, but not Spry1/4, has a role in the corneal epithelium. The 3′-UTR reporter assay confirmed that miR-21 directly targets the 3′-UTR of Spry2 and inhibits its expression in corneal epithelial cells. Furthermore, we inhibited the expression of miR-21 and SPRY2 simultaneously, and the results showed that p-ERK, HIF-1α, and VEGF were significantly induced, and the corneal epithelial markers CK3 and CK12 were considerably reduced compared to those in cells treated with miR-21 inhibitor. These results implied that high levels of SPRY2 were essential for suppressing the expression of p-ERK, HIF-1α, and VEGF and maintaining the expression of CK3 and CK12. In agreement with our results, Impagnatiello et al. reported that Spry1 and Spry2 are membrane-anchored proteins that negatively regulate angiogenesis-associated receptor tyrosine kinase signaling and inhibit angiogenesis. Taken together, our results indicated that the miR-21/SPRY2 axis is vital for the proangiogenic phenotype of corneal epithelial cells. In summary, our results showed that TGF-β1 or hypoxia failed to induce tube formation. It was vital for the proangiogenic phenotype of corneal epithelial cells. The miR-21/SPRY2 axis modulated epithelial phenotypes, as indicated by CK3 and CK12 expression. In addition, we demonstrated that TGF-β1 or hypoxia promoted the migration of corneal epithelial cells and increased wound healing via miR-21.

Acknowledgments

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