miRNA-181a Inhibits the Proliferation, Migration, and Epithelial–Mesenchymal Transition of Lens Epithelial Cells

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Purpose. MicroRNA-181a (miR-181a) is thought to be involved in posterior capsule opacification (PCO). This study investigated the role of miR-181a in the proliferation, migration, and epithelial–mesenchymal transition (EMT) of lens epithelial cells (LECs).

Methods. The expression of miR-181a was detected in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts by quantitative RT-PCR (qRT-PCR). The proliferation of SRA01/04 cells transfected with miR-181a mimics was analyzed by MTT assays and bromodeoxyuridine (BrdU)-incorporation assays. The migration of SRA01/04 cells was evaluated by wound-healing assays and Transwell migration. Luciferase reporter assays were used to validate the regulation of a putative target of miR-181a.

Results. The expression of miR-181a is decreased in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts. A significant decrease in proliferation was observed in SRA01/04 cells transfected with miR-181a mimics. The overexpression of miR-181a inhibited the migration ability of LECs. Downregulation of fibronectin, Slug, and cyclooxygenase-2 (COX-2) expression and upregulation of E-cadherin expression were induced in human PCO-attached LECs transfected with miR-181a mimics and miR-181a–overexpressing LECs obtained from patients with anterior polar cataracts. Furthermore, luciferase assays using a reporter carrying a putative miR-181a target site in the 3′ untranslated region of c-Met, Slug, and COX-2 revealed that miR-181a directly targets c-Met, Slug, and COX-2.

Conclusions. These data reveal that miR-181a can inhibit the proliferation, migration, and EMT of LECs and suggest that the restoration of miRNA-181a expression may be a potential novel therapeutic target for the prevention and treatment of posterior capsule opacification.

Keywords: lens epithelial cells, proliferation, migration, epithelial–mesenchymal transition, miR-181a

Posterior capsule opacification (PCO) is the most common complication of cataract surgery, resulting in a secondary loss of vision.[1] Posterior capsule opacification is generally associated with the pathologic progression of postoperative residual lens epithelial cells (LECs), including proliferation, migration, and epithelial–mesenchymal transition (EMT).[1–3] Epithelial–mesenchymal transition results in loss of cell adhesion and apical–basal polarity toward the mesenchymal phenotype, giving rise to fibroblasts and myofibroblasts. Additionally, LECs can also transdifferentiate into mesenchyme-like cells during the formation of anterior subcapsular cataracts.[4–6]

Many cytokines and growth factors, such as hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), transforming growth factor β (TGF-β), and epidermal growth factor (EGF), are involved in the development of PCO, and the levels of these cytokines and growth factors in the aqueous humor increase after cataract surgery.[3,7] A large number of studies have shown that HGF and its receptor c-Met are expressed in primary cultures of human lens cells and LECs.[8–10] HGF and c-Met perform a multitude of functions in LECs, such as stimulating proliferation, protein synthesis, and the migratory responses of LECs.[8–10]

The molecular mechanisms of EMT appear to be multifold and cell-type specific, but one of the key events in EMT is a loss of cadherin junctions between epithelial cells, which results from the suppression of the molecular hallmarks that compose the junctional complexes, such as E-cadherin. Several transcriptional repressors of E-cadherin have now been identified, including the zinc-finger transcription factor Slug, which belongs to the Snail superfamily.[11–13] Increasing evidence indicates that Slug, which binds specifically to a subset of E-box motifs (E2-box: CAGGTG/CACCTG) in target promoters, such as the E-cadherin promoter, plays an important role in EMT.[11–13]

Cyclooxygenase (COX) is the rate-limiting enzyme in prostanoid synthesis. *Cyclooxygenase-2* (*COX-2*) is an immediate early response gene that is typically not expressed in resting cells, but its expression is highly inducible by growth factors and inflammatory cytokines; it is well known to be involved in inflammation, carcinogenesis, and EMT.[14,15] Recent studies have demonstrated that COX-2 was upregulated in canine cataracts and PCO.[16] Additionally, inhibiting its enzymatic activity effectively prevented the EMT of LECs in vitro by decreasing migration and proliferation and increasing apoptosis in an ex vivo model of PCO.[16]

MicroRNAs (miRNAs) are small noncoding RNAs consisting of 22 nucleotide base pairs, which could regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions (UTRs) of target mRNAs, resulting in mRNA either translational repression or transcript degradation.[17] Evidence also indicates that some miRNAs play a role in regulating lens differentiation,[18] cataractogenesis,[19,20] and PCO.[21] Specifically, we previously reported that the expression of miR-26b is downregulated in human PCO-attached LECs and that miR-26b can inhibit the proliferation, migration, and EMT of LECs.[22] MicroRNA-181a (miR-181a) belongs to the miR-181s family, which includes four highly conserved mature miR-181s (miR-181a, miR-181b, miR-181c, and miR-181d).[23] Recent findings indicate that miR-181a is a multifunctional miRNA that participates in many biological processes, such as apoptosis, cell proliferation, and cellular invasion, and functions as a tumor suppressor.[24] Furthermore, a recent study has shown that miR-181a expression is downregulated in human PCO tissues when comparing the miRNA profile in human PCO tissues with that of normal-attached LECs[25]; however, the mechanism for dysregulation of miR-181a expression that is involved in PCO has not been reported to date.

Therefore, the aim of the present study was to explore the ability of miRNA-181a to affect the PCO. In this study, we present the first evidence demonstrating that miR-181a can inhibit the proliferation, migration, and EMT of LECs by directly silencing c-Met, Slug, and COX-2.

Materials and Methods

Patient Lens Epithelial Cell Collection and Culture

Fresh lens capsules with adherent LECs were obtained during cataract surgery from 46 patients with the clinical diagnosis of nuclear or anterior polar cataracts. The ages of the patients ranged from 42 to 86 years. The study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University, Beijing, People's Republic of China, and the study was performed in accordance with the Declaration of Helsinki. Each participant received detailed information and provided informed written consent before being included in the study. In brief, after injecting viscoelastic agent into the anterior chamber, continuous curvilinear capsulorrhexis was performed. The anterior capsules were carefully removed with forceps and spread onto tissue culture dishes with the epithelium side up. Then, a single drop of fetal bovine serum (FBS) was placed on the epithelial surface to allow for adequate adherence of the tissue to the dish. Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS was added, and the cells were incubated in a 5% CO_2 humidified atmosphere at 37°C until further experimentation.

Fresh PCO tissues and normal-attached LEC samples from organ donors were provided by the Eye Bank of Beijing, China (Beijing, China). Lens capsules were spread onto tissue culture dishes, epithelium side up. Then, a single drop of FBS was placed on the epithelial surface to allow for adequate adherence of the tissue to the dish. Dulbecco's modified Eagle's medium supplemented with 20% FBS was added, and the cells were incubated in a 5% CO_2 humidified atmosphere at 37°C until further experimentation.

SRA01/04 Cell Culture

The human lens epithelial cell line SRA01/04 was obtained from the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). The method for SRA01/04 cell culture has been described in detail previously.[22] In brief, cells were routinely cultured in Eagle's minimum essential medium (Life Technologies Corp., Gibco/Brl, Grand Island, NY, USA) with 10% FBS in a 5% CO_2 humidified atmosphere at 37°C. Cells were passaged at approximately 80% confluence.

Transfection

The method for cell transfection has been previously described in detail.[22] In brief, miR-181a mimics and the scrambled control microRNA were obtained from GenePharma (Shanghai, China). For 1×10^6 cells, 0.4 nmol microRNA mimics or miR-181a control mimic were mixed with 15 μL GenePORTER transfection reagent (GTS, Inc., San Diego, CA, USA) and transfected into the cells. After 6 hours, the supernatant was removed, and fresh medium was added.

Quantitative RT-PCR (qRT-PCR)

The qRT-PCR method has been described in detail previously.[22,26] The primers are as follows: miR-181a sense, 5′-CGCCTCGAGCCCAATATATGTTAATCTCTTACC-3′; miR-181a antisense, 5′-GCGCGCGTCGACTTTTTAATAAATTTTTACTTGCTA-3′; E-cadherin sense, 5′-GTCATCCAACGGGAATGCA-3′; E-cadherin antisense, 5′-TGATCGGTTACCGTGATCAAAA-3′; fibronectin sense, 5′-AGCGGACCTACCTAGGCAAT-3′; fibronectin antisense, 5′-GGTTTGCGATGGTACAGCTT-3′.

MTT Cell Proliferation Assay

The method for the MTT cell proliferation assay has been described in detail previously.[22] In brief, the optical density value of each well was measured at 570 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Cell Proliferation Detected by 5-Bromodeoxyuridine (BrdU) Incorporation Assay

The method for the BrdU incorporation assay has been described in detail previously.[22] In brief, cells (5×10^3) were plated into 96-well plates in 10% FBS/DMEM in the presence of miR-181a mimics or scrambled control microRNA. After 24, 48, and 72 hours, the culture medium was replaced with fresh medium. The cells were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich Corp., St. Louis, MO, USA) for 2 hours. After the cells were fixed in 0.4% paraformaldehyde for 20 minutes and washed with PBS, they were incubated in 1.5 M HCl for 10 minutes. Then, the cells were washed with PBS and incubated with mouse anti-BrdU–fluorescein isothiocyanate (Roche, Indianapolis, IN, USA) for 2 hours. The cells were washed four times with PBS. Then, the cells were incubated with the secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour and washed with PBS. Finally, the cells were counterstained with 4′,6-diamino-2-phenylindole (DAPI, 1 μg/mL; Invitrogen, Carlsbad, CA, USA), mounted, and examined, and then counted using fluorescence light microscopy (BX50; Olympus, Inc., Tokyo, Japan).

Wound-Healing Assay

The method of wound-healing assay has been described in detail previously.[22] In brief, SRA01/04 cells were seeded in a 24-well plate (1×10^5 cells/well). After 24 hours of transfection with microRNA mimics or miR-181a control mimics, the cell density of each well reached 90% confluence, and the confluent monolayers of the SRA01/04 cells were scratched

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with a sterile, 20-μL pipette tip. Wounded monolayers were washed with PBS to remove detached cells and debris, and fresh medium was added to each well. The wounds in each well were photographed at 0, 24, 48, and 72 hours. The length of the remaining wound in each image was measured five times using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The data were quantified based on the percentage of average gap (AG); the wound at 0 hours was considered 100% AG. The results were analyzed in triplicate.

Transwell Migration Assay

The method of Transwell migration assay has been described in detail previously.[22] In brief, SRA01/04 cells were seeded into the inner chamber of tissue culture inserts (Transwell Assay System; Corning, High Wycombe, UK) of 24-well plates. Cells transfected with microRNA mimics or miR-181a control mimics at a density of 5×10^5 cells/mL were added to the upper polycarbonate membrane insert and allowed to migrate through the 8-μm pores, and in the lower well, 300 μL DMEM with 20% FBS was used as chemoattractant. After 48 hours of incubation at 37°C in 5% CO_2, the membranes were fixed with 10% formaldehyde and stained with hematoxylin. The cells that had migrated to the bottom of the insert were counted five times in random microscope fields.

Western Blot Analysis

The method used for Western blot analysis has previously been described in detail.[22,26,27] The primary antibodies are anti-c-Met, anti-Slug, anti–cyclin-dependent kinase 2 (CDK2), anti-CDK4, anti-CDK6, anti–E-cadherin, anti-fibronectin, anti-actin (all from Cell Signaling Technology, Beverly, MA, USA), and anti–COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Luciferase Assay

The method of luciferase assay has been described in detail previously.[22] In brief, the 3′-UTRs of c-Met, Slug, and COX-2 containing the predicted miR-181a binding or mutant sites were amplified by PCR using the following primers: c-Met sense 5′-TCGAGAGCTCAGGAAATATTGAGGGCTTCT-3′; c-Met antisense, 5′-TCGAGTCGACCTATTCGGGAGGCTTGAG-3′; Slug sense 5′-ATACTCGAGATGACAAATAAAGTCCAAAGG-3′; Slug antisense, 5′-ATACTCGAGATGACAAATAAAGTCCAAAGG-3′; COX-2 sense 5′-CGGGGTACCGAGTCATACTTGTGAAG-3′; COX-2 antisense, 5′-GCACTCGAGCCTGTTTTTGTTTGATG-3′; mutant c-Met sense 5′-GTCATTCACCCATTAGGTATTGTAAG CCTTTTAAATGTTTGTTT-3′; mutant c-Met antisense, 5′-CAA AAAACAAACAAACATTTAAAAGGCTTACAATACCTAATGGG-3′; mutant Slug sense 5′-GCCAGACGCGAACTCAGCTCCGTAAA AAAGTATTCCAAGT-3′; mutant Slug antisense, 5′-ACTTGGAA TACTTTTTTACGGAGCTGAGTTCGCGTCTGGC-3′; mutant COX-2 sense 5′-GTGGTTTCAACTTATATTATAAGAACG-3′; and mutant COX-2 antisense, 5′-GACGAAAAGACGTCAAAACT CATTT-3′. Fragments were subcloned into the Not I and Xho I sites in the 3′-UTR of Renilla luciferase of the psiCHECK-2 reporter vector (psiCHECK-2; Promega, Madison, WI, USA). The psiCHECK-2/c-Met 3′-UTR or psiCHECK-2/c-Met 3′-UTR mutant, psiCHECK-2/Slug 3′-UTR or psiCHECK-2/Slug 3′-UTR mutant, and psiCHECK-2/COX-2 3′-UTR or psiCHECK-2/COX-2 3′-UTR mutant reporter plasmids (200 ng) were cotransfected with the miR-181a mimics or miR-181a control mimics into SRA01/04 cells (60% confluence). After 48 hours, the cells were lysed, and reporter activity was assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance with the manufacturer's protocols.

Statistical Analysis

All experiments were performed at least three times. Quantitative data are presented as the mean ± SE and were analyzed by 1-way ANOVA or Student's *t*-test. A P value of <0.05 was considered statistically significant.

Results

Expression of miR-181a Is Decreased in Human PCO-Attached LECs and LECs Obtained From Patients With Anterior Polar Cataracts

To investigate the involvement of miR-181a in PCO development, we analyzed the level of miR-181a in human PCO-attached LECs and normal-attached LECs by qRT-PCR. MicroRNA-181a expression was significantly decreased in human PCO-attached LECs compared with normal-attached LECs (3.8-fold, $P < 0.01$) (Fig. 1A). The expression levels of EMT markers were also determined in human PCO-attached LECs and normal-attached LECs by qRT-PCR (Figs. 1B, 1C). Downregulation of E-cadherin and upregulation of fibronectin expression were detected in human PCO-attached LECs.

During the formation of anterior polar cataracts, LECs can transdifferentiate and proliferate into mesenchyme-like cells or myofibroblasts through the EMT.[4–6] To investigate the involvement of miR-181a in anterior polar cataract development, we determined the level of miR-181a in LECs obtained from patients with anterior polar cataracts and nuclear cataracts by qRT-PCR. MicroRNA-181a expression was significantly decreased in LECs obtained from patients with anterior polar cataracts compared with patients with nuclear cataracts (3.2-fold, $P < 0.01$) (Fig. 1D). Downregulation of E-cadherin and upregulation of fibronectin expression were also detected in the LECs obtained from patients with anterior polar cataracts (Figs. 1E, 1F).

miR-181a Inhibits the Proliferation and Migration of SRA01/04 Cells

MicroRNA-181a was downregulated in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts, suggesting a potential role in the biological properties of LECs. MicroRNA-181a expression was significantly higher in the SRA01/04 cells transfected with miR-181a mimics compared with the cells transfected with miR-181a control mimics, as shown by qRT-PCR (2640-fold, $P < 0.01$) (Fig. 2A). In addition, miR-181a control expression was significantly higher in the SRA01/04 cells transfected with miR-181a control mimics (Fig. 2B). As shown in Figure 2C, the overexpression of miR-181a inhibited SRA01/04 cell growth at 24, 48, and 72 hours after miR-181a mimics transfection, as demonstrated by the MTT assay ($P < 0.01$). Furthermore, to confirm that the effects of miR-181a on cell proliferation were not related with high expressed levels of microRNA, the overexpression of miR-498 did not affect SRA01/04 cell growth at 24, 48, and 72 hours after miR-498 mimics transfection (Supplementary Fig. S1). Additionally, SRA01/04 cells were immunostained for BrdU (Fig. 2D, red), and their nuclei were stained with DAPI (Fig. 2D, blue). The BrdU assay revealed that the overexpression of miR-181a inhibited SRA01/04 cell growth at 24, 48, and 72 hours after transfection ($P < 0.05$, Figs. 2D, 2E).

To confirm the effects of miR-181a on cell migration, SRA01/04 cells were transfected with miR-181a mimics or miR-181a control mimics. After 48 hours of incubation, SRA01/04 cells transfected with miR-181a mimics demonstrated reduced

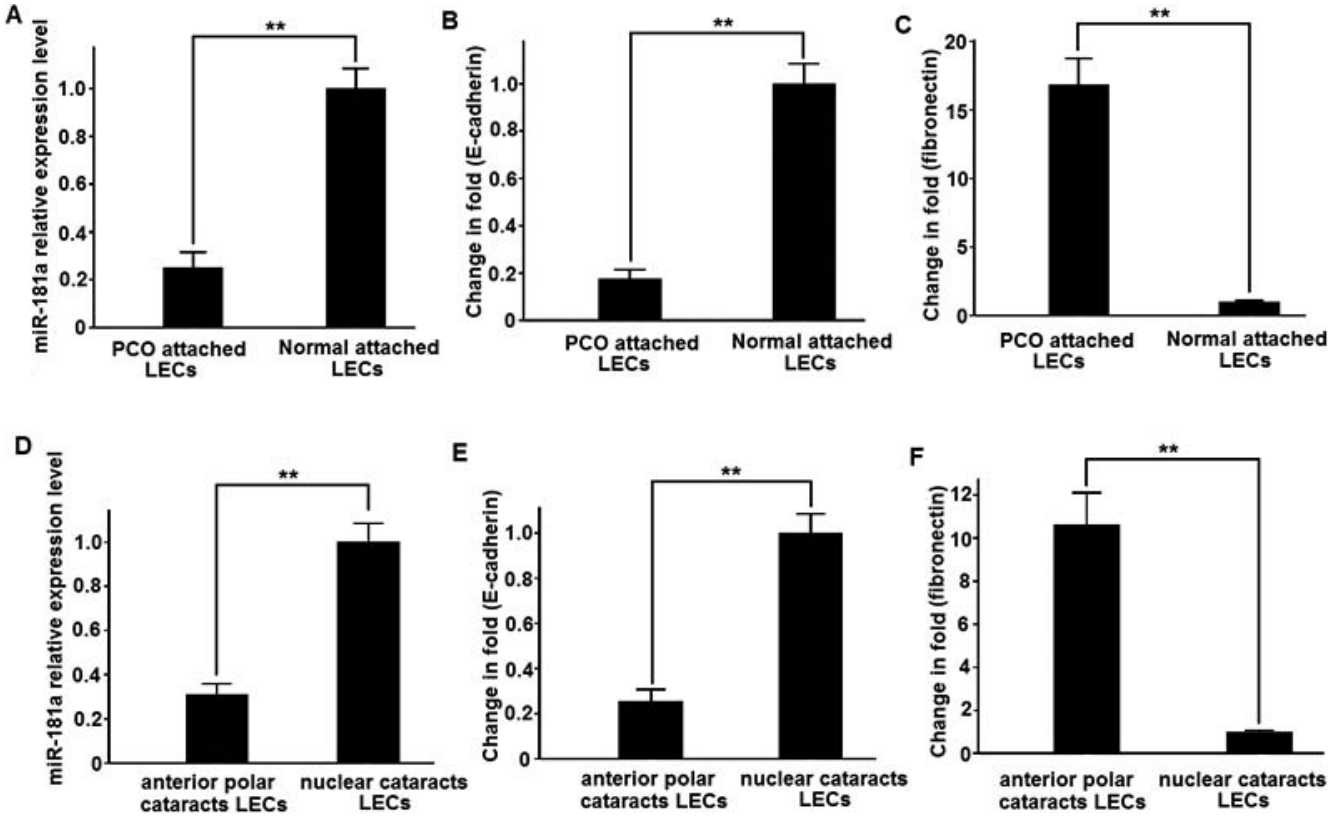

FIGURE 1. MicroRNA-181a was relatively downregulated in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts. (**A**) miR-181a expression was significantly decreased in human PCO-attached LECs compared by qRT-PCR with normal-attached LECs (3.8-fold, $P < 0.01$). (**B**, **C**) Downregulation of E-cadherin and upregulation of fibronectin were detected in human PCO-attached LECs. (**D**) MicroRNA-181a expression was significantly decreased in LECs obtained from patients with anterior polar cataracts compared by qRT-PCR with nuclear cataracts (3.2-fold, $P < 0.01$). (**E**, **F**) Downregulation of E-cadherin and upregulation of fibronectin were detected in LECs obtained from patients with anterior polar cataracts. Results are statistically significant ($^{**}P < 0.01$). *Error bars* denote SEM.

migration across the polycarbonate membrane compared with the control cells (Fig. 2F). Additionally, the wound-healing assay revealed that the miR-181a–overexpressing cells moved slower than the SRA01/04 cells transfected with the miR-181a control mimics and the mock SRA01/04 cells (Figs. 2G, 2H).

miR-181a Downregulated c-Met Expression and Other Cell Cycle–Related Proteins via Regulation of c-Met

The c-Met and cell cycle–related proteins have been found to be associated with the proliferation, protein synthesis, and migratory responses of LECs.[8–10,28] To investigate the mechanism through which miR-181a affects LEC proliferation, we evaluated the expression of c-Met and other cell cycle–related proteins in SRA01/04 cells transfected with miR-181a mimics or miR-181a control mimics. Western blot analysis revealed that c-Met expression was reduced by miR-181a transfection in SRA01/04 cells (Fig. 3A). Additionally, the overexpression of miR-181a also downregulated the expression of CDK2, CDK4, and CDK6 (Fig. 3A).

To determine whether miR-181a inhibits LEC proliferation by downregulating c-Met and its downstream molecules, we altered the expression level of c-Met using specific small interfering RNA (siRNA) in SRA01/04 cells (Fig. 4B). Western blot analysis revealed that the expression levels of CDK2, CDK4, and CDK6 were significantly decreased in the c-Met siRNA-transfected cells compared with the c-Met control siRNA-transfected cells and the mock SRA01/04 cells (Fig. 3B).

We used miRanda to search for the 3′-UTR sequences of the mRNAs encoding c-Met, CDK2, CDK4, and CDK6 and found that only the c-Met mRNA contained a seed sequence for miR-181a, which suggests that miR-181a binds directly to its 3′-UTR (Fig. 3C). To test this proposal, we performed a luciferase reporter assay to verify that miR-181a directly targets c-Met (Fig. 3D).

miR-181a Inhibits the EMT of Human PCO-Attached LECs and LECs Obtained From Patients With Anterior Polar Cataracts via Regulation of Slug and COX-2

To determine the potential ability of miR-181a to downregulate fibronectin and upregulate E-cadherin, human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts were transfected with miR-181a mimics or miR-181a control mimics. When comparing the human PCO-attached LECs to normal-attached LECs, the expression of E-cadherin was decreased, and the expression of fibronectin, Slug, and COX-2 was increased in the PCO-attached LECs, as shown by Western blot analysis (Fig. 4A). However, a downregulation of fibronectin, Slug, and COX-2 expression and an upregulation of E-cadherin expression were observed in the miR-181a–overexpressing human PCO-attached LECs.

As shown in Figure 4B, the expression of E-cadherin was decreased, and the expression of fibronectin, Slug, and COX-2 was increased in LECs obtained from patients with anterior polar cataracts. Furthermore, a downregulation of

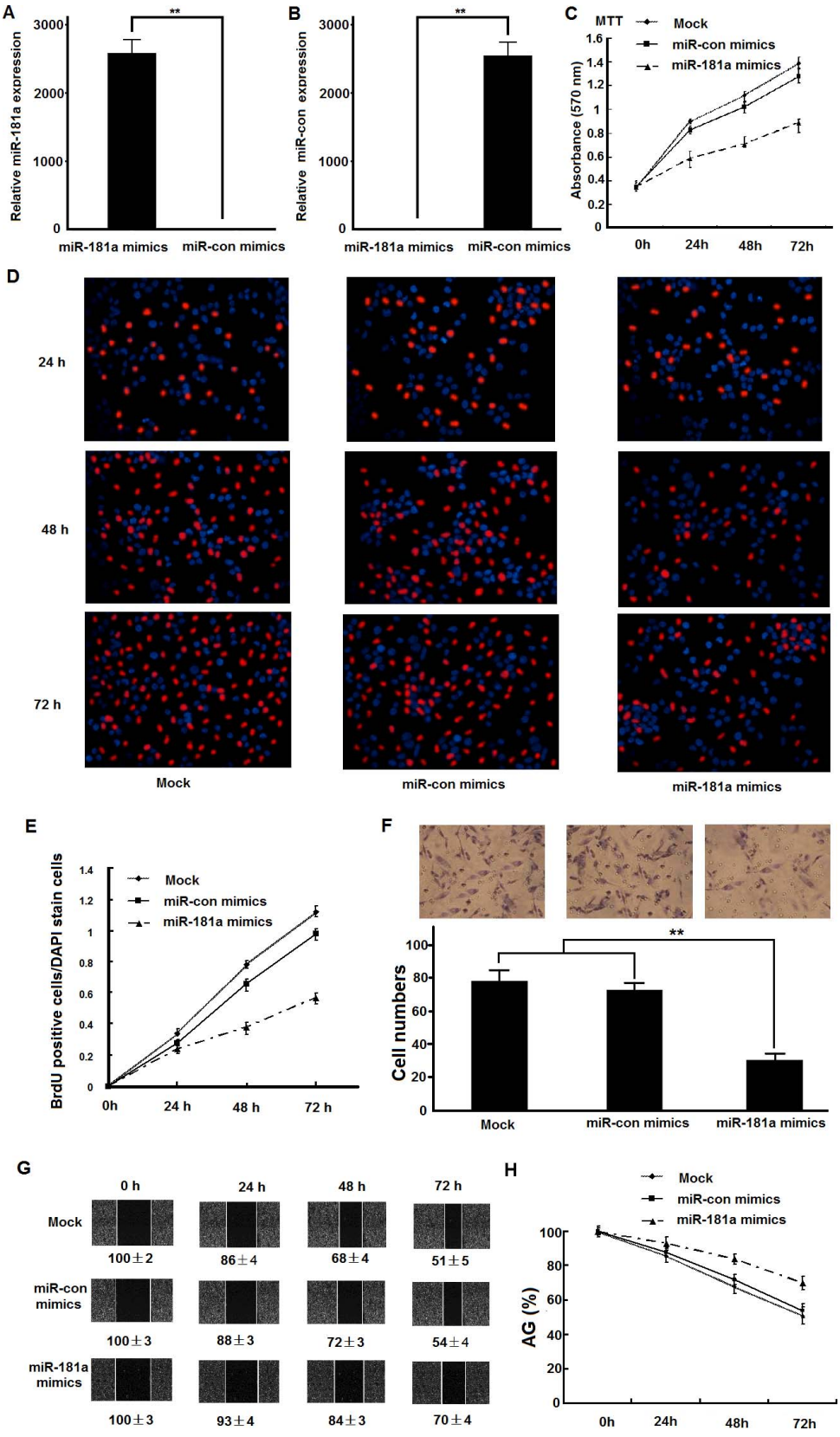

Figure 2. MicroRNA-181a inhibited proliferation and migration of SRA01/04 cells. (**A**) MicroRNA-181a expression was significantly increased in the SRA01/04 cells transfected with miR-181a mimics compared by qRT-PCR with the SRA01/04 cells transfected with miR-181a mimics control (2640-fold, $P < 0.01$). (**B**) MicroRNA-181a control expression was significantly higher in the SRA01/04 cells transfected with miR-181a control mimics (2580-fold, $P < 0.01$). (**C**) MTT assay results revealed that overexpression of miR-181a inhibited SRA01/04 cell growth at 24, 48, and 72 hours compared with the SRA01/04 cells transfected with miR-181a control mimics and mock SRA01/04 cells. (**D**) The SRA01/04 cells labeled with BrdU

were immunostained for BrdU (*red*) and counterstained for nuclei (*blue*). (**E**) BrdU assay results revealed that the overexpression of miR-181a inhibited SRA01/04 cell growth at 24, 48, and 72 hours after transfection. (**F**) Transwell migration assays were performed and analyzed to investigate the effect of miR-181a on cell migration. Cells on Transwell inserts were stained (original magnification: ×200). The number of migratory miR-181a–overexpressing SRA01/04 cells was decreased compared with that of control cells. (**G**, **H**) The effects of miR-181a on SRA01/04 cell motility shown by wound-healing assay. The cells were incubated in growth medium containing 1% FBS. The migration ability of SRA01/04 cells transfected with miR-181a mimics after 72 hours was significantly decreased compared with that of control cells. The data were quantified based on the percentage of AG; the wound at 0 hours was considered 100% AG. Results are statistically significant (**$P < 0.01$). *Error bars* denote SEM.

fibronectin, Slug, and COX-2 expression and an upregulation of E-cadherin expression were induced in miR-181a–overexpressing LECs obtained from patients with anterior polar cataracts.

We used miRanda to search for the 3′-UTR sequences of the mRNAs encoding E-cadherin, fibronectin, Slug, and COX-2 and found that Slug and COX-2 mRNA contained a seed sequence for miR-181a, which suggests that miR-181a binds directly to these 3′-UTRs (Figs. 4C, 4E). To test this proposal, we performed a luciferase reporter assay to verify that miR-181a directly targets Slug and COX-2 (Figs. 4D, 4F).

Discussion

Posterior capsule opacification is the most common postoperative complication of cataract surgery that causes vision loss; its incidence is 30% to 50% in adults and 100% in children who receive surgery.[1] Although PCO is known to be associated with wrinkling/contraction of the posterior capsule and the pathologic progression of postoperative residual LECs, including proliferation, migration, and EMT, the mechanism underlying this process is not yet clearly understood. MicroRNAs affect essential biological processes such as metabolism, cell

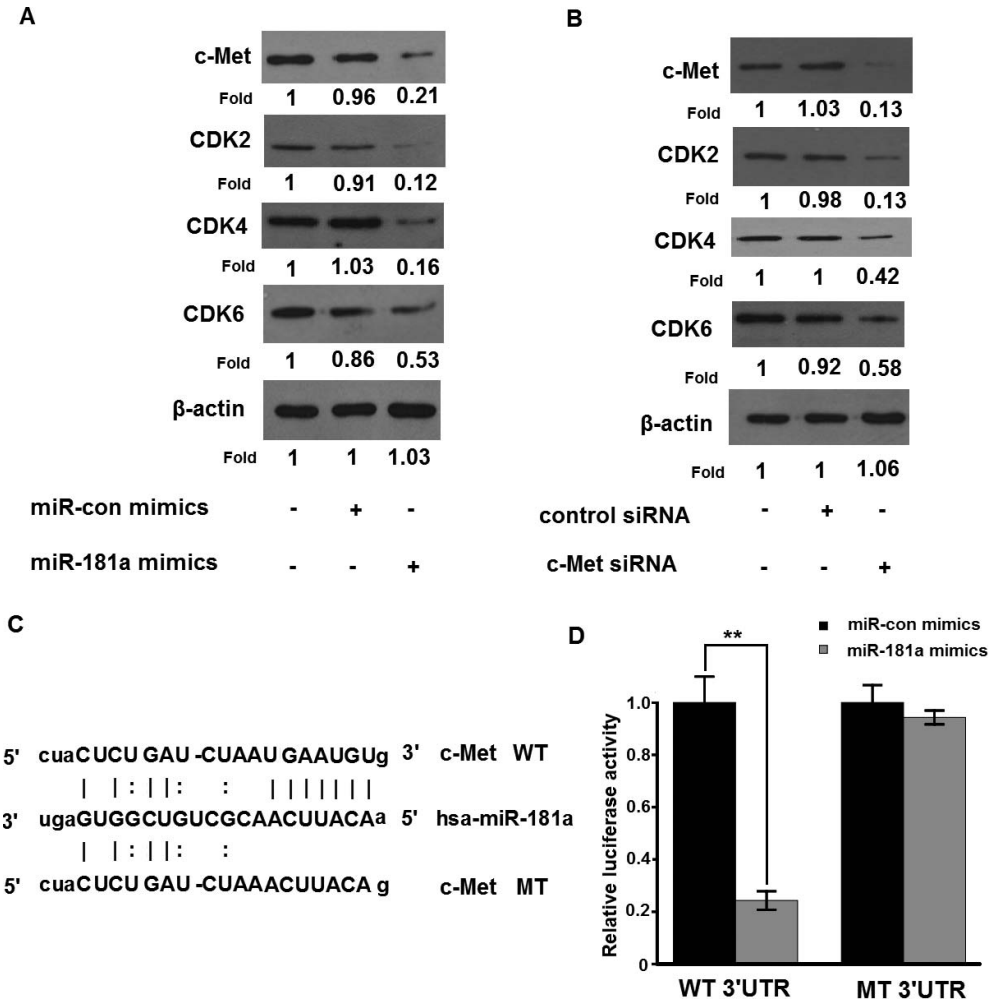

Figure 3. Introduction of miR-181a downregulated c-Met expression and other cell cycle–related proteins. (**A**) SRA01/04 cells were transfected with miR-181a mimics or miR-181a control mimics. Cell lysates were prepared and used for Western blot analysis of c-Met, CDK2, CDK4, and CDK6. β-actin was used as a loading control. Overexpression of miR-181a downregulated the expression of c-Met, CDK2, CDK4, and CDK6 after miR-181a mimics transfection. (**B**) Direct knockdown of c-Met downregulated the expression of c-Met and cell cycle–related molecules, by Western blot analysis. (**C**) The region of the c-Met mRNA 3′-UTR predicted to be targeted by miR-181a. (**D**) Dual-luciferase report assays were performed on SRA01/04 cells. The luciferase activity of wild-type (WT) reporter transfected with miR-181a mimics was significantly decreased compared with miR-181a mimics control ($P < 0.01$). However, the luciferase reporter activity was not inhibited by miR-181a mimics when the seeding sites were mutated ($P > 0.05$). Results are statistically significant (**$P < 0.01$). *Error bars* denote SEM.

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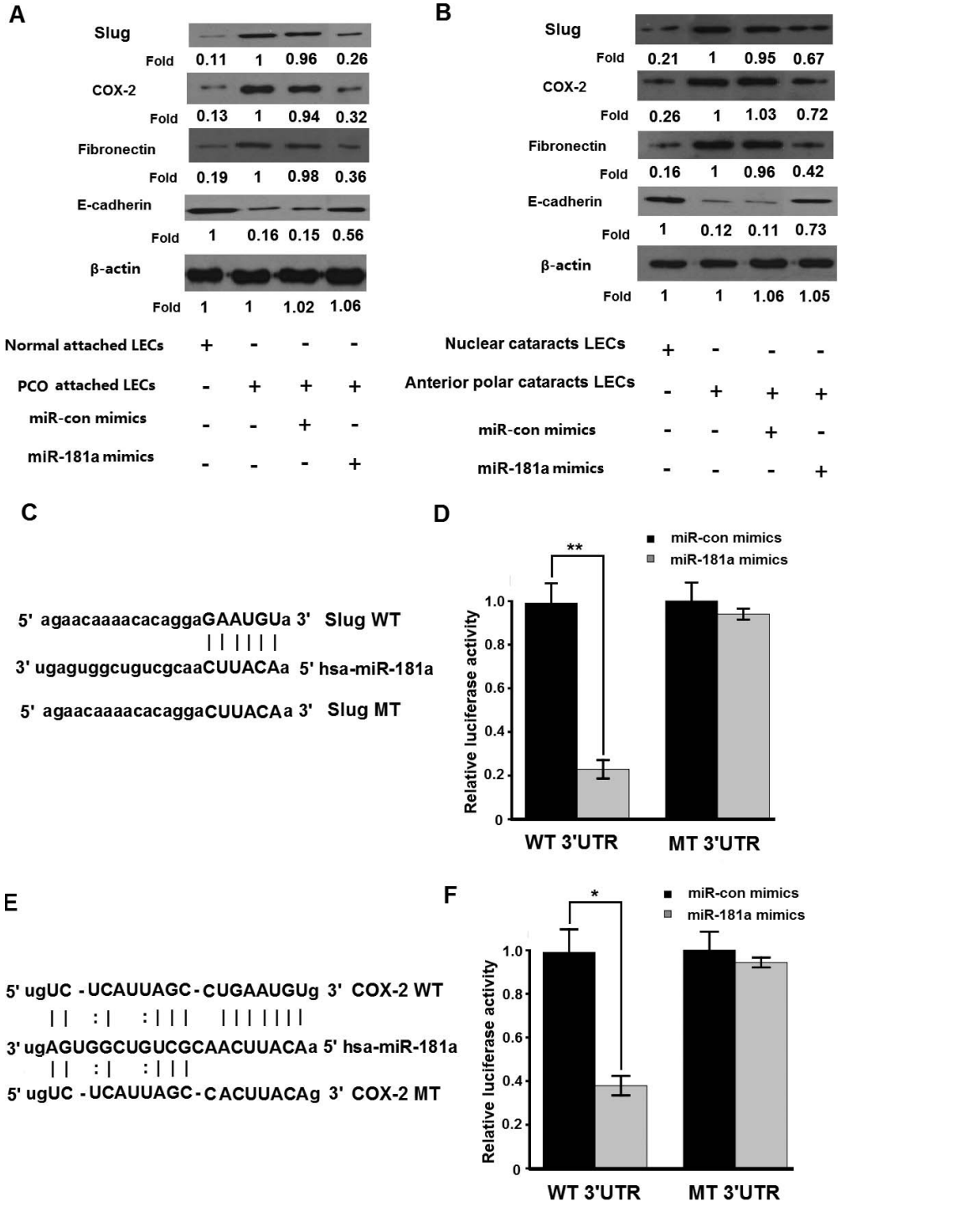

FIGURE 4. MicroRNA-181a inhibited the EMT of human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts via regulation of Slug and COX-2. (**A**) Western blot analysis detected downregulated E-cadherin and upregulated fibronectin, Slug, and COX-2 in human PCO-attached LECs compared with that of normal-attached LECs. However, the expression of E-cadherin was increased and the expression of fibronectin, Slug, and COX-2 was decreased in human PCO-attached LECs transfected with miR-181a mimics. (**B**) The expression of E-cadherin was downregulated, and the expression of fibronectin, Slug, and COX-2 was increased in LECs obtained from patients with anterior polar cataracts, by Western blot analysis. However, downregulation of fibronectin, Slug, and COX-2 and upregulation of E-cadherin were induced in miR-181a–overexpressing LECs obtained from patients with anterior polar cataracts. (**C**, **E**) The region of the Slug and COX-2 mRNA 3′-UTR predicted to be targeted by miR-181a. (**D**, **F**) Dual-luciferase report assays were performed on SRA01/04 cells. The luciferase activity of WT reporter transfected with miR-181a mimics was significantly decreased compared with miR-181a mimics control. However, the luciferase reporter activity was not inhibited by miR-181a mimics when the seeding sites were mutated ($P > 0.05$). Dual-luciferase reporter assay verified that miR-181a directly targets Slug and COX-2. Results are statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$). *Error bars* denote SEM.

proliferation, migration, differentiation, development, and EMT by negatively regulating the translation and stability of their target mRNAs.[29] In this study, we discovered that miR-181a expression was downregulated in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts, which implies a potential role in PCO and anterior polar cataracts.

Within a few hours after cataract extraction surgery, proliferation of the remaining LECs may begin. Concurrently, the majority of anterior LECs that reside on the internal surface of the anterior lens capsule migrate to the previously cell-free posterior capsule. Thus, inhibition of LEC proliferation and migration may be an effective strategy in preventing PCO. The MET proto-oncogene encodes the receptor tyrosine kinase (RTK) c-MET.[30,31] In the canonical HGF/c-MET signaling pathway, c-MET dimerizes and autophosphorylates upon ligand binding, which in turn creates active docking sites for proteins that mediate the downstream signaling, resulting in activation of the mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K), and Rac1-Cdc42 pathways.[29,30] Previous studies have demonstrated that LECs normally express c-Met and that this expression is upregulated after mechanical trauma.[8] Therefore, the autocrine feedback system may regulate normal LECs and modulate the epithelial migration and growth that is associated with the development of PCO.[8] Hence, the HGF/c-Met signaling pathway has been shown to be involved in LECs proliferation and migration.[8–10]

A large number of studies have shown that microRNAs affect essential biological processes such as metabolism, cell proliferation, migration, differentiation, and development by regulating c-MET.[32–34] Consistent with previous studies, our study demonstrated that miR-181a inhibits the proliferation and migration of SRA01/04 cells, using MTT, BrdU incorporation, wound-healing, and Transwell migration assays. Furthermore, miR-181a downregulated c-Met expression via regulation of c-Met mRNA. The expression levels of cell cycle–regulatory proteins were determined to investigate the mechanism underlying the antiproliferative activity of miR-181a inhibition. Our study revealed that cell cycle–related molecules, including CDK2, CDK4, and CDK6, were downregulated in miR-181a–transfected LECs. Cyclin-dependent kinases are the catalytic subunits of a family of mammalian heterodimeric serine/threonine kinases that have been implicated in controlling cell-cycle progression and transcription.[35] CDK2, CDK4, and CDK6 are all members of the CDK family, which are essential for controlling the G1/S phase transition.[35] Consequently, to determine whether miR-181a inhibits LEC proliferation by downregulating c-Met and its downstream molecules, we altered the expression level of c-Met using specific siRNA in SRA01/04 cells. Using a c-Met knockdown approach, we demonstrated that the expression levels of CDK2, CDK4, and CDK6 were significantly decreased in c-Met siRNA–transfected cells compared with c-Met control siRNA–transfected cells and mock SRA01/04 cells. This finding suggests that miR-181a regulates the proliferation of LECs by negatively regulating c-Met and thereby also regulates its downstream molecules.

Increasing evidence indicates that the LECs of anterior subcapsular cataracts and anterior polar cataracts are transdifferentiated into fibroblast-like cells and produce a large amount of extracellular components that are not normally present in the lens capsules.[4–6,36–38] Previous studies have demonstrated that EMT not only plays a role in the transformation of LECs but also influences the healing process after cataract surgery.[1–3,39] Epithelial–mesenchymal transition is characterized by a loss of epithelial cell–cell adhesion caused by the suppression of the molecular hallmarks that compose the junctional complexes, such as E-cadherin. Additionally, these cells also acquire mesenchymal features, such as the production of matrix proteins (e.g., fibronectin, type I collagen), reorganization of the actin cytoskeleton to activate the motility machinery, and production of metalloproteases, which also facilitate cell migration. Cyclooxygenase-2 and Slug are considered to be important mediators in the mechanism of PCO and the regulation of the EMT of LECs.[11,16,22,40] Our study showed that the expression level of E-cadherin was decreased and the expression levels of fibronectin, Slug, and COX-2 were increased in human PCO-attached LECs and LECs obtained from patients with anterior polar cataracts. Furthermore, downregulation of fibronectin, Slug, and COX-2 and upregulation of E-cadherin expression were induced in human PCO-attached LECs transfected with miR-181a mimics and miR-181a–overexpressing LECs obtained from patients with anterior polar cataracts. Furthermore, luciferase assays revealed that miR-181a inhibits the EMT of LECs by directly targeting Slug and COX-2.

In summary, the current study provides new insights regarding the ability of miRNA-181a to inhibit the proliferation, migration, and EMT of LECs. MicroRNAs are believed to have potential therapeutic value for many diseases. These data indicate that miR-181a may have important clinical consequences in the treatment of PCO by directly silencing c-Met, Slug, and COX-2.

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References

1. Awasthi N, Guo S, Wagner BJ. Posterior capsular opacification: a problem reduced but not yet eradicated. *Arch Ophthalmol*. 2009;127:555–562.
2. Apple DJ, Solomon KD, Tetz MR, et al. Posterior capsule opacification. *Surv Ophthalmol*. 1992;37:73–116.
3. Wormstone IM, Wang L, Liu CS. Posterior capsule opacification. *Exp Eye Res*. 2009;88:257–269.
4. Novotny GE, Pau H. Myofibroblast-like cells in human anterior capsular cataract. *Virchows Arch A Pathol Anat Histopathol*. 1984;404:393–401.
5. Pau H, Novotny GE, Kern W. The lenticular capsule and cellular migration in anterior capsular cataract. *Graefes Arch Clin Exp Ophthalmol*. 1986;224:118–121.
6. Lee EH, Seomun Y, Hwang KH, et al. Overexpression of the transforming growth factor-beta-inducible gene betaig-h3 in anterior polar cataracts. *Invest Ophthalmol Vis Sci*. 2000;41:1840–1845.
7. Huang WR, Fan XX, Tang X. SiRNA targeting EGFR effectively prevents posterior capsular opacification after cataract surgery. *Mol Vis*. 2011;17:2349–2355.
8. Wormstone IM, Tamiya S, Marcantonio JM, Reddan JR. Hepatocyte growth factor function and c-Met expression in human lens epithelial cells. *Invest Ophthalmol Vis Sci*. 2000;41:4216–4222.
9. Weng J, Liang Q, Mohan RR, Li Q, Wilson SE. Hepatocyte growth factor, keratinocyte growth factor, and other growth factor-receptor systems in the lens. *Invest Ophthalmol Vis Sci*. 1997;38:1543–1554.
10. Fleming TP, Song Z, Andley UP. Expression of growth control and differentiation genes in human lens epithelial cells with extended life span. *Invest Ophthalmol Vis Sci*. 1998;39:1387–1398.

11. Choi J, Park SY, Joo CK. Transforming growth factor-beta1 represses E-cadherin production via slug expression in lens epithelial cells. *Invest Ophthalmol Vis Sci*. 2007;48:2708–2718.
12. Molina-Ortiz P, Villarejo A, MacPherson M, et al. Characterization of the SNAG and SLUG domains of Snail2 in the repression of E-cadherin and EMT induction: modulation by serine 4 phosphorylation. *PLoS One*. 2012;7:e36132.
13. Villarejo A, Cortés-Cabrera A, Molina-Ortíz P, Portillo F, Cano A. Differential role of Snail1 and Snail2 zinc fingers in E-cadherin repression and epithelial to mesenchymal transition. *J Biol Chem*. 2014;289:930–941.
14. Stoll SW, Rittié L, Johnson JL, Elder JT. Heparin-binding EGF-like growth factor promotes epithelial-mesenchymal transition in human keratinocytes. *J Invest Dermatol*. 2012;132:2148–2157.
15. Kirane A, Toombs JE, Larsen JE, et al. Epithelial-mesenchymal transition increases tumor sensitivity to COX-2 inhibition by apricoxib. *Carcinogenesis*. 2012;33:1639–1646.
16. Chandler HL, Barden CA, Lu P, Kusewitt DF, Colitz CM. Prevention of posterior capsular opacification through cyclooxygenase-2 inhibition. *Mol Vis*. 2007;13:677–691.
17. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522–531.
18. Wolf L, Gao CS, Gueta K, et al. Identification and characterization of FGF2-dependent mRNA: microRNA networks during lens fiber cell differentiation. *G3 (Bethesda)*. 2013;3:2239–2255.
19. Varma SD, Kovtun S, Hegde K, Yin J, Ramnath J. Effect of high sugar levels on miRNA expression: studies with galactosemic mice lenses. *Mol Vis*. 2012;18:1609–1618.
20. Varma SD, Kovtun S. Protective effect of caffeine against high sugar-induced transcription of microRNAs and consequent gene silencing: a study using lenses of galactosemic mice. *Mol Vis*. 2013;19:493–500.
21. Hoffmann A, Huang Y, Suetsugu-Maki R, et al. Implication of the miR-184 and miR-204 competitive RNA network in control of mouse secondary cataract. *Mol Med*. 2012;18:528–538.
22. Dong N, Xu B, Benya SR, Tang X. MiRNA-26b inhibits the proliferation, migration, and epithelial-mesenchymal transition of lens epithelial cells. *Mol Cell Biochem*. 2014;396:229–238.
23. Giordano S, Columbano A. MicroRNAs: new tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma? *Hepatology*. 2013;57:840–847.
24. Korhan P, Erdal E, Atabey N. MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. *Biochem Biophys Res Commun*. 2014;450:1304–1312.
25. Wang Y, Li W, Zang X, et al. MicroRNA-204-5p regulates epithelial-to-mesenchymal transition during human posterior capsule opacification by targeting SMAD4. *Invest Ophthalmol Vis Sci*. 2013;54:323–332.
26. Dong N, Chang L, Wang B, Chu L. Retinal neuronal MCP-1 induced by AGEs stimulates TNF-α expression in rat microglia via p38, ERK, and NF-κB pathways. *Mol Vis*. 2014;20:616–628.
27. Dong N, Li X, Xiao L, Yu W, Wang B, Chu L. Upregulation of retinal neuronal MCP-1 in the rodent model of diabetic retinopathy and its function in vitro. *Invest Ophthalmol Vis Sci*. 2012;53:7567–7575.
28. Awasthi N, Wagner BJ. Suppression of human lens epithelial cell proliferation by proteasome inhibition, a potential defense against posterior capsular opacification. *Invest Ophthalmol Vis Sci*. 2006;47:4482–4489.
29. Hou Q, Tang J, Wang Z, et al. Inhibitory effect of microRNA-34a on retinal pigment epithelial cell proliferation and migration. *Invest Ophthalmol Vis Sci*. 2013;354;6481–6488.
30. Goyal L, Muzumdar MD, Zhu AX. Targeting the HGF/c-MET pathway in hepatocellular carcinoma. *Clin Cancer Res*. 2013;19:2310–2318.
31. Morishita A, Gong J, Masaki T. Targeting receptor tyrosine kinases in gastric cancer. *World J Gastroenterol*. 2014;20:4536–4545.
32. Zhao H, Li M, Li L, Yang X, Lan G, Zhang Y. MiR-133b is downregulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. *PLoS One*. 2013;8:e83571.
33. Huang J, Dong B, Zhang J, et al. miR-199a-3p inhibits hepatocyte growth factor/c-Met signaling in renal cancer carcinoma. *Tumour Biol*. 2014;35:5833–5843.
34. Wan L, Zhu L, Xu J, et al. MicroRNA-409-3p functions as a tumor suppressor in human lung adenocarcinoma by targeting c-Met. *Cell Physiol Biochem*. 2014;34:1273–1290.
35. Malumbres M, Barbacid M. Mammalian cyclin-dependent kinases. *Trends Biochem Sci*. 2005;30:630–641.
36. Hatae T, Ishibashi T, Yoshitomi F, Shibata Y. Immunocytochemistry of types I-IV collagen in human anterior subcapsular cataracts. *Graefes Arch Clin Exp Ophthalmol*. 1993;231:586–590.
37. Font RL, Brownstein S. A light and electron microscopic study of anterior subcapsular cataracts. *Am J Ophthalmol*. 1974;78:972–984.
38. Lee EH, Joo CK. Role of transforming growth factor-beta in transdifferentiation and fibrosis of lens epithelial cells. *Invest Ophthalmol Vis Sci*. 1999;40:2025–2032.
39. Xie L, Santhoshkumar P, Reneker LW, Sharma KK. Histone deacetylase inhibitors trichostatin A and vorinostat inhibit TGFβ2-induced lens epithelial-to-mesenchymal cell transition. *Invest Ophthalmol Vis Sci*. 2014;55:4731–4740.
40. Chen X, Xiao W, Chen W, Luo L, Ye S, Liu Y. The epigenetic modifier trichostatin A, a histone deacetylase inhibitor, suppresses proliferation and epithelial-mesenchymal transition of lens epithelial cells. *Cell Death Dis*. 2013;4:e884.

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