

# Knockdown of lncRNA XIST prevents the epithelial-mesenchymal transition of TGF- $\beta$ 2-induced human lens epithelial cells via miR-124/Slug axis

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**Key words:** LncRNA XIST, miR-124, Slug, Posterior capsular opacification, Epithelial-Mesenchymal Transition

**Abstract:** Posterior capsular opacification (PCO) is linked to the pathological process of lens epithelial cells, which includes proliferation, migration, and epithelial-mesenchymal transition (EMT). Our goal was to investigate whether long noncoding RNA (lncRNA) XIST contributes to EMT via targeting miR-124/Slug axis in TGF- $\beta$ 2-induced SRA01/04 cells. EMT was induced by stimulating SRA01/04 cells with 10 ng/mL TGF- $\beta$ 2 for 24 h, and PCO microenvironment was simulated. The expression levels of lncRNA XIST, miR-124, and Slug were measured by real-time polymerase chain reaction (RT-PCR) and western blot. The role and mechanism of lncRNA XIST in promoting EMT of TGF- $\beta$ 2-treated SRA01/04 cells were investigated by using cell transfection, cell counting kit-8 (CCK-8), immunofluorescence staining, transwell assay, wound-healing assay, RT-PCR, western blot and dual-luciferase reporter assay. The expression of Slug and lncRNA XIST was markedly increased, while miR-124 was downregulated in TGF- $\beta$ 2-treated SRA01/04 cells compared with the control group. Knockdown of lncRNA XIST reduced EMT, migration, and cell viability in TGF- $\beta$ 2-induced SRA01/04 cells; moreover, it adversely modulated miR-124 and adjusted the expression of Slug in SRA01/04 cells, while these effects were diminished by co-transfection with AMO-miR-124. Our data demonstrated that lncRNA XIST functioned as a competitive endogenous RNA (ceRNA) of miR-124 to modulate the expression level of Slug, thereby modulating EMT, migration, and cell viability in SRA01/04 cells. In conclusion, lncRNA XIST has a crucial role in promoting TGF- $\beta$ 2-induced EMT via modulating the miR-124/Slug axis in SRA01/04 cells, and it may provide a novel therapeutic option for PCO treatment.

## Introduction

Posterior capsular opacification (PCO) is one of the most common complications in recent cataract surgeries; it is also known as “secondary cataract” (Awasthi *et al.*, 2009). Within 2–5 years after the operation, 20%–40% of the patients experience vision decline due to PCO (Awasthi *et al.*, 2009; Nibourg *et al.*, 2015). Currently, the only effective treatment for PCO is Nd: YAG laser posterior capsulotomy, but the risk of complications still exists (Zhang and Xie, 2020). PCO mainly results from the pathological process of residual lens epithelial cells after cataract surgery and includes epithelial-mesenchymal transition (EMT), proliferation, and migration. EMT is the primary causal factor for PCO development (Dong, 2019a; Zhang *et al.*, 2017). In the process of EMT, lens

epithelial cells (LECs) transdifferentiate into myofibroblasts, losing cell polarity, cell-cell contact, and epithelial cell characteristics, and obtaining mesenchymal properties, such as motility, invasiveness, and antiapoptotic ability (Ashrafizadeh *et al.*, 2020; Kim *et al.*, 2014; Wernecke *et al.*, 2018; Zhang *et al.*, 2017). Therefore, inhibition of EMT can prevent the development of PCO.

Transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) is considered to be a crucial trigger for the changes associated with EMT in PCO. It plays a vital role in the activation and promotion of EMT in LECs through the Smads pathway, PI3K/Akt pathway, and other pathways. Besides, TGF- $\beta$ 2 is the primary subtype of the TGF- $\beta$  family in aqueous humor (Dong, 2020; Zhang *et al.*, 2017). Therefore, TGF- $\beta$ 2-induced human lens epithelial cells (HLECs) can be used to simulate the PCO microenvironment (Wang *et al.*, 2019; Zhang *et al.*, 2017).

To date, a large number of noncoding RNAs (ncRNAs) have been considered to control gene expression and

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pathway activation at almost all levels, including the activation and inhibition of the EMT process (Beermann *et al.*, 2016; Zhao *et al.*, 2017). Among them, lncRNAs are non-protein coding transcripts that accommodate over 200 nucleotides, they are involved in cancer and other diseases, including PCO (Bach and Lee, 2018; Zhang *et al.*, 2017; Zhang *et al.*, 2018). One study showed that 775 lncRNAs were anomalously expressed in HLEB-3 cells in total during TGF- $\beta$ 2-induced EMT compared with the control group, a total of 325 lncRNAs were upregulated, whereas 450 were downregulated (Zhang *et al.*, 2017). Furthermore, lncRNAs have been proven to regulate gene expression by acting as microRNA sponges or in competition with endogenous RNAs (Li *et al.*, 2018). Nevertheless, the role of most lncRNAs in PCO has not been explored.

Recent studies have demonstrated that lncRNA X inactive specific transcript (XIST), is the primary regulator of X chromosome inactivation (XCI)—an epigenetic process that results in an equal dose of X-linked genes in female and male mammals (Loda and Heard, 2019). XIST is located on chromosome Xq13.2. LncRNA XIST is associated with the modulation of EMT in various malignant tumors (Chen *et al.*, 2017; Shen *et al.*, 2019). Moreover, the latest study confirmed that the expression level of lncRNA XIST was upregulated in TGF- $\beta$ 2-induced primary HLECs (Dong, 2019b). However, to the best of our knowledge, the possibility that lncRNA XIST regulates the EMT process in PCO remains unclear and needs to be further explored.

The lncRNA XIST has been reported to interact with miR-124 to regulate the growth of laryngeal squamous cell carcinoma and bladder cancer (Xiao *et al.*, 2019; Xiong *et al.*, 2017). Decreased expression of miR-124 was found in LECs with cataracts (Wu *et al.*, 2017a; Wu *et al.*, 2017b). Meanwhile, miR-124 has a significant role in the modulation of EMT by focusing on Slug in regulating gastric cancer progression (Li *et al.*, 2017). In addition, Slug is upregulated in TGF- $\beta$ 2-treated HLECs and participates in the regulation of EMT in PCO (Dong *et al.*, 2015). Consequently, we postulated that the existence of the lncRNA XIST/miR-124/Slug axis was involved in the EMT process of PCO.

No studies have explored the regulatory roles of lncRNA XIST and miR-124 in the EMT process of PCO. Therefore, we analyzed the regulatory relationship between lncRNA XIST, miR-124, and Slug in SRA01/04 cells induced by TGF- $\beta$ 2 to identify a new potential target for the treatment of PCO.

## Materials and Methods

### Cell culture and treatment

SV40 T-antigen-human lens epithelial cell line SRA01/04 (Otwo Biotech, Shenzhen, China) was cultured overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. SRA01/04 cells were divided into experimental groups and the control group. After the cells had reached approximately 80%–90% confluence, those in the experimental groups were treated by adding TGF- $\beta$ 2 at different concentrations (0, 1, 5, 10 ng/mL) at different times (0, 6, 12, 24 h).

### Cell transfection

The siRNA (siXIST) and its negative control (NC) were designed to target lncRNA XIST. MiR-124 mimics, anti-miR-124 oligodeoxyribonucleotide (AMO-miR-124), and its negative control (AMO-NC) were obtained from RIBOBIO (Guangzhou, China). X-treme GENE siRNA Transfection Reagent was used in line with the manufacturer's instructions (GTS, CA, USA).

### RNA isolation and qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA), and the extraction procedure complied with the instructions. The concentration of the extracted total RNA was monitored by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). U6 was detected as the internal reference for miR-124, and GAPDH were detected as the internal reference for lncRNA XIST, and Slug. Quantitative statistics were calculated using the 2<sup>-ΔΔCt</sup> method calculation. The primer sequences used are shown in Table 1.

### Western blot

Total protein was extracted from SRA01/04 cells that had been prepared for immunoblotting analysis. The membranes were capped with 5% skimmed milk for 2 h at room temperature and then incubated with primary antibodies at 4°C overnight. The following antibodies were used: GAPDH (ZSGB-BIO, China), Slug (C19G7; Cell Signaling), E-cadherin (4065; Cell Signaling), Fibronectin (FN) (ab2413, Abcam), α-SMA (ab32575, Abcam), and Vimentin (5741; Cell Signaling). The bands on the membrane were captured and quantified by the Odyssey fluorescence scanning system (LI-COR).

### Cell viability

Cell counting kit-8 (CCK-8) was designed to analyze cell viability in line with the manufacturer's instructions. SRA01/04 cells were seeded into 96-well plates at a density of 1 × 10<sup>4</sup> cells/well and cultured for 24 h. We added 10-μL CCK-8 solution to the cells and incubated them at 37°C. After 2 h of incubation, the absorbance was measured at 450 nm using a microplate reader (BMG LABTECH, Durham, NC, USA). Each sample was prepared in triplicate, and the experiment was repeated three times.

### Immunofluorescence staining

The location and expression level of Slug in SRA01/04 cells were verified by immunofluorescence staining after treating the cells in different experimental settings, as explicitly described in the previous study (Du *et al.*, 2020). The nuclei were stained with Hoechst dye.

### Luciferase reporter assay

Online bioinformatics databases including Starbase v2.0, TargetScan, LncBase, and RNAhybrid, were exploited for target prediction. The sequences of target binding sites to be detected were cloned into pmiR-RB-REPORT™ Vectors (GenePharma, Shanghai, China), i.e., wt-XIST, mut-XIST, wt-Slug, or mut-Slug. MiR-124 mimics, AMO-miR-124, and their negative control (NC) were transfected into HEK293T cells. After treatment, a dual-luciferase reporter assay system

(Promega, Madison, Wisconsin, USA) was utilized to detect luciferase activities. The specific experimental procedure was also described in other studies (Du *et al.*, 2017). The related primer sequences are listed in Table 1.

#### Transwell assay

Cell migration capacity was monitored using Transwell platelets (Corning, New York, USA), i.e., 24 h after siXIST, siNC, AMO-miR-124, and AMO-NC had been transfected, SRA 01/04 cells were treated with TGF- $\beta$ 2 for another 24 h at 37°C. The cells with a density of  $5 \times 10^5$  cells/mL were seeded onto the upper chambers of 24-well plates and cultured in serum-free DMEM. Specific experimental details adhered to the manufacturer's instructions. The number of cells undergoing migration was measured with five random fields under an inverted microscope (Olympus Corporation, Japan).

#### Wound-healing assay

SRA01/04 cells were cultured into a monolayer and placed in a serum-free medium for 24 h. After removing the medium, we scratched a straight line across the cell layer using the tip of a pipette, and then washed the cells with phosphate-buffered saline (PBS) to remove the suspended cells. siXIST, siNC, AMO-miR-124, and AMO-NC were transfected into the cells, and then the cells were induced by TGF- $\beta$ 2 (10 ng/mL) for 24 h. The data were measured according to the percentage of average distance. The scratch gap was recorded and photographed with an inverted microscope (Olympus Corporation, Japan).

#### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD) and analyzed in SPSS 16.0 software (IBM, Armonk, NY, USA) using a two-tailed Student's *t*-test and one-way Analysis of Variance (ANOVA). *p* value  $< 0.05$  was classified as statistically significant.

## Results

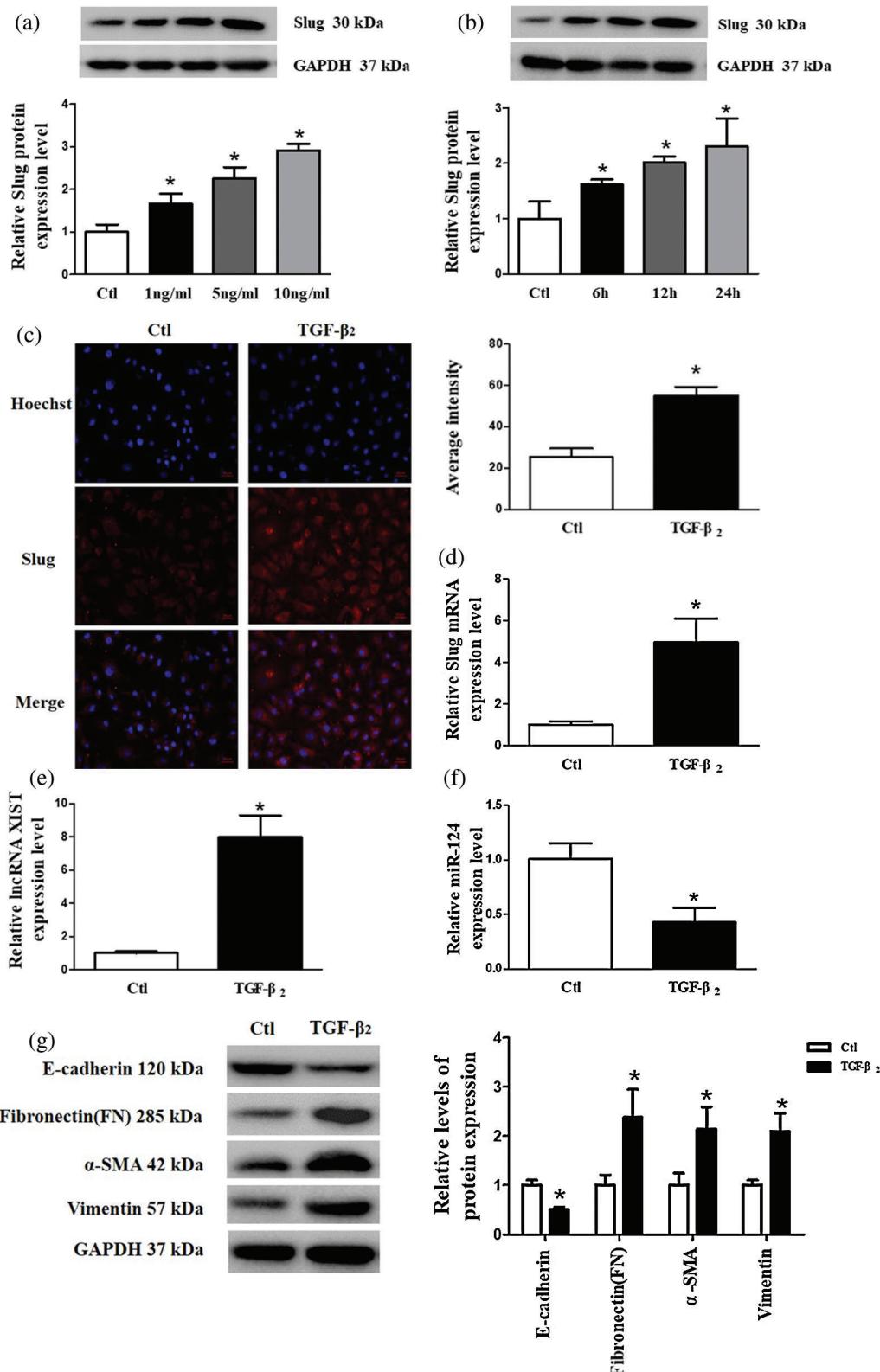
*The expression levels of Slug and lncRNA XIST were increased, whereas miR-124 was decreased in TGF- $\beta$ 2-induced in SRA01/04 cells*

To explore whether EMT takes part in the pathogenesis of PCO and to determine the expression of various substances in the EMT cell model, we used different concentrations (0, 1, 5, 10 ng/mL) of TGF- $\beta$ 2 for treating SRA01/04 cells for 0, 6, 12, and 24 h to establish the EMT cell model *in vitro*. Slug protein expression was upregulated in a concentration- and time-dependent manner with TGF- $\beta$ 2 (Figs. 1a and 1b). According to the peak expression of Slug, the cells were treated with 10 ng/mL TGF- $\beta$ 2 for 24 h in the follow-up experiment. Immunofluorescence results indicated that Slug protein expression increased after the cells had been induced by TGF- $\beta$ 2 (Fig. 1c). The expression of Slug mRNA and lncRNA XIST increased, while the expression of miR-124 decreased when treated with 10 ng/mL TGF- $\beta$ 2 for 24 h (Figs. 1d-1f). We used western blot to examine the expression of EMT-related markers, including epithelial markers (E-cadherin), and mesenchymal markers (FN,  $\alpha$ -SMA, and vimentin). The results reflected that the protein

TABLE 1

Primers for expressing vectors, luciferase reporter plasmids and miR-124 quantification

Name	Sequence (5'-3')
Slug-F	ATAGAATTCCCAGACCCGCTGGCAAGATG
Slug-R	AATGGATCCCGAGTAAACATTGATTGCGTCA
Slug-UTR-F	ATACTCGAGATGACAATAAGTCAAAGG
Slug-UTR-R	ATACTCGAGATGACAATAAGTCAAAGG
Slug-UTR-Mut-F	GCCAGACGCGAACTCAGCTCCGTAAAAAGTATTCCAAGT
Slug-UTR-Mut-R	ACTTGAATACTTTACGGAGCTGAGTCGCGTCTGGC
miR-124-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCCTGGATACGACGGCATTCT
miR-124-F	GATACTCATAGGCACCGCG
miR-124-R	GTGCAGGGTCCGAGGT
U6-F	CGCTTCGGCAGCACATATAC
U6-R	CAGGGGCCATGCTAACCTT
XIST-F	AGCTCCTCGGACAGCTGTAA
XIST-R	CTCCAGATAGCTGGCAACC
XIST siRNA-1-F	AUACUUGGGCUUCUAUCCATT
XIST siRNA-1-R	GAUAGAAGGCCAAA GUUAATT
XIST siRNA-2-F	UUUUGAAGCAUAUUUUGGUUUTT
XIST siRNA-2-R	GCCAAAAAU AUGCUUCAAAGATT
XIST siRNA-3-F	UAAUGAUGCAUAUUUUGGUUUTT
XIST siRNA-3-R	GCUAGAAUGUCGUUCAAAGGATT
GAPDH-F	AGAAGGCTGGGCTCATTTG
GAPDH-R	AGGGGCCATCCACAGTCTTC

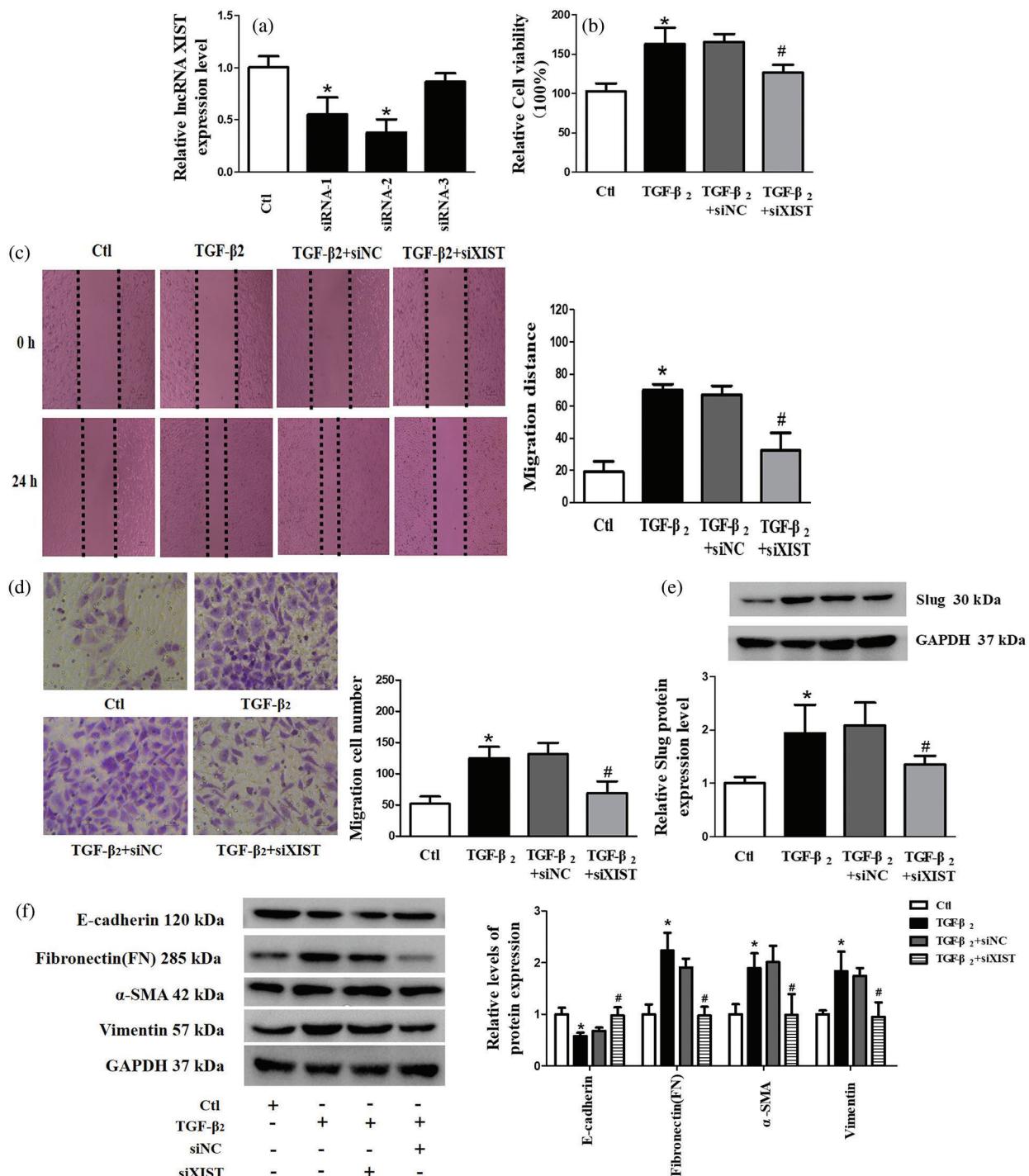


**FIGURE 1.** The expression levels of Slug and lncRNA XIST increased, whereas miR-124 decreased after treatment of SRA01/04 cells with TGF- $\beta$ 2. (a) SRA01/04 cells were induced with different concentrations (0, 1, 5, 10 ng/mL) of TGF- $\beta$ 2 for 24 h. (b) TGF- $\beta$ 2 (10 ng/mL) was used to induce SRA01/04 cells for 0, 6, 12, and 24 h. (c) The average intensity of Slug protein expression increased in SRA01/04 cells induced by TGF- $\beta$ 2 compared with the control (Ctl) group. (d) The expression of Slug mRNA in SRA01/04 cells induced by 10 ng/mL TGF- $\beta$ 2 for 24 h. (e) The expression of lncRNA XIST in SRA01/04 cells induced by 10 ng/mL TGF- $\beta$ 2 for 24 h. (f) The expression of miR-124 in SRA01/04 cells induced by 10 ng/ml TGF- $\beta$ 2 for 24 h. (g) EMT markers in SRA01/04 cells induced by TGF- $\beta$ 2. \* $p$  < 0.05 vs. Ctl group. Data are expressed as mean  $\pm$  SD. N = 3 batches of cells for each group.

expression of E-cadherin decreased, while the protein levels of FN,  $\alpha$ -SMA, and vimentin increased in SRA01/04 cells (Fig. 1g).

*Knockdown of lncRNA XIST had a reverse effect on TGF- $\beta$ 2 induced EMT, cell viability, and migration in SRA01/04 cells*. To further investigate the biological function of lncRNA XIST, we transfected XIST siRNA (siXIST) and screened for the best knockdown efficiency to alleviate endogenous lncRNA XIST

in SRA01/04 cells (Fig. 2a). The role of lncRNA XIST on cell viability was evaluated using CCK-8 assay, which indicated that inhibition of lncRNA XIST effectively suppressed the increased cell viability induced by TGF- $\beta$ 2 stimulation of SRA01/04 cells (Fig. 2b). Additionally, the wound healing assay and transwell assay revealed that the knockdown of lncRNA XIST remarkably inhibited the SRA01/04 cell migration induced by TGF- $\beta$ 2 (Figs. 2c and 2d).



**FIGURE 2.** Knockdown of lncRNA XIST had a reverse effect on TGF- $\beta_2$  induced EMT, cell viability, and migration in SRA01/04 cells. (a) We constructed the cell transfection model by silencing lncRNA XIST and verified the effectiveness of transfection. (b) CCK-8 assay was used to detect cell viability. (c) Wound-healing assay was used to test cell migration. (d) Transwell assay was used to test cell migration. (e) The expression of Slug protein in the TGF- $\beta_2$  group was significantly increased compared with control (Ctl) group, while Slug protein expression in the TGF- $\beta_2$ +siXIST group was considerably lower than that in the TGF- $\beta_2$ +siNC group. (f) Western blot was used to detect the expression of EMT-related markers (E-cadherin, FN,  $\alpha$ -SMA, and vimentin). \* $p < 0.05$  vs. Ctl group. # $p < 0.05$  vs. TGF- $\beta_2$  + siNC group. Data are expressed as mean  $\pm$  SD. N = 3 batches of cells for each group.

As shown in Fig. 2e, suppressed lncRNA XIST reversed the elevated protein expression of Slug induced by TGF- $\beta_2$ . E-cadherin expression was obviously downregulated, while the expression levels of FN,  $\alpha$ -SMA and vimentin were significantly upregulated in TGF- $\beta_2$ -treated SRA01/04 cell line. Still, the interference of lncRNA XIST could reverse these effects on EMT-related markers (Fig. 2f). These results

suggested that lncRNA XIST had a regulatory role in TGF- $\beta_2$ -induced EMT, cell viability, and migration in SRA01/04 cells.

#### Target prediction between lncRNA XIST and miR-124, as well as between miR-124 and Slug

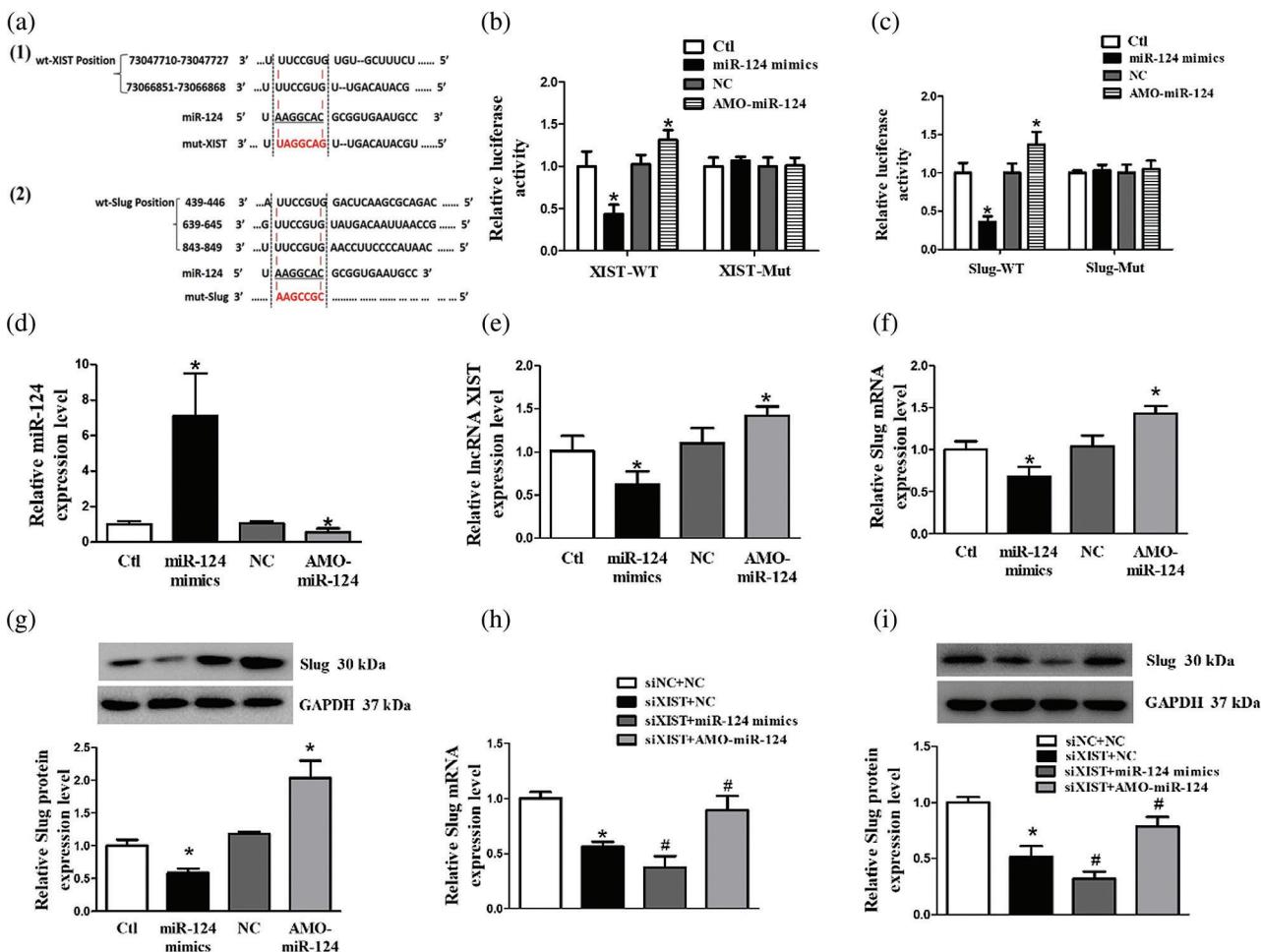
According to the results presented above, we noticed that lncRNA XIST expression highly correlated with Slug.

Previous studies showed that MiR-124 was able to target lncRNA XIST and Slug. Therefore, we speculated that there was a potential regulatory relationship between lncRNA XIST, miR-124, and Slug. We used biological prediction tools to examine the potential binding sites between lncRNA XIST and miR-124, as well as between miR-124 and Slug (Fig. 3a). The relative luciferase activities significantly decreased when miR-124 mimics and wt-XIST or wt-Slug-3'UTR were co-transfected into the cells. Conversely, the relative luciferase activities increased when AMO-miR-124 and wt-XIST or wt-Slug-3'UTR were co-transfected into the cells. No significant changes were found in fluorescence activity at the mutant binding sites of XIST or Slug-3'UTR (Figs. 3b and 3c). To further explore the regulatory role of miR-124 on Slug expression, with miR-124 mimics and AMO-miR-124 transfected, Slug expression was detected by western blot. As shown in Figs. 3d-3g, overexpressed miR-124 decreased the

expression of lncRNA XIST as well as Slug mRNA and protein. Moreover, the expression levels of lncRNA XIST and Slug mRNA and protein were upregulated with AMO-miR-124 transfection. As shown in Figs. 3h and 3i, silencing lncRNA XIST inhibited Slug expression, while miR-124 mimics was able to further inhibit Slug expression. AMO-miR-124 reversed the decrease of Slug expression caused by the knockdown of lncRNA XIST. Collectively, the results demonstrated that lncRNA XIST acted in a competing endogenous RNA (ceRNA) manner by targeting miR-124 to affect miR-124 expression, thereby regulating Slug expression.

*AMO-miR-124 had a reversed effect of silencing lncRNA XIST on the expression of Slug, EMT, cell viability and migration induced by TGF- $\beta$ 2*

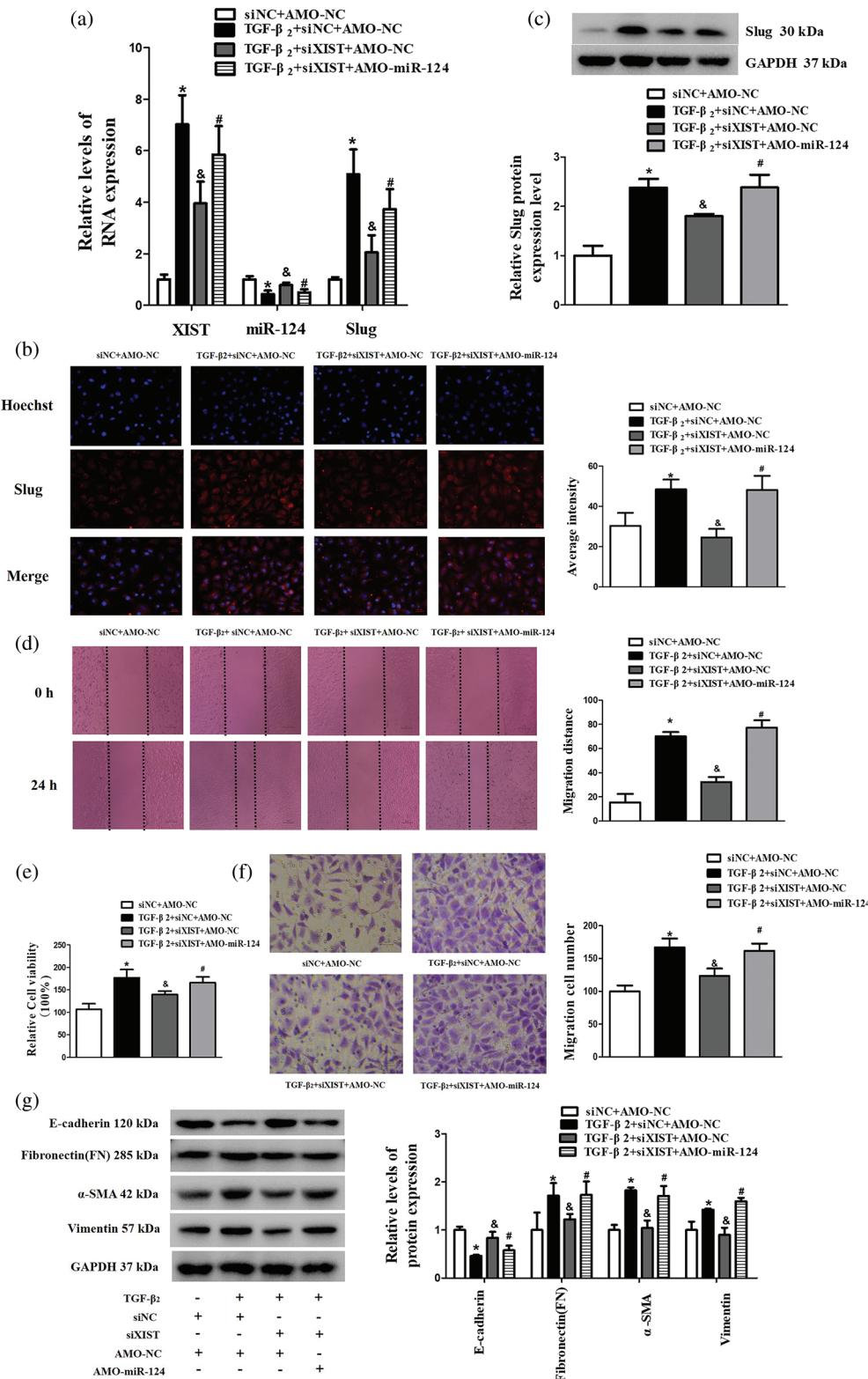
To verify that the influence of lncRNA XIST on the regulation of Slug was mediated by miR-124, SRA01/04 cells were



**FIGURE 3.** Target prediction between lncRNA XIST and miR-124. Slug directly targeted miR-124. (a) Target prediction between lncRNA XIST and miR-124, as well as between miR-124 and Slug, by bioinformatic analysis. (a-1) The bioinformatic analysis demonstrated that two predicted binding sites of wt-XIST (wild type) directly targeted with miR-124. Mut-XIST (mutant) contained mutations in the binding site within the XIST sequence. (a-2) Bioinformatic analysis indicated that miR-124 directly targeted three predicted binding sites of 3'UTR of the Slug gene (wild type). Mut-Slug (mutant) contained mutations in the binding site within the Slug sequence. (b) LncRNA XIST targeted with miR-124 directly by dual-luciferase reporter assay. (c) Slug targeted miR-124 directly by dual-luciferase reporter assay. (d) The expression level of miR-124 transfected with miR-124 mimics or AMO-miR-124. (e) The expression level of lncRNA XIST transfected with miR-124 mimics or AMO-miR-124. (f) The expression level of Slug mRNA transfected with miR-124 mimics or AMO-miR-124. (g) The expression level of Slug protein transfected with miR-124 mimics or AMO-miR-124. (h) The expression level of Slug mRNA with co-transfection of siXIST and miR-124 mimics, or siXIST and AMO-miR-124. (i) The expression level of Slug protein with co-transfection of siXIST and miR-124 mimics, or siXIST and AMO-miR-124. \* $p < 0.05$  vs. NC group. # $p < 0.05$  vs. siXIST + NC group. Data are expressed as mean  $\pm$  SD. N = 3 batches of cells for each group.

transfected with siXIST accompanied by TGF- $\beta$ 2 stimulation. The expression level of miR-124 decreased, while Slug increased under TGF- $\beta$ 2 treatment of SRA 01/04 cells. However, the suppression of lncRNA XIST alleviated the effect of TGF- $\beta$ 2 on miR-124 and Slug, these effects were reversed with AMO-miR-124 co-transfection (Figs. 4a–4c). Besides, AMO-miR-124 inverted the role of suppressing lncRNA XIST on cell viability, migration, and EMT in

TGF- $\beta$ 2-treated SRA01/04 cells (Figs. 4d–4g). These findings demonstrated that the knockdown of lncRNA XIST diminished the TGF- $\beta$ 2-induced influence on EMT via miR-124/Slug axis in SRA01/04 cells, and AMO-miR-124 was able to reverse the changes in EMT-related proteins induced by silencing lncRNA XIST. Therefore, the lncRNA XIST/miR-124/Slug axis participated in the progression of EMT in TGF- $\beta$ 2-treated SRA01/04 cells.



**FIGURE 4.** AMO-miR-124 reversed the effect of silencing lncRNA XIST on the expression of Slug, EMT, cell viability, and migration induced by TGF- $\beta$ 2. (a) The RNA expression level of lncRNA XIST, miR-124, and Slug transfected with siXIST and AMO-miR-124. (b) The average intensity of Slug protein expression transfected with siXIST and AMO-miR-124 was detected by immunofluorescence. (c) The expression of Slug protein transfected with siXIST and AMO-miR-124 was detected by western blot. (d) Wound-healing assay was used to detect the migration distance in SRA01/04 cells transfected with siXIST and AMO-miR-124. (e) CCK-8 assay was used to detect the cell viability in SRA01/04 cells transfected with siXIST and AMO-miR-124. (f) Transwell assay was used to detect the migration cell number in SRA01/04 cells transfected with siXIST and AMO-miR-124. (g) Western blot was used to detect the expression of EMT-associated markers in SRA01/04 cells transfected with siXIST and AMO-miR-124. \* $p < 0.05$  vs. siNC+AMO-NC group. & $p < 0.05$  vs. TGF- $\beta$ 2+siNC+AMO-NC group. # $p < 0.05$  vs. TGF- $\beta$ 2+siXIST+AMO-NC group. Data are expressed as mean  $\pm$  SD. N = 3 batches of cells for each group.

## Discussion

PCO is the most common complication after cataract surgery. It mainly develops due to cell viability, migration, and EMT of residual HLECs postoperatively (Li *et al.*, 2019); specifically, EMT likely plays a core role in the PCO formation (Dong, 2019a), but the underlying mechanisms of molecular regulation in the development of PCO remain unclear. In the current study, we showed that the expression of EMT-associated markers, cell migration, and cell viability in SRA01/04 cells induced by TGF- $\beta$ 2 were modulated through a lncRNA XIST-dependent mechanism. Specifically, lncRNA XIST negatively regulated miR-124, and miR-124 targeted Slug (snail 2) directly. Finally, we proved that the lncRNA XIST/miR-124/Slug axis participated in the EMT of HLECs induced by TGF- $\beta$ 2.

LncRNA XIST is the first lncRNA identified on chromosome Xq13.2. It is dysregulated in a wide variety of human cancers, and it plays an integral role in the development and progression of tumors by regulating cell viability, invasion, migration, and EMT (Hai *et al.*, 2020; Hu *et al.*, 2018; Shen *et al.*, 2019; Wang *et al.*, 2018; Xiao *et al.*, 2019). For example, elevated lncRNA XIST was reportedly found in pancreatic cancer cells and tissues, and the knockdown of lncRNA XIST in pancreatic cancer cell lines inhibited EMT by suppressing mesenchymal markers ( $\beta$ -catenin and Snail) and upregulating epithelial markers (E-cadherin and Claudin-1) (Shen *et al.*, 2019). Another study showed that elevated lncRNA XIST expression was closely associated with poor overall survival in colorectal cancer patients and promoted metastasis and EMT in colorectal cancer (Chen *et al.*, 2017). LncRNA XIST was upregulated in TGF- $\beta$ 2-treated HLECs (Dong, 2019b), which is in agreement with our findings. Therefore, we demonstrated that silencing lncRNA XIST can inhibit EMT in TGF- $\beta$ 2-induced SRA01/04 cells.

LncRNA XIST acts as a sponge and can represent biological characteristics via a ceRNA mechanism (Hai *et al.*, 2020). Several previous findings indicated that lncRNA XIST could adversely modulate miR-124 via sponging miR-124, thus regulating various cancers, including laryngeal squamous cell carcinoma and bladder cancer (Xiao *et al.*, 2019; Xiong *et al.*, 2017). The action of miR-124 in PCO has not been investigated, except that some previous studies showed that miR-124 expression was significantly decreased in LECs of cataract (Wei and Sun, 2019; Wu *et al.*, 2017a; Wu *et al.*, 2017b). In the current study, we demonstrated that the expression level of miR-124 decreased in TGF- $\beta$ 2-induced SRA01/04 cells. Interestingly, by luciferase reporter assay, we also found that lncRNA XIST was able to adversely modulate miR-124 via functioning as a ceRNA of miR-124, which is consistent with the previous report (Du *et al.*, 2020).

In addition to the TGF- $\beta$  signaling pathway, the activation of crucial EMT transcriptional repressors, such as Snai1, Slug, Twist1, Twist2, ZEB1, and ZEB2, could suppress CDH1(E-cadherin promoter) in a variety of different routes in cancers (Derynick *et al.*, 2021; Puisieux *et al.*, 2014). CDH1 expression deficiency has been recognized as a characteristic feature of EMT (Liang *et al.*, 2018). However,

the underlying molecular events that drive the EMT process during the progression of PCO are still largely unknown. Slug is a biomarker of EMT and has been reported to be upregulated in PCO-attached LECs and HLECs induced by TGF- $\beta$  (Chandler *et al.*, 2007; Dong *et al.*, 2015; Wang *et al.*, 2016; Zhang *et al.*, 2018), which is in agreement with our results. Meanwhile, E-cadherin expression was downregulated, while the expression levels of FN,  $\alpha$ -SMA, and vimentin were increased, which is in agreement with the former findings (Dong *et al.*, 2014; Zhang *et al.*, 2018). The data showed the EMT cell model was successfully established. We confirmed that Slug was participated in the etiopathogenesis of PCO. Besides, we found binding sites between miR-124 and Slug by bioinformatic prediction, consistent with previous reports (Cui and Hu, 2016).

We further demonstrated that the knockdown of lncRNA XIST can upregulate miR-124 expression and downregulate the expression of Slug protein in TGF- $\beta$ 2-treated SRA01/04 cells. Our findings further indicated that co-transfection of AMO-miR-124 can reverse the downregulation of Slug induced by siXIST, which is consistent with the result that miR-124 regulates Slug expression (Cui and Hu, 2016; Liang *et al.*, 2013). In addition, siXIST attenuated the impact of TGF- $\beta$ 2 on the expression of miR-124 and Slug and the process of cell viability, migration, and EMT, but these effects were reversed by co-transfection of AMO-miR-124. These findings suggested that lncRNA XIST regulated TGF- $\beta$ 2-induced Slug protein expression by competitively interacting with miR-124. Overall, these results indicated that the knockdown of lncRNA XIST can protect HLECs treated by TGF- $\beta$ 2 from EMT progression via regulating the miR-124/Slug pathway.

The limitation of this study is that although the TGF- $\beta$ 2-induced SRA01/04 cell line is a common *in vitro* cell model for PCO research (Wang *et al.*, 2019; Zhang *et al.*, 2018), it may not reflect normal lens biology because it does not express many normal lens markers; thus, we cannot determine whether there are potential regulatory differences when compared with the normal lens (Weatherbee *et al.*, 2019). Besides, we only verified the regulatory axis at the cellular level, but not in the animal model. We will do further research in the follow-up experiments. Another interesting point that needs to be discussed is that this study's purpose was to reveal a downstream regulatory signaling pathway of lncRNA XIST in the pathological development of PCO, which is the common mechanism of PCO in female and male individuals. LncRNA XIST is an X chromosome-related lncRNA. Its differential expression is tightly associated with the gender-related incidence of diseases, such as breast cancer and cervical cancer (Chen *et al.*, 2019; Zong *et al.*, 2020). Still, lncRNA XIST is also involved in some non-gender-related diseases, such as diabetic retinopathy (Dong *et al.*, 2020). One research study showed that the sex ratio of diabetic retinopathy was not uniform. It was reported that the incidence rate in men and women was similar in the study of the Nakuru (Bastawrous *et al.*, 2017; Sabanayagam *et al.*, 2019). Several epidemiologic studies have suggested that the incidence of PCO is higher in elderly women than in age-matched men (Congdon *et al.*, 2008; Fong *et al.*, 2014;

[Lee et al., 2016](#)). Nevertheless, this is not a uniform conclusion, and other studies do not support this view ([Westling and Calissendorff, 1991](#)). Therefore, the existence of gender differences in PCO should be studied. In addition, there are many factors affecting PCO, such as intraocular lens material and design, surgical techniques, and age ([Awasthi et al., 2009; Nibourg et al., 2015](#)). We will further explore the activation mechanism of lncRNA XIST upstream in X chromosome based on this pathway to indirectly explain whether there is a gender difference trend in PCO, which will have important clinical significance.

In conclusion, the current study revealed that the knockdown of lncRNA XIST alleviated EMT-associated Slug protein alterations by sponging miR-124. We demonstrated the existence of the lncRNA XIST/miR-124/Slug axis in the TGF- $\beta$ 2-induced SRA01/04 cell line. TGF- $\beta$ 2-induced SRA01/04 cell line is a common cell model to study EMT progression in PCO. Therefore, we speculated that the lncRNA XIST/miR-124/Slug axis may be related to the pathogenesis of PCO. Together, the results disclosed novel lncRNA and miRNA participating in the EMT cell model of PCO, suggesting that the lncRNA XIST/miR-124/Slug axis has the potential to be a promising target for clinical treatment of PCO.

**Availability of Data and Materials:** All data generated or analysed during this study are included in this article.

**Authors' Contributions:** Xue Jiang conceived and designed the experiments. Xue Jiang carried out the experiments and contributed to the writing of the manuscript. Xue Jiang and Hong Zhang participated in the design and implementation of the project, raised funds, and completely revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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