• Basic Research •

TGF-β2-induced NEAT1 regulates lens epithelial cell proliferation, migration and EMT by the miR-26a-5p/FANCE axis

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Abstract

● **AIM:** To explore the regulatory mechanism of nuclear paraspeckle assembly transcript 1 (NEAT1) in the pathogenesis of posterior capsule opacification (PCO).

● **METHODS:** Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was executed to analyze NEAT1 and microRNA (miR)-26a-5p expression in transforming growth factor-beta 2 (TGF-β2)-disposed lens epithelial cells (LECs). The proliferation, cell cycle progression, apoptosis, and migration of TGF-β2-disposed LECs were evaluated. The relationship between NEAT1 or fanconi anemia (FA) complementation group E (FANCE) and miR-26a-5p was verified by dual-luciferase reporter assay.

● **RESULTS:** TGF-β2 induced NEAT1 expression in LECs. NEAT1 inhibition accelerated apoptosis, cell cycle arrest, decreased proliferation, epithelial-mesenchymal transition (EMT), and migration of TGF-β2-disposed LECs. NEAT1 sponged miR-26a-5p to further regulate FANCE expression. Rescue experiments presented that miR-26a-5p downregulation overturned NEAT1 silencing-mediated impacts on TGF-β2-disposed LEC biological behaviors. Additionally, FANCE overexpression reversed miR-26a-5p mimic-mediated impacts on TGF-β2-disposed LEC biological behaviors.

● **CONCLUSION:** TGF-β2-induced NEAT1 facilitates LEC proliferation, migration, and EMT by upregulating FANCE *via* sequestering miR-26a-5p.

● **KEYWORDS:** posterior capsule opacification; transforming growth factor-beta 2; nuclear paraspeckle assembly transcript 1; miRNA-26a-5p; fanconi anemia complementation group E

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INTRODUCTION

Posterior capsule opacification (PCO), also termed as a secondary cataract, is a common complication after cataract surgery that can lead to secondary vision loss[1]. Elevated transforming growth factor-beta 2 (TGF-β2) in ocular tissues is one of the main factors of post-cataract in patients undergoing cataract surgery[2]. The progression of PCO is related to the migration, proliferation, and epithelial-mesenchymal transition (EMT) of residual lens epithelial cells (LECs) on the lens capsule[3].

LncRNAs are related to a series of cellular functions[4]. Also, lncRNAs are associated with pathological conditions, such as inflammatory diseases, cardiovascular diseases, and cancers[5-6]. Moreover, lncRNAs exert important roles in ocular diseases[7]. For instance, lncRNA MALAT1 could facilitate cell oxidative stress and apoptosis in LECs in diabetic cataract[8]. LncRNA nuclear paraspeckle assembly transcript 1 (NEAT1), also termed as LINC00084[9], has been uncovered to be implicated in the advancement of various diseases, such as endocrine diseases[10] and tumors[11]. Also, NEAT1 accelerated the EMT of LECs[12]. Nevertheless, the mechanism of NEAT1-mediated PCO progression remains largely indistinct.

Generally, lncRNAs participate in the advancement of some diseases by competitively binding to microRNAs (miRs)[13]. MiRs can maintain cell homeostasis through regulating gene expression[14]. MiR-26a-5p exerts a suppressive role in multiple tumors, such as melanoma[15] and bladder cancer[16]. Also, miR-26a-5p plays a repressive influence on cell EMT in LECs under TGF-β2 stimulation[17]. At present, the mechanism of miR-26a-5p dysregulation in LECs is still unclear.

Fanconi anemia (FA) is an inherited cancer susceptibility disease[18]. FA complementation group E (FANCE), a member of the FA core complex, promotes the FA DNA repair pathway by recruiting FANCD2 into the FA E3 ligase complex[19]. Moreover, the phosphorylation of FANCE is involved in the FA/BRCA pathway[20]. Wang *et al*[21] unmasked the abnormal expression of FANCE in the lens cortex of age-related cortical cataract. However, the role of FANCE in PCO progression has not been reported.

Herein, we verified the elevation of NEAT1 in TGF-β2-disposed LECs. Importantly, NEAT1 sponged miR-26a-5p to elevate FANCE expression, thereby accelerated proliferation, migration, EMT of LECs.

MATERIALS AND METHODS

TGF-β2 Treatment Human SRA01/04 cells (BNCC, Suzhou, China) were cultured in DMEM (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Solarbio, Beijing, China) in a 5% CO_2 incubator at 37°C. For TGF-β2 treatment, SRA01/04 cells were grown in DMEM with different concentration of TGF-β2 (Procell, Wuhan, China; 0, 1, 5, and 10 ng/mL) for 48h or in DMEM with TGF-β2 (5 ng/mL) for different times (0, 12, 24, and 48h).

Cell Transfection si-NEAT1#1, si-NEAT1#2, NC, miR-26a-5p inhibitor (anti-miR-26a-5p), inhibitor NC (anti-NC), miR-26a-5p mimic (miR-26a-5p), mimic NC (miR-NC), pcDNA3.1 vector (Thermo), and pcDNA3.1-FANCE (FANCE) plasmid were transfected into SRA01/04 cells using Lipofectamine 3000 reagent (Thermo, Waltham, MA, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction The miRNeasy Mini Kit (Qiagen, Hilden, Germany) was applied to isolate total RNA. Total RNA was utilized for generation of cDNA together with a HiScript Q RT SuperMix for qPCR kit (Vazyme, Nanjing, China) or miRNA cDNA synthesis kit (GeneCopoeia, Guangzhou, China). The complementary DNA was utilized for quantitative reverse transcription polymerase chain reaction (RT-qPCR) with an SYBR Green PCR master mix (Vazyme). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH for NEAT1 and U6 for miR-26a-5p were used as internal controls.

Cell Proliferation Assessment SRA01/04 cells were cultured in DMEM containing 5 ng/mL TGF-β2 for different time. After co-incubation with the CCK-8 solution (10 μL), the absorbance at 450 nm was assessed using a Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Apoptosis and Cycle Analysis SRA01/04 cells (1×10^6) were collected and digested with trypsin (Sigma). The apoptotic rate and cell cycle of SRA01/04 cells were analyzed with an Annexin V-FITC/PI apoptosis detection kit (Becton Dickinson, San Jose, CA, USA) and DNA content quantitation assay kit (Solarbio) following the manufacturer's steps, respectively. The samples were analyzed using the FACS Calibur Flow Cytometer (Becton Dickinson).

Cell Migration Assessment Cell migration was evaluated using 24-well transwell chambers (Corning, NY, USA) with a fibronectin-coated polycarbonate membrane. In short, the medium (600 μL) containing 10% FBS and 5 ng/mL TGF-β2 was supplemented to the lower chamber. About 1×10^5 SRA01/04 cells were uniformly tiled to the top chamber. After 24h, the migrating cells were stained with 0.25% of crystal violet (Solarbio), followed by counting by using an inverted microscope (MTX Lab Systems, Bradenton, FL, USA) at 100 × magnification.

Western Blotting All antibodies used in the research were displayed as follows: α-SMA (ab265588, 1:1000, Abcam, Cambridge, Massachusetts, USA), E-cadherin (ab1416, 1:1000, Abcam), vimentin (ab8978, 1:1000, Abcam), FANCE (sc-398558, 1:100, Santa Cruz, CA, USA), GAPDH (ab8245, 1:1000, Abcam), and goat anti-mouse (ab205719, 1:20000, Abcam) or anti-rabbit (ab205718, 1:20000, Abcam) IgG. Extraction of total protein was conducted using the RIPA lysis buffer (Thermo). Western blotting (WB) was executed as previously described[22]. Enhanced chemiluminescence was performed based on the manufacturer's procedures (Thermo).

Dual-Luciferase Reporter Assay SRA01/04 cells were co-transfected with miR-NC or miR-26a-5p and a luciferase reporter. The luciferase activities were analyzed with the luciferase reporter assay kit (Promega, Madison, WI, USA). The sequences of wild type NEAT1 (NEAT1-WT) and 3' untranslated regions (UTR) of FANCE (FANCE-WT) and their mutant (MUT) sequences NEAT1-MUT and FANCE-MUT were inserted into the pMIR-REPORT vectors (Applied Biosystems, Foster, CA, USA) to construct luciferase reporters, respectively.

Statistical Analysis All experiments were repeated at least 3 times, and each experiment was carried out in triplicate. Results were analyzed by GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) and presented as the mean±standard deviation. One-way analysis of variance followed by Turkey's post hoc test was applied to analyze the differences among 3 or more groups. Unpaired Student's *t*-test was executed to determine the difference between 2 groups. Statistical significance was set at $P<0.05$

RESULTS

TGF-β2 Induced NEAT1 Expression in SRA01/04 Cells We performed RT-qPCR to verify NEAT1 expression in PCO. As exhibited in Figure 1A, NEAT1 expression was gradually increased in SRA01/04 cells with the elevation of TGF-β2. Also, TGF-β2 stimulation resulted in an overt elevation in SRA01/04 cells in a time-dependent manner (Figure 1B).

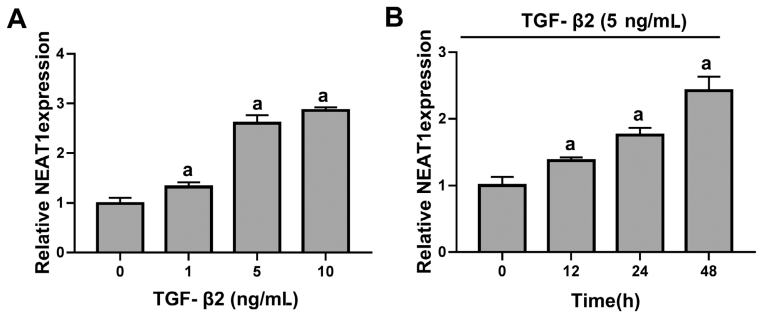

Figure 1 TGF-β2 stimulation elevated NEAT1 levels in SRA01/04 cells RT-qPCR exhibiting NEAT1 levels in SRA01/04 cells with TGF-β2 treatment. [a]$P<0.05$.

Because the expression of NEAT1 did not change significantly under 5 and 10 ng/mL TGF-β2 treatments, 5 ng/mL of TGF-β2 was utilized for further study.

NEAT1 Silencing Reduced TGF-β2-Indcued PCO Progression After si-NEAT1#1 or si-NEAT1#2 transfection, NEAT1 expression was observably decreased in SRA01/04 cells, particularly si-NEAT1#2 (Figure 2A). CCK-8 assay exhibited that NEAT1 downregulation reduced cell proliferation in SRA01/04 cells under TGF-β2 treatment (Figure 2B). Moreover, NEAT1 silencing partly counteracted the inhibitory effect of TGF-β2 treatment on SRA01/04 cell apoptosis in flow cytometry assay (Figure 2C and 2D). In addition, NEAT1 silencing impaired TGF-β2-induced cell cycle progression in SRA01/04 cells (Figure 2E). Also, silenced NEAT1 expression reversed the promoting effect on SRA01/04 cell migration mediated by TGF-β2 treatment (Figure 2F). WB revealed that TGF-β2 treatment reduced E-cadherin levels and elevated α-SMA and vimentin levels, but these tendencies were restored after NEAT1 silencing (Figure 2G). Together, these data indicated that NEAT1 knockdown impaired PCO progression.

NEAT1 Served as a miR-26a-5p Sponge Subsequently, we sought for miRs that possessed a sequence complementary to NEAT1. Starbase database prediction presented that NEAT1 might interact with miR-26a-5p (Figure 3A). Luciferase assay displayed a lower luciferase activity in cells with NEAT1-WT reporter and miR-26a-5p mimic than the control group (Figure 3B). Furthermore, we discovered that TGF-β2 treatment led to a decrease in miR-26a-5p expression (Figure 3C and 3D). Also, NEAT1 silencing weakened the decrease in miR-26a-5p levels in cells caused by TGF-β2 stimulation, but this effect mediated by NEAT1 silencing was weakened by knockdown of miR-26a-5p (Figure 3E). Collectively, NEAT1 severed as a miR-26a-5p sponge.

NEAT1 Modulated PCO Progression Through Adsorbing miR-26a-5p Subsequently, we executed rescue experiments to analyze whether NEAT1 modulated SRA01/04 cell behaviors through miR-26a-5p under TGF-β2 treatment. We observed that miR-26a-5p inhibitor weakened the suppressive role of NEAT1 downregulation on SRA01/04 cell proliferation under TGF-β2 treatment (Figure 4A). Also, miR-26a-5p downregulation overturned the promoting impact of NEAT1 inhibition on cell apoptosis under TGF-β2 stimulation (Figure 4B, 4C). Furthermore, the inhibiting impacts of NEAT1 silencing on SRA01/04 cell migration and cell cycle progression under TGF-β2 stimulation were impaired after anti-miR-26a-5p introduction (Figure 4D-4G). In addition, miR-26a-5p inhibition overturned NEAT1 knockdown-mediated effects on α-SMA, E-cadherin, and Vimentin protein levels in cells with TGF-β2 stimulation (Figure 4H). Collectively, NEAT1 adsorbed miR-26a-5p to modulate PCO development.

FANCE Acted as Target for miR-26a-5p Through the online tool Starbase, we discovered that miR-26a-5p might target FANCE (Figure 5A). We also observed that miR-26a-5p mimic declined the luciferase activity in SRA01/04 cells with the FANCE-WT reporter (Figure 5B). Moreover, TGF-β2 stimulation caused an increase in FANCE protein levels (Figure 5C, 5D). Data in Figure 5E displayed the overexpression efficiency of miR-26a-5p mimic. Under TGF-β2 treatment, miR-26a-5p overexpression reduced FANCE protein levels, but this decrease was reversed after FANCE introduction (Figure 5F). All in all, miR-26a-5p directly targeted FANCE.

MiR-26a-5p Targeted FANCE to Regulate PCO Progression To verify whether miR-26a-5p regulates SRA01/04 cell behaviors through targeting FANCE, we conducted executed rescue experiments. The results exhibited that FANCE overexpression overturned the inhibiting role of miR-26a-5p upregulation on SRA01/04 cell proliferation under TGF-β2 treatment (Figure 6A). Moreover, FANCE upregulation reversed the elevation in the apoptotic rate of SRA01/04 cells induced by miR-26a-5p overexpression under TGF-β2 treatment (Figure 6B, 6C). Also, FANCE elevation overturned the suppressive impact of miR-26a-5p upregulation on SRA01/04 cell migration and cycle progression under TGF-β2 stimulation (Figure 6D-6G). Additionally,

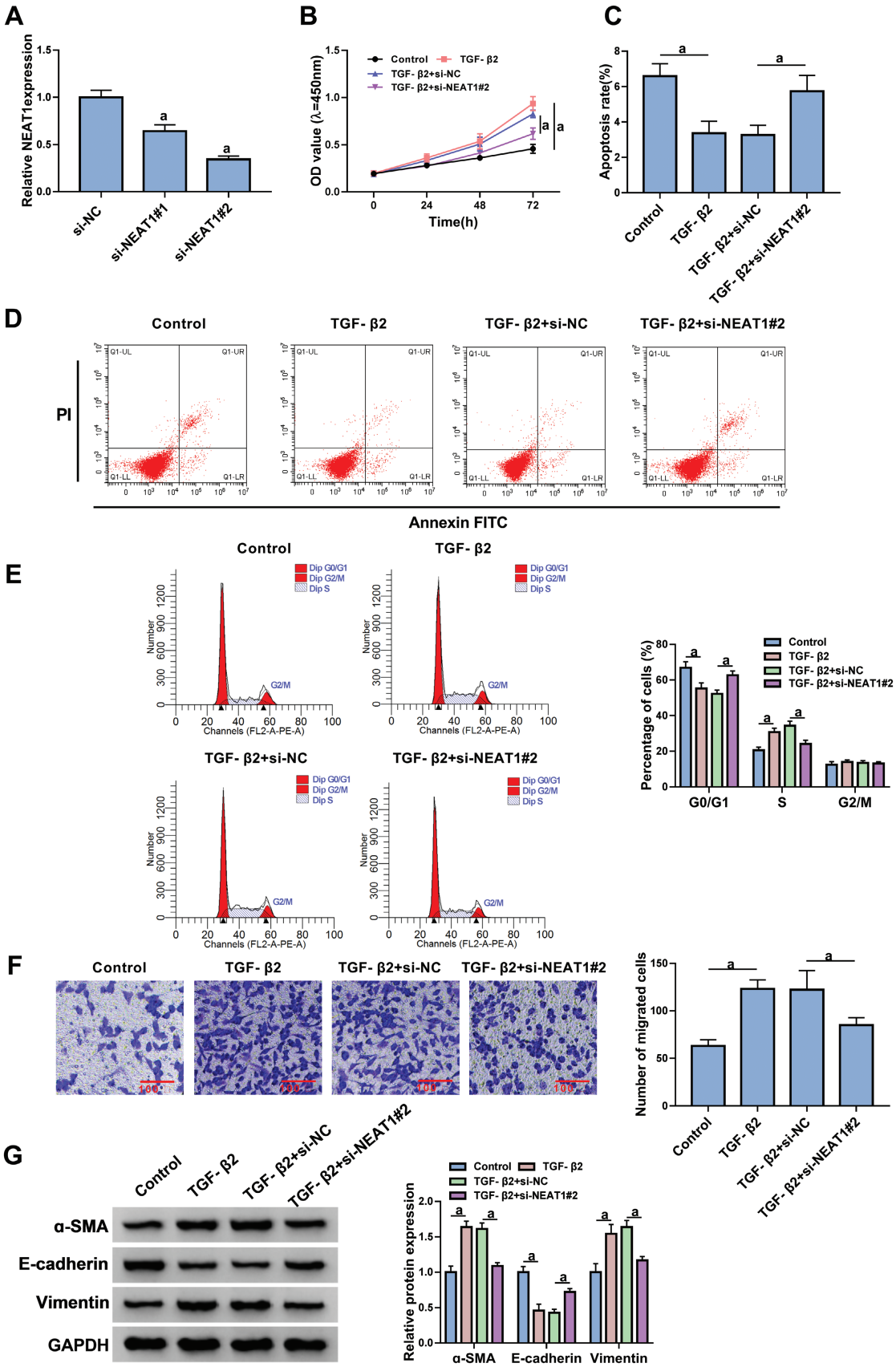

Figure 2 NEAT1 silencing weakened PCO progression A: The knockdown efficiencies of si-NEAT1#1 and si-NEAT1#2 on NEAT in SRA01/04 cells were verified by RT-qPCR. B-G: After transfection with si-NEAT1#2 or si-NC, SRA01/04 cells were treated with 5 ng/mL of TGF-β2. B-F: The proliferation, apoptosis, cycle progression, and migration of the above-mentioned cells were assessed. G: WB exhibiting α-SMA, E-cadherin, and vimentin levels in the above-mentioned cells. [a]P<0.05.

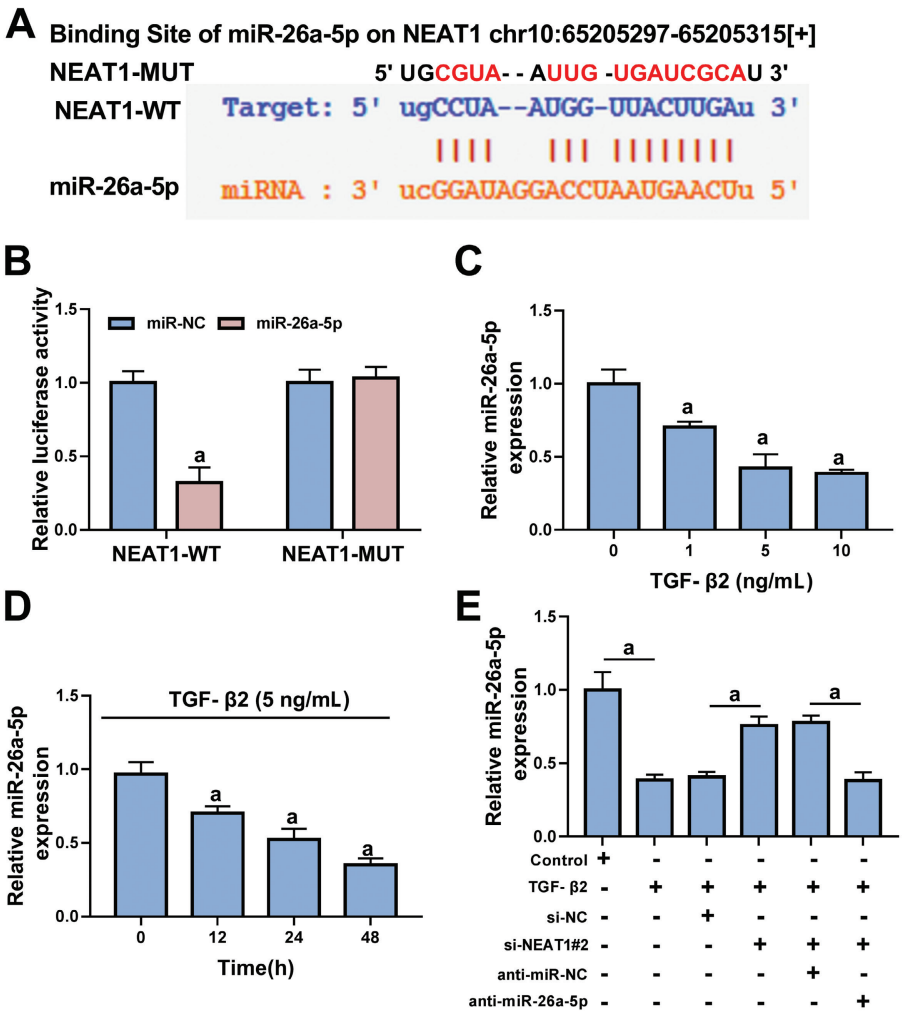

Figure 3 NEAT1 was a miR-26a-5p sponge A: The complementary sequences between NEAT1 and miR-26a-5p. B: The luciferase activities of NEAT1-MUT and NEAT1-WT reporters in cells with miR-26a-5p mimic or miR-NC. C, D: RT-qPCR presenting the miR-26a-5p level in SRA01/04 cells after TGF-β2 treatment. E: RT-qPCR showing the miR-26a-5p level in SRA01/04 cells with different treatments. [a]P<0.05.

miR-26a-5p elevation reduced α-SMA and vimentin protein levels and elevated E-cadherin protein levels in cells with TGF-β2 stimulation, whereas these tendencies were restored after FANCE upregulation (Figure 6H). These results manifested that miR-26a-5p regulated PCO progression *via* targeting FANCE.

NEAT1 Regulated FANCE Expression *via* Sponging miR-26a-5p We further explored whether NEAT1 modulated FANCE expression *via* miR-26a-5p. Data in Figure 7 presented that NEAT1 inhibition decreased FANCE protein levels in cells with TGF-β2 stimulation, but this tendency was abolished after miR-26a-5p silencing. These data indicated that NEAT1 modulated FANCE expression by sponging miR-26a-5p.

DISCUSSION

TGF-β2 exerts a vital role in modulating various biological processes[23]. Increasing researches have revealed that TGF-β2 accelerates LEC proliferation, migration, and EMT in PCO[24-26]. Herein, we verified that TGF-β2-mediated NEAT1 upregulation facilitated LEC proliferation, migration, and EMT.

Mounting studies have proved that lncRNAs participate in PCO development. Zhang *et al*[27] pointed out that TGF-β2-induced HOTAIR contributed to proliferation, viability, migration, and EMT of LECs. Also, FEZF1-AS1 dysregulation was involved in the proliferation and migration of LECs induced by TGF-β2[28]. In the present research, TGF-β2 treatment elevated NEAT1 expression in LECs. Moreover, NEAT1 silencing reversed TGF-β2-induced proliferation, apoptotic repression, cell cycle progression, and migration of LECs. The upregulation of α-SMA and vimentin is a typical event in the EMT process, leading to impaired epithelial integrity[29]. E-cadherin, a calcium-dependent cell-cell adhesion molecule, is usually downregulated during EMT[30]. Herein, NEAT1 inhibition overturned the downregulation of E-cadherin and the upregulation of α-SMA and vimentin in TGF-β2-treated LECs, indicating that NEAT1 participated in TGF-β2-induced EMT. Recent research had uncovered that TGF-β2-mediated NEAT1 facilitated cell EMT through elevating Zeb1 or Snail 1 expression by sponging miR-204

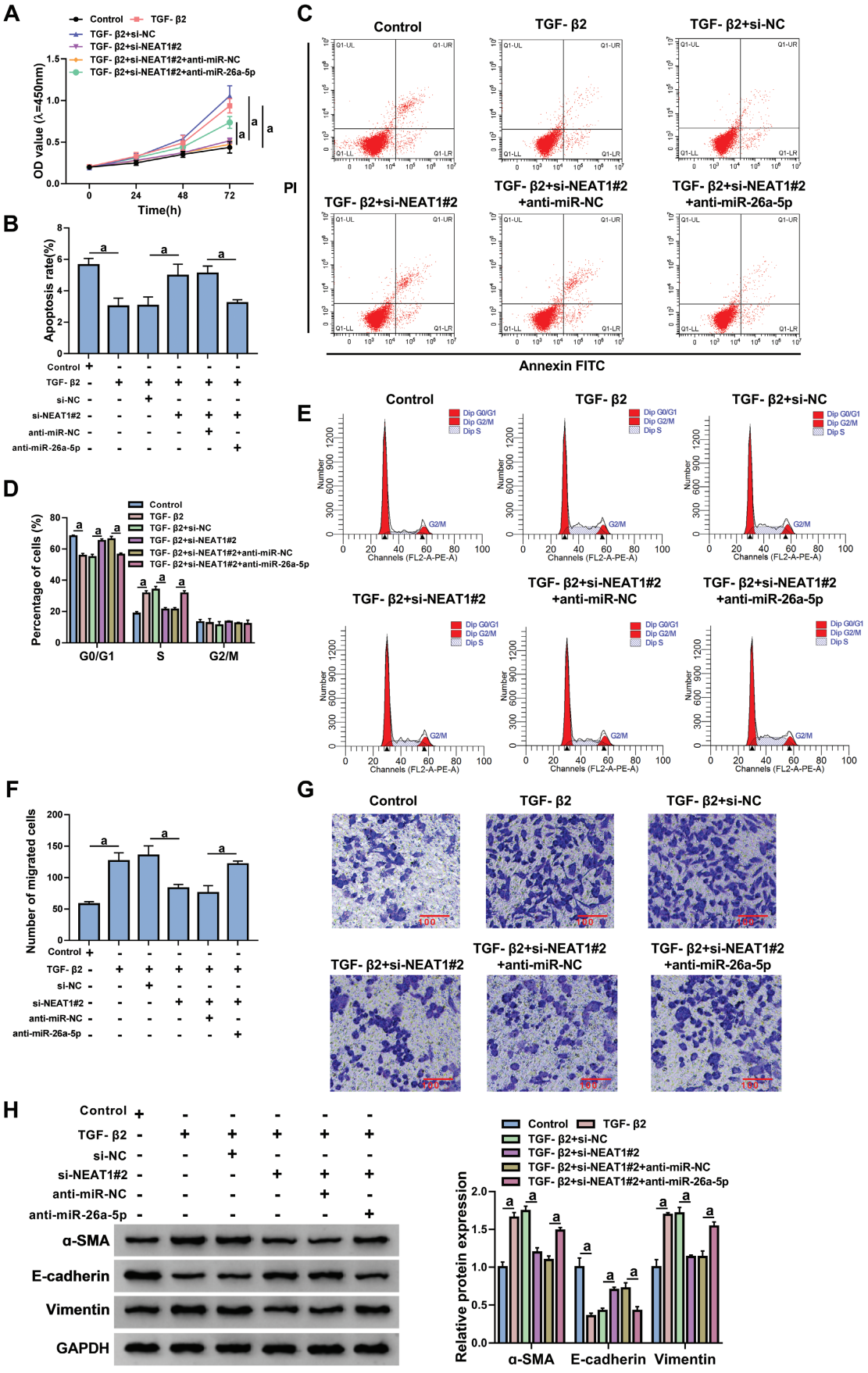

Figure 4 MiR-26a-5p inhibitor reversed the impacts of NEAT1 downregulation on PCO progression SRA01/04 cells were transfected with si-NC, si-NEAT1#2, si-NEAT1#2+anti-miR-NC, or si-NEAT1#2+anti-miR-26a-5p and then treated with TGF-β2 (5 ng/mL). A-G: Analysis of the proliferation, apoptosis, cell cycle progression, and migration of the above-mentioned cells. H: WB exhibiting α-SMA, E-cadherin, and vimentin protein levels in the above-mentioned cells. [a]P<0.05.

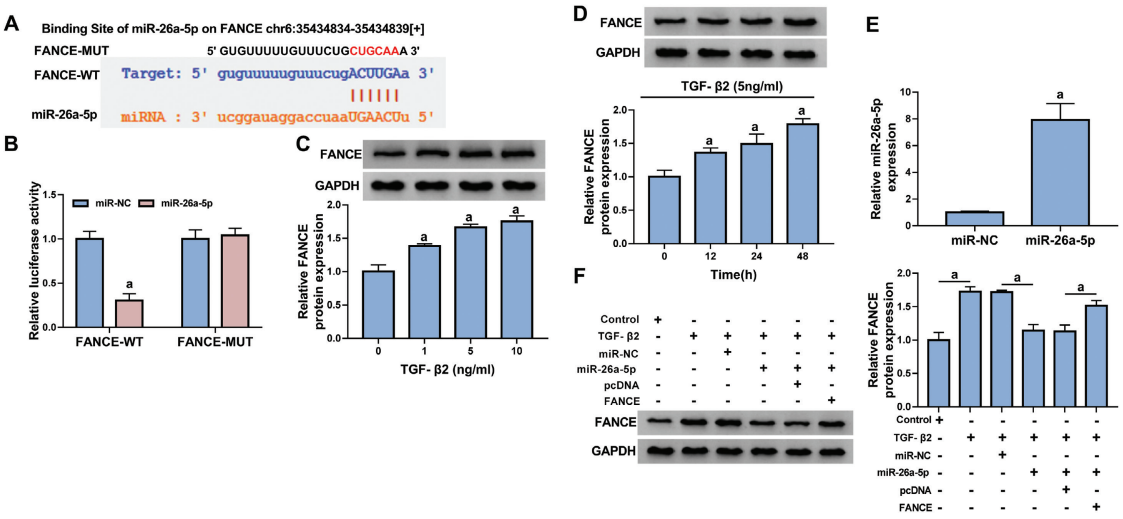

Figure 5 MiR-26a-5p directly targeted FANCE A: The sites of miR-26a-5p complementary to FANCE. B: Dual-luciferase reporter assay exhibiting the targeting relationship between FANCE and miR-26a-5p mimic. C, D: WB detection of FANCE protein levels in SRA01/04 cells with TGF-β2 treatment. E: The overexpression efficiency of miR-26a-5p mimic was verified by RT-qPCR. F: After TGF-β2 treatment, the level of FANCE protein in SRA01/04 cells transfected with miR-NC, miR-26a-5p, miR-26a-5p+pcDNA, or miR-26a-5p+FANCE was assessed by WB. $^{a}P<0.05$.

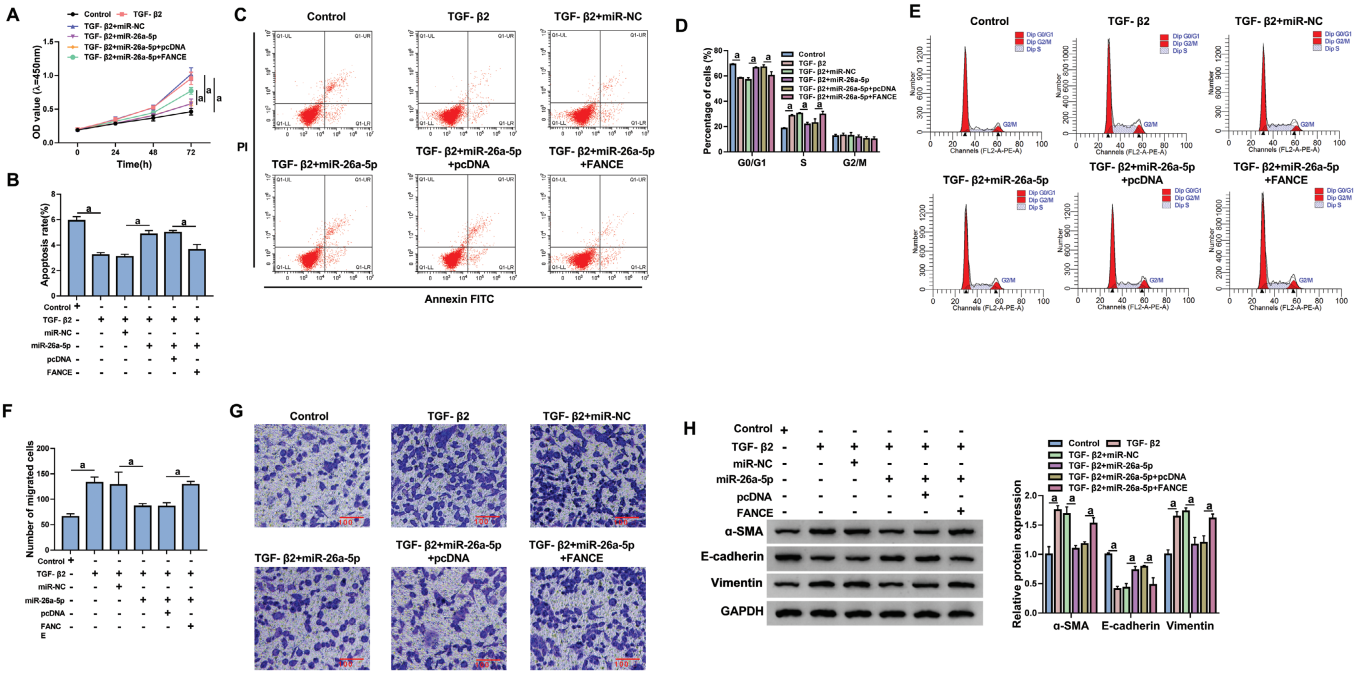

Figure 6 FANCE overexpression overturned miR-26a-5p mimic-mediated impacts on PCO progression After transfection, SRA01/04 cells were treated with TGF-β2 (5 ng/mL). A-G: The proliferation, apoptosis, cell cycle progression, and migration of the above-mentioned cells were detected. H: WB exhibiting α-SMA, E-cadherin, and vimentin protein levels in the above-mentioned cells. $^{a}P<0.05$.

or miR-34a in LECs[12]. Together, TGF-β2-induced NEAT1 participated in PCO progression.

According to the ceRNA hypothesis, lncRNAs can modulate the downstream targets of miRs *via* adsorbing miRs by miR response elements[31]. Herein, we proved NEAT1 as a miR-26a-5p sponge. Moreover, the downregulation of miR-26a-5p overturned NEAT1 silencing-mediated effects on LEC proliferation, apoptosis, migration, and EMT under TGF-β2 stimulation. Report of Chen *at al*[32] revealed that miR-26a-5p exerted an inhibitory role in cataract and lens fibrosis through the Jagged-1/Notch pathway. Moreover, TGF-β2-induced MALAT1 adsorbed miR-26a-5p to accelerate cell EMT in LECs[17]. Thus, NEAT1 regulated TGF-β2-induced PCO progression through adsorbing miR-26a-5p.

Additionally, we demonstrated FANCE as a miR-26a-5p target. A previous study had uncovered that FANCE was highly

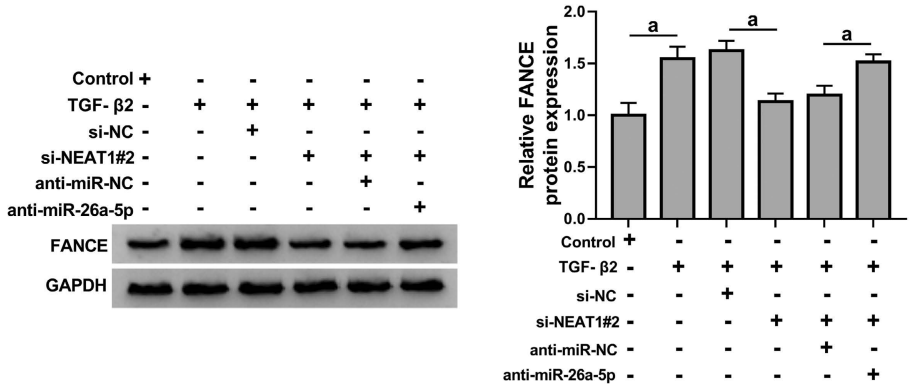

Figure 7 NEAT1 sponged miR-26a-5p to regulate FANCE expression WB presenting the impact of miR-26a-5p silencing on FANCE protein levels in NEAT1-inhibting SRA01/04 cells under TGF-β2 treatment. [a]P<0.05.

expressed in the lens cortex of age-related cortical cataract[21]. Herein, FANCE expression was elevated in TGF-β2-disposed LECs. Also, FANCE overexpression overturned miR-26a-5p mimic-mediated impacts on LEC proliferation, apoptosis, migration, and EMT under TGF-β2 stimulation, manifesting that miR-26a-5p targeted FANCE to regulate PCO progression. Furthermore, NEAT1 sponged miR-26a-5p to regulate FANCE expression. Thus, we concluded that TGF-β2-induced NEAT1 regulated LEC proliferation, migration, and EMT through modulating FANCE expression through binding to miR-26a-5p under TGF-β2 stimulation.

In conclusion, TGF-β2 induced NEAT1 expression in LECs. Notably, TGF-β2-induced NEAT1 sponged miR-26a-5p to increase FANCE expression, thereby facilitating PCO progression, which offered a new mechanism for comprehension of PCO progression.

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Conflicts of Interest: Yu XH, None; **Liu SY,** None; **Li CF,** None.

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