Abstract. Long noncoding RNAs (lncRNAs) are associated with various diseases including cataracts. The role of lncRNA potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1 (KCNQ1OT1) on lens epithelial cell (LEC) proliferation and epithelial-mesenchymal transition (EMT) in cataracts disease remains unclear. In the present study, KCNQ1OT1 and mothers against decapentaplegic homolog (SMAD)4 expression levels were upregulated in human cataract lens posterior capsular samples and in transforming growth factor (TGF)−β2-treated SRA01/04 cells, as demonstrated by reverse transcription-quantitative polymerase chain reaction, immunohistochemical staining and western blot analyses. A further loss of function test revealed that suppression of KCNQ1OT1 inhibited the proliferation and EMT of TGF-β2-treated SRA01/04 cells. Additionally, the present study reported that increase and decrease of KCNQ1OT1 regulated SMAD4 expression, which indicated that SMAD4 may be a downstream gene of KCNQ1OT1. Finally, a constructed SMAD4 RNA interference experiment confirmed that the function of KCNQ1OT1 was to act on LEC proliferation and EMT, and this was achieved via the SMAD4 signaling pathway. The findings of the present study may provide a novel target for molecular therapy of cataracts disease.

Introduction

As one of the common age-associated diseases, cataracts is a troublesome disease and is a leading cause of non-traumatic blindness worldwide. The global progression of aging has been associated with cataracts and resulting visual impairment, which has placed a burden on society. According to an epidemiological study published in 2011, ~96% of people aged >60 were reported to exhibit varying lens opacity (1). It is well known that various morphological and functional alterations of lens cells, including disordered cell cycle, DNA damage, lens epithelial cells (LECs) excessive proliferation and abnormal epithelial-mesenchymal transition (EMT) participate in the pathological formation of cataracts (2-5). Therefore, investigation of a key regulator associated with LECs’ proliferation and EMT may considerably contribute to the development of molecular therapy for cataracts disease.

Long noncoding RNAs (lncRNAs) are macromolecules grouped under noncoding RNAs, with length usually >200 nucleotides. They extensively exhibit varied roles in numerous physiological and pathological processes, including cell proliferation, cell differentiation, apoptosis, tumorigenesis and EMT (6-8). LncRNA potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1 (lncKCNQ1OT1) is located on human chromosome 11p15.5 and is associated with the development of a wide array of diseases. Ren et al (9) reported that knockdown of KCNQ1OT1 suppresses the proliferation and invasion of A549 cells, as well as advanced cellular apoptosis of A549 cells. Jin et al (10) suggested that KCNQ1OT1 may promote cataractogenesis, which may be dependent upon microRNA-214 and activation of the caspase-1 pathway.

In the present study, the expression levels of KCNQ1OT1 were investigated in cataract tissue specimens and in a constructed cataract cell model induced by transforming growth factor (TGF)-β2. In addition, the function of KCNQ1OT1 in LEC proliferation and EMT was evaluated. The findings of the present study may provide insight into a novel contributor to the process of cataract formation.
Materials and methods

Patients and tissue samples collection. A total of 30 cases (17 female and 13 male; mean age 63.5 years, range 58-73 years) of fresh posterior lens capsule specimens with age-associated cataracts during phacoemulsification and paired fresh posterior lens capsule specimens without cataracts caused by ocular trauma during ophthalmectomy were collected at 4th People's Hospital of Shenyang between July 2015 and July 2017. Written informed consent was obtained from all patients for all clinical investigations conducted and the present study was approved by the Institute Research Medical Ethics Committee of 4th People's Hospital of Shenyang (Shenyang, China).

Cell culture and TGF-β2 intervention. Human LEC cell line SRA01/04 was purchased from the American Type Culture Collection (Manassas, VA, USA) and was cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) staining, and hematoxylin (Amresco, LLC) was used as a counterstain at room temperature for 1 min. All sections were washed in PBS 3 times, an ECL Western Blotting Substrate kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) was used for chemiluminescence detection. Immunohistochemical (IHC) staining. The procedure was carried out as previously described (12). Briefly, the collected cataract tissues were treated orderly: 4% paraformaldehyde fixation, paraffin-embedding, section (4 µm thickness), deparaffinization, a descending series of 100, 95, 80 and 70% alcohol were applied for rehydration followed by 3% hydrogen peroxide incubation for 10 min, antigen retrieval, blocking with 5% BSA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Subsequently, membranes were incubated with antibodies against SMAD4 (1:5,000; cat. no. ab40759; Abcam) and GAPDH (1:10,000; cat. no. ab128915; Abcam) at 4˚C overnight. The following day, the membranes were incubated with secondary antibodies [goat anti-rabbit IgG horseradish peroxidase (HRP), 1:2,000; cat. no. ab287915; Abcam] at room temperature for 1 h. Following washing with TBS 3 times, an ECL Western Blotting Substrate kit (cat. no. ab65623; Abcam) was used for chemiluminescence imaging with Image J software version 2X (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The procedure was carried out as previously described (10). In brief, total RNAs of tissue specimens and of the cells following different interventions were extracted by TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNAs were synthesis by PrimeScriptTM RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). qPCR was performed in a total reaction volume of 50 µl with 35 cycles (denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and extension 72°C for 5 sec) using a SYBR Premix Ex Taq II kit (TaKaRa Biotechnology Co., Ltd.) and an Applied Biosystems 7500 Fluorescent Quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of KCNQ1OT1 and SMAD4 were calculated using GAPDH as an internal control by the 2-ΔΔCt method (11). The primers used were as follows:

KCNQ1OT1 forward, 5'-TGACGAAGACGGAGACCTGG-3'; reverse 5'-CTTTTGGGGAGGAGCACAAGA-3'; SMAD4 forward, 5'-GGTGAAGATCCCCTGTC-3'; reverse 5'-CCCAACGGTAAAGAGCCTCA-3'; GAPDH forward, 5'-GACCCGTCAGGCTGAGAAC-3'; reverse 5'-TTGTTGAGACGCACAGTGGA-3';

Western blot analysis. Total protein of tissue specimens and cells were lysed by ice-cold radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, Merck KGaA). Following protein concentration quantification with a Bicinchoninic Acid protein assay kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 40 µg protein samples were subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF; Amresco, LLC, Solon, OH, USA) and then blocked by 5% BSA (Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. Subsequently, membranes were incubated with antibodies against SMAD4 (1:5,000; cat. no. ab40759; Abcam, Cambridge, MA, UK), E-cadherin (1:100; cat. no. ab76055; Abcam), fibronectin (1 µg/ml; cat. no: ab23750; Abcam) and GAPDH (1:10,000; cat. no. ab128915; Abcam) at 4°C overnight. The following day, the membranes were incubated with secondary antibodies [goat anti-rabbit IgG horseradish peroxidase (HRP), 1:2,000; cat. no. ab205718, Abcam] at room temperature for 1 h. Following washing with TBST 3 times, an ECL Western Blotting Substrate kit (cat. no. ab65623; Abcam) was used for chemiluminescence imaging with Image J software version 2X (National Institutes of Health, Bethesda, MD, USA).
an EdU detection kit (cat. no. KGA331-500; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The nuclei were observed under a fluorescent microscope at an excitation wavelength of 350 nm (Leica Microsystems GmbH, Wetzlar, Germany). Images were analyzed using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The quantitative data were expressed as the percentage of EdU-positive nuclei relative to total number of nuclei counted. In addition, a CCK-8 assay was performed as previously described (14). SRA01/04 cells (2x10^3) were cultured with DMEM supplemented with 10% FBS in a 96-well plate for 24 h and followed by transfection of ddH_2O (mock group), KCNQ1OT shRNA (KCNQ1OT shRNA) and negative control shRNA (NC shRNA group) for 24 h. At days 1, 2, 3, 4 and 5 following transfection, 10 µl CCK-8 solution was added into each well and incubated at 37 °C for 4 h. The absorbance was measured at an optical density of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All experiments were repeated in triplicate and all data from three independent experiments were expressed as mean ± standard deviation. GraphPad Prism v5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. For paired groups, the expression level of KCNQ1OT1 and SMAD4, as well as alterations in proliferative ability were analyzed by a two-tailed Student’s t-test. For the comparison of more than two groups, one-way analysis of variance was conducted followed by a Student-Newman-Keuls post hoc test. Spearman correlation analysis was performed to determine the correlation between KCNQ1OT1 and SMAD4. P<0.05 was considered to indicate a statistically significant difference.

Results

Increased KCNQ1OT1 expression levels are observed in human cataract lens posterior capsular samples and in TGF-β2 induced cataract cell models.

The present study investigated the expression levels of KCNQ1OT1 in 30 fresh specimens of posterior lens capsules with age-associated cataract and 30 paired posterior lens capsules specimens without age-associated cataracts via RT-qPCR analysis. As presented in Fig. 1A and B, KCNQ1OT1 was significantly upregulated in tissue specimens with cataract as detected by RT-qPCR, *P<0.01 vs. non-cataract group. (C) KCNQ1OT1 was elevated in SRA01/04 cells with the presence of TGF-β2 as observed via RT-qPCR, *P<0.01 vs. control group. All data were presented as the mean ± standard deviation from three independent experiments. ID, identification; KCNQ1OT1, potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF-β2, transforming growth factor-β2.

SMAD4 is overexpressed in human cataract lens posterior capsular samples and in TGF-β2 treated SRA01/04 cells. The associated effects of SMAD4, including the mediation of proliferation and EMT have been widely reported in a variety of diseases, such as cataracts (15-17). In the present study, the
expression levels of SMAD4 in collected tissue specimens and in cataract cell models induced by TGF-β2 were investigated. The results were in accordance with previous studies (17). As presented in Fig. 2A-C, the overexpression of SMAD4
was detected only in tissue specimens with cataract disease, confirmed by IHC, RT-qPCR and western blot analyses. In addition, a positive correlation between KCNQ1OT1 and SMAD4 expression in cataract tissue specimens was determined by Spearman correlation analysis (Fig. 2D). Furthermore, significant upregulation of SMAD4 expression levels were detected within SRA01/04 cells treated with TGF-β2 compared with in cells not treated with TGF-β2, as determined via RT-qPCR and western blot assays (Fig. 2E and F).

**Knockdown of KCNQ1OT1 suppresses the proliferation and EMT of TGF-β2-treated SRA01/04 cells.** As previously mentioned, the expression of KCNQ1OT1 in tissue and at the cellular levels were analyzed. The present study also investigated the effects of KCNQ1OT1 on LEC proliferation and EMT. Firstly, KCNQ1OT1 in TGF-β2 treated SRA01/04 cells was suppressed via transfection with KCNQ1OT1 shRNA, which was confirmed by RT-qPCR (Fig. 3A). Secondly, CCK-8 and EDU assays were performed to determine alterations in proliferative ability following the suppression of KCNQ1OT1 expression. As demonstrated in Fig. 3B and C, the downregulation of KCNQ1OT1 significantly suppressed SRA01/04 cell proliferation in the presence of TGF-β2. Lastly, the effects of KCNQ1OT1 on SRA01/04 cells EMT with TGF-β2 treatment were investigated. As presented in Fig. 3D, TGF-β2 induced the increase of fibronectin but the suppression of E-cadherin expression levels in SRA01/04 cells. This phenomenon was reversed by knockdown of KCNQ1OT1 (transfection of KCNQ1OT1 shRNA), suggesting that the suppression of KCNQ1OT1 expression inhibited EMT in TGF-β2-induced cataract cell models.

**KCNQ1OT1 promotes proliferation and EMT via upregulation of SMAD4.** As both KCNQ1OT1 and SMAD4 have been revealed to be involved in LEC proliferation and EMT, the association between KCNQ1OT1 and SMAD4 was investigated in the present study. The results revealed that upregulated and downregulated KCNQ1OT1 may increase or reduce the expression levels of SMAD4, respectively (Fig. 4A-C). Subsequently, RNAi was employed to determine the function of SMAD4, which may be involved in KCNQ1OT1-induced promotion of proliferation and EMT. The silencing effect of SMAD4 shRNA was firstly confirmed by RT-qPCR and western blot analyses (Fig. 4D and E). As expected, the results of the present study revealed that KCNQ1OT1-induced proliferation of SRA01/04 cells in the presence of TGF-β2 was reversed by silencing SMAD4 (co-transfection of pcDNA-KCNQ1OT1 and SMAD4 shRNA; Fig. 4F). Furthermore, the same trend was observed in the analysis of EMT, characterized by fibronectin and E-cadherin expression within SRA01/04 cells in the presence of TGF-β2 (Fig. 4G). These findings suggested that KCNQ1OT1 enhanced SRA01/04 cell proliferation and EMT via the SMAD4 signaling pathway.

**Discussion**

Cataracts are generally classified into two types of disease: Anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO). ASC is a primary cataract disease characterized by star-shaped or irregular fibrotic plaques beneath the anterior capsule, resulting in notable reduction in vision due to visual axis involvement (18). PCO, known as a secondary lens opacification, is usually caused by aberrant growth of lens epithelial cells that remain in the capsular bag following cataract surgery (19). The cellular mechanism of ASC and PCO involves the proliferation, migration and EMT of LECs, leading to the transition from epithelial cells to fibroblasts, and the production of extracellular matrix proteins (collagens I, IV and fibronectin), which finally contributes to the formation of subcapsular plaques beneath the lens anterior or posterior capsule (20). At present, increasing evidence indicates that numerous factors and pathways mediate the proliferation, migration and EMT of residual LECs, and may contribute to the pathology of age-associated cataracts (21-23). Liu and Xiao (24) reported that hypoxia promotes hypoxia inducible factor-1α and facilitates EMT via the Notch homolog 1, translocation-associated (Drosophila)/snail family transcriptional repressor 1/E-cadherin pathway in SRA01/04 cells. Zhang et al (25) revealed that the silencing of mammalian target of rapamycin significantly inhibits the proliferation and migration of NEC. In the present study, the proliferation and EMT process of cataracts were investigated at the cellular level via a TGF-β2-induced cataract cell model; KCNQ1OT1 overexpression and suppression may affect the proliferation and EMT in SRA01/04 cells in the presence of TGF-β2.

LncRNA KCNQ1OT1 is located at human chromosome 11p15.5 and is generally reported as an oncogene in a variety of cancers types, including lung adenocarcinoma, hepatocellular carcinoma and breast cancer (25-27). Aberration of KCNQ1OT1 transcription is observed at a high frequency in patients with colorectal cancers (28). Gong et al (29) demonstrated that the knockdown of KCNQ1OT1 inhibits the proliferation and migration/invasive abilities of glioma cells via microRNA-370/cyclin E2 pathway. Jin et al (10) revealed that KCNQ1OT1 expression levels are elevated in human cataract lens anterior capsular samples and in SRA01/04 cell lines treated with H2O2. In the present study, the expression levels of KCNQ1OT1 were measured in the collected cataract tissue specimens. A previous report demonstrated an elevation in KCNQ1OT1 expression levels in cataract tissue specimens (30). Ectogenic TGF-β2 is widely used in cataract-associated research, particularly in LEC-associated EMT (16,31,32). In the same manner, the present study employed TGF-β2-treated SRA01/04 cells to generate a cataract cell model. The expression levels of KCNQ1OT1 were upregulated in the induced cataract cell models, as reported in the present study, which indicated that KCNQ1OT1 may function as an initiator in the formation of cataracts. Additionally, using a constructed functional experiment, the downregulation of KCNQ1OT1 inhibits SRA01/04 cell proliferation and EMT, which are the key phases in cataract formation (19).

SMAD4 belongs to the SMAD family and is a critical intracellular mediator in the TGF-β/SMAD4 signaling pathway. The effects of SMAD4 on the mediation of proliferation and EMT have been reported to be associated with cataractogenesis. Wang et al (19) suggested that microRNA-204-5p regulates EMT during human PCO by targeting SMAD4. In a SMAD4 knockout mouse study, Li et al (33) revealed that loss of SMAD4 suppresses E-cadherin expression, but upregulates N-cadherin expression, which is associated with
congenital cataracts. In a cataract study, Nahomi et al (18) suggested that LEC cells treated with TGF-β2 present marked upregulation of SMAD4 and EMT-associated markers. In the present study, the expression levels of SMAD4 in cataracts and the function of KCNQ1OT1 on SMAD4 were investigated. The results of the present study revealed that SMAD4 was significantly upregulated in cataract tissue specimens and within induced cataract cell models. Furthermore, the results indicated that an increase and decrease of KCNQ1OT1 may correspondingly regulate SMAD4 expression levels. In addition, via antisense investigations, the present study proposed that SMAD4 was a downstream target of KCNQ1OT1. The effects of KCNQ1OT1 on cell proliferation and EMT were achieved via SMAD4 signaling. KCNQ1OT1 may promote...
Figure 4. KCNQ1OT1 promotes proliferation and EMT via the upregulation of SMAD4. (A) Elevation and suppression of KCNQ1OT1 was confirmed by RT-qPCR. **P<0.01 vs. empty vector group, ##P<0.01 vs. NC shRNA group. Elevation and suppression of KCNQ1OT1 positively regulated SMAD4 mRNA and protein expression levels as determined by (B) RT-qPCR and (C) western blotting, respectively. **P<0.01 vs. empty vector group, ##P<0.01 vs. NC shRNA group. SMAD4 expression levels were significantly knocked down by transfection of SMAD4 shRNA, as confirmed by (D) RT-qPCR (**P<0.01 vs. NC shRNA group) and (E) western blot analyses (Relative SMAD4 protein expression in SMAD4 shRNA group was 0.34 comparing with NC shRNA group, which was 1.00). (F) Upregulation of KCNQ1OT1 (pcDNA-KCNQ1OT1 group) promoted SRA01/04 cell proliferation, this effect was reduced by silencing SMAD4 expression (pcDNA-KCNQ1OT1+SMAD4 shRNA group) as detected by EdU, **P<0.01, ##P<0.01 vs. pcDNA-KCNQ1OT1 group, P>0.05. (G) Elevation of KCNQ1OT1 (pcDNA-KCNQ1OT1 group) promoted the progress of EMT (elevation of fibronectin, but suppression of E-cadherin expression levels), however the facilitative effect was decreased by silencing SMAD4 expression (pcDNA-KCNQ1OT1+SMAD4 shRNA group) as detected by western blotting, **P<0.01, #P<0.01 vs. pcDNA-KCNQ1OT1 group, ##P<0.01 vs. pcDNA-KCNQ1OT1+SMAD4 shRNA group. All data were presented as the mean ± standard deviation from three independent experiments. EdU, 5-Ethynyl-20-deoxyuridine; KCNQ1OT1, potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1; NC, negative control; shRNA, short hairpin RNA; SMAD4, mothers against decapentaplegic homolog 4; TGF-β2, transforming growth factor-β2.
cell proliferation and EMT via the regulation of SMAD4 in SRA01/04 cells.

In conclusion, the formation of age-associated cataracts is an extremely complex issue associated with several mechanisms and complex networks comprising of numerous molecules. The findings of the present study provide a novel target and insight into the pathogenesis of age-associated cataract.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

BC was responsible for the analysis and interpretation of data of the manuscript; JM and CL were responsible for statistical analysis; LZ was responsible for design and drafting of the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients for all clinical investigations conducted and the present study was approved by the Institute Research Medical Ethics Committee of 4th People’s Hospital of Shenyang (Shenyang, China).

Consent for publication

Written informed consent was obtained.

Conflict of interest

The authors declare that they have no competing interests.

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