· **Basic Research** ·

Celastrol inhibits migration, proliferation and transforming growth factor-β2-induced epithelial-mesenchymal transition in lens epithelial cells

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Abstract

● **AIM: To investigate the mechanism of celastrol in inhibiting lens epithelial cells (LECs) fibrosis, which is the pathological basis of cataract.**

● **METHODS: Human LEC line SRA01/04 was treated with celastrol and transforming growth factor-β2 (TGF-β2). Wound-healing assay, proliferation assay, flow cytometry, real-time polymerase chain reaction (PCR), Western blot and immunocytochemical staining were used to detect the pathological changes of celastrol on LECs. Then, we cultured Sprague-Dawley rat lens in medium as a semi-*in vivo* model to find the function of celastrol further.**

● **RESULTS: We found that celastrol inhibited the migration of LECs, as well as proliferation (*P*<0.05). In addition, it induced the G2/M phase arrest by cell cycle-related proteins (*P*<0.01). Moreover, celastrol inhibited epithelial-mesenchymal transition (EMT) by the blockade of TGF-β/Smad and Jagged/Notch signaling pathways.**

● **CONCLUSION: Our study demonstrates that celastrol could inhibit TGF-β2-induced lens fibrosis and raises the possibility that celastrol could be a potential novel drug in prevention and treatment of fibrotic cataract.**

● **KEYWORDS:** lens; cataract; fibrosis; transforming growth factor-β2; celastrol

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INTRODUCTION

Cataract is the world's number one blinding eye disease[1]. At present, the treatments of cataract are cataract phacoemulsification and Nd:YAG laser capsulotomy. These surgeries are mature, but the complications couldn't be avoided among a large number of patients, which brings a heavy economic burden to society[2]. Lens fibrotic disorder, including anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO), is the main cause of cataract. This study intends to explore the pathogenesis of fibrotic cataract and bring new directions for the prevention and treatment of cataract.

ASC and PCO are fibrotic cataract sharing a similar pathological process[3-4]. ASC is a primary cataract, which is caused by *in situ* proliferation, abnormal fibrosis of lens epithelial cells (LECs)[5]. PCO is a common complication of cataract surgery. It is caused by postoperative residual LECs migrating to the posterior capsule, undergoing abnormal fibrosis. Then, the posterior capsule begins to shrink and be cloudy[6]. Pathological epithelial-mesenchymal transition (EMT) plays an important role in the development of fibrotic cataract. During EMT process, LECs lose their original epithelial morphology, gradually elongate to form mesenchymal-like cells and secrete extracellular matrix (fibronectin, collagens IV). At the molecular level, the expression of EMT marker (α-smooth muscle actin) has also increased[4]. EMT is affected by many growth factors and signaling pathways. Transforming growth factor-β2 (TGF-β2) is the main component in the aqueous humor and the most important growth factor in the occurrence of EMT[7]. Therefore, suppressing the migration, proliferation and TGF-β2 induced EMT maybe an effective measure.

Celastrol is an active compound extracted from the roots of Tripterygium wilfordii. It has a wide range of biological functions, including anti-oxidant, anti-inflammatory, anti-tumor and immunomodulatory[8]. Recently, studies have also found that celastrol can reduce pain and cartilage damage caused by osteoarthritis[9]. Moreover, it can protect myocardium from ischemia-reperfusion injury[10], help improving learning and memory[11]. Many studies have confirmed that celastrol can inhibit the proliferation, migration and EMT of tumor

cells[12-13]. For example, it can inhibit EMT of lung cancer cells through TGF-β/Smad signaling pathway[14]. However, the research of celastrol on the eye is mainly confined to the retina. It can protect the retinal ganglion cell from degeneration caused by high intraocular pressure[15]. Also, it is helpful to the treatment of optic neuritis and retina experiencing light damage by inhibiting oxidative stress and inflammation[16]. Moreover, celastrol can regulate the innate immune response of retinal pigment epithelial cells through the NF-κB and Hsp70[17]. However, the role of celastrol in lens fibrosis is still unclear. This study first confirms that celastrol could inhibit the fibrosis of LECs induced by TGF-β2. It can significantly inhibit the migration and proliferation of LECs and cause cell cycle arrest. Moreover, EMT in LECs is inhibited by inactivation of the classical TGF-β2/Smad. Also, celastrol could regulate the Jagged/Notch pathways. In summary, this study demonstrates that celastrol could be a new drug for the treatment of fibrotic cataract in the future.

MATERIALS AND METHODS

Reagents and Antibodies Celastrol (≥98%) was purchased from Sigma-Aldrich. Recombinant human TGF-β2 was purchased from R&D SYSTEMS. Antibodies against cdc2 (polyclonal), cycling B1 (monoclonal), p-Smad2/3 (monoclonal), Smad2/3 (polyclonal), Notch1 (monoclonal), Notch3(monoclonal), Jagged1(monoclonal), GAPDH (monoclonal) were purchased from Cell Signaling Technology. α-SMA (polyclonal), Col IV (polyclonal) and FN (polyclonal) were purchased from Abcam.

Cell Culture and Treatment The human LEC line SRA01/04 was provided by professor Fu Shang from Nutrition and Vision Research Laboratory, Tufts University (Boston, MA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Life Technologies) at 37°C in humidified atmosphere with 5% CO_2 and dissociated with 0.25% trypsin-0.02% EDTA solution (Gibco, Life Technologies). TGF-β2 was dissolved in 1% Albumin from bovine serum (BSA) at a stock concentration of 20 mmol/L. Celastrol was dissolved in dimethyl sulfoxide at a stock concentration of 44 mmol/L. LECs were treated with 5 ng/mL TGF-β2 and various concentrations of celastrol at different time points.

Wound-Healing Assay LECs were seeded in the six-well plate. When the cell density of each well reached 90% confluence, cells were starved by DMEM containing 1% FBS overnight. Then LECs were scratched with a 200 µL pipette tip. Wounded monolayers were washed with phosphate buffer saline (PBS) to remove detached cells, and fresh medium was added to each well. The wound in each well was photographed at 0, 4, 8 and 12h. The length of the remaining wound in each image was measured 3 times using Zeiss software.

Cell Proliferation Assay LECs were seeded in the 96-well plate in triplicate. When the cell density of each well reached 50% confluence, we pipeted 20 µL CellTiter 96® AQ_{ueous} One Solution Reagent (Promega) into each well containing 100 µL samples and incubated the plate at 37°C for 1h in a humidified, 5% CO_2 atmosphere. Then, we recorded the absorbance at 490 nm by 96-well plate reader.

Cell Cycle Analysis Cells were treated with increasing concentrations of celastrol for 24h. After harvested and fixed in cold 70% ethanol overnight at -20°C, cells were washed by PBS and stained by propidium iodide solution (Thermo Scientific) for 30min at dark place. Then, the cells were measured by flow cytometry (BD Biosciences, San Jose, CA, USA).

Western Blot Analysis Cells were lysed in radio immunopreci pitation assay (RIPA) buffer and protein was mixed with 5×SDS sample buffer. An equal amount of total protein from each group was separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk for 1h at room temperature and incubated overnight at 4°C with different primary antibodies. Then, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Finally, the protein bands were detected by chemiluminescence detection reagents.

Real-time Polymerase Chain Reaction Analysis Total RNA was isolated from LECs according to the manufacturer's protocol (QIAGEN). cDNA was synthesized with reverse transcription kit (Thermo Scientific). For real-time polymerase chain reaction (PCR), the SYBR (Roche) was used according to the manufacturer's protocol with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Immunofluorescence LECs were fixed by 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in 3% BSA for 1h. Then LECs were incubated overnight at 4°C with different primary antibodies. Next day, the LECs were incubated with Alexa Flour 488 or Alexa Flour 555-conjugated secondary antibodies for 1h at dark place. The nuclei were stained by DAPI, and the images were captured by fluorescence confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

Lens Culture and Treatment Animals included in this study were approved by the Animal Use and Care Committee of Zhongshan Ophthalmic Center (Guang Zhou, People's Republic of China) and has been performed in accordance with the ARVO statement. Lens from 250 g Sprague-Dawley rats were cultured in DMEM containing 1% FBS, 5 ng/mL TGF-β2 to induce turbidity. Then, celastrol (1 µmol/L) was added to the medium from one of them randomly. Medium was changed every two days. Then, they were photographed and fixed on the seventh day.

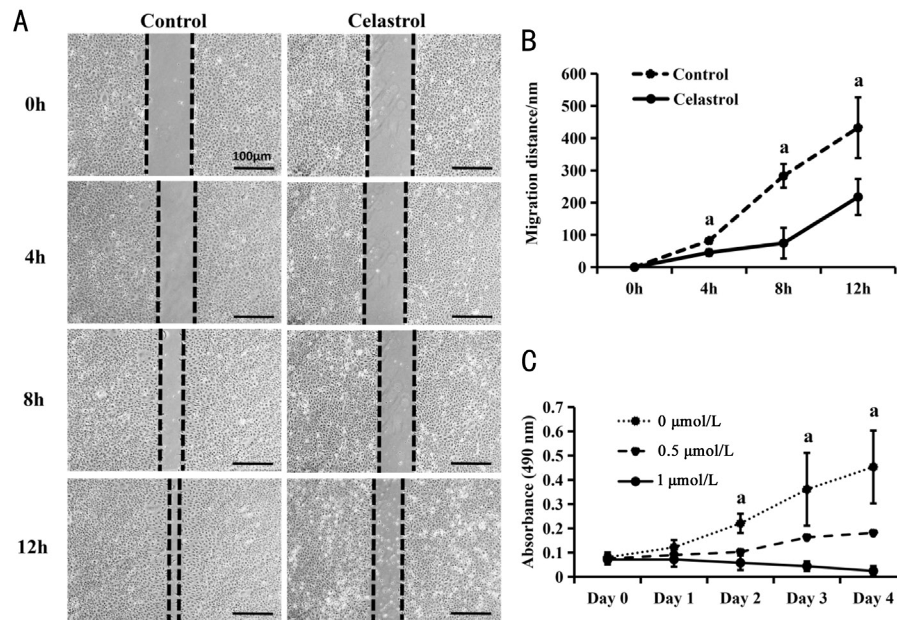

Figure 1 Celastrol inhibited the migration and proliferation of LECs A: LECs were treated with or without celastrol (1 μmol/L) for 12h. Pictures were captured every 4h and the black lines represented the wound edges (scale bar 100 μm); B: The distance of LECs migration was measured at different time points; C: LECs were treated with 0, 0.5, 1 μmol/L celastrol for 0, 1, 2, 3, 4d. Data were the mean±SD from an experiment which was repeated three times. $^aP<0.05$ *vs* the control group.

Statistical Analysis Each experiment was repeated at least three times. All data were expressed as mean±SD and analyzed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Two tailed Student's *t*-test was used to calculate the *P* value between the groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

Celastrol Inhibited the Migration and Proliferation of LECs The migration and proliferation of residual LECs is the original cause of PCO. To evaluate the effect of celastrol on the migration of LECs, we performed wound-healing assay. The distance of cell migration was observed by an inverted phase contrast microscope at 0, 4, 8 and 12h (Figure 1A-1B). In the control group, the migration of LECs were 0.0 nm, 82.1±7.7, 283.3±36.5, 432.3±94.1 nm during these four time points. In the celastrol group, the migration of LECs were 0.0 nm, 45.3±9.7, 74.6±47.5, 217.8±55.8 nm, respectively. In the wound-healing assay, we found that the inhibition of LECs migration increased with the increasing concentration of celastrol ($P<0.05$ *vs* the control group). Proliferation of LECs plays an important role during the pathogenesis of fibrotic cataract. In cell proliferation assay, the absorbance value at 490 nm in control group were 0.08±0.02, 0.12±0.03, 0.22±0.04, 0.36±0.15, 0.45±0.15 for 0, 1, 2, 3, 4d, respectively. For the celastrol group (1 μmol/L), they were 0.07±0.02, 0.07±0.03, 0.06±0.03, 0.04±0.02, 0.02±0.02, respectively. We found cell proliferation was attenuated under the concentration-dependent effect of celastrol and almost completely inhibited in the 1 μmol/L group after 2d ($P<0.05$ *vs* the control group; Figure 1C).

Celastrol Induced G2/M Phase Arrest by Regulating Cell Cycle-related Proteins Cell cycle progression has influence on cell proliferation. Then, we further examined the effect of celastrol on cell cycle. Distribution of cell cycle in LECs treated with different concentration of celastrol was detected. The percentages of LECs in G2 phase were 14.8%±3.1%, 19.6%±3.6%, 27.6%±2.1% for the control, 1, 2 μmol/L groups ($P<0.01$ *vs* the control group; Figure 2A-2B). To elucidate the preliminary mechanisms, we detected the expression of cell cycle-related proteins. As shown in Figure 2C and 2D, celastrol decreased the expression of cdc2 and cyclin B1. All in all, these results suggested that celastrol might inhibit the proliferation of LECs by inducing G2/M phase arrest.

Celastrol Inhibited TGF-β2-induced EMT in LECs by Suppressing the Phosphorylation of Smad2/3 TGF-β2 is crucial in the formation of fibrotic cataract[4]. To detect the role of celastrol in LECs EMT, we used TGF-β2 to establish an *in vitro* model. TGF-β2 increased the expression of FN and Col IV at mRNA levels. On the contrary, celastrol abrogated their upregulation ($P<0.05$ *vs* the TGF-β2 group; Figure 3A). Then, the expression of FN protein was detected by western blot, and its trend is the same as mRNA levels ($P<0.01$ *vs* the TGF-β2 group; Figure 3B-3C). Moreover, immunoflourescence assay was used by confocal microscopy to further detect the expression of FN, Col IV and α-SMA. The results shown that TGF-β2 up-regulated their expression compared with the control group. However, FN, Col IV and α-SMA were significantly down-regulated after treatment with celastrol (Figure 3D). TGF-β2 can activate the Smad pathways to induce

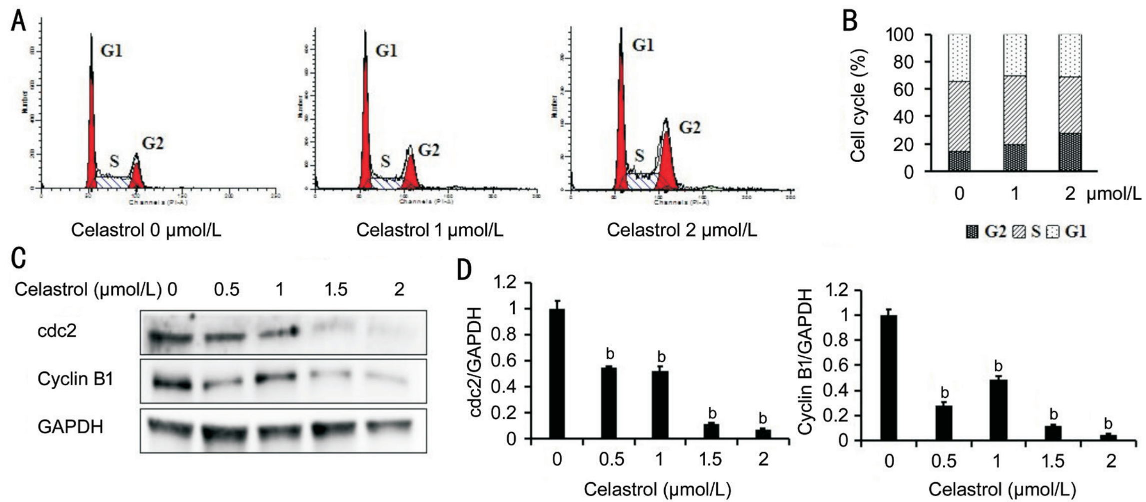

Figure 2 Celastrol induced G2/M phase arrest by cell cycle-regulated proteins A, B: LECs were treated with 0, 1, 2 μmol/L celastrol for 24h. Cell cycle was detected by flow cytometry. The percentage of LECs in G2 phase was calculated; C: LECs were treated with 0, 0.5, 1, 1.5, 2 μmol/L celastrol for 48h. The protein expression levels of cdc2 and cyclin B1 were detected by Western blot analysis. D: Quantitative analysis of protein levels were detected three times. Data were derived by three independent experiments. $^bP<0.01$ *vs* the control group.

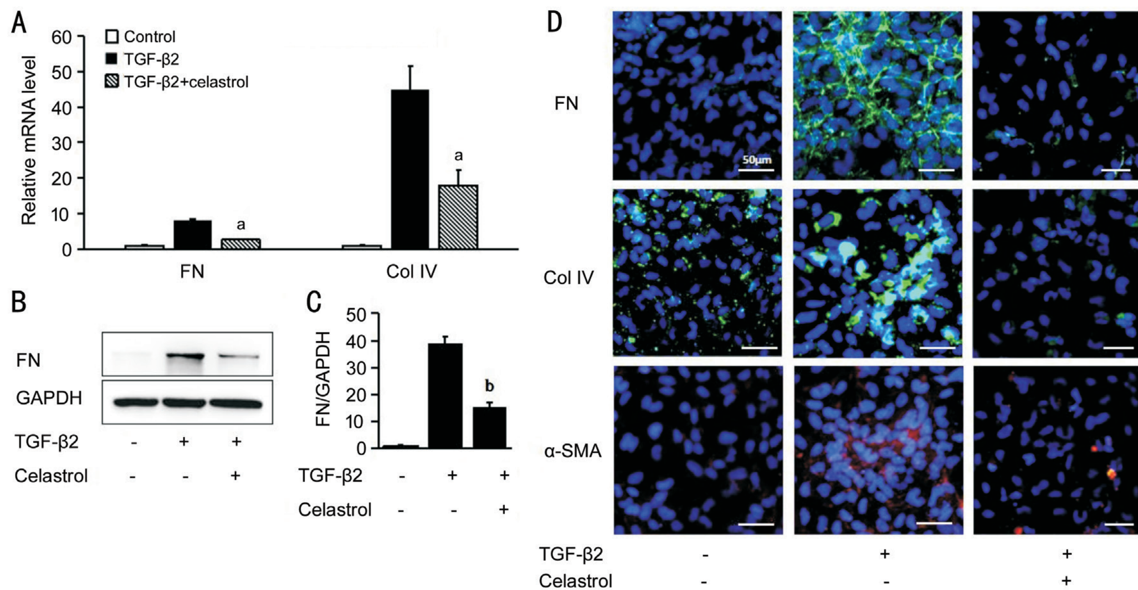

Figure 3 Celastrol inhibited TGF-β2-induced EMT in LECs LECs were cultured in the absence or presence of TGF-β2 (5 ng/mL) with celastrol (1 μmol/L) for 24-48h. A: The mRNA expression level of FN and Col IV in LECs was detected by real-time PCR; B, C: The protein expression level of FN was determined by Western blot analysis; D: Immunofluorescence analysis of FN (green), Col IV (green) and α-SMA (red) were observed using confocal microscopy (scale bar 50 μm). Data were derived by three independent experiments. $^aP<0.05$, $^bP<0.01$ *vs* the TGF-β2 group.

EMT, and contribute to the formation of fibrotic cataract[18]. Then, we detected the impact of celastrol on the activation of Smad2/3. As shown in Figure 4B and 4C, TGF-β2 activated Smad2/3 by phosphorylation. On the contrary, co-treatment with celastrol inhibited the phosphorylation of Smad2/3 in LECs. To sum up, these results indicated that TGF-β2-induced EMT could be inhibited by celastrol by suppressing phosphorylation of Smad2/3.

Celastrol Regulated the Jagged/Notch Signaling Pathway in LECs The Jagged/Notch signaling pathway plays an important role in embryonic development, fibrotic diseases and cancer metastasis[19]. To investigate the effect of celastrol on LECs fibrosis further, we explored the impact of celastrol on the Jagged/Notch signaling pathway. The TGF-β2 group significantly increased the expression of Notch2, Notch3, Jagged1 at mRNA levels (Figure 4A) and Notch1, Notch3, Jagged1 at protein levels (Figure 4B-4D). However, celastrol could attenuate the TGF-β2-induced upregulation in LECs. Altogether, these results suggested that celastrol could down-regulate the Jagged/Notch signaling pathway.

Celastrol Suppressed TGF-β2-induced ASC To further investigate whether celastrol could prevent TGF-β2-induced

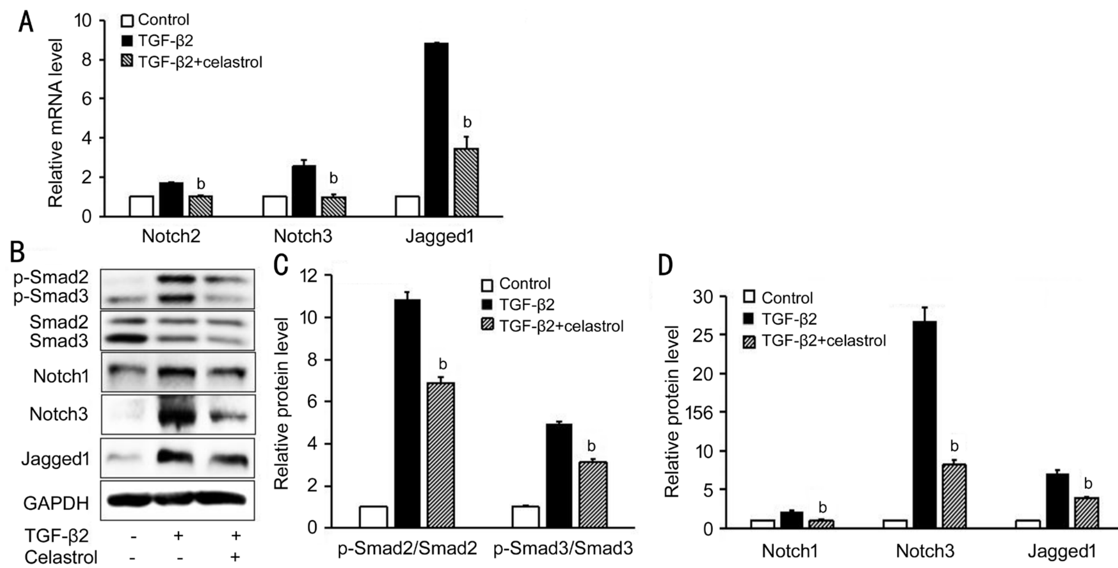

Figure 4 Celastrol inhibited TGF-β2 signaling pathway by suppressing the phosphorylation of Smad2/3 and downregulating the Jagged/Notch signaling pathway LECs were cultured in the absence or presence of TGF-β2 (5 ng/mL) with celastrol (1 μmol/L) for 24-48h. A: The mRNA expression levels of Notch2, Notch3 and Jagged1 in LECs were detected by real-time PCR; B-D: The protein expression levels of p-Smad2/3, Smad2/3, Notch1, Notch3 and Jagged1 were determined by Western blot analysis. Data were derived by three independent experiments. $^{b}P<0.01$ *vs* the TGF-β2 group.

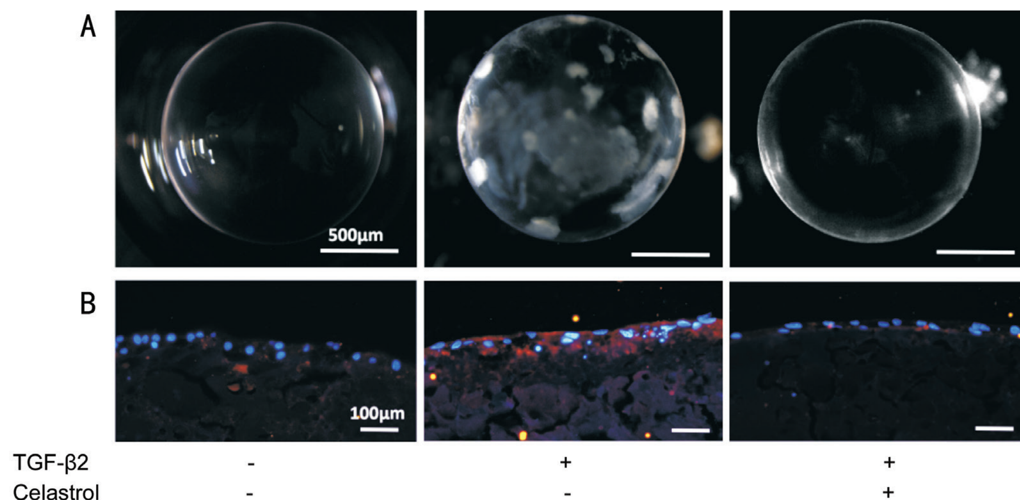

Figure 5 Celastrol suppressed TGF-β2-induced ASC in the whole lens culture semi-*in vivo* mode LECs were cultured in the absence or prescence of TGF-β2 (5 ng/mL) with celastrol (1 μmol/L) for a week. A: The morphology of lens was captured by dissecting microscope (scale bar 500 μm); B: The staining of parafin section of α-SMA (red) was captured by confocal microscopy (scale bar 100 μm).

fibrosis in lens, we cultured the whole lens in semi-*in vivo* model. Lens had obvious anterior opacity treated with TGF-β2 (Figure 5A). As shown in Figure 5B, LECs treated with TGF-β2 contained the accumulation of α-SMA. In contrast, the lens co-cultured with TGF-β2 and celastrol remained transparent and did not have the accumulation of α-SMA. Taken together, these data indicated that celastrol could abrogate TGF-β2-induced ASC in Sprague-Dawley rat lens.

DISCUSSION

Celastrol is a natural product with a variety of biological activities. It can inhibit the EMT of many tumor cells[20], but its role in fibrotic cataract is not clear. Fibrotic cataract shows an increase in cell migration, proliferation and pathological changes of EMT[21]. Therefore, we explore from these aspects to find the pathomechanisms of fibrotic cataract. In this study, we first discovered that celastrol could inhibit TGF-β2-induced LECs fibrosis. We demonstrated that celastrol could 1) inhibit the migration and proliferation of LECs; 2) cause G2/M phase arrest; 3) inhibit the process of EMT by inactivating Smad2/3 phosphorylation; 4) regulate the Jagged/Notch signaling pathway.

During the process of EMT, migratory mesenchymal cell types were produced, which have the ability of enhanced motility and proliferation[22-23]. Increased LECs migration and proliferation is the primary cause of fibrotic cataract. There are many factors that affect cell proliferation, and cell cycle arrest has a close

relationship with it[24]. The progression of cell cycle depends on the coordination of cell cycle-related proteins and signaling pathways, which ultimately affect cell proliferation[25]. For example, cdc2 binds to cyclin B. The complex promotes cell cycle progression from G2 to M[26]. Celastrol can cause G2/M arrest in a variety of tumor cells[27-28], but its function on LECs is unclear. We found that it could reduce the expression of cyclin B1 and cdc2. Therefore, this may be another mechanism of celastrol inhibiting the proliferation of LECs.

EMT plays an important role in LECs fibrosis. TGF-β2 signaling pathway is crucial in many fibrotic diseases. It can activate phosphorylation of Smad2/3[29]. At present, more and more studies have found that the Jagged/Notch signaling pathway plays an important role in fibrotic diseases, such as fibrotic cataract[30-31]. We found that the phosphorylation of Smad2/3 was increased when LECs were induced by TGF-β2, but the phosphorylation level was decreased treated with celastrol. In addition, it could inhibit the Jagged/Notch signaling pathway. During the development of EMT, there is a change in related markers, such as FN, Col IV and α-SMA. We found celastrol reduced the expression of FN, Col IV and α-SMA induced by TGF-β2. Therefore, we confirm that celastrol could inhibit the EMT of LECs.

The lens consists of LECs, cortex and capsular membranes. It is a good model for studying LECs. Many studies have shown that TGF-β2 induces lens to undergo morphological and molecular changes which are similar to fibrotic cataract[32]. In order to verify the function of celastrol on lens fibrosis, we cultured the whole Sprague-Dawley rat lens with TGF-β2 to simulate the *in vivo* environment. We found that lens treated with celastrol was more transparent and the expression of α-SMA was decreased. Through these results, we find that celastrol can keep the transparency of lens and inhibit the formation of cataract.

In summary, our study confirms that celastrol can inhibit the fibrosis of LECs for the first time. Celastrol not only inhibits the migration and proliferation of LECs, but also leads to cell cycle arrest. At the same time, celastrol inhibits EMT by inactivating TGF-β/Smad and regulate the Jagged/Notch signaling pathways. Our study provides a new direction for the prevention and treatment of fibrotic cataract, and it could benefit a majority of cataract patients in the future.

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