

Klotho attenuates epithelial-mesenchymal transition of retinal pigment epithelial cells in subretinal fibrosis by suppressing the ERK1/2 and Wnt/ β -catenin signaling pathways

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Abstract. Retinal pigment epithelial (RPE) cells undergoing epithelial-mesenchymal transition (EMT) are a key factor in promoting the progression of subretinal fibrosis. The klotho protein and gene exert anti-fibrotic effects in multiple fibrotic diseases. However, the mechanisms involved in the role of klotho are unclear in subretinal fibrosis. The aim of the present study was to explore the effects of klotho on subretinal fibrosis induced by laser photocoagulation in mice and EMT induced by TGF- β 1 in RPE cells and the underlying molecular mechanisms. *In vitro*, klotho overexpression or knockdown was performed in ARPE-19 cells (adult retinal Pigment Epithelial-19), then TGF- β 1 treatment was applied. Using western blotting, expression of epithelial markers (zonula occludens-1), mesenchymal signs (α -smooth muscle actin, α -SMA, N-cadherin, N-cad and collagen I), and the ERK1/2 and Wnt/ β -catenin signaling pathways were assessed. The proliferative ability of ARPE-19 cells was examined by CCK-8 and EdU test, and the migratory ability was examined by wound healing and Transwell assays. Furthermore, to explore the underlying molecular pathway of klotho overexpression, RNA-sequencing (seq) was performed. *In vivo*, photocoagulation was used to induce subretinal fibrosis in mice, which occurs as a result of choroidal neovascularization (CNV), then recombinant mouse klotho protein was administered intravitreally. Upregulation of epithelial and downregulation of mesenchymal markers demonstrated that klotho overexpression prevented TGF- β 1-induced EMT; klotho knockdown resulted in the opposite effects. Additionally, klotho overexpression suppressed cell proliferation and migration and attenuated ERK1/2 and Wnt/ β -catenin signaling activated by

TGF- β 1. RNA-seq results demonstrated that several signaling pathways, including cellular senescence and the TNF signaling pathway, were associated with anti-fibrotic effects of klotho overexpression. *In vivo*, subretinal fibrotic areas were attenuated following klotho treatment in laser-induced CNV lesions, as illustrated by immunofluorescence and Masson staining of the mouse eyes. Western blotting results that the protein levels of mesenchymal markers were significantly downregulated and those of epithelial markers were upregulated. In summary, the present study suggested that klotho may have therapeutic value in management of fibrotic vitreoretinal disorders such as subretinal fibrosis.

Introduction

Age-related macular degeneration (AMD) contributes to severe vision impairment or loss of vision in the elderly. It is predicted that 288 million individuals worldwide will have AMD by 2040 (1). Its wet form, also termed neovascular age-related macular degeneration (nAMD), is responsible for $\geq 80\%$ of all cases of vision loss and blindness caused by AMD worldwide (2,3). Choroidal neovascularization (CNV) and development of subretinal fibrosis beneath the macula are the primary pathological features of AMD (4,5). Currently, anti-VEGF treatment is the most widely applied therapeutic strategy for CNV but subretinal fibrosis can still develop (6). The retinal pigment epithelium and photoreceptors are permanently destroyed by subretinal fibrosis, which is associated with poor visual prognosis for patients with nAMD.

Fibrosis refers to overaccumulation of extracellular matrix (ECM) released by myofibroblasts, resulting from an exaggerated response to several triggers, such as inflammation, injury and aging. Subretinal fibrosis is seen predominately in nAMD (7). As there are no resident myofibroblasts in healthy retina, the myofibroblasts in AMD are derived from various myofibroblast precursor cell types, including macrophages (8) and retinal pigment epithelium (RPE) (9) and Müller cells (10). These cellular components contribute to subretinal fibrosis in AMD by interacting with inflammatory cytokines and growth factors through their trans-differentiation into myofibroblasts (11). RPE cells are a key source of myofibroblasts by transdifferentiating to the mesenchymal phenotype via epithelial-mesenchymal transition (EMT) (12). When EMT occurs in RPE cells, the expression of epithelial cell

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markers is downregulated, while mesenchymal cell markers are upregulated. RPE cells acquire fibrotic properties, such as morphological transformation, ECM remodeling and increased cell proliferation and migration (7).

Retinal fibrosis can develop in numerous types of proliferative vitreoretinal disease, such as nAMD (7), proliferative diabetic retinopathy (13), retinopathy of prematurity (14) and proliferative vitreoretinopathy (15,16). Age is a primary risk factor for fibrosis (17), however, the precise molecular mechanism causing or alleviating fibrosis remains unclear and effective treatments for curing or preventing fibrosis are currently lacking.

The anti-aging gene klotho was first identified and analyzed in 1997 (18). Klotho is also termed α -klotho to distinguish it from β - and γ -klotho, the two other members of the klotho family (19). Klotho is here used to refer to α -klotho. Klotho protein has been used to treat fibrotic diseases such as atrial fibrillation (20), cardiovascular fibrosis (21), diabetic kidney disease (22) and fibrosis of the peritoneum (23) or the lung (24). Klotho protein is observed in the healthy retina and klotho deficiency leads to a reduction in retinal function as measured by electroretinography (25). Further study has revealed the presence and protective effects of klotho in RPE cells and a decrease in klotho expression may lead to age-associated retinal pathologies (26). According to our previous research, recombinant human klotho protects the RPE against damage caused by oxidative stress (27). Another study reported that klotho inhibits RPE degeneration, suggesting that it may have therapeutic value for the dry type of AMD (28). Furthermore, it has been previously shown that klotho is involved in the hypoxia-inducible factor (HIF)-1 α -induced fibrotic process in the retina (29). Hypoxia-induced P53 activation results in upregulation of expression of microRNA (miRNA/miR)-34a, which inhibits the expression of klotho and induces EMT in ARPE-19 cells (adult Retinal Pigment Epithelial Cell Line-19). Additionally, blocking the HIF-1 α /p53/miRNA-34a/klotho axis can inhibit development of subretinal fibrosis *in vivo* (29). Nonetheless, the exact mechanism by which klotho modulates subretinal fibrosis in nAMD remains unclear.

In the present study, the mechanism by which klotho attenuates the EMT of ARPE-19 cells and inhibits subretinal fibrosis in a mouse model was examined.

Materials and methods

Cell culture. ARPE-19 cells were acquired from American Type Culture Collection. Cells were cultured in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.), containing 1% penicillin/streptomycin (HyClone, Cytiva.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained at 37°C with 5% CO₂ for all cultures/incubation and the culture media was changed every 3 days.

Lentiviral infection. Lentiviral plasmids (Hanbio Biotechnology Co., Ltd.) were used for human full-length klotho-mediated overexpression and short hairpin (sh) RNA-mediated klotho knockdown, according to the manufacturer's protocol. Lv-NC was the negative control for overexpression; sh-NC was the negative control for knockdown. Lentiviral vectors carrying shRNA targeting

the klotho gene (Table SI) were constructed to knock down klotho expression. For lentivirus packaging, 5x10⁶ 293T cells (Hanbio Biotechnology Co., Ltd.) were cultured in a 12-well plate and transfected with a plasmid system once at 30-50% confluence to produce recombinant or negative lentiviruses. The 2nd generation plasmid system included 10 μ g pSPAX2 (packaging plasmids), 5 μ g pMD2G (envelope plasmids), and 10 μ g lentivirus plasmid containing exogenous target genes or shRNA. The duration of transfection was 16 h, then culture media were replaced with fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and the culture was continued at 37°C, 5% CO₂. After 48 h, the media were collected. For lentivirus transduction, ARPE-19 cells were cultured in 6-well plates at 37°C for 24 h. Once at 30-50% confluence, cells were transduced with lentivirus (MOI=5) and polybrene (2 μ g/ml) at 37°C for 24 h. The culture medium carrying the lentivirus was removed and cells were cultured in fresh medium for 48 h. The lentiviral-infected cells were passaged and cultured in the presence of 1 μ g/ml puromycin to select successfully transfected cells.

Cell counting kit (CCK)-8 assay. A total of 3x10³ cells were plated on 96-well plates (100 μ l culture medium/well) and then cultured for 24 h. Next cells were cultured without serum for 24 h, then treated with 10 ng/ml recombinant human TGF- β 1 (cat. no. HY-P7118, MedChemExpress) in DMEM/F12 with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 24, 48 and 72 h to induce EMT. Medium was replaced with 10 μ l CCK-8 solution (GLPbio) in 90 μ l fresh medium for 2 h. Finally, the absorbance at 450 nm was detected using an ELISA reader (Thermo LabSystems, Inc.).

EdU assay. Cells were cultured at 37°C for 24 h on 6-well plates (1.2x10⁵ cells in 2 ml medium/well), then cells were deprived of serum at 37°C for 24 h, treated with 10 ng/ml TGF- β 1 at 37°C for 24 h, exposed to 10 μ M EdU in 10% FBS DMEM/F12 at 37°C for 2 h, treated with 4% paraformaldehyde at room temperature, washed three times with 3% BSA (cat. no. 4240GR005, Biofrox GmbH Co., Ltd.) for 5 min each, exposed to 0.3% Triton-X100 in PBS at room temperature for 15 min, then washed with 3% BSA again. Cells were incubated for 30 min using a 500 μ l prepared click reaction solution (BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 488 (cat. no. C0071S, Beyotime Institute of Biotechnology) following the manufacturer's instructions. Cells were washed three times with 3% BSA, incubated at room temperature for 10 min with Hoechst 33342 (1:1,000 in PBS), washed three times with BSA and then examined using a fluorescence microscope (magnification, x200, Nikon Corporation).

Scratch assay. Cells were cultured for 24 h on 6-well plates (8x10⁵ cells in 2 ml medium/well). Following serum deprivation for 24 h, cells were exposed to 10 ng/ml TGF- β 1 for 48 h. A scratch was created in the monolayer of cells at 95% confluence using a thin 200- μ l pipette tip. The cells were treated with TGF- β 1 in DMEM/F12 without serum for 48 h. The wound area was assessed at 0 and 48 h after scratching using an inverted light microscope (magnification, x200, Nikon Corporation). Wound closure was quantified using ImageJ 1.53c (National Institutes of Health).

Transwell migration assay. Transfected cells were administered with 10 ng/ml TGF- β 1 for 48 h. A total of 4×10^4 cells in 200 μ l serum-free medium was added to the upper chamber of a Transwell insert in a 24-well plate and 1 ml 20% FBS-containing medium was added to the lower chamber. Cells were incubated at 37°C for 24 h. Cells remaining in the upper chamber were wiped away and cells that had migrated were stained with 0.1% crystal violet at room temperature for 15 min and imaged using an inverted light microscope (magnification, x200, Nikon Corporation). Analysis was performed using ImageJ.

RNA sequencing (-seq) and analysis. Transfected cells were lysed using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and stored in liquid nitrogen. RNA-seq and analysis were performed by IGENEBOOK Biotech Corporation. Total RNA was extracted using RNAprep Pure kit (cat. no. DP432, TIANGEN Biotech Co., Ltd.), following the manufacturer's instructions. RNA samples were assessed for their integrity using Qsep1 instrument (Bioptic, Inc). To construct RNA libraries with the VAHTS mRNA-seq V3 Library Prep Kit for Illumina (Nova seq 6000, cat. no. PE150), 10 pM of total RNA was used (30). The procedure included polyA-selected RNA extraction, RNA fragmentation, random hexamer primed reverse transcription, and 150nt paired-end sequencing by Illumina Novaseq 6000 reagent kit (300 cycles; cat. no. 20028312; Illumina Inc.). Genes with log2 (fold-change) >1 and false discovery rate (FDR) <0.05 between (Lv-NC and Lv-klotho) were considered differentially expressed genes (DEGs) with edgeR (31). GO (32) (Gene Ontology, <http://geneontology.org/>) and KEGG (33) (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>) enrichment analyses were implemented to analyze DEGs using ClusterProfiler in R package (version 4.2.0) (32). GO and KEGG enrichment analysis were calculated using hypergeometric distribution with a q value cutoff of 0.05. R package was also employed to perform Pearson's correlation coefficient analysis and principal component analysis and to create the volcano plots and heatmap of DEGs.

Animals. A total of 65 mice (male, C57BL/6J, age, 6-8 weeks; weight, 20-25 g) was obtained from Kunming Medical University Laboratory Animal Center (Kunming, China) and raised in a specific-pathogen-free facility. Mice were housed in cages with a 12/12-h light/dark cycle at temperatures of 18-22°C and humidity of 50-60% and had free access to water and food. The Animal Experiment Ethics Review Committee of Kunming Medical University approved the experimental protocol (approval no. kmmu20221787), and all animal studies were performed in compliance with Association for Research in Vision and Ophthalmology guidelines (34).

Establishment of the laser-induced subretinal-fibrosis model. The laser-induced subretinal-fibrosis model was induced as described previously (35). Tropicamide-phenylephrine eye drops (0.5%, Santen Pharmaceutical Co., Ltd.) were applied topically to dilate pupils. Sodium pentobarbital (Millipore Sigma) was administered intraperitoneally (70 mg/kg body weight) to anesthetize mice for laser photocoagulation and intravitreal injection. Proparacaine hydrochloride (0.5%, Alcon

Laboratories, Inc.) was used for local corneal anesthesia. Eyes received laser burns (250 mW, 100 msec duration, 100 μ m spot size, 2-3 papilla diameters surrounding the optic disc) using a 532 nm YAG laser (Topcon Corporation) with a slip-lamp delivery system. A total of 4-6 laser burns were made in each eye for immunofluorescence and 20 laser burns were made for western blotting. Only laser burns where bubbles appeared right after the laser induction were included in the study. Eyes were excluded if there were hemorrhages.

Intravitreal injection. Recombinant mouse klotho protein (cat. no. 1819-KL, R&D Systems, Inc.) or PBS was administered intravitreally 3 days after photocoagulation. Pupil dilation and animal anesthesia were performed as aforementioned. Following the creation of an incision with a 30-gauge needle at the limbus, 1 μ l 10 or 20 nM recombinant klotho protein or an equivalent volume of PBS was injected into the vitreous cavity using a 33-gauge microneedle (Hamilton). The dose was determined based on a previous study (28). One drop of antibiotic ointment was applied after the injection. Eyes were collected for analysis 4 days after the intravitreal injection. Mice were euthanized by cervical dislocation following being intraperitoneally injected with 200 mg sodium pentobarbital/kg body weight.

Western blotting. Total protein from ARPE-19 cells or RPE-choroid-sclera complexes was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitor cocktail (Beijing Solarbio Science & Technology Co., Ltd.). RPE-choroid-sclera complexes were homogenized using a freezing grinder. Lysate from cells or the RPE-choroid-sclera complex was centrifuged at 12,000 x g at 4°C for 15 min. BCA kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to measure the protein concentration. Proteins were then denatured using SDS-PAGE loading buffer (Beijing Solarbio Science & Technology Co., Ltd.) and stored at -80°C. A total of 20 μ g protein/lane was loaded on 10% SDS-PAGE, and then transferred to PVDF membranes. The membranes were blocked with 5% BSA for 2 h at room temperature. Membranes were incubated with the primary antibodies at 4°C overnight followed by incubation with secondary antibody at room temperature for 1 h. Signals were developed using enhanced chemiluminescent kit (cat. no. BL520A, LABSELECT Co., Ltd.) and visualized with an iBright CL 1500 system (Thermo Fisher Scientific, Inc.). The ImageJ software (version 1.53c, National Institution of Health) was used for densitometry.

Anti- α -smooth muscle actin (SMA; cat. no. ab124964, 1:25,000), anti-N-cadherin (Cad; cat. no. ab76011, 1:10,000) and anti-klotho (cat. no. ab181373, 1:500) were obtained from Abcam. Anti-phosphorylated (p)ERK1/2 (cat. no. GB113492, 1:1,000), anti-ERK/2 (cat. no. GB12087, 1:500), anti-zona occludens (ZO)-1 (cat. no. GB111402, 1:1,000), anti-GAPDH (cat. no. GB15004, 1:5,000) and anti-Collagen I (cat. no. GB11022, 1:500) were obtained from Wuhan Servicebio Technology Co., Ltd. Goat anti-rabbit IgG (H+L) HRP (cat. no. S0001, 1:6,500), and anti-mouse IgG (H+L) HRP (cat. no. S0002, 1:6,500) were purchased from Affinity Biosciences.

Reverse transcription-quantitative (RT-q)PCR. Total RNA from cells was extracted using TRIzol (Invitrogen; Thermo

Fisher Scientific, Inc.). An RT kit (cat. no. K1622, Thermo Fisher Scientific, Inc.) was used to generate cDNA from 1,000 ng RNA according to the manufacturer's protocol. qPCR was performed using a Roche FastStart Universal CYBR Green Master kit (Roche Diagnostics) in the ABI QuantStudio 5 system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles including denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. mRNA levels were standardized to the levels of GAPDH. The comparative Cq method was employed for relative quantification (36). The sequences of primers are listed in Table SII.

Immunofluorescence staining of mouse eye sections. Mice were euthanized by excessive anesthesia. Eyeballs were removed intact. The eyeballs were treated with the eyeball fixative solution (cat. no. G1109, Wuhan Servicebio Technology Co., Ltd.) at room temperature for >24 h. Subsequently, eyeballs were dehydrated and then embedded in paraffin. The embedded eyes were dissected into 3- μ m-thick sections. Sections with the largest lesion under a light microscope were used for immunofluorescence staining. The sections were dewaxed with deparaffinizing agent (cat. no. G2118, Wuhan Servicebio Technology Co., Ltd.), washed with anhydrous ethanol and double distilled water, subjected to antigen retrieval by immersion in sodium citrate solution (cat. no. G1219, Wuhan Servicebio Technology Co., Ltd.) and microwave on medium heat for 8 min and low heat for 7 min, blocked with 3% hydrogen peroxide at room temperature for 20 min, washed with PBS, blocked with 3% BSA (cat. no. 4240GR005, Biofroxx GmbH Co., Ltd.) at room temperature for 1 h, then incubated with primary antibody at 4°C overnight, washed with PBS, incubated with secondary antibody at room temperature for 1 h. The primary antibodies included anti-RPE65 antibody (1:300, cat. no. 17939-1-AP, ProteinTech Group, Inc.) and anti- α -SMA antibody (1:300, cat. no. GB111364, Wuhan Servicebio Technology Co., Ltd.). The secondary antibody was a goat anti-rabbit IgG (H+L) HRP (1:200; cat. no. S0001, Affinity Biosciences). iF-488-tyramide and iF-555-tyramide (TSAPLus fluorescent double staining kit, cat. no. G1226, Wuhan Servicebio Technology Co., Ltd.) was used for fluorescent immunolabeling according to the manufacturer's protocol. The nuclei were stained with DAPI at room temperature for 5 min. Sections were imaged using a confocal microscope (magnification, x400, 3DHISTECH Co., Ltd.) and analyzed using CaseViewer (version 2.4, 3DHISTECH Co., Ltd.).

Immunofluorescence staining of RPE-choroid-sclera flat mounts. Immunofluorescence assay was performed on RPE-choroid-sclera flat mounts to assess the area of subretinal fibrosis. The eyeballs were immersed in 4% paraformaldehyde in PBS for a duration of 1 h at room temperature. The RPE-choroid-sclera complexes were manually isolated from the eyeballs and treated with a 5% BSA solution (cat. no. 4240GR005, Biofroxx GmbH Co., Ltd.) containing 0.3% Triton-X100 at room temperature for 1 h. RPE-choroid-sclera complex was incubated with Alexa Fluor 488-conjugated collagen I antibody (1:100, cat. no. bs-10423R-AF488, BIOSS)

overnight at 4°C. The RPE-choroid-sclera complexes were rinsed with PBS three times, placed on slides with mounting medium and imaged using a confocal microscope (magnification, x200, Nikon Corporation). The area of collagen I-positive region (indicated by Alexa Fluor 488) was quantified using ImageJ.

Masson staining. At 7 days after laser photocoagulation, eyes were fixed and embedded as aforementioned. Paraffin-embedded eyes were cut into 3 μ m-thick sections. Sections with the largest area of visible CNV were used for Masson staining using the Masson's trichrome stain kit (cat. no. G1006, Wuhan Servicebio Technology Co., Ltd.) according to the manufacturer's protocol. Collagen fibers were stained blue, imaged using light microscope (magnification, x200, PerkinElmer Co., Ltd.) and examined using ImageJ.

Statistical analysis. Quantitative data are presented as the mean $\pm$ SEM of ≥ 3 repeats. The analyses were conducted using GraphPad Prism 10.3.0 (GraphPad Software, Inc.; Dotmatics). Comparisons between two groups were assessed using unpaired Student's t-test and differences between >2 groups were analyzed using a one-way ANOVA followed by Tukey's multiple comparisons post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TGF- β 1 induces EMT and downregulates klotho expression in ARPE-19 cells. TGF- β 1 is a crucial mediator of fibrosis (37). Therefore, TGF- β 1 was used to induce EMT and develop a fibrotic cell model. Following treatment with 10 ng/ml TGF- β 1 for 48 h, ARPE-19 cells transformed from a short cobblestone-like morphology to an elongated spindle shape with whirlpool-like growth (Fig. 1A). Western blotting results showed that TGF- β 1 treatment resulted in upregulation of mesenchymal markers (α -SMA, N-cad and collagen I), accompanied by downregulation of epithelial marker ZO-1 (Fig. 1B and C). Furthermore, cell proliferation was enhanced by TGF- β 1 treatment, as demonstrated by CCK-8 and EdU tests (Fig. 1D-F). Subsequently, wound healing and Transwell assays were performed, revealing TGF- β 1 markedly promoted the migration of ARPE-19 cells (Fig. 1G-J). Collectively, the data demonstrated that treating ARPE-19 cells with 10 ng/ml TGF- β 1 for 48 h effectively triggered EMT. Moreover, the RT-qPCR and western blotting data showed significant decreases in klotho mRNA and protein expression levels in TGF- β 1-treated cells (Fig. 1K-M).

Klotho overexpression attenuates EMT in ARPE-19 cells induced by TGF- β 1. As the expression of klotho was significantly decreased in ARPE-19 cells treated with TGF- β 1, klotho was overexpressed to establish a cause-and-effect relationship. Fluorescence microscopy showed transfection efficiency of lentivirus (Fig. S1). Expression of EMT markers were assessed using western blotting. TGF- β 1-induced reduction in epithelial marker ZO-1 and increase in mesenchymal markers (α -SMA, N-cad and collagen I) was reversed following klotho overexpression (Fig. 2A-E). CCK-8 and Edu assay revealed that TGF- β 1 promoted the proliferation of ARPE-19 cells, and

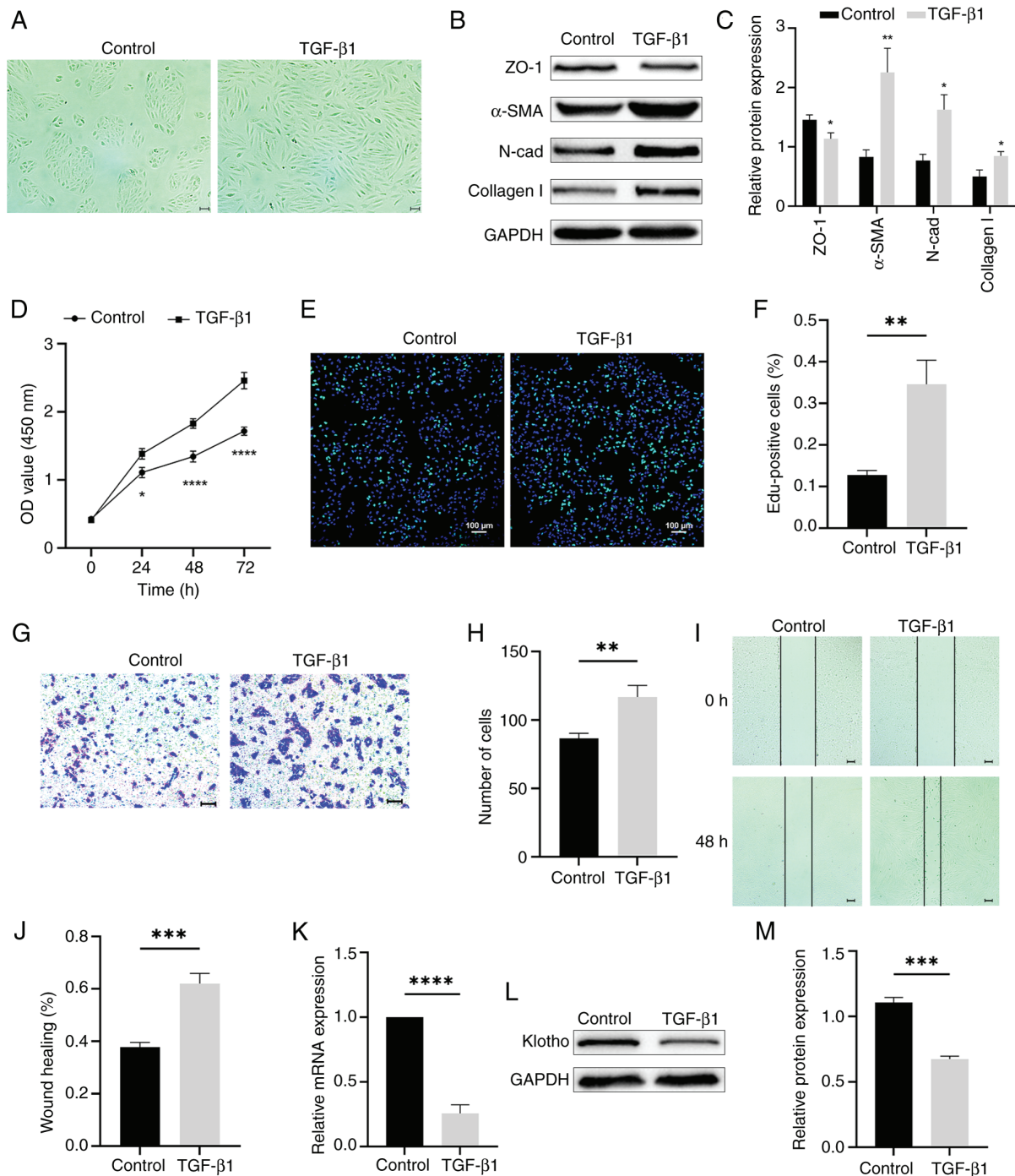


Figure 1. TGF-β1 induces EMT and downregulates klotho expression in ARPE-19 cells. (A) TGF-β1-induced morphological changes in ARPE-19 cells. Scale bar, 200 μm. (B) Expression levels of EMT-associated markers in response to TGF-β1 stimulation were detected by western blotting. (C) Relative protein expression (normalized to GAPDH) in western blot. (D) Cell Counting Kit-8 assay was used to determine the proliferation of ARPE-19 cells treated with 10 ng/ml TGF-β1. (E) EdU assays were used to confirm proliferation of ARPE-19 cells treated with 10 ng/ml TGF-β1 for 24 h. Scale bar, 100 μm. (F) Quantification of the proliferative ability of cells in EdU assays. (G) Representative migration of ARPE-19 cells treated with TGF-β1. (H) Quantification of the migratory ability of cells in Transwell migration assay. Scale bar, 400 μm. (I) Wound healing of ARPE-19 cells treated with TGF-β1. Scale bar, 200 μm. (J) Quantification of the migratory ability of cells in scratch assay. (K) Relative mRNA expression (normalized to GAPDH) of klotho in cells treated with or without TGF-β1. (L) Relative expression of klotho in cells treated with or without TGF-β1 were detected by western blotting. (M) Quantification of the relative protein expression (normalized to GAPDH) in western blot. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control group. EMT, epithelial-mesenchymal transition; SMA, smooth muscle actin; ZO, zona occludens; Cad, cadherin; OD, optical density.

this increase in proliferation was mitigated by overexpression of klotho (Fig. 2F-H). Wound healing and Transwell assay demonstrated that cell migration increased in TGF-β1-treated cell groups, while the overexpression of klotho reversed these effects (Fig. 2I-L). These data suggest that klotho

overexpression attenuated EMT in ARPE-19 cells induced by TGF-β1. RT-qPCR showed that the klotho mRNA increased to ≥2,000-fold after klotho overexpression (Fig. 3A). Western blotting results showed that klotho protein increased to 19-fold after klotho overexpression (Fig. 3B and C). These suggested

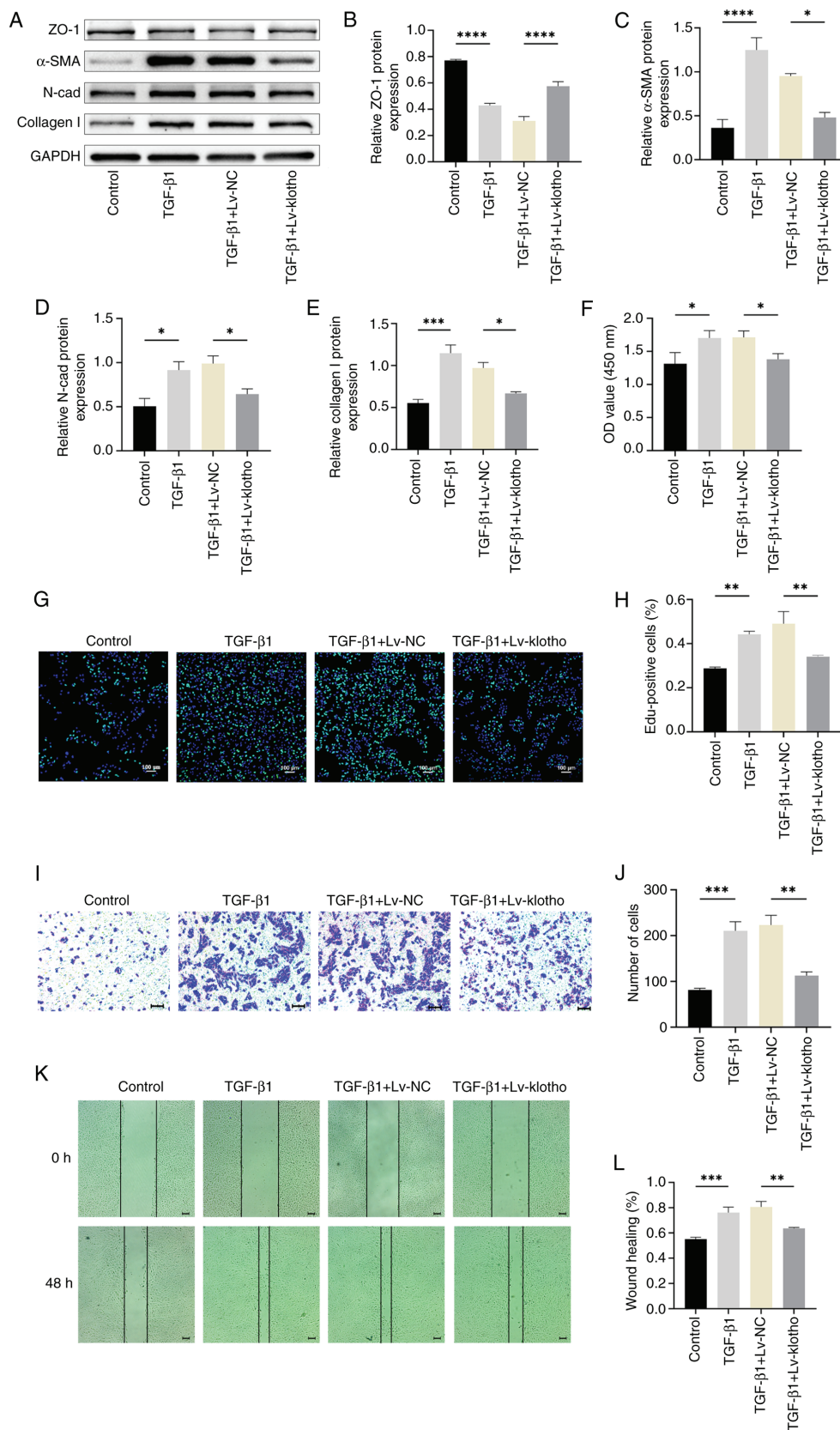


Figure 2. Klotho overexpression attenuates EMT in ARPE-19 cells induced by TGF- β 1. ARPE-19 cells were infected with Lv-klotho or Lv-NC, followed by treatment with or without TGF- β 1. (A) Protein levels of EMT-associated markers were analyzed using western blotting. (B) Relative ZO-1 protein expression (normalized to GAPDH) in western blot. (C) Quantification of the relative α -SMA protein expression (normalized to GAPDH) in western blot. (D) Quantification of the relative N-cadherin protein expression (normalized to GAPDH) in western blot. (E) Quantification of the relative collagen I protein expression (normalized to GAPDH) in western blot. (F) Cell Counting Kit-8 assays were used to determine the proliferation of ARPE-19 cells. (G) EdU assays were used to assess the proliferation of ARPE-19 cells. Scale bar, 100 μ m. (H) Quantification of the proliferative ability of cells in EdU assays. (I) Representative Transwell cell migration assay. Scale bar, 200 μ m. (J) Quantitative analysis of the migratory ability of cells. (K) Wound healing assay was used to assess cell migration. Scale bar, 200 μ m. (L) Quantification of the migratory ability of cells in wound healing assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Lv-NC, lentiviral vector negative control; EMT, epithelial-mesenchymal transition; SMA, smooth muscle actin; ZO, zona occludens; Cad, cadherin; OD, optical density.

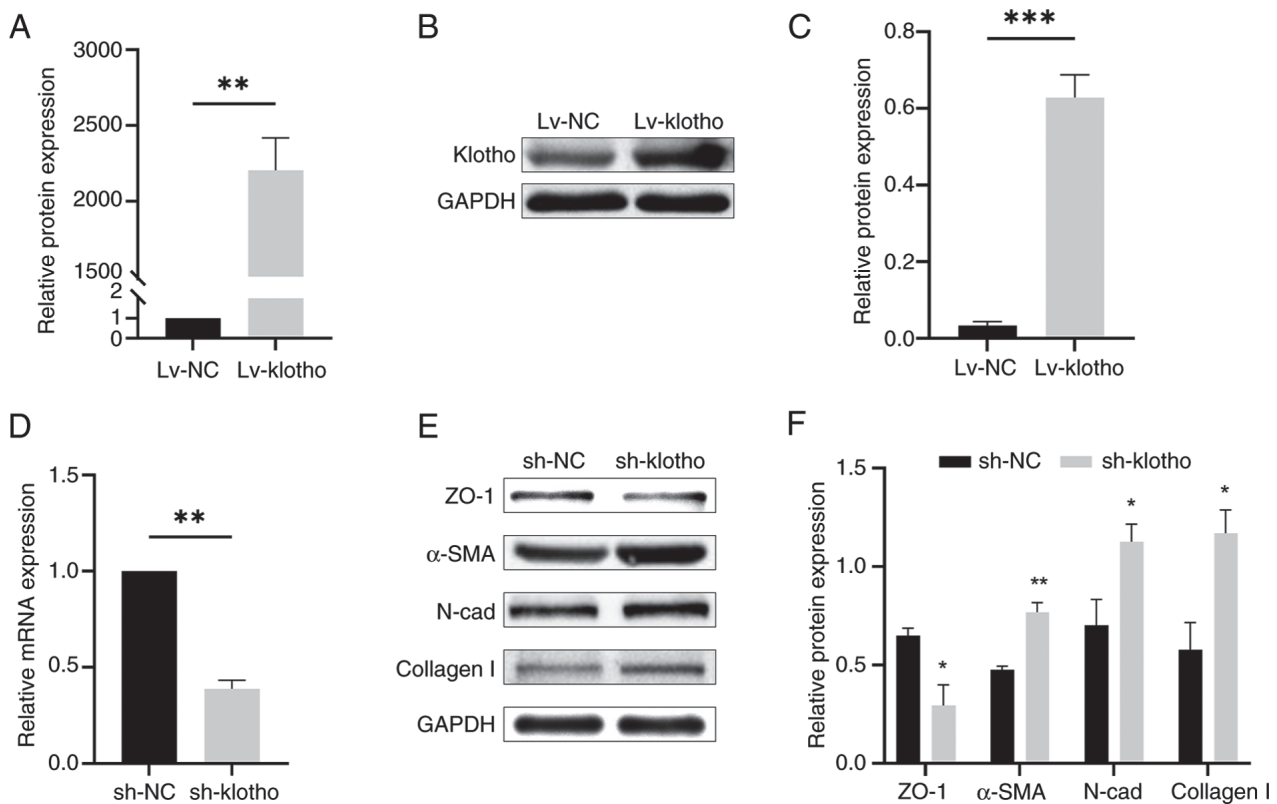


Figure 3. Knockdown of klotho induces EMT in ARPE-19 cells. ARPE-19 cells were infected with lentiviral plasmids harboring sh-klotho or empty vector. (A) Klotho expression was measured using RT-qPCR following klotho overexpression. (B) Protein expression levels of klotho. (C) Quantification of the relative protein expression (normalized to GAPDH) in western blot. (D) Klotho expression was measured using RT-qPCR following knockdown. (E) Protein expression levels of EMT-related proteins were detected following klotho knockdown. (F) Quantification of the relative protein expression (normalized to GAPDH) in western blot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. sh-NC. sh, short hairpin; RT-q, reverse transcription-quantitative; NC, negative control; EMT, epithelial-mesenchymal transition; SMA, smooth muscle actin; ZO, zona occludens; Cad, cadherin; Lv, lentiviral vector.

that the lentiviral transfection was an effective method for klotho overexpression.

Klotho knockdown induces EMT in ARPE-19 cells. Having shown that klotho overexpression attenuated EMT in ARPE-19 cells induced by TGF- β 1, whether klotho knockdown results in mesenchymal transdifferentiation of the cells was assessed. Lentiviral plasmids were used for shRNA-mediated klotho knockdown in ARPE-19 cells. RT-qPCR showed effective knockdown of klotho (Fig. 3D). Western blotting showed that klotho knockdown significantly increased expression of mesenchymal markers (α -SMA, N-cad and collagen I) and decreased expression of the epithelial marker (ZO-1) compared with sh-NC group (Fig. 3E and F).

Bioinformatic analysis following klotho overexpression. To explore the alterations in activity of signaling pathways following klotho overexpression, bioinformatic analyses were used to compare gene expression between Lv-klotho and Lv-NC groups. In the RNA-seq analysis, $\geq 95\%$ of the raw data had a quality score $> Q30$ and the clean data accounted for $\geq 99.62\%$ of the raw data (data not shown) suggesting that the data were of high quality. Biological reproducibility was demonstrated by Pearson's correlation coefficient analysis (Fig. S2) and principal component analysis (Fig. 4A). The heatmap of DEGs demonstrated notable differences between groups (Fig. S3). The volcano plot of

the significant DEGs (Fig. 4B) revealed 1,374 DEGs in the Lv-klotho group compared with the Lv-NC group. Among these, 669 DEGs were up- and 705 were downregulated. GO enrichment suggested that 'positive regulation of cell migration', 'positive regulation of cell motility', 'response to cytokine', 'positive regulation of locomotion' and 'positive regulation of cellular component movement' were downregulated, whereas 'negative regulation of cell proliferation' and 'negative regulation of cell adhesion', 'regulation of T cell mediated immunity' and 'blood vessel morphogenesis' were upregulated (Fig. 4C). Using KEGG analysis, 18 significantly enriched pathways were identified (Fig. 4D); these included 'TNF signaling pathway', 'cellular senescence', 'AGE-RAGE signaling pathway in diabetic complications' and 'MAPK signaling pathway'.

Klotho regulates EMT in ARPE-19 cells via ERK1/2 and Wnt/ β -catenin signaling. The Wnt/ β -catenin pathway (35,38) and MAPK (ERK1/2) (39) promote EMT via crosstalk with the TGF β signaling pathway. Klotho has also been reported to suppress these pathways to inhibit EMT (22,40). Additionally, RNA-seq results showed that overexpression of klotho downregulated the MAPK signaling pathway. Therefore, whether these pathways were affected by TGF- β 1 treatment or klotho overexpression was assessed. Western blotting demonstrated that TGF- β 1 enhanced ERK1/2 phosphorylation. However,

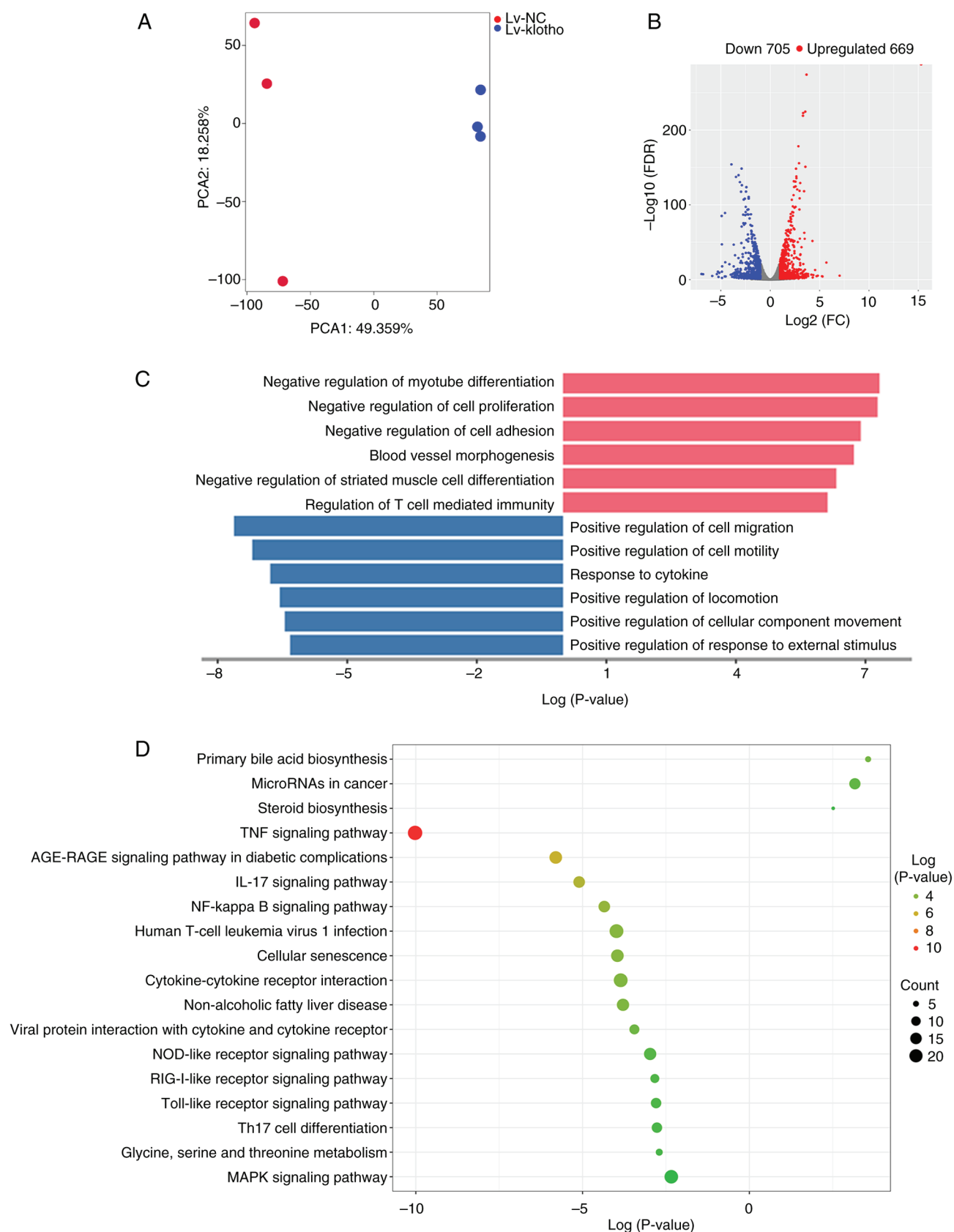


Figure 4. Bioinformatics analysis following klotho overexpression. (A) Principal component analysis and (B) volcano plot of significant DEGs. (C) GO and (D) enrichment of genes and pathways. DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Lv, lentiviral vector; NC, negative control; FDR, false discovery rate; FC, fold-change; PCA, principal components analysis.

overexpression of klotho significantly reversed this effect (Fig. 5A and B). Furthermore, TGF- β 1-induced activation of

the Wnt/ β -catenin pathway (indicated by c-Myc, cyclinD1 and β -catenin expression) was also reversed following

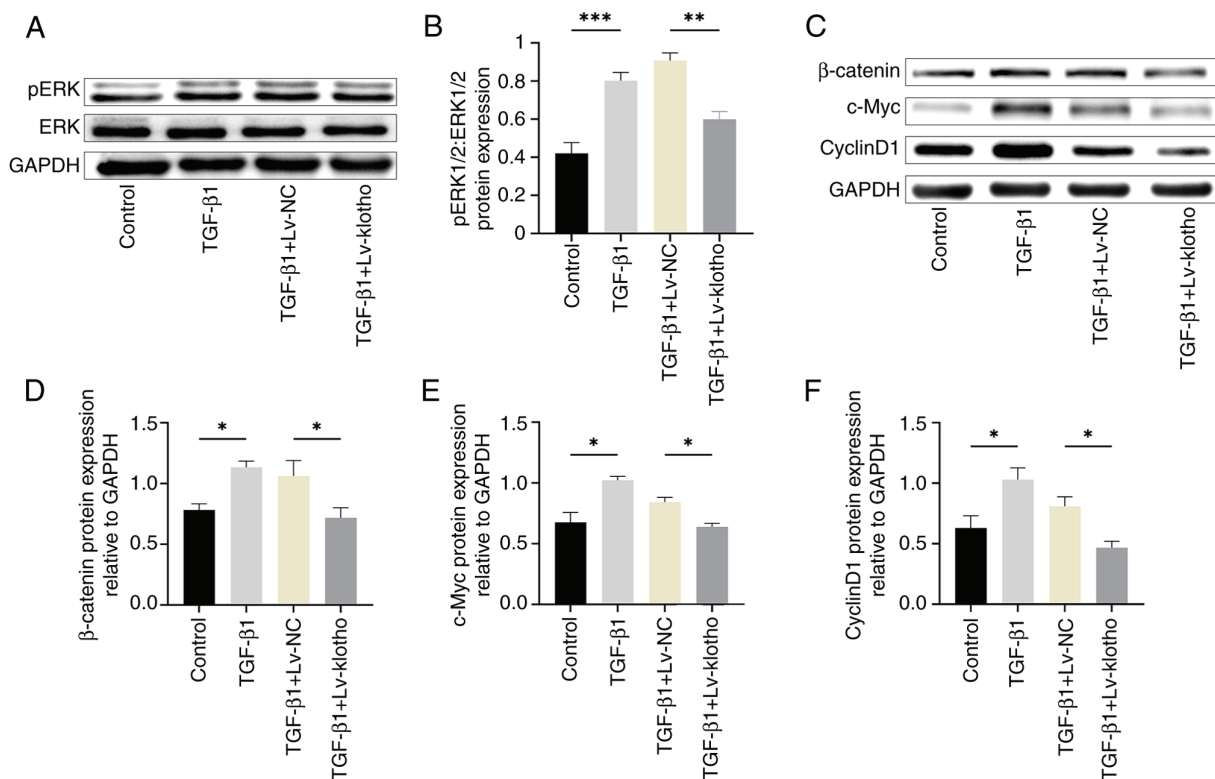


Figure 5. Klotho regulates EMT of ARPE-19 cells via the ERK1/2 and Wnt/ β -catenin signaling pathway. Protein expression levels of (A) ERK signaling pathway were analyzed using western blotting. (B) Relative protein expression. (C) β -catenin pathway were analyzed using western blotting. (D) Quantification of the relative protein expression of β -catenin. (E) Quantification of the relative protein expression of c-Myc. (F) Quantification of the relative protein expression of CyclinD1 (normalized to GAPDH) in western blot. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. EMT, epithelial-mesenchymal transition; Lv, lentiviral vector; NC, negative control; p, phosphorylated.

klotho overexpression (Fig. 5C-F). Collectively, these findings suggested that overexpression of klotho prevented APRE-19 cells from undergoing EMT by suppressing the Wnt/ β -catenin and ERK1/2 pathways.

Klotho alleviates progression of subretinal fibrosis in a mouse model of CNV. Subretinal fibrosis was induced in 6-8-week-old male mice. There was a notable subretinal fibrotic formation following laser induction, as illustrated by Masson staining of collagen deposition in mouse eye sections and immunofluorescence staining of collagen I (a marker of fibrosis) (7) in RPE-choroid-sclera flat mounts. In the control group, RPE was intact and distinguishable and there was no observable formation of subretinal fibrotic lesions; therefore, comparison of area of subretinal fibrosis could not be made between the control (without subretinal fibrosis) and mice with subretinal fibrosis (Fig. S4). Laser-induced subretinal fibrosis mice that received PBS treatment were used as vehicle control. To explore the potential influence of klotho on subretinal fibrosis, the CNV mice were treated with recombinant klotho protein (1 μ l) intravitreally 3 days after laser surgery. A total of 4 days after intravitreal injection (7 days after photocoagulation), eyes were collected for further examination. Klotho significantly attenuated the area of subretinal fibrosis, as revealed by immunofluorescence staining of collagen I in RPE-choroid-sclera flat mounts (Fig. 6A and B). A total of 10 and 20 nM klotho was used as there was a slight but not significant increase in cell size when 30 nM was used (Fig. S5). Compared with the

vehicle group, the anti-fibrotic effect of 10 and 20 nM klotho was significant (Fig. 6A); 10 nM was selected for subsequent experiments. Masson staining showed a significant decrease in subretinal collagen deposition in the 10 nM klotho group compared with the vehicle group (Fig. 6C and D).

To detect the difference in EMT-associated protein levels between the klotho-treated and vehicle group, immunofluorescence staining using antibodies specific to RPE65 (a marker for RPE cells) and α -SMA were performed. RPE cells in mice treated with 10 nM klotho expressed low levels of α -SMA in the subretinal fibrotic lesion area compared with the group treated with the vehicle (Fig. 6E). Next, western blotting was performed for proteins from the RPE-choroid-sclera complex. Klotho significantly suppressed α -SMA, N-cad and collagen I and increased ZO-1 expression (Fig. 6F-J) compared with the vehicle group. These findings showed that klotho inhibited subretinal fibrosis *in vivo*.

Next, whether klotho inhibited EMT process in mouse RPE cells via regulation of ERK1/2 and the Wnt/ β -catenin pathway was assessed. Western blotting demonstrated that klotho significantly suppressed phosphorylation of ERK1/2 and the activation of the Wnt/ β -catenin pathway (Fig. 7A-F).

Discussion

Overexpression of klotho not only reversed the alterations in cell proliferation, migration and EMT-related proteins but also attenuated the ERK1/2 and Wnt/ β -catenin pathways in

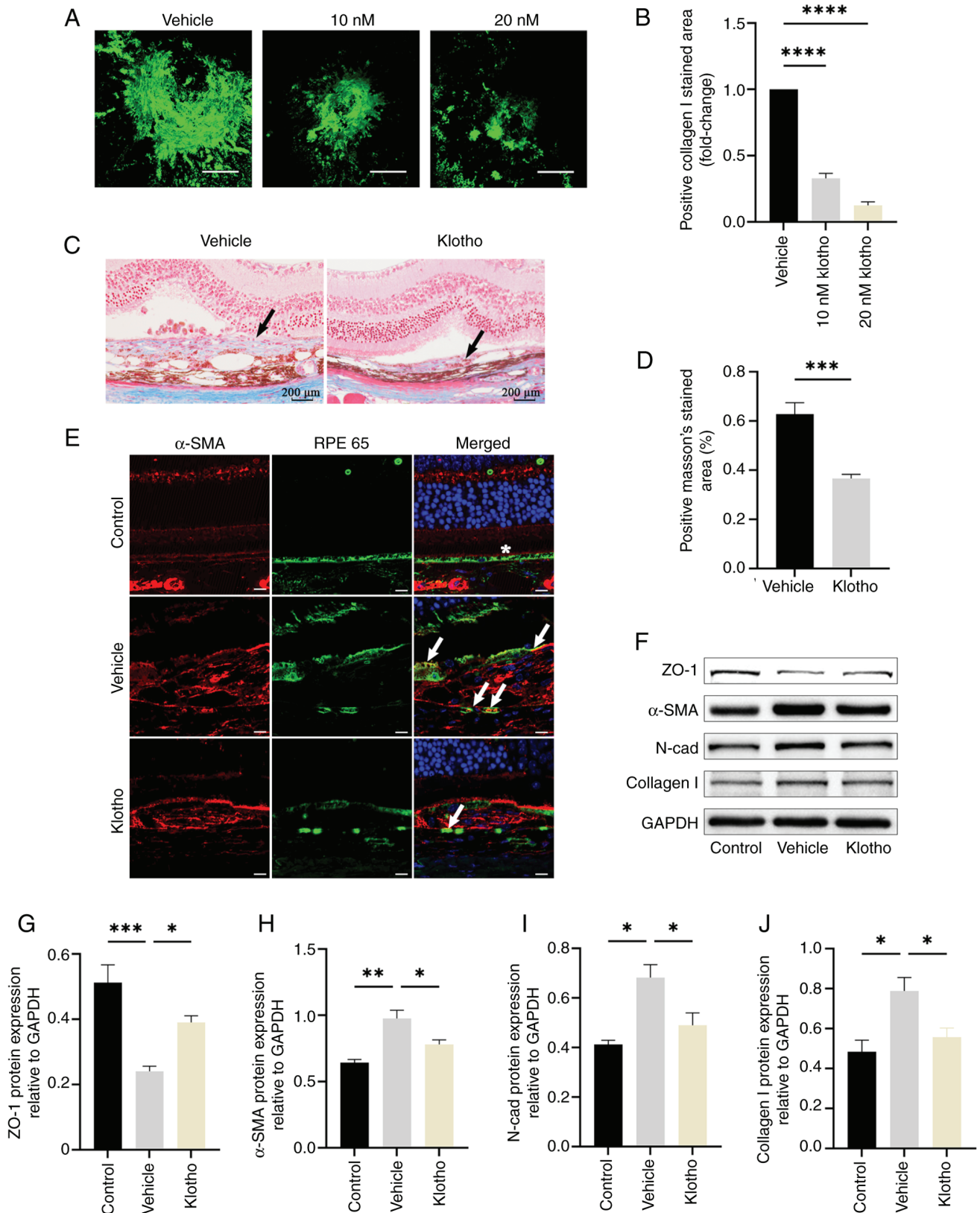


Figure 6. Klotho alleviates progression of subretinal fibrosis in a mouse model of choroidal neovascularization. (A) Representative confocal images of RPE-choroid-sclera flat-mounts stained for collagen I in mice with subretinal fibrosis treated with 10 or 20 nM klotho. Scale bar, 100 μ m. (B) Quantitative analysis of collagen I-positive lesion area in the mice. (C) Representative Masson staining of collagen fibers (black arrow). Magnification, x200, Scale bar, 200 μ m. (D) Semiquantitative analyses of the Masson staining showed that subretinal deposition of collagen was alleviated following klotho treatment. (E) Representative confocal images of eye sections stained for RPE65 (green) and α -SMA (red), showing the presence of RPE65⁺ α -SMA⁺ cells (white arrows) in the fibrotic lesion. Cell nuclei were stained with DAPI (blue). Scale bar, 10 μ m. (F) Western blotting was used to determine protein expression levels of epithelial-mesenchymal transition-related markers. (G) Relative ZO-1 protein expression (normalized to GAPDH) in western blot. (H) Quantification of relative α -SMA protein expression (normalized to GAPDH) in western blot. (I) Quantification of the relative N-cad protein expression (normalized to GAPDH) in western blot. (J) Quantification of the relative collagen I protein expression (normalized to GAPDH) in western blot. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. RPE, retinal pigment epithelium; SMA, smooth muscle actin; ZO, zona occludens; cad, cadherin.

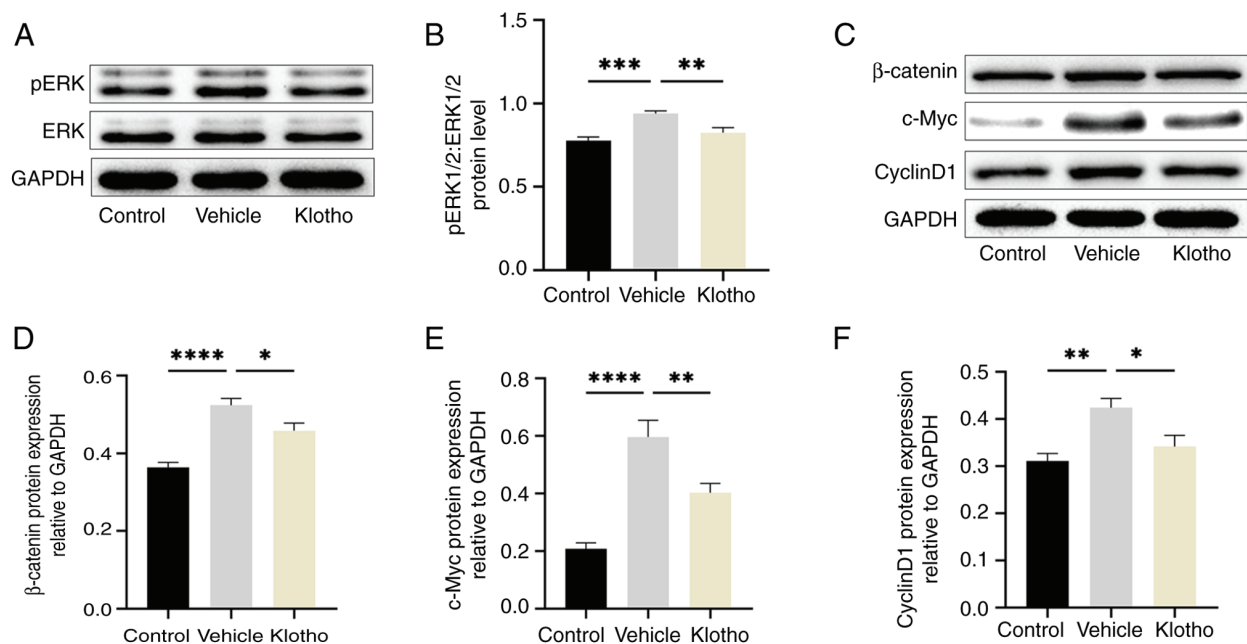


Figure 7. Klotho alleviates progression of subretinal fibrosis *in vivo* via ERK1/2 and Wnt/β-catenin signaling pathway. (A) Protein expression levels of ERK pathway were analyzed using western blotting. (B) Quantification of the relative protein expression (normalized to GAPDH) in western blot. (C) β-catenin pathway were analyzed using western blotting. (D) Relative β-catenin protein expression (normalized to GAPDH) in western blot. (E) Quantification of the relative c-Myc protein expression (normalized to GAPDH) in western blot. (F) Quantification of the relative cyclinD1 protein expression (normalized to GAPDH) in western blot. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. p, phosphorylated.

ARPE-19 cells induced by TGF-β1. Moreover, the *in vivo* experiments demonstrated that intravitreal injection of recombinant klotho protein prevented laser-induced subretinal fibrosis. These results indicated that the anti-fibrotic activity of klotho was partially achieved by inhibiting ERK and Wnt signaling.

Multiple studies have demonstrated that anti-VEGF therapy is ineffective in preventing formation of subretinal fibrosis and contributes to its development (41,42). Therefore, it is important to explore novel therapeutic methods for inhibiting subretinal fibrosis other than anti-angiogenic approaches for the treatment of nAMD. Emerging evidence has revealed that EMT of RPE cells serves an important role in the pathogenesis of fibrosis (43) and inhibition of EMT is considered a promising therapeutic approach for treating subretinal fibrosis. TGF-β1 is considered the primary mediator in EMT of RPE cells (44-46). TGF-β1 expression is increased in the retina of laser-induced mouse CNV and subretinal fibrosis model (47), vitreous and aqueous humor (48) and CNV membranes of patients with nAMD (49). Here, a fibrotic cell model was developed by treating ARPE-19 cells with TGF-β1 to induce EMT. The present findings showed that TGF-β1-stimulated transdifferentiation of ARPE-19 cells into myofibroblast-like cells and transformation from an epithelial to a mesenchymal phenotype. Following exposure to 10 ng/ml TGF-β1 for 48 h, the expression of the epithelial cell marker ZO-1 was downregulated, whereas the mesenchymal markers (α-SMA, N-cad and collagen I) were upregulated. Migration, proliferation and ECM remodeling of epithelial cells are key processes during EMT (50). Here, enhanced cell migration and proliferation and morphological changes accompanied the TGF-β1-mediated EMT of RPE cells.

The anti-aging gene klotho is essential for several pathophysiological processes, including inflammation, aging and

oxidation reactions (51,52). It exerts its anti-fibrotic properties in numerous organs and tissues, including the kidneys (53) and lungs (24). The mouse and human klotho gene encode three variants: Secreted, soluble and the full-length transmembrane protein. The secreted form of the protein is translated from an alternatively spliced mRNA, while the soluble form of the protein is the result of shedding of the extracellular domain of the membrane protein (54). Klotho can shed its soluble extracellular domain to prevent EMT; this shed protein functions as a potent fibrosis inhibitor in fibrotic disease (28). However, the underlying mechanisms remain unclear. Klotho was previously reported to inhibit VEGF secretion and regulate the expression of AMD-associated genes, suggesting klotho may serve as a potential treatment for nAMD (26). In our previous study, klotho expression was decreased under H₂O₂-induced oxidative stress in the dry form of AMD (27). Consistently, the present study showed that expression of klotho was also inhibited in ARPE-19 cells by the presence of TGF-β1, indicating that RPE-derived klotho may have a protective effect on TGF-β1-induced fibrosis during nAMD.

Earlier reports show that klotho alleviates renal fibrosis (55,56) via attenuating miR-34a-induced EMT in tubular epithelial cells (57,58). Klotho is also reported to reverse EMT in cervical cancer, as illustrated by alterations in EMT-associated protein expression, thereby blocking tumor invasion (40). Hypoxia inhibits expression of klotho in ARPE-19 cells and patients with nAMD, facilitating the hypoxia-induced EMT process (29,59). This demonstrates the key role of klotho in HIF-1α-mediated EMT (29). In addition, cell viability and metabolism of retinal pigment epithelial cell are seriously impacted by a lack of klotho (60). In line with the aforementioned studies, the present *in vitro* experiments showed that klotho overexpression reversed changes in

cell proliferation and migration and attenuated alterations in EMT-associated markers (downregulation of epithelial and upregulation of mesenchymal markers) induced by TGF- β 1. Conversely, klotho knockdown promoted transdifferentiation from epithelial to mesenchymal phenotype.

To obtain more comprehensive understanding of the anti-fibrotic effects of klotho overexpression, RNA-seq analysis was performed to explore downstream biological pathways and signaling mechanisms. GO enrichment analysis showed that 'positive regulation of cell migration' and 'positive regulation of cell motility' were downregulated, while 'negative regulation of proliferation' and 'negative regulation of cell adhesion' were upregulated, demonstrating that cell proliferation and migration during EMT were inhibited by klotho overexpression. The enriched pathways included 'TNF signaling pathway', 'cellular senescence', 'AGE-RAGE in diabetic complications' and 'MAPK signaling pathway'. As the MAPK (ERK1/2) (39) and the Wnt/ β -catenin pathway (38) promote EMT via cross-talk with TGF β signaling pathways and klotho is known to inhibit these pathways to prevent EMT(22,40), the expression of the ERK1/2 and the Wnt/ β -catenin pathway members in APRE-19 cells infected with Lv-klotho or Lv-NC was examined. These pathways are also involved in TGF- β 1 induced EMT in ARPE-19 cells. Western blotting demonstrated that TGF- β 1 led to phosphorylation of ERK1/2 and resulted in increased c-Myc, cyclinD1 and β -catenin expression, while klotho overexpression abolished these effects. These results suggested that klotho blocked the ERK1/2 and Wnt/ β -catenin pathways to prevent EMT of ARPE-19 cells.

The fibrous scar in nAMD is characterized by abnormal accumulation of ECM-associated proteins, including collagen, α -SMA and fibronectin (9). Collagen I deposition has been identified in samples from individuals diagnosed with AMD (61). In the present *in vivo* experiments, reduced collagen I and α -SMA immunostaining signals and decreased deposition of collagen fibrils were detected in the subretinal fibrotic region of klotho-treated CNV mice, indicating that klotho inhibited mesenchymal-like phenotypes of PRE induced by CNV; results of western blotting supported this: The upregulation of mesenchymal markers and downregulation of the epithelial markers were detected in the RPE-choroid-sclera complex of the mouse model with subretinal fibrosis, while intravitreal injection of klotho reversed this EMT-associated protein change, which further indicated the anti-fibrotic effects of klotho on subretinal fibrosis.

In conclusion, the present study demonstrated that klotho could partially prevent TGF- β 1-induced EMT of RPE via ERK1/2 and Wnt/ β -catenin signaling pathways. This may be the mechanism by which klotho exerts its anti-fibrotic function in subretinal fibrosis. The findings of the present study improve understanding of the pathogenesis of subretinal fibrosis and provide an innovative therapeutic approach for preventing and treating subretinal fibrosis secondary to nAMD.

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Availability of data and materials

The data generated in the present study may be found in the Sequence Read Archive under accession number SUB14887211 or at the following URL: ncbi.nlm.nih.gov/sra/PRJNA1190151.

Authors' contributions

YJ and YL conceived the study. YJ, QC, XW and XJ conducted the experiments. YJ and XW performed the data analysis. YJ wrote the manuscript. YL revised the manuscript and supervised the study. All authors have read and approved the final manuscript. YJ and XW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The Animal Experiment Ethics Review Committee of Kunming Medical University (approval no. kmmu20221787) approved the experimental protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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