A HGF-derived peptide suppresses EMT in human lens epithelial cells via the TGF-β/Smad and Akt/mTOR signaling pathways

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Abstract. Posterior capsule opacification (PCO) as a result of proliferation and fibrogenesis of lens epithelial cells (LECs) is the most frequent long-term complication of modern cataract surgery. LECs may undergo epithelial-mesenchymal transition (EMT) that resembles the morphological and molecular characteristics of PCO. A pre-identified novel, hepatocyte growth factor (HGF)-derived peptide H-RN, was reported to exhibit anti-angiogenic activity and anti-inflammatory effects in ocular cells both *in vitro* and *in vivo*. However, the role of H-RN in the promotion of the development of EMT in LECs is unknown. In the present study, the effects of H-RN on the development of EMT induced by transforming growth factor (TGF)-β in human LECs, and the possible signaling pathways participating in this process were investigated. The results showed that H-RN promoted the expression of the EMT-associated markers, α-smooth muscle actin and fibronectin, whereas the expression of E-cadherin and connexin 43 were reduced. The morphological changes typically associated with EMT seen in LECs induced by TGF-β2 were inhibited by H-RN, which was consistent with the effects of a TGF-β2 inhibitor, SB431542. Smad2 and Smad3 phosphorylation induced by TGF-β2 were reduced by H-RN, and phosphorylation of Akt, mTOR and P70S6K induced by TGF-β2 were also notably reduced by H-RN in LECs. Therefore, the results of the present study showed that H-RN treatment significantly suppressed the development of EMT induced by TGF-β2, at least partially through the TGF-β/Smad and Akt/mTOR signaling pathways in human LECs. The present study highlights that H-RN, a novel HGF-derived peptide, may be a novel therapeutic agent for prevention and treatment of PCO.

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Introduction

Posterior capsule opacification (PCO), which is the most frequent long-term complication of modern cataract surgery, is the result of proliferation and fibrogenesis of lens epithelial cells (LECs) following surgical trauma, and may also result in anterior capsular constriction and capsular bag fibrosis (1-4). Inhibition of proliferation and fibrogenesis of LECs may significantly improve the results of refraction cataract surgery.

Transforming growth factor-β (TGF-β) is an extensively well studied polypeptide growth factor, and consists of three subtypes in humans, TGF-β1, TGF-β2 and TGF-β3. TGF-β1 and TGF-β2 are both highly expressed in the human lens and ocular media (5), and serve key roles in regulating proliferation, migration and epithelial-mesenchymal transition (EMT) of LECs in PCO (6-8). TGF-β2 is most closely associated with trans-differentiation and histopathological fibrosis of LECs, and is an important regulatory factor in the pathogenesis and growth of LECs (9,10). TGF-β2 expression is upregulated in aqueous humor and vitreous bodies following surgical trauma (11). Binding of TGF-β2 to its receptor activates the Smad protein family and results in the translocation of membrane kinase receptors to the cell nucleus (12). Smad3 is a key protein involved in EMT induced by TGF-β following trauma, and LEC-EMT is dependent on the Smad3 pathway (13). Wormstone *et al* (14) showed that the increase in the expression of α-smooth muscle actin (SMA) induced by TGF-β2 in LECs was significantly inhibited by a human monoclonal anti-TGF-β2 antibody, CAT-152. Sun *et al* (15) also demonstrated that the anti-TGF-β2 (anti-T) antibody significantly reduced migration and EMT in LECs.

Research on peptides has increased significantly in the past decade (16,17), and numerous therapeutic peptides are in pre-clinical or clinical trials and ~60 peptide drugs have already been approved (18,19). These peptide-based therapeutics have certain advantages over drugs based on small molecules or protein antibodies, including higher biological activity, higher specificity and low levels of toxicity (20). It has been demonstrated that peptide drugs have potential applications in clinical fields, including in metabolic diseases, oncology and cardiovascular disease (19). Currently, a number of peptides have been developed for treating ocular pathologies (21,22). In previous studies, a small molecule peptide H-RN has been demonstrated to exert anti-inflammatory effects in an endotoxin-induced uveitis model, and a decrease in the infiltration of inflammatory cells and protein transudation, inhibition in the production of pro-inflammatory mediators in aqueous humor and ocular tissues (23-26). Furthermore, pathological changes in the ocular tissues as a result of inflammation was ameliorated by H-RN, and it exhibited no toxic effects on macrophages and human umbilical vein endothelial cells (HUVECs), and significantly suppressed lipopolysaccharide (LPS)-induced phosphorylation of NF-κB-p65, possibly via the PI3K/Akt signaling pathway (23-26). Furthermore, other similar functional small peptides have also been identified by our laboratory (27-30).

In our previous studies, the peptide H-RN, derived from hepatocyte growth factor (HGF) kringle 1 domain was designed and demonstrated to exhibit anti-angiogenic activity in a mouse model of vascular endothelial growth factor (VEGF)-induced corneal neovascularization (23-26). In the present study, the effects of H-RN on the development of EMT induced by TGF-β2 via the TGF-β/Smad and Akt/mTOR signaling pathways in human LECs were investigated.

Materials and methods

Cell culture and treatment. Human LEC line SRA01/04 was obtained from American Type Culture Collection, and the cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS (Thermo Fisher Scientific, Inc.) in a humidified 37˚C incubator with 5% CO_2. A total of 24 h prior to treatment, cells were seeded in cell culture plates, and 30 min before treatment, 10 ng/ml recombinant human TGF-β2 (Cell Signaling Technology, Inc.) was added to the cells with or without 50 μM H-RN (ChinaPeptides Co., Ltd.) for 24 h (25), and TGF-β2 inhibitor SB431542 (Sigma-Aldrich; Merck KGaA) was added combined with TGF-β2 as a positive control or to untreated cells as a negative control.

Reverse transcription-quantitative (RT-q)PCR. Following the various aforementioned treatments, cells with harvested and total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The mRNA levels of different genes in the cells were measured using qPCR using an EzQuick™ One-Step qPCR kit (Biomics Biotechnologies Co., Ltd.) according to the manufacturer's protocol. Reverse transcription was performed at 42˚C for 30 min, and the following thermocycling conditions: Initial denaturation at 95˚C for 10 min, followed by 45 cycles of 95˚C for 20 sec and final extension at 60˚C for 1 min. The housekeeping gene β-actin was used as an internal control. The results of RT-qPCR were analyzed using the $2^{-\Delta\Delta Cq}$ method (31). The primer sequences used are presented in Table I.

Western blotting. A total of $1x10^5$ cells/well were plated in a 6-well plate and grown for 24 h, cells were treated as described above, and total proteins were extracted using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) on ice. Denatured proteins were quantified using the BCA method and then 50 μg protein per lane was loaded on a 12% SDS-gel, resolved using SDS-PAGE and transferred to a PVDF membrane (GE Healthcare). Subsequently, membranes were blocked using 5% non-fat milk for 2 h at room temperature, then incubated with rabbit anti-human α-SMA (1:2,000; ab5694), fibronectin (FN) (1:1,000; ab2413), E cadherin (E-cad) (1:500; ab15148), connexin 43 (Cx43) (1:1,000; ab11370), Akt (1:1,000, ab179463), phosphorylated (p)-Akt (1:5,000; ab81283), mTOR (1:1,000; ab134903), p-mTOR (1:1,000; ab109268), p70S6K1 (1:5,000; ab32529), p-p70S6K1 (1:1,000; ab5231), Smad2 (1:2,000; ab40855), p-Smad2 (1:300; ab53100), Smad3 (1:1,000; ab40854) or p-Smad3 (1:2,000; ab52903) antibody or a mouse anti-human β-actin antibody (1:2,000; ab115777; all from Abcam) at 4˚C overnight. After washing with TBST (0.05% Tween-20), the membranes were incubated with a horseradish peroxidase-conjugated IgG secondary antibody (1:2,000; cat. no. ab205718; Abcam) for 2 h at room temperature. After washing with TBST again, signals were visualized using ECL Western Blotting Substrate (Promega Corporation). Densitometry analysis was performed using ImageJ v1.51 (National Institutes of Health).

Immunofluorescence staining. Cells were seeded in 24-well plates that contained a glass coverslip in each well and treated as described above. Once cells had adhered, they were fixed with 4% paraformaldehyde at 4˚C for 30 min, washed with PBS, permeabilized with 0.5% Triton X-100 for 10 min at room temperature, washed with PBS and blocked with 1% BSA in PBS for 30 min at room temperature. Subsequently, the cells were incubated with α-SMA (1:50; cat. no. ab5694), FN (1:50; cat. no. ab2413), Cx43 (1:1,000; cat. no. ab11370) or E-cad (1:50; cat. no. ab15148) antibodies (all from Abcam) overnight at 4˚C. The following day, the cells were washed with PBS, and incubated with an Alexa Fluor® 594 conjugated goat anti-rabbit IgG (1:1,000; cat. no. ab150080; Abcam) at room temperature for 30 min. The nucleus was stained using Hoechst 33258 (Thermo Fisher Scientific, Inc.) for 10 min at room temperature. Fluorescent cells were visualized using a fluorescence microscope (magnification, x40).

Cell proliferation assay. Cell proliferation was assessed using the MTT method. Briefly, $1x10^3$ cells/well were plated in a 96-well plate and grown for 24 h. After cells were treated for 24, 48 and 72 h as described above, 10 μl of MTT (Promega Corporation) was added to each well, and incubated at 37˚C for 4 h away from light. Subsequently, 150 μl DMSO was added to each well and incubated at 37˚C for 10 min. Fluorescence intensity of each well was measured at 490 nm using a microplate reader (BioTek Instruments, Inc.).

Table I. Sequences of reverse transcription-quantitative PCR primers.

Gene name	Sequences (5'−3')
α-SMA	F: CTCCGGAGCGCAAATACTCT
	R: TGCTAGAGACAGAGAGGAGCA
FN	F: ACAAGCATGTCTCTCTGCCA
	R: TCAGGAAACTCCCAGGGTGA
Cx43	F: AGCCACTAGCCATTGTGGAC
	R: CCACCTCCACCGGATCAAAA
E-cad	F: TTGTCTGGCCACATCTTGACT
	R: CTGCAGCACTTTAGGCACTAT
β-actin	F: TTGCCGACAGGATGCAGAAGGA
	R: AGGTGGACAGCGAGGCCAGGAT

F, forward; R, reverse. α-SMA, α-smooth muscle actin; FN, fibronectin; E-cad, E-cadherin; Cx43, connexin 43.

Wound-healing assay. Cell migration was assessed using a wound-healing assay. Briefly, $1x10^5$ cells/well were plated into a 96-well plate and grown for 24 h. A wound was created in the confluent monolayer of cells using a 1-ml pipette tip and cell culture medium was replaced with fresh DMEM containing 1% FBS (32), then cells were cultured in a humidified 37˚C incubator with 5% CO_2. After been treated as described above for 4 h, cells were washed with PBS, and images were taken using a light microscope after 0, 24 and 48 h (magnification, x4), and cell migration was calculated by relative migration rate (%) = [1-(wound area at Tt/wound area at T0)] x 100.

Hematoxylin and eosin (H&E) staining. Cells were seeded into 24-well plates containing glass coverslips, and treated as described above. Subsequently, the glass coverslips were washed with PBS, fixed in 95% pre-chilled ethanol for 10 min at room temperature and washed with PBS. This was followed by staining with Harris hematoxylin solution for 10 min at room temperature, washing with PBS, and then staining with eosin solution for 10 min, following which they were washed again with PBS. Finally, cells were mounted using antifade mounting medium, and the stained cells were observed under a light microscope (magnification, x20).

Statistical analysis. All experiments were performed three times independently. Data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS version 19.0 (IBM Corp.). A comparison between two groups was performed using a Student's t-test and the differences between multiple groups were determined using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H-RN suppresses the development of TGF-β2-induced EMT. To investigate the effect of H-RN on TGF-β2-induced EMT in human LECs, the mRNA and protein expression levels of EMT markers: α-SMA, FN, E-cad and Cx43 were detected by RT-qPCR and western blotting, respectively. Compared with the untreated cells, the mRNA and protein expression levels of α-SMA and FN were significantly increased, and the expression levels of E-cad and Cx43 were significantly decreased in LECs induced by TGF-β2. The expression of these EMT markers was increased by TGF-β2 and decreased by H-RN significantly in LECs, and the same results were observed when cells were treated with the TGF-β2 inhibitor SB431542 (Figs. 1 and 2).

Expression of the EMT markers, α-SMA, FN, E-cad and Cx43, in LECs were also determined by immunofluorescence, and the results showed that the expression levels of these EMT markers were consistent with the western blotting and RT-qPCR results (Fig. 3). Therefore, H-RN inhibited TGF-β2 induced EMT in LECs.

Proliferation and migration of LECs is modulated by H-RN. MTT assays were used to assess the proliferation of LECs treated with TGF-β2 and H-RN. The results showed that the proliferation of LECs was increased by TGF-β2 significantly ($P<0.05$), and the TGF-β2-induced increase in cell proliferation was significantly inhibited by H-RN and SB431542 ($P<0.05$; Fig. 4A), compared with the untreated cells. The migration of LECs treated with TGF-β2 and H-RN was assessed using a wound-healing assay. Compared with the untreated cells, the migration of LECs was significantly increased by TGF-β2 ($P<0.05$), and TGF-β2-induced cell migration was significantly inhibited by H-RN or SB431542 ($P<0.05$; Fig. 4B).

Effect of H-RN on the morphology of LECs. H&E staining was used to observe the morphology of LECs treated with TGF-β2 and H-RN. The results showed that the morphology of the cells exhibited a more mesenchymal-like phenotype when treated with TGF-β2. LECs exhibited altered morphologies, appearing flattened and stretched instead of their typical oval-like shape and additionally, adherence was reduced resulting in an increase in the number of floating cells, and the gaps between cells were increased as well. Treatment with H-RN or SB431542 in conjunction with TGF-β notably reduced the morphological changes (Fig. 5).

H-RN suppresses the development of EMT via the TGF-β/Smad signaling pathway. To clarify the regulatory mechanism by which H-RN regulated EMT in LECs, the phosphorylation of Smad2 and Smad3, members of the TGF-β/Smad signaling pathway, were assessed using western blotting. The results showed that Smad2 and Smad3 phosphorylation were increased by TGF-β2, and H-RN or SB431542 significantly inhibited this (Fig. 6). The results suggest that H-RN suppressed the development of EMT, possibly via inhibition of the TGF-β/Smad signaling pathway.

H-RN suppresses the development of EMT via the Akt/mTOR signaling pathway. The effects of H-RN on the Akt/mTOR signaling pathway were further investigated. The results showed that levels of p-Akt, mTOR and

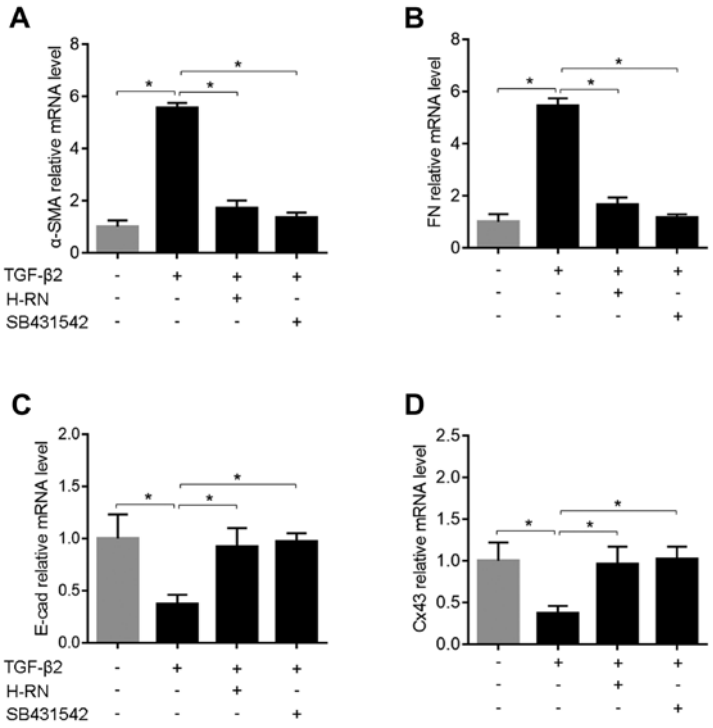

Figure 1. H-RN regulates mRNA expression levels of EMT markers. mRNA levels of EMT-associated markers (A) α-SMA and (B) FN induced by TGF-β2 were downregulated by H-RN. mRNA levels of (C) E-cad or (D) Cx43 were increased by H-RN. *P<0.05 vs. untreated or TGF-β2 treated cells. EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; FN, fibronectin; E-cad, E-cadherin; CX43, connexin 43; TGF-β2, transforming growth factor-β2.

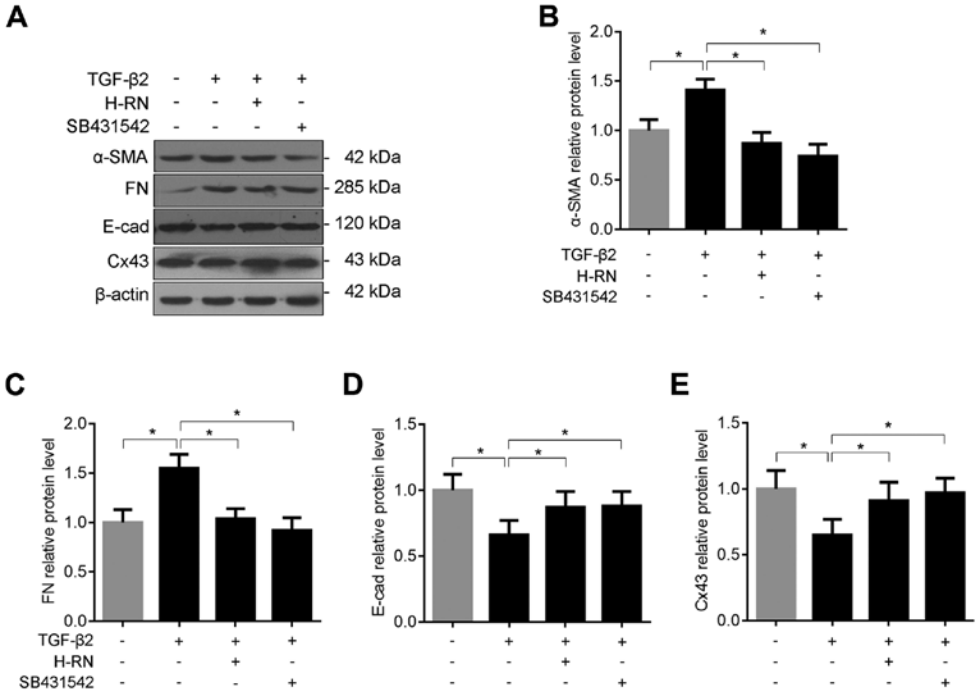

Figure 2. H-RN regulates the protein expression levels of EMT markers. (A) Western blotting showed that H-RN led to the downregulation of EMT-associated markers induced by TGF-β2. Protein expression levels of (B) α-SMA and (C) FN were decreased by H-RN. (D) E-cad and (E) Cx43 were increased by H-RN. *P<0.05 vs. untreated or TGF-β2 treated cells. EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; FN, fibronectin; E-cad, E-cadherin; CX43, connexin 43; TGF-β2, transforming growth factor-β2.

P70S6K were increased significantly in TGF-β2 treated LECs, and H-RN or SB431542 significantly inhibited the TGF-β-induced increase (Fig. 7). The results further support the hypothesis that H-RN suppressed the development of EMT, possibly via inhibition of the Akt/mTOR signaling pathway.

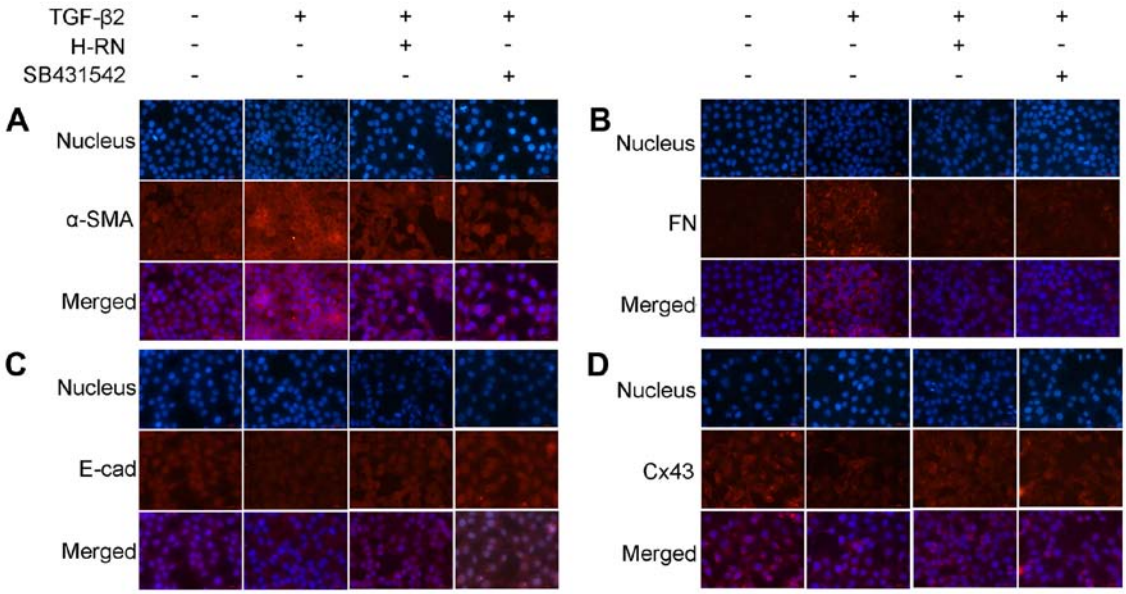

Figure 3. Immunofluorescence of EMT markers affected by H-RN. H-RN reduced the expression of (A) α-SMA and (B) FN, while increasing the expression of (C) E-cad and (D) Cx43 induced by TGF-β2 in LECs. Scale bar indicates 50 μm. EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; FN, fibronectin; E-cad, E-cadherin; CX43, connexin 43; LEC, lens epithelial cells; TGF-β2, transforming growth factor-β2.

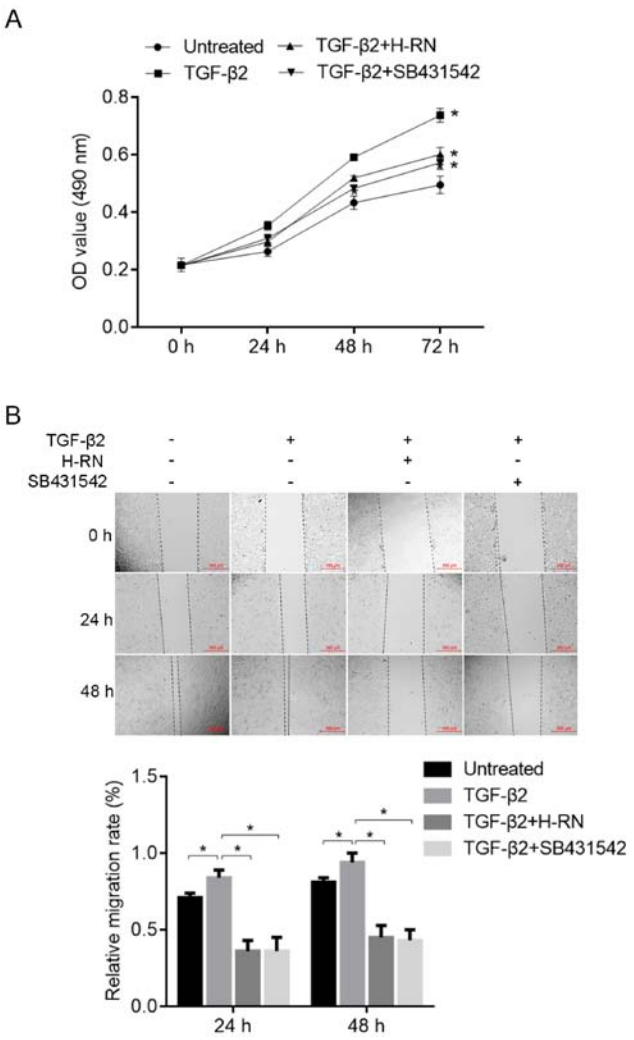

Figure 4. H-RN reduces the proliferation and migration of LECs. H-RN treatment significantly reduced the (A) proliferation and (B) migration of LECs. *P<0.05 vs. untreated or TGF-β2 treated cells. LECs, lens epithelial cells; TGF-β2, transforming growth factor-β2.

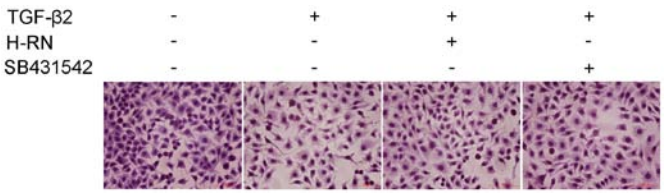

Figure 5. Hematoxylin and eosin staining of lens epithelial cells induced by TGF-β2 treated with or without H-RN. TGF-β2, transforming growth factor-β2.

Discussion

Lens epithelial cells (LECs) are essential for the normal development and homeostatic maintenance of the lens (33). Aberrant growth, proliferation, migration, trans-differentiation and secretion of components of the extracellular matrix of LECs following cataract surgery may contribute to the development of various diseases of the lens, such as Posterior capsule opacification (PCO) (1,2). Numerous studies have shown that LECs can undergo epithelial-mesenchymal transition (EMT), and these transformed cells bear a morphological and molecular resemblance to PCO (34-36). During EMT, there are a series of changes, such as the formation of spindle-like cells accompanied by wrinkling of the lens capsule, accumulation of extracellular matrix and cell death as a result of apoptosis (37,38). However, EMT is a complex mechanism, and involves different signaling pathways from the microenvironment *in vitro* and *in vivo* (39-41). The TGF-β subtypes, including TGF-β1 and TGF-β2 have been demonstrated to promote EMT in LECs (6-8). In the present study, it was demonstrated that TGF-β2 treatment significantly increased α-SMA and fibronectin expression levels, decreased E-cadherin and Cx43 expression levels, and that EMT in LECs was induced by TGF-β2. Also, the proliferation and migration of LECs were increased by TGF-β2 significantly, although

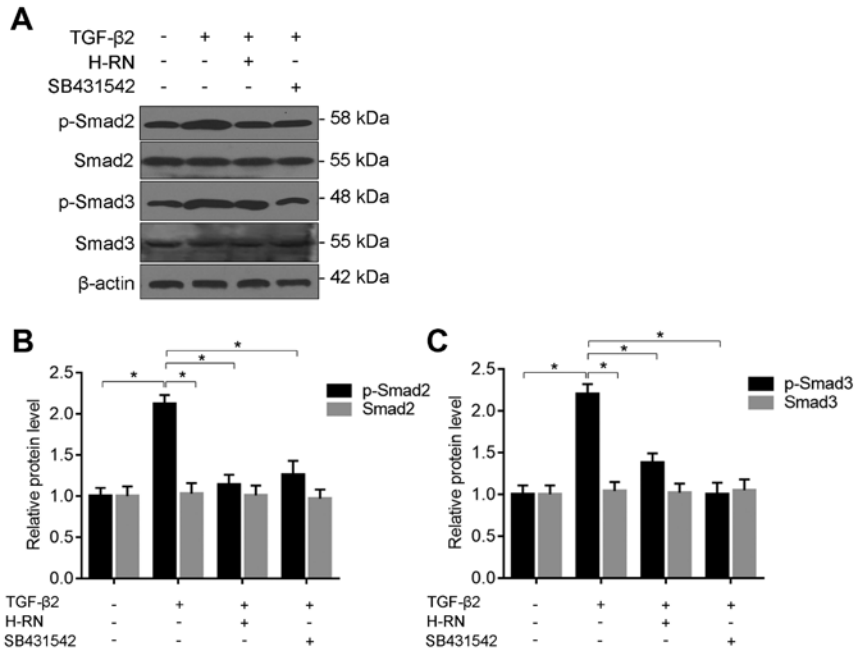

Figure 6. H-RN reduces the development of EMT via a TGF-β/Smad signaling pathway. (A) Western blots of p-Smad2, Smad2, p-Smad3 and Smad3. Densitometry analysis of (B) p-Smad2 and total Smad2 expression levels; and (C) p-Smad3 and total Smad3 expression levels. *P<0.05 vs. untreated or TGF-β2 treated cells. EMT, epithelial-mesenchymal transition; p-, phospho-; TGF-β2, transforming growth factor-β2.

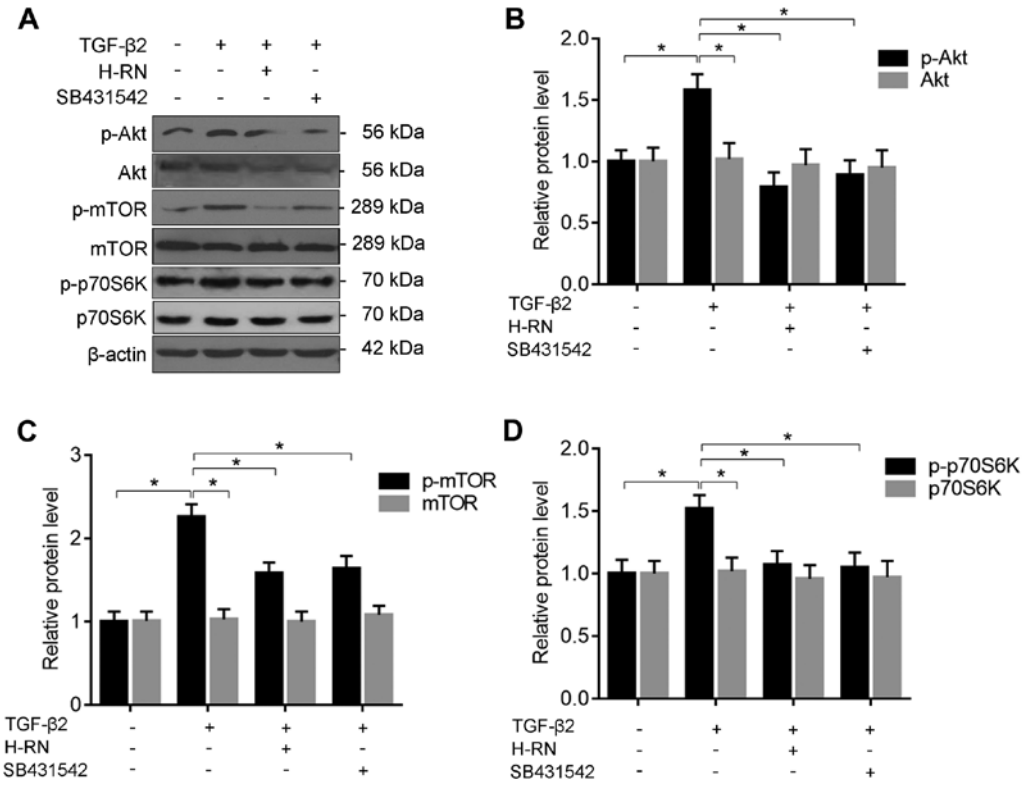

Figure 7. H-RN suppresses the development of EMT via an Akt/mTOR signaling pathway. (A) Western blots of p-Akt, Akt, p-mTOR, mTOR, p-p70S6K and p70S6K. Densitometry analysis of (B) p-Akt and total Akt expression levels; (C) p-mTOR and total mTOR expression levels; and (D) p-p70S6K and total p70S6K expression levels. *P<0.05 vs. untreated or TGF-β2 treated cells. EMT, epithelial-mesenchymal transition; p-, phospho-; TGF-β2, transforming growth factor-β2.

the cell migration experiments may have been affected by the low confluence of cells. Furthermore, the morphology of LECs was significantly altered to a more mesenchymal-like phenotype when LECs were treated with TGF-β2.

Hepatocyte growth factor (HGF) is a paracrine cellular growth factor that regulates motility and morphogenic development, and it has been shown to possess a major role in embryonic organ development, specifically in myogenesis, in

adult organ regeneration and in wound healing (42,43). HGF is also a potent stimulator of neo-angiogenesis and an important angiogenic factor in vascular retinopathies where it acts as a retinal angiogenesis regulator (44). HGF is comprised of an amino-terminal domain (N), four kringle domains (K1-K4), and a serine proteinase homology (SPH) domain (45). The kringle 1 domain, was reported to exhibit antiangiogenic, antitumor and anti-inflammatory effects (46-48). H-RN (amino acid sequence, RNPRGEEGGPW; molecular weight: 1254.34 Da), is a novel peptide derived from HGF kringle 1 domain, which was identified by Xu *et al* (23). H-RN effectively inhibited the proliferation, migration and tube formation of RF/6A cells stimulated by VEGF, and was also shown to exhibit antiangiogenic activity *in vitro* and *in vivo* (23). Sun *et al* (24) also confirmed that H-RN exhibited anti-angiogenic activity in HUVECs, and in a mouse model of VEGF-induced corneal neovascularization the anti-angiogenic activity of H-RN was associated with apoptosis and cell cycle arrest, indicating a strategy for anti-angiogenic treatment in the cornea. Wang *et al* (25) further demonstrated that intravitreal treatment of H-RN suppressed clinical manifestation, this included inhibiting ocular inflammatory cytokine production and improving histopathological scores in a concentration dependent manner, and H-RN was shown to suppress tumor necrosis factor-α-induced adhesion molecule expression and E-selectin. H-RN significantly suppressed LPS-induced phosphorylation of NF-κB-p65 and inhibited PI3K-p85 and Akt (Ser473) phosphorylation, which may result in the attenuation of LPS-induced IκB kinase (IKK) complex activation and IκB degradation, this study suggested that H-RN exhibits anti-inflammatory effects possibly via the PI3K/Akt/IKK/NF-κB signaling pathway (25). Several signaling pathways mediate TGF-β induced EMT of LECs, and canonical TGF-β/Smad signaling has been demonstrated to serve a crucial role in the networks regulating EMT in LECs (13). In addition, noncanonical signaling pathways including RhoA (5,41), Akt (7,25) and ERK (7) are also involved. In the present study, the effect of H-RN on the development of EMT induced by TGF-β2 via Smad-dependent and Smad-independent pathways in human LECs were investigated. Smad2 and Smad3 phosphorylation were induced by TGF-β2, and phosphorylation of Akt, mTOR and P70S6K was increased significantly, suggesting that H-RN suppressed the development of EMT via the TGF-β/Smad and Akt/mTOR signaling pathways.

In conclusion, to the best of our knowledge, the present study is the first to report that H-RN exhibits anti-EMT activity in human LECs induced by TGF-β2. EMT suppression by H-RN may be mediated via the TGF-β/Smad and Akt/mTOR signaling pathways, indicating a potential strategy for PCO treatment.

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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

XH and HZ conceived and designed the study. XH, YW and PZ performed the experiments. XH and HZ analyzed and interpreted the data. XH drafted the manuscript. YW, PZ and HZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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