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LncRNA HOTTIP improves diabetic retinopathy by regulating the p38-MAPK pathway

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Abstract. – OBJECTIVE: To explore the mechanism of HOTTIP in diabetic retinopathy.

MATERIALS AND METHODS: The diabetic rat model was established by a single intraperitoneal injection of streptozocin (STZ). The expression of HOTTIP in the retina of diabetic mice and wild-type mice was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). The wild-type and diabetic rats were injected with HOTTIP shRNA or Scr shRNA adenovirus, and the down-regulated expression of HOTTIP was accessed by RT-PCR. Visual electrophysiology (ERG) was performed to detect the effect of HOTTIP on visual function in rats. Western blot was carried out to detect the expressions of ICAM-1 (intercellular cell adhesion molecule-1), VEGF (vascular endothelial growth factor) and TNF-α (tumor necrosis factor-α) in the retina of rats in each group. Small RNA interference decreased the expression of HOTTIP in RF/6A cells, and then, stimulated with high glucose (or H_2O_2). The viability of RF/6A cells was detected by MTT. Cell apoptosis was determined by flow cytometry. Western blot was carried out to determine the activation of p38, JNK (c-Jun N-terminal kinase) and ERK1/2 (extracellular regulated protein kinases) in RF/6A cells after high glucose and HOTTIP downregulation, and to investigate whether HOTTIP could activate Mitogen-activated protein kinase (MAPK) Signaling thus regulating the function of retinal endothelial cells.

RESULTS: HOTTIP was significantly upregulated in the retina of diabetic rats and mice. RT-PCR showed that the expression of HOTTIP in the retina of diabetic rats injected with HOTTIP shRNA adenovirus was down-regulated. There was no significant change after injection of shRNA NC adenovirus. Down-regulation of HOTTIP can reduce the visual function decline and apoptosis of retinal cells caused by diabetes. It also reduced the expression of ICAM-1 and VEGF inflammatory factors in the retina. After high glucose or H_2O_2 treatment, the viability of RF/6A cells decreased, and the viability of living cells was further decreased after HOTTIP was reduced. Down-regulation of HOTTIP resulted in decreased phosphorylation of p38, but had no effect on phosphorylation of ERK1/2 or JNK1/2. Upregulated HOTTIP could increase the viability of RF/6A cells, which was reversed by pretreatment of a p38 inhibitor, SB23580. However, ERK inhibitor or JNK inhibitor had no effects on cell viability.

CONCLUSIONS: HOTTIP improves diabetic retinal microangiopathy through the p38-MAPK pathway. HOTTIP is expected to become a new target for the treatment of diabetic microangiopathy.

Key Words:
lncRNA, HOTTIP, Diabetic retinopathy, p38-MAPK.

Introduction

Diabetes is now a global health problem that affects the health of children, adolescents and adults, and its incidence is becoming more prevalent. According to the epidemiological survey, there are more than 240 million people with diabetes in the world[1]. At present, there are over 100 million diabetic patients in our country. In 2013, the prevalence of diabetes in China was 11.6%[2]. Diabetes can lead to vascular complications through increased production of reactive oxygen species, inflammatory reactions and damage to the vascular barrier[3]. Diabetic vascular complications are serious and varied, mainly include the microvascular and macrovascular dysfunction in multiple tissues and organs, such as muscle, eye, skin, heart, brain, and kidney[4]. In most diabetic patients, the main diabetic vascular complications of diabetic retinopathy, which is the main cause of blindness in diabetic patients[5].

In the human genome, less than 2% of the gene sequences are protein-coding genes and about 98% of the remaining genomic sequences are non-coding protein sequences[6]. Most of the transcripts of these sequences are non-coding RNAs. In non-coding RNA, RNAs with over 200 nucleotides in length are long non-coding RNAs

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(lncRNA). LncRNAs are similar in structure to messenger RNAs (mRNAs) but are not involved in protein coding. They are capable of modulating the expression of protein-coding genes at the transcriptional and post-transcriptional levels[7].

Researches[8,9] have shown that lncRNAs are widely participated in lots of important biological functions in the body, such as epigenetic regulation, cell cycle regulation, apoptosis and induction of pluripotent stem cell reprogramming. Its abnormal expression is also closely related to the occurrence and development of many human diseases such as cardiovascular diseases and cancers[10]. In recent investigations[11], the analysis focused on human β cell transcriptome revealed that lncRNAs can be dynamically modulated and aberrantly expressed in type 2 diabetes. Genome-wide association studies[12] have shown that lncRNA-ANRIL is highly related to type 2 diabetes. The above data demonstrated that lncRNA may participate in the pathogenesis of diabetes, the underlying effect of which in diabetes-induced vascular dysfunction, however, is not fully elucidated.

LncRNA HOTTIP, is a non-coding RNA transcribed from the HOXA family and its function has been reported in many diseases, but its mechanism in retinopathy remains unclear. Therefore, the study of HOTTIP can further understand the mechanism of retinopathy, which provides a theoretical basis for its prevention and treatment.

Materials and Methods

Cell Culture

The RF/6A cell line used in this experiment was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 10% fetal calf serum (FBS) and 1% penicillin (Gibco, Grand Island, NY, USA), and then placed in a 37°C incubator with 5% CO_2. Fresh medium was changed every 2-3 days.

Cell Transfection

Cells seeded in 6-well plates were transfected when fusion degree reached 50%. 5 μL of lipotap transfection reagent and 5 μL of siRNA solution (Invitrogen, Carlsbad, CA, USA) were added in 135 μL of glutamate-free DEME medium, and were then placed in 1.5 mL EP tube at room temperature for 15 min. The cell upper medium was removed, each dish was re-added 1.5 mL of FBS-free DMEM medium, the mixed siRNA was then added to the dish, and gently shaking. The normal medium was changed 6 h later.

Visual Electrophysiology

Visual electrophysiological examination of each group of rats was performed after establishment of diabetic rat model 1, 3, 5 months later. Impairment of visual function was accessed. Rats were acclimatized for 2 h under the dark light, with light anesthesia, mydriasis, put on a platform, their heads were fully extended into the ganzfeld and electrophysiological procedures were performed.

Experimental Animals

Two-month-old healthy male SD (Sprague-Dawley) rats weighing from 180 to 200 g were purchased from the Animal Science Center, Affiliated Hospital of Weifang Medical University. This study was approved by the Animal Ethics Committee of Weifang Medical University Animal Center. Rats were fed with the particulate food, free drinking water, and raised in the environment with temperature at 22-26°C, and humidity at 50-60%. The environment was in good ventilation and circadian rhythm. 60 rats were randomly assigned into 4 groups: normal control group, simple diabetic group, diabetes + HOTTIP shRNA group, and diabetes + shRNA NC group. Congenital diabetes mice db/db mice were purchased from the Pengsheng biotechnology company (Dalian, China) in China[13].

Establishment of Diabetic Rat Model

After 12 h of fasting, SD rats were injected intraperitoneally with STZ (0.1 mol/L sodium citrate buffer, pH 4.6) at 60 mg/kg body weight. A blood sample from rat tail vein was harvested for glucose detection 72 h later. Rat diabetes model was considered to be successful when the random blood glucose was higher than 16.7 mmol/L and maintained for over 1 week. Weight and random blood sugar were recorded weekly.

Intraocular Injection of shRNA

Rats were anesthetized with 10% chloral hydrate solution (Yeasen, Shanghai, China) at 3.5 mL/kg body weight, mydriatic and ocular surface anesthesia were performed. 5 μm of adenovirus was extracted with a microinjector under a surgical microscope. The flattened portion of the eye ciliary body was inserted into the vitreous cavity

for injection. Antibiotic gel smeared eye surface, rats were put the insulation board to wake up. After 3 d, postoperative fundus hemorrhage was observed. Antibiotic eye drops were given 3 times daily for an eye infection.

MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Discard the cell culture medium, add 500 μL of DMEM and 100 μL of 5 mg/mL MTT solution (Beyotime, Shanghai, China). Cells were incubated for 2 h at 37°C. Discard the supernatant and add 500 μL of isopropanol. Shake well for 10 min. 100 μL of samples were taken to 96-well plates and the absorbance was recorded by spectrophotometer (Hitachi, Tokyo, Japan) at 490 nm.

Flow Cytometry Analysis of Apoptosis

The treated cells, culture medium and the rinse solution were collected in separated centrifuge tubes, and after trypsinization, the cells were collected. 100 μL of 1 × Binding Buffer was added for suspension of cells in each tube, and uniform single cell suspension was prepared to adjust the cell density to (5 - 10) × 10^6/mL. 5 μL of Annexin V-FITC and 5 μL of PI (propidium iodide) were added to gently mix and incubate in the dark at room temperature for 10 min. Add 400 μL of 1 × Binding Buffer (Invitrogen, Carlsbad, CA, USA) to each reaction tube and mix gently. Flow cytometry (Partec AG, Arlesheim, Switzerland) was used to detect and analyze the results.

RNA Extraction and qRT-PCR

We used qRT-PCR to determine the RNA expression of rat eyeballs and cells. Total RNA of tissue or cultured cells were extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). The QRT-PCR analysis was performed using a PrimeScript reverse transcription kit and SYBR Premix Ex Taq, and the results were normalized with the expression level of phosphoglycerate dehydrogenase (GAPDH).

Western Blot

Retinal tissues or cells were added to the protein lysate, mechanical or ultrasound homogenization for extracting total protein. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (Merck Millipore, Billerica, MA, USA), primary antibodies (Cell Signaling Technology, Danvers, MA, USA) were added and incubated overnight at 4°C. HRP-labeled (horseradish peroxidase) goat anti-rabbit IgG (1:4000) secondary antibody was used. Gene Tools software was used to analyze the protein expression.

Statistical Analysis

We used statistical product and service solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) for statistical analysis. All data were expressed as mean ± SD; independent sample *t*-test was used to analyze the difference between two groups. Comparison between groups was performed using One-way ANOVA test followed by Post- Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant. Graph Prism V6.0 software (Version X; La Jolla, CA, USA) was utilized for graph editing.

Results

HOTTIP Was Up-Regulated in Diabetic Animal Model

Here we studied the expression of HOTTIP in a diabetic rat model. QRT-PCR results indicated that the expression level of HOTTIP in diabetic rats retina induced by STZ was significantly higher than those of non-diabetic rats (Figure 1A). HOTTIP expression level was remarkably enhanced in the retina of db/db mice, the type 2 diabetes model, in comparison to the control group with same age (Figure 1B). The up-regulation of HOTTIP in diabetic retina indicated that HOTTIP may be involved in the regulation of diabetic microvascular complications.

To further investigate the effects of downregulated HOTTIP on retinal function, we performed intraocular shRNA NC or HOTTIP shRNA adenovirus in wild-type and diabetic rats, respectively. QRT-PCR demonstrated that HOTTIP shRNA significantly reduced the expression level of HOTTIP while shRNA NC did not alter the expression level of HOTTIP (Figure 1C and 1D).

Effect of HOTTIP on Visual Function in Diabetic Rats

After diabetes induction for 5 months, we performed a visual electrophysiological examination on rats from each group. A significant decrease in the amplitude of a waves, b waves and OPs waves in rats were observed after 5 months of diabetes induction. Downregulation of HOTTIP can significantly improve retinal function and against downward trend of a waves, b waves and OPs waves (Figure 2A, 2B, 2C). The results in-

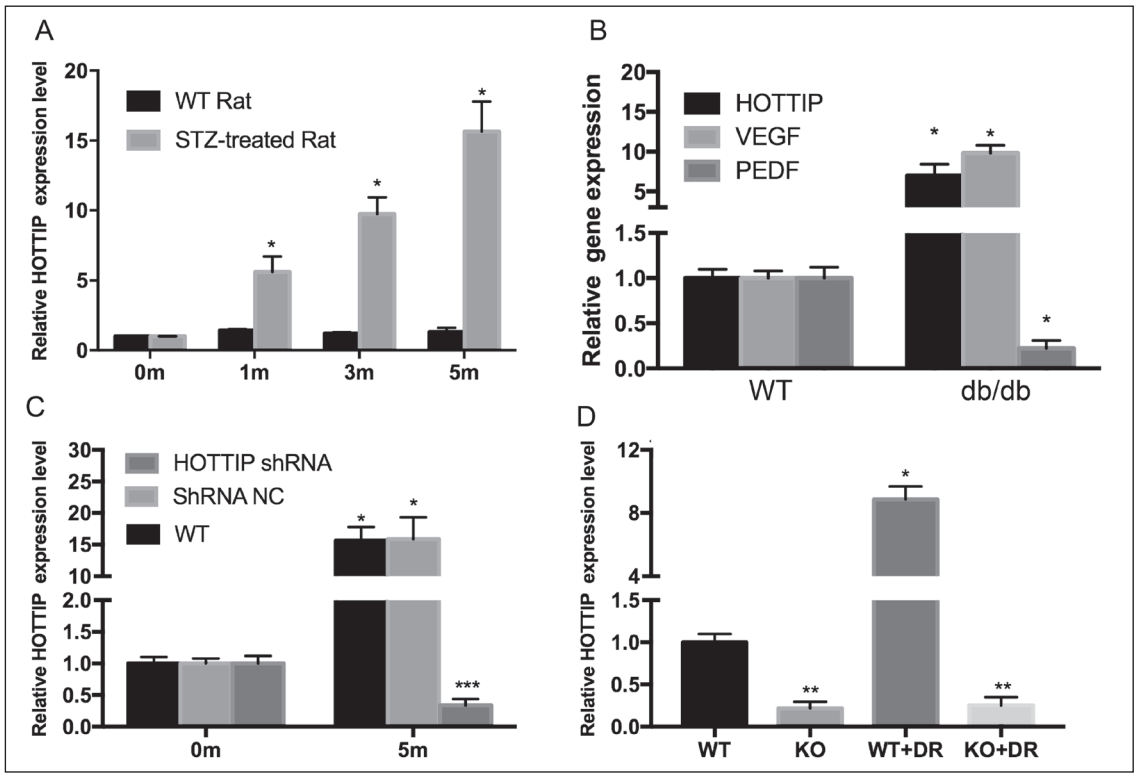

Figure 1. HOTTIP expression was significantly increased in the retina of diabetic mice. **A,** The expression of HOTTIP in STZ-induced diabetic rats and non-diabetic control rats was increased after 1 month, 3 months, and 5 months in a time-dependent manner, and the expression of HOTTIP was doubled as compared with the control. **B,** Expressions of HOTTIP and VEGF in Db/db mice was higher than those of the control group, while PDGF expression was significantly decreased. **C,** The expression of HOTTIP in the retina of diabetic mice induced by STZ was decreased after the injection of HOTTIP shRNA for 5 months. **D,** The expression of HOTTIP in mice from diabetic group was higher than that of wild-type, and the expression of HOTTIP in wild-type or diabetic group was decreased after knockdown of HOTTIP.

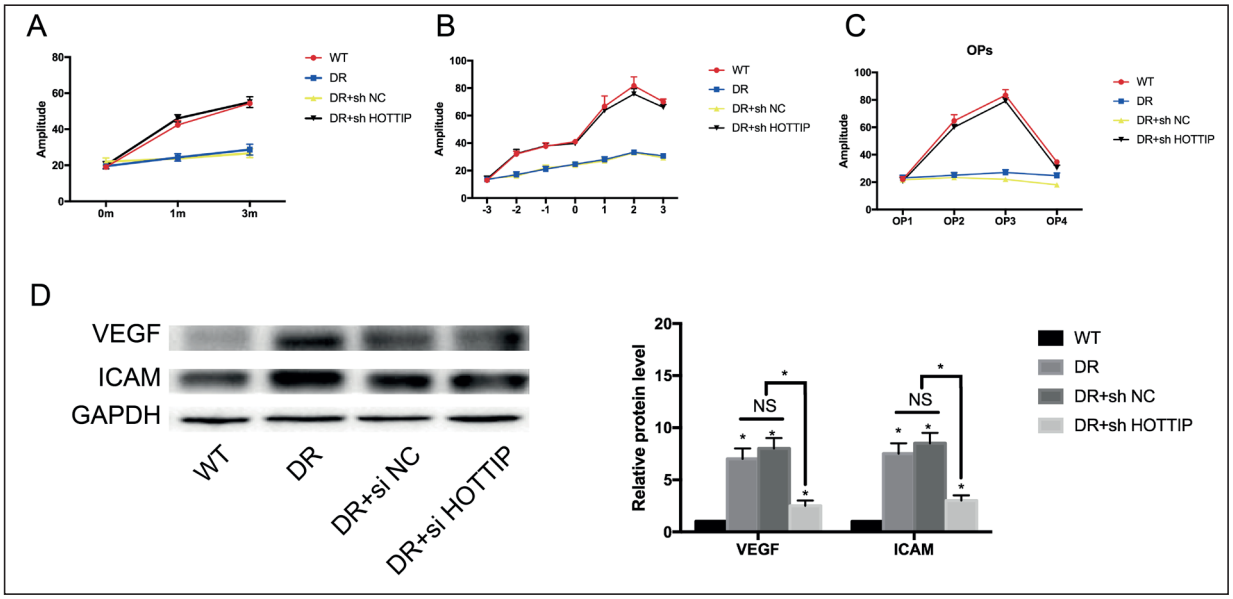

Figure 2. HOTTIP affected the retinal potential thus suppressing inflammation. **A, B** and **C,** After diabetes induction for 5 months, downregulated HOTTIP can significantly improve retinal function and fight against the downward trend of the a wave, b wave, OPs. **D,** Diabetes led to a significant increase in the expressions of ICAM-1 and VEGF. However, down-regulation of HOTTIP can significantly reduce the expressions of ICAM-1 and VEGF.

dicated that downregulated HOTTIP can prevent ERG (electroretinogram) abnormalities induced by diabetes.

Downregulation of HOTTIP Attenuated Retinal Inflammation in Diabetic Rats

Retinal inflammation is responsible for the diabetic microvascular syndrome, and expressions of proinflammatory proteins were significantly increased during the development of the diabetic complications, including ICAM-1, VEGF, and TNF-α[14]. Western blot was used to determine the expressions of ICAM-1 and VEGF in the retina. Diabetes caused a significant increase in the expressions of ICAM-1 and VEGF. However, downregulation of HOTTIP significantly decreased the expressions of ICAM-1 and VEGF (Figure 2D).

Effect of Downregulated HOTTIP on RF/6A Under High Glucose Environment/Oxidative Stress

To further explore the functional association of diabetes-induced up-regulation of HOTTIP. We examined the effect of downregulated HOTTIP on the viability of endothelial cells. We found that the transfection of HOTTIP siRNA led to a remarkable decrease in the level of HOTTIP (Figure 3A). Next, to investigate whether HOTTIP regulates the progression of hyperglycemia-induced apoptosis, we treated the RF/6A cells in high glucose medium with HOTTIP siRNA, si NC or untreated, respectively. We experimentally added H_2O_2 to RF/6A cells to simulate oxidative stress and downregulation was also performed.

Downregulation of HOTTIP can reduce the viability of RF/6A cells (Figure 3B and 3C). MTT results illustrated that high glucose/H_2O_2 can apparently decrease the amount of cell viability (Figure 3D and 3E). Meanwhile, downregulation of HOTTIP could significantly reduce the cell viability in high glucose/ H_2O_2 treated cell lines.

HOTTIP exacerbated apoptosis induced by high glucose/H_2O_2 compared with simple high glucose/H_2O_2 treatment (Figure 3F and 3G).

HOTTIP Regulated Retinal Endothelial Cells Via the p38-MAPK Pathway

In previous studies, bioinformatics analysis confirmed that MAPK signaling pathway participates in the pathogenesis of retinal neovascular disorders. As we speculated, there may be an association between HOTTIP and MAPK signal-

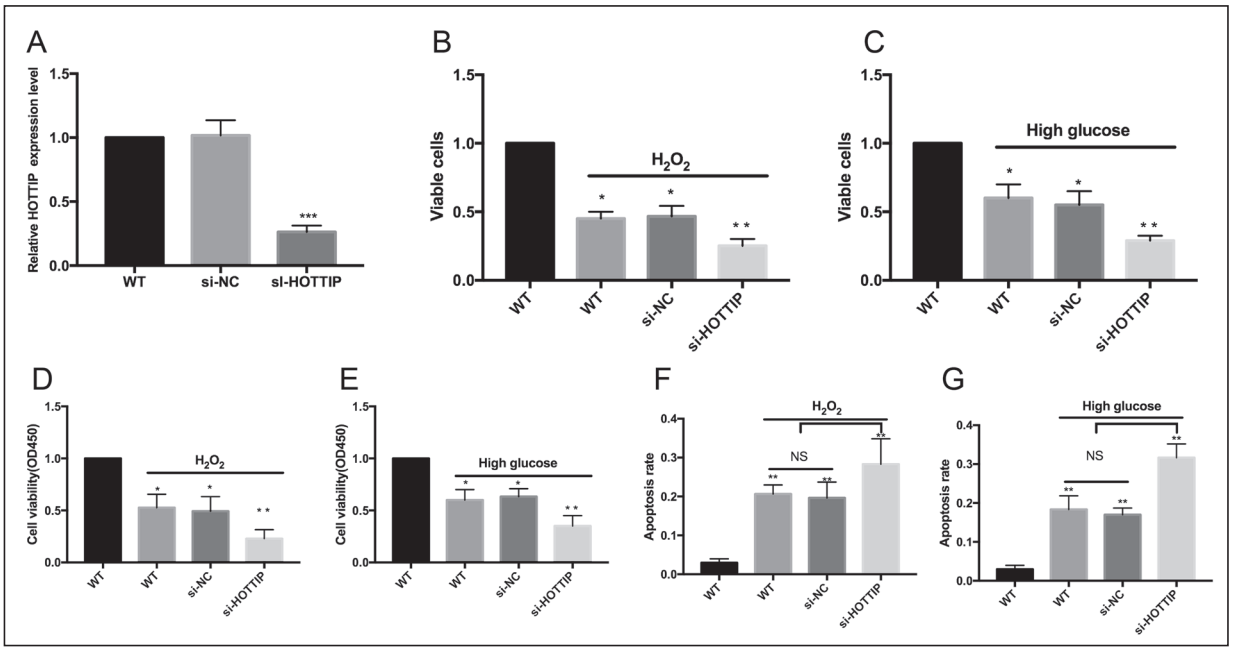

Figure 3. HOTTIP promoted the apoptosis of RF/6A cells and inhibited cell viability. **A,** The expression of HOTTIP was decreased after transfected with HOTTIP siRNA. **B** and **C,** Trypan blue staining results showed that high glucose/H_2O_2 can significantly reduce the number of living cells, downregulated HOTTIP can further reduce the number of RF/6A viable cells. **D** and **E,** MTT experimental results showed that high glucose/H_2O_2 can significantly reduced cell viability, while down-regulation of HOTTIP can further reduce the viability of RF/6A cells. **F** and **G,** Flow cytometry showed that high glucose/H_2O_2 can significantly promote apoptosis, while down-regulation of HOTTIP can further promote the apoptosis of RF/6A cells.

ing in diabetic-induced retinal vascular injury[15]. Here, we utilized Western blot to determine the activation of the MAPK pathway. We found that downregulated HOTTIP resulted in a significant decrease in phosphorylated p38; however, no effect on phosphorylated ERK1/2 or JNK1/2 was observed (Figure 4A, 4B). Downregulation of HOTTIP led to inactivation of the p38-MAPK pathway (Figure 4A, 4B). To further examine the relationship between the p38 MAPK pathway and the activity of HOTTIP-regulated cells, the pretreatment of RF/6A cells with p38 inhibitor SB203580 blocked the over-expression of HOTTIP, thereby causing increased viability of RF/6A cells. However, the ERK inhibitor U0126 or the JNK inhibitor SP600125 did not help to change in the viability mediated by HOTTIP (Figure 4C). In addition, p38-MAPK activation was suppressed by p38 siRNA, but not by ERK siRNA or JNK siRNA (Figure 4D). In conclusion, the abovementioned results suggested that HOTTIP modulates retinal endothelial cells *via* the p38-MAPK signaling pathway.

Discussion

So far, the effect of lncRNA in vascular biology has been well recognized. 31 unknown lncRNAs are identified in RNA sequences of human vascular smooth muscle cells. Among them, researches found that lncRNA-SENCR is a lncRNA that exists in vascular cells, and its downregulation can lead to decreased expression of genes related to cardiac muscle and smooth muscle contraction[16]. Lnc-Ang362 is an Ang II regulatory lncRNA

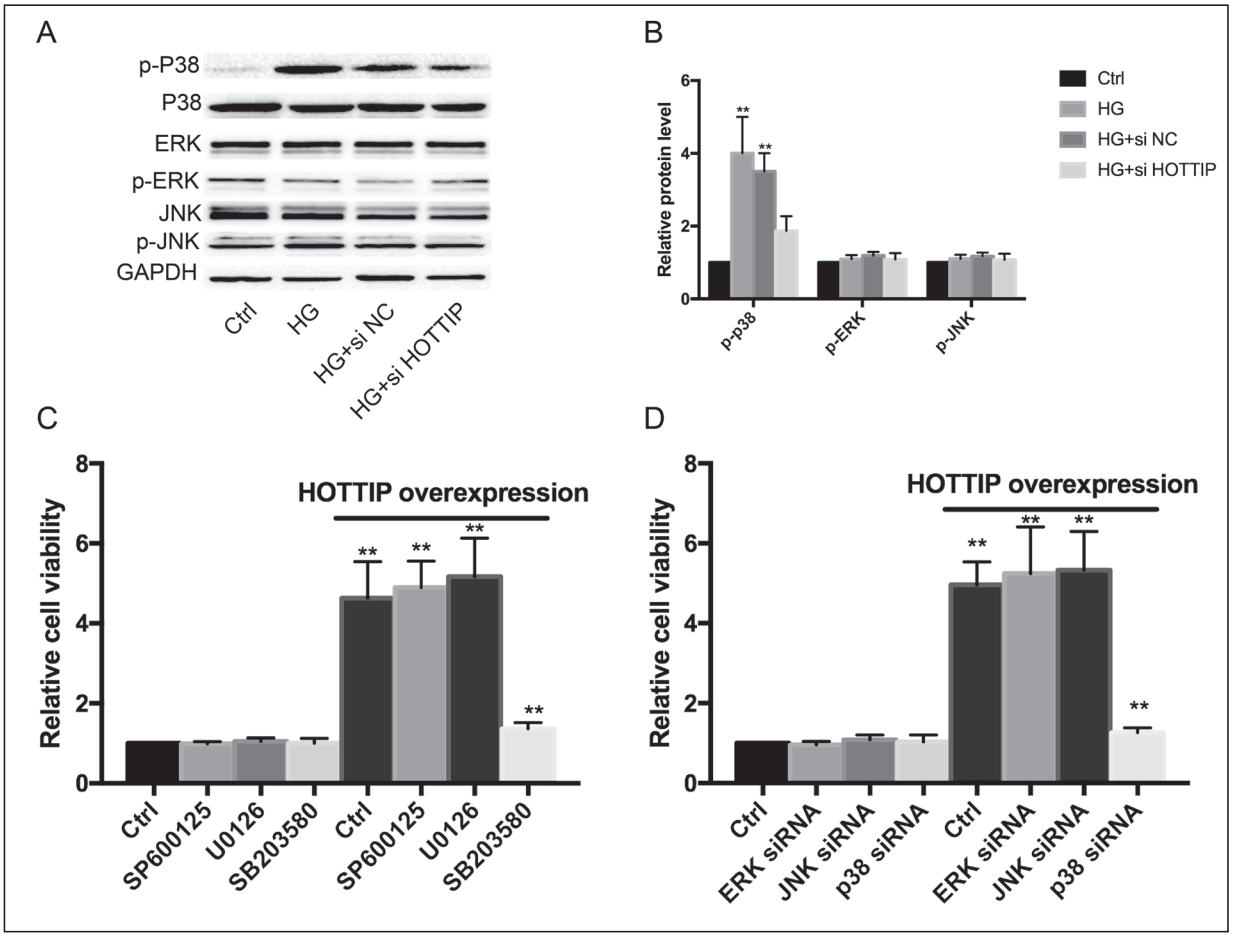

Figure 4. HOTTIP regulated retinal endothelial cells via the p38-MAPK pathway. **A** and **B,** Down-regulation of HOTTIP resulted in a significant decrease of phosphorylated p38, but had no effect on phosphorylated ERK1/2 or JNK1/2. **C,** Viability of RF/6A cells were increased after pretreatment of RF/6A cells with p38 inhibitor SB203580. However, ERK inhibitor U0126 or JNK inhibitor SP600125 had no effect on the change of cell viability induced by HOTTIP. **D,** P38-MAPK activity was inhibited by p38 siRNA, but not by ERK siRNA or JNK siRNA.

whose downregulation affects the proliferation of vascular smooth muscle. This suggested that lncRNAs also play essential roles in Ang-II-related (Angiopoietin-II) cardiovascular disease[17]. Downregulation of MALAT1 (metastasis associated lung adenocarcinoma transcript 1) can affect the proliferation and migration of vascular endothelial cells and reduce the growth of blood vessels through knockdown of genes or drug inhibition of MALAT1 expression[18]. Many studies have shown that lncRNA is greatly involved in the process of vascular disease.

As one of the most common vascular complications, diabetic retinopathy will occur in long-term diabetic patients and has a high rate of blinding[19]. Diabetic retinopathy has a complex pathophysiological mechanism. Sustained hyperglycemia in the retinal vessels leads to the accumulation of advanced glycation end products (AGEs), inflammatory reactions, oxidative stress and neuronal dysfunction[20]. Dysregulated biochemical activities resulted from retinal vessels further lead to increased retinal vascular permeability, apoptosis of vascular endothelial cells or pericytes, and retinal inflammatory response, which in turn bring severe visual impairment in diabetic patients[21-23]. Hyperglycemia is the most severe feature of diabetes and adversely affects vascular cells in diabetic vascular complications. Our results illustrated that high glucose-induced upregulation of HOTTIP expression in retinal vascular cells and the retina of diabetic animals. HOTTIP silencing can significantly reduce retinal neovascularization and retinal inflammation caused by diabetes. In addition, knockdown of HOTTIP gene can inhibit the proliferation and tubule formation capacity of vascular endothelial cells, as well as various aspects of cell function. In summary, our findings demonstrated that HOTTIP can rescue exacerbated retinal function caused by high glucose and significantly improve visual function.

Multiple extracellular stimuli can activate the MAPK signaling pathway, including high glucose stress. The MAPK signaling pathway activates a cascade of physiological outcomes, such as apoptosis, proliferative ability, cell mitosis, and the transcription of certain genes[24,25]. In the present study, we found that downregulation of HOTTIP can effectively alter the expression level of phosphorylated p38-MAPK, but not on phosphorylation of ERK1/2 or JNK1/2. Cell proliferation induced by HOTTIP can be inhibited by p38-MAPK inhibitor or p38 siRNA. All evidence indicated that a close relationship between HOTTIP expression and activation of p38-MAPK signaling pathway existes. HOTTIP is capable of regulating retinal vascular endothelial cells from various aspects via MAPK pathway. In summary, an accurate understanding of the molecular mechanisms of lncRNA in vascular disease processes is of great significance. It is a key step in exploring new therapeutic strategies.

Conclusions

We showed that HOTTIP promotes retinal cell inflammatory response by activating p38-MAPK, thereby promoting the progression of diabetic retinopathy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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