RNA sequencing reveals BMP4 as a basis for the dual-target treatment of diabetic retinopathy

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Abstract

Background Diabetic retinopathy (DR), currently considered as a neurovascular disease, has become the major cause of blindness. More and more scholars believe that DR is no longer just a kind of microvascular disease, but accompanied by retinal neurodegenerative changes. Intravitreal injection of anti-vascular endothelial growth factor (VEGF) drugs is a classic treatment for DR; however, anti-VEGF drugs can exacerbate fibrosis and eventually lead to retinal detachment. The aim of this study was to explore the pathogenesis of DR and identified new treatments that can provide dual-target intervention for angiogenesis and fibrosis.

Methods We explored changes in gene expression in high glucose–induced vascular endothelial cells using RNA sequencing (RNA-seq) technology. We identified bone morphogenetic protein 4 (BMP4) and SMAD family member 9 (SMAD9) among 449 differentially expressed genes from RNA-seq data and confirmed the expression of these two genes in the blood of diabetes patients by RT-PCR and in streptozotocin-induced rat retinas by RT-PCR, immunofluorescence, and western blot. Moreover, considering that DR is a multifactorial and multicellular disease, we used hydrogen peroxide (H₂O₂), advanced glycation end products (AGEs), CoCl₂, 4-hydroxynonenal (4-HNE), and hypoxia to induce three human retinal cell types (Müller, retinal pigment epithelium, and human retinal capillary endothelial cells) to simulate the pathogenesis of DR, and MTT experiment, scratch experiment, Transwell experiment, and lumen formation experiment were used to test whether the model was successfully established. Then, we verified the overexpression of these two genes in the cell models by RT-PCR, immunofluorescence, and western blot. We further tested the effects of BMP4 on retinal cells. We use BMP4 to stimulate retinal cells and observe the effect of BMP4 on retinal cells by MTT experiment, scratch experiment, and RT-PCR.

Results The results demonstrated that BMP4 and SMAD9 were highly expressed in both in vivo and in vitro models, while BMP4 could significantly upregulate the expression of SMAD9 and promote the expression of VEGF and fibrosis factors.

Conclusions This study is the first to analyze the mechanism by which high glucose levels affect retinal vascular endothelial cells through RNA transcriptome sequencing and indicates that BMP4 may be a potential target for the dual-target treatment (anti-VEGF and anti-fibrosis) of DR.

Key messages
• High-glucose effect on vascular endothelial cell was analyzed by RNA-seq.
• KEGG analysis revealed enrichment of TGF-beta signaling pathway.
• SMAD9 and BMP4 expression was upregulated in all samples.
• Dual-target therapy of PDR by antagonizing BMP4.

Keywords Diabetic retinopathy · RNA sequencing technology · High glucose · BMP4 · Dual-target treatment
Introduction

As a common and serious complication of diabetes, diabetic retinopathy (DR) has become the leading cause of blindness worldwide [1, 2]. In the last decade, increasing numbers of studies have shown that DR, which is accompanied by retinal neurodegenerative changes, can no longer be regarded simply as a microvascular disease but as a disruption of the retinal neurovascular unit [3, 4]. Epidemiological results suggest that long-term inadequate glycemia in cell dysfunction, oxidative stress, and inflammatory response in the course of DR [5–11].

Intravitreal injection of anti-VEGF antibodies has been widely used in the treatment of proliferative diabetic retinopathy (PDR) [12, 13]. However, the progression and development of fibrovascular membranes and traction retinal detachment followed by elevated connective tissue growth factor (CTGF) levels after anti-VEGF injection cannot be ignored [14, 15]. It is suggested that the effects of both VEGF and CTGF should be considered simultaneously in the treatment of DR to achieve the ultimate goal of dual-target therapy. Therefore, finding new targets to balance the effects of VEGF and CTGF has become an urgent task.

RNA sequencing (RNA-seq) technology is widely used in basic research, clinical diagnosis, and drug research, taking advantage of the ability to quickly obtain comprehensively transcribed sequence information from specific tissues or organs [16, 17].

In this study, we applied RNA-seq technology to high glucose–treated vascular endothelial cells, extracted the hidden information from an in-depth analysis of the data, and performed repeated verification in multiple models and functional assays. We detected the expression of bone morphogenetic protein 4 (BMP4) and SMAD family member 9 (SMAD9) in a series of models of diabetic retinopathy, such as streptozotocin (STZ)-induced rat retinas, blood of diabetes patients, and H₂O₂, advanced glycation end products (AGEs), CoCl₂, 4-hydroxynonenal (4-HNE), and hypoxia-induced cell models. The results showed that these two genes were highly expressed in the pathological state of diabetic retinopathy. We further evaluated the function of BMP4 and SMAD9 and found that BMP4 could promote the proliferation and migration of retinal cells and promote the expression of VEGF. The results clearly demonstrated that BMP4 and SMAD9 are potential novel targets for dual-target treatment of DR. The further role of BMP4 in diabetic retinopathy remains to be explored.

Methods and materials

Cell culture

We used RF/6A cells (Tianjin Medical University Eye Institute, China) for most experiments. RF/6A cells were grown in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and an antibiotic mixture of 100 U/mL penicillin (Gibco, Thermo Fisher Scientific, USA) and 100 U/mL streptomycin (Gibco, Thermo Fisher Scientific, USA). We also used human retinal capillary endothelial cells (HRCECs), Müller cells, and ARPE-19 cells in follow-up validation experiments. HRCECs, Müller cells, and RPE cells were stored at the Tianjin Medical University Eye Institute. HRCECs and Müller cells were grown in high-glucose DMEM (Gibco, Thermo Fisher Scientific), and RPE cells were grown in DF/12 (Gibco, Thermo Fisher Scientific), which supplemented with 10% fetal bovine serum and an antibiotic mixture of 100 U/mL penicillin and 100 U/mL streptomycin. Cells were passaged when they reached 90% confluence, and cells at or before six passages were used for experiments. All cells were incubated in a sterile humidified atmosphere at 37 °C in 5% CO₂.

Cell stimulus treatments

We used high glucose to treat RF/6A cells. RF/6A cells were cultured to nearly 50% confluence in 6-well dishes before being starved in serum-free medium overnight. The cells were then divided into two groups: a control group (N) and a high-glucose group (HG). Previously, Wang et al. [16] reported the plasma glucose concentration to be approximately 5.8 mM. Thus, we used 5 mM glucose in N. Most in vitro studies use 25 mM glucose for 48 h.

We also used H₂O₂ to stimulate the cells. Cultured cells were incubated with different concentrations of H₂O₂ (0 μmol/L, 100 μmol/L, 200 μmol/L, 400 μmol/L, 800 μmol/L, and 1000 μmol/L) for different time periods (1 h, 2 h, 3 h, and 4 h), followed by 24 h of recovery.

A hypoxic environment was achieved using a dedicated incubator. Hypoxic cell culture conditions were maintained under 2% oxygen atmosphere (via injection of N₂). Cells were maintained under hypoxic conditions for 3 h or 6 h, after which the culture medium was replaced, and the cells were maintained under normal conditions for follow-up experiments.

Cells were treated with different concentrations of 4-HNE (5 μM, 10 μM, 20 μM, 40 μM, 80 μM, and 100 μM), of CoCl₂ (10 μM, 25 μM, 50 μM, 100 μM, and 200 μM), or
Oxidative stress is associated with DR, so we used H2O2 to stimulate cells. The effects of hypoxia and CoCl2 represent the physical and chemical characteristics of ischemic hypoxia in DR. In addition, 4-HNE was used to simulate the accumulation of lipids during the DR process, and AGES were used to simulate the accumulation of glycosylated end products in the DR process.

**MTT assay**

A total of $3 \times 10^4$ cells per well were seeded in 96-well plates. After treatment with different stimulants, 110 μL of MTT solution (Solarbio, China) was added into each well and incubated for 4 h at 37 °C. The MTT in each well was then replaced with 150 μL DMSO (Solarbio, China), and the optical density (OD) at 490 nm was measured with a microplate reader (Thermo, USA).

**Wound healing assay**

A total of $2 \times 10^5$ RF/6A cells per well were cultured in 12-well plates. When the cells grew to approximately 80% confluence, a linear wound was generated by scratching the cells with a sterile pipette tip. The cells were washed twice with PBS (Gibco, Thermo Fisher Scientific, USA) and then incubated in RPMI 1640 medium. Cell migration toward the wounds was imaged in the same visual field after 0 h and 24 h with a phase-contrast microscope (Olympus, Tokyo, Japan). Cell migration was measured by cellSens Standard software, and the migration rate (%) was defined as follows: migration rate = ($S_T - S_0$) / $S_0 \times 100\%$ (where $S_0$ is the raw bare area and $S_T$ is the bare area at each time point).

**Transwell assay**

A total of $1 \times 10^5$ cells per well were seeded into a Transwell insert (Corning, Tewksbury, MA) and then cultured with culture media without serum. Different stimuli were added to the lower chambers. Cells were subsequently allowed to migrate across the polycarbonate filter for 24 h at 37 °C. Nonmigrated cells on the top side of the filter were removed by scraping. Migrated cells on the bottom side of the filter were subsequently fixed with 4% paraformaldehyde (PFA) (Solarbio, China) for 30 min and stained with 0.1% crystal violet solution (Solarbio, China) for 20 min. The number of stained cells in five random fields of each well was counted using ImageJ software to determine the average number of migrated cells.

**Tube formation assay**

Twenty-four-well plates were coated with 300 μL Matrigel (BD, San Jose, CA, USA) per well and incubated at 37 °C for 20 min to allow the Matrigel to solidify. A total of $1 \times 10^5$ cells per well were seeded into the plate and incubated with different stimuli at 37 °C for 6 h. The cells in five randomly selected fields per well were then imaged using a Zeiss digital camera. Tube formation was quantified by measuring the length of capillary structures using ImageJ software. The average tube length in five fields was taken as the value for each sample.

**RNA-seq**

RNA-seq was performed as previously described [16]. Briefly, total RNA was extracted from cells in different groups, and various parameters reflecting the quality of the extracted total RNA were determined. After determining that the total RNA samples were of high quality, magnetic beads were used to enrich and fragment the mRNA, which has a poly(A) tail, and a library was constructed according to the common New England Biolabs (NEB) library construction method and a chain-specific library construction method. Following library construction, fluorescence quantitative reverse transcription was applied to accurately quantify the concentration of the library (the concentration of the library was greater than 2 nmol/L) on the basis of preliminary quantification by a fluorometer to ensure the quality of the library. After the library was qualified, data from the different libraries were collected according to the effective RNA concentration and the demand of the operation data quantity. The Illumina HiSeq/MiSeq sequencer captured the fluorescence signal and converted the optical signal into a sequencing peak through computer software to obtain sequence information on the tested fragments.

**Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses**

Differential gene expression was determined from the obtained gene expression profiles with the edgeR function in Bioconductor software. The edgeR function assumed that the count of sequencing reads for each gene was a negative binomial distribution, and the hypothesis test was conducted based on this theory. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted based on the protein sequences encoded by genes exhibiting significant differential expression ($P \leq 0.001$).

Blast software was used to compare the gene sequences to the nonredundant (nr) database and the KEGG database, followed by the extraction of GO and pathway annotation information for all genes. The top GO function and
hypergeometric test in Bioconductor software were used to analyze the GO and KEGG pathways of the differentially expressed genes. The data were screened for genetic variations, log-2 to log2 interval meaningless data were screened and removed, and the data were listed one by one according to the $P$ value for identification. Then, through consulting the relevant literature, we investigated the role of each gene in different tissues and its mechanisms. In addition, according

Fig. 1 Differentially expressed genes (DEGs) between the two groups were determined through RNA-seq analysis. a Volcano plot of the DEGs. The $X$-axis represents the log2-fold change in genes expressed at high (positive values) and low (negative values) levels. Significantly upregulated genes (log2 (FC) > 2 and $P < 0.05$) are represented by red spots, while downregulated genes (log2 (FC) < −2 and $P < 0.05$) are represented by green spots. b Results of GO enrichment analysis of DEGs categorized by biological process (BP), molecular function (MF), and cellular component (CC). DEGs enriched in the BP, MF, and CC terms by GO enrichment analysis with the smallest significance level using Fisher’s exact test are listed. c Global view of KEGG enrichment pathways
to the role of the genetic pathways, we investigated the relationships between significantly differentially expressed genes.

**RNA extraction**

Then, 200 μL chloroform was added to the samples with TRizol (Invitrogen, USA). After centrifugation at 1200×g for 10 min at 4 °C, approximately 500 μL of the supernatant was transferred into another EP tube. Then, 500 μl of isopropanol was added to the EP tube and mixed well. After centrifugation at 1200×g for 5 min at 4 °C, the supernatant was discarded, 1 mL of 75% ethanol was added, and the sample was centrifuged at 1200×g for 5 min at 4 °C. The supernatant was discarded, and the EP tube was left open in the fume hood for 30 min. When the precipitate in the EP tube was nearly completely dry, 30 μl DEPC water was added to dissolve the RNA precipitate.

**Reverse transcription quantitative PCR**

We chose six significantly differentially expressed genes for reverse transcription quantitative PCR (RT-qPCR) validation. RT-qPCR was performed using purified RNA from six kinds of samples: high glucose–cultured RF/6A cells, diabetic...
patient blood, HRCECs, Müller cells, and RPE cells under different stimuli.

Total RNA was extracted, and the RNA concentration was detected by ultramicro-spectrophotometer. An A260/A280 ratio between 1.8 and 2.0 was considered sufficient to use the RNA in subsequent reverse transcription reactions. The total RNA was reverse transcribed into complementary DNA (cDNA) by a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. GAPDH was used as a reference control for RT-qPCR, and Primer Express 3.0 was used to design primer sequences, which are listed in Table S1. Two microliters of cDNA, 2 μl of primers, and 4 μl of SYBR (Roche, Switzerland) were mixed and added to 384-well plates, after which RT-qPCR was performed in a real-time PCR instrument (Life Technologies, USA).

The thermal cycling conditions for qPCR were as follows: 40 cycles of 50 °C for 2 min, 95 °C for 10 min, and 95 °C for 15 s, followed by 55 °C for 15 s and 72 °C for 30 s, followed by a dissociation stage (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s), Relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ formula.

**Peripheral blood mononuclear cell isolation**

Blood was isolated from patients in Tianjin Medical University Eye Hospital. The process was approved by the Ethics Committee of Tianjin Medical University Eye Hospital (Tianjin, China). Blood was collected in an EDTA anticoagulant tube. After centrifugation at 1800×g for 5 min, the lower red liquid was diluted with an equal amount of PBS at room temperature. Ficoll peripheral blood mononuclear cell (Solarbio, China) was added to the diluted blood.

After centrifugation at 1200×g for 20 min, the liquid in the centrifuge tube was layered. The middle white film layer was carefully aspirated with a pipette and placed in another 15-mL centrifuge tube, and 1 mL TRIzol was added to the white precipitate after washing. Finally, the samples were stored at −80 °C.

**Immunofluorescence**

Cells or tissues were fixed in 4% PFA for 20 min and then incubated in 0.1% Triton X-100 (Solarbio, China) for 10 min, followed by a 1-h incubation in blocking solution consisting of 10% goat serum (Solarbio, China), 0.1% Tween 20 (Sigma, USA), and 1% BSA (Solarbio, China) in PBS. Cells or tissues were incubated with rabbit anti-SMAD9 (Bioss, China) diluted 1:400 in goat serum blocking solution and rabbit anti-BMP4 (Boster, China) diluted 1:20 in goat serum blocking solution at 4 °C, followed by the addition of goat anti-rabbit IgG H&L (Alexa Fluor® 488) (1:500, Abcam, UK). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Solarbio, China). The cells or tissues were observed under a fluorescence microscope (U-LH100L-3; Olympus Corporation, Tokyo, Japan).

**Western blot**

Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate). Thirty micrograms of protein lysate was separated by a 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Millipore, USA). The primary antibodies used were anti-SMAD9 (1:1000; Bioss, China) and anti-BMP4 (1:1000; Boster, China). Anti-GAPDH antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA) was used as a loading control. After washing, the membrane was incubated with a horseradish peroxidase (HRP)–conjugated secondary antibody (1:3000; GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. Protein bands were visualized by ECL (Amersham Pharmacia Biotech, Arlington Height, IL, USA) on an X-ray film, which was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**STZ-induced diabetes in mice**

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Diabetes was induced in 6- to 8-week-old adult male Sprague Dawley (SD) rats (200–220 g). The rats were housed 3 per cage under a 12/12-h light/dark cycle at a temperature of 19 to 23 °C. The rats were randomly divided into two groups: a normal group and a diabetic group. Then, rats in the diabetic group received a caudal injection of a freshly prepared solution of streptozotocin at 55 mg/kg bodyweight. Three days after injection, we measured the blood glucose levels of the rats, and rats with a blood glucose level of 16.7 mmol/L or higher were considered to have diabetes.
Statistical analysis

One-way analysis of variance (ANOVA) and independent samples t tests were used to assess the significance of differences between groups, and the data are presented as the mean ± standard deviation (SD). All experiments were repeated at least three times, and $P < 0.05$ indicated statistically significant differences.

Results

Effects of high glucose–induced cell proliferation, migration, and tube formation in RF/6A cells

Hyperglycemia is an important factor leading to DR, so we constructed a model of retinal vascular endothelial cells induced by hyperglycemia in vitro to explore the pathogenesis of DR. An in vitro MTT assay was carried out to examine the proliferative ability of RF/6A cells after the cells had been treated with high glucose (25 mM) for 24 h. The results showed that 25 mM glucose treatment significantly increased the proliferation of RF/6A cells compared with 5 mM glucose treatment.

A wound scratching assay and Transwell assay were conducted to examine whether high glucose levels were able to modulate the migration of RF/6A cells. The results showed that 25 mM glucose significantly accelerated the wound closure and increased the number of migrating cells compared with the 5 mM glucose treatment.

To examine the effect of glucose (25 mM) on angiogenesis, the tube formation of RF/6A cells was evaluated by Matrigel assay. The results showed that 25 mM glucose significantly increased the number of capillary-like structures compared with the 5 mM glucose treatment.

Upon stimulation with high glucose, the proliferation ability, migration ability, and lumen formation ability of retinal vascular endothelial cells were significantly improved, indicating that the high-glucose model was successfully established and could be used for the RNA-seq analysis in the next step (Fig. S1).

Sample quality and gene expression levels

To obtain precise results, three RF/6A cell samples were selected from the control and high-glucose groups. The total number of reads from the control group and the high-glucose group were 47,865,006 and 46,731,260, respectively, and there was no significant difference between the numbers of reads between the two groups. On average, 61.59% of the total reads were mapped to the reference genome. Detailed sample quality data are listed in Table S2.

We analyzed differences in gene expression between the two different groups. Compared with gene expression in the control group, high-glucose treatment changed the expression of 449 genes. Of the 449 differentially expressed genes, 297 were upregulated and 152 were downregulated, and the top 20 significantly differentially expressed genes are listed in Table S3. To further investigate differentially expressed genes in the high glucose–treated RF/6A cells, we explored pathway enrichment with the KEGG database. KEGG pathway enrichment analysis revealed the involvement of the differentially expressed genes in 207 pathways, including the TGF-beta signaling pathway. KEGG pathway enrichment results are presented in Fig. 1.

Confirmation of differentially expressed genes in glucose-induced RF/6A cells

To confirm the RNA-seq findings, we validated the expression of six genes by RT-qPCR in the sequenced samples. These six genes were SMAD9, BMP4, DAB1, ATP1A3, CHST5, and UNC5C. These genes are involved in cancer, cardiovascular disease, and other diseases, but their involvement in DR has not been shown. BMP4 was previously shown to be involved in regulating VEGF. BMP4, SMAD9, DAB1, and CHST5 are related to extracellular matrix metabolism. ATP1A3 regulates the Na+/K+ ATP enzyme system, causing ischemia and apoptosis in ganglion cells. Moreover, UNC5C can also cause apoptosis in ganglion cells (Fig. 2).

From the RT-qPCR data, we found that the expression of SMAD9 and BMP4 was significantly increased in the sequenced samples, which is consistent with their expression levels determined by RNA-seq, indicating that high glucose can upregulate the expression of the SMAD9 and BMP4 genes. Moreover, we used immunofluorescence and western blot to determine the expression and distribution of SMAD9 and BMP4 in high glucose–treated RF/6A cells. From the immunofluorescence and western blot results, we found that BMP4 and SMAD9 expression was significantly higher in
high glucose–treated cells than in the 5 mM glucose treatment group (Fig. 2).

**Confirmation of differentially expressed genes in blood samples**

To characterize the expression of these differentially expressed genes in DR patients, we collected blood samples from 20 diabetic patients and 20 normal people and extracted peripheral blood monocytes to detect the expression of these differentially expressed genes. Before testing gene expression, we conducted statistical analysis on the baseline data on gender, age, BMI, and other clinical data of the study subjects, and the results showed that there were no significant differences in the baseline data except for HBA1c and fasting blood glucose (Tables S4 and S5).

We detected the expression of the six selected differentially expressed genes in patients through RT-qPCR, and the results showed that BMP4 and SMAD9 were highly expressed, which was consistent with the RNA-seq results and the results verified in the original model. We further analyzed BMP4 and SMAD9 in each patient with DR. The details of each patient are listed in the table below, and the results showed that the expression of these two genes was enhanced in the patient blood samples (Fig. 3).

**Characterization of the expression of BMP4 and SMAD9 in the retinas of STZ-induced diabetic rats**

To better understand the possible role of BMP4 and SMAD9 in DR, we attempted to determine the expression and distribution of these two genes in the retina. Since human retinal specimens are difficult to obtain, we selected rat retinas to observe the expression of these two genes. We first established a STZ-induced DR rat model, and by measuring changes in blood glucose and body weight, we found that all STZ-induced rats displayed typical diabetic symptoms of polyphagia, polydipsia, polyuria, and emaciation, and the blood sugar concentration was significantly higher in the STZ-induced rats than in the control rats. From HE staining, we observed that compared to that of the retinas of normal rats, the arrangement of retinal cells of diabetic rats became disordered, and microvascular expansion and microvascular lesions were observed. These changes indicated that our diabetic rat model was successfully established. To validate the expression of BMP4 and SMAD9, retinas from diabetic and normal rats were prepared for RT-qPCR, immunofluorescence, and western blot experiments. As shown in Fig. 4, we found that BMP4 and SMAD9 were distributed throughout the rat retina and were more highly expressed in diabetic rats than in normal rats. To further investigate the distribution and expression of these two genes in the retina, we examined them in an in vitro cell model.

**Characterization of the expression of BMP4 and SMAD9 in an HRCEC DR model**

Since the blood of diabetic patients and the retina of diabetic rats can only be used to study the expression of differentially expressed genes at the overall level, we constructed a DR cell model in vitro to further study the distribution and expression of BMP4 and SMAD9. Since DR is also a vascular disease, we first observed the expression of these two genes in HRCECs. In addition, DR is a multifactorial disease, so HRCECs were stimulated in H2O2, hypoxia, 4-HNE, AGEs, and CoCl2.

We used the MTT assay, wound scratching assay, and tube formation assay to explore the effects of these five stimulation methods on HRCECs. The results showed that hypoxia, 4-HNE, and CoCl2 could promote the proliferation, migration, and tube formation of HRCECs, while H2O2 and AGE stimulation could suppress these angiogenesis processes. The results were statistically significant, indicating that these five HRCEC DR models had been successfully established. Next, we aimed to verify the expression of differentially expressed genes in the HRCEC DR cell models (Fig. S2).

Based on the successful establishment of the model, we detected the expression of BMP4 and SMAD9 in the five HRCEC models by PCR, western blotting, and immunofluorescence. The results showed that the expression of these two genes was increased at both the RNA and protein levels, with statistically significant differences. Next, we aimed to verify the expression of these two genes in other cell models (Fig. 5).

**Characterization of the expression of BMP4 and SMAD9 in Müller DR cell models**

Since DR is a neurovascular disease that can cause neuropathy in addition to microvascular lesions, we explored whether BMP4 and SMAD9 could be detected in Müller cells and investigated their expression in the Müller cell models of DR. We used the MTT assay, wound scratching assay, and Matrigel assay to observe the effect of H2O2, hypoxia, 4-HNE, AGEs, and CoCl2 on the proliferation and migration ability of Müller cells.

The results showed that H2O2, hypoxia, and 4-HNE could promote the proliferation and migration of Müller cells, while
AGES and CoCl₂ inhibited the proliferation and migration of Müller cells. These results indicated the establishment of DR in Müller cells, and next, we verified BMP4 and SMAD9 expression in these five cell models (Fig. S3).

The proliferation, migration, and other assays showed that a DR Müller cell model had been successfully established, based on the detection of the distribution and expression of BMP4 and SMAD9 in these models by PCR, western blotting, and immunofluorescence. The results showed that these two genes were highly expressed in the DR Müller cell model (Fig. S4).

**Characterization of the expression of BMP4 and SMAD9 in DR RPE cell models**

RPE is located between the choroid and the neurosensory retina and forms the external retinal barrier, and RPE cells also play a significant role in DR. Therefore, we measured the expression of the two genes in RPE cells affected by DR. We treated RPE cells with H₂O₂, hypoxia, 4-HNE, AGEs, and CoCl₂ to simulate the DR eye microenvironment, and we used the MTT assay, wound scratching assay, and Matrigel assay to observe whether these cell models were successfully established. From the results, we can see that these five stimuli significantly promoted the proliferation and migration of RPE cells. Next, we detected the expression of BMP4 and SMAD9 in these established cell models (Fig. S5).

We determined that the RPE cell model for DR was successfully established by a variety of methods. Next, we examined the expression of the differentially expressed genes BMP4 and SMAD9 in these five cell models. The results showed that by PCR, immunofluorescence, and western blotting, BMP4 and SMAD9 were significantly increased at both the protein and RNA levels in the RPE cell model, with statistically significant differences (Fig. S6).

**Exploring the effect of BMP4 on DR in retinal cells**

In a previous work, to eliminate the false-positive results of RNA-seq, we verified the expression of BMP4 and SMAD9 in the blood of diabetic patients, in vivo animal models, and a variety of cell models in vitro, and the results showed that these two molecules were highly expressed in a variety of models. Next, to further clarify the role of these two genes in DR, we first explored the relationship between BMP4 and SMAD9 through immunofluorescence, western blotting, and PCR, and the results showed that BMP4 could promote the expression of SMAD9. Next, we stimulated RF/6A cells, HRCECs, Müller cells, and RPE cells with BMP4 and determined that BMP4 could significantly promote the proliferation and migration of retinal cells, as demonstrated by MTT and wound scratch assays. In addition, we explored the function of retinal vascular endothelial cells induced by BMP4, and the results showed that BMP4 could significantly promote the expression of VEGF and fibrosis-related factors in vascular endothelial cells (Fig. 6).

**Discussion**

It is known that retinal neovascularization and fibrotic membranes are hallmarks of PDR [18]. Anti-VEGF drugs have gradually become an effective way to assist the treatment of PDR due to their significant effect on neovascular and exudative lesions [13]. However, a large number of studies have found that anti-VEGF drug therapy can lead to increased expression of fibrotic factors and aggravate the fibrotic process [19, 20]. Previous studies have found that CTGF expression in the proliferative membrane of PDR patients treated with anti-VEGF drugs was significantly decreased, but fibrotic factor expression was significantly upregulated [21]. Therefore, we also hope to explore novel dual-target therapies for DR by mining its pathogenesis.

In the present study, we used RF/6A cells stimulated with high glucose levels to simulate the ocular environment in DR patients to some extent. Stimulation with high glucose levels could promote the proliferation and migration of cells, indicating the remarkable influence of a high glucose state on the biological characteristics of RF/6A cells. To further understand the mechanism by which high glucose levels affect vascular endothelial cells, we used RNA-seq to comprehensively sequence transcripts in a DR cell model. With the accurate and quantitative recognition of molecular markers by RNA-seq, we successfully identified two differentially expressed genes, BMP4 and SMAD9, which belong to the TGF-β pathway.

Bone morphogenetic proteins (BMPs), members of the TGF-β family, are involved in many cellular functions [22]. BMP4, a member of the BMP family, is involved in eye development, including protecting Müller glial cells in the chick retina, promoting cell invasion and migration in malignant melanoma and smooth muscle, regulating the transcription and secretion of the VEGF gene in ARPE-19 cells and zebrafish embryos, and contributing to renal fibrosis [23–27]. In detail, BMP4 increased VEGF secretion in a dose- and time-dependent manner by binding to BMP-activated SMAD-binding elements, affecting the phosphorylation of R-SMADs (SMAD1, SMAD5, and SMAD9) and forming complexes with Smad4, which ultimately activate BMP target genes [28–31]. BMP4 affects the phosphorylation of SMAD9 and forms complexes with Smad4, which finally induces BMP target genes. Many studies have shown the up-regulation of SMAD9 expression by BMP4 in many cell types, such as C2C12, H9c2, 3T3-L1, HepG2, B16 cells, and primary fibroblasts [32, 33]. Moreover, BMP4 significantly increased the collagen production of fibroblasts and induced the differentiation of fibroblasts into myofibroblasts [34]. In a variety of cells, high expression of BMP4 significantly upregulates extracellular components, including FN,
Fig. 6 The function of BMP4 was validated in retinal cells. a Wound scratch assays were performed to measure cell migration under BMP4 stimulation. Scale bars: 200 μm. b Under BMP4 stimulation, SMAD9 expression in various cells was detected by immunofluorescence. Scale bars: 50 μm. c Quantitative analysis of wound scratch assay results. d Quantitative analysis of cell immunofluorescence experiment results. e TT assay was performed to assess survival of different cells under BMP4 stimulation. f The expression of SMAD9 in various cells stimulated by BMP4 was detected by PCR. g The expression of SMAD9 in various cells stimulated by BMP4 was detected by western blot. h Quantitative analysis of western blot results. i The effect of BMP4 on the expression of VEGF and fibrosis-related factors in retinal vascular endothelial cells was detected by PCR. All values represent the mean ± standard deviation. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control group.
BMP4 and SMAD9 may function as partners to play a role in angiogenesis and fibrosis in DR.

Binding of BMP4 to the TGF-β receptor initiates SMAD9 phosphorylation and translocation into the nucleus from the cytoplasm. Afterwards, a functional protein complex consisting of SMAD4 and SMAD9 is formed, which leads to upregulate the transcription and secretion of the VEGF gene and the promotion of extracellular matrix formation.

Considering that RNA-seq may contain false-positive results, in order to minimize this possibility, we tried our best to confirm the expression of the identified differential genes by establishing multiple DR models in diverse cell types. We first measured the expression of these two genes in the blood of people with diabetes. In DR, blood is the most practical and clinically significant experimental sample. The expression of the two factors in patient blood samples was measured to fully represent the true distribution of these two factors in DR patients. We collected blood from 20 diabetic patients and 20 healthy subjects and isolated monocytes. In the RNA extracted from peripheral blood lymphocytes, we detected the expression of these two genes by PCR. We studied the expression level of these two genes in the 20 patients on average and in each diabetic patient. The results showed that the expression of BMP4 and SMAD9 was increased in peripheral blood lymphocytes of diabetic patients, and the expression was increased in each diabetic patient.

Then, we detected the expression of these two genes in STZ-induced rat retinas. Since these two genes are highly expressed in DR, it is necessary and meaningful to detect the expression and distribution of these two genes in retinal samples. However, since it is difficult to obtain human retinal samples, we chose rat retinas as the most genetically similar experimental model available to us. The immunofluorescence results on rat retinas demonstrated that BMP4 and SMAD9 are highly expressed in all layers of the retina. Then, we extracted RNA and protein from the rat retinas, and the results showed that the expression of BMP4 and SMAD9 was upregulated at both the RNA and protein levels.

By studying the expression of BMP4 and SMAD9 in the blood of DR patients and in rat retinas, we adopted a comprehensive approach to understand the distribution of these molecules. The abovementioned results demonstrate that DR is a disease resulting from multiple factors that affect multiple cells. Subsequently, we designed a series of assays to verify the expression of BMP4 and SMAD9 in diverse disease models with multiple cell types. Therefore, considering the involvement of the neurovascular unit, we studied the expression of BMP4 and SMAD9 in HRCEC, Müller, and RPE cell models, which are the representative cellular components of the neurovascular unit. DR is a multifactorial disease, and classical theories suggest that it is caused by the accumulation of glycosylated end products, lipid metabolism disorders, oxidative stress, and so on [35, 36]. Therefore, in this study, we used five different methods to stimulate cells to mimic the microenvironment of retinal cells in DR from different perspectives. Oxidative stress is associated with DR, so we used H2O2 to stimulate cells [37]. The effects of hypoxia and CoCl2 represent the physical and chemical characteristics of ischemic hypoxia in DR [38, 39]. In addition, 4-HNE was used to simulate the accumulation of lipids during the DR process, and AGEs were used to simulate the accumulation of glycosylated end products in the DR process [40, 41]. We first used cell viability, migration, and lumen formation assays to observe the effects of these stimuli on cell proliferation, migration, and other biological characteristics and to determine whether each cell model was successfully established. Then, we examined the BMP4 and SMAD9 expression in these cell models, and the results showed that the increased expression of these two genes in the different cell models was consistent with the RNA-seq results.

We also stimulated different retinal cells (RF/6A, HRCEC, Müller, and RPE) with BMP4 to observe the effect of BMP4 on multiple retinal cells. We found that BMP4 significantly affected the proliferation, migration, and lumen formation of a variety of retinal cells. Moreover, we also observed the regulatory effect of BMP4 on SMAD9 and the effect of these two factors on the cellular functions of multiple retinal cells. From the experimental results, we found that after treating cells with BMP4, the expression of SMAD9 was significantly increased at both the RNA and protein levels. Under the condition of high levels of both BMP4 and SMAD9, the expression of fibrosis-related factors and VEGF in cells was significantly upregulated, which further confirmed our previous hypothesis. Therefore, BMP4 and SMAD9 may function together to play a role in angiogenesis and fibrosis in DR.

In recent years, a consensus has been reached in the field of ophthalmology, whereby the inhibition of the formation of new blood vessels and the control of fibrosis are considered equally important in the treatment of DR, and some pioneers have proposed the idea of dual-target therapy and have exerted considerable efforts in putting it into practice. They used VEGF inhibitors and anti-CTGF shRNA simultaneously to treat diabetic rats and found that the therapy could help restore normal transcription levels of CTGF and VEGF and improve retinal vascular dysfunction [42]. In comparison, BMP4, which is the focus of our study, is capable of killing two birds with one stone, namely regulating the expression of CTGF and VEGF simultaneously. Another significant advantage is that the factors related to fibrosis affected by BMP4 are far more than just CTGF, FN, and laminin α. Both SMA and collagen I are also under its control.

Conclusions

In conclusion, effectively antagonizing BMP4 can not only inhibit the expression of VEGF, that is, the formation of new blood
vessels and the occurrence of leakage, but it will also certainly play a more extensive role in anti-fibrosis, that is, the formation of fibrous membranes. In summary, we propose a new perspective to achieve dual-target therapy of PDR by antagonizing BMP4.

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

Compliance with ethical standards Competing interests The authors declare that they no conflicts of interests. Ethical approval and consent to participate All human studies have been approved by the Ethics Committee of Tianjin Medical University Eye Hospital, and animal experimentation conforms to protocols were approved by the Animal Ethical and Welfare Committee of Tianjin Medical University Eye Institute.

Consent for publication Not applicable

Abbreviations DAPI, 4’,6-diamidino-2-phenylindole; DR, diabetic retinopathy; GO, Gene Ontology (GO); HRCECs, human retinal capillary endothelial cells; KEGG, Kyoto Encyclopedia of Genes and Genomes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEB, New England Biolabs (NEB); nr, nonredundant; PFA, paraformaldehyde; RNA-seq, sequencing; RT-qPCR, reverse transcription quantitative PCR; SD, Sprague Dawley; STZ streptozotocin

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