Long noncoding RNA Hotair facilitates retinal endothelial cell dysfunction in diabetic retinopathy

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Running title: Role of Hotair in diabetic retinopathy
Abstract

**Background:** Retinal endothelial cell (REC) dysfunction induced by diabetes mellitus (DM) is an important pathological step of diabetic retinopathy (DR). Long noncoding RNAs (lncRNAs) have emerged as novel modulators in DR. This study aimed to investigate the role and mechanism of lncRNA Hotair in regulating DM-induced REC dysfunction.

**Methods:** The retinal vascular preparations and immunohistochemical staining assays were conducted to assess the role of Hotair in retinal vessel impairment in vivo. The EdU, transwell, cell permeability, CHIP, luciferase activity, RIP, RNA pull-down, and Co-IP assays were employed to investigate the underlying mechanism of Hotair-mediated REC dysfunction in vitro.

**Results:** Hotair expression was significantly increased in diabetic retinas and high glucose (HG)-stimulated REC. Hotair knockdown inhibited the proliferation, invasion, migration, and permeability of HG-stimulated REC in vitro and reduced the retinal acellular capillaries and vascular leakage in vivo. Mechanistically, Hotair bound to LSD1 to inhibit VE-cadherin transcription by reducing the H3K4me3 level on its promoter and to facilitate transcription factor HIF1α-mediated transcriptional activation of VEGFA. Furthermore, LSD1 mediated the effects of Hotair on REC function under HG condition.

**Conclusion:** The Hotair exerts its role in DR by binding to LSD1, decreasing VE-cadherin transcription, and increasing VEGFA transcription, leading to REC dysfunction. These findings revealed that Hotair is a potential therapeutic target of DR.

**Keyword:** lncRNA Hotair, diabetic retinopathy, LSD1, VEGFA, VE-cadherin
**Clinical Perspectives**

(1) Background as to why the study was undertaken:

LncRNAs have emerged as novel modulators in DR, while the role and mechanism of IncRNA Hotair in DM-induced REC dysfunction is still unknown.

(2) A brief summary of the results:

Hotair exerts its role in DR by binding to LSD1, decreasing VE-cadherin transcription, and increasing VEGFA transcription, ultimately leading to REC dysfunction.

(3) The potential significance of the results to human health and disease:

Our study highlights the important link between Hotair, LSD1, VE-cadherin, and VEGFA, and provides a deeper understanding of DR pathogenesis. This interplay may provide a potentially targeted method for DR treatment.
Introduction

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes mellitus of diabetes mellitus (DM) characterized by pericytes loss, acellular capillaries increase, and blood-retinal barrier (BRB) hyperpermeability (1). Despite its mortality rate is lower than that of macrovascular complications, DR has become the leading cause of vision loss among working-age people (20-65-year-old) (2). The pathogenesis of DR is complicated, and the dysfunction of retinal endothelial cells (REC) has been considered as a major pathological step of DR. Under pathological conditions of DM or high glucose (HG), the permeability of REC cells is increased, which leads to the leakage of BRB, thereby resulting in retinal bleeding, exudation, and detachment (3). Besides, the abnormal proliferation and migration of REC induced by DM or HG can cause capillary occlusion and pathological angiogenesis, and finally promoting the progression of DR (4). Thus, an in-depth study of molecular mechanisms that regulate REC dysfunction is important for understanding the pathogenesis of DR and its treatment.

Long noncoding RNAs (lncRNAs), a class of transcripts with a length longer than 200 nt, are involved in the transcription, translation, and epigenetic regulation of target genes (5). It has been proven that the aberrant expression of lncRNAs is closely associated with the development of many diseases, especially these proliferative diseases (6, 7). At present, although the roles of certain lncRNAs such as lncRNA myocardial infarction associated transcript (MIAT), lncRNA imprinted maternally expressed transcript (H19), lncRNA FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR), and lncRNA CDKN2B antisense RNA 1 (ANRIL) (8-11) have been elucidated, the roles of the most lncRNAs in DR have not been determined, including HOX transcript antisense intergenic RNA (Hotair). Hotair is a lncRNA with ~2000 nt in length and highly conserved among species (12) and its function in promoting angiogenesis has been indicated by many studies (13, 14). As reported, Hotair is abnormally expressed in many diabetes-related diseases and plays an important role in the development and progression of these diseases, including diabetic kidney disease and diabetic cardiomyopathy (12, 15, 16). It also has been reported that the expression of Hotair is up-regulated in the serum of DR patients and can be considered as a marker of DR diagnosis and prognosis (17). Inspired by these shreds of evidence, we speculated that Hotair might exert its role in DR by regulating the function of REC cells.

In this study, we aimed to investigate whether Hotair exerts its role in DR by regulating REC cell
dysfunction and if yes, what is the molecular regulation mechanism? Here, we found that Hotair was up-regulated in retinas of DM mice and HG-stimulated REC cells. As expected, the in vitro experiments showed that the knockdown of Hotair protected the function of REC cells, and the in vivo experiments revealed that its knockdown relieved the retinal vessel impairment of DM mice. We then clarified a molecular mechanism by which Hotair acted as a scaffold of histone lysine-specific demethylase 1 (LSD1) to regulate the transcriptional expressions of VE-cadherin and vascular endothelial growth factor A (VEGFA), thereby affecting the function of REC cells.

Materials and methods

Animals and DM model establishment

Adult C57BL/6 mice (male, 5-7 weeks old) were purchased from the Laboratory Animal Center of Zhengzhou University and kept in a standard condition. All animal experiments were approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University and performed in the Laboratory Animal Center of Zhengzhou University (approval no. 2020-KY-350). After 1 week acclimation, mice were intraperitoneally injected with 130 mg/kg streptozotocin (STZ) to induce the DM model. Mice in the control group were intraperitoneally injected with the vehicle. The fasting blood glucose of mice was determined by using a glucometer at 3 days after STZ injection. Mice with blood glucose >11.1 mmol/l was considered as a successful model and subjected to further study. 20 mice (10 DM mice and 10 control mice) were sacrificed by intraperitoneal injection of an overdose of pentobarbital at 4 weeks, 12 weeks, and 20 weeks after STZ injection (Fig. 1A) and their eyeballs were collected for further study.

Adeno-associated virus injection

Recombinant adeno-associated virus (AAV) carrying Sh-Hotair (AAV-Sh-Hotair) was obtained from the FITGENE company (Guangzhou, China). The scramble sh-RNA was used as a negative control. The sequences of sh-Hotair and scramble sh-RNA were shown in Table S1. At the beginning of STZ injection, mice were anesthetized with ketamine (20 mg/kg) and xylazine (6 mg/kg) via intraperitoneal injection. Then mice were intravitreally injection with 1 µl (1×10^10 viral particles/mL) of AAV-Sh-Hotair or scramble sh-RNA. 10 weeks after the STZ injection, mice received the second injection of AAV-Sh-Hotair or scramble sh-RNA. All mice were sacrificed at 20 weeks after STZ injection (Fig. 4A) and their eyeballs were collected for further study.

Immunohistochemical assay
The immunohistochemical staining of retinal paraffin-embedded sections with an HRP-labeled IgG antibody was used to detect retinal permeability as previously described (9). Mouse retinal tissues were used to prepare paraffin-embedded cross-sections. The paraffin-embedded cross-sections were immersed in a sodium citrate buffer to perform antigen repair. After blocked with H₂O₂, sections were incubated with HRP-labeled IgG antibody. Later, the DAB chromogenic solution was dropped onto sections. The sections were observed under a microscope (Leica, Japan) after stained with hematoxylin.

**Retinal vascular preparations**

The retinal vascular preparations were prepared according to previously described (18). In brief, the eyes of mice were enucleated and then fixed in paraformaldehyde for 24 h. Then the whole retinas were isolated from eyes under a microscope and then digested with 5% pepsin and 2.5% trypsin to isolate the retinal vasculature. The samples were stained with periodic acid Schiff (PAS) and hematoxylin to evaluate the changes of capillaries and pericytes. The acellular capillaries and pericytes in ten random fields per retina were counted and averaged.

**Cell culture, treatment, and transfection**

Mouse retinal endothelial cells (mREC) were isolated as previously described (19) and cultured in the basal medium containing 10% fetal bovine serum (FBS). mREC were treated with high glucose (HG, 25 mmol/l) or normal glucose (NG, 5.5 mmol/l). The overexpression vectors (Hotair and LSD1) and silence vectors (sh-Hotair, sh-LSD1, and sh-HIF1α) were synthesized by GenePharma company. Plasmids were used for these overexpression and silence vectors. These vectors were transfected into cells using Lipofectamine 2000 (Invitrogen, USA).

**EdU assay**

EdU assay was used to detect the proliferation of mREC cells. All experimental producers were according to the manufacture instructions of the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime, China).

**Transwell assay**

mREC cells were suspended in the FBS-free medium. Then cells (5×10⁴ cells/chamber) were seeded into the upper transwell chambers. The FBS-contained medium (700 μl/ chamber) was added into the lower transwell chambers. After 24 for incubation, cells in the lower transwell chambers were fixed with paraformaldehyde, stained with crystal violet, and then observed under a microscope.
(Leica, Japan).

**Wound healing assay**

The mREC cells were cultured in FBS-free basal medium for 24 h and then seeded into the collagen-coated 6-well plate. Then a linear wound was generated within the monolayers by scraping the cells using the sterile pipette tip. Cells were observed under an inverted microscope at 0 h and 24 h after incubation.

**Permeability detection assay**

The permeability of mREC cells was detected as previously described (20). Briefly, mREC cells were suspended in the basal medium and then were seeded into the upper transwell chambers (1x $10^5$ cells/chamber). Later, FITC-conjugated bovine serum albumin was added into each upper chamber at a final concentration of 10 μg/ml and 500 μl medium was added into each lower chamber. After incubation for 30 min, the fluorescence intensity of each lower chamber was detected by a microplate reader (BioTek, USA) with the excitation wavelength of 490 nm and an emission wavelength of 525 nm. The concentration of permeabilized FITC-albumin in the lower chamber was assessed according to the fluorescence intensity.

**Fluorescence in situ hybridization (FISH)**

The fluorescent probe targeting Hotair (cy3-labeled) was used to perform in situ hybridization. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Hybridization was performed using Fluorescent in Situ Hybridization kit (GenePharma, China) according to its manufacturer’s instructions. Cells were observed under a fluorescence microscope (Nikon, Japan).

**Immunofluorescence assay**

Retinas for the whole-mount were isolated from mice. The paraffin-embedded sections of mouse retinas were prepared. mREC cells were seeded onto the coverslips. Then the retinal whole-mount, retinal cross-sections, or mREC cells were washed with PBS, fixed with 4% formaldehyde, and permeated with 0.2% Triton X-100. After blocked with normal serum, cells were incubated with primary antibodies: anti-VE-cadherin, anti-ZO-1, and anti-VEGF at 4°C overnight, followed by incubation with FITC-conjugated secondary antibody for 1.5 h. Then cells were stained with DAPI for 5 min at room temperature and the immunofluorescence density and area of cells were observed under a fluorescence microscope (Nikon, Japan).

**RNA pull-down assay**
RNA pull-down assay was conducted using Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo, USA). mREC cells were lysed using a standard lysis buffer and Hotair was labeled with biotin. The biotin-labeled Hotair was incubated with Streptavidin Magnetic Beads and cell lysate. Then protein levels of LSD1 and HIF1α in Hotair pulled-down products were detected by western blot.

**RNA immunoprecipitation (RIP) assay**

The RIP assay was performed using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). mREC cells were lysed by RIP buffer. Then cell lysates were incubated with magnetic beads and anti-LSD1 antibody at 4°C overnight. Later, RNA was extracted from the complex and used for qPCR analysis.

**Chromatin immunoprecipitation (CHIP) assay**

The CHIP assay was performed using the Magna ChIP™G Tissue Kit (Magna, USA). In brief, mREC cells were fixed with formaldehyde for 10 min at 37°C, followed by incubation with glycine to stop cross-linking. Then cells were lysed with CHIP lysis buffer and sonicated to generate DNA fragments of 200–1000 bp. The 20 µL cell lysate was stored at -80°C as input and remaining cell lysate was incubated with the anti-H3K4me3, anti-H3K27me3, anti-H3K9me3, anti-LSD1, anti-HIF1α, or IgG (negative control) at 4°C for 24 h. After then, Protein A/G magnetic beads were added into lysates to prepare the immunoprecipitated protein-DNA complexes. The DNA was extracted from complexes and the expressions of VEGF and VE-cadherin in DNA samples were detected by qPCR. The sequences of primer used for Chip-qPCR were shown in Table S2.

**Co-immunoprecipitation (Co-IP) assay**

The Co-IP assay was used to assess the interaction between LSD and HIF1α. mREC cells were lysed by RIPA lysis. The cell lysates were incubated with anti-LSD1 antibody and Protein A agarose beads. Then the protein level of HIF1α in immunoprecipitation complex was detected by western blot.

**Luciferase reporter assay**

The luciferase reporter assay was used to assay the activities of VEGFA and VE-cadherin promoters. Briefly, the promoter sequences of VEGFA and VE-cadherin were subcloned into the upstream of a luciferase reporter gene in a pGL3 plasmid. Each recombinant vector was
co-transfected with sh-Hotair or sh-HIF1α into mREC cells. Then the luciferase activity was detected by the Dual-Luciferase Reporter Assay System (Promega, USA).

**Real-time quantitative PCR (qRT-PCR)**

Total RNA was isolated from mouse retinas and mREC cells and reversely transcribed to cDNA using BeyoRT™ First Strand cDNA Synthesis Kit (Beyotime, China). Then cDNA was utilized to perform qRT-PCR using BeyoFast™ SYBR Green qPCR Mix (Beyotime, China). The relative expressions of genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primer used for qRT-PCR were shown in Table S2.

**Western blot**

mREC were used to extract protein. The concentrations of protein samples were measured using the BCA Protein Assay Kit (Beyotime, China) and 30 μg protein of each sample was fractionated on an SDS-PAGE. After then, proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were incubated with the primary antibodies: anti-VEGF (1:500, Abcam), anti-VE-cadherin (1:1000, Abcam), anti-HIF1α (1:1000, Abcam), and anti-GAPDH (1:2500, Abcam) at 4°C for 24 h, followed by the incubation with the secondary antibody at room temperature for 1.5 h. The immune blots were visualized using BeyoECL Moon (Beyotime, China) and an Odyssey Infrared Imaging System.

**Statistical analysis**

All data were analyzed using Graphpad Prism 7.0 software and expressed as mean ± standard deviation (SD). One-way ANOVA combined with Tukey post-test and Student t-test was applied to determine the significance between groups. The p-value <0.05 indicated statistical significance.

**Results**

**Hotair expression pattern in the retina of DM mice and HG-stimulated REC**

To investigate the role of Hotair in DR, we first established an STZ-induced mouse DM model (Fig. 1A). 12 and 20 weeks after STZ injection, the retinal vascular preparations showed the decreased pericytes and increased acellular capillaries in the retinas of DM mice (Fig. 1B). Since IgG extravasation is considered as a marker of increased vessel permeability (21), we then stained the retinas of mice with IgG. The results revealed an increase in vascular leakage of DM mice. Moreover, the vascular leakage increased with the increase of diabetes weeks (Fig. 1C). As shown in Fig. 1D, Hotair is highly conserved among mouse, rat, and human. We then detected the
expression of Hotair in the retinas of DM mice and HG-stimulated mouse endothelial cells. The results showed that Hotair was up-regulated in DM mice and HG-stimulated mREC cells (Fig. 1E-F). Collectively, these results suggested that the expression of Hotair is increased in DM or HG-stimulated REC.

**Hotair knockdown improves the function of REC under HG condition**

We then explored the role of Hotair in regulating the function of REC in vitro. Firstly, we assessed the effects of Hotair knockdown on the proliferation of REC by EdU fluorescence staining. As shown in Fig. 2A, Hotair knockdown had no significant effect on the proliferation of mREC cells under NG condition, while it significantly inhibited the proliferation of mREC cells under HG condition. Secondly, we evaluated the impacts of Hotair silence on the invasion and migration of endothelial cells by transwell and wound healing assays, respectively. The results showed Hotair silence had no significant impact on the invasion and migration of mREC cells under NG condition, but it abolished the HG-induced increase of mREC cell invasion and migration (Fig. 2B & C). Thirdly, the influence of Hotair knockdown on the permeability of endothelial cells was evaluated by FITC-albumin assay. As shown in Fig. 2D, the permeability of mREC cells was not altered by the knockdown of Hotair under NG condition. However, HG condition led to the hyperpermeability in mREC cells and Hotair knockdown reversed this effect. These results indicated that Hotair knockdown improved the function of HG-stimulated REC.

**Hotair knockdown regulates the expressions of VE-cadherin and VEGF in REC under HG condition**

It is has been reported that VEGF plays an important role in the pathological angiogenesis of REC in DR (22). Besides, VE-cadherin also plays an important role in DR by regulating the permeability of REC (9). To further clarify the mechanism of Hotair in regulating the function of REC under HG condition, we then detected the effects of Hotair knockdown on the expression of VEGF and VE-cadherin. As shown in Fig. 3A-B, the immunofluorescence staining showed that knockdown of Hotair had no significant effects of ZO-1 and VE-cadherin expressions in mREC cells under NG condition, while it abrogated the HG-induced down-regulation of ZO-1 and VE-cadherin expressions in RECs. The qRT-PCR and western blot assays revealed that Hotair knockdown did not change the mRNA expressions of VE-cadherin and VEGFA (a encode gene of VEGF protein) in mREC cells under NG condition, but significantly increased the mRNA and
protein levels of VE-cadherin and decreased VEGFA mRNA level and VEGF protein level (Fig. 3C-D). These results suggested that Hotair may exert its role in REC dysfunction by regulating the expression of VE-cadherin and VEGF.

**Hotair knockdown relieves retinal vessel impairment in vivo**

We then addressed whether Hotair can affect retinal vessel impairment in vivo. As shown in Fig. 4A, the AAV vectors carrying sh-Hotair or scramble sh-RNA (negative control) were injected into DM mice. The retinal vascular preparations showed that Hotair knockdown decreased the acellular capillaries and increased the pericytes in the retinas of DM mice (Fig. 4B). The immunohistochemistry analysis of IgG revealed that Hotair silence reduced the vascular leakage of DM mice (Fig. 4C). The qRT-PCR assay showed that AVV-sh-Hotair effectively suppressed the expression of Hotair in the retinas of DM mice (Fig. 4D). Furthermore, the immunofluorescent staining of retinal whole-mount revealed that the silence of Hotair caused an increase of VE-cadherin expression in retinas of mice (Fig. 4E). The results of Fig. 4F showed that Hotair knockdown resulted in a decrease of VEGF expression in retinas of mice. Together, these data indicated that Hotair knockdown can relieve retinal vessel impairment of DM mice.

**Hotair/LSD1 inhibits the transcriptional expression of VE-cadherin via reducing the H3K4me3 level on its promoter**

Based on the RNA-FISH assay, we found that Hotair was mainly located in the nuclei of mREC (Fig. 5A). The qRT-PCR analysis showed increased Hotair expression and decreased mRNA level of VE-cadherin in HG-stimulated mREC cells. The correlation analysis further indicated that the expression of Hotair was negatively correlated with VE-cadherin mRNA level (Fig. 5B). Besides, the luciferase reporter gene assay showed the knockdown of Hotair elevated the promoter activity of VE-cadherin in mREC cells under HG condition (Fig. 5C). These findings indicated that Hotair may regulate VE-cadherin expression by modulating its transcriptional expression.

The previous studies have shown that Hotair can regulate the transcriptional activity of target genes by binding to histone methylase PRC2 and histone demethylase LSD1 (23). Among the two enzymes, PRC2 is mainly responsible for the methylation of H3K27, and LSD1 is mainly responsible for the demethylation of H3K4 or H3K9 (23, 24). Hence, we then detected the effects of Hotair on the levels H3K4me3, H3K9me3, and H3K27me3 on the VE-cadherin promoter. The CHIP assay showed that although HG decreased the H3K4me3 level and increased the H3K27me3
level on the promoter of VE-cadherin, the knockdown of Hotair only altered the H3K4me3 level on VE-cadherin promoter under HG condition (Fig. 5D). Because of this, we hypothesized that Hotair might regulate the H3K4me3 level on the VE-cadherin promoter through binding to LSD1, thereby affecting the transcription of VE-cadherin. To confirm this speculation, we first assessed the effect of Hotair on the expression of LSD1 under DM or HG conditions. As shown in Fig. 5E-F, the mRNA expression of LSD1 was significantly increased in the retinas of DM mice, and the knockdown of Hotair had no significant effect on the LSD1 mRNA level. Meanwhile, the in vitro experiments also showed that the silence of Hotair did not affect the mRNA and protein levels of LSD1 in mREC cells under HG condition (Fig. 5G), suggesting that Hotair has no effect on LSD1 expression. We then verified whether Hotair can bind to LSD1 in mREC cells. The RNA pull-down assay showed that Hotair was enriched in the complex immunoprecipitated by LSD1 antibody, and the RIP assay revealed that LSD1 was enriched in the product pulled-down by Hotair probe (Fig. 5H). Next, we determined the impact of Hotair on the binding capacity of LSD1 to the VE-cadherin promoter in mREC cells. The CHIP assay confirmed that the overexpression of Hotair enhanced the binding capacity of LSD1 to the VE-cadherin promoter under NG condition, and the silence of Hotair repressed the binding capacity of LSD1 to VE-cadherin promoter under HG condition (Fig. 5I). At last, we verified whether LSD1 mediates the regulatory effect of Hotair on the H3K4me3 of VE-cadherin promoter. The result revealed that the force expression of Hotair reduced the H3K4me3 level on the VE-cadherin promoter, while the knockdown of LSD1 reversed this effect (Fig. 5J). Therefore, these results demonstrated that Hotair binds to LSD1 to inhibit the H3K4me3 on the VE-cadherin promoter, thereby suppressing the transcriptional expression of VE-cadherin.

**Hotair/LSD1 promotes HIF1α-mediated transcription activation of VEGFA**

The qRT-PCR assay also showed that the expression of Hotair and the mRNA level of VEGFA were both increased in HG-stimulated mREC. The correlation analysis showed a significant positive correlation between Hotair expression and VEGFA mRNA level (Fig. 6A). We then explored whether Hotair can affect the histone methylation on VEGFA promoter in REC under HG condition. As shown in Fig. 6B, HG reduced the H3K9me3 and H3K27me3 levels on VEGFA promoter, while the knockdown of Hotair had no significant effect on them, implying that Hotair
does not affect the transcriptional expression of VEGFA via regulating the histone methylation on its promoter.

To further investigate the mechanism of Hotair in regulating VEGFA transcriptional expression, we determined the effects of Hotair on the activity of the VEGFA promoter. The results showed that the silence of Hotair reduced the activity of VEGFA promoter at -1983bp, -1340bp, and -643bp regions, while had no significant effect on the activity of VEGFA promoter at -325 bp regions (Fig. 6C). In view of this, we hypothesized that the target of Hotair regulating VEGFA promoter activity may be located at -643bp to -325bp. We then predicted the possible binding sites of transcription factors on this region of VEGFA promoter using the JASPAR software and found possible HIF1α binding sites in this region (Fig. 6D). Since many studies have reported that HIF1α can act as a transcription factor to promote the expression VEGFA (25, 26), we then investigate whether HIF1α mediates the role of Hotair in regulating VEGFA transcriptional expression under HG condition. As shown in Fig. 6E-F, the mRNA and protein expression of HIF1α was elevated in the retinas of DM mice and HG-stimulated mREC cells. The silence of HIF1α reduced VEGFA promoter activity at -643bp region under HG condition, while had no significant effect on -325 bp region (Fig. 6G), indicating that HIF1α mainly regulates the VEGFA promoter activity between -643bp and -325bp region. Besides, our results showed that the knockdown of HIF1α decreased the mRNA level of VEGFA in mREC cells under HG condition (Fig. 6H). The CHIP assay revealed that the silence of Hotair or LSD1 suppressed the binding of HIF1α to the VEGFA promoter (Fig. 6I). The knockdown of Hotair or LSD1 reduced the protein level of HIF1α and the mRNA and protein levels of VEGF, but had no significant effect on HIF1α mRNA level (Fig. 6J), implying that Hotair or LSD1 regulates HIF1α expression at the post-transcriptional level. The RNA pull-down and Co-IP assays showed that HIF1α could bind to Hotair and LSD1 (Fig. 6K), making us speculating that HIF1α, Hotair, and LSD1 might form a complex. Our further results revealed the overexpression of Hotair increased the mRNA level of VEGFA and protein level of HIF1α in mREC cells under HG condition, while the knockdown of LSD1 canceled this effect (Fig. 6L), suggesting the role of Hotair in regulating expressions of VEGFA and HIF1α depends on LSD1. Furthermore, the overexpression of LSD1 elevated the mRNA level of VEGFA and protein level of HIF1α in mREC cells under HG condition, while this effect was partially reversed by Hotair knockdown (Fig. 6M). Hence, these data demonstrated that
Hotair/LSD1 regulates HIF1α at the protein level, thereby affecting HIF1α-mediated transcription activation of VEGFA.

**LSD1 mediates the effects of Hotair on the function of HG-stimulated retinal REC**

We then verified whether LSD1 mediates the effects of Hotair on the function of HG-stimulated retinal REC. As shown in Fig. 7A-B, the overexpression of Hotair promoted the invasion and increased the permeability of HG-stimulated mREC, while these effects were reversed by LSD1 knockdown. Besides, Hotair overexpression increased VEGF protein level and decreased VE-cadherin protein level in HG-stimulated mREC cells, while LSD1 silence abrogated these impacts (Fig. 7C). Therefore, these results showed that LSD1 mediates the effects of Hotair on the function of HG-stimulated retinal REC.

**Discussions**

Hotair has been proven to play important roles in many diseases, such as cancers, Parkinson's disease, osteonecrosis of the femoral head, and diabetic cardiomyopathy (12, 27-29). The previous study found that the expression of Hotair was significantly up-regulated in the serum of DR patients (17), whereas its role in DR has bot been clarified. Similarly, we found the expression of Hotair was evidently up-regulated in the retinas of DM mice and HG-stimulated REC cells in this study. Thus, we then focused on the role of Hotair in DR in the present study. The dysfunction of REC has been considered as an important pathological step since it can induce abnormal angiogenesis and BRB hyperpermeability of retinas (3, 4). Interestingly, our *in vitro* results revealed that the knockdown of Hotair protects the function of REC under HG condition by inhibiting the proliferation, invasion, migration, and permeability of REC. The *in vivo* results further showed that the silence of Hotair relieved the retinal microvascular disorder of DM mice which was indicated by the reduced acellular capillaries and permeability of retinas. Thus, our current results demonstrated that Hotair plays a role in DR by promoting REC dysfunction.

VEGF and VE-cadherin are described as the major regulators in DR by regulating REC function (3). Under DM or HG conditions, the expression of VEGF is up-regulated and then binds to its receptors to facilitate the proliferation and migration of REC cells, leading to pathological angiogenesis and aggravated progression of DR (30, 31). The VE-cadherin is responsible for the integrity of the adherens junctions between adjacent REC cells, while its down-regulated expression induced by DM or HG can destroy this integrity, thereby increasing the permeability of
REC and causing BRB injury (32, 33). In this study, the up-regulated expression of VEGF and downregulated expression of VE-cadherin was found in the diabetic retinas of mouse and HG-stimulated REC cells. Besides, our *in vitro* and *in vivo* experiments showed that knockdown of Hotair inhibited VEGF and promoted VE-cadherin under DM and HG conditions, implying that VEGF and VE-cadherin mediate the role of Hotair in regulating REC function in DR.

The histone methylation is an important epigenetic regulatory mechanism of gene expression. The methylation of H3K4 is associated with gene activation, while the methylations of H3K9 and H3K27 are associated with gene repression (34). LSD1, as an important lysine-specific histone demethylase, can trigger the demethylation of H3K4 and H3K9 (35). The reported studies indicated that LSD1 is involved in DR by regulating the histone demethylation on the promoters of target genes (36, 37). Zhong et al. reported that the expression of LSD1 is increased in the retinas of DM rats and patients, and its knockdown inhibited the expression of MMP-9 by increasing the H3K9me2 level on MMP-9 promoter in REC cells, thereby relieving mitochondria injury of REC cells in DR (37). Another study of them showed that the silence of LSD1 also can promote the expression of SOD2 to ameliorate mitochondria dysfunction by increasing the H3K4me2 and H3K4me1 levels on SOD2 promoter (36). In the current study, we also found that the expression of LSD1 was up-regulated in retinas of DM mice and HG-stimulated REC cells. Our CHIP assay showed that HG decreased the H3K4me3 but not H3K9me3 on the VE-cadherin promoter. Besides, HG enhanced the binding capacity of LSD1 to VE-cadherin promoter in REC cells. These results revealed that LSD1 might repress the transcription of VE-cadherin by reducing the H3K4me3 level on its promoter in REC cells under HG condition.

HIF1α is a hypoxia-modulated transcription factor and can act as a transcriptional activator to induce the expression of VEGF in DR (38, 39). In the present study, we found that HIF1α expression was increased in the retinas of DM mice and HG-stimulated REC cells. The silence of HIF1α significantly reduced the promoter activity and mRNA level of VEGFA in REC cells under HG condition. These data confirmed that HIF1α can participate in DR by promoting VEGFA transcriptional activation.

In this study, we found that the expression of Hotair was positively correlated to the VEGFA mRNA level, while it was negatively correlated to VE-cadherin in REC cells under HG condition. The knockdown of Hotair reduced VEGFA promoter activity and increased VE-cadherin in
HG-stimulated REC cells, indicating that Hotair can regulate the transcriptions of VEGFA and E-cadherin. Since emerging studies provide a hypothesis that Hotair can act as a scaffold of histone modification complexes to regulating the expression of target genes (23, 40)(41), we then investigate whether Hotair can regulate the transcriptions of VEGFA and E-cadherin by scaffolding to histone modification complexes. Our results showed that Hotair silence increased the H3K4me3 level on the E-cadherin promoter. Our RIP and RNA pull-down assays showed that Hotair could bind to LSD1 in REC cells. Although the knockdown of Hotair did not affect the expression of LSD1, it inhibited the binding capacity of LSD1 to E-cadherin promoter in HG-stimulated REC cells. Moreover, our results showed that LSD1 mediated the effect of Hotair on the H3K4me3 level of the VE-cadherin promoter. Hence, these studies suggested that Hotair can scaffold to LSD1 to repress VE-cadherin transcription by reducing the H3K4me3 level on its promoter in HG-stimulated REC cells. We also explored the effect of Hotair on the histone methylation on the VEGFA promoter. The results showed that Hotair had no effects on the H3K4m3, H3K27me3, and H3K9me3 levels on VEGFA promoter, suggesting that Hotair might affect VEGFA transcription by other mechanisms rather histone methylation. Based on further study, we found that the knockdown of Hotair or LSD1 reduced the recruitment of transcription factor HIF1α to VEGFA promoter in HG-stimulated REC cells. The RNA pull-down and Co-IP assays revealed that HIF1α could bind to LSD1 and Hotair, implying that LSD1, HIF1α, and Hotair might form a complex. Furthermore, our present results showed that the role of Hotair in regulating expressions of VEGFA and HIF1α depended on LSD1. These results indicated that Hotair binds to LSD1 to promote HIF1α-mediated transcriptional activation of VEGFA in HG-stimulated REC cells. Taken together, these findings demonstrated that Hotair regulates the transcriptions of VE-cadherin and VEGFA by acting as a scaffold of LSD1. Interestingly, our results showed that the silence of Hotair and LSD1 had no effect on the mRNA level of HIF1α in HG-stimulated REC cells, while caused a decrease in its protein level. This evidence suggested that Hotair and LSD1 regulate the expression of HIF1α at the protein level. Since the previous study has pointed out that LSD1 can mediate the demethylation of HIF1α protein at K391, thereby protecting HIF1α protein against ubiquitin-mediated protein degradation in tumor angiogenesis (42), we thus speculated that Hotair and LSD1 might regulate the protein level of HIF1α in DR by this mechanism. We will further investigate whether Hotair/ LSD1
regulates HIF1α by modulating its protein methylation and ubiquitination in DR in the future.

In conclusion, our findings in the current work demonstrated that Hotair serves as a scaffold of LSD1 to decrease VE-cadherin transcription and increase VEGFA transcription, which leads to REC dysfunction, thereby resulting in microvascular dysfunction and aggravating the progression of DR (Fig. 7D). This study provides a deeper understanding of DR pathogenesis and a potentially targeted method for DR treatment.

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Authors’ contributions

ZD: Methodology, Writing Original Draft, and Data curation; ZYY, WLN, LYL, ZSY, and GF: Investigation; MXJ, ZHH, LZZ, and MDD: Visualization and Supervision; XLJ and ZLX: Writing-Reviewing and Editing Manuscript and Software; LJQ and QGJ: Conceptualization and Methodology.

Conflict of interest

All authors declare that they have no conflicts of interest in this work.

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Figure legends

Fig. 1 The relative expression of Hotaira in DR. (A) The schematic diagram of mice treatment. (B) The changes of acellular capillaries and pericytes in the retina of mice were detected by the PAS staining of retinal vascular preparations. Black arrows: acellular capillaries; asterisks: pericyte. (n=10; **p<0.01 vs. control). (C) The retinal permeability of mice was detected by immunohistochemical staining of IgG. The black arrows indicated IgG (n=10). (D) The schematic diagram showed the conservation of Hotair among species. (E) The expression of Hotair in the retina of DM mice was detected by qRT-PCR (n=10; *p<0.05, **p<0.01 vs. control). (F) mREC cells were treated with 5 mmol/l D-glucose and 25 mmol/l D-glucose (HG) 24 h or 48 h. Cells treated with 25 mmol/l L-glucose as an osmotic control (Osm). The expression of Hotair in cells was determined by qRT-PCR (**p<0.01 vs. NG; ##p<0.01 vs. HG.)

Fig. 2 The effects of Hotair knockdown on HG-stimulated retinal endothelial cell function in vitro. mREC cells were transfected with sh-Hotair for 24 h and then were exposed under NG or HG conditions for another 24 h. (A) The proliferation of mREC cells was detected by the EdU assay. (B-C) The invasion and migration of mREC cells was detected by transwell and wound-healing assays, respectively. (D) The permeability of monolayer mREC cells was detected using FITC-conjugated albumin. n=3. **p<0.01 vs. NG; #p<0.05, ##p<0.01 vs. HG.

Fig. 3 The effects of Hotair knockdown on the expressions of VE-cadherin and VEGF in HG-stimulated retinal endothelial cells. mREC cells were transfected with sh-Hotair for 24 h and then were exposed under NG or HG conditions for another 24 h. (A-B) The representative images of VE-cadherin and ZO-1 immunofluorescent staining and the quantitative analysis of fluorescence intensity. (C) The mRNA levels of VE-cadherin and VEGFA were detected by qRT-PCR. (D) The protein levels of VEGF and VE-cadherin were detected by western blot. n=3. *p<0.05, **p<0.01 vs. NG; #p<0.05, ##p<0.01 vs. HG.

Fig. 4 The effects of Hotair knockdown on retinal endothelial cell function of DR mice in vivo. (A) The schematic diagram of mice treatment and AAV vectors injection. (B) The changes of acellular capillaries and pericytes in the retina of mice were detected by PAS staining of retinal vascular preparations. The black arrows indicated acellular capillaries. (C) The retinal permeability of mice was detected by immunohistochemical staining of IgG. The black arrows indicated IgG. (D) The expression of Hotair in the retina of mice was detected by qRT-PCR. (E)
The expression of VE-cadherin was detected by the immunofluorescence staining of the retinal whole-mount. (F) The expression of VEGF was detected by the immunofluorescent staining of retinal cross-sections. The expression of VEGF in the retina of mice was detected by qRT-PCR and western blot. n=10. *p<0.05, **p<0.01 vs. control; *p<0.05, **p<0.01 vs. STZ+AAV-scramble.

Fig. 5 Hotair/LSD1 regulated the transcriptional expression of VE-cadherin via reducing the H3K4me3 level. (A) RNA-FISH assay showed the location of Hotair in mREC cells. (B) mREC cells were treated with HG for 0, 6, 12, and 24 h and then the expressions of VE-cadherin and Hotair were detected by qRT-PCR (*p<0.05, **p<0.01 vs. Hotair expression at 0 h; *p<0.05, **p<0.01 vs. VE-cadherin expression at 0 h). (C-D) mREC cells were transfected with sh-Hotair and then treated with HG. (C) The relative promoter activity of VE-cadherin was detected (**p<0.01 vs. NG; *p<0.05 vs. HG). (D) The enrichment of H3K4me3, H3K9me3, and H3K27me3 on the promoter of VE-cadherin was detected by CHIP assay (**p<0.01 vs. NG; *p<0.05 vs. HG). (E-F) The mRNA level of LSD1 in the retina of mice was detected by qRT-PCR (**p<0.01 vs. control). (G) mREC cells were transfected with sh-Hotair and then treated with HG. The expression of LSD1 was detected by qRT-PCR and western blot (**p<0.01 vs. control). (H) mREC cells were treated with NG or HG. Then the binding of Hotair to LSD1 was detected by RNA pull-down and RIP assays (**p<0.01 vs. IgG). (I) mREC cells were transfected with Hotair or sh-Hotair and then treated with NG or HG. The enrichment of LSD1 on the promoter of VE-cadherin was determined by CHIP assay (**p<0.01 vs. NG; *p<0.05 vs. HG). (J) mREC cells were transfected with Hotair or and sh-LSD1 and then treated with NG. The enrichment of H3K4me3 on the promoter of VE-cadherin was determined by the CHIP assay (**p<0.01).

Fig. 6 Hotair/LSD1 regulated HIF1α-mediated transcriptional activation of VEGFA. (A) mREC cells were treated with HG for 0, 6, 12, and 24 h and then the expressions of Hotair and VEGF were detected by qRT-PCR (*p<0.05, **p<0.01 vs. Hotair expression at 0 h; *p<0.05, **p<0.01 vs. VE-cadherin expression at 0 h). (B-C) mREC cells were transfected with sh-Hotair and then treated with HG. Then the enrichment of H3K4me3, H3K9me3, and H3K27me3 on the promoter of VEGF was detected by CHIP assay (**p<0.01 vs. NG). (C) The relative activity of VEGFA promoter (-1983bp, -1340bp, -643bp, and -325bp) was detected (**p<0.01 vs. NG; *p<0.05,
**p<0.01 vs. HG). (D) The scores of possible transcription factors on the VEGFA promoter were predicted by JASPAR software. (E-F) The expression of HIF1α in the retinas of mice and mREC cells was detected by qRT-PCR and western blot (p<0.05, **p<0.01 vs. control or 0 h). (G-H) mREC cells were transfected with sh-HIF1α and then treated with HG. (G) The relative activity of the VEGFA promoter (-643bp and -325bp) was detected (**p<0.01 vs. HG). (H) The mRNA level of VEGFA was detected by qRT-PCR (**p<0.01 vs. HG). (I-J) mREC cells were transfected with sh-Hotair or sh-LSD1 and the treated with HG. (I) The enrichment of HIF1α on the VEGFA promoter was detected by ChIP assay (**p<0.01 vs. HG). (J) The expressions of HIF1α and VEGFA were detected by qRT-PCR and western blot (**p<0.01 vs. HG). (K) The binding of HIF1α to Hotair or LSD1 was detected by RNA pull-down and Co-IP assays. (L) mREC cells were transfected with Hotair or/and sh-LSD1 and then treated with HG. The expressions of VEGFA and HIF1α were detected by qRT-PCR and western blot, respectively (**p<0.01 vs. HG; ##p<0.01 vs. HG+Hotair). (M) mREC cells were transfected with LSD1 or/and sh-Hotair and then treated with HG. The expressions of VEGFA and HIF1α were detected by qRT-PCR and western blot, respectively (**p<0.01 vs. HG; ##p<0.01 vs. HG+LSD1).

**Fig. 7 Hotair/ LSD1 axis changed the effects of HG on retinal endothelial cell function.** mREC cells were transfected with Hotair or/and sh-LSD1 and then treated with HG. (A) The invasion of mREC cells was detected by transwell assay. (B) The permeability of monolayer mREC cells was detected using FITC-conjugated albumin. (C) The protein levels of VEGF and VE-cadherin in mREC cells were detected by western blot. (D) The summary figure of this study.

*p<0.05, **p<0.01 vs. HG; #p<0.05, ##p<0.01 vs. HG+Hotair.
