• Basic Research •

Down-regulation of histone deacetylase 7 reduces biological activities of retinal microvascular endothelial cells under high glucose condition and related mechanism

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Abstract

● **AIM:** To investigate the expression and effect of histone deacetylase 7 (HDAC7) in human retinal microvascular endothelial cells (HRMECs) under high glucose condition and related mechanism, and the expression of HDAC7 in the retinal tissue in diabetic rats.

● **METHODS:** The expression of HDAC7 in HRMECs under high glucose and the retinal tissue from normal or diabetic rats were detected with immunohistochemistry and Western blot. LV-shHDAC7 HRMECs were used to study the effect of HDAC7 on cell activities. Cell count kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), flow cytometry, scratch test, Transwell test and tube formation assay were used to examine the ability of cell proliferation, migration, and angiogenesis. Finally, a preliminary exploration of its mechanism was performed by Western blot.

● **RESULTS:** The expression of HDAC7 was both up-regulated in retinal tissues of diabetic rats and high glucose-treated HRMECs. Down-regulation of HDAC7 expression significantly reduced the ability of proliferation, migration, and tube formation, and reversed the high glucose-induced high expression of CDK1/Cyclin B1 and vascular endothelial growth factor in high glucose-treated HRMECs.

● **CONCLUSION:** High glucose can up-regulate the expression of HDAC7 in HRMECs. Down-regulation of HDAC7 can inhibit HRMECs activities. HDAC7 is proposed to be involved in pathogenesis of diabetic retinopathy and a therapeutic target.

● **KEYWORDS:** human retinal microvascular endothelial cells; histone deacetylase 7; high glucose; diabetic rat; vascular endothelial growth factor

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INTRODUCTION

Diabetic retinopathy (DR) is a prevalent ocular complication characterized by retinal microvascular lesions in patients with diabetes mellitus[1]. It is a leading cause of irreversible blindness and visual impairment among working-age individuals worldwide[2]. By 2040, an estimated 600 million people will have diabetes, with one-third of them expected to suffer from DR[3]. However, injection of anti-vascular endothelial growth factor (VEGF) drugs always leads to several injection risks and adverse events[4], and is also expensive. In addition, there are limited clinical methods for the early diagnosis and treatment of DR[5-6]. Thus, there is an urgent need to investigate effective therapeutic targets for early intervention in this blinding disease.

The cellular pathology of DR involves at least nine types of retinal cells, including photoreceptors, horizontal cells, bipolar cells, amacrine cells, retinal ganglion cells, glial cells, endothelial cells, pericytes, and retinal pigment epithelial cells[7]. Among them, endothelial cells play a crucial role in the early stage of DR and the early stage of neovascularization[8]. High glucose stimulation leads to endothelial damage and death, forming dead cell capillaries that cannot support blood flow[9]. Moreover, high glucose can induce proliferation, endothelial cell-mesenchymal cell transition (EndMT) and vulnerable neovascularization[8,10]. However, the mechanism of high glucose stimulating endothelial cell proliferation, causing EndMT transition and forming defective blood vessels is not clear.

Histone deacetylases (HDACs) plays a crucial role in regulating the development of various retinal cells, including retinal progenitor cells, photoreceptors, bipolar cells, ganglion cells and Müller glial cells[11]. HDACs IIa (HDAC4/5/7/9) regulate many aspects of cell biology, including proliferation, differentiation, autophagy, apoptosis, migration and angiogenesis[12-13]. Furthermore, HDAC7 has been identified as a key regulator of endothelial cell migration and angiogenesis[14]. However, the role of HDAC7 in diabetic retina remain poorly understood.

This study aims to investigate the expression of HDAC7 in human retinal microvascular endothelial cells (HRMECs), and the retina of diabetic rat.

MATERIALS AND METHODS

Ethical Approval All animal experiments were conducted upon ethical approval by the Animal Committee of the Air Force Military Medical University (IACUC-20230040). All animal experiments were performed according to the principles outlined in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals In this study, 20 rats were utilized, consisting of 10 middle-aged (8 months old) Goto-Kakizaki (GK) rats (a non-obese model that spontaneously develop type 2 diabetes mellitus early in life), and 10 age-matched Wistar rats. The Wistar rats and GK rats were obtained from Enswell Biotechnology Co., Ltd. (Xi'an, China), and maintained in the Animal Research Center of the Air Force Military Medical University under controlled environmental conditions, including light (12h day/night cycle) and humidity (45%–65%), and were given standard hard pellet chow and sterile water *ad libitum*. The selection of rats was based on a glucose tolerance test.

Immunohistochemistry Twenty pairs of eyeball tissues were fixed with eyeball fixative solution (G1109, Service Bio) and then embedded in paraffin. Immunohistochemical staining was performed according to standard procedures, using HDAC7 [1:100, # 33418, cell signaling technology (CST)].

Cell Culture and Lentivirus Infection HRMECs (purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) were cultured in endothelial cell medium (ECM) with 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution (ScienCell Research Laboratories, Carlsbad, CA, USA) at 37°C under 5% CO_2 atmosphere. Cells between passages three and eight were used in this study. Cells treated with normal glucose (5.5 mmol/L) as a control or high glucose (30 mmol/L). Culture normally in a cell culture incubator for 48h. The medium was changed daily to maintain cell viability and continuous high concentration of sugar stimulation. After the cells were treated with high glucose, the HDAC IIa antibody was used to screen downstream molecules (Table 1). The HDAC7 and respective control lentiviruses were obtained from Genechem Corporation (Shanghai, China), both containing green fluorescent protein (GFP), puromycin resistance gene. Before the cells are treated with high glucose, HRMECs were infected with lentiviral vectors encoding shRNA targeting HDAC7, as well as a control lentivector encoding a nonspecific shRNA sequence according to the protocol of the Genechem Recombinant Lentivirus Operation Manual. Finally, after puromycin drug screening, green fluorescence expression was checked by fluorescence microscope and image were taken. After the knockdown effect of HDAC7 was verified by Western blot, follow-up experiments were carried out.

Table 1 Antibodies used in Western blot

Antibodies	Catalogue number	Company
HDAC4	17449-1-Ap	Proteintech
HDAC5	16166-1-Ap	Proteintech
HDAC7	33418	CST
HDAC9	67364-1-Ig	Proteintech
CDK1	19532-1-AP	Proteintech
Cyclin B1	55004-1-AP	Proteintech
VEGF	19003-1-AP	Proteintech
Anti-beta actin	AT0001	Engibody

CST: Cell Signaling Technology; VEGF: Vascular endothelial growth factor.

Western Blot Total protein was extracted from cells using RIPA kit (AWB0136, Abiowell). The proteins were separated using 10% SDS-PAGE and then electrotransferred to a nitrocellulose filter membrane. The membrane was blocked with TBST containing 5% bovine albumin for 2h. The primary antibodies were incubated with the membrane at 4°C for 12h. The membrane was then incubated with the secondary antibody at room temperature for 2h. The expression of the target protein was detected using enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA) and ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). β-actin was used as a loading control. Quantification of Western blot results, using the band densitometry analysis, was performed with Image J software.

Analysis of Cell Viability Cell viability was assessed using cell counting kit-8 (CCK-8, 7Sea) in accordance with the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a microplate reader (SpectraMax M5, Molecular Device).

5-Ethynyl-2'-Deoxyuridine Incorporation Assay Cell proliferation was assessed by a 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. The BeyoClick EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Shanghai, China) was used according to the manufacturer's instructions.

Nuclear morphology was assessed by staining with 4',6-diamino-2-phenylindole (DAPI, Leagene, Beijing, China). Finally, the cells were imaged with an Olympus FV1000 confocal microscope (Olympus). EdU-positive cells were counted using Image J software.

Cell Cycle Flow Cytometry Cell suspensions of control, HG, HG+LV-shHDAC7 control, and HG+LV-shHDAC7 groups were collected, centrifuged at 500× g for 3min, and washed twice in phosphate-buffered saline (PBS), fixed with 70% ice-ethanol, the cell flow cytometry were detected about 10min after PI Flow cytometry. MODFIT software was used to analyze the cell cycle proportion.

Scratch Wound Assay Cells were seeded into six-well plates with routine culture medium containing 5% FBS. When the cells had grown to confluence, a linear scratch was created in the middle of each well using a 200 μL micropipette tip. The floating cells were washed with PBS 3 times, normal medium was added, and image were immediately captured. The gaps between the wound edges were monitored and photographed under a microscope after 24h.

Transwell Assay The Transwell assay was used to quantify cell migration capacity. Briefly, cells were inoculated into the upper Transwell chamber (Corning, Costar 3422) that contained FBS-free ECM (150 μL). ECM supplemented with 5% FBS (600 μL) was added to the lower chamber. After 24h, formalin was added to fix migrated cells, followed by staining with 0.1% crystal violet (Solarbio), imaging, and counting.

Tube Formation Assay Tube formation of HRMECs was tested using a previously described protocol[15]. Growth Factor Reduced Matrigel matrix (ABW & Matrigengel, 082704) was laid on the bottom of a 48-well plate, and HRMECs were seeded on top of it. After 6h, capillary-like structures were observed under a Nikon Eclipse Ti inverted microscope (Nikon). At least six different fields were randomly selected and observed in each well. Meshes, branches, and the branching length of the capillary-like structures were analyzed using Image J software.

Statistical Analysis All experiments were repeated at least 3 times. The quantitative data of each group were compared with *t*-test, and categorical data were analyzed by χ^2 test or Fisher's exact test. $P<0.05$ was considered statistically significant. The data were analyzed using SPSS 24.0 software (IBM Corp).

RESULTS

HDAC7 Expression in Retinal Endothelial Vessels of Diabetic Rats and HRMECs After High Glucose Treatment The expression of HDAC7 in retina of Wistar rats and GK rats (animal model of spontaneous type 2 diabetes mellitus) rats was detected by immunohistochemistry (Figure 1A) and Western blot (Figure 1B) respectively. It was found that the expression level of HDAC7 in retina microvascular of GK rats was higher than that in Wistar rats. After the HRMECs were stimulated with high glucose, Western blot detected the change level of HDAC IIa and found that HDAC4 and HDAC9 have no significant changes. The HDAC5 and HDAC7 showed increased expression which was statistically significant, while pronounced increase was seen in HDAC7 (Figure 1C). This result is consistent with the results of animal experiments. Based on the above results, we established HDAC7-knocdown HRMECs and control HRMECs using lentivirus. The protein expression level of HDAC7 was significantly down-regulated, and the fluorescence expression of the control group and LV-shHDAC7 cells was good, indicating that the cells in both groups were normally infected (Figure 1D, 1E).

Down-Regulation of HDAC7 Attenuated the Proliferation of HRMECs Stimulated by High Glucose We used CCK-8, EdU incorporation assays, and cell cycle flow cytometry to detect cell proliferation. In the CCK-8 experiment, we found that after high glucose treatment, the proliferation activity of HRMECs was enhanced. And compared with the HG+LV-shHDAC7 control group, HDAC7 knockdown attenuated high glucose-induced cell proliferation activity (Figure 2A). Then, we used the EdU experiment to show the changes in the cell proliferation ability. Under high glucose stimulation, the proportion of proliferating cells increased significantly. After HDAC7 lentivirus knockdown, the proportion of cells in the proliferating phase also decreased significantly (Figure 2B). Finally, we examined the cell cycle by flow cytometry. The ratio of G2+S/G1 cells in the high glucose group was significantly increased, and this result was partially reversed after HDAC7 knockdown. Compared with the HG+LV-shHDAC7 control group, the HRMECs in the HG+LV-shHDAC7 group were G2+S/G1 ratio is significantly reduced (Figure 2C). The results showed that the knockdown of HDAC7 decreased the proliferation of HRMECs induced by high glucose.

Down-Regulation of HDAC7 Attenuates the Migration and Angiogenesis of HRMECs Stimulated by High Glucose We tested the migration ability of HRMECs by scratch test and Transwell test, and the angiogenesis ability of HRMECs by tube formation test. The scratch test results showed that the migration ability of HRMECs was enhanced under the stimulation of high glucose. Down-regulation of HDAC7 expression partially reversed the pro-migration effect under high glucose stimulation (Figure 3A). The Transwell results also further confirmed this result (Figure 3B). We further evaluated tube formation in different groups of HRMECs. As shown in Figure 3C, high glucose significantly increased the network formation of HRMECs. And under high glucose, network formation was also reduced in HDAC7-knockdown HRMECs compared with the HG+LV-shHDAC7 control group.

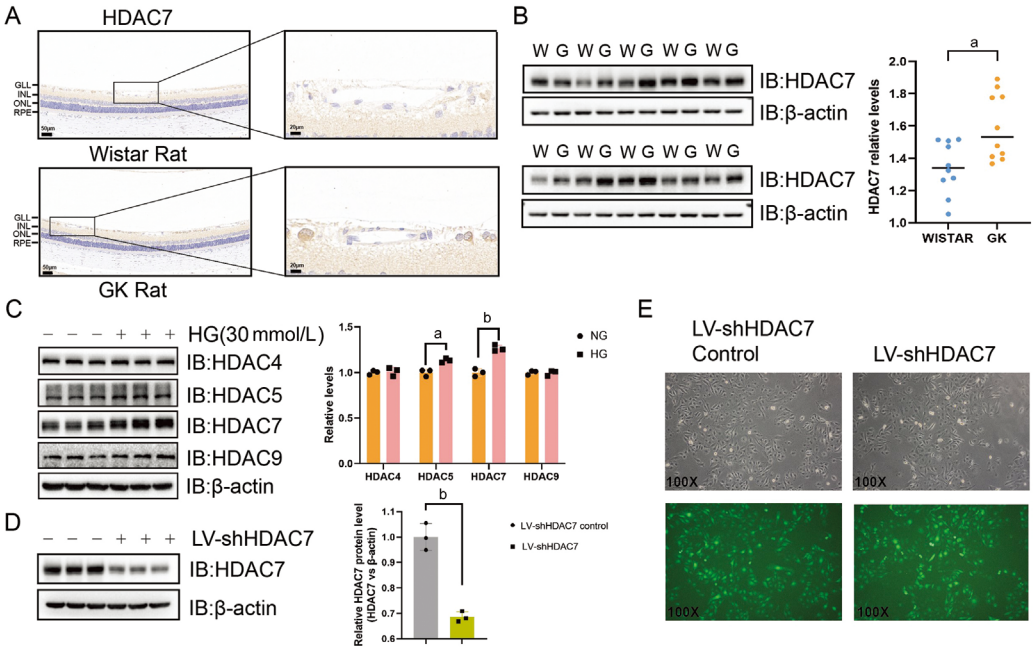

Figure 1 HDAC7 is high expressed in retinal endothelial vessels of diabetic rats and HRMECs after high glucose treatment A: Representative immunohistochemical image showing HDAC7 expression in retinal endothelial vessels of Wistar rat and GK rat; B: Western blot of HDAC7 expression in retinal tissues of 10 pairs of Wistar rats and GK rats and statistical analysis; C: Western blot image of HDAC IIa expressions in HRMECs after high glucose treatment and statistical analysis; D: Western blot image of HDAC7 expression in HRMECs after lentivirus transfection and statistical analysis; E: Bright field image and green fluorescence image of cells under fluorescence microscope after lentivirus transfection and puromycin screening. [a]*P*<0.01, [b]*P*<0.001. LV: Lentivirus. HG: High glucose; HDAC7: Histone deacetylase 7; HRMECs: Human retinal microvascular endothelial cells; GK: Goto-Kakizaki.

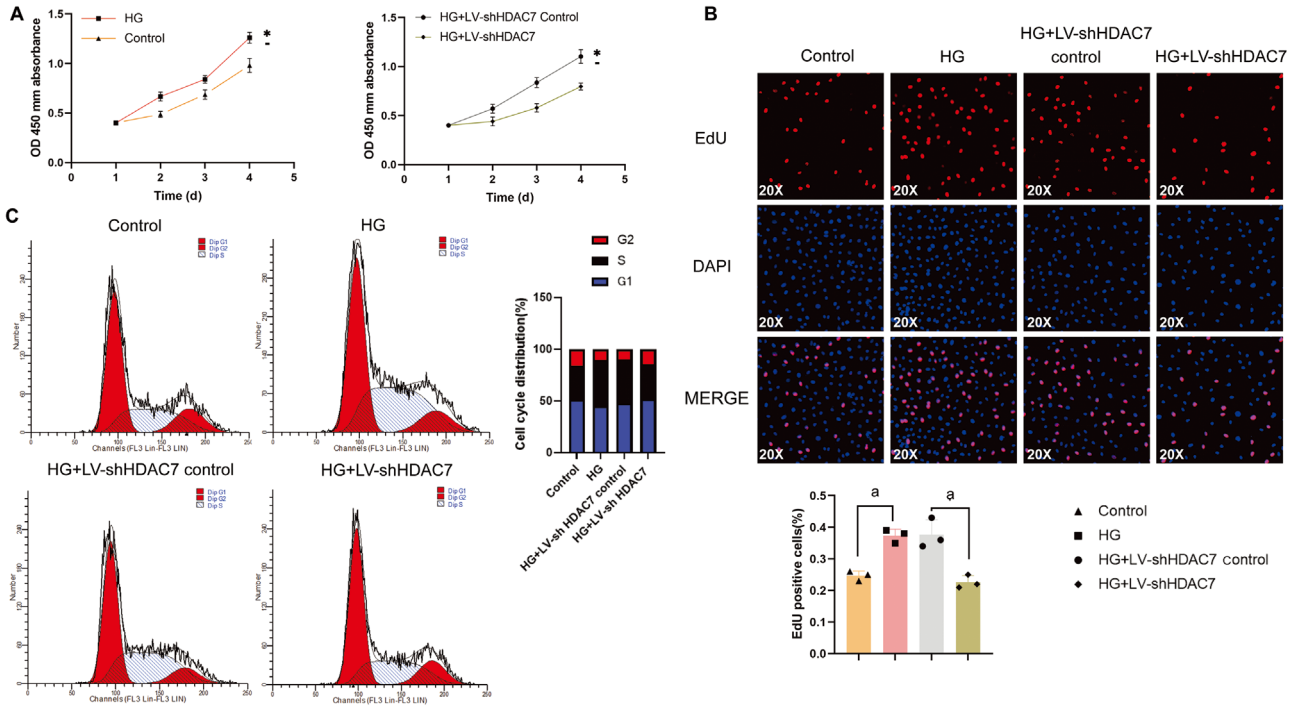

Figure 2 Down-regulation of HDAC7 attenuated the proliferation of HRMECs stimulated by high glucose A: The results of the CCK-8 assays in HRMECs with or without high glucose treatment, and the results of the CCK-8 assays in HRMECs with or without high glucose and lentivirus treatment. B: Representative image of EdU incorporation analysis of HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). The result was calculated as the ratio between the number of EdU-stained cells (red fluorescence) and the total number of DAPI-stained cells (blue fluorescence). C: The cell cycle distribution in HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7), and the data analyzed by flow cytometry. [a]*P*<0.05. LV: Lentivirus. HG: High glucose; HDAC7: Histone deacetylase 7; HRMECs: Human retinal microvascular endothelial cells; CCK-8: Cell count kit-8; EdU: 5-ethynyl-2'-deoxyuridine; DAPI: 4',6-diamino-2-phenylindole.

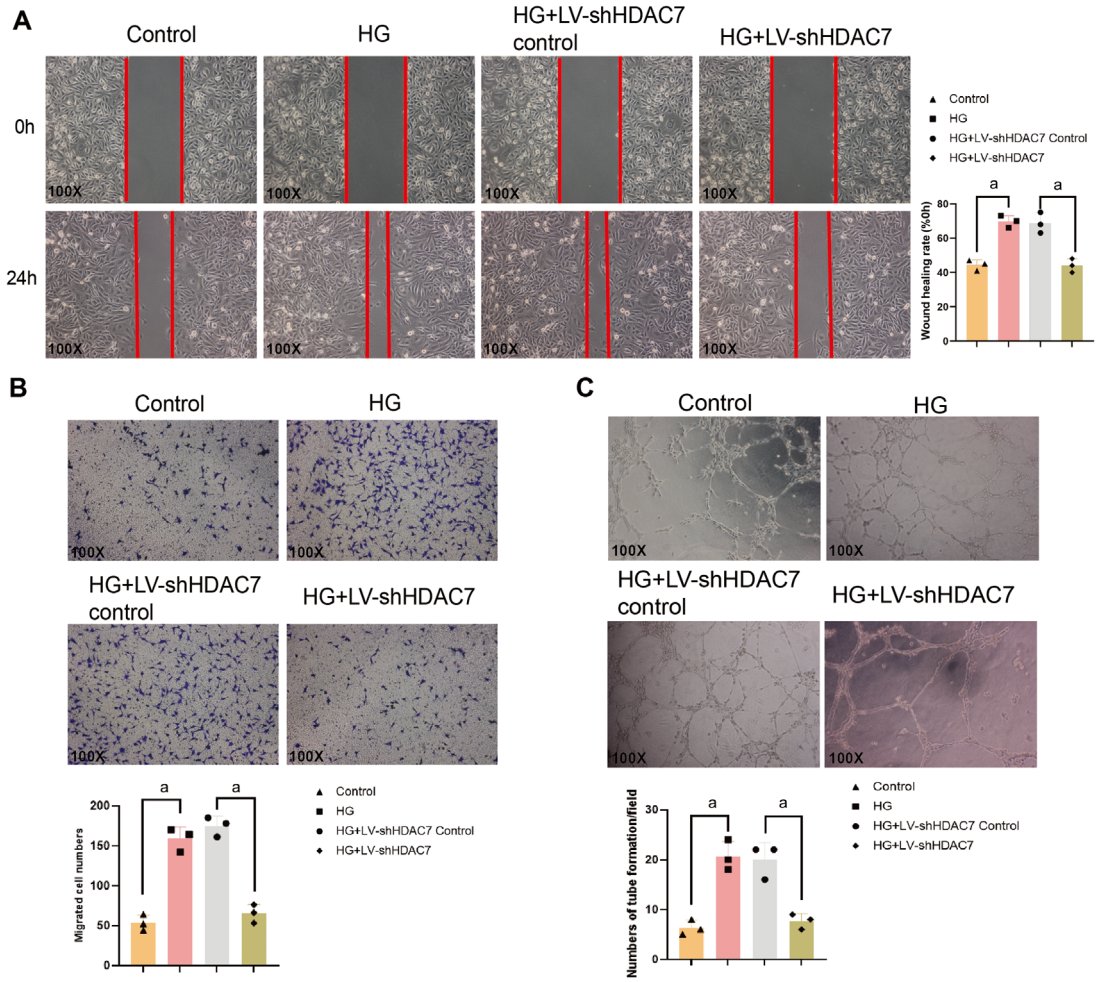

Figure 3 Down-regulation of HDAC7 attenuates the migration and angiogenesis of HRMECs stimulated by high glucose A: Representative image and statistical analysis of cell scratch test using HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). Wound healing rate=the migration distance [scratch width (0h) – scratch width (24h)] of the experimental group was calculated relative to that of the control group. B: Representative image and statistical analysis of transwell migration assays using HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). C: Representative image and statistical analysis of tube formation assay migration assays using HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). [a]*P*<0.05 compared with the control group. LV: Lentivirus; HG: High glucose; HDAC7: Histone deacetylase 7; HRMECs: Human retinal microvascular endothelial cells.

High Glucose Induces Molecular Changes in Proliferation and Angiogenesis by Regulating HDAC7 We detected the representative proteins of cell cycle and angiogenesis by Western blot. It was found that under different experimental treatment conditions, some of these marker proteins changed with the change of HDAC7 (Figure 4A). Under the action of high glucose, the expression of HDAC7 in HRMECs was significantly increased. And our transfected lentivirus also successfully down-regulated the expression level of HDAC7 in HRMECs stimulated by high glucose (Figure 4B). CDK1 and its related cell cycle protein cyclinB1 were significantly increased under high glucose, and their expression levels were down-regulated as the expression of HDAC7 was down-regulated by lentivirus (Figure 4C, 4D). The key molecule of neovascularization, VEGF, was significantly increased under the action of high glucose, and this phenomenon could be weakened with the down-regulation of HDAC7 expression (Figure 4E). The above results indicated that high glucose could up-regulate the expression level of HDAC7, and then affect the key molecules of HRMECs proliferation, migration, and angiogenesis.

DISCUSSION

The mechanism of retinal angiogenesis is very complex, and retinal neovascularization plays a crucial role in the pathological progression of diabetic eye disease. However, there is a lack of relevant research[8,16-17]. Many studies have shown that there is a close relationship between HDAC functions and diabetes. Not only HDAC dysfunction is related to β-cell dysfunction, HDAC inhibitors are also an important target for the treatment of diabetes and some

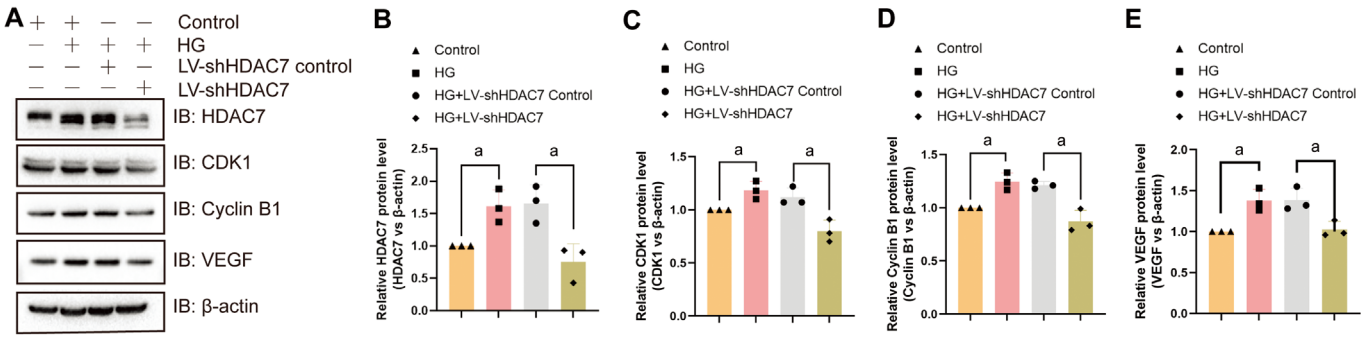

Figure 4 HDAC7 affects the expression levels of markers of cell proliferation in HRMECs after high glucose treatment Representative Western blot (A) showing the levels of HDAC7, CDK1, cyclinB1, and VEGF in HRMECs under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). Statistical analysis of HDAC7 (B), CDK1 (C), cyclinB1 (D), and VEGF (E) level under different treatment (control, HG, HG+LV-shHDAC7 control, HG+LV-shHDAC7). $^{a}P<0.05$ compared with the control group. LV: Lentivirus; HG: High glucose; HDAC7: Histone deacetylase 7; HRMECs: Human retinal microvascular endothelial cells; VEGF: Vascular endothelial growth factor.

diabetic complications[18-19]. HDAC3 inhibitors have potential value in treating diabetes mellitus[20]. The O-GlcNA acylation of HDAC4 in Ser-642 counteracts the pathologic calcium 2 receptor/calmodulin dependent protein kinase II signal transduction in diabetes, which can protect the heart of diabetic patients[21]. HDAC11 is also closely related to the pathogenesis of some metabolic diseases, including obesity, diabetes and diabetic complications[22]. Increased expression of HDAC9 in fibrovascular membrane of patients with proliferative DR was found by retinal immunohistochemistry in 25 patients with diabetes mellitus[23]. It also has been found that HDAC7 plays an important role in the pathological mechanism of diabetes. Overexpression of HDAC7 resulted in impaired insulin secretion, decreased insulin content in β-cells, mitochondrial respiration and cellular ATP levels[24-25]. Overexpression of HDAC7 also leads to an increase in the expression of Tcf7l2 and a decrease in the expression of gene sets that regulate DNA replication and repair and nucleotide metabolism[24]. These injuries were reversed by HDAC7 inhibitor MC1568, which also increased insulin secretion and had no effect on islets in non-diabetic individuals[25]. However, there is a lack of research on the role of HDAC7 in DR. In this study, by analyzing the immunohistochemical results of the retina of diabetic and normal rats, we found that HDAC7 was highly expressed in the retina of diabetic rats. And under the stimulation of high glucose, the expression of HDAC7 in HRMECs was significantly increased, and the proliferation and angiogenesis of HRMECs were enhanced. After we knocked down HDAC7 or added HDAC inhibitor, the corresponding ability of cells was weakened. This finding indicates that HDAC7 may play a detrimental role in DR by promoting endothelial dysfunction.

Diabetic damage can promote the growth of neovascularization, abnormal growth of neovascularization can cause bleeding, scar, which can lead to retinal detachment[26-27]. A large number of studies have shown that HDACs can promote cell proliferation by regulating the expression and activity of cyclin proteins. For example, HDAC1 is essential for unrestricted cell proliferation by inhibiting the expression of selective cell cycle suppressor genes[28]. Overexpression of HDAC4 can significantly inhibit the expression of cyclin dependent kinase (CDK) inhibitors p21 and p27, and then promote the proliferation and invasion of glioma cells[29]. Zhou *et al*[30] found that HDAC5 loss could induce CDK4/6 inhibitor resistance in cancer. In order to further explore the molecular mechanism of HDAC7 promoting the proliferation of HRMECs, we detected the cell cycle related proteins by Western blot assay. The results showed that the high expression of HDAC7 mediated by high glucose promoted the proliferation of HRMECs by up-regulating the expression of CDK1/CyclinB1. This result is consistent with previous findings of HDAC7 in esophageal squamous cell carcinoma cells, where the effects on proliferation, migration and invasiveness of esophageal squamous cell carcinoma cells were positive or negative in the HDAC7 overexpression or knockdown groups, respectively[31]. Flow cytometry showed that the percentage of cells in G2 phase and S phase increased after HRMECs was treated with high glucose. In addition, LV-shHDAC7 group reversed this effect of promoting proliferation. Therefore, our study proves that HDAC7 promotes the proliferation of HRMECs.

Long-term diabetes causes ocular tissue ischemia and hypoxia, which will induce the up-regulation of VEGF factor[32-33]. VEGF induces multiple inflammatory releases and neovascularization through a variety of pathways, such as NF-κB and STAT3[34-35]. Some studies have found that HDAC7 plays an important mediating role in VEGF activation of proliferation and angiogenesis pathway. VEGF induces phosphorylation of HDAC7 at Ser178/344/479 residues in endothelial cells *via* PKD1-dependent mechanism and subsequent nuclear exclusion of HDAC7 and MEF2 transcriptional activation[36]. In addition, HDAC7 also acts

as an intermediary in the VEGF signal to assist with early endothelial cell migration[36]. In this study, our results showed that high glucose-induced high-level VEGF could be reversed by HDAC7 knockdown. This finding indicates that VEGF and HDAC7 constitute a reciprocal feedback circuit that modulates angiogenesis in HRMECs.

In summary, this study shows that high glucose can up-regulate the expression of HDAC7 and affect the proliferation and angiogenesis of HRMECs. HDAC7 may play an important role in the early diabetic-induced changes in retinal endothelial cells, which may further lead to vascular damage and DR progression. Further experiments are needed to elucidate the specific mechanism and the potential therapeutic value of HDAC7 in DR management.

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