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Heparanase mediates vascular endothelial growth factor gene transcription in high-glucose human retinal microvascular endothelial cells

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Purpose: To observe the nuclear expression and interaction of heparanase and RNA polymerase II (RNA Pol II), an enzyme that catalyzes the transcription of DNA in eukaryotic cells) in human retinal microvascular endothelial cells (HRECs) under high glucose condition and to investigate the association of heparanase with the transcription activity of the vascular endothelial growth factor (*VEGF*) gene promoter.
Methods: Cultured HRECs were maintained for 3 days in media with high or normal glucose. The expressions of heparanase and RNA Pol II in each group were analyzed with immunofluorescence. Co-immunoprecipitation was applied to detect the interaction of heparanase and Pol II proteins. Cells in both groups were used for chromatin immunoprecipitation (ChIP) with anti-heparanase and anti-RNA Pol II antibodies to identify high-confidence heparanase-binding regions across the entire *VEGF* gene promoter. Moreover, real-time PCR was used to demonstrate the interaction between heparanase and the *VEGF* gene promoter region.
Results: The immunofluorescence studies showed that the nuclear expression of heparanase was intense in high-glucose HRECs but faint in the normal group; RNA Pol II in the nucleus was also intense in high glucose HRECs, and the distribution of heparanase was consistent with that of RNA Pol II. The co-immunoprecipitation data showed that heparanase combined with RNA Pol II in HRECs cells treated with high glucose, and the molecular size of HPA interacted with RNA Pol II was 50 kDa, while no combination of two proteins was evident in normal HRECs cells. Real-time PCR–based ChIP results showed that the high-confidence HPA-binding region was −1155 to −1018 (containing hypoxia response element) in the VEGF gene promoter, and the cells treated with high glucose showed increases in heparanase and RNA Pol II in the *VEGF* gene promoter region compared with the normal glucose treated cells ($t = -3.244$, $p = 0.032$; $t = -6.096$, $p = 0.004$, respectively).
Conclusions: Nuclear heparanase combines directly with the *VEGF* gene promoter and is involved in the regulation of *VEGF* gene transcription in high-glucose HRECs.

Heparanase is a mammalian endoglucuronidase localized primarily in a perinuclear pattern within lysosomes, late endosomes, and occasionally, cell surfaces. It is responsible for heparan sulfate (HS) degradation, yielding relatively large, biologically potent HS fragments (5–10 kDa) [1]. The degradation of HS side chains releases HS-binding angiogenic growth factors, including β fibroblast growth factor and vascular endothelial growth factor (VEGF), in addition to HS fragments [2]. These fragments play a decisive role in fundamental biological processes, such as angiogenesis and cancer metastasis, that are associated with remodeling of the extracellular matrix, generally by decreasing the activity of HS [2]. Heparanase expression has been associated with an aggressive malignant phenotype and an adverse prognosis in cancer patients [2]. Heparanase has been observed in the nucleus, as well as the cytoplasm [3]. Studies of heparanase in the nuclei of various human tumor cells showed that it can participate in the gene regulation of angiogenesis-related proteins associated with an aggressive malignant phenotype and play an important role in tumor angiogenesis [4,5]. Recently, studies have demonstrated that a novel class of signal transduction kinases translocates into the nucleus and associates with chromatin to directly modulate the transcription of target genes, in addition to the traditional function of heparanase [6-8].

Heparanase has also been implicated in the pathogenesis of diabetes, with studies reporting elevated levels in the serum and urine of patients with diabetic nephropathy [9] and the expression of the heparanase protein in renal glomerular cells of some diabetic patients [10]. Heparanase expression was upregulated and associated with an increase in VEGF expression in the streptozotocin-induced diabetic rat retina. The study suggested that the expression of heparanase increased in response to high glucose and that it was highly correlated with VEGF levels in human retinal vascular endothelial cells

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in vitro [11]. However, the mechanism by which heparanase enhances VEGF expression is not entirely clear.

In the present study, using RNA polymerase II (RNA Pol II)—a key enzyme in the active gene transcription of eukaryotes—as a marker, we examined the possibility that heparanase in the nucleus directly participates in *VEGF* gene regulation by affecting the transcription of the VEGF promoter. The present study was designed to observe the expression of heparanase and RNA Pol II, detect the interaction of heparanase and RNA Pol II in the nuclei of human retinal endothelial cells (HRECs), and investigate the effect of heparanase on the transcription activity of the VEGF promoter in human retinal microvascular endothelial cells (HRECs) induced by high glucose.

METHODS

Culture and treatment of HRECs: Human eyes were obtained from the Eye Bank of Zhongshan Ophthalmic Center of Sun Yat-sen University within 24 h postmortem. All the donors of the eyes were healthy accident victims. The acquisition of all human materials complied with the ethical principles of the World Medical Association (Declaration of Helsinki) for medical research. The cell culture procedures were carried out as previously described [12]. Briefly, retinal tissues were removed by dissection and digested with 2% trypsogen and 0.1% collagenase I (Sigma Chemical Co, St. Louis, MO) for 20 min at 37 °C and then subjected to centrifugation (1,000 ×*g* for 10 min). The pellets were cultured in a 21.5-mm^2 culture dish coated with 5 mg/ml of fibronectin (Gibco, Grand Island, NY) for 1 h in human endothelial serum-free medium (Gibco), supplemented with 10% fetal bovine serum and 5 ng/ml β-endothelial cell growth factor (Sigma), in a humidified atmosphere of 5% CO_2 at 37 °C. The expression of the endothelial marker of cultured HRECs, anti-VIII factor antibody (Biosynthesis Biotechnology Co, Beijing, China) was determined by immunofluorescent staining. Only cells at passages 3 to 5 were used for the experiments. The HRECs were incubated with normal medium (control group: 5 mmol/l of glucose) or high glucose (high-glucose group: 30 mmol/l of glucose) for 72 h.

Immunofluorescence: The HRECs were seeded on glass coverslips precoated with 5 mg/ml of fibronectin (Gibco) and allowed to grow to semiconfluence in a culture dish. The cells were washed with phosphate buffered saline (PBS) three times and fixed in fresh 4% paraformaldehyde (pH 7–8) for 10 min at room temperature. PBS was composed of 2.89 g of $Na_2HPO_4 \cdot 12H_2O$, 8 g of NaCl, 0.2 g of KCl, 0.2 g KH_2PO_4 and 80 ml of ddH_2O. Next, the HRECs were permeabilized in 0.1% of Triton X-100 (Sigma, St. Louis, MO) for 5 min and blocked with 1% albumin from bovine serum (BSA; Sigma) in PBS containing 0.1% Tween 20 (blocking solution) for 60 min at room temperature. The cells were then incubated with rabbit anti-human heparanase antibody (1:300 dilution; Abcam, Cambridge, MA) and mouse anti-human RNA Pol II antibody (1:200 dilution; Abcam) overnight at 4 °C for the expression of heparanase and RNA Pol II, followed by incubation with appropriate secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 555 (1:200 dilution; Boster Biologic Technology, Ltd., Wuhan, China) for 2 h at room temperature. The cells were stained with 100 ng/ml of 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min, mounted with an antifading fluorescence medium (Vector Laboratories, Burlingame, CA), and imaged using a laser scanning confocal microscope (Carl Zeiss, Jena, Germany).

Co-immunoprecipitation assays: The HRECs were harvested and lysed in lysis buffer containing 50 mM of HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.125% TritonX-100, and a protease inhibitor cocktail (Sigma), followed by spinning to collect the supernatants (nuclear extract). A total of 300 µl of extract was resuspended in 1 ml of protein inhibiting buffer and precleared for 1 h at 4 °C with settled protein-A Sepharose beads (Upstate Biotechnology, Lake Placid, NY). After removal of the beads, the precleared sample was incubated with rabbit anti-human/mouse heparanase (Insight Biopharmaceuticals, Rehovot, Israel), rabbit anti-human RNA Pol II antibody (Abcam) and normal rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. The coupled protein/antibody complexes were adsorbed onto protein-A Sepharose beads by incubation of the sample for 2 h at 4 °C. The protein/antibody complex beads were then resuspended in buffer containing sodium dodecyl sulfate (SDS). The immunoprecipitated proteins were released from the beads by boiling at 95–100 °C for 3–5 min and spinning briefly to collect the supernatants. They were then investigated with western blot analysis. Ten percent of the volume of the cell extract was the input DNA. The experiments were conducted in quadruplicate and repeated three times.

Western blot analyses: The immunoprecipitated material from each sample was dissolved in sample buffer and boiled for 5 min before loading with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% Tris-glycine gel (Invitrogen, Paisley, UK) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) at 250 mA for 90 min. Each membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.5% Tween-20 for 1 h at room temperature. The membranes were then incubated with polyclonal mouse anti-human heparanase antibody

(1:800 dilution; Abcam) or mouse anti-human RNA Pol II antibody (1:200 dilution; Abcam) at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature after washing with PBS containing 0.1% Tween 20. Finally, the blots were developed with a chemiluminescence reagent (Cell Signaling Technology Inc., Danvers, MA).

Chromatin Immunoprecipitation: The HRECs were treated for 72 h before harvesting for chromatin immunoprecipitation (ChIP) following the manufacturer's instructions. ChIP assays were performed using the provided ChIP assay kit (Upstate Biotechnology). The cells were crosslinked by adding formaldehyde to a final concentration of 1% and then incubated at 37 °C for 10 min. They were then collected and pelleted at 4 °C and resuspended in 0.3 ml of cell lysis buffer (50 mmol/l Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS) containing a complete protease inhibitor mixture (Sigma). The chromatin lysate was sonicated on ice to an average DNA length of 300–800 bp. Immunoprecipitation was performed with rabbit anti-heparanase (Insight Biopharmaceuticals) and rabbit anti-RNA polymerase II antibodies (Abcam) overnight at 4 °C, with rabbit IgG (Santa Cruz Biotechnology) as the negative control, followed by the addition of 50 μl of a 50% slurry of protein-A agarose/salmon sperm DNA and incubated at 4 °C for an additional 2 h. The beads were pelleted and reversed protein-DNA crosslinked by heating at 65 °C overnight. The DNA was then extracted by phenol:chloroform. For quantification of the ChIP analysis, real-time PCR was performed as described by the manufacturers (TaKaRa, Kusatsu, Japan). The formation of PCR products was detected by incorporation of SYBR green I using ROX as a passive reference (TaKaRa). For real-time PCR, the primer specific to the VEGF promoter (forward, 5′-GAC GTT CCT TAG TGC TGG CGG GTA GGT TTG A −3′; reverse, 5′-GGC ACC AAG TTT GTG GAGCT GAG AAC- 3′) was used. Quantitative PCR was performed in an ABI PRISM7000 (Applied Biosystems, Grand Island, NY) sequence detector. Ten percent of testis extract was loaded as input. The experiments were conducted in quadruplicate and repeated three times.

Statistical analysis: The relative expression software tool was used to evaluate the quantitative statistical value of PCR group-wise comparisons based on PCR efficiency and the mean crossing point deviation between the sample and control group. The above experimental procedures were repeated and analyzed three times. Statistical significance was analyzed by performing a one-way analysis of variance (ANOVA) or the Student *t* test with SPSS software 13.0 for Windows (SPSS Inc., Chicago, IL). A *p*-value<0.05 was considered statistically significant.

RESULTS

Subcellular localization of heparanase and RNA Pol II: The expression of the endothelial marker of cultured primary HRECs, anti-VIII factor antibody, was determined by immunofluorescent staining to confirm the primary quality isolation (Figure 1). The expression of heparanase in the cytoplasm and nuclei was intense and higher in the high-glucose HREC group (Figure 2F,I) but faint in the control group (Figure 2B,I). The intensity of heparanase staining was greatest in the cell nuclei, nucleoli, or perinuclear areas of the cells treated with high glucose (Figure 2F,H). However, the relative distribution of heparanase in these locations varied somewhat. The intensity of nuclei staining and protein expression for RNA pol II were increased in the high-glucose HREC group (Figure 2C,G,I). Heparanase and RNA Pol II were distinctly colocalized in the cells treated with high glucose (Figure 2H,I) in contrast to the control group (Figure 2D,I).

Interaction of heparanase with RNA Pol II in high-glucose-induced HRECs: RNA Pol II catalyzes the transcription of DNA to synthesize precursors of mRNA. The nuclear colocalization of heparanase and RNA Pol II led us to investigate the function of nucleus heparanase colocated with RNA Pol II in HRECs. The data showed that heparanase combined with RNA Pol II in the HRECs treated with high glucose. The molecular size of heparanase that interacted with RNA Pol II was 50 kDa (Figure 3A). No protein–protein combination was observed in the control HRECs (Figure 3B). The nuclear colocalization of heparanase and RNA Pol II suggests that heparanase may fulfill functions (i.e., regulation of gene expression and signal transduction) other than its well documented involvement in cancer metastasis, angiogenesis, and inflammation [3].

Effect of high glucose treatment on the expression level of the VEGF gene promoter in vitro: A previous study reported that heparanase increased the expression level of VEGF [13]. We first conducted ChIP and real-time PCR to examine the association of heparanase with the transcription activity of the *VEGF* gene promoter. The real-time PCR–based ChIP results showed that the occupancy of the heparanase binding region in the VEGF gene promoter was −1155 to −1018 (containing the hypoxia response element). Heparanase and RNA Pol II increased in the *VEGF* gene promoter region of the cells treated with high glucose compared with the control group ($t = -3.244$ and $p = 0.032$; $t = -6.096$ and $p = 0.004$, respectively, Figure 4). Figure 4 shows the effect of nuclear heparanase on *VEGF* gene transcription in the high-glucose-induced condition.

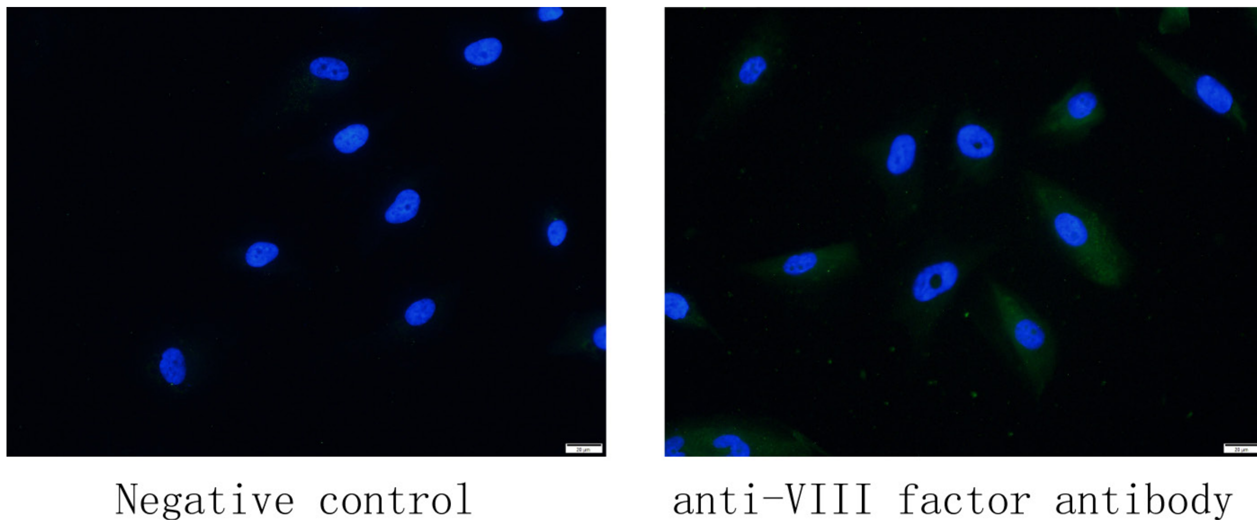

Figure 1. Image of human retinal endothelial cell (HREC) marker immunofluorescence. The HRECs were cultured in normal media. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; blue) or rabbit anti-VIII factor antibody (green).

DISCUSSION

Diabetic retinopathy is a leading cause of adult vision loss and blindness. Much of the retinal damage was induced by neurodegenerative changes, retinal vascular leakage, and nonperfusion. Uncontrolled hyperglycemia is the main risk factor in the development of diabetic vascular complications, and the endothelial cells are important cells targeted in hyperglycemia [14]. VEGF is a secreted angiogenic mitogen from retinal endothelial cells, retinal pigment epithelium cells, and Müller cells [15-17]. The importance of VEGF in ocular neovascularization has been established by more than a decade of research, beginning in the early 1990s with the identification of elevated levels in several ocular neovascular syndromes [16,18,19]. VEGF has been shown to play a crucial role during critical angiogenic processes involved in the pathogenesis of cancer, diabetic vascular diseases, and other vascular diseases [19-22]. Elevated levels of VEGF-A have been found in the aqueous humor and vitreous of patients with proliferative diabetic retinopathy [23]. Our previous study showed that heparanase expression is upregulated by high glucose and that it has a close relationship with VEGF levels in high-glucose-induced HRECs in vitro [11].

Heparanase is synthesized as a 65-kDa inactive precursor that undergoes proteolytic cleavage, yielding 8-kDa and

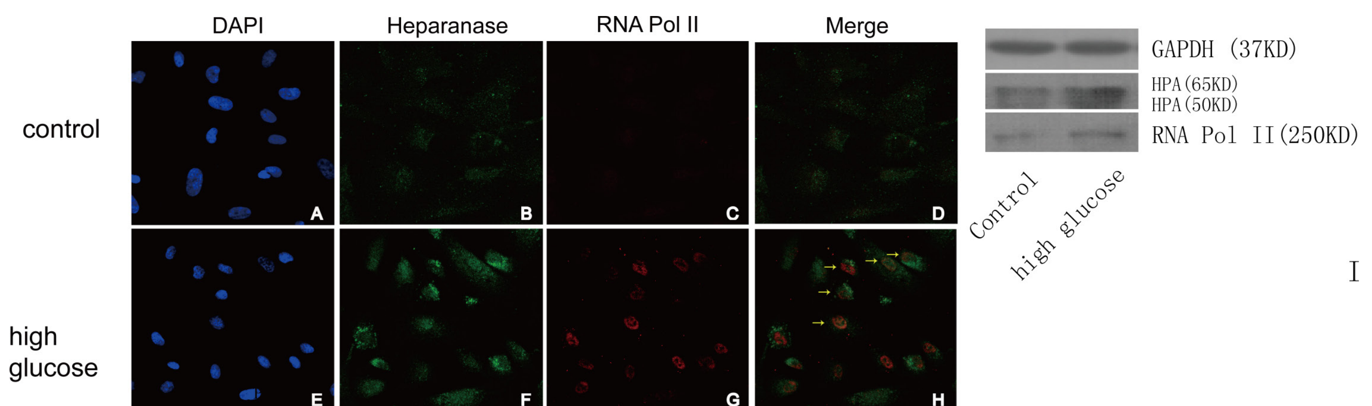

Figure 2. Localizations of heparanase and RNA polymerase II (Pol II) in high-glucose-induced human retinal endothelial cells (HRECs). The HRECs were cultured in normal media (control: **A**–**D**) or treated with high glucose (**E**–**H**) for 72 h. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; blue: **A**, **E**), rabbit anti-heparanase antibody (green: **B**, **F**), and mouse anti-RNA Pol II antibodies (red: **C**, **G**). Low levels of heparanase (**B**) and RNA Pol II (**C**) were observed in control HRECs, whereas high levels were observed in the high-glucose cells, with colocalization of heparanase and RNA Pol II (**H**, arrows). Western blot analysis indicated that the expressions of heparanase and RNA Pol II were higher in the high-glucose cells (**I**).

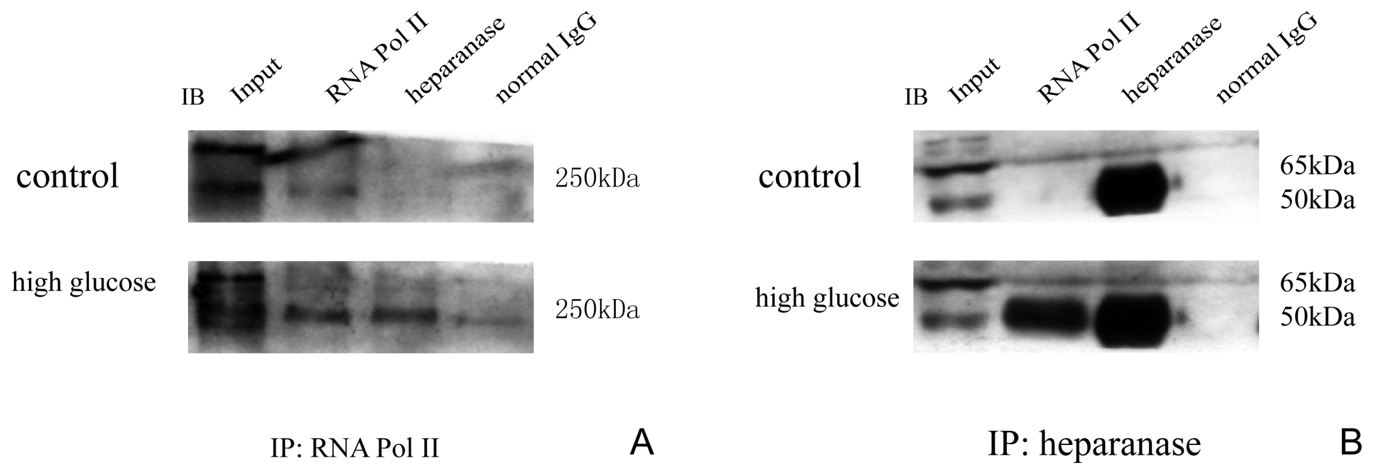

Figure 3. Interaction of heparanase with RNA polymerase II (Pol II) in high-glucose-induced human retinal endothelial cells (HRECs). The HRECs were incubated with normal medium and high glucose for 72 h. The immunoprecipitated antibody is shown in the top row; the immunoblotted antibody is shown in the bottom row. **A**: The cell lysates were immunoprecipitated with anti-heparanase antibody and immunoblotted with anti-RNA Pol II antibody. **B**: The cell lysates were immunoprecipitated with anti-RNA Pol II antibody and immunoblotted with anti-heparanase antibody. The second lane in **A** and the third lane in **B** both meet heparanase bound with RNA Pol II together in high glucose HRECs. All experiments were performed in triplicate.

50-kDa protein subunits that heterodimerize to form an active enzyme [24]. Early studies suggested that human heparanase is localized primarily in a perinuclear pattern in lysosomes and late endosomes [3]. Later studies indicated that it also occurs in the nuclei of various human tumor cells [3,4,25,26]. Studies have also suggested that enhanced heparanase expression is correlated with shorter postoperative survival of cancer patients, where it participates in gene regulation of angiogenesis-related proteins, such as cyclooxygenase-2 and fibroblast grow factor-2 (FGF-2) [27-29]. Consistent with

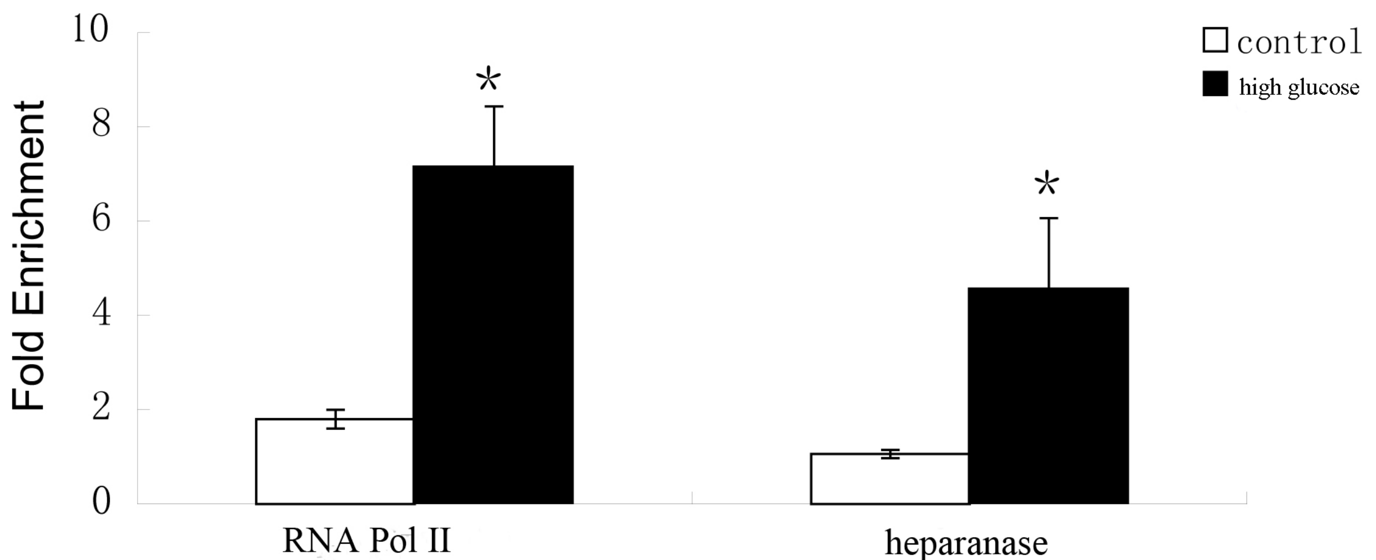

Figure 4. High-glucose-induced increase of vascular endothelial growth factor (*VEGF*) gene transcription by recruitment of heparanase binding to the VEGF gene promoter. Human retinal endothelial cells (HRECs) incubated with normal medium or high glucose for 72 h were subjected to a chromatin immunoprecipitation (ChIP) assay with the indicated antibodies. The *VEGF* gene promoter of the RNA polymerase II (Pol II) binding protein A agarose DNA–antigen–antibody complex increased in the high-glucose group compared with the control group, denoting transcription upregulation of the *VEGF* gene. The expression of the *VEGF* gene promoter of the heparanase antibody binding protein A agarose DNA–antigen–antibody complex was higher in the high glucose group than in the control group. ChIP data were quantified by real-time PCR analysis. *p<0.05 versus control.

previous reports, our data showed that human heparanase is localized in both the cell cytoplasm and the nucleus. Schubert described two feasible mechanisms for heparanase nuclear localization. One is the presence of two potential nuclear localization signals (residues 271–277, PRRKTAK; and residues 427–430, KRRK) that can mediate nuclear localization of the enzyme. The other is the occurrence of nuclear HS proteoglycans (HSPG), which can mediate the nuclear localization of heparanase by using HS as a vehicle to bind the enzyme. In the present study, heparanase and RNA Pol II staining was intense in the nuclei of high glucose-induced HRECs, suggesting that the function of heparanase is possibly associated with the start of target gene transcription.

Analysis of the interaction of heparanase and RNA Pol II in the high-glucose-induced HREC group showed that the molecular size of heparanase that interacted with RNA Pol II was 50 kDa, illustrating that the enzyme is active and capable of degrading both nuclear- and extracellular matrix–derived HS. Kovalszky demonstrated that nuclear HSPGs played a fundamental role in the regulation of topoisomerase-I-mediated DNA, a nuclear enzyme localized at active sites of transcription [30]. This enzyme can change the superhelical state of duplex DNA by transiently breaking single strands to allow relaxation of both positively and negatively supercoiled DNA [30]. Therefore, nuclear heparanase combined with RNA Pol II may contribute to the transcription of the *VEGF* gene by degrading HS to liberate the inhibitory effect of HS on topoisomerase-I DNA relaxation. Likewise, heparanase cleavage of nuclear HS may affect the transcriptional activity associated with nuclear FGF-2 [28,29]. The real-time PCR–based ChIP results demonstrated that occupancy of heparanase-binding regions (1155 to −1018 from the ATG initiation codon containing the hypoxia response element) were more numerous in the *VEGF* gene promoter. The results also revealed an increase in heparanase and RNA Pol II in the *VEGF* gene promoter region of the high-glucose-induced HRECs compared with control HRECs.

Another study demonstrated that the human *VEGF* gene promoter (full-length 1.5 kb) has a single major transcriptional start site (nucleotides −749 to −720 and −714 to −685) 1,038 bp upstream from the ATG initiation codon [31], which is consistent with our findings. Several characterized transcription factors, such as hypoxia inducible factor-1, activator protein-1, and specificity protein-1, that bind to the VEGF promoter were also shown to initiate and activate the transcription of a gene directly [31]. Based on our results, we propose that nuclear heparanase can likely mediate *VEGF* gene transcription. Under normal conditions, DNA in the *VEGF* proximal promoter region exists in a duplex form. The regulatory region of the *VEGF* gene contains several transcription factor binding sites, and transcriptional regulation of this gene appears to be extremely complex, with levels of control at the transcriptional and translational level [32]. Heparanase binds to the *VEGF* proximal promoter region and activates transcription by recruiting and binding RNA Pol II and regulating topoisomerase-I-mediated DNA relaxation. The capacity of heparanase to combine with RNA Pol II may be attributed to its molecular properties and structure, as stated above. Following the recruitment and binding of RNA Pol II by heparanase, it may link to the *VEGF* gene promoter to initiate *VEGF* gene transcription. However, the detailed mechanism underlying the involvement of heparanase in *VEGF* gene transcriptional regulation remains unclear and requires further study.

The mechanisms of heparanase upregulation under high glucose conditions remain unclear. Increased heparanase in vitreous samples from patients with proliferative diabetic retinopathy has been suggested to originate from active local endothelial cells and leukocytes [33]. The results from our previous studies and the present research have also confirmed the ability of HRECs to produce heparanase in response to high glucose [11,34]. In cells that produce heparanase, the latent form of heparanase is synthesized and secreted out of the cells, followed by reuptake into the cells and proteolytic cleavage to become the active form in lysosomes. The active heparanase may then undergo translocation to the nucleus or secretion out of the cells [35,36]. Therefore, upregulation of heparanase under high glucose condition in our study may have involved one or more steps in the synthesis, secretion, reuptake, and proteolytic cleavage of the latent heparanase and nuclear translocation of the active heparanase. In our previous study, we showed that high glucose can induce heparanase mRNA synthesis [34]. Secretion of the latent form of heparanase by endothelial cells can also be increased by high glucose [37], and reuptake of the latent heparanase usually occurs through endocytosis [36]. Studies have shown that endocytosis can be increased by high glucose in retinal vascular endothelial cells [38]. It is possible that endocytosis of HRECs can be increased by high glucose too, although this needs to be further investigated. High glucose can also induce lysosomal cleavage of latent heparanase and secretion of the active form of heparanase from the lysosomes [9,11,34,37]. Finally, our study also suggests that nuclear translocation of heparanase may also be increased by high glucose. Taken together, high glucose conditions may be involved in heparanase upregulation through multiple mechanisms.

In summary, the current study demonstrated that heparanase is localized in the nuclei of HRECs and not restricted

to cancer cell lines maintained in culture. It also confirmed that nuclear heparanase directly combines with the *VEGF* gene promoter and that it is involved in *VEGF* gene transcription with RNA Pol II in high-glucose-induced HRECs. In previous studies, we stated that heparanase protein levels are positively associated with increased VEGF mRNA and protein levels, which were reduced by the heparanase inhibitor, PI-88, in vitro and in vivo [11,39,40]. Taken together, the findings imply that heparanase actively mediates *VEGF* gene transcription in high-glucose HRECs. These results provide an innovative background for further studies of pharmacological interventions for diabetic vascular disease, thereby supporting our understanding of the complications associated with diabetes.

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