Lentivirus-mediated overexpression of CD97/ADGRE5 reverses dysregulated high glucose-induced endothelial cell migration

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Abstract. Elevated blood glucose levels contribute to a series of complications in patients with diabetes mellitus, including chronic ulcers and accelerated atherosclerosis. Dysregulated endothelial migration induced by high glucose is important in vascular-associated complications. In the present study, cluster of differentiation (CD)97/adhesion G protein-coupled receptor 5 (ADGRE5), a member of the G protein-coupled receptor protein family, stimulated angiogenesis was investigated to determine its role in cell models of diabetes mellitus using lentivirus-mediated overexpression and siRNA transfection. The results revealed reduced expression in high glucose-treated human umbilical vein endothelial cells and in the endothelium of diabetic mice. Among the three CD97 isoforms, the majority of the expression of CD97 (EGF1,2,5) in the endothelial cells was regulated by high levels of glucose. Using stable lentivirus-mediated transfection and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology, the present study constructed CD97-overexpression and CD97-knockout endothelial cell lines. Migration assays showed that the lentivirus-mediated overexpression of CD97/ADGRE5 improved the inhibition of high glucose-induced endothelial cell migration. In addition, using cytoskeleton staining, it was found that CD97 promoted membrane ruffling and lamellipodia formation. Cell division cycle 42, a small GTP-binding protein, and its downstream factor, actin-related protein 2, were involved in CD97-induced actin reorganization in endothelial cells. Additionally, the use of transcription factor filter plate assays revealed that the nuclear translocation of signal transducer and activator of transcription 1 stimulated by high glucose contributed to the inhibited transcription of CD97. In conclusion, the present study established that the overexpression of CD97 improved high glucose-induced dysfunction of endothelial cell migration. These findings provide insight to assist in identifying therapeutic targets with potential to ameliorate certain vascular complications of diabetes.

Introduction

Endothelial dysfunction associated with type 2 diabetes mellitus and insulin resistance (1) is involved in multiple complications of diabetes. Numerous studies have suggested that endothelial cell dysfunction increases macromolecule permeability (2,3), causes abnormal vasodilation/vasoconstriction (4-6) and results in procoagulant activation (7).

Endothelial migration contributes to vascular repair and is inhibited under conditions of high glucose (8). Furthermore, previous data have shown that cluster of differentiation (CD)97, a surface molecule abundantly expressed in endothelial cells, can stimulate migration, invasion and angiogenesis (9). Therefore, the present study hypothesized that CD97 may act to promote endothelial cell migration under high glucose treatment conditions.

CD97 is encoded by the adhesion G protein-coupled receptor 5 (ADGRE5) gene (10) and is a member of the epidermal growth factor (EGF)-seven transmembrane family, which belongs to the adhesion family of G protein-coupled receptors (GPCRs) (11-13). CD97 is widely expressed on the surface of lymphoid cells, macrophages, smooth muscle cells and several types of tumor cell (14-19). A previous study also found that CD97 enhances cell invasion via Ras homolog (RHO) and extracellular signal-regulated kinase activation by associating with lysophosphatidic acid receptor 1 in prostate cancer cells (19).

In the present study, a lentivirus-mediated endothelial cell line overexpressing CD97 was constructed and the effects of CD97 on cell migration were investigated. It was found that CD97 ameliorated the inhibition of high glucose-induced endothelial cell migration. In addition, the molecular mechanism whereby high levels of glucose regulate the expression of CD97 was also characterized in detail.

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Materials and methods

Cell culture. The human umbilical vein endothelial cell (HUVEC) line was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in an incubator at 37°C, 5% CO_2 in complete medium containing 10% FBS (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and Dulbecco's modified Eagle's medium (DMEM; 5.5 mM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Prior to exposure to glucose at concentrations of 5.5, 10 or 33 mM for 24 h, $1x10^5$ cells were starved of FBS for 12 h at 37°C in the incubator. A 5.5 mM concentration of glucose was used as a control.

Small interfering (si)RNA and plasmid transfection. Transfection of the cells was performed using Polyplus transfection reagent (jetPPRIME; Polyplus Transfection, Illkirch, France). In brief, 1 μl of siRNA (50 μM; Ruibo Biotechnology Co., Ltd., Guangzhou, China) or 2.5 μg plasmid cDNA (ViGene Biosciences, Inc., Shandong, China) was added to 200 μl of jetPRIME buffer. Following mixing with 4 μl of jetPRIME, the solution was vortexed for 10 sec. Following incubation for 10 min at room temperature, the mixture was added into one well of a 6-well plate with $1x10^6$ cells cultured in 1 ml complete medium. Following culture for an additional 24 h, the cells were harvested and used in subsequent assays.

Western blot analysis. The cells were lysed on ice in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), which included a cocktail of protease inhibitors (Cell Signaling Technology, Inc., Danvers, MA, USA) and protein levels was determined by bicinchoninic acid assay method. Subsequently, 40 μg of total protein was loaded onto 10% SDS-PAGE gels, electrophoresed and transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). Following blocking using 5% non-fat milk (Nestlé, Vevey, Switzerland), antibody incubation and immunoblotting using ECL (Kangwei, Beijing China) were used to detect fluorescence. The following primary antibodies were used: anti-human CD97 (cat. no. ab108368; 1:1,000; Abcam, Cambridge, MA, USA), anti-human rhodopsin (RHO; cat. no. ab5417; 1:1,000; Abcam), anti-human Ras-related C3 botulinum toxin substrate 1 (RAC; cat. no. ab33186; 1:1,000; Abcam), anti-human cell division cycle 42 (CDC42; cat. no. ab155940; 1:1,000; Abcam), anti-human actin-related protein 2 (ARP2; cat. no. ab47654; 1:1,000; Abcam), anti-human signal transducer and activator of transcription 1 (STAT1; cat. no. ab3987; 1:1,000; Abcam) and anti-human GAPDH (cat. no. CW0101M; 1:2,000; Nuoyang, Hangzhou, China). All primary antibodies were incubated at 4°C overnight. The relevant secondary antibody was goat anti-rabbit antibody (cat. no. A0208; 1:5,000; Beyotime Institute of Biotechnology) incubated at room temperature for 1 h.

Flow cytometry. Following treatment, the cells were washed twice with PBS and then incubated with FITC-conjugated anti-CD97 (BD Biosciences, Franklin Lakes, NJ, USA). The subsequent analysis of the expression of CD97 was performed using a flow cytometer (BD Biosciences).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the endothelial cells using an RNAsimple total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized using a Primescript RT reagent kit (Takara Bio, Inc., Otsu, Japan). A total of 5 ng cDNA of each sample was subjected to PCR reactions consisting of 40 cycles of 95°C for 10 sec, 68°C for 30 sec and 72°C for 30 sec using SYBR-Green Premix Ex Taq (Takara Bio, Inc., Otsu, Japan) and detected by ABI PRISM 7500 Sequence Detection System (Thermo Fisher Scientific, Inc.). The relative expression level results were analyzed using the $2^{-\Delta\Delta Cq}$ method (20). Finally, the PCR products were examined using DNA agarose gel electrophoresis. The following primers were used for RT-PCR analysis: CD97, forward 5'-ACTCTGCCGGGAGCTGAAAC-3' and reverse 5'-TGGATGGTGACCTCGGCTGA-3'; 18S, forward 5'-CCG CACTTGATACGGTTCCT-3' and reverse 5'-CCAGGCTGA TCTATCCCACTG-3'.

Wound healing assay. Endothelial cells were seeded at density $1x10^5$ and cultured in 6-well plates to 80-90% confluence and were serum-starved for 24 h. Two scratches were then introduced to the cell layer in each well using a 100-1,000 μl tip. Following washing twice with PBS, the cells were incubated in DMEM with glucose (33 mM), and 5.5 mM glucose DMEM treatment was used as a control. Images of the same regions were captured at 0 and 24 h following stimulation with light microscope (Stemi 2000; Zeiss GmbH, Jena, Germany); the paired images were analyzed.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed according to the manufacturer's protocol using a kit from Cell Signaling Technology, Inc. In brief, for each group, $1x10^7$ cells were fixed with 1% formaldehyde (Aladdin Industrial, Inc., Nashville, TN, USA). Subsequently, chromatin DNA was sheared using micrococcal nuclease (Cell Signaling Technology, Inc.) to yield DNA fragments ranging between 300 and 900 bp. Following preclearance with 10 μl protein A/G agarose beads, the samples were incubated with rabbit anti-STAT1 monoclonal antibody (2 μg; cat. no. ab3987; 1:100; Abcam) or control rabbit IgG antibody (2 μg; cat. no. 2729; 1:100; Cell Signaling Technology, Inc.). The samples were then immunoprecipitated by incubation with 30 μl protein A/G agarose beads, and complexes were reverse cross-linked by protease K and NaCl (5 M) treatment. Finally, DNA was purified using a DNA purification kit (Cell Signaling Technology, Inc.). The content of the purified DNA was assessed using RT-quantitative PCR analysis as aforementioned. The following primers were used to amplify the STAT1 binding element in the promoter of the CD97 gene: Forward 5'-TAG CGCTAAGACACAGTTGGACC-3' and reverse 5'-ACTCGC CAGTTGCAACAGTTC-3'.

Generation of the CD97-Cas9 endothelial cell line. To knockout CD97 in an endothelial cell line, experiments were performed according to a previously published protocol (21). In brief, a custom designed gRNA for CD97 was cloned into the Pep-ko (Pep-330x) plasmid. Then the plasmid was transfected into endothelial cells at a density $3x10^5$ using Polyplus transfection reagent (JetPRIME; Polyplus Transfection) at 37°C for 24 h,

the endothelial cells were filtered using puromycin (2 μg/ml; Sigma-Aldrich; Merck Millipore). The surviving cells were seeded into a 96-well plate and cultured into monoclonal cell lines for further assessment of the expression of CD97. The CD97 gRNA sequences were as follows: Forward 5'-accgTCCGGTGGACGAGGCGGCGG-3' and reverse 5'-accgCGGCCGACCACCACCGCTTC-3'.

Construction of stable CD97-expressing endothelial cells by lentivirus transfection. A customized CD97-overexpression lentivirus vector was obtained from ViGene Biosciences, Inc. Endothelial cells were transfected with the CD97 lentivirus and screened/selected using puromycin (2 μg/ml; Sigma-Aldrich; Merck Millipore). The surviving cells were cultured into multiple monoclonal cell lines and were assessed for the expression of CD97 using western blot analysis.

Immunofluorescence staining. Endothelial cells were seeded at a density of 1×10^5 in a cell culture dish (Nest Scientific, Rahway, NJ, USA). Following treatment with glucose (control, 5.5 mM; high, 33 mM) for 24 h, the cells were fixed using 1% formaldehyde and incubated with FITC-phalloidin (Thermo Fisher Scientific, Inc.). Finally, the cells were observed using a Zeiss Confocal Imaging system (Zeiss GmbH).

Animal model. A total of 20 male C57BL/6 J mice (age, 4 weeks; weight, 20±4 g, maintained at 20°C, normal lighting) were purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). Diabetes was induced in these mice by intraperitoneal injection of STZ (70 mg/kg; Sigma-Aldrich; Merck Millipore), and the mice were continually fed a high-fat diet for 3 months. Mice with glucose levels >16.4 mM were considered a successful diabetic mouse model. Three months later, mice were sacrificed by cervical dislocation. The aortic endothelium was harvested and subjected to analysis of the expression of CD97 using immunohistochemistry (IHC).

IHC. Following deparaffinization, hydration and blocking, the paraffin-embedded tissue transverse sections (thickness, 5 μm) were incubated with primary antibodies (CD97; cat. no. ab108368, 1:200; Abcam) for 2 h at 37°C. Following incubation, the tissue sections were washed with PBS for 15 min, followed by incubation with anti-digoxigenin-conjugated secondary antibodies (1:200; cat. no. ZX300, Nuoyang, Beijing, China) for 1 h at room temperature. Subsequently, the sections were washed again with PBS and incubated with DAB reagent (ViGene Biosciences, Inc., China). Finally, images of the stained samples were captured using an optical microscope (Olympus, Tokyo, Japan).

Dual luciferase reporter assay. Genomic DNA was extracted from the endothelial cells using a Genome DNA Extract kit (Kangwei, China). The 2,000, 1,500, 1,000 and 500 bp upstream regions from the transcription initiation site of the ADGRE5 promoter were amplified, gel-purified and sub-cloned into a pGL3-basic luciferase reporter vector (Promega Corporation, USA) between the *Hind*III and *Sac*I sites. The primer sequences used were as follows: 2 k-promoter, forward 5'-CAAGTCACGCCGAATCCAATA-3'

and reverse 5'-CGGTCCTGAACTTTCCGAGATG-3'; 1.5k-promoter, forward 5'-GACGGCTCAGGACCTACATAA-3' and reverse 5'-CGGTCCTGAACTTTCCGAGATG-3'; 1k-promoter, forward 5'-GAATCCCAATACGTCAAGCCA-3' and reverse 5'-CGGTCCTGAACTTTCCGAGATG-3'; 0.5k-promoter, forward 5'-CTAAGCAACCGTGTCGAACAC-3' and reverse 5'-CGGTCCTGAACTTTCCGAGATG-3'. Each vector was transfected into the endothelial cells with glucose treatment (control, 5.5 mM; high, 33 mM) for 24 h. The activity of each was determined using a dual luciferase reporter assay system.

Transcription factor (TF) filter plate assay. TF filter plate (Signosis, Santa Clara, CA, USA) assays were performed according to the manufacturer's protocol. In brief, endothelial cells were collected to extract nuclear proteins. TF DNA complexes were created by mixing TF probes with a 500 bp sequence of the ADGRE5 promoter. Subsequently, complexes were separated from free probes and the bound probes were eluted. Following hybridization of the eluted probes on a hybridization plate, which included nuclear proteins, the relative activity was detected using a luminometer (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. All significant differences between the mean were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Comparisons were performed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CD97/ADGRE5 in high glucose-induced HUVECs and diabetic mice. The expression of CD97 in endothelial cells subjected to glucose treatment was assessed using western blot analysis and flow cytometry; a basal concentration (5.5 mM) of glucose was used as a control. As shown in Fig. 1A and B, the expression of CD97 was reduced when exposed to an increasing glucose concentration gradient. Alterations in the three CD97 isoforms, CD97 (EGF1,2,5), CD97 (EGF1,2,3,5) and CD97 (EGF1,2,3,4,5), were also analyzed in high glucose-induced endothelial cells. As shown in Fig. 1C, the endothelial cells predominantly expressed CD97 (EGF1,2,5), which was ~600 bp in length, and its pattern was altered in response to high glucose treatment. Furthermore, staining of the aortic endothelial tissues from the diabetic mice using anti-CD97 antibody showed lower expression of CD97, compared with the physiological saline-treated group (Fig. 1D).

Overexpression of CD97 (EGF1,2,5) in HUVECs attenuates high glucose-induced dysregulation of migration. To determine whether CD97 (EGF1,2,5) enhanced the dysregulation of endothelial cell migration induced by high glucose, the present study initially constructed a CD97 (EGF1,2,5)-overexpression endothelial cell line via lentivirus transfection (Fig. 2A). As shown in Fig. 2B, high glucose stimulation reduced the mobility ratio of the endothelial cells, whereas the overexpression of CD97 partially attenuated this.
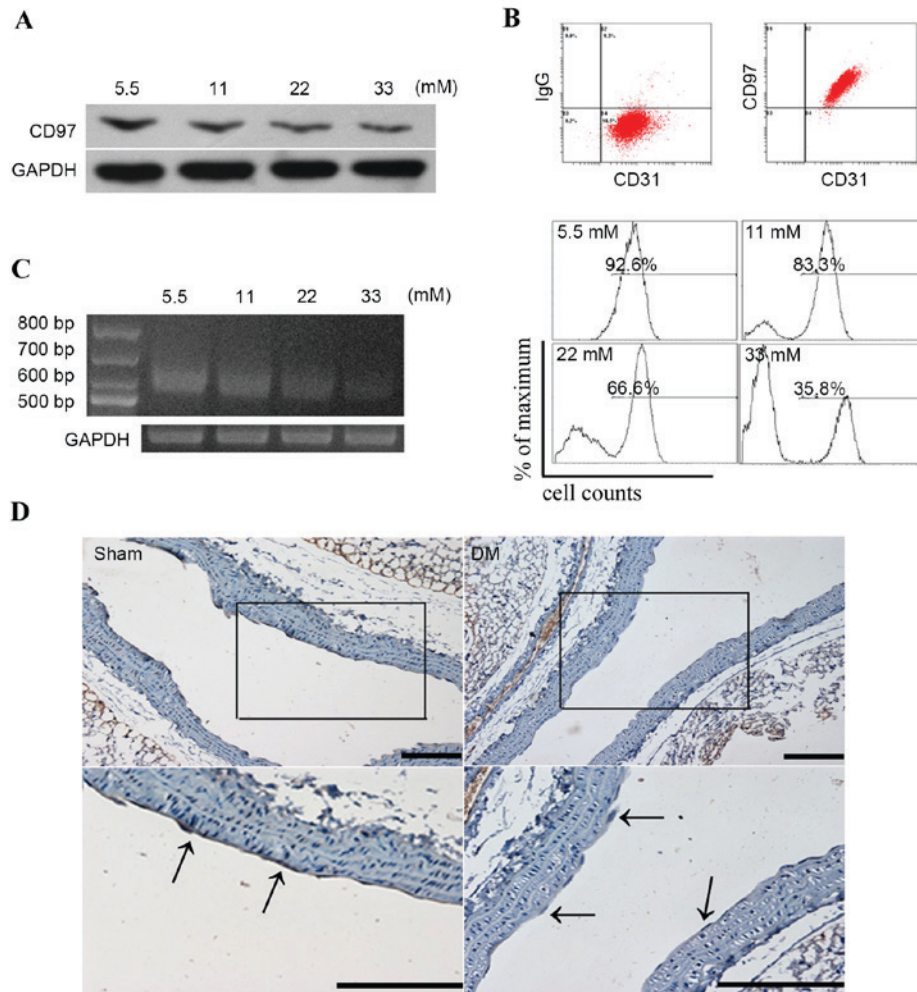
Figure 1. Expression of CD97 is reduced in high glucose-treated endothelial cells and mice with DM. (A) Analysis of the protein expression ofCD97 in response to high glucose (11, 22 and 33 mM); the basal control condition was 5.5 mM glucose. GAPDH was used as a loading control. (B) Analysis of the expression of CD97 using flow cytometry combined with anti-CD31; IgG was used as a control to treat the endothelial cells (above), or the cells were treated with high glucose (11, 22 and 33 mM) with a control of 5.5 mM basal glucose (lower). (C) CD97 mRNA transcript levels were assessed using reverse transcription-polymerase chain reaction analysis in cells treated under conditions of high glucose (11, 22 and 33 mM) and using a basal control of 5.5 mM glucose. Subsequently, CD97 isoforms were separated using agarose gel electrophoresis. GAPDH was used as a loading control. (D) Aortic endothelium samples from mice with DM and healthy control mice were stained using anti-CD97 antibody. Endothelial cells are indicated by black arrows. DM, diabetes mellitus. CD97, cluster of differentiation 97. Scale bar, 60 µm.
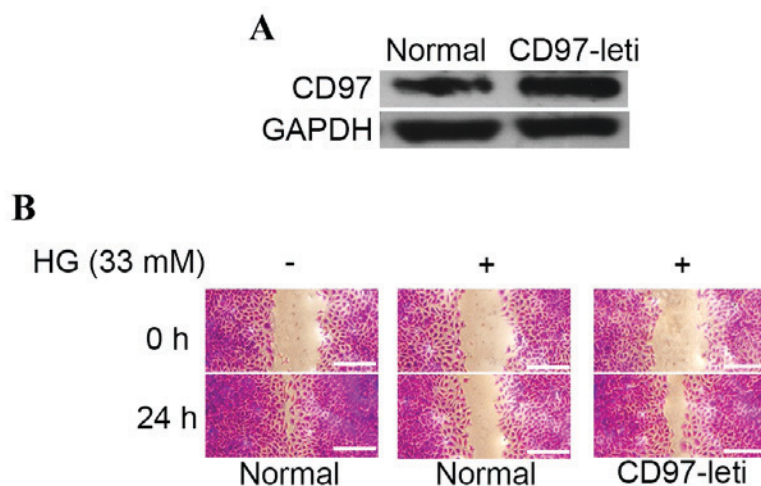

Figure 2. Overexpression of CD97 reverses the dysregulation of high glucose-induced endothelial cell migration. (A) Confirmation of the overexpression of CD97 in endothelial cells transfected with a lentivirus using western blot analysis. (B) Representative images from wound healing assays prior to (0 h) and following (24 h) high glucose (33 mM) or basal glucose (5.5 mM; control) treatment of normal and CD97-lentivirus endothelial cells. Scale bar, 70 µm. CD97, cluster of differentiation 97; HG, high glucose; leti, lentivirus; HG, high glucose.
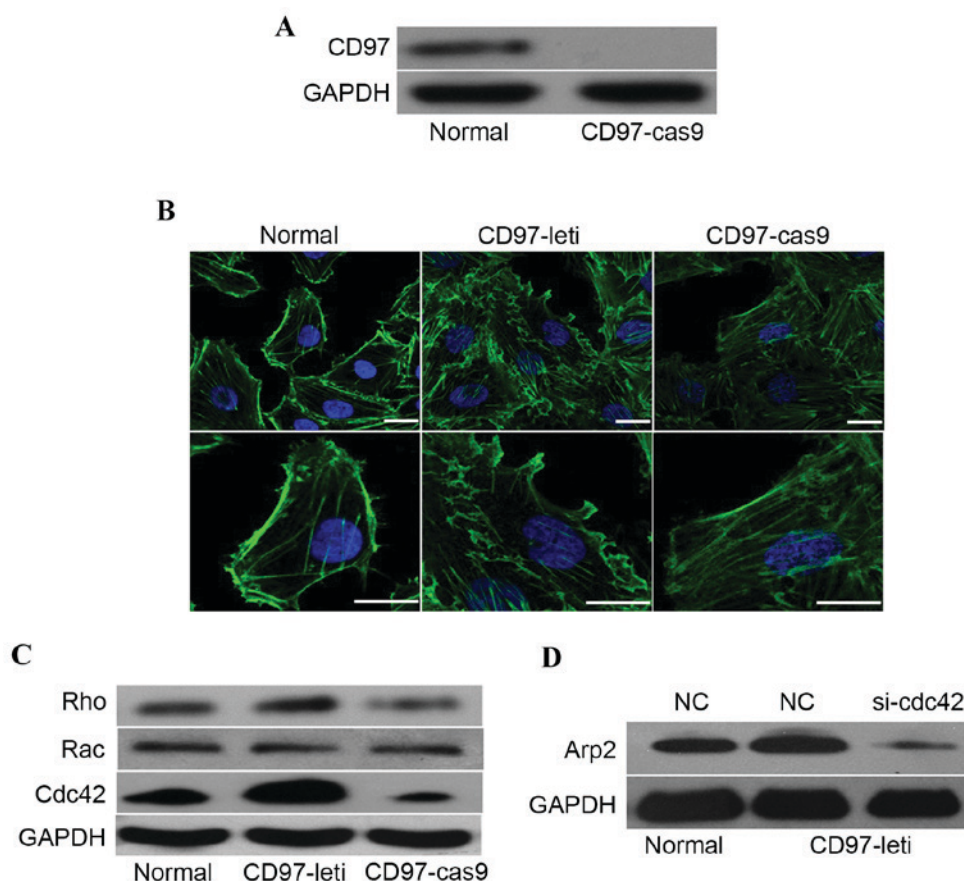
Figure 3. CD97 promotes membrane ruffling and lamellipodia formation in endothelial cells. (A) Validation of the CD97 knockout status of endothelial cells generated by clustered regularly interspaced short palindromic repeats/Cas9 using western blotting. (B) Alterations in the distribution of F-actin in endothelial cells, CD97-Cas9 endothelial cells or CD97-lentivirus endothelial cells. Stress fibers or lamellipodia are indicated by white arrows. Scale bar, 3 μm. (C) Protein levels of RHO, RAC and CDC42 in endothelial cells, CD97-Cas9 endothelial cells or CD97-lentivirus endothelial cells. (D) Analysis of protein expression levels ofCD97 in endothelial cells, CD97-lentivirus endothelial cells or CD97-lentivirus endothelial cells transfected with siRNA to knockdown the expression of CDC42. CD97, cluster of differentiation 97; Cas9, clustered regularly interspaced short palindromic repeats-associated protein9; Rho, Ras homolog; Rac, Ras-related C3 botulinum toxin substrate; Cdc42, cell division cycle 42; Arp2, actin-related protein 2; NC, negative control; si, small interfering RNA; leti, lentivirus.

CD97 promotes actin enrichment and reorganization in a CDC42-ARP2 dependent manner. To analyze the effects of CD97 in the cytoskeleton of endothelial cells, a CD97-knockout endothelial cell line (CD97-Cas9) was constructed using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology (Fig. 3A). Cytoskeletal staining revealed that the expression level ofCD97 was associated with membrane ruffling and lamellipodia formation (Fig. 3B). In the previous study by Wojciak-Stothard *et al* (22), RHO, RAC and CDC42 were found to control reorganization of the actin cytoskeleton and to promote migration in endothelial cells. The present study hypothesized that these GTP-binding proteins are also activated in the CD97-overexpressing cell line. As shown in Fig. 3C, the expression of CDC42 was positively regulated by CD97. Furthermore, to elucidate the mechanism underlying the effects of CD97 on cytoskeletal alterations, an evolutionarily conserved actin nucleation factor, the ARP2/3 complex, which is necessary for lamellipodia extension and cell migration in fibroblasts, was examined (23). As shown in Fig. 3D, the upregulation of CD97 increased the levels of ARP2, whereas the downregulation of CDC42, induced by using siRNA to target the mRNA transcripts encoding CDC42, abrogated the increase levels of ARP2. This suggested that CD97 promoted lamellipodia formation, which was dependent upon the activation of CDC42 and ARP2.

High glucose inhibits CD97 transcription via the regulation of STAT1. The mechanism underlying the regulatory effect of high glucose on the expression of CD97 was also examined in detail. To characterize the promoter region of CD97, a series of luciferase reporter plasmids, including the 500, 1,000, 1,500 and 2,000 bp sequences upstream of the transcription start point (24), were constructed (Fig. 4A). Dual luciferase reporter assays revealed that the promoters, which included a 500 bp sequence, represented the minimal length required to suppress CD97 transcription. Subsequently, TF filter plate assays were performed using this 500 bp sequence of the CD97 promoter. As shown in Fig. 4B, STAT1 was the most prominent factor to be activated by high glucose concentrations; this factor also bound to the 500 bp promoter region upstream of CD97. Therefore, the present study aimed to characterize the role of STAT1 in the regulation of CD97 transcription. Nuclear extracts from high glucose-induced endothelial cells showed higher expression levels of STAT1 (Fig. 4C). Additionally,
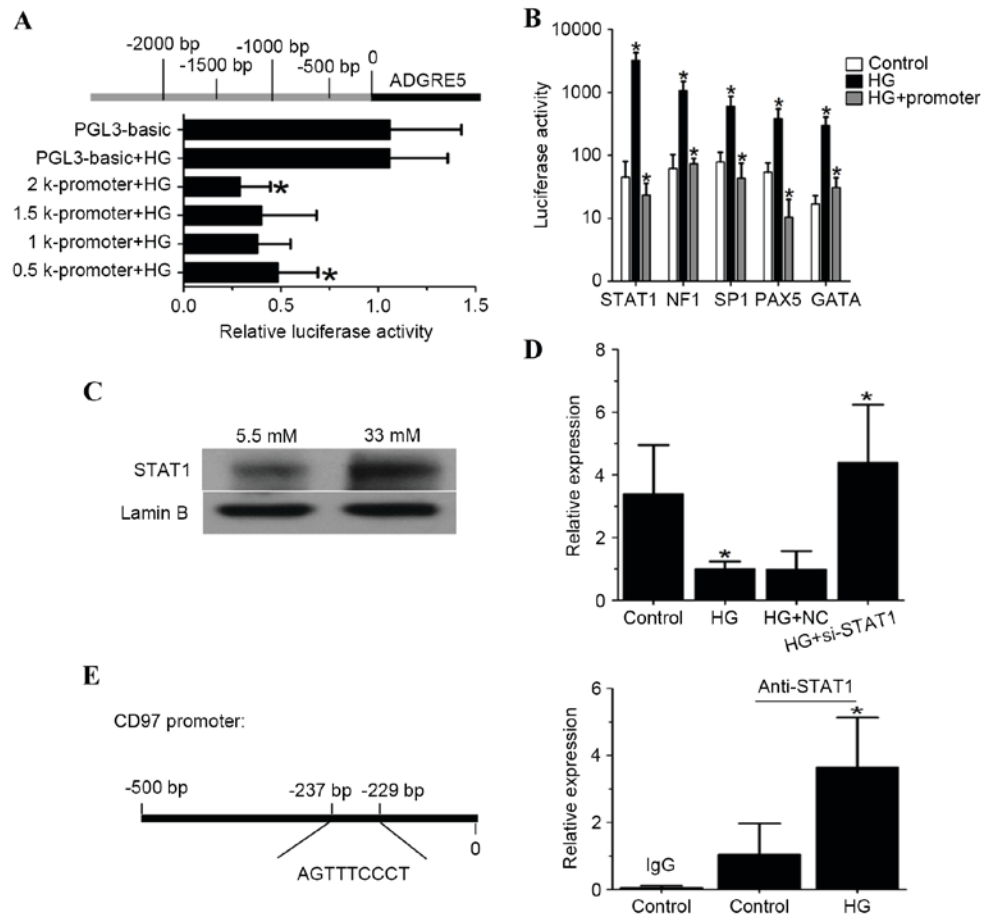
Figure 4. High glucose concentrations inhibit CD97 transcription via the upregulation of STAT1. (A) Schematic representation of the promoter regions (upper), which were sub-cloned into the pGL3-basic luciferase reporter. Activation of the promoter-luciferase reporters in response to high glucose concentrations in endothelial cells is shown below. (B) Top five most robust binding transcription factors in the CD97 promoter region, determined using TF filter plate assays. (C) High glucose (33 mM) stimulation promoted STAT1 nuclear translocation; 5.5 mM glucose stimulation was used as a control. (D) Analysis of the mRNA transcript expression of CD97 in endothelial cells transfected with siRNA targeting STAT1 prior to high glucose (33 mM) treatment (5.5 mM glucose served as a control). (E) Schematic illustration of potential binding sites in the CD97 promoter for STAT1. High glucose (33 mM) treatment increased STAT1 binding to its binding site in the CD97 promoter (5.5 mM glucose served as a control). *P<0.05. CD97, cluster of differentiation 97; ADGRE5, adhesion G protein-coupled receptor 5; STAT1, signal transducer and activator of transcription 1; NF1, neurofibromatosis type 1; SP1, specificity protein 1; PAX5, paired box 5; GATA, GATA binding protein; HG, high glucose; si, small interfering RNA.

transfection with siRNA targeting STAT1 under high glucose conditions revealed that high glucose resulted in reduced expression levels of CD97 via the upregulation of STAT1 (Fig. 4D). The effect of high glucose on the binding activity of STAT1 to the CD97 promoter was also examined using ChIP assays. As shown in Fig. 4E, high levels of glucose increased the binding activity of STAT1 at the CD97 promoter.

Discussion

CD97/ADGRE5 belongs to the GPCR family (11-13) and has been found to regulate migration in multiple cell types, including granulocytes (25), prostate cancer cells (19) and endothelial cells (9). In the present study, the role of CD97 in high glucose-induced dysregulation of endothelial cell migration was reported. By activating CDC42 and ARP2, CD97 promotes the formation and extension of lamellipodia by endothelial cells. Unlike prostate cancer cells, for which CD97-mediated invasion is primarily RHO-dependent (19), CD97 primarily regulates the expression of CDC42 rather than RHO in endothelial cells. This difference may be attributed to

differences in cell type or treatment. In addition, the present study found that specificity protein 1 (SP1) controlled the transcription of CD97 in smooth muscle cells (26) and regulated the transcription of CD97 under conditions of high glucose treatment. However, compared with the STAT1 transcription factor, SP1 exhibits lower activity in high glucose-stimulated endothelial cell assays. However, in endothelial cells stimulated by high glucose concentrations, whether these two factors can promote the transcription of CD97 in a cooperative manner remains to be elucidated. Additionally, whether CD97 acts in high glucose-induced apoptosis or other modes of dysfunction in endothelial cells remains to be elucidated. The present study is the first, to the best of our knowledge, to describe a link between CD97 and the dysregulation of high glucose-induced endothelial migration, which may provide insights in the identification of novel therapeutic targets for the treatment of diabetic complications.

In conclusion, the overexpression of CD97 reversed the dysregulation of high glucose-induced endothelial cell migration by activating CDC42, which acts via its downstream signaling adaptor ARP2.

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