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ORIGINAL ARTICLE

BASP1 promotes high glucose-induced endothelial apoptosis in diabetes *via* activation of EGFR signaling

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ABSTRACT

Aims: Diabetes mellitus is a common chronic disease of glucose metabolism. Endothelial dysfunction is an early event in diabetes complicated by cardiovascular disease. This study aimed to reveal the expression of BASP1 and its biological roles in endothelial cell dysfunction in diabetes complicated by cardiovascular disease.

Materials and Methods: By analyzing the databases related to diabetes complicated with coronary heart disease, BASP1 was screened out as an upregulated gene. Human umbilical vein endothelial cells (HUVECs) and primary mouse aortic endothelial cells were treated with high glucose to establish cell models of diabetes-related endothelial dysfunction, and the expression changes of BASP1 were verified by RT-qPCR, western blot, and immunofluorescence. BASP1 was silenced or overexpressed by siRNA or overexpression plasmid, and its effects on cell migration, apoptosis, tube formation, inflammatory response, and ROS were detected. The possible signaling pathway of BASP1 was found and the mechanism of BASP1 on promoting the progression of endothelial dysfunction was explored using the EGFR inhibitor, gefitinib.

Results: Bioinformatics analysis indicated that the expression of BASP1 in patients with diabetes mellitus and concomitant coronary heart disease was increased. High glucose induced the upregulation of BASP1 expression in endothelial cells, and showed a time-dependent relationship. Silencing of BASP1 alleviated the damage of high glucose to endothelial cells. BASP1 regulated EGFR positively. The promoting effect of BASP1 on endothelial cell apoptosis may be achieved by regulating the EGFR pathway.

Conclusion: BASP1 promotes endothelial cell injury induced by high glucose in patients with diabetes, which may be activated by activating the EGFR pathway.

INTRODUCTION

Diabetes mellitus is a chronic disease that leads to a long-term hyperglycemia in the body due to abnormal glucose metabolism caused by insufficient insulin secretion or decreased tissue sensitivity to insulin. Type 1 and type 2 diabetes are the two most common types. The most frequent kind of diabetes in the elderly is type 2 diabetes[1,2]. With the improvement of people's living standards and the gradual aging of the population, the number of patients with diabetes is gradually increasing. Over a long period of time, abnormal glucose metabolism in the human body may cause various complications including coronary heart disease.

As the first barrier between the blood vessels and blood, endothelial cells undertake a variety of autocrine, paracrine, and endocrine functions, and endothelial dysfunction is an early pathogenic event of vascular dysfunction. The long-term high blood glucose environment caused by diabetes can induce serious vascular disease[3], which further leads to stroke, myocardial infarction, kidney failure, blindness, limb gangrene, and other serious complications[4]. Therefore, studying the metabolic and

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physiological changes in vascular endothelial cells under high-glucose conditions may shed light on the pathogenesis of diabetes complicated with cardiovascular disease and provide a theoretical basis for finding potential drug targets.

Endothelial cells in the vasculature play vital roles in maintaining vascular shape and function[5,6]. Human umbilical vein endothelial cells (HUVECs) are commonly used to study central vascular injury in diabetes mellitus. The culture of HUVECs under high glucose conditions can effectively mimic the extracellular environment of vascular endothelial cells under diabetic conditions. In recent years, a large number of studies have explored the pathogenesis of diabetic vascular endothelial dysfunction[7–9]. Hyperglycemia impairs insulin signaling and eNOS activity in endothelial cells, induces reactive oxygen species (ROS) accumulation, and promotes the production of pro-inflammatory factors through polyols, hexosamines, and advanced glycation end products[10]. Hyperglycemia can also lead to ROS accumulation by damaging the mitochondrial respiratory chain, activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and by inhibiting the expression of antioxidant enzymes[11]. In addition, ROS activates the nuclear transcription factor NF-κB to regulate the expression of pro-inflammatory genes, and ultimately leads to inflammatory damage of vascular endothelial cells[12,13]. Mining transcriptome sequencing data, looking for endothelial cell altered genes under the condition of diabetes complicated with cardiovascular disease, and further studying their effects on endothelial cells, will be helpful to understand the mechanism of diabetes-induced vascular endothelial cell inflammatory damage.

The transcriptional cofactor brain acid soluble protein 1 (BASP1) is mostly expressed in the cytoplasm and nucleus. BASP1 has been found to be highly expressed in nerve terminals, testicles, lymphatic organs, kidneys, and other tissues[14–16]. The main biological role of BASP1 is to enhance axon growth and development *via* controlling the movement of cytoskeleton-related proteins[14]. In recent years, BASP1-related reports have been related mostly to cancer[17,18]. Overexpression of BASP1 has different effects on survival in different cancers. BASP1 is down-regulated in melanoma[19], hepatocellular carcinoma[20], and pancreatic cancer[21] with a poor prognosis. This could be linked to aberrant BASP1 promoter methylation. Similarly, in breast cancer tissues, high expression of BASP1 is associated with better patient outcomes[18]. High BASP1 expression, on the other hand, was associated with a lower overall survival rate in cervical cancer patients, which is possibly due to its role in cell cycle and proliferation regulation[22]. Studies have also shown that BASP1 is associated with diabetes and endothelial dysfunction[16,23].

Given that vascular endothelial cells are closely related to diabetes complicated with cardiovascular disease, we speculated that there were significantly differentially expressed genes (DEGs) in endothelial cells in diabetic patients with cardiovascular disease and in normal controls. These DEGs may be involved in its pathogenesis and may serve as potential diagnostic and therapeutic targets. This study screened DEGs in diabetic patients with cardiovascular disease and in normal controls. In addition, the effects of BASP1 in endothelial cells under high-glucose conditions were detected. The findings of the present study provide theoretical support for finding new targets for the diagnosis and treatment of diabetes complicated with cardiovascular disease.

MATERIALS AND METHODS

Bioinformatic analysis

In the GEO database (http://www.ncbi.nlm.nih.gov/geo), the datasets related to diabetes complicated with coronary heart disease and endothelial injury were retrieved and GSE132651, GSE144803, and GSE113969 were screened out. In the GSE132651 dataset, six participants with normal coronary endothelial function and 13 subjects with poor coronary endothelial function, endothelial gene expression linked with early coronary atherosclerosis were found. In the GSE144803 dataset, analysis of the global expression of primary endothelial cells before and after TNF-α stimulation was processed. In the GSE113969 dataset, patient-derived induced pluripotent stem cells were collected from seven type 2 diabetes mellitus patients without cardiovascular disease and seven type 2 diabetes mellitus patients with cardiovascular disease. The differential expression genes in the endothelial cells differentiated from the patient-derived induced pluripotent stem cells were analyzed.

HUVEC culture

HUVECs were purchased from Procell (Wuhan, China), seeded in 12 mm dishes, and cultured in Ham's F-10 (cat. no.: 11550043; Gibco, Grand Island, NY, USA) supplemented with 0.1 mg/mL heparin (Sigma, St. Louis, MO, USA; cat. no.: H3393), 0.03 mg/mL endothelial cell growth supplement (ECGS; cat. no.: CB-40006; Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS; cat. no.: 10099141; Gibco), and 1% penicillin–streptomycin solution (cat. no.: 15140122; Gibco) at 37°C and 5% CO_2. The complete culture medium contains 6.11 mM glucose.

Primary culture of mouse aortic endothelial cells

Mouse aortic endothelial cells were isolated from male C57BL/6 mice (Jinan Pengyue Experimental Animal Breeding Co., Ltd, Jinan, China) as described previously[24]. In brief, the thoracic aorta was isolated from mice using micro-dissection forceps. After washing with ice-cold PBS, the aorta was cut into ~1 mm rings and incubated with endothelial cell growth medium at 37°C under 5% CO_2 for 4 h. Four days later, the aorta sections were removed and fresh culture medium was added for another 2 days of incubation. Primary mouse aortic endothelial cells were collected by centrifugation at 900 × g for 5 min at room temperature. Cells at the second to the third passages were used in the following studies. This experimental procedure was approved by the Animal Research Committee of our hospital.

High glucose treatment

To induce endothelial cell dysfunction, 25 mM glucose (cat. no.: A2494001, Gibco) was added to the culture medium and incubated at 37°C for 24 h. Cells in the hypertonic group were treated with 25 mM mannitol at 37°C.

Cell transfection

Transfection of siRNA or overexpression plasmids was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the endothelial cells were seeded into 96-well plates and cultured for 1 day, 4 μL of Lipofectamine 2000 reagent and 1.5 μg of plasmid DNA were added to 50 μL of culture medium, incubated for 5 min, and added to the cells. After 6 h, the medium was replaced with a normal medium, and subsequent experiments were conducted after 24 h of culture. For HUVEC transfection, BASP1 siRNA-1 (5′-GAGGCAAGCTCAGCAAGAAGA-3′), BASP1 siRNA-2 (5′-GCAAGCTCAGCAAGAAGAAGA-3′), and scrambled siRNA (5′-GGAGGCACAAGCGAGCATAAA-3′) were synthesized by RiboBio (Guangzhou, China). pcDNA3.1-BASP1 and the pCDNA3.1-HA-C empty vector were purchased from Shanghai Hewu Biotechnology Co., Ltd (Shanghai, China). For primary mouse aortic endothelial cell transfection, BASP1 siRNA-1 (5′-GAGGCAAGCTGAGCAAGAAGA-3′), BASP1 siRNA-2 (5′-GCAAGCTGAGCAAGAAGAAGA-3′), and scrambled siRNA (5′-GAAAGGCGCAGACGGAGTAAA-3′) were synthesized by RiboBio.

Immunofluorescence analysis

HUVECs were inoculated on 14 mm slides, cultured for 24 h, washed with PBS three times, and then fixed with 4% paraformaldehyde at room temperature for 1 h. The cells were infiltrated in PBS supplemented with 0.4% Triton X-100 and 3% BSA for 30 min and then labeled with appropriate anti-BASP1 antibody (1:100, cat. no.: 703692; Invitrogen) and secondary antibody (1:200, cat. no.: A32732; Invitrogen) at room temperature for 1 h. Finally, the tablets were sealed with a DAPI-containing tablet, and the subsequent imaging analysis was carried out using a fluorescence microscope.

CCK-8 assay

A CCK-8 Cell Counting Kit (CCK-8; cat. no.: C0037; Beyotime, Shanghai, China) served to evaluate the cellular activities. The endothelial cells were inoculated into 96-well plates at a density of 5×10^3 cells per well. After treatment for 24 h, the cells were washed with PBS and incubated with CCK-8 for 2 h. The optical density was measured at 450 nm using a microplate reader (Varioskan LUX; Thermo Scientific, Rockford, IL, USA). The results are expressed as a percentage of control values based on the absorbance of untreated cells.

Transwell assay

To confirm the effect of high glucose on endothelial cell migration, transwell migration experiments were performed. In brief, endothelial cells (1×10^5) were implanted into the upper transwell chamber (cat. no.: 7910; Costar-Corning, New York, NY, USA) with an aperture of 8.0 μm. After 1 h, hyperosmotic or high glucose medium was added into the lower chamber and incubated for 6 h to allow the cells to migrate. The transwell chamber was removed and the cells on the upper side of the membrane were removed with a cell scraper, then fixed with 4% fresh paraformaldehyde and washed with PBS. After crystal violet staining, the migration of endothelial cells was observed in five random microscopic fields (10×) for each membrane.

Tube formation

Following the indicated treatment, endothelial cells (2×10^4) were plated in 48-well plates which were pre-coated with Matrigel (cat. no.: 3562373034; Costar-Corning). After incubation at 37°C for 24 h, tube formation was captured and the percentage of tube/cord length of capillary-like structures was calculated using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Flow cytometry was used to detect cell apoptosis. As described above, the cells (1×10^5) were inoculated on 6-well culture plates and treated after 24 h. Cell death was quantified by Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (cat. no.: 40305ES20; Yeason, Shanghai, China), according to the manufacturer's protocol. Briefly, the cells were detached using 0.25% trypsin solution (cat. no.: 15050065; Gibco) and floated in the 100 μL binding-buffer containing 5 μL Annexin V-Alexa Fluor 488 and 10 μL PI. After incubation for 15 min in the dark at room temperature, 400 μL binding buffer was added and the sample was immediately analyzed by BD LSRFortessa X-20 (BD Biosciences, San Jose, CA, USA).

ROS detection

Intracellular ROS levels were measured using a ROS measuring kit (cat. no.: S0033S; Beyotime). In all, 1×10^6 cells were seeded in a 6-well plate. Twenty-four hours after seeding, the cells were washed with PBS and loaded with 10 mM carboxy-H2DCFDA for 30 min. After cleaning with PBS, images were taken and recorded by a fluorescence microscope.

ELISA

Enzyme-linked immunosorbent assay was performed using ELISA kits (cat. no.: PT518, PI330, and PI305, respectively; Beyotime). Logarithmically grown cells were seeded in 1×10^4 cells per well of microtiter plate wells (96 wells, flat bottom) with 100 μL of medium per well. After overnight incubation, the manufacturer's procedures were followed. The spectrophotometric absorbance of the sample was measured at a wavelength of 450 nm using a microplate reader. Each independent experiment was performed three times.

RT-qPCR

The PCR amplification for quantification of BASP1 and cytokine mRNA in HUVEC was performed in the LightCycler System using a SYBR Green RT-PCR kit (cat. no.: AH0104-A, Sparkjade, Shandong, China) as described previously[25]. Initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 72°C (60°C for β-actin) for 10 s, and elongation at 72°C for 10 s. A melting curve analysis was used to identify specific from nonspecific products and primer dimers. The concentrations of each sample were calculated *via* the $2^{-\Delta\Delta t}$ method. To avoid amplification of contaminating genomic DNA, the primers spanned more than two exons, designed using the Primer 3 tool.

The oligoribonucleotide primers were human BASP1 sense: 5′-GCAACTCGTTTGCAGCGG-3′, antisense 5′-CCCATCTTG GAGTTCTCGGC-3′; human TNF-α sense: 5′-CCCCAGGGA CCTCTCTCTAA-3′, antisense: 5′-GCTT GAGGGTTTGCTAC AACA-3′; human IL-6 sense: 5′-CCTTCTCCACAAGCGCCT TC-3′, antisense: 5′-GGAAGGCAGCAGGCAACA-3′; human IL-1β sense: 5′-TT CGAGGCACAAGGCACAA-3′, antisense: 5′-TGGCTGCTTCAGACACTTGAG-3′; human β-actin sense: 5′-GCACAGAGCCTCGCCTTT-3′, antisense: 5′-CACA GGA CTCCATGCCCAG-3′; mouse BASP1 sense: 5′-GAGAGCCT TTGCTGAGCG AC-3′, antisense 5′-CCATCTTGGAGTTC GGCTT TG-3′; mouse TNF-α sense: 5′-ACCCTCACACTCA CAAACCA-3′, antisense: 5′-ACCCTGAGCCATAATCCCC T-3′; mouse IL-6 sense: 5′-GCCTTCTTGGGA CTGATGCT-3′, antisense: 5′-TGTG ACTCCAGCTTATCTCTTGG-3′; mouse IL-1β sense: 5′-TGCCACCTTTTGACAG TGATG-3′, antisense: 5′-TTCTTGTGACC CTGAGCGAC-3′; mouse β-actin sense: 5′-CCAGCCTTCCTTCTTGGGTAT-3′, antisense: 5′-GGGTGT AAAACGCAGCTC AG-3′.

Western blot

Western blot assays were performed as described previously. Briefly, 150 μg of protein was extracted from endothelial cells using RIPA lysis buffer (cat. no.: R0010; Solarbio, Beijing, China), subjected to SDS-PAGE (10%), and transferred. They were blocked for 1 h in 0.2–0.4% TBST containing bovine serum albumin (3%). The membranes were then incubated overnight at 4°C with primary anti-BASP1 (ab214322), anti-CD68 (ab283654), anti-SLC7A11 (ab175186), anti-cleaved caspase-3 (ab32042), anti-Bax (ab32503), anti-Bcl-2 (ab32124), anti-TNF-α (ab183218), anti-IL-6 (ab233706), anti-IL-1β (ab254360), anti-EGFR (ab52894), Wnt (ab15251), β-catenin (ab223075), Dll4 (ab183532), and Notch1 (ab167441, dilution 1:1,000; Abcam, Cambridge, MA, USA), followed by an HPR-conjugated secondary antibody, and imaged in a chemiluminescence imaging system using ECL luminescent solution. Using β-actin (1:10,000, ab213262; Abcam) as an internal reference, the protein levels were normalized according to β-actin band density and analyzed in ImageLab software.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software. Each measure is expressed as the mean ± SD. Comparisons between two groups were analyzed using a *t*-test. Comparisons between multiple groups were analyzed using one-way ANOVA followed by Tukey's *post hoc* test, with the significance set at $P < 0.05$.

RESULTS

Increased BASP1 expression in high glucose-induced endothelial cells

Firstly, we found three gene sets related to diabetes complicated with cardiovascular disease through the GEO database, which were GSE132651, GSE144803, and GSE113969, respectively. In these three gene sets, a number of genes were significantly upregulated in patients with diabetes complicated with cardiovascular disease. Three overlapping genes were screened by the overlapping analysis tool, namely: BASP1, CD68, and SLC7A11, as shown in Figure 1a. In the GSE113969 dataset, the expression of BASP1 was higher in the tissues of patients with type 2 diabetes complicated with coronary heart disease than in those without coronary heart disease ($P < 0.05$, Figure 1b). HUVECs were cultured with high glucose to mimic an *in vitro* model of endothelial cell dysfunction in diabetic patients. Mannitol was used as a negative control to exclude the effect of elevated osmotic pressure caused by high glucose. The results of RT-qPCR and western blot results showed that high glucose upregulated BASP1 expression at transcriptional and protein levels, and the effect was time-dependent, while mannitol did not affect BASP1 expression ($P < 0.05$, Figure 1c,d). Neither high glucose nor mannitol had any effects on the expression of CD68 and SLC7A11 protein in HUVECs (Figure 1d). Immunofluorescence staining results showed that high glucose (16 h) promoted BASP1 expression, and BASP1 mainly was expressed in the membrane of endothelial cells (Figure 1e).

Silencing of BASP1 improves the proliferation and migration of endothelial cells

siRNA was used to downregulate BASP1 expression in HUVECs. Western blot experiments showed that both siRNA-1 and siRNA-2 could significantly downregulate the expression of BASP1 in HUVECs under a high glucose environment ($P < 0.05$, Figure 2a). The CCK-8 assay was used to detect cell viability, and it was found that high glucose inhibited cell viability, and silencing of BASP1 by siRNAs alleviated the effects of high glucose on the HUVEC viability loss ($P < 0.05$, Figure 2b). In addition, high glucose inhibited cell migration and tube formation, while silencing of BASP1 alleviated the effect of high glucose on HUVEC migration and tube formation ($P < 0.05$, Figure 2c–f).

Moreover, the effects of BASP1 on endothelial cell proliferation and migration were confirmed in primary mouse aortic endothelial cells. The expression of BASP1 was increased in primary mouse aortic endothelial cells under high glucose

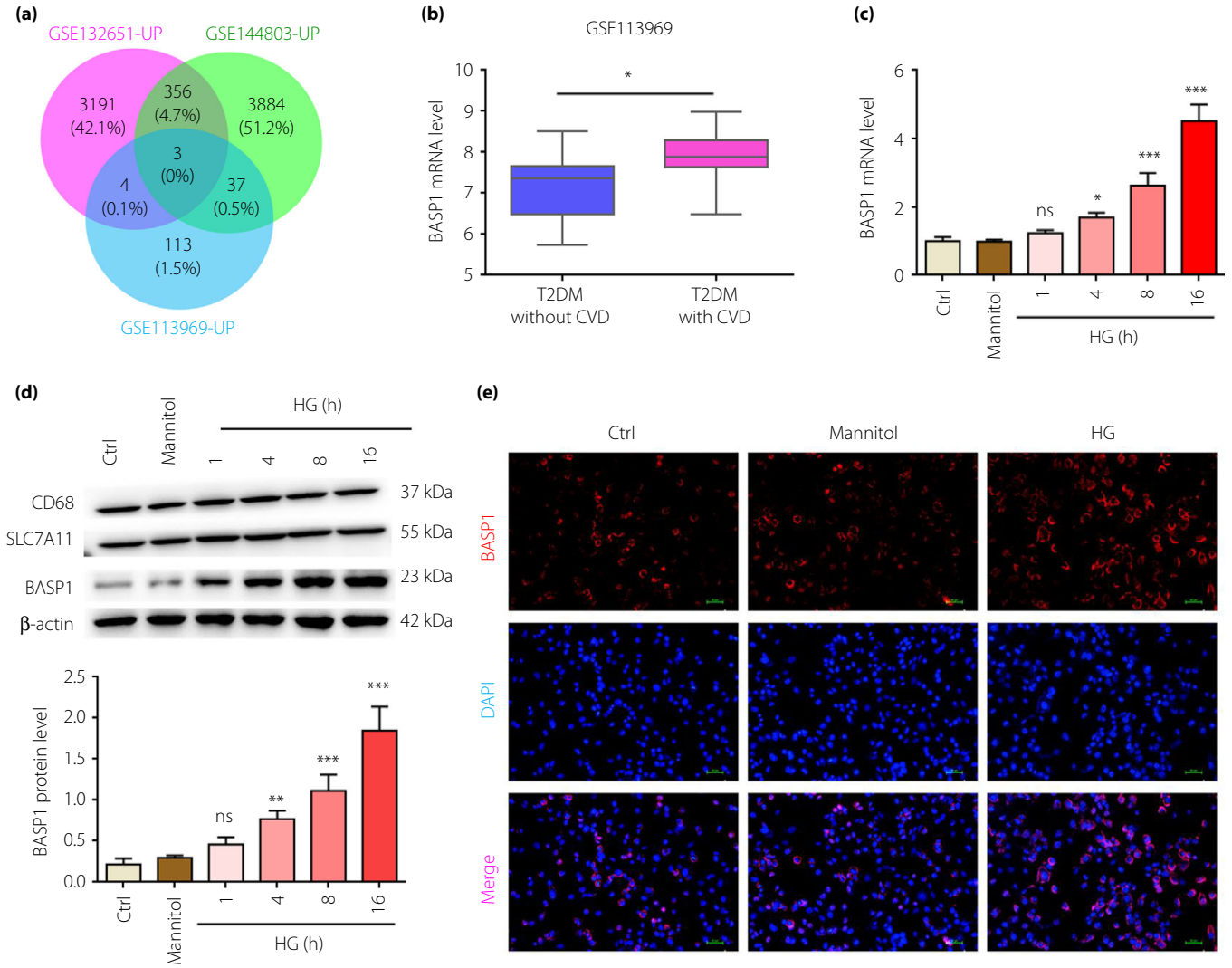

Figure 1 | Increased BASP1 expression in high glucose-induced endothelial cells. (a) GSE132651, GSE144803, and GSE113969 datasets were used for overlapping analysis and three overlapping genes were identified, namely: BASP1, CD68, SLC7A11. (b) In the GSE113969 dataset, the expression of BASP1 in the tissues of patients with type 2 diabetes complicated by coronary heart disease and those without coronary heart disease. (c) HUVECs were treated with mannitol or high glucose, respectively, and then the transcriptional expression of BASP1 was detected by RT-qPCR. (d) The expression of BASP1, CD68, and SLC7A11 proteins was detected by western blot in HUVECs treated with mannitol or high glucose. (e) The expression of BASP1 in HUVECs was detected by immunofluorescence following the treatment of mannitol or high glucose (16 h). **P* <0.05, ***P* <0.01, and ****P* <0.001.

stimulation, whilst it was decreased by siRNA transfection ($P < 0.05$, Figure 3a). Silencing of BASP1 in primary mouse aortic endothelial cells reversed the effects of high glucose on viability loss ($P < 0.05$, Figure 3b). In addition, the inhibitory effects of high glucose on mouse aortic endothelial cell migration and tube formation were partially reversed by BASP1 silencing ($P < 0.05$, Figure 3c–f).

Silencing of BASP1 inhibits high glucose-induced apoptosis in endothelial cells

Next, flow cytometry was used to detect the apoptosis of HUVEC in a high glucose environment. As shown in Figure 4a ($P < 0.05$), the apoptosis ratio of HUVECs was increased under a high glucose environment, while apoptosis was alleviated by BASP1 silencing. The expression of apoptosis-related proteins was detected by western blot, and the results showed that high glucose increased the ratio of Bax/Bcl-2 and the cleavage of caspase-3. Silencing of BASP1 by siRNAs alleviated the effect of high glucose on Bax/Bcl-2 ratio and caspase-3 cleavage ($P < 0.05$, Figure 4b). High glucose promoted ROS generation, and silencing of BASP1 alleviated the high glucose-induced ROS accumulation (Figure 4c).

The effects of BASP1 on endothelial cell apoptosis were also confirmed in primary mouse aortic endothelial cells.

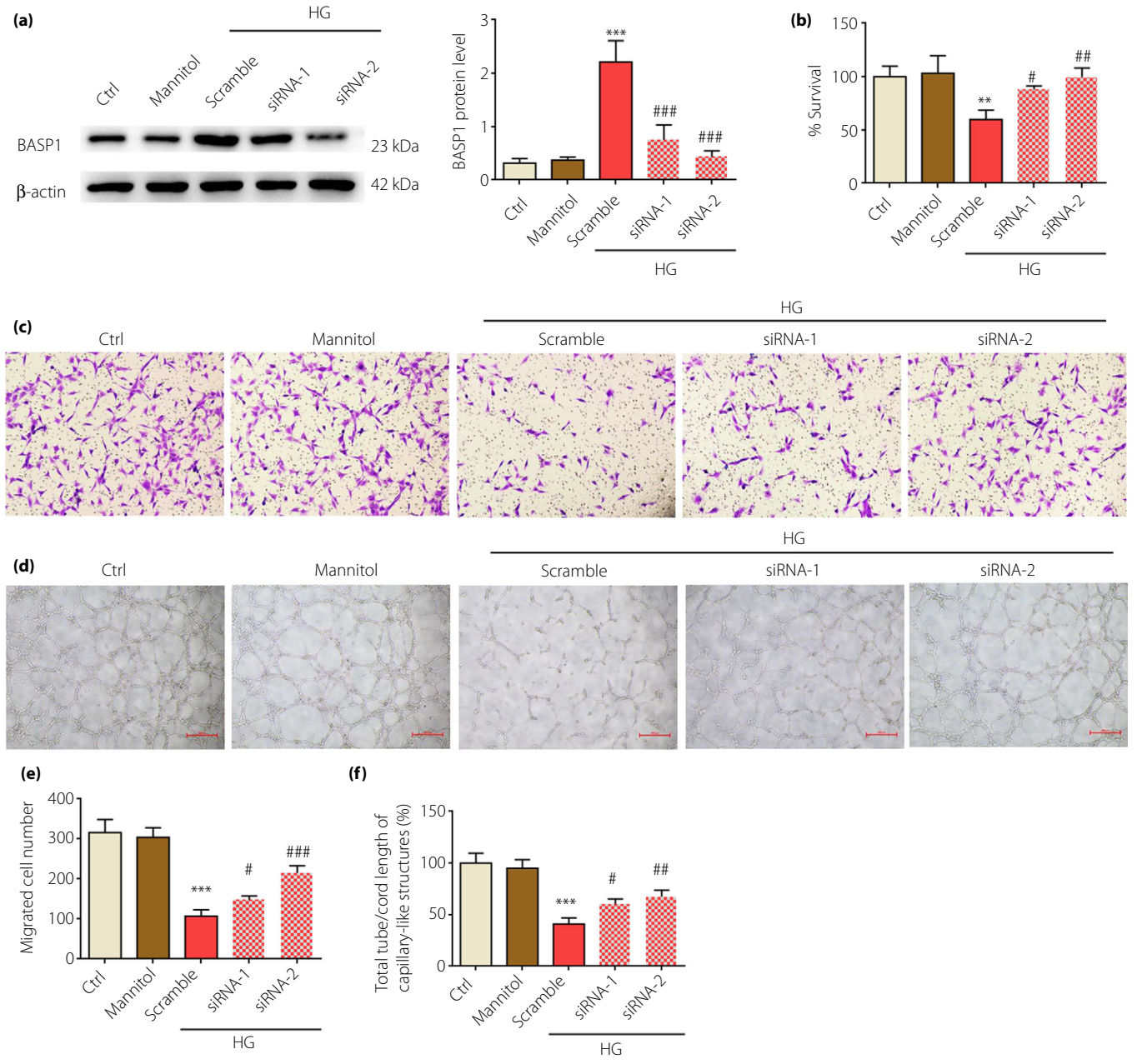

Figure 2 | Silencing of BASP1 improves the migration and tube formation of HUVECs. (a) BASP1-specific siRNAs (siRNA-1 and siRNA-2) were transfected into HUVECs, after which the cells were treated with mannitol or high glucose. Western blot assay showed that both siRNA-1 and siRNA-2 could significantly downregulate the expression of BASP1 in HUVECs. (b) Cell viability was detected by CCK-8 assay. (c and e) A transwell chamber assay was used to detect cell migration. (d and f) Tube formation of HUVECs was detected. ***P*-value <0.01 and ***<0.001 compared with the Control group. #*P*-value <0.05, ##<0.01, and ###<0.001 compared with the Scrambled group.

Primary mouse aortic endothelial cells that were treated with high glucose showed a remarkable apoptosis as was observed by the increases of apoptosis rate, Bax/Bcl-1 ratio, and caspase-3 cleavage ($P < 0.05$, Figure 5a,b). Silencing of BASP1 attenuated the effects of high glucose on primary mouse aortic endothelial cell apoptosis ($P < 0.05$). In addition, the ROS generation induced by high glucose in primary mouse aortic endothelial cells was attenuated by BASP1 silencing ($P < 0.05$, Figure 5c).

Silencing of BASP1 inhibits high glucose-induced inflammation in endothelial cells

The relationship between BASP1 and the expression of inflammatory factors was further detected in HUVECs. The RT-qPCR

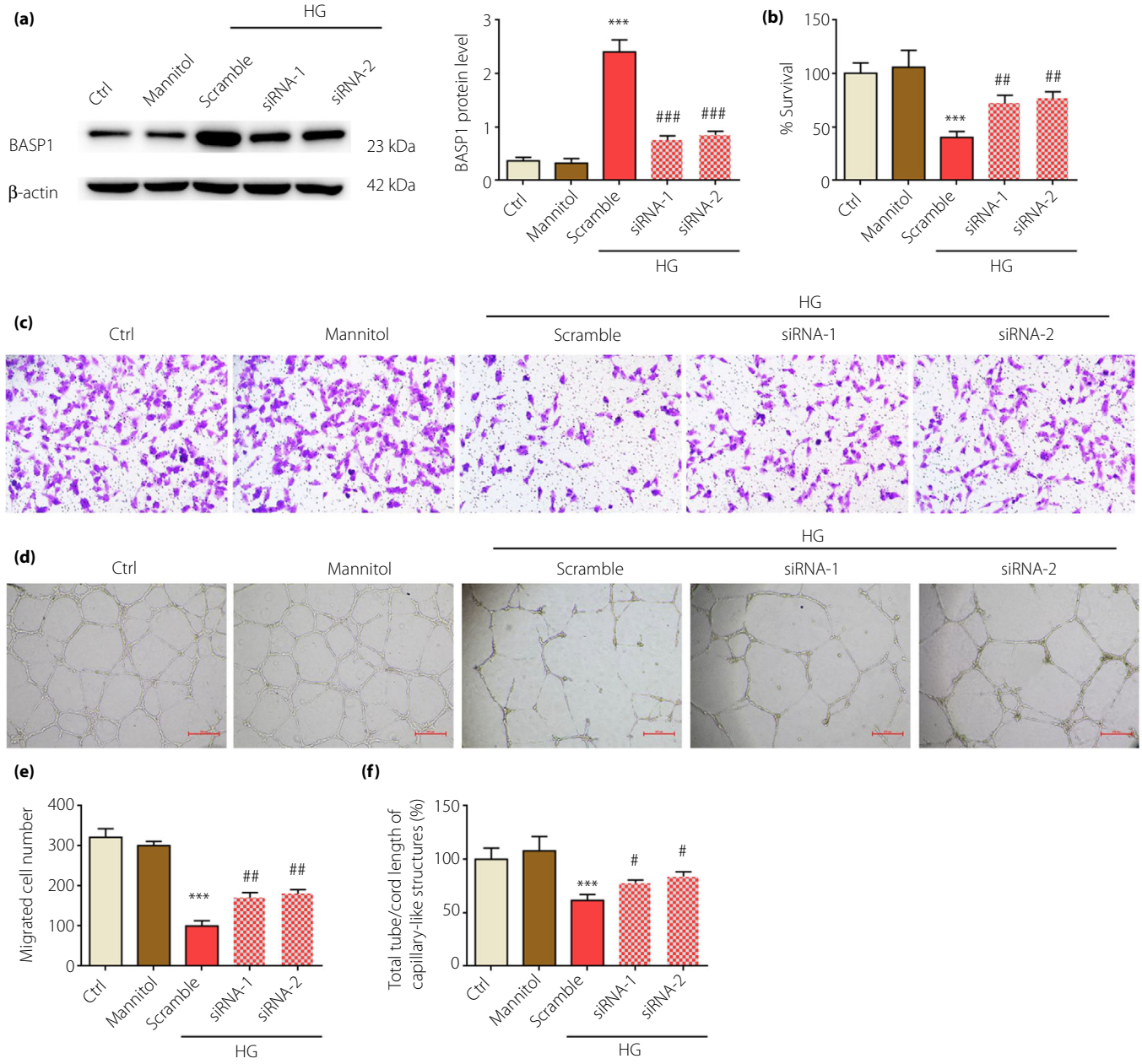

Figure 3 | Silencing of BASP1 improves the migration and tube formation of primary mouse aortic endothelial cells. (a) BASP1-specific siRNAs (siRNA-1 and siRNA-2) were transfected into primary mouse aortic endothelial cells, after which the cells were treated with mannitol or high glucose. Western blot assay showed that both siRNA-1 and siRNA-2 could significantly downregulate the expression of BASP1 in primary mouse aortic endothelial cells. (b) Cell viability was detected by CCK-8 assay. (c and e) A transwell chamber assay was used to detect cell migration. (d and f) Tube formation of primary mouse aortic endothelial cells was detected. ***P-value 0.001 compared with the Control group. $^{\#}P$-value <0.05, $^{\#\#}$<0.01, and $^{\#\#\#}$<0.001 compared with the Scrambled group.

data indicated that high glucose significantly promoted the expression of inflammatory factors, including TNF-α, IL-6, and IL-1β, and silencing of BASP1 alleviated the effect of high glucose on the expression of these inflammatory factors ($P < 0.05$, Figure 6a). The ELISA and western blot results showed that high glucose increased the levels of inflammatory factors in the supernatant and cell lysate of HUVECs, and silencing of BASP1 alleviated the effects of high glucose on the these inflammatory factors ($P < 0.05$, Figure 6b,c).

BASP1 enhances EGFR signaling in endothelial cells

It has been reported that BASP1 has a positive regulatory relationship with EGFR[26], and EGFR plays a key regulatory role in diabetes mellitus and endothelial dysfunction[27,28]. In

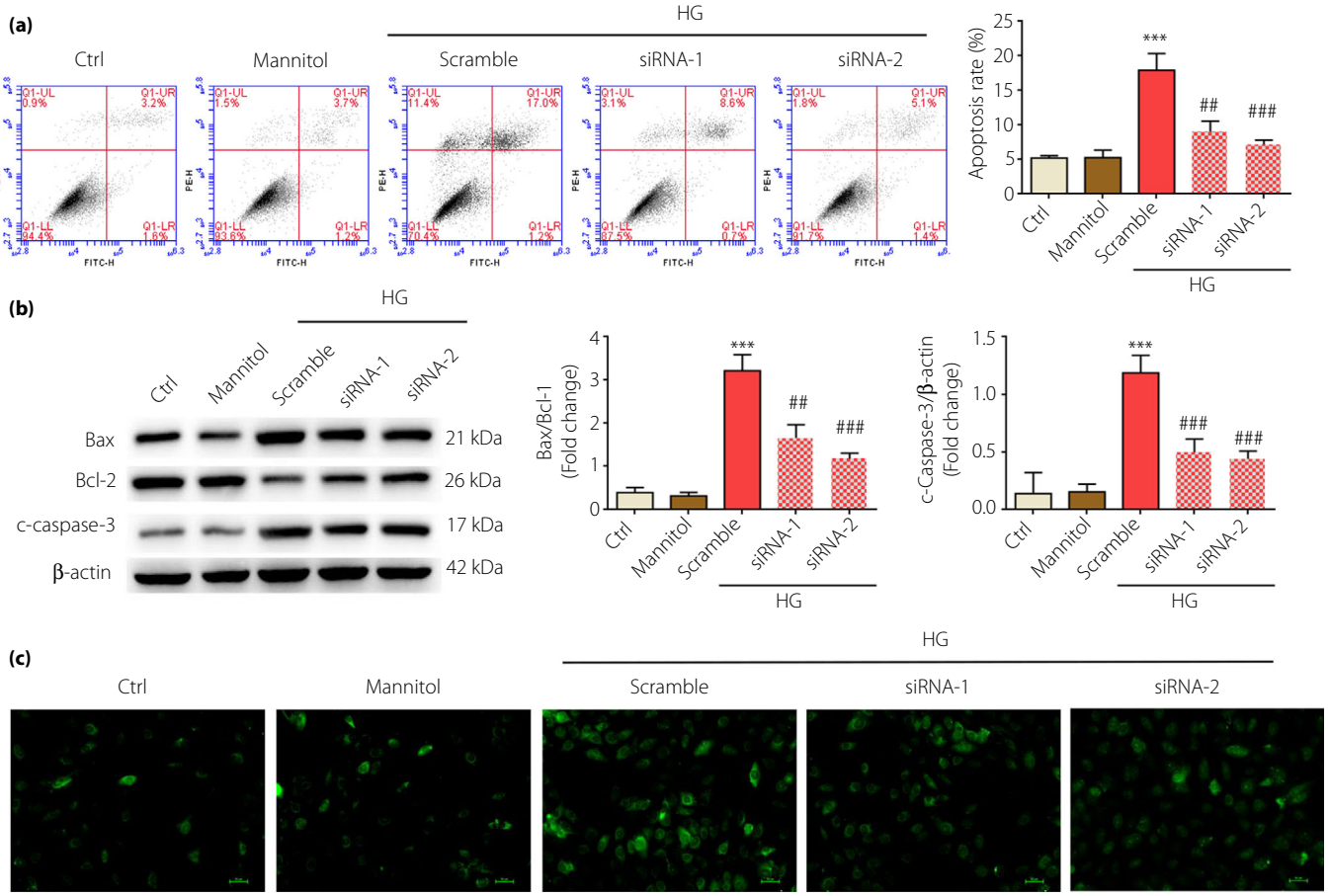

Figure 4 | Silencing of BASP1 inhibits high glucose-induced apoptosis in HUVECs. (a) BASP1-specific siRNAs (siRNA-1 and siRNA-2) were transfected into HUVECs, after which the cells were treated with mannitol or high glucose. Cell apoptosis was detected by flow cytometry. (b) The expression of apoptosis-related proteins was detected by western blot. (c) ROS levels were detected with DHE probes. ***P-value <0.001 compared with the Control group. ##P-value <0.01 and ###<0.001 compared with the Scrambled group.

endothelial cells, whether BASP1 regulates EGFR and plays a regulatory role in endothelial function was explored. The effect of high glucose on EGFR expression was detected by western blot. High glucose could promote EGFR expression, and this promotion was time-dependent (Figure 7a). The effect of BASP1 silencing on EGFR expression was detected. The results showed that EGFR expression was downregulated by BASP1 silencing (Figure 7b). EGFR protein degradation was detected by treating the cells with 100 μM cycloheximide (CHX) for 0–24 h. As shown in Figure 7c ($P < 0.05$), EGFR protein was degraded slowly in scramble transfected cells, while it was degraded quickly in BASP1-silenced cells, suggesting that BASP1 maintains the stability of EGFR protein. Figure 7d is a schematic diagram of the role of BASP1 in the regulation of EGFR signaling. In addition, silencing of BASP1 markedly inhibited the activation of Wnt/β-catenin and Dll4/Notch1 signaling pathway (Figure 7e), which are abnormally activated in high glucose-treated endothelial cells and contribute to diabetic endothelial dysfunction[29–31].

BASP1 promotes high glucose-induced apoptosis in endothelial cells *via* EGFR signaling

To further test our hypothesis, HUVECs were transfected with BASP1 overexpression vector and treated with the EGFR inhibitor gefitinib. The western blot results showed that the BASP1 overexpression vector upregulated the expression of BASP1 protein (Figure 8a) and gefitinib inhibited the expression of EGFR protein in HUVECs (Figure 8b). The CCK-8 assay showed that high glucose inhibited the viability of HUVECs. Overexpression of BASP1 further increased the inhibitory effect of high glucose on cell viability, while gefitinib alleviated the effect BASP1 on cell viability ($P < 0.05$, Figure 8c). BASP1 overexpression further increased the apoptosis rate induced by high glucose, while gefitinib inhibited the apoptosis rate ($P < 0.05$, Figure 8d). These results indicated

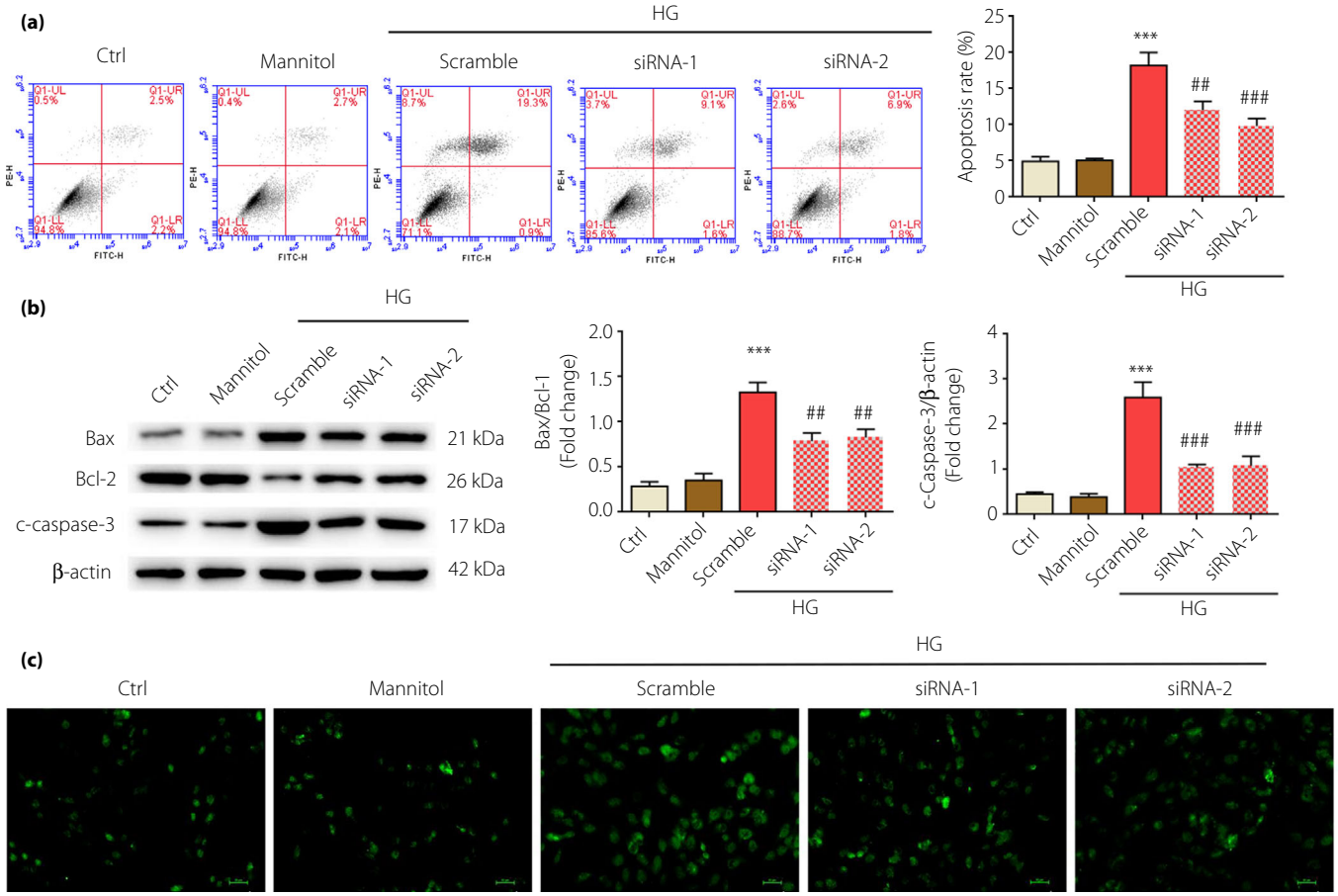

Figure 5 | Silencing of BASP1 inhibits high glucose-induced apoptosis in primary mouse aortic endothelial cells. (a) BASP1-specific siRNAs (siRNA-1 and siRNA-2) were transfected into primary mouse aortic endothelial cells, after which the cells were treated with mannitol or high glucose. Cell apoptosis was detected by flow cytometry. (b) The expression of apoptosis-related proteins was detected by western blot. (c) ROS levels were detected with DHE probes. ***P-value <0.001 compared with the Control group. $^{\#\#}P$-value <0.01 and $^{\#\#\#}$<0.001 compared with the Scrambled group.

that BASP1 inhibited cell proliferation and promoted apoptosis through the EGFR pathway.

DISCUSSION

In this study, the effect and related mechanism of BASP1 on endothelial dysfunction induced by high glucose were investigated. BASP1 may be involved in the incidence and progression of diabetic endothelium dysfunction, according to a bioinformatics analysis. HUVECs and primary mouse aortic endothelial cells were treated with mannitol or high glucose, respectively. It was found that mannitol had no significant effects on the viability, migration, tube formation, apoptosis, ROS generation, and inflammation of endothelial cells. However, high glucose significantly inhibited the viability, tube formation, and migration of endothelial cells, and induced significant apoptosis, ROS accumulation, and cellular inflammation, and this effect is time-dependent. Silencing of BASP1 alleviated the damage of high glucose on endothelial cells. Further research found that BASP1 was positively correlated with EGFR and contributed to maintaining the stability of EGFR protein. The promoting effect of BASP1 on the apoptosis of endothelial cells may be achieved by regulating the EGFR pathway. It has been reported that BASP1 knockdown protects podocyte injury after high glucose treatment through activation of the p53 pathway *via* WT1[16,23]. In this study, the similar results were obtained by high glucose treatment, and it was found that the degree of BASP1 upregulation was positively correlated with the time of high glucose treatment. Silencing of BASP1 played a protective role in endothelial cells against high glucose-induced cell dysfunction.

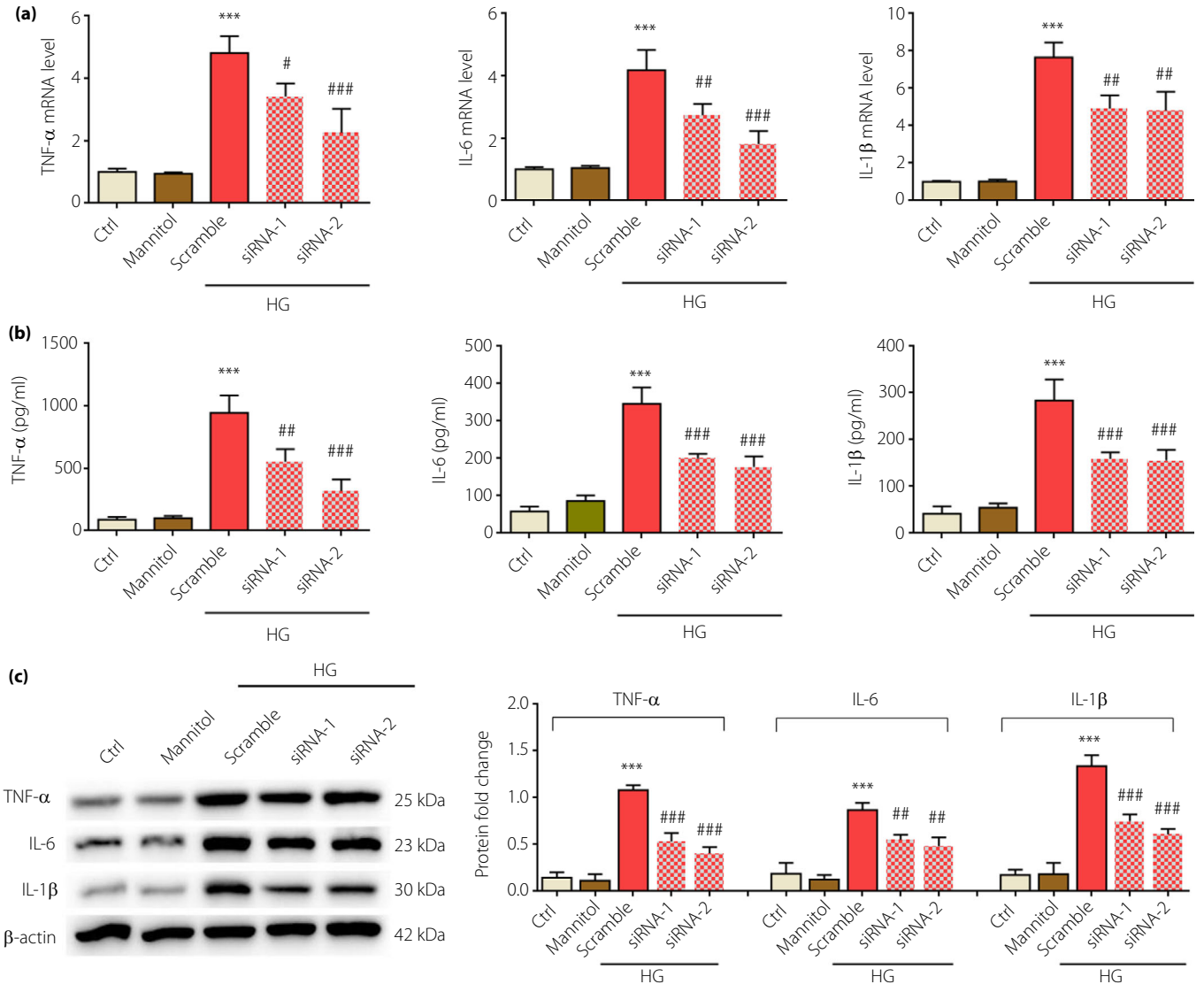

Figure 6 | Silencing of BASP1 inhibits high glucose-induced inflammation in HUVECs. (a) BASP1-specific siRNAs (siRNA-1 and siRNA-2) were transfected into HUVECs, after which the cells were treated with mannitol or high glucose. The expression of inflammatory factors was detected by RT-qPCR. (b) The contents of TNF-α, IL-6, and IL-1β in the cell supernatant were detected by the ELISA kit. (c) Western blot was used to detect the expression levels of TNF-α, IL-6, and IL-1β proteins in cell lysates. ***P-value <0.001 compared with the Control group. $^{\#}P$-value <0.05, $^{\#\#}$<0.01, and $^{\#\#\#}$<0.001 compared with the Scrambled group.

Previous studies have reported that BASP1 regulates a variety of signaling pathways, thereby regulating cell morphology and function. For example, BASP1 activates the p53 pathway through WT1 and leads to podocyte apoptosis in diabetic nephropathy[16]. It has also been reported that BASP1 inhibits the carcinogenic ability of MYC protein by interfering with the binding of MYC protein to calmodulin[32]. The expression of BASP1 can be induced by albumin, serum deprivation, high glucose, and cytokines, and its expression contributes to tubular cell apoptosis and chronic kidney disease[23,33]. Consistently, we observed here high expression of BAPS1 in high glucose-treated endothelial cells. Silencing of BASP1 attenuated high glucose-induced apoptosis in endothelial cells, suggesting that BASP1 is a pro-apoptotic factor in diabetic endothelial dysfunction. In addition, silencing of BASP1 improved the migration and tube formation of endothelial cells in high glucose conditions.

EGF has essential roles in regulating endothelial cell proliferation, permeability, and tube formation[34]. However, abnormal activation of EGFR contributes to the vascular dysfunction associated with type 1 and type 2 diabetes[27,28]. EGFR has been considered to be a potential target for diabetes-related

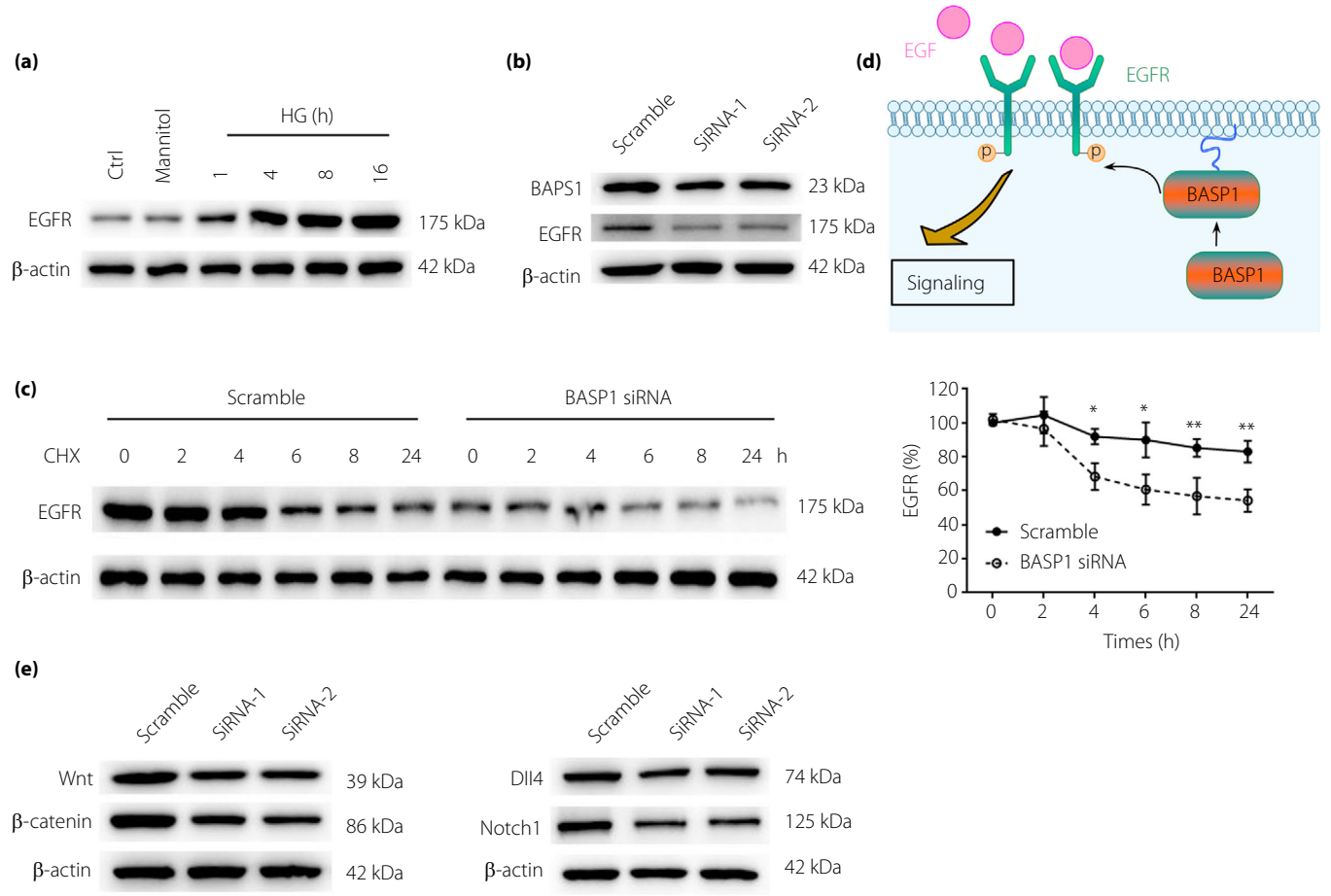

Figure 7 | BASP1 enhances EGFR signaling in HUVECs. (a) The effect of high glucose on EGFR expression was detected by western blot. (b) The effect of BASP1 silencing on EGFR expression was detected by western blot. (c) Cells were treated with 100 μM cycloheximide (CHX) for 0–24 h to detect the degradation of EGFR protein. (d) A schematic diagram of action of BASP1 in the regulation of EGFR signaling. (e) The effect of BASP1 silencing on Wnt/β-catenin and Dll4/Notch1 signaling pathway was detected by western blot. **P*-value <0.05 and ***<0.01 compared with the Scramble group.

complications[35–37]. Inhibition of EGFR in streptozocin-induced diabetes in rat reversed the gene expression in mesenteric vasculature and normalized vasodilator responses[38,39]. Endothelial dysfunction in diabetes is partly due to abnormal activation of EGFR and downstream oxidative stress[40]. The ability of glucose to activate EGFR to induce ROS formation and its contribution to vascular change have been well studied[41]. EGFR deletion protects animals from HFD-induced endothelial dysfunction, creatininemia, and albuminuria[41]. BASP1 has been shown to increase EGFR signal transduction and to stabilize EGFR protein by promoting EGFR protein escape from the ubiquitin-proteasome pathway[26]. In this study, BASP1 was found to regulate EGFR and to maintain the stability of EGFR protein. BASP1 may promote apoptosis of endothelial cells by regulating the EGFR pathway.

In addition to EGFR signaling, Wnt/β-catenin and Dll4/Notch1 signaling pathways are also abnormally activated in high glucose-treated endothelial cells and contribute to diabetic endothelial dysfunction[29–31]. Herein, silencing of BASP1 was found to be effective in inhibiting Wnt/β-catenin and Dll4/Notch1 signaling pathways, which was in line with a previously reported article[42]. It seems that BAPS1 regulates various signaling pathways in diabetic endothelial dysfunction. Further studies are required to explain its complexity.

In conclusion, BASP1 promotes endothelial cell injury induced by high glucose in patients with diabetes, which is mediated by activating the EGFR signaling pathway. This research gives a theoretical foundation for better understanding diabetic endothelial dysfunction.

DISCLOSURE

The authors declare that they have no conflicts of interest.
Approval of the research protocol: N/A.
Registry and the registration no. of the study/trial: N/A.

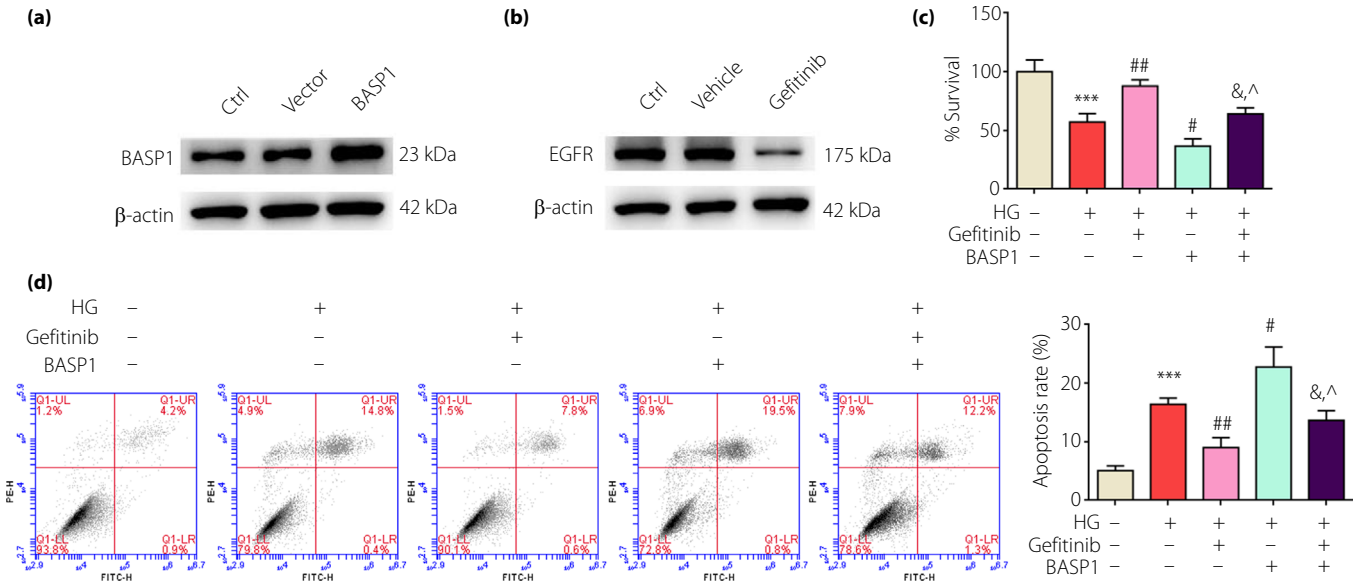

Figure 8 | BASP1 promotes high glucose-induced apoptosis in HUVECs *via* EGFR signaling. (a) Western blot assay showed that the BASP1 overexpression vector upregulated the expression of BASP1 protein in HUVECs. (b) Western blot assay showed that gefitinib inhibited the expression of EGFR protein in HUVECs. Gefitinib was dissolved in DMSO and diluted with culture medium (Vehicle refers to 0.1% DMSO). (c) HUVECs that were transfected with BASP1 overexpression vector or the empty control were treated with high glucose in the presence or absence of gefitinib. Cell viability was measured by CCK-8 assay. (d) Cell apoptosis was detected by flow cytometry. ***P-value <0.001 compared with the Control group. $^{\#}P$-value <0.05 and $^{\#\#}$<0.01 compared with the HG group. $^{\&}P$-value <0.05 compared with the HG + gefitinib group. ^P-value <0.05 compared with the HG + BASP1 group.

Animal studies: N/A.
Informed consent: N/A.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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