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Sprouty-related proteins with EVH1 domain (SPRED2) prevents high-glucose induced endothelial–mesenchymal transition and endothelial injury by suppressing MAPK activation

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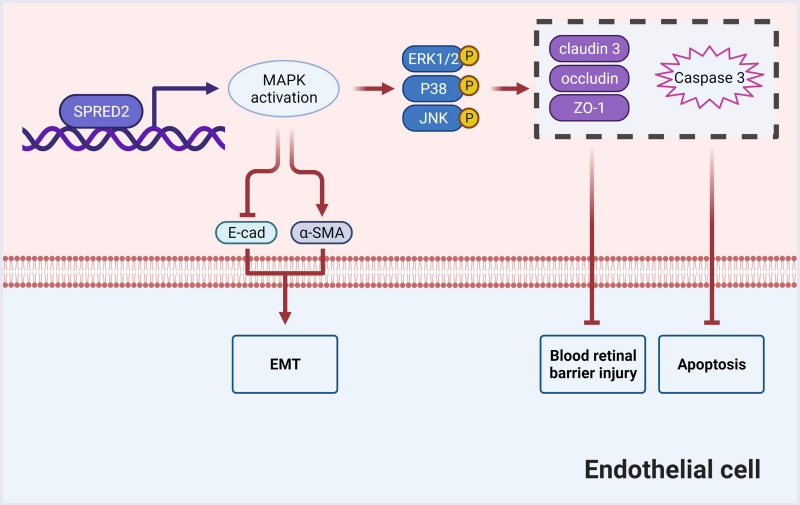
ABSTRACT

Diabetic retinopathy (DR) is a common complication of diabetes, and the leading cause of blindness in adults. Sprouty-related proteins with EVH1 domain (SPRED2) play an important role in diabetes and are closely related to the lens and eye morphogenesis. This study attempted to investigate the role and related mechanism of SPRED2 in DR. DR rat model was established by administration streptozocin. Human retinal endothelial cells (HRECs) were treated with high glucose (HG) to mimic DR. The results showed that SPRED2 expression was decreased in the retinal tissues of DR rats and HG-treated HRECs. MTT assay and flow cytometry data showed that SPRED2 overexpression reduced cell viability of HG-treated HRECs. SPRED2 overexpression enhanced Caspase-3 activity and promoted apoptosis of HG-treated HRECs. Furthermore, the expressions of endothelial cell markers CD31 and E-cad were down-regulated, whereas the expressions of mesenchymal cell markers FSP1, SM22, and α-SMA were up-regulated in the HG-treated HRECs. SPRED2 overexpression reversed HG-induced endothelial–mesenchymal transition in HRECs. The expressions of tight junction components claudin 3, occludin, and ZO-1 were increased in HG-treated HRECs following SPRED2 up-regulation. In addition, SPRED2 overexpression downregulated the expression of p-ERK1/2, p-p38, and p-JNK in the HG-treated HRECs. In conclusion, this study demonstrated that SPRED2 overexpression repressed endothelial–mesenchymal transition and endothelial injury in HG-treated HRECs by suppressing MAPK signaling pathway. These findings suggested that SPRED2 may be a novel potential therapeutic target implicated in DR progression.



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Highlights

- SPRED2 expression was decreased in the DR rats and HG-treated HRECs.
- SPRED2 promoted cell viability and repressed cell apoptosis in HG-treated HRECs.
- SPRED2 retarded EMT progress and alleviated BRB damage in HG-treated HRECs.
- SPRED2 inhibited MAPK signaling pathway in HG-treated HRECs.

Introduction

Diabetes is a serious public health problem worldwide, and its global prevalence is increasing year by year. Diabetes can cause various complications, including diabetic retinopathy (DR) [1,2]. Epidemiological data show that the overall prevalence of DR in diabetic patients is as high as 35%, which is the leading cause of blindness among working-age people [3,4]. In the development of DR, human retinal endothelial cells (HRECs) dysfunction is an important cause of multifactorial pathology [5,6]. Emerging evidence shows that HRECs are susceptible to high glucose (HG) that cause damage to HRECs, which leads to changes in their surface phenotype to obtain mesenchymal properties [7]. The mesenchymal properties can easily cause fibrosis on the surface of the retina and traction of the epiretinal membrane, which can cause vision-threatening complications and ultimately result in blindness [8]. In addition, the destruction of the blood-retinal barrier (BRB) is a typical event in the early stage of DR [9]. Exposure to HG leads to endothelial cell dysfunction, aggravates BRB imbalance and damage, and increases vascular leakage, thereby exacerbating the progression of DR [10,11]. In summary, inhibiting the endothelial–mesenchymal transition (EndMT) of HRECs and the destruction of BRB are the main options for the treatment of DR.

Sprouty-related proteins with EVH1 domain (SPRED2) are a member of the Sprouty/SPRED family, and they negatively regulate the Ras/Raf/ERK/MAPK signaling pathway [12]. High expression of SPRED2 is closely associated with the progression of various cancers, such as colorectal cancer, pancreatic cancer, and hepatocellular carcinoma [13–16]. Additionally, SPRED2 takes part in EndMT. For example, SPRED2 inhibits epithelial–mesenchymal transition (EMT) of colorectal cancer cells by inhibiting ERK signaling pathway, which contributes to alleviate colorectal cancer progression [17]. SPRED2 also inhibits the cell motility and EMT of keratinocytes induced by TGF-β1 [18]. Furthermore, SPRED2 negatively regulates high-fat diet-induced obesity, adipose tissue inflammation, metabolic abnormalities, and insulin resistance by inhibiting the ERK/MAPK pathway [12]. Wazin et al. have found that the levels of p-ERK1/2 are increased in SPRED2-deficient mice. High levels of p-ERK1/2 change the morphology of the lens and increase the activity of fibroblasts, thereby aggravating the damage of lens and eye morphogenesis [19]. Thus, these data indicate that SPRED2 plays an important role in high-fat diet-induced diabetes and is closely related to the lens and eye morphogenesis. In addition, SPRED2 has an inhibitory effect on the MAPK signaling pathway, and inhibition of MAPK signaling pathway alleviates the BRB destruction of DR, thereby protecting the homeostasis of BRB [20,21].

This study aimed to investigate the functions of SPRED2 and the regulatory relationship between SPRED2 and MAPK pathway in DR. The results revealed that SPRED2 expression was decreased in DR rats and HG-treated HRECs. SPRED2 overexpression repressed EndMT and endothelial damage in HG-treated HRECs by suppressing MAPK activation. These findings suggested that SPRED2 may be a novel potential treatment factor implicated in DR progression.

Materials and methods

Animals

Sprague Dawley (SD) rats (8 weeks; 180–200 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were housed in the animal house at 22–26°C and 50–60% humidity. Rats were supplied with adequate food and water. This study was approved by the Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western

Medicine (Approval No. 2022-L044) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

DR rat model

SD rats were randomly divided into two groups (n = 5) [1]: DR group: rats were intraperitoneally injected with 60 mg/kg of streptozocin (STZ; dissolved in 0.1 mol/L saline sodium citrate (SSC), pH 4.6) (V900890-1 G, Sigma-Aldrich, St. Louis, MO, USA) after 12 h of fasting [2]. Sham group: rats were intraperitoneally injected with the same quantity of 0.1 mol/L SSC. The blood glucose of the blood from tail vein of rats was monitored. When the random blood glucose was higher than 16.7 mmol/L and maintained for more than 1 week, the rat DR model was considered to be successful [22,23].

Cell culture and treatment

Human retinal endothelial cells (HRECs, purchased from Angio-Proteomie company, Boston, MA, USA) were cultured in an endothelial cell medium (Sciencell, Carlsbad, CA, USA) at 37°C and 5% CO_2. The medium contained 10% fetal bovine serum (FBS; 16,000,044, Gibco Laboratories, Grand Island, NY, USA) and 100 μg/mL penicillin/streptomycin (Solarbio, Beijing, China). HRECs were treated with 25 mmol/L glucose (HG) for 24 h [24]. HRECs were treated with 5 mmol/L glucose for 24 h as control.

Cell transfection

The overexpression vector pCEP4-SPRED2 and empty vector pCEP4-NC were constructed and purchased from GenePharma Co., Ltd. (Shanghai, China). HRECs were transfected with pCEP4-SPRED2 or pCEP4-NC plasmids (50 nM) utilizing Lipofectamine 2000 Reagent (11,668,019, Invitrogen, Carlsbad, CA, USA). The transfected cells were cultured in an endothelial cell medium for 48 h.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from HRECs and retinal tissues using TRIzol reagent (15,596,026, Invitrogen, Carlsbad, CA, USA) [25]. RNA was used to synthesize cDNA by using PrimeScrip RT reagent Kit (RR037A, Takara Bio Inc., Dalian, China). The expression of SPRED2 was detected by performing PCR reaction utilizing TB Green Premix Ex Taq. Primers were shown as follows: SPRED2: F-5'-GGAGGCTT TGATGTCGAAGCCCT-3' and R-5'-CCTCCG AAACTACAGCTTCGGGAG-3'; GAPDH (housekeeping gene): F-5'-GCAGTGGCAAAGTGGAG ATT-3' and R-5'-TGAAGTCGCAGGAGACAAC C-3'.

Western blot (WB)

HRECs and the sliced retinal tissues were treated with RIPA Lysis Buffer (P0013B, Beyotime, Shanghai, China), and total protein was extracted from the lysate [26]. The concentration of protein samples was examined by BCA Protein Assay Kit (P0010S, Beyotime) and then separated by 10% SDS-PAGE. The proteins were transferred onto the PVDF membranes. The membranes were incubated with the primary antibodies, including SPRED2 (#ab153700; 1:1000 dilution), CD31 (#ab76533; 1:5000 dilution), E-cad (#ab133597; 1:1000 dilution), FSP1 (#b124805; 1:1000 dilution), SM22 (#ab170902; 1:1000 dilution), α-SMA (#ab5694; 1:1000 dilution), claudin 3 (#ab214487; 1:1000 dilution), occludin (#ab216327; 1:1000 dilution), ZO-1 (#ab216880; 1:1000 dilution), p-ERK1/2 (#ab223500; 1:400 dilution), ERK1/2 (#ab184699; 1:1000 dilution), p-p38 (#44-680 G; 1:1000 dilution), p38 (#ab178867; 1:1000 dilution), p-JNK (#ab4821; 1:1000 dilution), JNK (#ab112501; 1:1000 dilution), or β-actin (#ab8227; 1:1000 dilution). The membranes were then incubated with the goat anti-rabbit HRP-IgG (#ab6721; 1:2000 dilution) secondary antibody. The p-p38 antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA), and other antibodies were purchased from Abcam (Cambridge, MA, USA). The densitometry of protein bands was analyzed by ImageJ software.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

HRECs were incubated with 25 mmol/L glucose and then seeded into 96-well plates at a concentration of 2000 cells/100 μL. Cells were incubated with 10 μL MTT reagent (C0009S, Beyotime) at 37°C for 4 h and then incubated with 100 μL DMSO to dissolve the Formazan. Finally, the absorbance of each well was detected on a microplate reader [27].

Flow cytometry

Annexin V-FITC Apoptosis Detection Kit (C1062S, Beyotime) was used to assess cell apoptosis of HRECs following 25 mmol/L glucose treatment. Cells were collected by centrifugation and washed with PBS several times. Cells (195 μL) at the concentration of 1×10^5 were stained with 5 μL Annexin V-FITC and 10 L PI in darkness for 20 min. Finally, the apoptotic cells were examined by flow cytometry (BD Biosciences, CA, USA) [28].

Caspase-3 activity analysis

Caspase-3 activity was examined using Caspase-3 activity Detection Kit (BC3830, Solarbio, Beijing, China). HRECs were incubated with 25 mmol/L glucose for 24 h. After that, the cells (1×10^6) were incubated with reagent II in the kit at 4°C for 15 min. The absorbance of each sample was detected on a microplate reader [29].

Immunofluorescence staining

Following 24 h of 25 mmol/L glucose treatment, HRECs were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 20 min. After washing with PBS several times, cells were blocked with normal goat serum. Cells were incubated with the primary antibodies against α-SMA (#ab124964; 1:500 dilution) or E-cad (#ab40772; 1:500 dilution) and then incubated the secondary antibody, including goat anti-rabbit Alexa Fluor® 488-IgG (#ab150077; 1:500 dilution) or Alexa Fluor® 647-IgG (#ab150083; 1500 dilution). Cells were stained with DAPI (Sigma-Aldrich) for 15 min to label cell nuclei. The fluorescence of the cells was observed under a confocal microscope (Olympus, Tokyo, Japan) [30].

HE staining

To assess the histologic characteristics of retinas in rats, hematoxylin and eosin (HE) staining was carried out [31,32]. Rats were intraperitoneally injected with sodium pentobarbital (lethal dose), then sacrificed under deep anesthesia by cervical dislocation, and the eyes were quickly taken out. The eyes were maintained in 4% paraformaldehyde solution for 24 h and then embedded in paraffin and cut into 5-μm sections. The paraffin sections were dyed through the HE staining. The thicknesses of the internal limiting membranes (ILM), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layers (ONL) were assessed. The images were acquired through a light microscope (Leica, Heidelberg, Germany).

Statistical analysis

Each assay was performed three times. The representative data were presented as mean ± standard deviation. Statistical analysis was carried out utilizing SPSS 22.0 statistical software (IBM, Armonk, NY, USA) and analyzed by Two-tailed Student's *t* test and one-way ANOVA. *P* value less than 0.05 indicates a significant difference.

Results

The study aimed to investigate the functions of SPRED2 and the regulatory relationship between SPRED2 and MAPK pathway in DR. This work uncovered that SPRED2 prevented HG-induced EndMT and BRB injury by suppressing MAPK activation.

SPRED2 expression was decreased in the DR rats and HG-treated HRECs

As displayed in Figure 1(a,b), the overexpression efficiency of SPRED2 was confirmed through qRT-PCR and western blot. To determine the role of SPRED2 in DR, we constructed DR rat model by

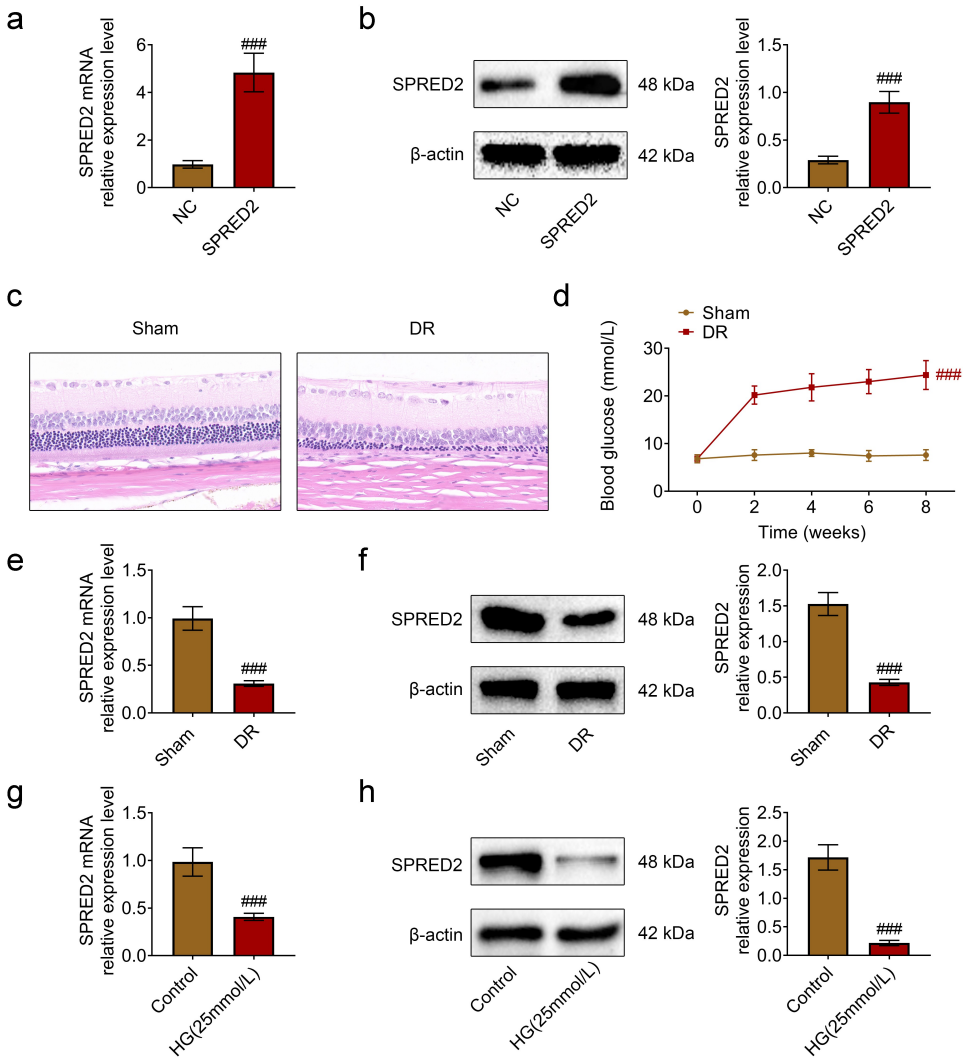

Figure 1. SPRED2 expression was decreased in the DR rats and HG-treated HRECs. DR rat model was constructed by injection of STZ. Sham rats were injected with SSC as control. (a- and b) The overexpression efficiency of SPRED2 was confirmed through qRT-PCR and western blot. (c) The retina changes were assessed through HE staining. (d) The blood glucose of DR and Sham rats was detected. The mRNA and protein expression of SPRED2 in the retinal tissues of DR and Sham rats was assessed by qRT-PCR (e) and WB (f). HRECs were treated with 25 mmol/L glucose (HG) or 5 mmol/L glucose (Control) for 24 h. The mRNA and protein expression of SPRED2 in the HRECs was examined by qRT-PCR (g) and WB (h). ***$P < 0.001$ vs. Control.

administration of STZ. The retina INL, OPL, and ONL in the DR group were significantly thinner, and the ILM in the DR group was thicker than those in the sham group (Figure 1(c)). Rats in the sham group exhibited a normal value of blood glucose. Two weeks after STZ administration, the blood glucose levels of DR rats were consistently higher than 16.7 mmol/L (Figure 1(d)). The results of qRT-PCR and western blot showed that SPRED2 mRNA and protein level were severely decreased in DR rats compared with rats in the sham group (Figure 1(e,f)). Moreover, we treated HRECs with HG to construct DR cell model. SPRED2 was also down-regulated in HG-treated HRECs (Figure 1(g,h)). Thus, SPRED2 expression was decreased in the DR rats and HG-treated HRECs.

SPRED2 overexpression promoted cell viability and repressed apoptosis of HG-treated HRECs

Subsequently, we overexpressed SPRED2 in HRECs, and examined the impact of SPRED2 overexpression on cell viability and apoptosis of HG-treated HRECs. SPRED2 protein expression

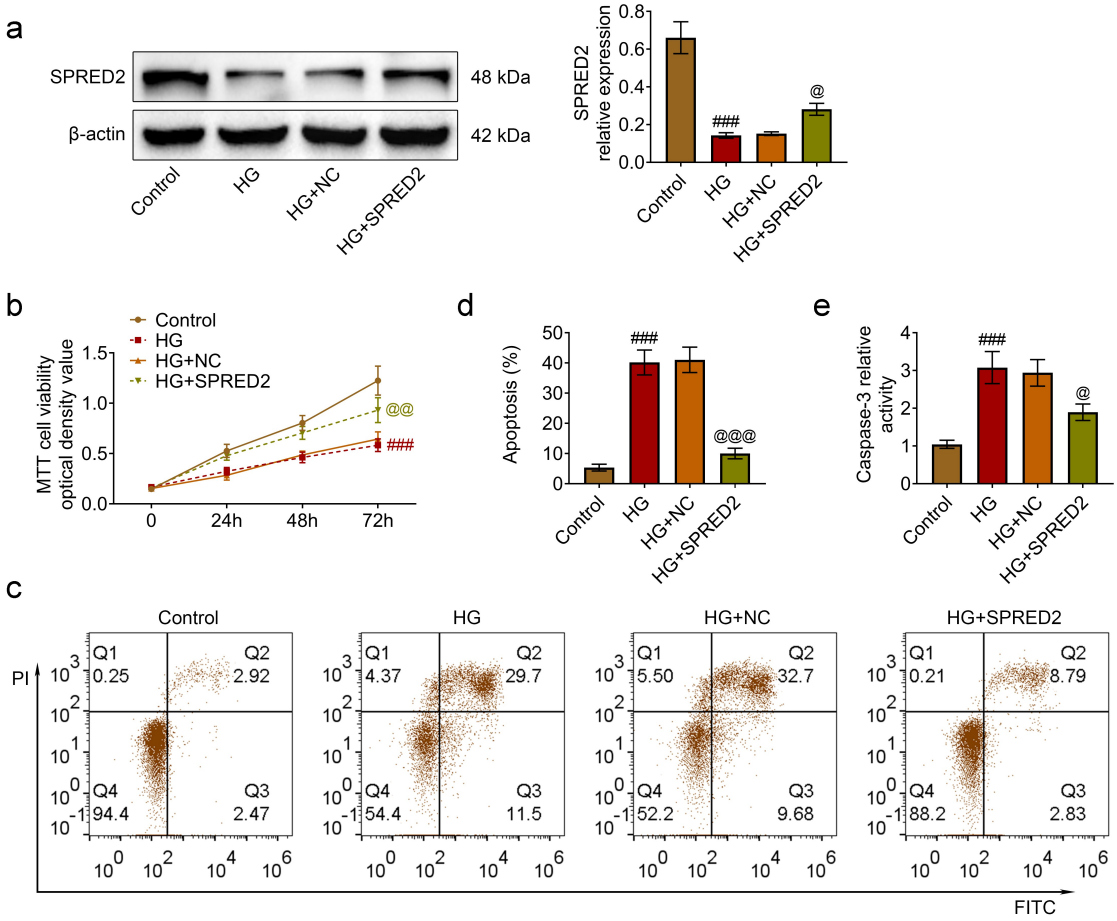

Figure 2. SPRED2 overexpression promoted cell viability and repressed apoptosis of HG-treated HRECs. HRECs were transfected with pCEP4-SPRED2 or pCEP4-NC, followed by 25 mmol/L glucose (HG) treatment for 24 h. (a) The protein expression of SPRED2 in the HRECs was examined by WB. (b) Cell viability of HRECs was detected by MTT assay. (c- and d) Cell apoptosis of HRECs was measured by flow cytometry. (e) Caspase-3 activity of HRECs was detected. $^{###}P < 0.001$ vs. Control; $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.001$ vs. HG + NC.

was elevated in HG-treated HRECs following transfection of pCEP4-SPRED2 (Figure 2(a)). The cell growth curve obtained from MTT assay showed that HG treatment suppressed cell viability of HRECs, which was partly rescued by SPRED2 overexpression (Figure 2(b)). The results of flow cytometry revealed that SPRED2 up-regulation impaired HG-induced increase in apoptosis of HRECs (Figure 2(c, d)). We also found that Caspase-3 activity was enhanced in HG-treated HRECs. SPRED2 overexpression led to a decrease in Caspase-3 activity in HG-treated HRECs (Figure 2(e)). Therefore, SPRED2 overexpression promoted cell viability and repressed apoptosis of HG-treated HRECs.

SPRED2 up-regulation inhibited HG-induced EndMT in HRECs

The expression of EndMT-related markers was detected by western blot analysis to determine the influence of SPRED2 on EndMT of HRECs. As shown in Figure 3(a), the expressions of the endothelial cell markers CD31 and E-cad were down-regulated, whereas the expressions of mesenchymal cell markers FSP1, SM22, and α-SMA were up-regulated in the HG-treated HRECs. SPRED2 overexpression resulted in up-regulation of CD31 and E-cad, and down-regulation of FSP1, SM22, and α-SMA in the HG-treated HRECs (Figure 3(a)). In addition, the immunofluorescence staining also demonstrated that E-cad expression was reduced and

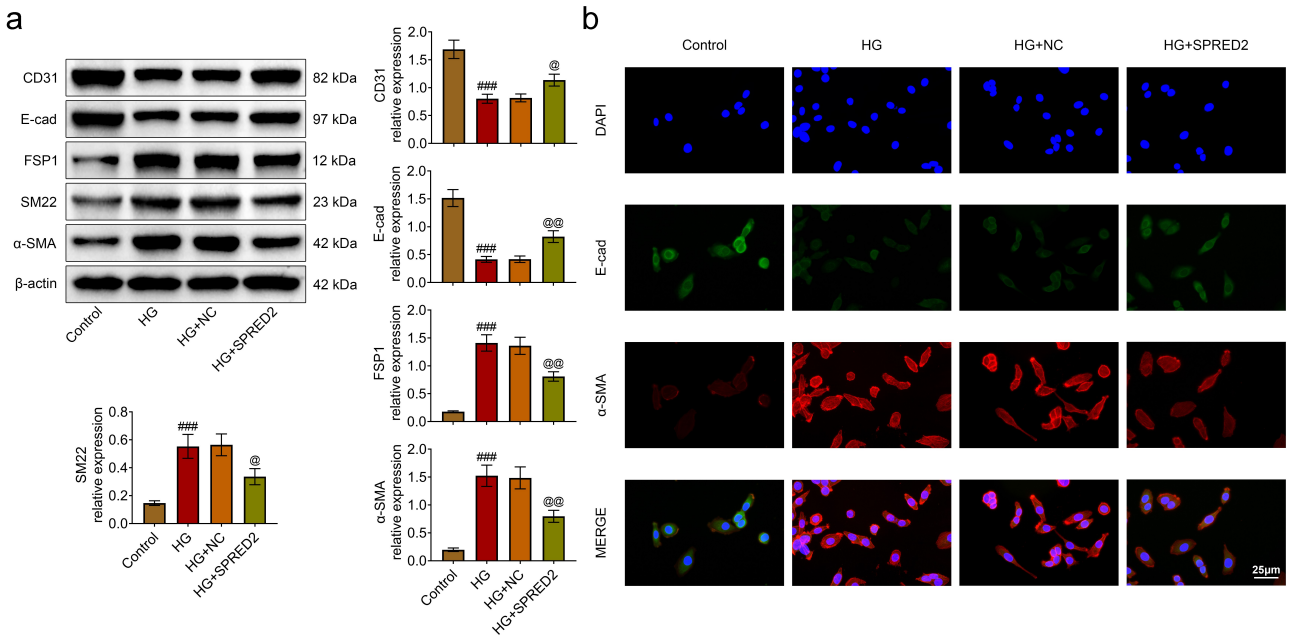

Figure 3. SPRED2 overexpression inhibited EndMT of HG-treated HRECs. HRECs were transfected with pCEP4-SPRED2 or pCEP4-NC, followed by 25 mmol/L glucose (HG) treatment for 24 h. (a) The expression of CD31, E-cad, FSP1, SM22, and α-SMA in HRECs was assessed by WB. (b) IF staining was performed to detect the expression of E-cad and α-SMA in HRECs. $^{###}P < 0.001$ vs. Control; $^{@}P < 0.05$, $^{@@}P < 0.01$ vs. HG + NC.

α-SMA expression was enhanced in the HG-treated HRECs. SPRED2 up-regulation reversed HG-induced EndMT in HRECs (Figure 3(b)). These data show that SPRED2 up-regulation inhibits HG-induced EndMT in HRECs.

SPRED2 overexpression alleviated BRB damage in HG-treated HRECs

To examine the impact of SPRED2 on HG-induced BRB injury in HRECs, we measured the expression of tight junction components claudin 3, occludin, and ZO-1 in HRECs by western blot analysis. The protein expressions of claudin 3, occludin, and ZO-1 were significantly decreased in HRECs in the presence of HG. However, these proteins were up-regulated by SPRED2 up-regulation in the HG-treated HRECs (Figure 4). Therefore, these results indicate that SPRED2 overexpression alleviates HG-induced BRB injury in HRECs.

SPRED2 inhibited MAPK signaling pathway

Finally, we examined whether SPRED2 can affect MAPK signaling pathway. Western blot was carried out to assess the expression of MAPK signaling pathway-related proteins. The results revealed that the total protein expression of ERK1/2, p38, and JNK had no change in the HRECs following HG treatment or combined with SPRED2 overexpression. HG treatment enhanced the expression of p-ERK1/2, p-p38, and p-JNK in HRECs. SPRED2 up-regulation reversed HG-mediated promoting of p-ERK1/2, p-p38, and p-JNK in HRECs (Figure 5). Thus, SPRED2 inhibited MAPK signaling pathway.

Discussion

Accumulating studies have shown that SPRED2 negatively regulates the Ras/Raf/ERK/MAPK signaling pathway and plays an important role in various pathophysiological processes such as embryonic development, tumorigenesis, and tumor development. For instance, SPRED2 deficiency activates ERK signaling pathway and impairs autophagy, which induces cardiomyocyte hypertrophy, cardiac fibrosis, and arrhythmias, and impairs electrical excitability in mice [33]. Inhibition of SPRED2 increases neutrophil infiltration and inflammation by activating the ERK1/2

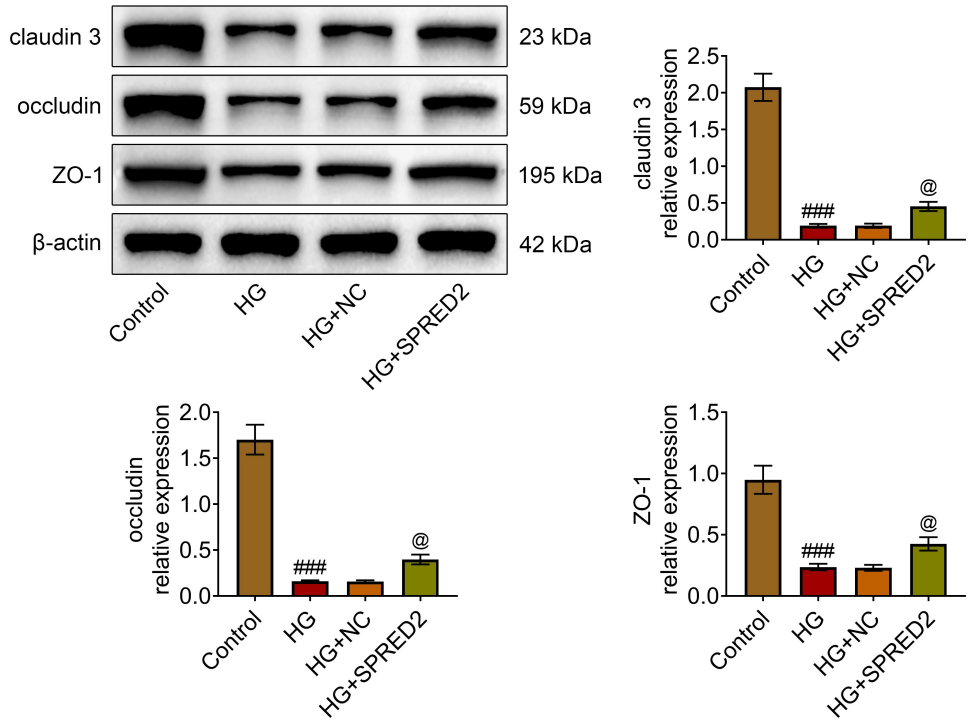

Figure 4. SPRED2 overexpression inhibited BRB damage. HRECs were transfected with pCEP4-SPRED2 or pCEP4-NC, followed by 25 mmol/L glucose (HG) treatment for 24 h. The expression of claudin 3, occludin, and ZO-1 in HRECs was assessed by WB. $^{###}P < 0.001$ vs. Control; $^{@}P < 0.05$ vs. HG + NC.

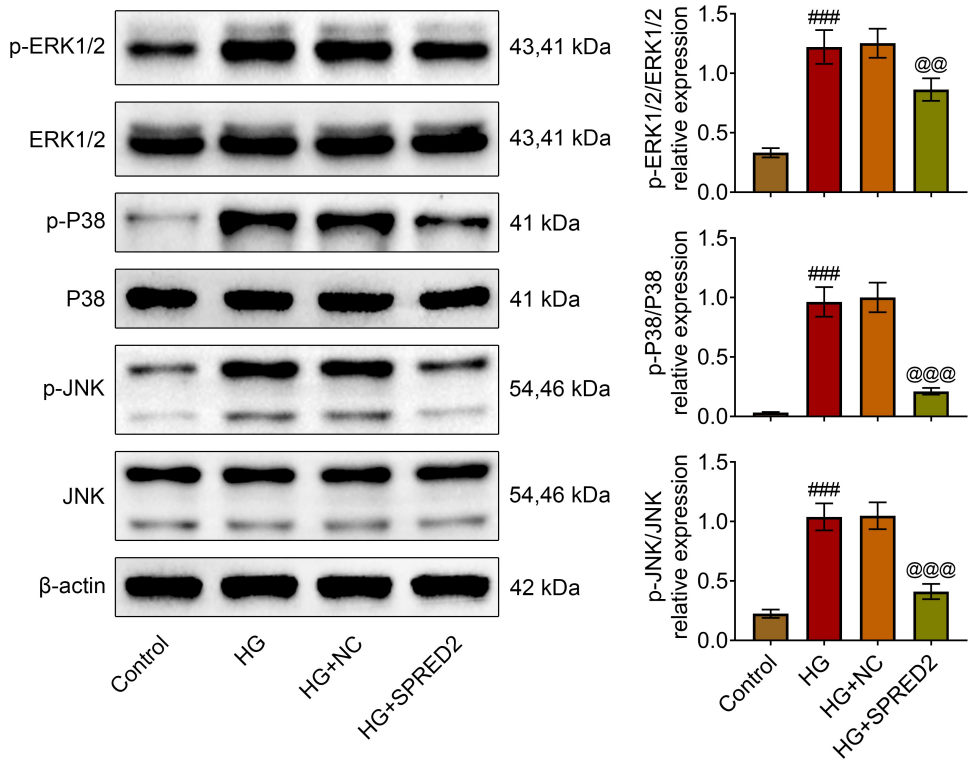

Figure 5. SPRED2 overexpression repressed MAPK signaling pathway. HRECs were transfected with pCEP4-SPRED2 or pCEP4-NC, followed by 25 mmol/L glucose (HG) treatment for 24 h. The expression of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, and JNK in HRECs was assessed by WB. $^{###}P < 0.001$ vs. Control; $^{@@}P < 0.01$, $^{@@@}P < 0.001$ vs. HG + NC.

signaling pathway, which contributes to accelerate the severity of lung ischemia-reperfusion injury [34]. Moreover, miR-142-3p/SPRED2 axis accelerates the development of diabetes through affecting apoptosis, inflammatory response, and oxidative stress of Gly-LDL-induced human vascular endothelial cells [35]. The present study determined the biological role of SPRED2 in the diabetic complication, DR. The results of this work showed that the mRNA and protein expression of SPRED2 was severely decreased in the retinal tissues of DR rats and HG-treated HRECs. SPRED2 overexpression promoted cell viability and reduced apoptosis of HG-treated HRECs. Thus, SPRED2 was closely associated the development of DR.

EndMT is an important process in the development of DR. For example, long non-coding RNA H19 inhibits HG-induced EndMT in HRECs through Smad/TGF-β signaling pathway, which contributes to prevent DR progression [7]. MEG3 overexpression suppresses EndMT in DR rat and cell models by inactivating the PI3K/Akt/mTOR signaling pathway [32]. Whether SPRED2 is related to EndMT remains unclear. This work showed that the expressions of endothelial cell markers CD31 and E-cad were down-regulated, whereas the expressions of mesenchymal cell markers FSP1, SM22, and α-SMA were up-regulated in HRECs following HG treatment, indicating that HG induced EndMT in HRECs. SPRED2 overexpression reversed HG-induced EndMT in HRECs.

BRB is an important barrier to maintain the normal shape and function of the retina, and has selective permeability for the exchange of materials between retinal tissues and blood circulation [36]. The destruction of the structure and function of the BRB is the early pathological basis of DR [10]. Erythropoietin enhances the expression of ZO-1 and occluding in retinal pigment epithelium cells to maintain the integrity of outer BRB, which attributes to regulate HIF-1α expression and JNK signaling pathway [37]. This work found that the expressions of claudin 3, occludin, and ZO-1 were significantly decreased in HRECs following HG treatment. In diabetes, the expressions of retinal tight junction proteins claudin 3, occludin, and ZO-1 are decreased, the integrity of the neurovascular regulatory barrier is destroyed, BRB is dysfunction, and vascular leakage is increased, which signifies the formation of DR [38]. However, SPRED2 overexpression increased the expression of claudin 3, occludin, and ZO-1 in HG-treated HRECs, indicating that SPRED2 overexpression inhibited BRB damage in HG-treated HRECs.

A previous study has confirmed that SPRED2 is a direct target of miR-19, and miR-19 inhibits EMT and metastatic phenotype of osteosarcoma cells by repressing SPRED2 expression [39]. SPRED2 as a negative regulator of the Ras/Raf/ERK/MAPK signaling pathway participates in the development of various diseases. For instance, SPRED2 deficiency activates ERK/MAPK pathway to enhance innate immune responses and protect mice from polymicrobial sepsis [40]. The deficiency of SPRED2 also induces obsessive-compulsive disorder-like behavior in mice through the overactivation of Ras/ERK-MAPK signaling pathway [41]. Importantly, MAPK pathway exhibits a key regulatory function in DR. On the one hand, inhibition of MAPK pathway suppresses inflammatory response and cell apoptosis in rat model, and alleviates DR symptoms [42]. On the other hand, inhibition of MAPK pathway can relieve BRB damage in DR, thus protecting BRB homeostasis [21,43]. This work demonstrated the function of SPRED2/MAPK signaling pathway in DR. SPRED2 overexpression repressed MAPK signaling pathway in HG-treated HRECs, suggesting that SPRED2 exerted a protective effect on DR rats through inhibiting MAPK signaling pathway.

Conclusion

Our work revealed that SPRED2 expression was decreased in DR rats and HG-treated HRECs. SPRED2 overexpression repressed EndMT and endothelial damage in HG-treated HRECs by suppressing MAPK activation. Thus, this discovery suggests that SPRED2 may be a novel potential therapeutic target involved in DR progression.

Contribution of authors

Tian Liu designed the study and carried them out, supervised the data collection, analyzed the data, and interpreted the data. Jing Zhao and Chengmin Lin prepared the manuscript

for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval

Ethical approval was obtained from the Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine (Approval No. 2022-L044).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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