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Research article

SIPA1 promotes angiogenesis by regulating VEGF secretion in Müller cells through STAT3 activation

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ABSTRACT

Diabetic retinopathy (DR) is a prevalent complication of diabetes that can lead to vision loss. The chronic hyperglycemia associated with DR results in damage to the retinal microvasculature. Müller cells, as a kind of macroglia, play a crucial role in regulating the retinal vascular microenvironment. The objective of this study was to investigate the role of signal-induced proliferation-associated protein 1 (SIPA1) in regulating angiogenesis in Müller cells. Through proteomics, database analysis, endothelial cell function tests, and Western blot detection, we observed an upregulation of SIPA1 expression in Müller cells upon high glucose stimulation. SIPA1 expression contributed to VEGF secretion in Müller cells and regulated the mobility of retinal vascular endothelial cells. Further investigation of the dependence of SIPA1 on VEGF secretion revealed that SIPA1 activated the phosphorylation STAT3, leading to its translocation into the nucleus. Overexpression of SIPA1 combined with the STAT3 inhibitor STATTIC demonstrated the regulation of SIPA1 in VEGF expression, dependent on STAT3 activation. These findings suggest that SIPA1 promotes the secretion of pro-angiogenic factors in Müller cells by activating the STAT3 signaling pathway, thereby highlighting SIPA1 as a potential therapeutic target for DR.

1. Introduction

Diabetic retinopathy (DR) is a serious complication of diabetes and continues to be the primary cause of vision loss in working-age individuals globally [1,2]. DR is the most common microvascular complication of diabetes, characterized by histological features such as blood-retinal barrier disruption, retinal hemorrhages, microaneurysms, macular edema, capillary occlusions and neovascularization, etc. [3–5]. Under hyperglycemic conditions, oxidative stress, hemodynamic changes, and inflammatory responses are all involved in the secretion of pro-angiogenic growth factors, especially VEGF, through its phosphorylation of tight junction proteins, increases vascular permeability, resulting in macular edema and promoting angiogenesis. Despite extensive research, the pathophysiology of DR is not fully understood. Currently, the primary approach to clinically treat diabetic retinopathy (DR) involves administering anti-VEGF drugs through intravitreal injections to decrease the abnormal levels of VEGF [6,7]. However, prolonged anti-VEGF treatment can lead to both effectiveness and potential side effects like retinal inflammation [8,9]. Hence, regulating the balance of endogenous VEGF may present a more promising therapeutic strategy.

Müller glial cells, as the main neuroglial cells, migrate from the outer to inner layers, and play a crucial role in providing nutrition and structural stability to the retina, thereby maintaining retinal homeostasis [10]. Damage to Müller cells in a hyperglycemic

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environment will inevitably disrupt the integrity of the retinal neurovascular unit [11,12]. Studies have shown that knockout of the VEGF gene in Müller cells significantly inhibits ischemia-induced retinal vascular proliferation and vascular leakage, indicating that VEGF secreted by Müller cells is a major proangiogenic factor in the retina [13–15]. Therefore, we can alleviate the progression of DR by inhibiting VEGF secretion in Müller cells.

Signal-Induced Proliferation Associated 1 (SIPA1) gene, was initially cloned in 1995 from a mouse lymphoblastic cell line called LFD 14^{16}. Its expression levels and localization varies across different tissues and cells in the human body [17,18]. SIPA1 has been found to be an important regulatory factor in multiple cancer-related signaling pathways, and SIPA1 has been identified as a negative modulator of cell adhesion [19–21]. Research has demonstrated that SIPA1 can interact with the ITGB1 promoter, promoting its transcriptional activity. This interaction results in phosphorylation alterations in the integrin-mediated FAK/Akt signaling pathway, consequently influencing the adhesion and invasiveness of cancer cell [22]s. Additionally, SIPA1 can regulate the tight junction protein ZO-1 through AF6, and its binding with Brd4 promotes the transition of the cell cycle from the M phase to the G1 phase, potentially regulating cancer development [23]. TCGA data analysis suggests that SIPA1 may regulate angiogenesis, but the specific role is still to be explored.

In this study, we observed a significant increase in SIPA1 expression in Müller cells under high glucose (HG) stimulation, further demonstrating that reducing SIPA1 in Müller cells decreases VEGF secretion via STAT3 activation, thus suppressing retinal vascular endothelial cell mobility. This discovery provides a new pathway for understanding the occurrence and development of angiogenesis and offers a potential novel target for the treatment of DR.

2. Methods

2.1. Animal

All experiments involving mice were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animal in ophthalmic and vision research, and were approved by the Internal Animal Committee Review Board of Chongqing University.

Researchers obtained 6 weeks male mice (C57BL/6J) from Hunan Slaike Jingda Laboratory Animal Co. LTD. We induced type I diabetes in the mice by administering intraperitoneal injections of streptozotocin (STZ) from Sigma-Aldrich over five consecutive days. The dose was 40 μg/g body weight and it was dissolved in a 0.1 mol/L citrate buffer. The control group of mice received injections of citrate buffer only. Diabetes was confirmed by measuring urine glucose levels, with values greater than 300 mg/dL considered indicative of diabetes.

2.2. Cell lines and antibodies

HEK293 cells were acquired from Procell Life Science&Technology Co., Ltd. (CL-0001), while Müller cells were obtained from Professor Jingfa Zhang at the Department of Ophthalmology, Shanghai First People's Hospital. HEK293 cells were cultured in DMEM medium (HG, 25 mM) with 10 % fetal bovine serum (FBS), Müller cells were cultured in DMEM medium (low glucose, 5.5 mM) with 10 % FBS, Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in ECM medium. All cells were maintained at a temperature of 37 °C and 5 % CO_2.

2.3. Gene set enrichment analysis

The Gene Set Enrichment Analysis (GSEA) method in conjunction with the "clusterProfiler" package were performed to run the set enrichment analysis. The analysis involved the utilization of Hallmark MSigDB gene sets as the reference gene sets for the enrichment analysis [24].

2.4. Western blot

To prepare the cells for protein analysis, we used a kit (P0013B, Beyotime) for cell lysis, along with a protease inhibitor cocktail (HY–K0013, MCE). Protein levels were measured using the BCA assay (P0009, Beyotime). The protein samples were separated using SDS-PAGE and transferred onto PVDF membranes (ISEQ00010, EMD Millipore). To eliminate non-specific binding, the PVDF membranes were blocked using 5 % non-fat milk in TBS buffer at room temperature for 30 min. The primary antibodies, anti-SIPA1 (A19867, ABclonal, 1:1000), anti-p-STAT3 (AP0070, ABclonal, 1:1000), anti-STAT3 (60199-1-Ig, Proteintech, 1:1000), anti-VEGF (A12303, ABclonal, 1:1000), anti-Lamin B1 (A16910, ABclonal, 1:1000) and anti-GAPDH (AC001, ABclonal, 1:10000), were applied to the membranes and incubated overnight. After washing with TBST, the membranes were treated with secondary antibodies (HRP Goat Anti-Rabbit IgG, AS014, ABclonal, 1:5000 and Anti-Mouse IgG, AS003, ABclonal, 1:5000) at room temperature for 1 h. The blots were visualized using the Tanon 5200 Multi imager.

2.5. Immunohistochemistry

Mouse eyes were fixed in 4 % paraformaldehyde and snap-frozen in OCT. 8-μm cryosections were placed on adhesion microscope slides, then washed in PBS and blocked in 5 % normal goat serum, 2 % BSA and 0.3 % Triton X-100 in PBS for 30 min at RT followed by

incubating with the following primary antibodies: anti-SIPA1 (1:200) overnight at 4 °C. After incubation with the appropriate biotin-conjugated secondary antibody, the sections were subsequently incubated with streptavidin solution (Beyotime) and DAB substrate kit (Beyotime). Imaging of the sections were performed using a microscope (Olympus BX53) and processed with ImageJ.

2.6. CCK8 assay

Müller cells were planted in 96-well plates at a density of 5×10^3 cells per well, with or without SIPA1 inhibition. Following a 48-h incubation period, 10 μL of CCK8 solution was introduced to each well. The plates were then incubated for an additional 2 h, and the absorbance at 450 nm was quantified using a microplate reader.

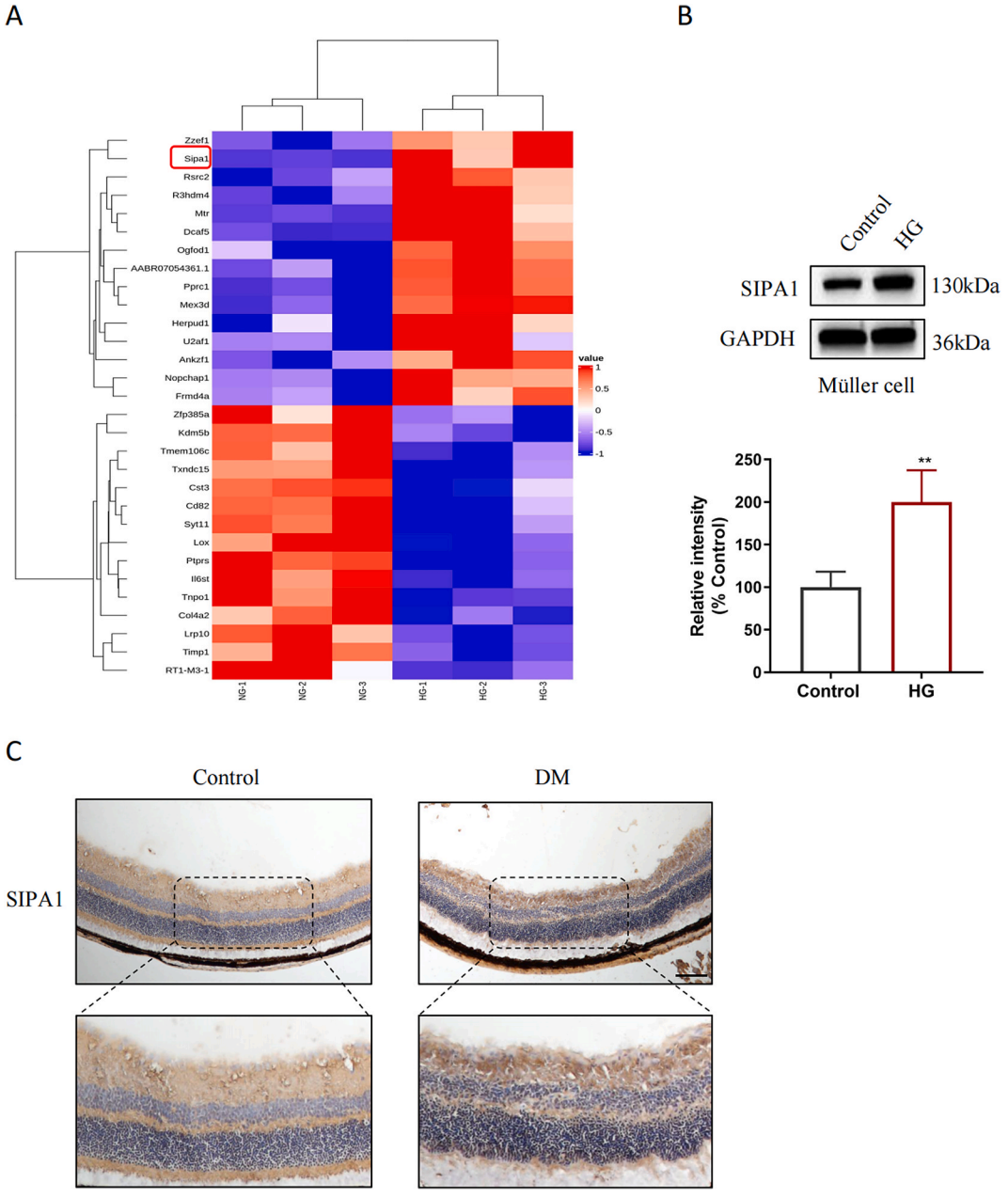

Fig. 1. SIPA1 is highly expressed in HG-induced Müller cells. (A) Proteomics changes from normal to HG-induced Müller cells. The Proteomics abundance was normalized by cell number. n = 3 per group. (B) Protein expression levels of SIPA1 were examined in HG-induced Müller cells. (C) Retinal SIPA1 immunohistochemical staining of 6 months diabetic mice. Scale bar: 100 μm $^{**}P < 0.01$, statistical analysis was performed with unpaired *Student's t*-test.

2.7. Immunofluorescence assay

Müller cells, both with and without SIPA1 inhibition, were cultured on coverslips and subsequently fixed with a 4 % PFA solution. The cells were then incubated overnight at a temperature of 4 °C with the specified primary antibodies at the indicated dilutions (anti-SIPA1, 1:200; anti-STAT3, 1:200). Following the primary antibody incubation, the cells were treated with appropriate secondary antibodies and incubated for 1 h. Subsequently, the cell nuclei were stained with DAPI for 15 min at room temperature. The imaging of the cells was conducted using a laser scanning microscope (Olympus BX53).

2.8. Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF (EK5870, SAB Signalway Antibody) and ANGPTL-4 (EM0721, Fine Test) secreted from the cells were quantified

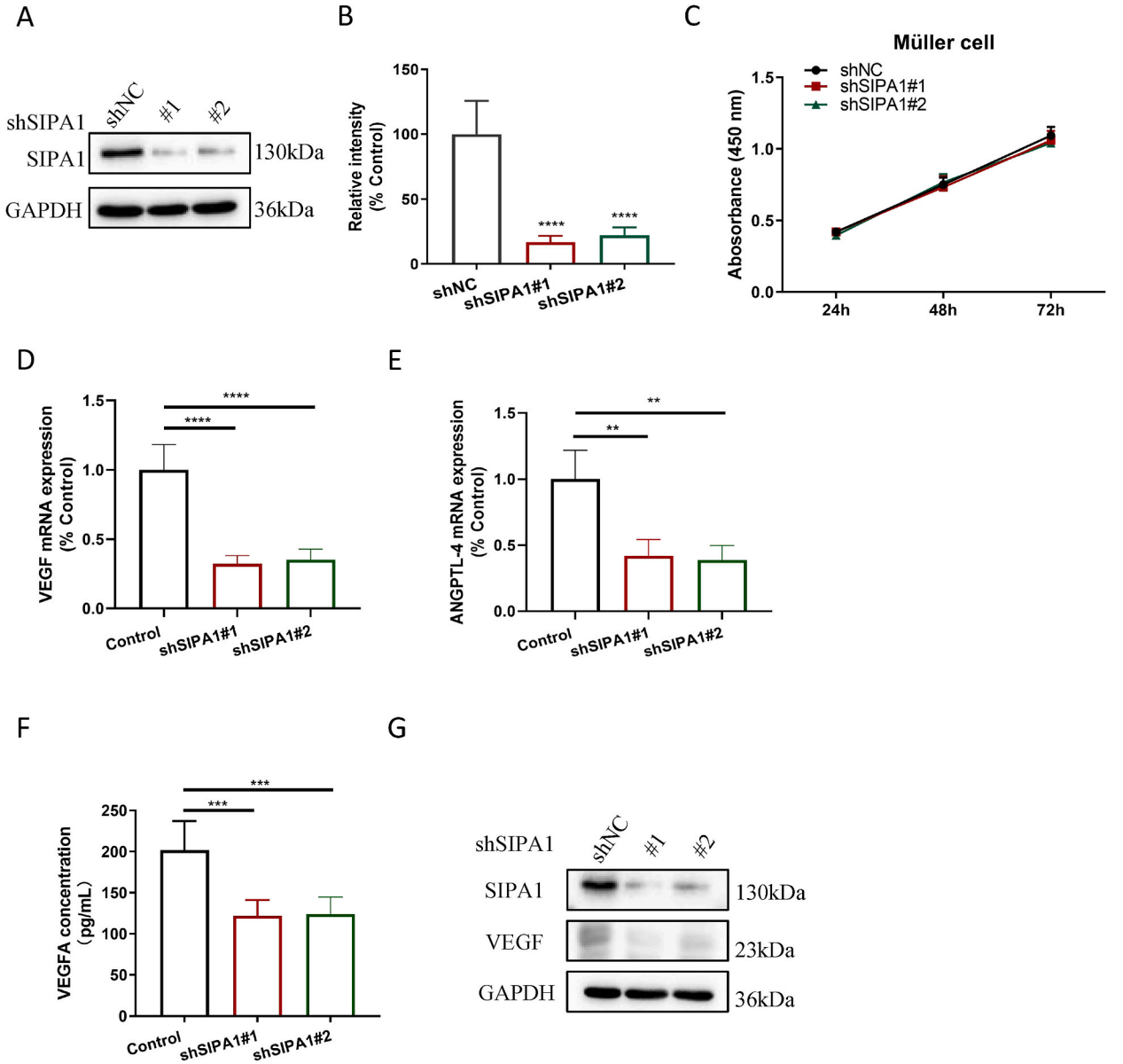

Fig. 2. SIPA1 knockdown inhibits expression of pro-angiogenic factors. (A, B) Cells were transfected with specific shRNA, and SIPA1 protein expression was analyzed using antibodies. (C) CCK8 assay assessed cell proliferation in shCON, shSIPA1#1, and shSIPA1#2 groups of Müller cells. (D, E) Müller cells were transfected with specific SIPA1 shRNA, the transcription of VEGF and ANGPTL-4 were detected by qPCR. (F, G) Protein expression was analyzed by ELISA and Western blot. n = 6 per group. $^{**}p < 0.01$; $^{***}p < 0.0005$; $^{****}p < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

using ELISA kits following the provided protocols and procedures.

2.9. Quantitative real-time PCR (RT-qPCR)

For extraction of total RNA, the RNA Easy Fast Tissue/Cell kit (DP451, TIANGEN) was utilized. Subsequently, cDNA synthesis was performed using the ReverAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific). The synthesized cDNA was combined with Genious 2X SYBR Green Fast qPCR Mix (RK21206, Abclonal Technology) and specific primers (Supplementary Table S1). The ABI 7300 QuantStudio3 PCR (RT-PCR) System was employed to quantify the mRNA levels of the target genes.

2.10. Statistical analyses

Prism V.9.0 (GraphPad Software, Inc.) was utilized for conducting the statistical analyses. The data are presented as mean ± standard deviation (SD), unless specifically indicated otherwise. Depending on the experimental design, either a *t*-test, one-way ANOVA, or two-way ANOVA was employed for data processing, as appropriate. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

3. Results

3.1. SIPA1 is highly expressed in Müller cells in diabetic retinopathy

Untargeted Proteomic analysis indicated a protein profile change in multiple Müller cell lines treated with HG (25 mM) versus

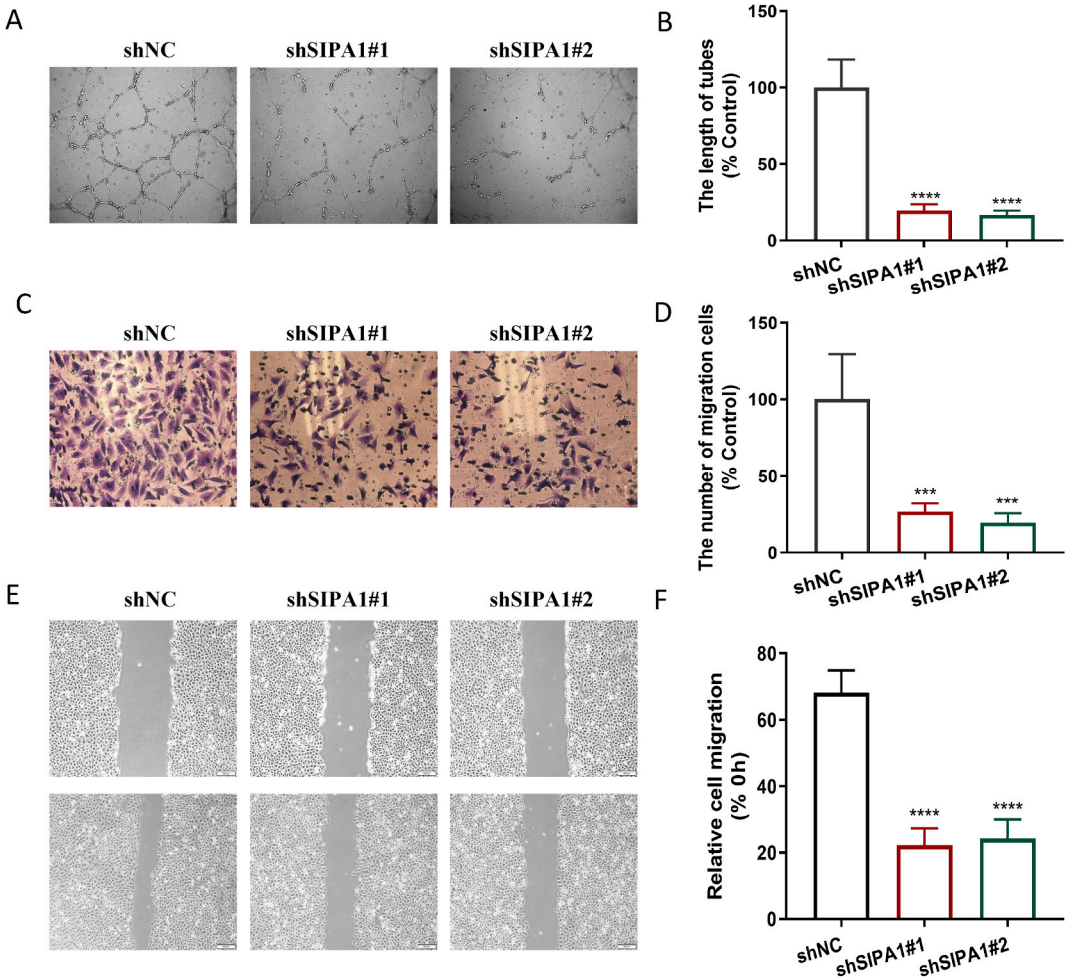

Fig. 3. SIPA1 regulates the mobility of retinal endothelial cells. (A, F) Conditioned media from Müller cells regulate vascular endothelial cell tube formation and migration ability. n = 6 per group. Scale bar: 200 μm $***p < 0.0005$; $****p < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

parental cell lines (Fig. 1A). The results showed that SIPA1 was significantly increased in HG induced Müller cells. SIPA1 expression in HG induced Müller cells was further confirmed by Western blot (Fig. 1B). In addition, we detected that the expression of SIPA1 in the retina of 6-month-diabetes mice after STZ induction, SIPA1 expression was significantly increased from the ganglion layer to the outer nuclear cell layer in the diabetic retina (Fig. 1C), which was consistent with the proteomics results. The findings of the study indicated a significant increase in SIPA1 expression in Müller cells when exposed to hyperglycemic levels.

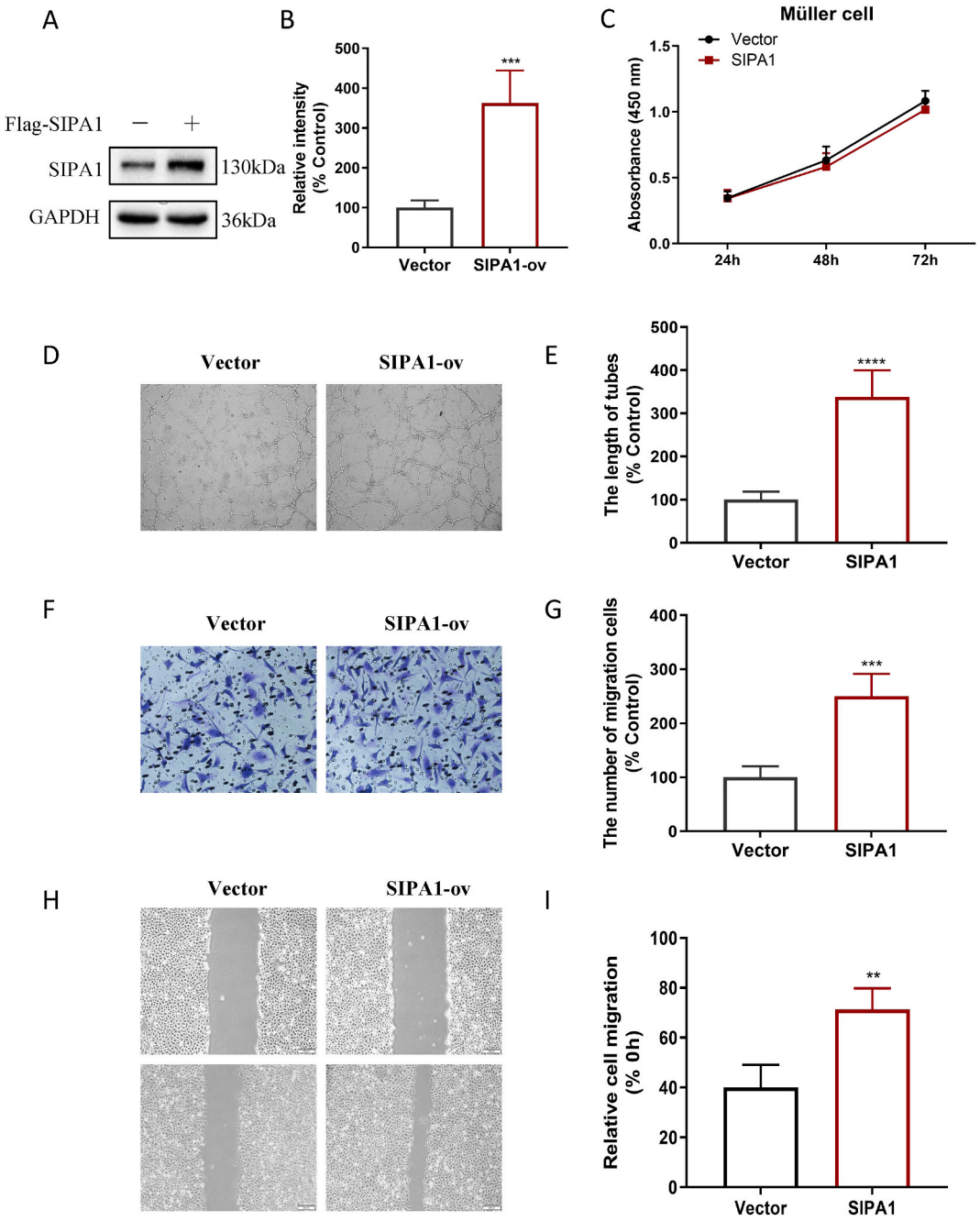

Fig. 4. SIPA1 over-express promotes expression of pro-angiogenic factors and the mobility of retinal endothelial cells. (A, B) Cells were transfected with SIPA1 over-express vector, and SIPA1 protein expression was analyzed using antibodies. (C) CCK8 assay assessed cell proliferation in vector and SIPA1 over-express groups of Müller cells. (D, I) Conditioned media from Müller cells regulate vascular endothelial cell tube formation and migration ability. n = 6 per group. Scale bar: 200 μm $^{**}p < 0.01$; $^{***}p < 0.0005$; $^{****}p < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

3.2. SIPA1 promotes the secretion of pro-angiogenic growth factors

Now that, we have convinced that SIPA1 was upregulated in HG-induced Müller cells. However, the role of SIPA1 in Müller cells has not been extensively studied thus far. To gain insights into its potential function, we conducted a GSEA using publicly available datasets. Interestingly, our analysis revealed a positive correlation between the upregulation of SIPA1 and angiogenesis, as shown in Supplementary Fig. 1. To gain a deeper understanding of SIPA1's involvement in angiogenesis regulation, We first constructed SIPA1 knockdown shRNA. The results showed a significant reduction in SIPA1 expression (Fig. 2A and B). Meanwhile, CCK-8 experiments indicated that the knockdown of SIPA1 did not affect the status of Müller cells (Fig. 2C). Then we examined the mRNA expression levels of two angiogenic factors, VEGFA and Angiopoietin-like protein 4 (ANGPTL-4) in the presence of SIPA1 intervention. Surprisingly, the loss of SIPA1 was found to inhibit the expression of VEGFA and ANGPTL-4 (Fig. 2D and E). ELISA analysis also revealed that SIPA1 gene knockdown reduced the secretion level of VEGFA (Fig. 2F). Western blot results demonstrated a decrease in VEGFA protein expression with SIPA1 intervention (Fig. 2G). This implies that SIPA1 holds significant importance in modulating the release of factors that promote angiogenesis.

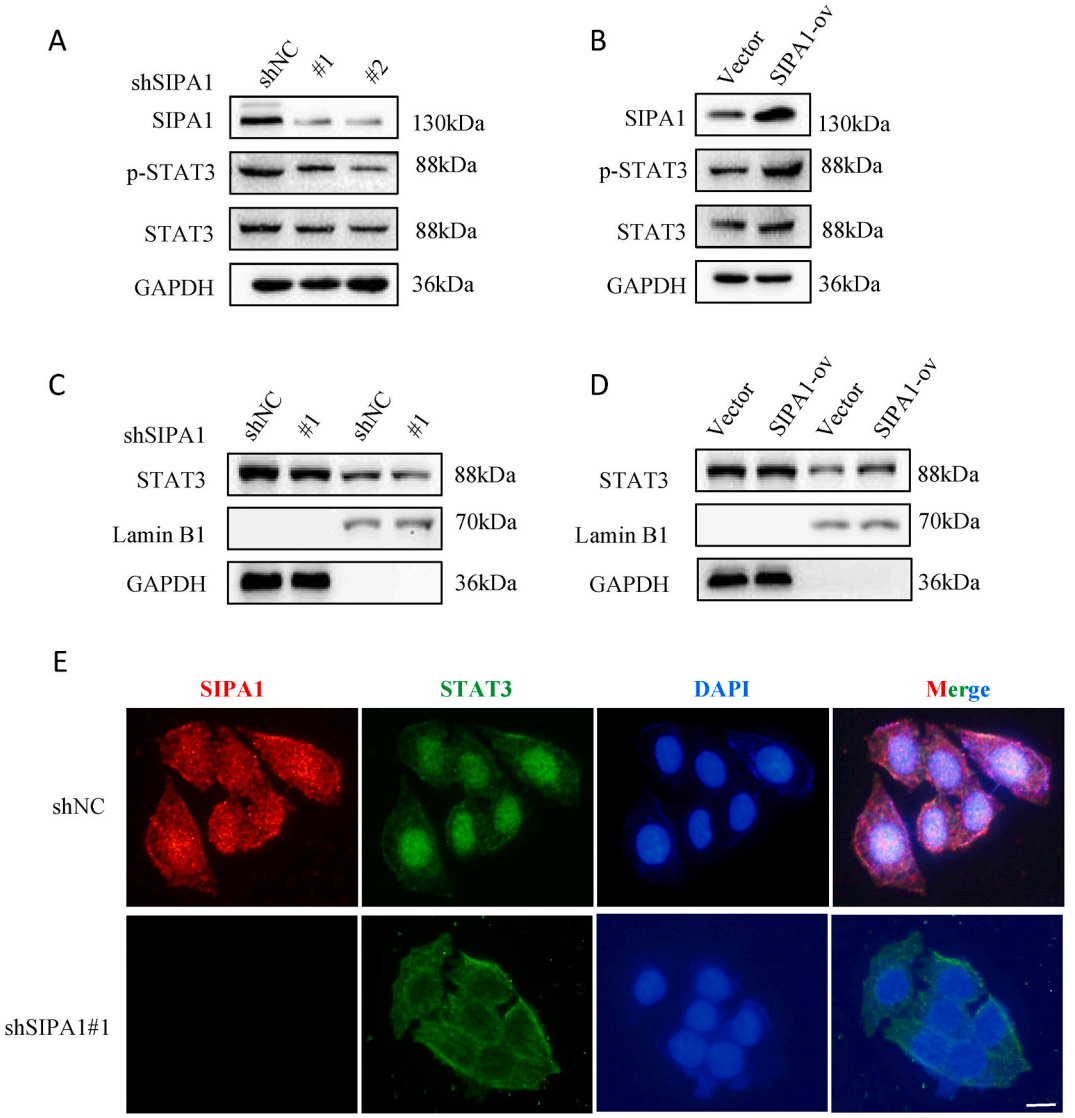

Fig. 5. SIPA1 influences the activation of the STAT3 signaling pathway. (A, B) Western blotting was used to detect changes in STAT3 phosphorylation upon SIPA1 intervention in Müller cells. (C, D) Nuclear and cytoplasmic proteins were separated and analyzed by western blotting after SIPA1 intervention in Müller cells. (E) An immunofluorescence assay was conducted to assess changes in the nuclear localization of STAT3 in response to SIPA1 intervention. Scale bar: 20 μm.

3.3. Inhibition of SIPA1 in Müller cells reduces the mobility of endothelial cells

Numerous research studies have provided evidence that pro-angiogenic growth factors originating from Müller cells play a significant role in the pathological alterations observed in retinal microvessels. Considering this, we employed retinal vascular endothelial cells to further evaluate the function of pro-angiogenic growth factors that are regulated by SIPA1. We collected conditioned medium from SIPA1 knockdown Müller cells and performed tube formation assays, migration assays, and HUVECs *trans*-well assays. The experimental results showed that conditioned medium from SIPA1 knockdown Müller cells significantly inhibited the formation of endothelial cell tubes and suppressed the migration ability of endothelial cells (Fig. 3A–F). In contrast, when we overexpressed SIPA1 in Müller cells, the proliferation of Müller cells remained unaffected (Fig. 4A–C). Moreover, conditioned medium from Müller cells overexpressing SIPA1 promoted endothelial cell angiogenesis and migration ability (Fig. 4D–I). These experiments aimed to shed light on the specific function of SIPA1 in modulating the release of pro-angiogenic factors and its potential impact on retinal vascular dynamics.

3.4. The activation of the STAT3 signaling pathway is modulated by SIPA1

To further explore how SIPA1 affects angiogenesis signaling pathway, GSEA analysis performed on TCGA database showed a significant correlation between SIPA1 and the IL-6/STAT3 pathway (Supplementary Fig. 2). Therefore, we hypothesized that SIPA1 activates the STAT3 signaling pathway, leading to the upregulation of VEGFA expression. To test this hypothesis, we conducted immunoblotting experiments, and the results revealed that inhibiting SIPA1 led to a reduction in STAT3 phosphorylation, while overexpressing SIPA1 resulted in increased STAT3 phosphorylation (Fig. 5A and B). The extent of STAT3 nuclear translocation reflects its functional state. Through cellular fractionation and immunoblotting analysis of nuclear and cytoplasmic proteins, we observed that SIPA1 promoted the nuclear translocation of STAT3 (Fig. 5C and D). Consistently, immunofluorescence analysis demonstrated a decrease in nuclear accumulation of STAT3 with SIPA1 intervention (Fig. 5E). These findings support SIPA1 affects the activation of the STAT3 signaling pathway.

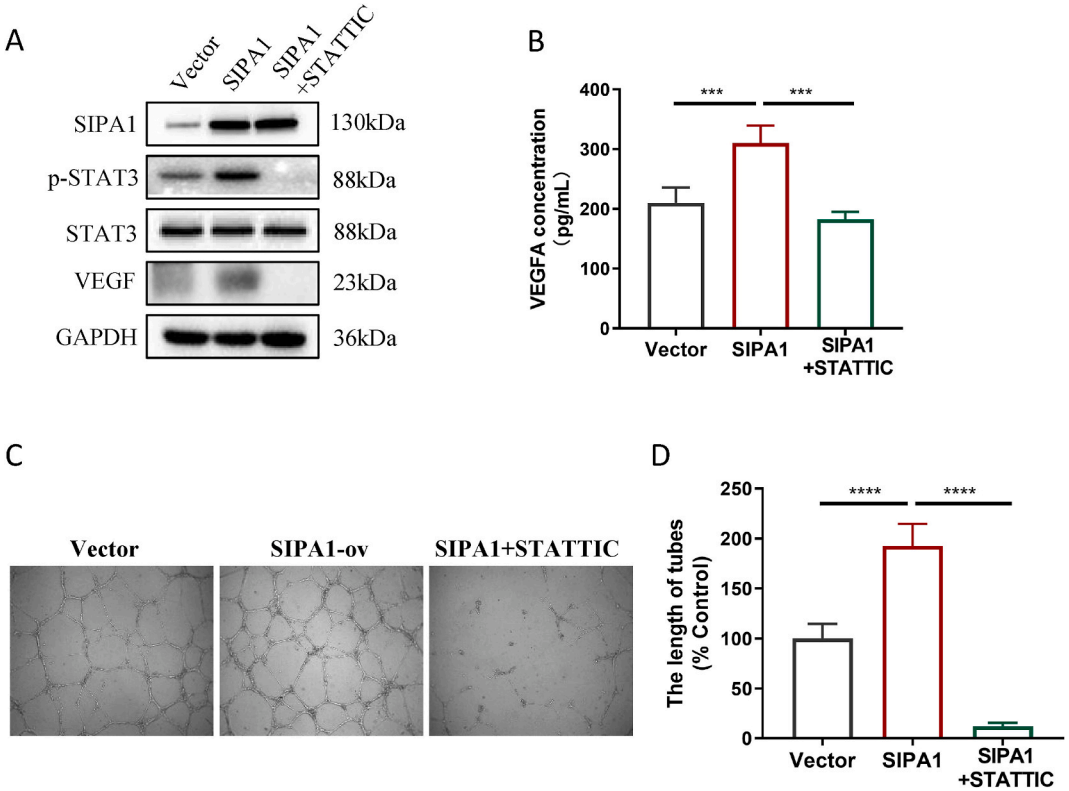

Fig. 6. The function of SIPA1 on VEGF secretion is mediated by STAT3 signaling pathway. (A, B) SIPA1 promotes VEGF expression through the activation of STAT3, and is abolished by the specific STAT3 inhibitor, STATTIC. (C, D) The tube formation assay demonstrated that SIPA1 influenced the function of endothelial cells, which were reversed upon treatment with STATTIC. n = 6 per group. *$p < 0.05$; **$p < 0.01$, ***$p < 0.0005$; ****$p < 0.001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

3.5. The angiogenic role of SIPA1 is facilitated through the mediation of the STAT3 signaling pathway

This study establishes the pivotal role of the STAT3 signaling pathway in regulating VEGFA secretion and angiogenesis. We further demonstrate that SIPA1 enhances angiogenesis, promotes VEGFA expression, and activates STAT3. To investigate whether the function of SIPA1 is reliant on the STAT3 pathway, we conducted a Western blot analysis. Our results reveal that the increased phosphorylation of STAT3 and upregulation of VEGFA induced by SIPA1 were attenuated by STATTIC, a known effective STAT3 inhibitor (Fig. 6A). Additionally, as STAT3 is a crucial transcription factor, it is essential to determine the impact of SIPA1 on mRNA expression levels of VEGFA. Real-time PCR results indicated that SIPA1 upregulates VEGFA expression. However, when STAT3 signaling was blocked using a STAT3 inhibitor, the upregulation of VEGFA was reversed (Fig. 6B). Using tube formation assay, we further demonstrated that SIPA1 regulated the tube formation of retinal vascular endothelial cells dependent on the phosphorylation of STAT3 (Fig. 6C and D). These results suggest that the function of SIPA1 to regulate the mobility of retinal vascular endothelial cells by regulating pro-angiogenic factors in a STAT3-dependent manner.

4. Discussion

In this study, we discovered that SIPA1 is expressed at high levels in Müller cells induced by hyperglycemia, which in turn regulates the mobility of vascular endothelial cells. The underlying mechanism is the upregulation of pro-angiogenic factors through activation of STAT3. Elucidating the SIPA1-mediated VEGF regulation mechanism could potentially lead to the development of novel therapeutic approaches for treating DR.

SIPA1, discovered in 1995, has been extensively researched for nearly three decades. Initially, it was believed that SIPA1 primarily functions as a specific GAP (GTPase-activating protein) targeting Ras-related proteins like Rap1, Rap2, Rsr1, and nuclear Ran [25]. However, recent studies have challenged this notion, indicating that SIPA1 does not act as a GAP for Ran or other small GTPases [26]. In the context of tumors and tumor metastasis, the majority of studies on SIPA1 have focused on breast cancer [16,21,27]. Furthermore, elevated SIPA1 expression has been correlated with poor prognosis and tumor metastasis in other types of cancer, including head and neck cancer [23], colorectal cancer [28], and cervical cancer [20]. Several studies have shown interactions between SIPA1 and various molecules involved in cell growth and division. These interactions offer a potential model to explore the migration process of endothelial cells [23]. Proteomic analysis of HG-stimulated Müller cells has revealed a significant increase in SIPA1 expression, indicating a potential role of SIPA1 in HG-stimulated Müller cells.

A large number of studies have shown a close relationship between SIPA1 and tumor invasion and metastasis [22,23,29]. However, so far, there has been no research exploring the role of SIPA1 in tumor vascularization. GSEA enrichment analysis of differentially expressed genes induced by SIPA1 using the TCGA public database suggests a positive correlation between SIPA1 and angiogenesis. Aberrant expression of VEGF is involved in the development of angiogenesis-dependent diseases, including cancer and retinal diseases [8,30,31]. Müller cells are the primary cells in the retina that secrete VEGF [13]. Our results are consistent with other studies, showing a significant increase in VEGF expression in Müller cells stimulated with HG. Concurrently, the expression of other pro-angiogenic factor ANGPTL-4 also increases [12,15]. By using shSIPA1 RNA, we also found a positive correlation between SIPA1 expression and VEGF secretion in HG-stimulated Müller cells. Real-time PCR results further suggest that SIPA1 regulates VEGF transcription. Furthermore, by constructing stable SIPA1 shRNA and high-expression SIPA1 Müller cell lines, which demonstrated that SIPA1 regulates the migration ability of Müller cells in angiogenesis and endothelial cells. Our findings also provide an additional explanation for SIPA1's regulation of tumor invasion and metastasis, suggesting that SIPA1 may promote tumor invasion and metastasis by regulating angiogenesis.

The transcription factor STAT3 plays an important role in angiogenesis [32,33]. GSEA enrichment analysis using public databases indicates a strong correlation between SIPA1 expression and activation of the STAT3 signaling pathway. Increasing evidence suggests that STAT3, to a large extent, is involved in vascular regulation through the modulation of VEGF expression [30]. For example, studies have shown that in the neovascularization process of blinding eye diseases, choroidal neovascularization (CNV) is typically accompanied by STAT3 activation in choroidal endothelial cells and macrophages [34]. Selective inhibition of STAT3 phosphorylation can be achieved by blocking the IL-6 receptor [35]. Similarly, pharmacological blockade of STAT3 inhibits CNV, suggesting that STAT3 plays a critical role as an intracellular signal induced by IL-6 in CNV generation [36]. Therefore, regulation of STAT3 activation is essential for conditional pathological neovascularization.

Our Western blot assay results revealed that SIPA1 promotes STAT3 activation, as evidenced by increased STAT3 phosphorylation. To further elucidate the dependence of SIPA1 function on the STAT3 pathway, we conducted STAT3 knockdown experiments. Remarkably, the effects of SIPA1 on STAT3 activation were reversed and by STATTIC. Further functional experiments on endothelial cells have also demonstrated that SIPA1's regulation of tube formation is dependent on phosphorylated STAT3. These findings suggest that SIPA1 regulates the expression of pro-angiogenic genes through STAT3-mediated transcriptional regulation.

5. Conclusion

In conclusion, our study provides important insights into the role of SIPA1 in HG-Müller cell through the regulation of angiogensis via the STAT3 signaling pathway. SIPA1 emerged as a key regulator of STAT3 activation, highlighting its potential as a therapeutic target in VEGF secretion. Further investigation into the underlying molecular mechanisms and exploration of SIPA1-targeted therapeutic strategies may pave the way for more effective treatments in DR.

Ethics statement

The project was supervised by the Experimental Animal Welfare Ethics Committee of Chongqing University and the ethical review number CQU-IACUC-RE-202305-002.

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Data availability statement

The data used to support findings of the study are available from the corresponding author upon request.

CRediT authorship contribution statement

Yanhong Fang: Writing – original draft, Project administration, Methodology, Investigation. **Qionghua Wang:** Software, Investigation, Data curation. **Lanyue Zhang:** Validation, Formal analysis. **Lin Xie:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24869.

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