Complement factor H inhibits endothelial cell migration through suppression of STAT3 signaling

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Abstract. Complement factor H (CFH), a major soluble inhibitor of complement, is a plasma protein that directly interacts with the endothelium of blood vessels. Mutations in the CFH gene lead to diseases associated with excessive angiogenesis; however, the underlying mechanisms are unknown. The present study aimed to determine the effects of CFH on endothelial cells and to explore the underlying mechanisms. The adenoviral plasmid expressing CFH was transduced into HepG2 cells, and the culture medium supernatant was collected and co-cultured with human umbilical vein endothelial cells (HUVECs). Cell proliferation was measured by CCK8 and MTT assays, and cell migration was measured by wound healing and Transwell assays. Reverse transcription-quantitative PCR was performed to detect gene transcription. Western blotting was used to determine protein levels. The results revealed that CFH can inhibit migration, but not viability, of HUVECs. In addition, CFH did not significantly alter MAPK or TGF-β signaling, whereas it decreased STAT3 phosphorylation in HUVECs. Furthermore, CFH failed to reduce migration of HUVECs, with inhibition of STAT3 signaling by STATTIC or activation of STAT3 signaling by overexpression of STAT3 (Y705D) compromising CFH-inhibited HUVEC migration. CFH also decreased the expression levels of vascular endothelial growth factor receptor 2, a downstream effector of STAT3 mediating endothelial cell migration. In conclusion, the present study suggested that CFH may be a potential therapeutic target for angiogenesis-related diseases.

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Introduction

Angiogenesis refers to the sprouting of capillaries from existing blood vessels (1). During angiogenesis, endothelial cells adopt different phenotypes, which allow them to proliferate and migrate, and to form tube-like structures that eventually result in the generation of new blood vessels (2). Physiological angiogenesis is essential for reproduction and placentation, whereas persistent abnormal angiogenesis drives tumor metastasis, blindness caused by ocular neovascularization and atherosclerotic plaque formation (3,4). The process of angiogenesis is tightly regulated by pro-angiogenic and anti-angiogenic molecules, and is orchestrated by the extracellular microenvironment (5). Multiple intracellular signaling pathways mediate the angiogenic response of endothelial cells, including vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling, TGF-β signaling and STAT signaling (4,6,7). In addition, all of the three major subfamilies of MAPK signaling, ERK, JNK and p38 MAPK, are involved in endothelial cell activation (8).

Complement factor H (CFH) is an abundant serum glycoprotein that is constitutively expressed in the liver (9). CFH controls the alternative pathway of complement activation in plasma (10). Mutations in the human CFH gene are associated with age-related macular degeneration characterized by disruption of the retina by excessive angiogenesis (11). Intravitreal injection of human CFH has been shown to suppress the laser-induced choroidal neovascularization in a rat model (12). In addition, $Cfh^{-/-}$ mice have been shown to exhibit a pro-angiogenic phenotype in a Matrigel plug assay and loss of CFH can increase endothelial cell migration (13). An *in vitro* Matrigel tube formation assay showed that an increased number of tubes were formed by endothelial cells co-cultured with ARPE-19 cells transfected with CFH-specific small interfering RNA (14). To date,

the molecular mechanisms underlying the anti-angiogenic effect of CFH remain unclear.

STAT3 belongs to the STAT family of transcription factors (15). Upon activation, STATs are phosphorylated on a tyrosine residue, leading to the formation of homo- or heterodimers, and translocation to the nucleus, where they activate the transcription of target genes (16,17). STAT3 can be phosphorylated on the Y705 site by the activation of JAK (6). Multiple studies have shown that STAT3 signaling mediates tumor angiogenesis (18-20). Membrane progesterone receptor α has been reported to promote endothelial cell migration and tube formation in lung adenocarcinoma through STAT3 signaling (21). Furthermore, STATTIC, a specific inhibitor of STAT3, was shown to reduce radiation-induced migration and invasion in hepatocellular carcinoma cells (22). Knockdown of SETD7, a tumor suppressor, can activate the STAT3 signaling pathway and enhance lung cancer cell migration, whereas STATTIC abrogates the effect of SETD7 on cell migration (23). In addition, STAT3 has been shown to upregulate the transcription of MMP-2, whereas blockade of STAT3 through the expression of a dominant-negative STAT3 significantly suppresses MMP-2 expression in metastatic tumor cells (24).

VEGF refers to a family of growth factors that have potent pro-angiogenic activity (25-27). VEGF family members bind to their tyrosine kinase cell receptors (VEGFRs) on endothelial cells (28). VEGFR2 is considered to have the strongest pro-angiogenic activity (29-31), and has been proven to be a downstream effector of STAT3 signaling (32,33). STAT3 has also been shown to directly bind the promoter of the VEGFR2 gene and activate its transcription (34).

The aim of this study was to evaluate the effect of CFH on HUVEC viability and migration. In addition, the effects of CFH on STAT3, MAPK and TGF-β signaling pathways in HUVECs were measured, as well as the effects of CFH on VEGFR2 expression. This study elucidated the molecular mechanisms underlying CFH-inhibited angiogenesis.

Materials and methods

Cell culture and transduction. HUVECs (cat. no. 8000) were purchased from ScienCell Research Laboratories, Inc. HUVECs were cultured in endothelial cell medium (ECM; ScienCell Research Laboratories, Inc.) supplemented with 5% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 1% endothelial cell growth supplements (ECGS; ScienCell Research Laboratories, Inc.) and 1% penicillin/streptomycin solution. The liver cancer cell line HepG2 (cat. no. CRL-10741) was purchased from American Type Culture Collection. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). Cells underwent STR genotyping. The CFH adenovirus (plasmid GV314 harboring CFH) and control virus (empty GV314 plasmid) expressing enhanced green fluorescent protein were obtained from Shanghai Jikai Gene Chemical Technology Co., Ltd. Adenovirus infection of HepG2 cells was carried out according to the manufacturer's protocol, as previously described (35). After 48 h, the culture medium supernatant and HepG2 cells were harvested and expression of CFH was tested by western blotting and reverse transcription-quantitative PCR (RT-qPCR). The supernatant of HepG2 cells was collected to perform subsequent experiments.

Inhibition and stimulation of STAT3 signaling. The culture medium for CFH adenovirus-infected HepG2 cells (CFH-containing conditioned medium) or non-infected HepG2 cells (control medium) was collected. HUVECs were seeded in 6-well plates at a density of $5x10^5$ cells/well and cultured in CFH-containing conditioned medium or control medium for 24 h at 37˚C. For STATTIC treatment, when cells reached 100% confluence, STATTIC (Apexbio Technology LLC) was added to the media and incubated with cells at different concentrations (0, 2.5, 5, 7.5 and 10 μM). Dimethyl sulfoxide (DMSO; Beijing Solarbio Science & Technology Co., Ltd.) was used as a control. Cells were harvested after 4 h of incubation at 37˚C and western blot analysis was used to detect the effects of STATTIC on phosphorylated (p)-STAT3 and the optimal dose. The optimal dose (7.5 μM) was used to perform the subsequent experiments.

GV230 is a eukaryotic expression plasmid.The full-length (NM_139276) gene of the Y705D-mutant STAT3 was cloned into the GV230 expression plasmid (Shanghai Genechem Co., Ltd.) by using *Xho*I and *Kpn*I restriction sites. The Y705D mutation is a phosphorylation mimicking form of STAT3 (6). For STAT3 (Y705D) transfection, when cells reached 40-90% confluence, Lipofectamine® 3000 (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) and 2.5 μg STAT3 (Y705D) vector were incubated at room temperature for 15 min and then added to each well. Empty GV230 plasmid was used as the control. Transfection was performed according to the Lipofectamine 3000 manufacturer's protocol. The culture medium was changed 6 h after transfection. After 24 h of transfection, subsequent experiments were performed.

Western blot analysis and Coomassie blue staining. The lysates of HepG2 or HUVECs were prepared with RIPA buffer containing 1 mM PMSF (Beyotime Institute of Biotechnology). Protein concentrations were quantified with a BCA protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Protein samples (20 μg) were loaded and separated by SDS-PAGE on a 10% SDS-polyacrylamide gel. The gels were incubated with Coomassie blue staining solution (Beijing Solarbio Science & Technology Co., Ltd.) and then completely destained in a 5% ethanol/10% acetic acid solution until clear bands appeared. For western blotting, proteins were transferred onto PVDF membranes (MilliporeSigma) and blocked with 5% nonfat dry milk buffer for 2 h at room temperature, followed by incubation with primary antibodies against CFH (1:1,000; cat. no. ab118820; Abcam), p-ERK1/2 (1:2,000; cat. no. 4370; Cell Signaling Technology, Inc.), ERK1/2 (1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), p-JNK (1:1,000; cat. no. 4668; Cell Signaling Technology, Inc.), JNK (1:1,000; cat. no. 9252; Cell Signaling Technology, Inc.), p-p38 MAPK (1:1,000; cat. no. 4511; Cell Signaling Technology, Inc.), p38 MAPK (1:1,000; cat. no. 8690; Cell Signaling Technology, Inc.), TGFβ receptor 1 (TGFβR1; 1:1,000; cat. no. ab235578; Abcam), p-Smad2 (1:1,000; cat. no. ab188334; Abcam), Smad2 (1:2,000; cat. no. ab40855; Abcam), p-Smad3 (1:1,000; cat. no. 9520; Cell Signaling Technology, Inc.), Smad3 (1:2,000;

cat. no. 9145; Cell Signaling Technology, Inc.), p-STAT3 (1:1,000; cat. no. 9520; Cell Signaling Technology, Inc.), STAT3 (1:1,000; cat. no. 9139; Cell Signaling Technology, Inc.), VEGFR2 (1:1,000; cat. no. 2479S; Cell Signaling Technology, Inc.) and GAPDH (1:10,000; cat. no. 10494-1-AP; Proteintech Group, Inc.) at 4˚C overnight. Subsequently, the membranes were cultivated with HRP-conjugated secondary antibody (Goat Anti-Rabbit IgG; 1:10,000; cat. no. 111-035-003; and HRP-Goat anti-Mouse IgG; 1:10,000; cat. no. 115-035-003; both from Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 2 h. Immobilon western chemiluminescent HRP substrate (MilliporeSigma) was used to detect chemiluminescence. The blots were visualized using an ECL imaging system and relative protein expression levels were calculated using ImageJ 1.8.0 (National Institutes of Health).

RNA extraction and RT-qPCR. Total RNA was isolated from the HUVECs and HepG2 cells using the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Inc.). cDNA synthesis and amplification were subsequently performed using HiScript®III RT Super Mix (cat no. R323-01; Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. The RT procedure was: 2 min at 42˚C, 15 min at 37˚C, 5 sec at 85˚C and then 4˚C for 30 min. ChamQ Universal SYBR qPCR Master Mix (cat no. Q711-02; Vazyme Biotech Co., Ltd.) was used for qPCR analysis with the following thermocycling conditions: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. GAPDH was used as an internal control and the relative expression levels of the target gene were calculated using the $2^{-\Delta\Delta Cq}$ method (36). The primers were designed as follows: hCFH, forward 5'-GTGAAGTGTTTACCAGTGACAGC-3', reverse 5'-AACCGTACTGCTTGTCCAAA-3'; hVEGFR2, forward 5'-TTAGTGACCAACATGGAGTCGTG-3', reverse 5'-TAGTAAAGCCCTTCTTGCTTTCC-3'; and hGAPDH, forward 5'-TGATGACATCAAGAAGGTGGTGAAG-3', reverse 5'-TCCTTGGAGGCCATGTGGGCCAT-3'.

Cell viability assays. Cell viability was evaluated using the MTT assay kit Beijing Solarbio Science & Technology Co., Ltd.) and the Cell Counting Kit-8 (CCK-8; Apexbio Technology LLC). For the MTT assay, HUVECs were seeded into a 96-well plate at a density of $5x10^4$ cells/well and cultured in CFH-containing conditioned medium (the supernatant of HepG2 cells) for 24 h at 37˚C, after which, 10 μl serum-free medium containing 5 mg/ml MTT solution was added to each well. After 4 h incubation at 37˚C, the supernatant was discarded, and 110 μl DMSO was added. The crystals were sufficiently dissolved and the optical absorbance value was measured at 490 nm using a microplate reader (Multiskan GO; Thermo Fisher Scientific, Inc.). For the CCK-8 assay, HUVECs were incubated with CFH-containing conditioned medium for 24 h at 37˚C, followed by the addition of 10 μl CCK-8 solution to each well and incubation of the plates for 4 h at 37˚C in a humidified incubator. Absorbance was measured at 450 nm using a microplate reader (Multiskan GO; Thermo Fisher Scientific, Inc.).

Wound healing assay. The HUVECs were seeded in 6-well plates at a cell density of $5x10^5$/well and at 37˚C where they reached 95-100% confluence. A 10-μl pipette tip was used to vertically scratch the 6-well plate to create a line across the surface, and the suspended cells were cleaned and removed with PBS. Cells were cultured in CFH-containing conditioned medium in a humidified 5% CO_2 incubator at 37˚C for 24 h. Images were captured at 0 and 24 h under a light microscope (Olympus Corporation). The migrated area was calculated using ImageJ software.

Transwell migration assay. A Transwell chamber (pore size, 8 μm; Corning, Inc.) was used. HUVECs were diluted to $10x10^4$/ml with CFH-containing conditioned medium or control medium, and a 200-μl cell suspension was added to the upper chamber. ECM (600 μl) supplemented with 5% FBS, 1% ECGS and 1% antibiotics was added to the lower chamber. The cells were allowed to migrate for 12 h at 37˚C. Subsequently, they were fixed with 4% formaldehyde solution (1 ml/well) for 10 min at room temperature and washed three times with PBS to remove the formaldehyde solution. They were then stained with 0.1% crystal violet (1 ml/well) for 30 min at room temperature and washed three times with PBS to remove the stain. Finally, migrated HUVECs were counted and images were captured from six random fields under an inverted light microscope.

Statistical analysis. Date analysis was performed using GraphPad Prism 8.0.2 software (Dotmatics). Data are presented as the mean ± standard deviation and all experiments were repeated at least three times. Statistical significance between two groups was assessed using an unpaired Student's t-test. Significance among multiple groups was calculated using one-way ANOVA and Bonferroni's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of CFH in HepG2 cells and collection of conditioned media. CFH is primarily expressed in hepatocytes. To imitate the *in vivo* environment, the culture medium of HepG2 cells transduced with a control vector, which contains HepG2 cell-secreted CFH, was used as the control to mimic the condition in normal blood. In addition, a recombinant adenovirus containing the full-length cDNA sequence of human CFH was constructed and transduced into HepG2 cells to induce overexpression of CFH, thus exogenously increasing the amount of CFH in the conditioned medium. Western blotting was used to detect the protein expression levels of CFH in HepG2 cells. The results revealed that the expression levels of CFH in the CFH adenovirus infection group was significantly higher than those in the control group (Fig. 1A and B). In addition, RT-qPCR results demonstrated that the mRNA expression levels of CFH were significantly elevated in HepG2 cells following CFH adenovirus transduction (Fig. 1C). HepG2 cell culture media were collected and subjected to western blot analysis. As shown in Fig. 1D, CFH protein expression in the conditioned medium of CFH-overexpressing HepG2 cells was markedly elevated.

CFH does not alter the viability of HUVECs but it does inhibit their migration. MTT and CCK-8 assays were applied to detect the viability of HUVECs. Both assays showed no significant difference in the viability of HUVECs between the

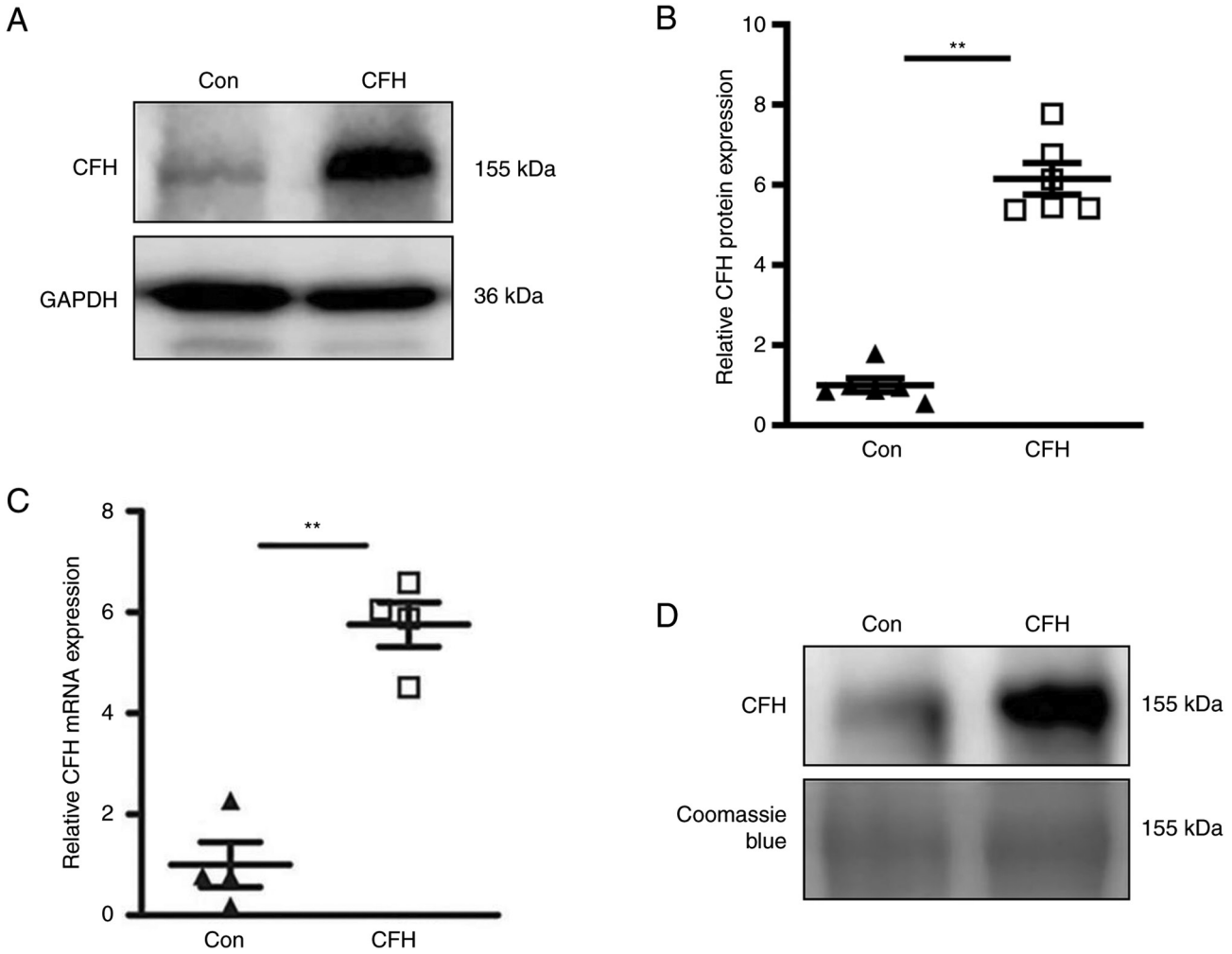

Figure 1. Overexpression of CFH in HepG2 cells and collection of CFH-containing conditioned medium. HepG2 cells were transduced with CFH-expressing adenovirus or control virus. (A) Analysis of protein levels of CFH via western blotting. (B) Relative protein levels of CFH. GAPDH was used as a loading control. (C) Analysis of mRNA levels of CFH via reverse transcription-quantitative PCR. (D) Secreted CFH protein in culture media was detected by western blot analysis. Coomassie blue staining of the gel was used as the loading control. $^{**}P<0.01$ vs. Con group; n=4. Con, control; CFH, complement factor H.

CFH-containing conditioned medium group and the control medium group (Fig. 2A and B). Therefore, CFH may not affect the viability of HUVECs.

Wound healing assay and Transwell migration assay were performed to determine the effect of CFH on the migration of HUVECs. The wound healing assay indicated that the migration of cells treated with the CFH-containing conditioned medium was decreased compared with that in the control group (Fig. 3A and B). Transwell migration assays indicated that the migration of HUVECs cultured in CFH-containing conditioned medium was significantly reduced compared with that in the control group (Fig. 3C and D). These results suggested that CFH inhibits the migration of HUVECs.

CFH does not affect the MAPK and TGF-β signaling pathways in HUVECs. To investigate the mechanisms underlying the effect of CFH on migration, HUVECs were incubated with CFH-containing conditioned medium for 24 h and proteins were extracted for western blotting of the major components of the MAPK and TGF-β pathways, which are involved in the regulation of cell migration (37,38). As shown in Fig. 4A-C, phosphorylation of ERK1/2, JNK and p38 MAPK, the three major members of the MAPK pathway, were not markedly changed in HUVECs treated with CFH-containing conditioned medium. The protein expression levels of TGFβR1 and the phosphorylation of Smad2/3 were also unchanged (Fig. 4D-F).

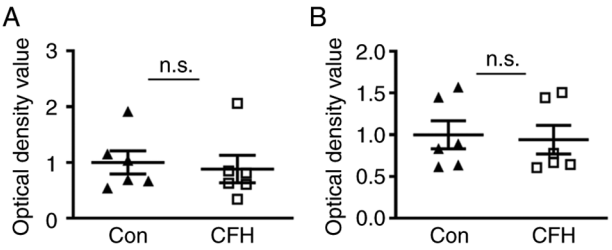

Figure 2. Effect of CFH on the viability of HUVECs. (A) Cell Counting Kit-8 assay of HUVECs incubated with CFH-containing conditioned medium or control medium. Absorbance was measured at 450 nm. (B) MTT assay of HUVECs incubated with CFH-containing conditioned medium or control medium. Absorbance was measured at 490 nm. n=6. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells; n.s., not significant.

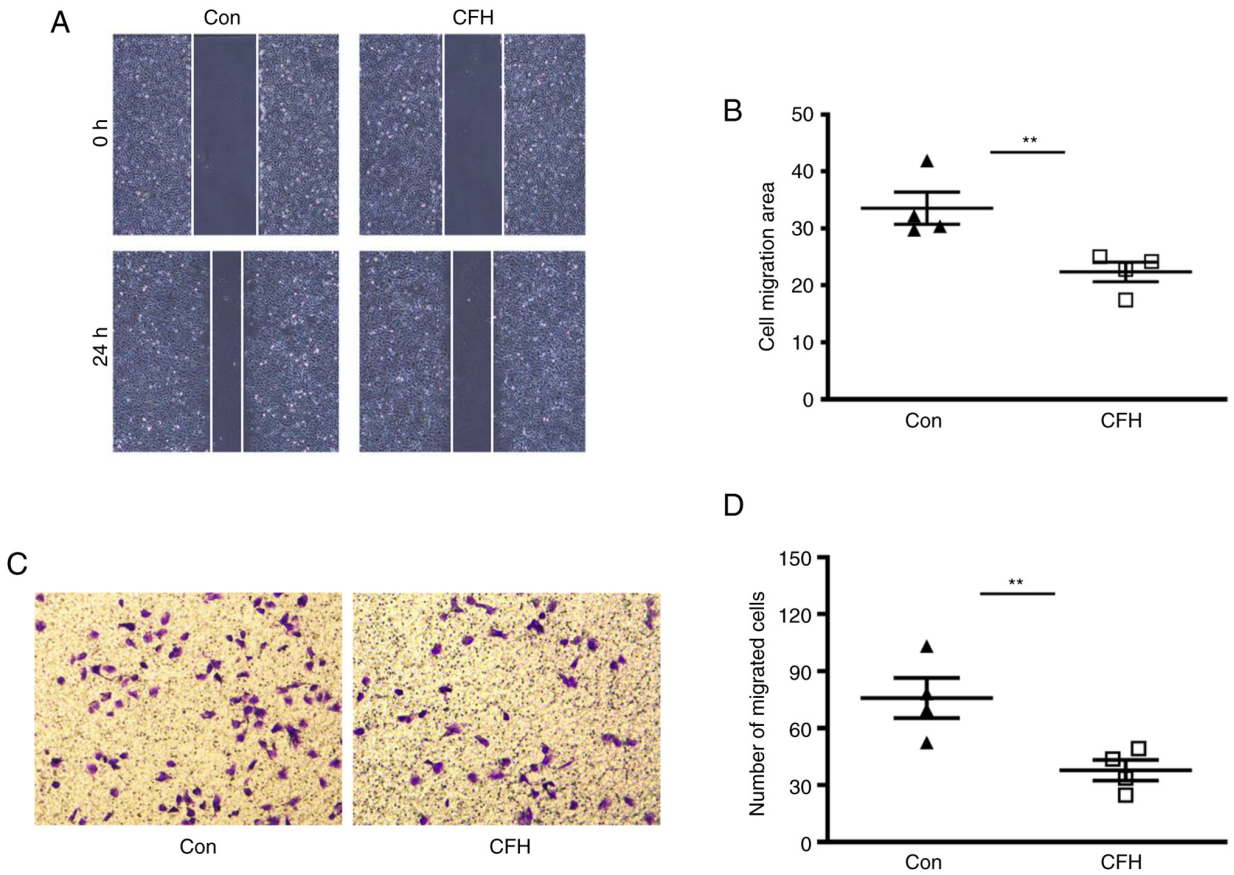

Figure 3. Effect of CFH on the migration of HUVECs. (A and B) Wound healing assay (magnification, x100) of HUVECs treated with CFH-containing conditioned medium or control medium. (C and D) Transwell migration assay (magnification, x400) of HUVECs treated with CFH-containing conditioned medium or control medium. **P<0.01 vs. Con group, n=4. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells.,

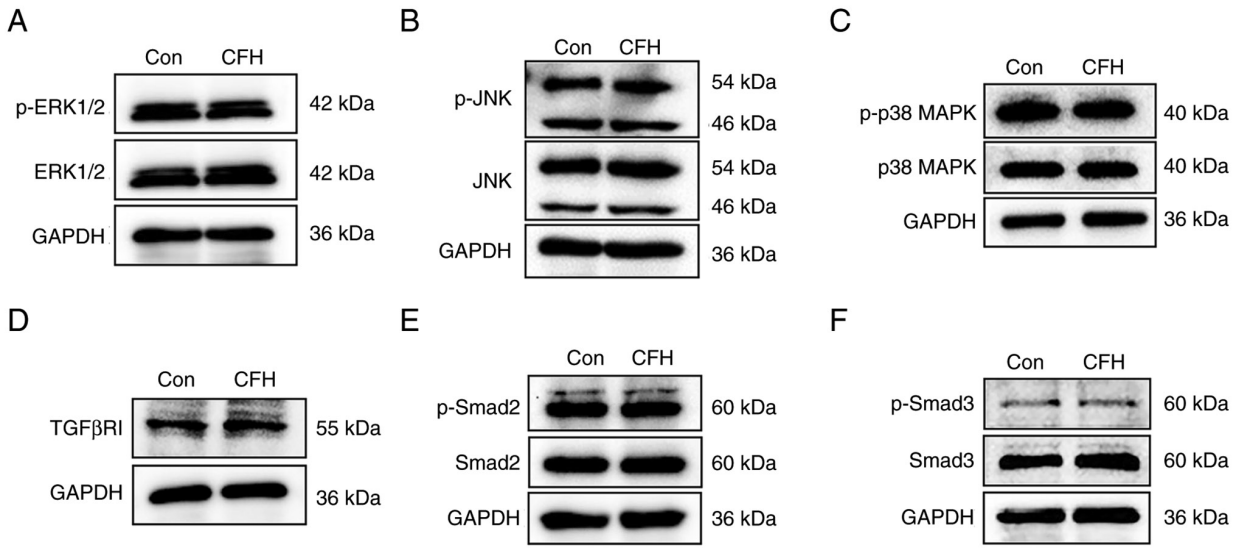

Figure 4. Effect of CFH on cell migration-related signaling pathways in HUVECs. Western blot analysis of proteins extracted from HUVECs incubated with CFH-containing conditioned medium or control medium. (A) ERK1/2 and p-ERK1/2, (B) JNK and p-JNK, (C) p38 MAPK and p-p38 MAPK, (D) TGF-βRI, (E) Smad2 and p-Smad2, and (F) Smad3 and p-Smad3 were detected. The p-ERK and ERK have bands at 42 and 44 kDa. For p-Smad, the band for the small molecule is correct. The other band is non-specific. GAPDH was used as a loading control. n=3. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells; p-, phosphorylated.

CFH inhibits the Y705 phosphorylation of STAT3 in HUVECs. The STAT3 signaling pathway regulates endothelial cell migration (23). After treatment with CFH-containing conditioned medium, the phosphorylation of STAT3 on Y705 was significantly decreased in HUVECs, whereas the total STAT3 protein contents remained unchanged compared with

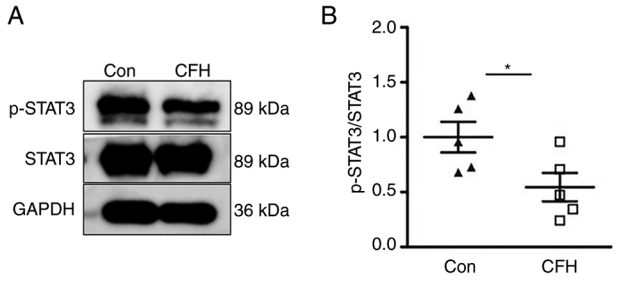

Figure 5. Effect of CFH on the STAT3 signaling pathway in human umbilical vein endothelial cells. HUVECs were incubated with CFH-containing conditioned medium or control medium. (A) Analysis of protein levels of total STAT3 and p-STAT3 via western blotting. GAPDH was used as a loading control. (B) Ratio of p-STAT3 and STAT3 protein levels. *P<0.05 vs. Con group, n=5. Con, control; CFH, complement factor H; p-, phosphorylated.

in the control group (Fig. 5A and B). These results suggested that CFH inhibits the Y705 phosphorylation of STAT3 in HUVECs.

Inhibition of STAT3 signaling compromises CFH-inhibited HUVEC migration. STATTIC is a small molecule that suppresses the physiological binding of tyrosine-phosphorylated peptides to the STAT3 SH2 domain, and reduces STAT3 dimerization and DNA binding (39). To confirm that the migration of HUVECs is dependent on STAT3, STATTIC was used to inhibit STAT3 signaling in HUVECs. As shown in Fig. 6A and B, 7.5 and 10 μM STATTIC completely inhibited STAT3 phosphorylation. After incubation with 7.5 μM STATTIC, the migration of HUVECs cultured in CFH-containing conditioned medium and control medium displayed no significant difference, as determined by wound healing and Transwell migration assays (Fig. 6C-F). These results indicated that CFH does not induce additional inhibition of the migration of HUVECs treated with STATTIC. If CFH inhibited HUVEC migration via other pathways, it would induce additional inhibition of the migration of STATTIC-treated HUVECs. Therefore, these results suggested that CFH decreases HUVEC migration through downregulation of STAT3 signaling.

Activation of STAT3 signaling compromises CFH-inhibited HUVEC migration. Phosphorylation-mimetic (constitutively active) STAT3 (Y705D) has a substitution of tyrosine to aspartate at position 705. The plasmid expressing STAT3 (Y705D) was transfected into HUVECs to mimic activation of STAT3 signaling (Fig. 7A). After transfection, wound healing and Transwell migration assays revealed no significant difference in migration between the CFH-containing conditioned medium group and the control group (Fig. 7B-E). Therefore, activation of STAT3 signaling also eliminated CFH-induced inhibition of HUVEC migration. These findings indicated that CFH inhibits migration of HUVECs in a STAT3-dependent manner.

CFH decreases the expression of VEGFR2. As a downstream target gene of STAT3, the expression of VEGFR2 is upregulated by phosphorylation of STAT3 (34). The present study detected the expression levels of VEGFR2 in HUVECs after incubation with CFH-containing conditioned medium. Western blotting and RT-qPCR demonstrated that the protein and mRNA expression levels of VEGFR2 in HUVECs cultured in CFH-containing conditioned medium were markedly reduced (Fig. 8A and B). Therefore, CFH may decrease the expression of VEGFR2 in HUVECs.

Discussion

Complement factor H has been reported to inhibit angiogenesis (40-42); however, the underlying molecular mechanisms remain unknown. The present study collected CFH-containing conditioned medium from HepG2 cells transduced with CFH-expressing adenovirus, and used this to culture HUVECs. Exogenous CFH did not affect viability, but it did significantly inhibit the migration of HUVECs. Western blotting results suggested that CFH had no significant effect on the MAPK and TGF-β signaling pathways, but it did inhibit the Y705 phosphorylation of STAT3 in HUVECs. It was further confirmed that the inhibitory effect of CFH on HUVECs migration was STAT3-dependent, which was verified by STATTIC treatment and STAT3 (Y705D) overexpression. In addition, CFH decreased the mRNA and protein expression levels of VEGFR2.

In the alternative complement pathway, CFH accelerates the decay of C3 and C5 convertases, and is also a cofactor for factor I-mediated inactivation of C3b, resulting in reduction of the generation of C3a and C5a, and the formation of the membrane attack complex (C5b-9) (43). CFH is a plasma protein that functions directly on endothelial cells in the circulatory system. The present findings suggested that exogenous CFH could inhibit HUVEC migration, but not viability. In $Cfh^{-/-}$ mice, no alteration was previously found in the aortic ring assay, but a significant increase in angiogenic vessels was shown in the Matrigel plug assay (13). In addition, plasma from $Cfh^{-/-}$ mice have been shown to significantly increase endothelial cell migration (13). Thus, CFH may directly interact with endothelial cells and inhibit their migration.

Endothelial cell migration is essential for angiogenesis. This dynamic and multistep process is directionally regulated by chemotactic, haptotactic and mechanotactic stimuli, and requires the activation of various signaling pathways (44). It has previously been shown that endothelial cell migration is regulated by a variety of intracellular signaling pathways. Reconstituted HDL-apoE3 can promote endothelial cell migration through ERK1/2 and p38 MAPK (45). In mice, downregulation of CFH expression can lead to increases in the expression levels of VEGF and TGF-β (46). Furthermore, signaling of TGF-β through ALK5 and subsequent Smad2/3 phosphorylation has been reported to lead to inhibition of endothelial cell proliferation and migration (47). The present study demonstrated that CFH-containing conditioned medium did not affect the MAPK and TGF-β signaling pathways; however, it inhibited the STAT3 signaling pathway. To further confirm whether CFH inhibited endothelial cell migration via STAT3 signaling, HUVECs were treated with a STAT3 inhibitor, STATTIC, or were transfected with phospho-mimetic STAT3 (Y705D) to reduce or activate the STAT3 signaling. STATTIC is a nonpeptidic small molecule shown to selectively inhibit the function of the STAT3 SH2 domain regardless of the STAT3 activation state *in vitro* (39). The Y705D mutation

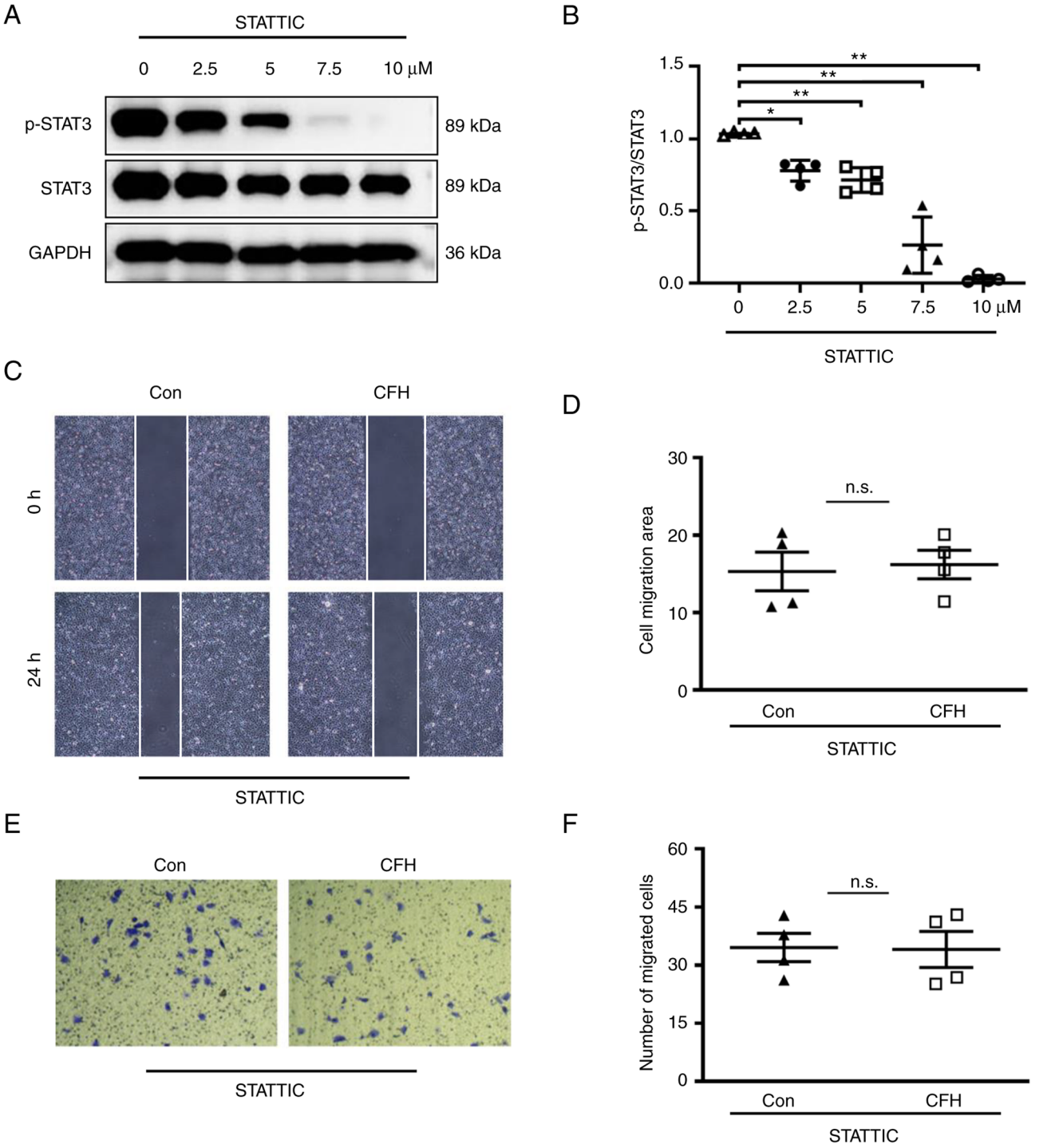

Figure 6. Effect of STATTIC treatment on CFH-regulated HUVEC migration. (A and B) Western blot analysis of total STAT3 and p-STAT3 in HUVECs treated with increasing doses of STATTIC for 4 h. GAPDH was used as a loading control. *P<0.05, **P<0.01 vs. Con group. (C and D) Wound healing assay (magnification, x100) of STATTIC-treated HUVECs incubated with CFH-containing conditioned medium or control medium. n=4. (E and F) Transwell migration assay (magnification, x400) of STATTIC-treated HUVECs incubated with CFH-containing conditioned media or control medium. n=4. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells; n.s., non-significant; p-, phosphorylated.

is a phospho-mimetic form of STAT3 (48). STAT3 is phosphorylated at Y705 within its SH2 domain, dimerizes and translocates into the nucleus to regulate target gene expression; dysfunction of the SH2 domain inhibits STAT3 activity (49). The results revealed that both inhibiting and increasing the phosphorylation of STAT3 abolished CFH-inhibited HUVEC migration. These results indicated that CFH inhibits the migration of endothelial cells in a STAT3-dependent manner. Yahata *et al* (16) also indicated that translocation of STAT3 into the nuclei is essential for triggering human dermal microvascular endothelial cell migration. Thus, the present study confirmed an important mechanism underlying CFH-inhibited endothelial cell migration.

VEGFR is expressed by endothelial cells and mediates angiogenic signaling (29-31). Upon binding with VEGF, VEGFR2 is phosphorylated, which then induces various

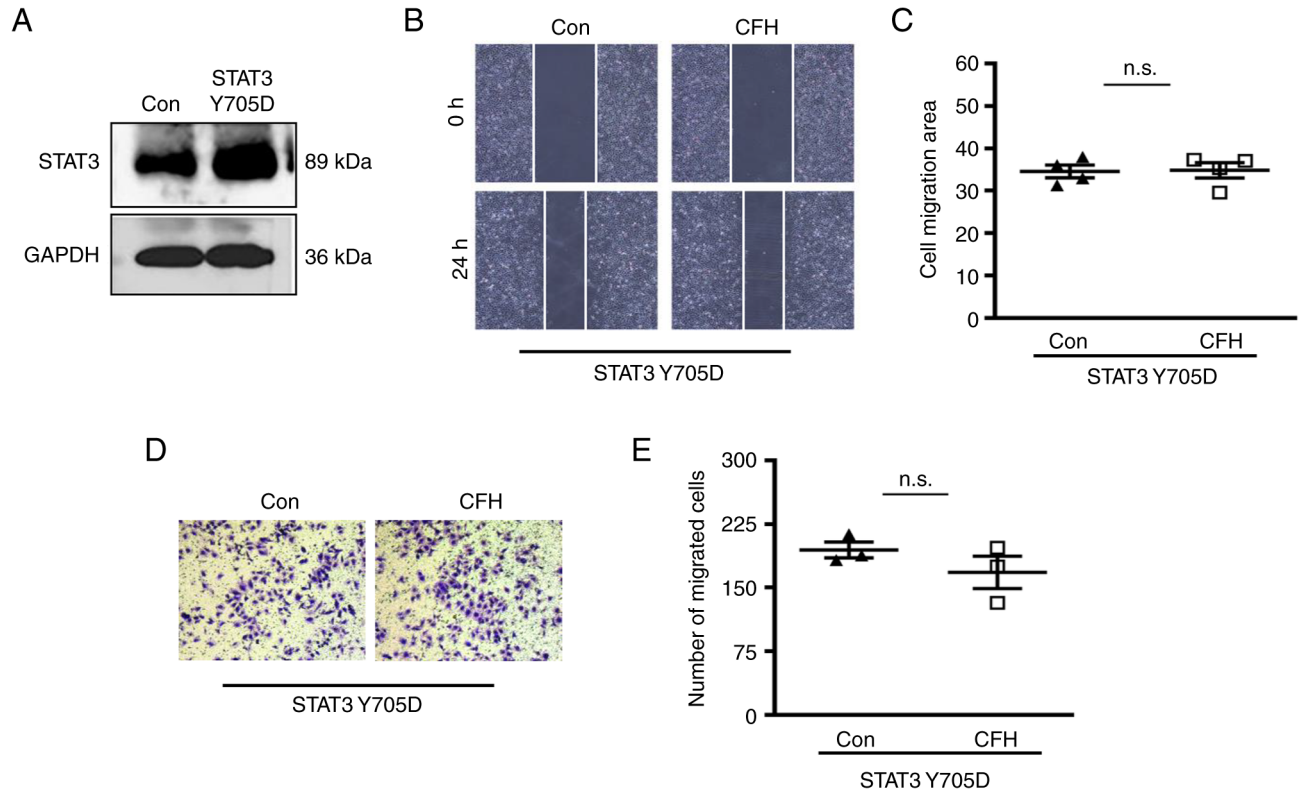

Figure 7. Effect of STAT3 (Y705D) on CFH-regulated HUVEC migration. (A) HUVECs were transfected with control vector or STAT3 (Y705D) vector, and STAT3 protein expression levels were detected by western blot analysis. GAPDH was used as a loading control. n=4. (B and C) Wound healing assay (magnification, x100) of STAT3 (Y705D)-transfected HUVECs incubated with CFH-containing conditioned medium or control medium. n=4. (D and E) Transwell migration assay (magnification, x400) of STAT3 (Y705D)-transfected HUVECs incubated with CFH-containing conditioned medium or control medium. n=3. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells; n.s., non-significant.

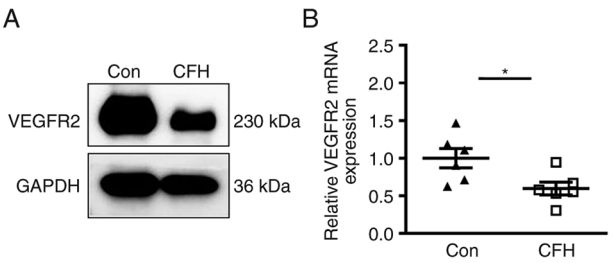

Figure 8. Effect of CFH on VEGFR2 expression in HUVECs. HUVECs were incubated with CFH-containing conditioned medium or control medium. (A) Analysis of protein levels of VEGFR2 via western blotting. GAPDH was used as a loading control. (B) Analysis of mRNA levels of VEGFR2 via reverse transcription-quantitative PCR. *P<0.05 vs. Con group, n=6. Con, control; CFH, complement factor H; HUVECs, human umbilical vein endothelial cells; VEGFR2, vascular endothelial growth factor receptor 2.

downstream angiogenic signaling pathways, including the MEK-ERK pathways, resulting in cell proliferation, survival, migration and increased vascular permeability (34,50). Lee *et al* (34) demonstrated that VEGF induced VEGFR2 expression in brain endothelial cells through STAT3 activation. Although p-STAT3 enters the nucleus, the direct interaction of STAT3 and the human VEGFR2 promoter has not been studied (16,17). The present study revealed that CFH decreases the expression of VEGFR2 in HUVECs, but the lack of chromatin immunoprecipitation on the VEGFR2 promoter is a limitation of this study. Collectively, the present results indicated that CFH may reduce VEGFR2 expression through inhibition of the phosphorylation of STAT3, and subsequently decrease endothelial cell migration.

In conclusion, the present study determined that CFH suppresses endothelial cell migration via inhibition of the STAT3 signaling pathway. As a plasma protein, CFH may be targeted directly through the blood circulation, thus it could be considered a new therapeutic target for anti-angiogenic therapy. Future work shall elucidate the detailed molecular mechanisms underlying the effects of CFH on endothelial cells.

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Availability of data and materials

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

Authors' contributions

JiL, HH, SX, MF, XW and JZ contributed to experimental design, acquisition of data and data analysis. KW, SH and AG contributed to the writing and editing of the manuscript, and the analysis and interpretation of the data. JuL contributed to the conception, experimental design, acquisition of data, data analysis, and the writing and editing of the manuscript. JiL and JuL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of the primary human cells in this study was approved by the Ethics Committee of Shandong Provincial Qianfoshan Hospital (approval no. 2017-106; Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Grant ZL, Whitehead L, Wong VH, He Z, Yan RY, Miles AR, Benest AV, Bates DO, Prahst C, Bentley K, *et al*: Blocking endothelial apoptosis revascularizes the retina in a model of ischemic retinopathy. J Clin Invest 130: 4235-4251, 2020.
2. Dahham SS, Tabana Y, Asif M, Ahmed M, Babu D, Hassan LE, Ahamed MBK, Sandai D, Barakat K, Siraki A and Majid AMSA: β-Caryophyllene induces apoptosis and inhibits angiogenesis in colorectal cancer models. Int J Mol Sci 22: 10550, 2021.
3. Unterleuthner D, Neuhold P, Schwarz K, Janker L, Neuditschko B, Nivarthi H, Crncec I, Kramer N, Unger C, Hengstschläger M, *et al*: Cancer-associated fibroblast-derived WNT2 increases tumor angiogenesis in colon cancer. Angiogenesis 23: 159-177, 2020.
4. Wang X, Abraham S, McKenzie JAG, Jeffs N, Swire M, Tripathi VB, Luhmann UFO, Lange CAK, Zhai Z, Arthur HM, *et al*: LRG1 promotes angiogenesis by modulating endothelial TGF-β signalling. Nature 499: 306-311, 2013.
5. Bell CL, Vandenberghe LH, Bell P, Limberis MP, Gao GP, Van Vliet K, Agbandje-McKenna M and Wilson JM: The AAV9 receptor and its modification to improve in vivo lung gene transfer in mice. J Clin Invest 121: 2427-2435, 2011.
6. Gao P, Wang LL, Liu J, Dong F, Song W, Liao L, Wang B, Zhang W, Zhou X, Xie Q, *et al*: Dihydroartemisinin inhibits endothelial cell tube formation by suppression of the STAT3 signaling pathway. Life Sci 242: 117221, 2020.
7. Li L, Dong F, Xu D, Du L, Yan S, Hu H, Lobe CG, Yi F, Kapron CM and Liu J: Short-term, low-dose cadmium exposure induces hyperpermeability in human renal glomerular endothelial cells. J Appl Toxicol 36: 257-265, 2016.
8. Yang X, Song W, Zhang K, Wang Y, Chen F, Chen Y, Huang T, Jiang Y, Wang X and Zhang C: p38 mediates T-2 toxin-induced Leydig cell testosterone synthesis disorder. Ecotoxicol Environ Saf 253: 114695, 2023.
9. Mao X, Zhou L, Tey SK, Ma APY, Yeung CLS, Ng TH, Wong SWK, Liu BHM, Fung YME, Patz EF Jr, *et al*: Tumour extracellular vesicle-derived complement factor H promotes tumorigenesis and metastasis by inhibiting complement-dependent cytotoxicity of tumour cells. J Extracell Vesicles 10: e12031, 2020.
10. Esparza-Gordillo J, Soria JM, Buil A, Almasy L, Blangero J, Fontcuberta J and Rodríguez de Córdoba S: Genetic and environmental factors influencing the human factor H plasma levels. Immunogenetics 56: 77-82, 2004.
11. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, *et al*: Complement factor H polymorphism in age-related macular degeneration. Science 308: 385-389, 2005.
12. Kim SJ, Kim J, Lee J, Cho SY, Kang HJ, Kim KY and Jin DK: Intravitreal human complement factor H in a rat model of laser-induced choroidal neovascularisation. Br J Ophthalmol 97: 367-370, 2013.
13. Liu J and Hoh J: Loss of complement factor H in plasma increases endothelial cell migration. J Cancer 8: 2184-2190, 2017.
14. Zhang Y, Huang Q, Tang M, Zhang J and Fan W: Complement factor H expressed by retinal pigment epithelium cells can suppress neovascularization of human umbilical vein endothelial cells: An in vitro study. PLoS One 10: e0129945, 2015.
15. Hu Z, Han Y, Liu Y, Zhao Z, Ma F, Cui A, Zhang F, Liu Z, Xue Y, Bai J, *et al*: CREBZF as a key regulator of STAT3 pathway in the control of liver regeneration in mice. Hepatology 71: 1421-1436, 2020.
16. Yahata Y, Shirakata Y, Tokumaru S, Yamasaki K, Sayama K, Hanakawa Y, Detmar M and Hashimoto K: Nuclear translocation of phosphorylated STAT3 is essential for vascular endothelial growth factor-induced human dermal microvascular endothelial cell migration and tube formation. J Biol Chem 278: 40026-40031, 2003.
17. Zhang M, Zhou L, Xu Y, Yang M, Xu Y, Komaniecki GP, Kosciuk T, Chen X, Lu X, Zou X, *et al*: A STAT3 palmitoylation cycle promotes T_H17 differentiation and colitis. Nature 586: 434-439, 2020.
18. Shen Y, Wang X, Liu Y, Singhal M, Gürkaşlar C, Valls AF, Lei Y, Hu W, Schermann G, Adler H, *et al*: STAT3-YAP/TAZ signaling in endothelial cells promotes tumor angiogenesis. Sci Signal 14: eabj8393, 2021.
19. Song D, Lan J, Chen Y, Liu A, Wu Q, Zhao C, Feng Y, Wang J, Luo X, Cao Z, *et al*: NSD2 promotes tumor angiogenesis through methylating and activating STAT3 protein. Oncogene 40: 2952-2967, 2021.
20. Huang J, Tang L, Zhao Y and Ding W: TRIM11 promotes tumor angiogenesis via activation of STAT3/VEGFA signaling in lung adenocarcinoma. Am J Cancer Res 9: 2019-2027, 2019.
21. Xia Z, Xiao J, Dai Z and Chen Q: Membrane progesterone receptor α (mPRα) enhances hypoxia-induced vascular endothelial growth factor secretion and angiogenesis in lung adenocarcinoma through STAT3 signaling. J Transl Med 20: 72, 2022.
22. Xu G, Zhu L, Wang Y, Shi Y, Gong A and Wu C: Stattic enhances radiosensitivity and reduces radio-induced migration and invasion in HCC cell lines through an apoptosis pathway. Biomed Res Int 2017: 1832494, 2017.
23. Cao L, Ren Y, Guo X, Wang L, Zhang Q, Li X, Wu X, Meng Z and Xu K: Downregulation of SETD7 promotes migration and invasion of lung cancer cells via JAK2/STAT3 pathway. Int J Mol Med 45: 1616-1626, 2020.
24. Xie TX, Wei D, Liu M, Gao AC, Ali-Osman F, Sawaya R and Huang S: Stat3 activation regulates the expression of matrix metalloproteinase-2 and tumor invasion and metastasis. Oncogene 23: 3550-3560, 2004.
25. Chen J, Wang Y, Wang S, Zhao X, Zhao L and Wang Y: Salvianolic acid B and ferulic acid synergistically promote angiogenesis in HUVECs and zebrafish via regulating VEGF signaling. J Ethnopharmacol 283: 114667, 2022.
26. Porter AM, Klinge CM and Gobin AS: Biomimetic hydrogels with VEGF induce angiogenic processes in both hUVEC and hMEC. Biomacromolecules 12: 242-246, 2011.
27. Cerezo AB, Hornedo-Ortega R, Alvarez-Fernandez MA, Troncoso AM and Garcia-Parrilla MC: Inhibition of VEGF-induced VEGFR-2 activation and HUVEC migration by melatonin and other bioactive indolic compounds. Nutrients 9: 248, 2017.
28. Mac Gabhann F and Popel AS: Dimerization of VEGF receptors and implications for signal transduction: A computational study. Biophys Chem 128: 125-139, 2007.
29. Rezzola S, Di Somma M, Corsini M, Leali D, Ravelli C, Polli VAB, Grillo E, Presta M and Mitola S: VEGFR2 activation mediates the pro-angiogenic activity of BMP4. Angiogenesis 22: 521-533, 2019.
30. Melincovici CS, Boşca AB, Şuşman S, Mărginean M, Mihu C, Istrate M, Moldovan IM, Roman AL and Mihu CM: Vascular endothelial growth factor (VEGF)-key factor in normal and pathological angiogenesis. Rom J Morphol Embryol 59: 455-467, 2018.

31. Nagarkoti S, Kim YM, Ash D, Das A, Vitriol E, Read TA, Youn SW, Sudhahar V, McMenamin M, Hou Y, *et al*: Protein disulfide isomerase A1 as a novel redox sensor in VEGFR2 signaling and angiogenesis. Angiogenesis 26: 77-96, 2023.
32. Zou S, Gao Y and Zhang S: lncRNA HCP5 acts as a ceRNA to regulate EZH2 by sponging miR-138-5p in cutaneous squamous cell carcinoma. Int J Oncol 59: 56, 2021.
33. Zhao M, Hu X, Xu Y, Wu C, Chen J, Ren Y, Kong L, Sun S, Zhang L, Jin R and Zhou X: Targeting of EZH2 inhibits epithelial-mesenchymal transition in head and neck squamous cell carcinoma via regulating the STAT3/VEGFR2 axis. Int J Oncol 55: 1165-1175, 2019.
34. Lee HT, Xue J, Chou PC, Zhou A, Yang P, Conrad CA, Aldape KD, Priebe W, Patterson C, Sawaya R, *et al*: Stat3 orchestrates interaction between endothelial and tumor cells and inhibition of Stat3 suppresses brain metastasis of breast cancer cells. Oncotarget 6: 10016-10029, 2015.
35. Zhang F, Hu G, Chen X, Zhang L, Guo L, Li C, Zhao H, Cui Z, Guo X, Sun F, *et al*: Excessive branched-chain amino acid accumulation restricts mesenchymal stem cell-based therapy efficacy in myocardial infarction. Signal Transduct Target Ther 7: 171, 2022.
36. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
37. Zhu T, Yao Q, Hu X, Chen C, Yao H and Chao J: The role of MCPIP1 in ischemia/reperfusion injury-induced HUVEC migration and apoptosis. Cell Physiol Biochem 37: 577-591, 2015.
38. Chen W, He S and Xiang D: Hypoxia-induced retinal pigment epithelium cell-derived bFGF promotes the migration and angiogenesis of HUVECs through regulating TGF-β1/smad2/3 pathway. Gene 790: 145695, 2021.
39. Schust J, Sperl B, Hollis A, Mayer TU and Berg T: Stattic: A small-molecule inhibitor of STAT3 activation and dimerization. Chem Biol 13: 1235-1242, 2006.
40. Lyzogubov VV, Tytarenko RG, Jha P, Liu J, Bora NS and Bora PS: Role of ocular complement factor H in a murine model of choroidal neovascularization. Am J Pathol 177: 1870-1880, 2010.
41. Rohrer B, Long Q, Coughlin B, Wilson RB, Huang Y, Qiao F, Tang PH, Kunchithapautham K, Gilkeson GS and Tomlinson S: A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. Invest Ophthalmol Vis Sci 50: 3056-3064, 2009.
42. Borras C, Delaunay K, Slaoui Y, Abache T, Jorieux S, Naud MC, Sanharawi ME, Gelize E, Lassiaz P, An N, *et al*: Mechanisms of FH protection against neovascular AMD. Front Immunol 11: 443, 2020.
43. Moore SR, Menon SS, Cortes C and Ferreira VP: Hijacking factor H for complement immune evasion. Front Immunol 12: 602277, 2021.
44. Siamwala JH, Reddy SH, Majumder S, Kolluru GK, Muley A, Sinha S and Chatterjee S: Simulated microgravity perturbs actin polymerization to promote nitric oxide-associated migration in human immortalized Eahy926 cells. Protoplasma 242: 3-12, 2010.
45. Valanti EK, Dalakoura-Karagkouni K, Fotakis P, Vafiadaki E, Mantzoros CS, Chroni A, Zannis V, Kardassis D and Sanoudou D: Reconstituted HDL-apoE3 promotes endothelial cell migration through ID1 and its downstream kinases ERK1/2, AKT and p38 MAPK. Metabolism 127: 154954, 2022.
46. Bora NS, Kaliappan S, Jha P, Xu Q, Sohn JH, Dhaulakhandi DB, Kaplan HJ and Bora PS: Complement activation via alternative pathway is critical in the development of laser-induced choroidal neovascularization: role of factor B and facto H. J Immunol 177: 1872-1878, 2006.
47. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S and ten Dijke P: Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol Cell 12: 817-828, 2003.
48. Min TR, Park HJ, Park MN, Kim B and Park SH: The root bark of Morus alba L. Suppressed the migration of human non-small-cell lung cancer cells through inhibition of epithelial-mesenchymal transition mediated by STAT3 and Src. Int J Mol Sci 20: 2244, 2019.
49. Coleman DR IV, Ren Z, Mandal PK, Cameron AG, Dyer GA, Muranjan S, Campbell M, Chen X and McMurray JS: Investigation of the binding determinants of phosphopeptides targeted to the SRC homology 2 domain of the signal transducer and activator of transcription 3. Development of a high-affinity peptide inhibitor. J Med Chem 48: 6661-6670, 2005.
50. Jin J, Yuan F, Shen MQ, Feng YF and He QL: Vascular endothelial growth factor regulates primate choroid-retinal endothelial cell proliferation and tube formation through PI3K/Akt and MEK/ERK dependent signaling. Mol Cell Biochem 381: 267-272, 2013.