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Research Paper

Loss of Complement Factor H in Plasma Increases Endothelial Cell Migration

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Abstract

Tumor growth depends on angiogenesis, the growth of new blood vessels. Complement factor H (CFH) is a plasma glycoprotein that functions as a regulator of the complement system. The aim of this study is to delineate the role of CFH in angiogenesis. A conditional null allele of the *Cfh* gene was generated in C57BL/6J mice by flanking the exon 3 with *loxP* sites. The $Cfh^{flox/flox}$ mice were crossed with *Rosa26-Cre* mice to obtain the mice homozygotes of *Cfh* deletion ($Cfh^{-/-}$). The $Cfh^{-/-}$ mice were examined by *in vivo* angiogenesis assays. Mouse endothelial cells were treated with media containing 5% of mouse plasma from the wildtype or $Cfh^{-/-}$ mice and assayed for proliferation, viability and migration. The $Cfh^{-/-}$ mice did not display any obvious abnormalities. They demonstrated a pro-angiogenic phenotype in matrigel plug assay, but not in aorta ring assay. *In vitro*, loss of Cfh in plasma does not affect proliferation or viability, but significantly increases migration of mouse endothelial cells. Our findings suggest that plasma CFH inhibits angiogenesis by reduction of endothelial cell migration. Thus the mutation of CFH might lead to excessive tumor angiogenesis.

Key words: complement factor H, angiogenesis, endothelial cell, migration

Introduction

Angiogenesis refers to the formation of new blood vessels from the preexisting vasculature [1]. Since newly-formed capillaries provide nutrients and oxygen to the proliferating tumor cells, angiogenesis is pivotal for maintenance and progression of solid tumors [2, 3]. Tumor cells secret specific growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to stimulate proliferation and migration of the naturally quiescent endothelial cells, leading to the formation of new vessel structures and blood circulation [4]. The process of angiogenesis is regulated by continuous interactions of its stimulators and inhibitors, including growth factors, enzymes, lipids and chemicals [1, 5]. Anti-angiogenesis therapy has recently emerged as an essential adjunct to the chemotherapy of certain cancers [6-8].

The complement system is an enzyme cascade that helps defend against infection and clear pathogens [9]. Three complement pathways, the classical, the alternative, and the mannose-binding lectin pathway, converge into a final common pathway when C3 convertase cleaves C3 into C3a and C3b [10]. C3 cleavage results in formation of the membrane attack complex (MAC), the cytotoxic component of the complement system [9]. Complement proteins were observed in angiogenic vessels [11], but their roles in angiogenesis has not been fully understood. Activation of the complement system is tightly regulated by complement control proteins, which are present at a high concentration in

the blood plasma [12].

Complement factor H (CFH), a serum glycoprotein mainly produced in the liver, is a negative regulator of the alternative pathway of complement activation [13]. Using genome-wide association study (GWAS), we firstly identified that a tyrosine to histidine change at amino acid 402 (Y402H has a T to C substitution at nucleotide 1277 in exon 9) of the *CFH* gene is significantly associated with age-related macular degeneration (AMD) [14]. Later, a number of other studies proved the strong association of the *CFH* polymorphism and AMD [15]. AMD, the leading cause of visual disability in the elderly, is characterized by formation of drusen and neovascularization of the choriocapillaris [16]. However, it is not clear whether the mutation of CFH leads to abnormal angiogenesis. In this study, we generated mice with deletion of the *Cfh* gene and explored the role of CFH on angiogenesis.

Materials and Methods

Animals

The mice with a *Cfh* floxed allele were generated by Ozgene (Bentley, Australia). Briefly, a FRT-flanked PGK-neomycin selection cassette was inserted downstream of exon 3 of the mouse *Cfh* gene. The *loxP* sites were inserted upstream of exon 3 and between exon 4 and the selection cassette. The construct was incorporated into the genomic DNA of embryonic stem (ES) cells from C57BL/6J mice by electroporation following selection. The targeted ES cells were injected into C57BL/6J blastocysts, which were transferred into the uterus of recipient mice to produce chimeric mice. The chimeric mice were crossed to C57BL/6J mice to generate $Cfh^{+/flox\text{-}neo}$ mice, which were crossed to *Rosa26-FlpE* mice (provided by Ozgene) to remove the selection cassette. The $Cfh^{+/flox}$ mice were crossed to *Rosa26-Cre* mice to create a *Cfh* null allele. All the mice were maintained on C57BL/6J background. The probes used for Southern blot analysis of ES cell clones and *Cfh* alleles in mice were summarized in Table 1. Animals were kept in a conventional, pathogen-free facility at the Yale University School of Medicine, and all experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Yale University. Genotyping was performed performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's directions. The following primers were used for PCR genotyping of *Cfh* alleles: sense, 5'-GCACATAAATAGCTGGATGGACCTG-3'; anti-sense, 5'-CCTTATGTAGGAGTCACGAAAGCAC-3'.

Gene expression analysis

The livers were collected from wildtype and $Cfh^{-/-}$ mice after intra-cardiac perfusion with cold PBS and homogenized in TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted by the RNeasy kit with optional DNaseI treatment (Qiagen, Hilden, Germany). cDNA synthesis was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Calsbard, CA) and the newly synthesised cDNA was used as a template for RT-qPCR. The 20 µl PCR reaction mix consisted of 10 µl 2X SYBR Green (ThermoFisher, Grand Island, NY), 1 µl cDNA template, 1 µl forward and reverse primers, and 8 µl deionised water. qRT-PCR was performed using CFX96 Real-Time PCR Detection System (Bio-Rad). The primer sequences were summarized in Table 2. RNA levels were normalized to the endogenous control gene mouse β-actin. All PCR reactions were repeated in triplicate.

Table 1: The primers used in the production the probes for Southern blotting.

Probe	Sequence	Size (bp)	Tm (^{0}C)
5′ probe			
Sense	TGACTGATGGACTACAGGAATGAACAG	510	60
Anti-sense	TTACTGAATATAGCTCACCTGGAATTTTCA		
3′ probe			
Sense	CCTTGAGGTCTTAGGTTTGACACAC	504	61
Anti-sense	CAATGGAAGTAGGACACAGCATTTG		
en probe			
Sense	AGCTCACTGCTGATCTCAAATACTTCAAAG	541	59
Anti-sense	GGAGTGCCTAAGCACAAATTGTAGG		

Note: All sequences are in the 5' to 3' orientation

Table 2: RT-PCR primer sequences

Gene	Sequence	Size (bp)	Tm (^{0}C)
mCFH			
Sense	CTCCTGGTCAGAACAACTATATCCAG	351	61.5
Anti-sense	AGTTCTGTCACAGGTAGACACTTCAC		
mβ-actin			
Sense	CGGTTCCGATGCCCTGAGGCTCTT	100	60
Anti-sense	CGTCACACTTCATGATGGAATTGA		

Note: All sequences are in the 5' to 3' orientation

Western blotting

The blood of wildtype and $Cfh^{-/-}$ mice was collected via the retro-orbital sinus, and the serum was prepared in sample buffer (Bio-Rad) with β-mercaptoethanol. The serum samples were electrophoresed on 7% Tris-HCl gradient gels, and the protein bands were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked overnight with 5% non-fat dry milk (EMD Millipore, Billerica, MA) and then incubated overnight with a sheep anti-Factor

H antibody (1:500; Abcam, Cambridge, MA) in PBS-T at 4°C. After washing, the membranes were probed with horseradish peroxidase (HRP)-conjugated donkey anti-sheep secondary antibody (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA). The blot signals were detected by enhanced chemiluminescence (ThermoFisher) and exposed on X-ray films.

Aortic ring assay

The two-month old WT and *Cfh*$^{-/-}$ mice were euthanized mice by perfusion with 10 ml cold PBS. The aortic ring was excised from mice, cut into 1 mm lengths and implanted in growth factor-reduced matrigel (BD Biosciences, San Jose, CA). The aortic explants were incubated with EBM-2 supplemented with 0.5% FBS for 6 days. Capillaries outgrowing from aortas was photographed with a digital camera linked to a Zeiss invert microscope with an objective lens and analyzed using ImageJ software (NIH, Bethesda, MA). Four rings were used for each assay that was repeated at least 3 times.

Matrigel plug assay

After thawing at 4°C overnight, 0.5 ml matrigel (BD Biosciences) was injected subcutaneously into each of the two-month old WT and *Cfh*$^{-/-}$ mice. At day 10, all the mice were sacrificed, and the matrigel plug was removed and embedded in OCT. The matrigel plugs were sectioned at 7μm and immunostained with a rat anti mouse CD31 antibody (BD Biosciences). The microvascular density count was carried out as previously described [17].

Cell culture

The immortalized mouse brain endothelial cells (bEnd.3) were purchased from ATCC (Manassas, VA) and cultured in basal endothelial cell medium (EBM2) supplemented using the EGM-2-MV bullet kit (Lonza, Basel, Switzerland). The cells were cultured in humidified air at 37°C with 5% CO_2.

Proliferation assays

Cell proliferation was evaluated with a MTT assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, cells were seeded at a density of 5x10^3 cells/well in a 96-well plate. The next day, the culture media was replaced with EBM2 basal media with 5% of mouse plasma from the WT and the *Cfh*$^{-/-}$ mice. After 24 hrs, cells were washed with PBS and MTT solution (10 μl of 5 mg/mL) was added to each well for 2 hrs. Formazan crystals were solubilized and colorimetric intensity was analyzed using a 96-well plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. Each experiment was repeated 4 times.

Cell viability assay

The viability of mouse endothelial cells was assessed after 24 hr incubation of EBM2 basal media with 5% of mouse plasma from the WT and the *Cfh*$^{-/-}$ mice. Cultures were washed and incubated in 0.05% Trypsin for 2 min at 37℃. After disaggregation, cell suspensions were diluted 1:1 in 0.4% Trypan blue (w/v in 0.9% NaCl) (Santa Cruz Technology) and the percentage of dye-free cells was calculated.

Boyden chamber migration assay

Cell migration assay was performed using modified 24-well Boyden chambers (Costar, New York, NY) containing a polycarbonate membrane with 5.0 μm pores. Mouse endothelial cells were starved in basic EBM2 (serum/growth factor-free) for 12 hours at 37°C before being harvested with trypsin and resuspended in EBM2 basal media with 5% of mouse plasma from the WT or the *Cfh*$^{-/-}$ mice. The single cell suspension was seeded at 1×10^5 cells/well in the upper chamber, and 0.5 ml EBM2 with 20 ng/ml of VEGF (Lonza) was added to the bottom chamber as chemoattractants. After 12 hrs, the migrated cells on the bottom surface were stained with 0.1% crystal violet and counted under microscope.

Wound-healing assay

A wound-healing assay was used to assess the migration process [18]. Mouse endothelial cells were seeded at a density of 5×10^5 cells/ml in 6-well flat-bottom plates and allowed to adhere overnight. At 95 % confluence, wounds were made using a sterile 10 μl pipette tip and the wells were washed twice with PBS to remove cellular debris. Then EBM basal medium with 5% of mouse plasma from the WT or the *Cfh*$^{-/-}$ mice was added, and photographs were taken immediately (time zero) through an inverted microscope (Leica, Wetzlar, Germany). The mouse endothelial cells were allowed to migrate for 12 hr and photographed again. The experiments were carried out 4 times. Measurements were performed on digital images using the ImageJ software (NIH). At least 10 images per treatment were analysed.

Electric Cell-Substrate Impedance Sensing (ECIS) Analysis

The real time wound healing assay was measured using the ECIS technique (ECIS model 1600; Applied Biophysics, Troy, NY) [19]. Briefly, 8-well ECIS arrays (8W10E+) were coated with L-cysteine. Mouse endothelial cells were plated in the wells and allowed to form monolayers. EBM2 basal media with 5% of mouse plasma from the WT or the *Cfh*$^{-/-}$ mice were added to the wells. Then the AC current was

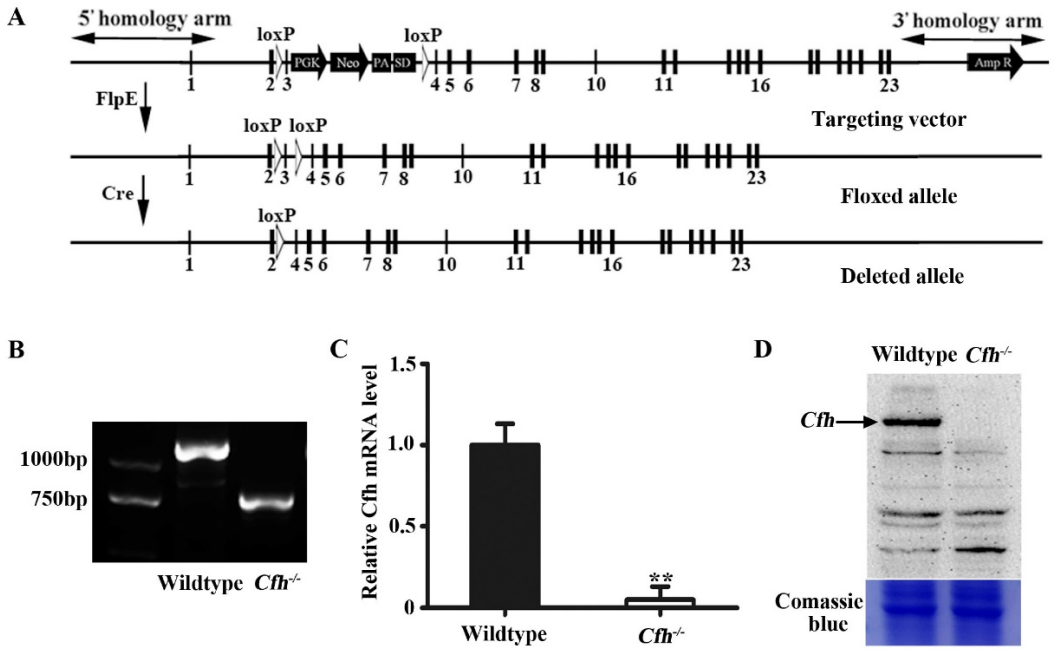

Figure 1: Validation and analyses of mice carrying *Cfh* null allele. (A) Schematic representation of the floxed and deleted alleles of *Cfh* conditional null mice. Exons are represented by rectangles. Two *loxP* sites (white triangles) were inserted between exon 2/3 and 3/4. The floxed sequences were deleted after Cre excision. (B) Genotyping of the ear tissue from WT and *Cfh*$^{-/-}$ mice. PCR produced a 1180bp band for the WT allele and a 730bp band for the *Cfh* allele. (C) Quantitative RT-PCR analysis of *Cfh* gene expression in the liver from WT and *Cfh*$^{-/-}$ mice. Each bar represents mean SE for 3 individual mice of same genotype. **, p<0.01. (D) Immunoblots of Cfh of the plasma from WT and *Cfh*$^{-/-}$ mice. Comassie blue staining of the gel was used as loading control.

given to the electrodes located at the center of each well, and the cells on the electrodes were killed. The wounded areas of each well were gradually healed by migration of viable cells and the migratory response was measured by recording the trans-endothelial resistance (TER). Data plots are representative of triplicate experiments.

Statistical analysis

Statistical significances were assessed by a paired-samples t test. A value of $p<0.05$ was considered significant.

Results

We generated a conditional null allele of *Cfh* gene in C57BL/6J mice by flanking the exon 3 with *loxP* sites (Fig. 1A). The *Cfh*$^{flox/flox}$ mice were crossed with *Rosa26*-mice to obtain the mice homozygotes of *Cfh* deletion (*Cfh*$^{-/-}$). The genotypes of the *Cfh*$^{-/-}$ mice were validated by PCR of the DNA extract from the ear tissue (Fig. 1B). The loss of Cfh expression was verified by RT-PCR analyses of the mouse *Cfh* mRNA from mouse liver and Western blotting of Cfh protein of the mouse plasma (Fig. 1C, D). Consistent to other studies [20], the *Cfh*$^{-/-}$ mice did not display any obvious phenotype in the vascular system.

Next, the effect of Cfh on angiogenesis in adult mice was examined by aorta ring assay and matrigel plug assay. The vessel growth was similar in the aorta rings from WT and *Cfh*$^{-/-}$ mice (p=0.22), suggesting that the loss of *Cfh* gene in vascular cells does not affect anigiogenesis (Fig. 2A, B). However, the number of capillaries penetrated into the matrigel plugs is significantly higher in *Cfh*$^{-/-}$ mice than those in WT mice (p<0.05) (Fig. 2C, D). CFH is a plasma protein and directly interacts with the endothelium of blood vessels. Thus, plasma Cfh contributes to angiogenesis in the matrigel plug assay but not the aorta ring assay, in which the aorta rings were cultured ex vivo and prevented from interaction with blood plasma.

To define the role of plasma Cfh, the mouse plasma was obtained by cardiac puncture and used for *in vitro* angiogenesis assays. The mouse endothelial cells were cultured with EBM2 basal media with 5% of mouse plasma from the WT and the *Cfh*$^{-/-}$ mice. As detected by MTT assay and Trypan blue exclusion assay, loss of Cfh in mouse plasma does not affect proliferation or viability of mouse endothelial cells (p=0.14, p=0.27 respectively; Fig. 3A, B). Cell migration was examined by Boyden chamber-type assays. The number of mouse endothelial cells migrating across the polycarbonate membrane was significantly increased in the groups using the plasma from *Cfh*$^{-/-}$ mice (p<0.01) (Fig. 4A, B). Cell migration during *in vitro* wound healing mimics the process of endothelial cell motility *in vivo*. After treatment with the media containing mouse plasma, the migrated area of mouse endothelial cells was significantly increased in *Cfh*$^{-/-}$ groups compared to WT groups

($p<0.05$) (Fig. 4C, D). To capture the subtle alterations of cell migration, we implemented ECIS system, which allows for real-time measurements of resistance caused by migration of endothelial cells. As shown on Fig. 4E, F, the transendothelial resistance was constantly increased in the wound area of mouse endothelial cells cultured with *Cfh*$^{-/-}$ mouse plasma ($p<0.01$). Taken together, these *in vitro* assays indicated that loss of Cfh in plasma increases endothelial cell migration.

Discussion

In this study, we generated a conditional null allele of the *Cfh* gene in C57BL/6J mice, and obtained Cfh$^{-/-}$ mice which did not display any obvious abnormalities. The *Cfh*$^{-/-}$ mice demonstrated a pro-angiogenic phenotype in matrigel plug assay. *In vitro,* the plasma from *Cfh*$^{-/-}$ mice did not affect endothelial cell growth, but significantly increases endothelial cell migration. By reconstitution of the microenvironment of choroid, Zhang et al. observed that knockdown of CFH in retinal pigmented epithelial cells (ARPE-19 cell line) promotes endothelial cell migration [21]. However, CFH has a relatively large size (139 kD) with a number of protein binding sites [22]. After produced by retinal pigmented epithelium, CFH might be difficult to migrate across peri-vascular tissues, particularly basement membrane, to interact with endothelial cells, the inner layer of the blood vessels. Our model system is unique as it precisely determines the roles of plasma Cfh on angiogenesis both *in vitro* and *in vivo*. The plasma Cfh, which is primarily produced from the liver, interacts with other plasma proteins and functions directly on the endothelial cells [23]. In addition, plasma proteins may be targeted by delivering their regulators directly into the blood circulation. In case of their deficiency, injection of recombinant proteins becomes an effective therapy [24]. Based on our findings, recombinant human CFH might be developed as a component for anti-angiogenesis therapy.

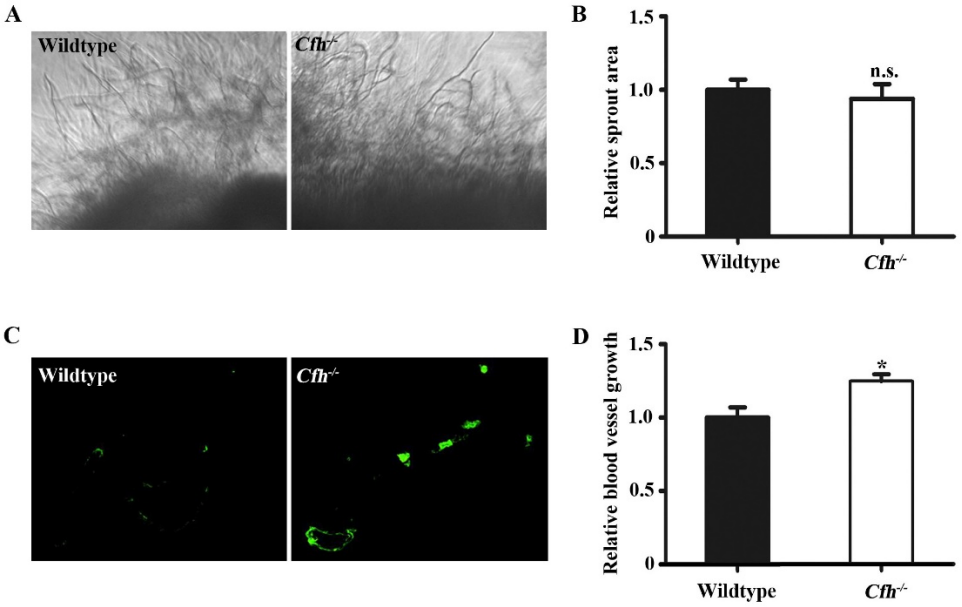

Figure 2: *In vivo* angiogenesis analyses of the WT and *Cfh*$^{-/-}$ mice. (A) Representative images of the capillary sprouts from the aorta rings of from WT and *Cfh*$^{-/-}$ mice. Magnification: 20X. (B) Relative sprout area of aorta rings from WT and *Cfh*$^{-/-}$ mice. n=6, n.s. non-significant. (C) Representative images of CD31 immunostaining on the sections of matrigel plugs injected into WT and *Cfh*$^{-/-}$ mice. Magnification: 200X. (D) Relative blood vessel growth of matrigel plugs injected into WT and *Cfh*$^{-/-}$ mice. n=6, *, $p<0.05$.

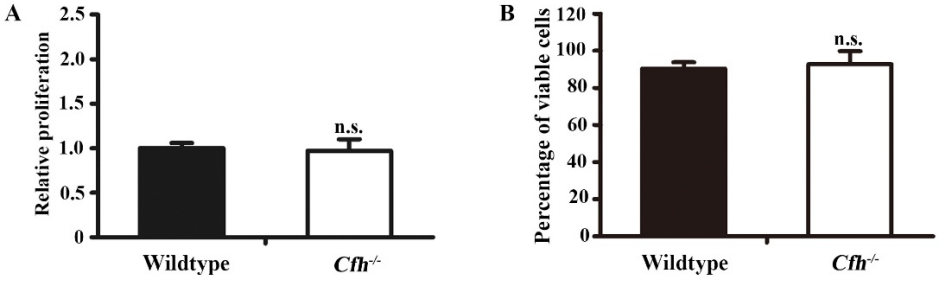

Figure 3: Proliferation and viability assays of mouse endothelial cells treated with plasma from the WT and *Cfh*$^{-/-}$ mice. MTT assay (A) and trypan blue viability assay (B) of mouse endothelial cells treated with EBM2 basal media with 5% of mouse plasma from the WT and the *Cfh*$^{-/-}$ mice for 24 hours; *n*=4; n.s., non-significant

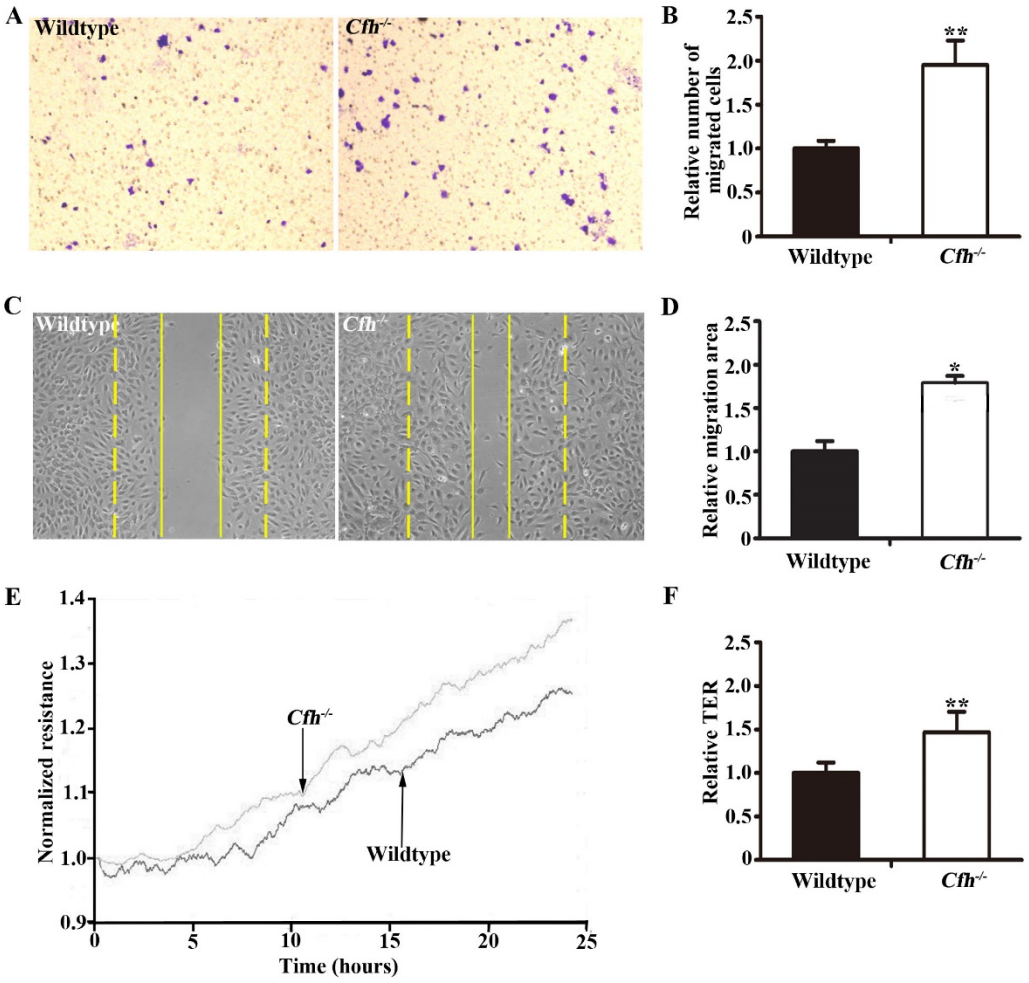

Figure 4: Migration assays of mouse endothelial cells treated with plasma from the WT and *Cfh*$^{-/-}$ mice. (A) Representative images of cell staining after transwell migration assay. Magnification: 40X. (B) The number of cells migrated through the transwell membrane. n=6, ** p<0.01; (C) Representative image of wound healing assay. Dash line: 0 hr; Solid line: 8 hr; Magnification: 100X. (D) Migrated area after wound healing. n=12, * p<0.05. (E) Representative image of real-time transendothelial resistance (TER) measurement of mouse endothelial cell monolayer; (F) Bar graph of the mean percentage of TER after 24 hr of measurement. n=4; **, p<0.01.

To date, no endothelial cell receptors of CFH have been identified. CFH functions as a negative regulator of complement activation in blood plasma, in part by binding to plasma proteins complement C3, heparin and C-reactive protein [13]. It functions as a cofactor in the inactivation of C3b by factor I, and increases the rate of dissociation of the C3bBb complex and the NBB complex in the alternative complement pathway [13]. Complement components C3a and C5a in drusen promote choroidal neovascularization [25]. C5a stimulates microvascular -like structure formation by endothelial cells [26]. Consistently, our results supported that CFH might block angiogenesis through inhibition of the complement pathway.

Angiogenesis is initiated from proliferation and migration of endothelial cells [27]. In this study, we dissected the effects of plasma Cfh on endothelial cells. Cfh does not affect endothelial cell proliferation, but inhibits migration. Endothelial cell migration is a dynamic and multistep process regulated by chemotactic, haptotactic, and mechanotactic stimuli [28]. Complement components induces migration of several cell types [29]. In addition, CFH might affect cell migration indirectly by regulating angiogenic effectors. In mice, down-regulation of Cfh expression leads to increase of the expressions of VEGF and transforming growth factor-β (TGF-β) [11]. VEGF is a pro-angiogenesis effector that activates endothelial cell proliferation and migration [1]. TGF-β inhibits the proliferation, but not migration of endothelial cells [30]. Thus, the increase of cell migration caused by loss of Cfh in plasma might be a result from the combination effects of VEGF and TGF-β.

In summary, we determined that plasma CFH inhibits endothelial cell migration and reduces angiogenesis. Thus CFH represents a novel target for anti-angiogenic therapy. Future work should elucidate the detailed molecular mechanisms underlying the effects of CFH on endothelial cells.

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Competing Interests

The authors have declared that no competing interests exist.

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