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RESEARCH ARTICLE

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Endothelial HNF4α potentiates angiogenic dysfunction via enhancement of vascular endothelial growth factor resistance in T2DM

Xubing Chai[1] | **Jun Yan**[1] | **Yaya Gao**[1] | **Jing Jin**[2]

[1]Department of Endocrinology, Xi'an Institute of Rheumatolog, Xi'an No. 5 Hospital, Xi'an, Shaanxi, China

[2]Department of Geriatric, Xi'an Institute of Rheumatolog, Xi'an No. 5 Hospital, Xi'an, Shaanxi, China

Correspondence
Jing Jin, Department of Geriatric, Xi'an Institute of Rheumatolog, Xi'an No. 5 Hospital, No. 112 Xi Guan Zheng Street, Lian Hu District, Xi'an, 710082 Shaanxi, China.
Email: ljjdoctor@163.com

Abstract

Although both hyperprocoagulant status, characterized by elevated thrombin levels, and vascular endothelial growth factor (VEGF) resistance, marked by attenuated expression of VEGFR2 (also called FLK1 or KDR), are known to contribute importantly to an increased risk of vascular events in diabetes mellitus type 2 (T2DM), it remains obscure whether these two biological events regulate angiogenic response in a coordinated manner. We show here that endothelial expression of hepatocyte nuclear factor 4α (HNF4α) was significantly upregulated in rodents and humans with T2DM, and HNF4α upregulation by thrombin was dependent on activation of multiple pathways, including protein kinase B, c-Jun N-terminal kinase, p38, oxidative stress, protein kinase C, and AMPK (5′-adenosine monophosphate (AMP)-activated protein kinase). Functionally, HNF4α inhibited VEGF-mediated endothelial proliferation and migration, and blunted VEGF-stimulated in vitro angiogenesis, thus rendering endothelial cells unresponsive to established angiogenic VEGF stimulation. Mechanistically, HNF4α potentiated the endothelial VEGF resistance through the direct transcriptional repression of *FLK1* gene. From a therapeutic standpoint, overexpression of the exogenous FLK1 successfully rescued HNF4α-inhibited angiogenic response to VEGF and potentiated VEGF-stimulated in vitro tube formation. Considering a strong association between *HNF4A* deregulation and increased risk of T2DM, our findings suggest that HNF4α may act as a critical converging point linking hyperprocoagulant condition to VEGF resistance in diabetic ECs, and repression of *FLK1* expression by thrombin-induced HNF4α mediates, at least partially, the vascular dysfunction caused by T2DM.

KEYWORDS

angiogenic dysfunction, diabetes mellitus type 2, endothelial cells, FLK1, hepatocyte nuclear factor 4α, vascular endothelial growth factor

1 | INTRODUCTION

Diabetes mellitus type 2 (T2DM), the most common long-term metabolic disorder, is currently a major worldwide cause of cardiovascular morbidity and mortality. Besides high blood sugar and insulin resistance, T2DM is also characterized by the platelet hyperprocoagulant activity.[1] In T2DM, platelets are hyperactive, and they are easy to

aggregate and release a number of antiangiogenic factors which contribute importantly to vascular endothelial damage, predisposing diabetic patients to various cardiovascular complications. Thrombin is a potent platelet agonist that signals predominantly through the protease-activated receptors.[2] Upon different stimuli, thrombin potentiates the selective releases of proangiogenic and antiangiogenic regulators from platelets via regulation of protease-activated receptor 1 (PAR1)/PAR4 receptor activation, thus leading to unbalanced angiogenic activities in T2DM.[3] A recent advance in this field has revealed an essential involvement of thrombin signaling in the induction of angiogenic dysfunction. For instance, thrombin promotes endothelial cells (ECs) growth through induction of neuron-derived orphan receptor-1 expression.[4] Very recently, Hao et al[5] demonstrate that thrombin impairs endotheliocyte proliferation, migration, and potentiates oxidative stress via KLF14-mediated transactivation of PLK1. Nevertheless, the mechanisms underlying the vascular effects triggered by thrombin remain poorly understood.

As a critical mediator of angiogenesis, vascular endothelial growth factor (VEGF) stimulates virtually all aspects of endothelial function, including proliferation, migration, permeability, and nitric oxide production.[6] VEGF binds and phosphorylates its receptors, namely VEGFR1 (also called FLT1) and VEGFR2 (also called FLK1 or KDR), leading to the activation of a series of intracellular signaling cascades. However, diabetic ECs show defective responses to VEGF, which is so-called VEGF resistance. T2DM induces VEGF resistance in various cell types including monocytes, cardiomyocytes, and ECs.[7] Mechanistically, attenuation of VEGFR expression, unspecific preactivation of intracellular pathways and unbalanced oxidative stress all contribute significantly to VEGF resistance,[8] but the molecular mechanisms by which VEGF resistance arises in T2DM are still largely unknown.

Hepatocyte nuclear factor 4α (HNF4α), encoded by the *HNF4A* gene, is a liver-enriched master regulator that regulates the expression of many genes critical for bile acid, lipid, glucose, and xenobiotic drug metabolism in hepatocytes.[9] Changes in the expression levels of *HNF4A* gene cause diabetes by reducing the amount of insulin that is produced by the pancreas. HNF4α-mediated diabetes is one of a group of familial diabetes types called maturity-onset diabetes of the young.[10] Besides its hepatic regulatory effects, growing evidence strongly suggests that additional, as yet poorly characterized, nonhepatic actions of HNF4α are very likely to take place. In concert with KLF4, CDX2 and C/EBPα, HNF4α regulates intestinal uptake of folates and analogs via transcriptional control of proton-coupled folate transporter expression.[11] Similarly, HNF4α and PKD1 form an integrated disease modifier network to regulate autosomal dominant polycystic brain disease.[12] However, the pathophysiological relevance of HNF4α-dependent signaling in such nonhepatic systems remains to be fully delineated.

Based on this rationale, we sought to investigate the expression profile of HNF4α in ECs from both nondiabetic and diabetic organisms. Using cell culture and a number of molecular biochemical approaches, we further show that the thrombin-elicited endothelial HNF4α substantially inhibits endothelial proliferation and migration and compromises tube formation, probably through transcriptional regulation of VEGF resistance.

2 | MATERIALS AND METHODS

2.1 | Animal model and human samples

Animal work was strictly conformed to the guidelines of the *Institutional Animal Care and Use* by NIH, and was approved by the IACUC of Xi'an No. 5 Hospital. Adult male C57BL/6 mice at ages of 10 weeks were obtained from the animal facility in our hospital. Mice were provided food pellets and water ad libitum in a 20 to 25°C environment, and were allowed to acclimatize for at least 1 week before experiments. To induce T1DM, mice were injected intraperitoneally with one dose of streptozotocin (STZ, 160 mg/kg/body weight; Sigma-Aldrich, Shanghai, China). The animals with blood glucose more than or equal to 250 mg/dL at 2 weeks after injection were considered diabetic.[5] Murine T2DM model was established by treatment with high-fat diet (HFD; Baotai Hongda Biotech, Beijing, China) for 3 weeks, followed by one single intraperitoneal injection of STZ (80 mg/kg/body weight). Mice with distinct hyperglycemia concomitant with insulin resistance (beyond 1-2 SEM values from the normal mice mean), at 3 weeks after STZ injection, were considered diabetic.[5]

Upon receipt of the written informed consent from all participants, peripheral blood samples were obtained from 10 healthy volunteers and 15 T2DM patients in our hospital from March 2015 to April 2017. Human $CD34^+$ cells were then isolated by FACS using EasySep Human CD34 Positive Selection Kit (STEMCELL Technologies, Shanghai, China). After collection, cells were immediately stored at −80°C until further biochemical analysis. The use and handling of the human samples, strictly conformed to the standards set by *2008 Revised Declaration of Helsinki*, was approved by the Human Research Committee in our hospital.

2.2 | Cell treatment

ECs were isolated and purified from mouse brain and liver according to previous reports.[5,13] Briefly, after a thorough mince and digestion with collagenase (200 U/mL; Thermo Fisher Scientific, Shanghai, China) at 37°C for 45 minutes, the tissue homogenates were filtered through a 70-μm cell strainer (BD Medical Technology, Bedford, MA). Cell pellets were then resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 4°C, followed by sequential incubation with anti-PECAM-1 or anti-ICAM-2 monoclonal antibody-coated beads (Thermo Fisher Scientific) at room temperature for 15 minutes. Final separation was performed on a magnetic separator (Bangs Lab, Fishers, IN). ECs were maintained in EGM2-MV media with 5% of FBS (Gibco, Shanghai, China), and were used for experiments at passages 2 to 3.

Human umbilical vein endothelial cells (HUVECs), obtained from ATCC (Rockville, MD), were routinely cultured in endothelial basal medium (EBM-2), supplemented with 1 mg/mL hydrocortisone, 12 mg/mL bovine brain extract, 50 mg/mL gentamycine, 50 ng/mL amphothericin-B, 10 ng/mL EGF, and 10% fetal calf serum (FCS), at 37°C in 5% CO_2 and 95% humidified air. To study the effects of hyperglycemia/hyperinsulinemia/thrombin on HNF4α expression, HUVECs were incubated with different doses of glucose/insulin/thrombin for 6 hours, followed by quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis. To investigate the signaling pathways potentially involved in the regulation of HNF4α expression by thrombin, HUVECs were stimulated with 5.0 U/mL of thrombin for 6 hours, in the presence of different pathway inhibitors including wortmannin (PI3K/Akt, 60 nM), SP600125 (JNK MAPK, 20 μM), SB203580 (p38 MAPK, 20 μM), diphenyleneiodonium chloride (DPI, oxidative stress, 20 μM), Go6976 (PKC, 1 μM), and WZ4003 (AMPK, 5 μM) (All inhibitors were from Selleck, Shanghai, China), followed by RT-qPCR analysis. HUVECs/HNF4A were established by transfection with pCMV3-His-HNF4A or empty vector (Sino Biological, Beijing, China) in HUVECs using FuGENE HD (Promega, Beijing, China), followed by selection with 200 μg/mL of hygromycin (Sigma-Aldrich). To transiently overexpress the exogenous FLK1, HUVECs/HNF4A were transfected with pCMV3-FLK1 or empty vector (Sino Biological) using FuGENE HD for 48 hours.

2.3 | Cell proliferation

HUVECs were plated in 96-well plates at a density of 1×10^4/mL in 100 mL of growth factor-free medium per well. Three hours later, cells were incubated with medium containing 50 ng/mL of VEGF (Sigma-Aldrich) for another 48 hours. Cell proliferation was assayed and determined using the Vybrant MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) at 570 nM on a microplate reader (Bio-Rad, Shanghai, China).

2.4 | Cell migration

Confluent HUVECs were starved in EBM-2 containing 0.5% bovine serum albumin for 3 hours. Cells were then seeded at the density of 1×10^5 cells/insert onto a 8-μm-pore Transwell precoated with gelatin (Sigma-Aldrich). The Transwell was then inserted into a well containing EBM-2 with or without 50 ng/mL of VEGF. The cells were allowed to migrate for another 12 hours, followed by staining with 4′,6-diamidino-2-phenylindole and calculation of endothelial migration in 16 high-power (×200) fields.[14]

2.5 | In vitro tube formation assay

HUVECs with different treatment were seeded at the density of 1×10^4 cells in 100 μL of M199 medium containing EBM-2 with or without 50 ng/mL of VEGF, in a 96-well plates which had been precoated with ECMatrix gel (BD Biosciences, Shanghai, China). After another 24-hours culture at 37°C, tube formation was observed and photographed.[14] ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify the branching points of newly formed tubes.

2.6 | Immunohistochemistry

Paraffin-embedded mouse tissue sections were routinely deparaffinized and hydrated. For antigen retrieval, slides were incubated with antigen retrieval buffer (R&D Systems, Minneapolis, MN) in a microwave oven for 10 minutes, followed by elimination of the endogenous peroxidase activity by incubation with 3% H_2O_2 in methanol for 10 minutes. Sections were then incubated with primary antibody at 4°C overnight.[15] Final peroxidase activity was developed with the aid of VECTASTAIN Elite Kit (Vector Laboratories, Shenzhen, China). The details of the primary antibody used for immunohistochemistry were provided in Table S1.

2.7 | Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated and purified using MagMAX Nucleic Acid Isolation Kit (Thermo Fisher Scientific). We then subjected RNA samples to reverse-transcription reaction using Superscript III Synthesis System (Thermo

Fisher Scientific), following the manufacturer's instructions. qPCR was performed according to the Promega protocol.[16] The primers used were *Hnf4a*, 5′-AGAAGATTGCCAACA TCAC-3′ and 5′-GGTCATCCAGAAGGAGTT-3′; *HNF4A*, 5′-GCCATCATCTTCTTTGACCCA-3′ and 5′-GATGTAGT CCTCCAAGCTCAC-3′; *FLT1*, 5′-AATCACCTACGTGC CGGACT-3′ and 5′-TGGCCAATGTGGGTCAAGAT-3′; *FLK1*, 5′-CGGTCAACAAAGTCGGGAGA-3′ and 5′-CA GTGCACCACAAAGACACG-3′; *VEGFA*, 5′-GTCCT GGAGCGTGTACGTTG-3′ and 5′-CAAATGCTT TCTCCGCTCTGA-3′; *18S*, 5′-CTCGCCGCGCTCTA CCTACCTA-3′ and 5′-ATGAGCCATTCGCAGTTTC ACTGTA-3′. The relative abundance of each target transcript was quantified using the comparative $2^{-\Delta\Delta C_t}$ method,[17] with 18S as an internal control.

2.8 | Western blot analysis

Total protein samples were isolated using radioimmunoprecipitation buffer (Roche, Shanghai, China). Approximately thirty micrograms of protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Thermo Fisher Scientific). After blocking with a 5% nonfatty milk, the blots were probed with different antibodies (Table S1) at 4°C overnight. Final immunocomplexes were developed using a Chemiluminescent Western blots Imager (LI-COR Biotechnology, Lincoln, NE), with β-actin serving as the loading control.

2.9 | Luciferase reporter assay

The human FLK1 promoter region (Nucleotides: −1824 to +102; Genbank accession number: NC_000004.12) was amplified by PCR and cloned into pGL3-basic reporter plasmids (Promega, Beijing, China) using In-Fusion HD Cloning Kit (Takara, Dalian, China). Site-directed mutagenesis was achieved using QuikChange II Site-Directed Mutagenesis Kit (Agilent, Beijing, China). For promoter luciferase assay, pCMV3-His-HNF4A or empty vector, the WT or Mu luciferase reporter plasmids, and pRL-TK *Renilla* reporter plasmid (Promega) were cotransfected into HEK/293/T cells using FuGENE HD. Twenty-four hours later, cells were stimulated with 5.0 U/mL of thrombin or/and VEGF (50 ng/mL) for another 6 hours, followed by measurement of the relative luciferase activity using Dual-Luciferase Activity System (Promega).[16]

2.10 | Chromatin immunoprecipitation

HUVECs/HNF4A were treated with 5.0 U/mL of thrombin for 6 hours, followed by being crosslinked with 1% formaldehyde and sonication. Soluble chromatin was then immunoprecipitated or double-immunoprecipitated using HNF4α or MTA1 antibodies, as described previously.[18] Recovery and preparation of DNA was amplified by PCR using the following primers: P1, 5′-TC ATAAACTGATTATATATT-3′ and 5′-CAGTTAAACTTT AAACCAGAC-3′; P2,5′-ATTAGAGAAGGTCTTTCCAA TTC-3′ and 5′-AGAATAAAGTTAGACTCCT-3′.

2.11 | Statistical analysis

Quantitative data, presented as mean ± SEM, were analyzed for statistically significant differences using analysis of variance with post hoc tests wherever appropriate. The correlation between *FLK1* expression and *HNF4A* messenger RNA (mRNA) levels was evaluated using the Pearson χ^2 test. $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | Upregulation of endothelial HNF4α in T2DM

To study the potential involvement of HNF4α in the regulation of endothelial dysfunction, we firstly established different murine DM models (Figure S1). Subsequent immunohistochemistry on liver and brain sections showed that HNF4α expression was significantly elevated in ECs from T2DM mice, compared with the negligible expression levels in ECs from Ctrl or T1DM mice (Figure 1A). To validate this observation, we isolated ECs from mouse liver and brain (Figure S2). The expression levels of *Hnf4a* transcripts evoked more than doubled in ECs isolated from liver and brain of T2DM mice, compared with those in Ctrl or T1DM mice (Figure 1B). This unique upregulation of HNF4α expression was further confirmed by Western blot analysis in primary cultured ECs (Figure 1C). Human circulating $CD34^+$ cells contain large numbers of endothelial progenitor cells.[13] Intriguingly, *HNF4A* expression was ~3.4 fold higher in $CD34^+$ cells from T2DM patients, compared with Ctrl volunteers (Figures S3 and 1D). T2DM thus induces the endothelial expression of HNF4α in mice, and likely in humans as well.

3.2 | Distinct regulation of HNF4α expression in ECs by thrombin signaling

Subsequent efforts were directed toward the characterization of the upstream regulation of HNF4α expression in ECs. The mean levels of *HNF4A* mRNA were unchanged in the HUVECs treated for 6 hours with different doses of

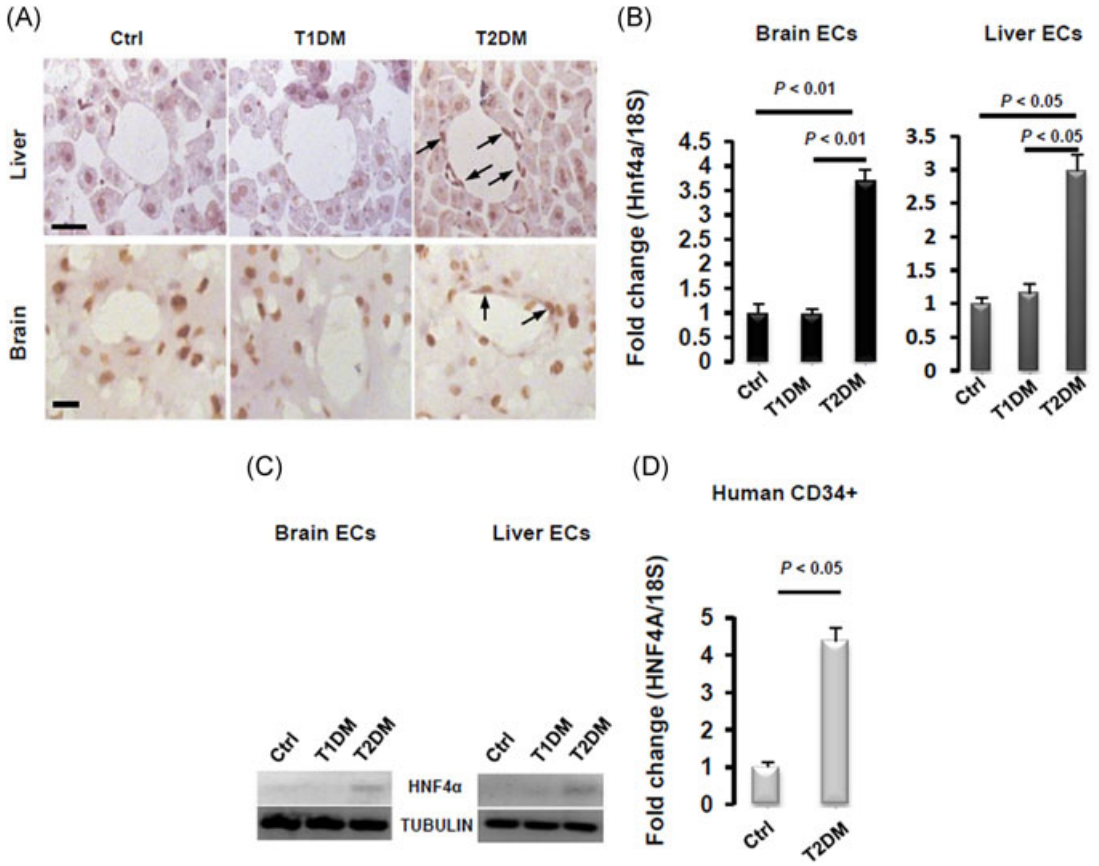

FIGURE 1 T2DM induces endothelial HNF4α expression. A, Expression of HNF4α (black arrows) in different tissues from Ctrl and diabetic mice was determined using immunohistochemistry. Scale bar = 15 μM. B, Relative levels of *Hnf4a* mRNA in freshly isolated ECs from mouse liver and brain were evaluated using RT-qPCR analysis, with parallel amplification of 18S as internal control. C, Relative levels of HNF4A protein in freshly isolated ECs from mouse liver and brain were evaluated using Western blot analysis. TUBULIN served as loading control. D, Relative *HNF4A* mRNA abundance in vasculogenic circulating $CD34^+$ cells isolated from patients with T2DM, vs matched normal subjects. EC, endothelial cell; HNF4α, hepatocyte nuclear factor 4α; mRNA, messenger RNA; RT-qPCR; quantitative reverse transcription polymerase chain reaction; T2DM, diabetes mellitus type 2

glucose (Figure 2A) or insulin (Figure 2B). By contrast, endothelial expression levels of *HNF4A* mRNA were significantly stimulated in the HUVECs treated with high concentration of thrombin in a dose-dependent manner (Figure 2C). To gain mechanistic insights into the regulation of endothelial *HNF4A* expression by thrombin signaling, we treated HUVECs with 5.0 U/mL of thrombin for 6 hours, in the presence of different inhibitors including wortmannin (PI3K/AKT), SP600125 (JNK MAPK), SB203580 (p38 MAPK), DPI (oxidative stress), Go6976 (PKC), and WZ4003 (AMPK). Apparently, coincubation with wortmannin, SP600125, SB203580, DPI, or WZ4003 all successfully reduced the induction of *HNF4A* transcription by thrombin, and DPI appeared to be the strongest inhibitor of thrombin-induced *HNF4A* expression (Figure 2D). Together, thrombin-elicited endothelial *HNF4A* induction was dependent on the activation of protein kinase B (AKT), c-Jun N-terminal kinase (JNK), p38, oxidative stress, protein kinase C (PKC), and 5′-adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathways.

3.3 | HNF4α inhibits endothelial function through potentiation of VEGF resistance

To directly ask whether HNF4α upregulation has a causal effect on endothelial dysfunction, we established the HUVECs/HNF4A that stably expressed the exogenous HNF4α (Figure 3A). VEGF-induced endothelial proliferation was effectively suppressed by HNF4α overexpression in HUVECs (Figure 3B). Likewise, VEGF-promoted endothelial migration in HUVECs was substantially abolished by HNF4α upregulation (Figure 3C and 3D). To further test the inhibitory effects of HNF4α on angiogenesis, we performed in vitro tube formation assay. HUVECs cultured on Matrigel containing 50 ng/mL of VEGF

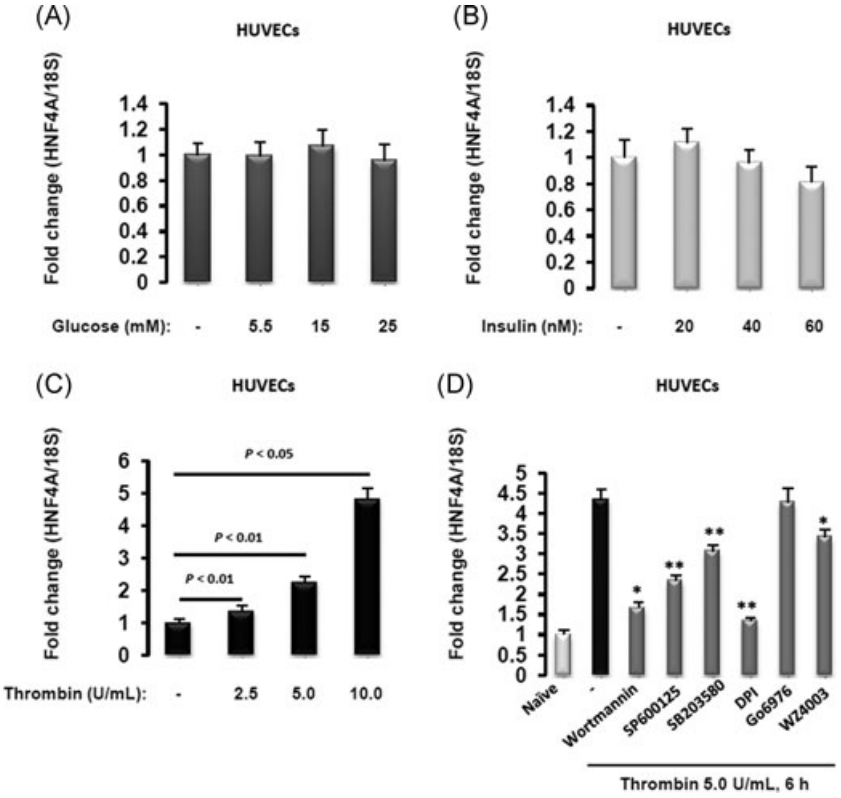

FIGURE 2 Regulation of endothelial HNF4α expression by thrombin through activation of multiple pathways. A-C, HUVECs were incubated with different doses of glucose/insulin/thrombin as indicated for 6 hours, followed by measurement of *HNF4A* expression using RT-qPCR analysis. D, HUVECs were stimulated with 5.0 U/mL of thrombin for 6 hours, in the presence of different pathway inhibitors including wortmannin (PI3K/Akt, 60 nM), SP600125 (JNK MAPK, 20 μM), SB203580 (p38 MAPK, 20 μM), diphenyleneiodonium chloride (DPI, oxidative stress, 20 μM), Go6976 (PKC, 1 μM), and WZ4003 (AMPK, 5 μM), followed by measurement of *HNF4A* expression using RT-qPCR analysis. $*P < 0.05$ and $**P < 0.01$ when compared with the values in cells treated alone with thrombin. HNF4α, hepatocyte nuclear factor 4α; HUVEC, human umbilical vein endothelial cell; RT-qPCR, quantitative reverse transcription polymerase chain reaction

showed morphological tubule formation, and HNF4α overexpression completely neutralized the VEGF-stimulated in vitro tube formation (Figure 3E). In line with these morphological changes, the VEGF-related signal transduction defects (AKT, ERK, and eNOS pathways)[7,19] could be detected in the HUVECs/HNF4A (Figure 3F). Based on the available data, we reason that HNF4α may promote endothelial dysfunction via enhancement of VEGF resistance.

3.4 | HNF4α inhibits FLK1 expression to desensitize ECs to VEGF stimulation

In an effort to search for the potential mechanism(s) underlying our observation, we found a significant negative correlation between *HNF4A* expression and *FLK1* expression in human $CD34^+$ cell samples (Figure 4A). Consistently, HNF4α overexpression significantly inhibited FLK1 expression at both protein and mRNA levels, but had no effects on FLT1 expression levels (Figure 4B and 4C). Given that the role of FLK1 in angiogenesis is more essential than FLT1 and has been well established,[20] we hypothesized that the promoting-effects of HNF4α on VEGF resistance are mainly mediated through negative regulation of FLK1 signaling. To verify this, we transiently transfected the HUVECs/HNF4A with pCMV3-FLK1 or empty vector. Transient overexpression of pCMV3-FLK1 successfully abolished HNF4α-induced inhibition of FLK1 expression in the HUVECs/HNF4A, evidenced by Western blot analysis (Figure 4D). Subsequent functional analysis regarding angiogenic function revealed that elevation of FLK1 expression partially but effectively rescued endothelial proliferation (Figure 4E) and migration (Figure 4F), and potentiated VEGF-stimulated in vitro tube formation (Figure 4G) in the HUVECs/HNF4A. Thus, deregulation of endothelial HNF4α diminishes neovascularization, at least in part through inhibition of FLK1 signaling pathway.

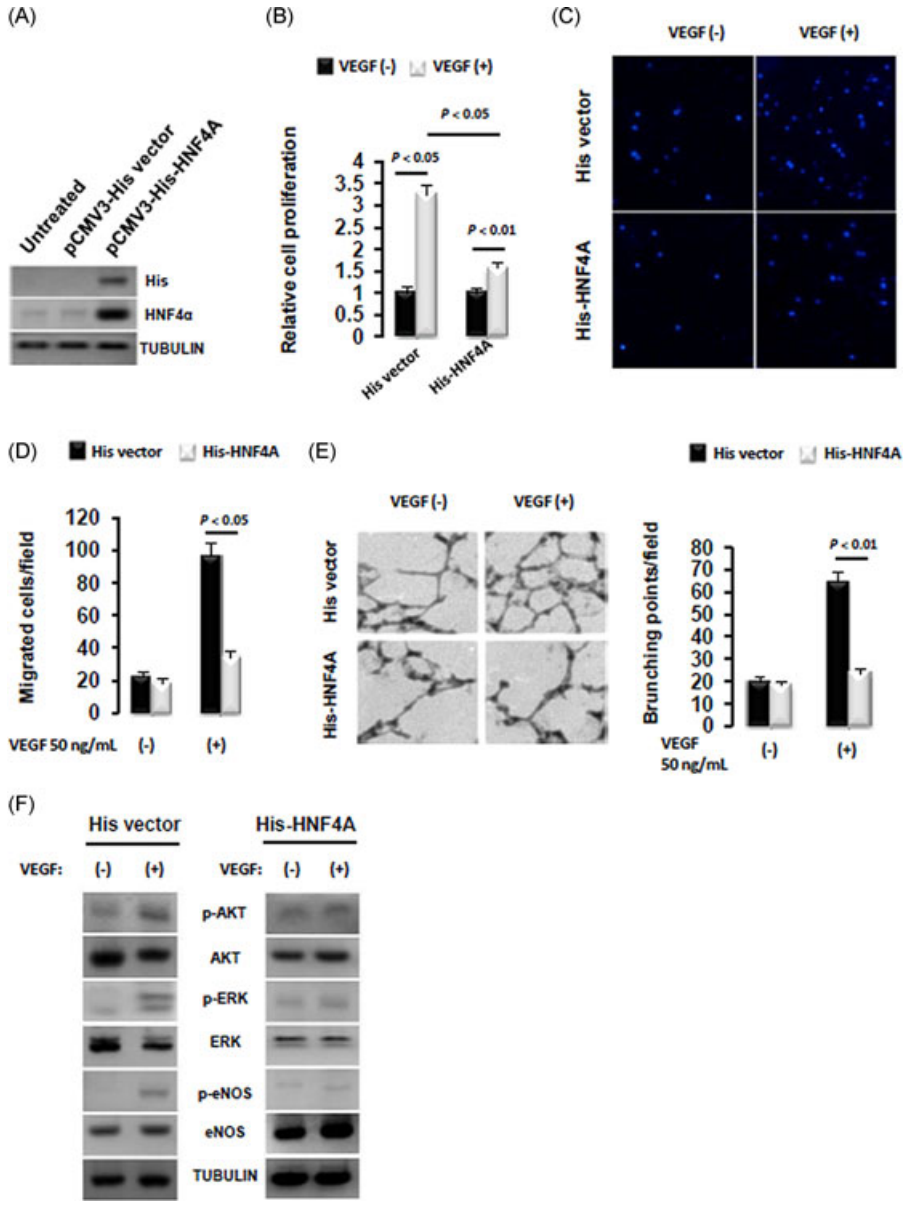

FIGURE 3 Endothelial HNF4α impairs angiogenic response to VEGF stimulation. A, Stable expression of the exogenous HNF4α in HUVECs was validated using Western blot analysis. B, HUVECs with different transfections were incubated with medium containing 50 ng/mL of VEGF for 48 hours, followed by measurement of endothelial proliferation using the Vybrant MTT Cell Proliferation Assay Kit. C, Representative images of HUVECs that migrated in the Transwell migration assay. D, Quantification of total migrated HUVECs in Transwell migration assay. E, Stable expression of the exogenous HNF4α inhibits the tube-forming activity in HUVECs. Left, representative images of HUVECs that were subjected to Matrigel tubule formation assay. Right, Quantification of total branching points in Matrigel tubule formation assay. F, HUVECs with different transfections were incubated with medium containing 50 ng/mL of VEGF for 48 hours, followed by measurement of VEGF signaling activation using Western blot analysis. HNF4α, hepatocyte nuclear factor 4α; VEGF, vascular endothelial growth factor

3.5 | Direct regulation of FLK1 transcription by HNF4α

In the last experimental setting, we further ascertained the specificity of our findings at the molecular levels by measuring the influence of HNF4α on *FLK1* transcription. Treatment with VEGF alone stimulated the *FLK1* promoter activity by ~27.2 fold. This stimulatory effect was substantially compromised by cotreatment with thrombin and pCMV3-His-HNF4A transfection. To be noted, thrombin treatment alone had no effects on *FLK1* transcription. Moreover, in the presence of pCMV3-His-HNF4A transfection, thrombin supplementation exhibited the inhibitory effects on the *FLK1* promoter activity in a dose-dependent manner (Figure 5A). Sequence analysis of the *FLK1* major promoter revealed two

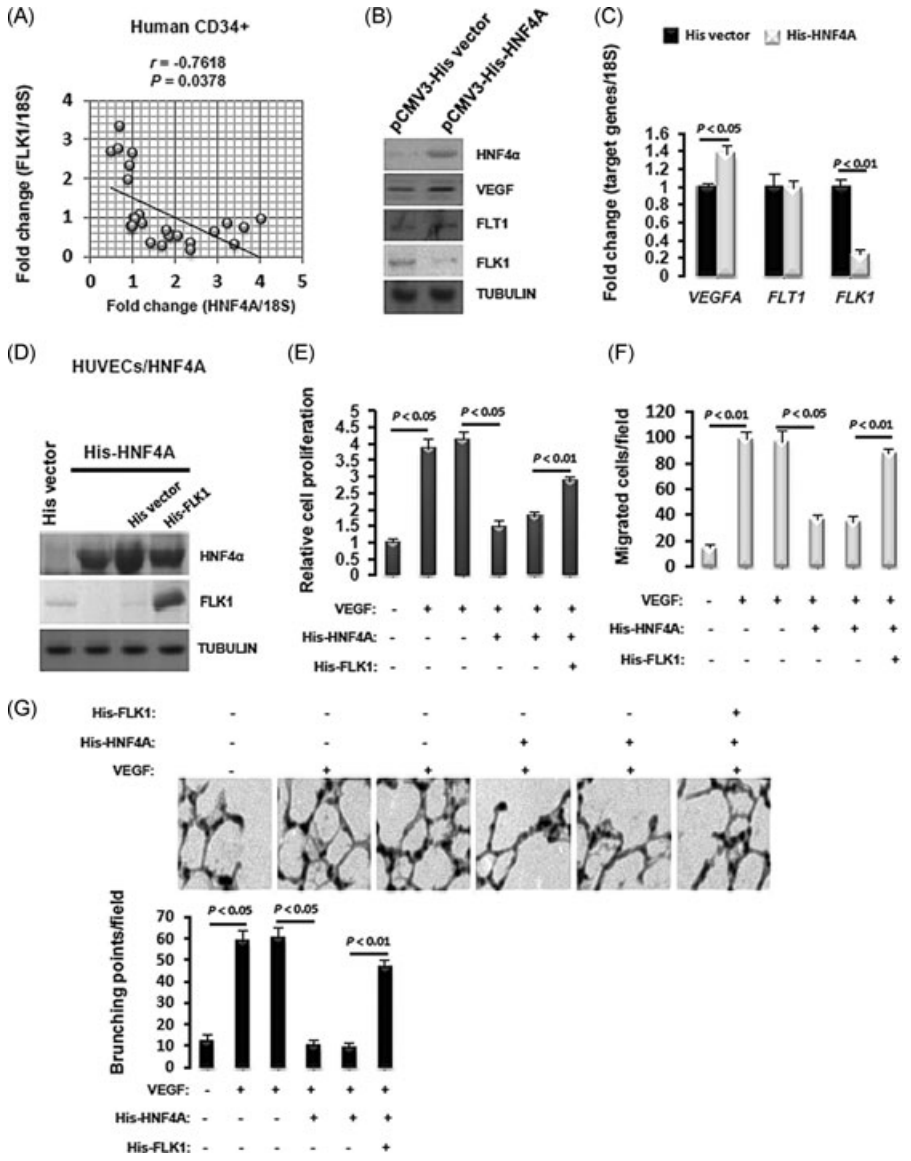

FIGURE 4 HNF4α inhibits endothelial function through potentiation of VEGF resistance. A, Expression levels of FLK1 and HNF4A transcripts in vasculogenic circulating $CD34^+$ cells were evaluated using RT-qPCR analysis, followed by the Pearson χ^2 test. B, Western blot analysis of expression levels of HNF4α, VEGF, FLT1, and FLK1 in the HUVECs transfected with pCMV3-His-HNF4A or empty vector. C, RT-qPCR analysis of HNF4A, VEGF, FLT1, and FLK1 in the HUVECs transfected with pCMV3-His-HNF4A or empty vector. D, HUVECs/HNF4A were transiently transfected with pCMV3-FLK1 or empty vector. Forty-eight hours later, cells were harvested and subjected to Western blot analysis. E, HUVECs with different transfections were incubated with medium containing 50 ng/mL of VEGF for 48 hours, followed by measurement of endothelial proliferation using the Vybrant MTT Cell Proliferation Assay Kit. F, Quantification of total migrated HUVECs in Transwell migration assay. G, In vitro tube formation assay showing that transient overexpression of the exogenous FLK1 effectively rescued HNF4α-inhibited in vitro angiogenesis. HNF4α, hepatocyte nuclear factor 4α; RT-qPCR, quantitative reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor

potential binding sites, located downstream from the start codon (Figure 5B). Subsequent chromatin immunoprecipitation (ChIP) assay showed that HNF4α was recruited to the −447 to −442 basepairs (bps) regions of the *FLK1* promoter (Figure 5C), and this recruitment was further enhanced by thrombin supplementation in a dose-dependent manner (Figure 5D). To further solid this observation, we mutated the binding site that was responsible for the binding of HNF4α onto the *FLK1* promoter (Figure 5E). As expected, cotreatment with thrombin and pCMV3-His-HNF4A abolished VEGF-stimulated *FLK1* promoter activity, and this inhibitory effect was substantially reversed when WT WT pGL3-*FLK1* reporter plasmid was replaced with Mu pGL3-*FLK1* reporter plasmid (Figure 5E). Taken together, we have demonstrated a direct co-association between HNF4α

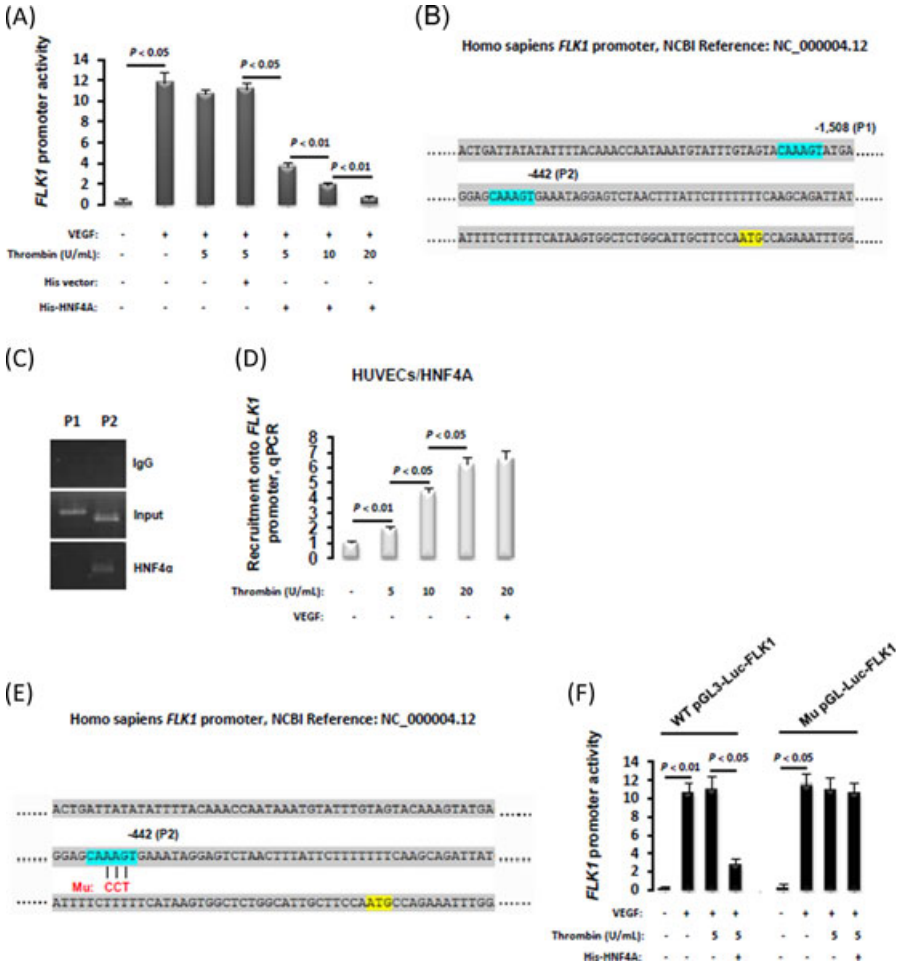

FIGURE 5 HNF4α directly inhibits HNF4A transcription. A, The pCMV3-His-HNF4A or empty vector and pRL-TK *Renilla* reporter plasmid were cotransfected into HEK/293/T cells using FuGENE HD. Twenty-four hours later, cells were stimulated with 5.0 U/mL of thrombin or/and VEGF (50 ng/mL) for another 6 hours, followed by measurement of the relative luciferase activity. B, Schematic of predicted HNF4α binding sites on the *FLK1* promoter region. C, ChIP analysis showing recruitment of HNF4α onto a specific region of the *FLK1* promoter. D, HUVECs/HNF4A were incubated with 5.0 U/mL of thrombin or/and VEGF (50 ng/mL) for 6 hours, followed by qPCR-ChIP assay. E, Schematic of the mutated HNF4α binding site on the *FLK1* promoter region. F, The WT or mutated pGL3-Luc-*FLK1* reporter plasmid and pRL-TK *Renilla* reporter plasmid were cotransfected with pCMV3-His-HNF4A or pCMV3-His vector into HEK/293/T cells using FuGENE HD. Twenty-four hours later, cells were stimulated with 5.0 U/mL of thrombin or/and VEGF (50 ng/mL) for another 6 hours, followed by measurement of the relative luciferase activity. ChIP, chromatin immunoprecipitation; HNF4α, hepatocyte nuclear factor 4α; qPCR, quantitative polymerase chain reaction; VEGF, vascular endothelial growth factor

and the *FLK1* chromatin in the thrombin-challenged ECs.

4 | DISCUSSION

The current communication provides the first expression and functional analysis of HNF4α in ECs obtained from human T2DM patients and murine T2DM model. Three original observations have been made in this proof-of-concept study: (i) HNF4α expression is upregulated in ECs from T2DM organisms. (ii) HNF4α upregulation, controlled by thrombin through regulation of multiple signaling pathways, predisposes ECs to vascular dysfunctions including impaired proliferation and migration, and blunted in vitro tube formation. (iii) HNF4α represses the *FLK1* transcription to potentiate the VEGF resistance and inhibit endothelial function (Figure 6). These data collectively indicate that HNF4α mediates, at least in part, the vascular dysfunction caused by the procoagulant state in T2DM.

We have shown that the induction of HNF4α by thrombin is dependent on the activation of AKT, JNK, p38, oxidative stress, PKC, and AMPK pathways (Figure 2D). These pathways have been functionally linked to thrombin function. For example, as a potent platelet agonist, thrombin causes a robust phosphorylation of AKT on Thr^{308} and Ser^{473}, and this

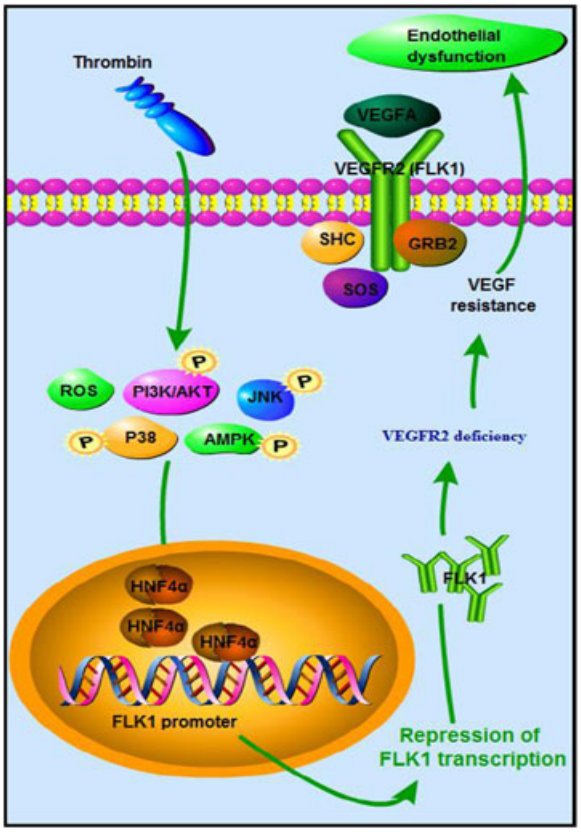

FIGURE 6 Schematic representation of HNF4α regulation of FLK1 and its impact on VEGF-stimulated angiogenic response. HNF4α, hepatocyte nuclear factor 4α; VEGF, vascular endothelial growth factor

thrombin-mediated phosphorylation of AKT and activation of its downstream effectors p38 MAPK and JNK, in addition to activation of PKC kinase, potentiates collagen-induced platelet aggregation.[2] AMPK is a serine-threonine kinase that acts as a metabolic sensor in multiple systems. Thrombin directly increases basal glucose uptake in human skeletal muscle cells through phosphorylation of AMPK.[2] Likewise, hypercoagulation (a state with elevated thrombin levels) has been identified as a feature of T2DM, and this increased coagulation is frequently paralleled with an elevation of reactive oxygen species (ROS) levels in cardiovascular system.[21,22] Previously, work from BelAiba et al[23] verifies that nicotinamide adenine dinucleotide phosphate oxidases-derived ROS plays an important detrimental role in thrombin-stimulated human microvascular endothelial cells.[23] Despite these advances, the downstream effector that mediates the deleterious effects of the above-mentioned pathways in thrombin-stimulated ECs remains unidentified, although interestingly. The data shown here suggest that HNF4α may serve as such an important effector in thrombin-challenged ECs. It will be of future interest to deconvolute how multiple pathways stimulated by thrombin converge at HNF4α, and whether HNF4α regulates biological effects of different pathways via transcriptional regulation of differential target genes.

Metabolic regulation of ECs is attracting much attention, especially in the context of cardiovascular diseases.[24] To this end, the results from our gain-of-function study support the importance of HNF4α upregulation in the induction of endothelial dysfunction. Alanine-glyoxylate aminotransferase 2 (AGXT2) is a mitochondrial pyridoxal phosphate-dependent aminotransferase, and deregulation of AGXT2 is frequently observed in diabetic samples. Changes in the AGXT2 activity affect several metabolites, including endogenous methylarginines, nitric oxide, and lipids, which are all known to be deregulated in diabetes. Interestingly, HNF4α has been shown to directly bind to the *AGXT2* chromatin and to regulate *AGXT2* transcription.[25] It is thus a logical hypothesis that HNF4α may predisposes ECs to antiangiogenic stimulation (eg, thrombin) through direct regulation of central regulators of energy metabolism. Of note, in contrast to our results, HNF4α in hepatocytes positively regulates metabolic changes.[10] Thus, HNF4α may exert its differential regulatory roles in a cell-context dependent manner.

As one of the most essential angiogenic growth factors described so far, VEGF has been confirmed by compelling evidence to regulate angiogenesis critically. VEGF promotes endothelial cell differentiation, proliferation, migration, and cord formation.[7] The proangiogenic effects of VEGF are mediated mainly through specific interactions with VEGF receptors VEGFR1 (FLT1) and VEGFR2 (FLK1). Unlike the bidirectional ability to modulate angiogenesis by FLT1, the role of FLK1 is strictly conserved, and FLK1 activation is essential for angiogenesis.[20] Intriguingly, FLK1 expression is tightly controlled at the transcriptional level. For instance, FOXF1 positively regulates VEGF signaling by direct binding onto the *FLK1* chromatin, and therefore plays an important role during the formation of embryonic vasculature.[26] Mixl1 promotes the transactivation of *Flk1* in a DNA binding-dependent manner. The Mixl1/Flk1 cascade is thus believed to function as a key regulator of mesendoderm development during mammalian gastrulation.[27] Similarly, during embryonic stem differentiation, *Flk1* transcription in murine hematocardiovascular progenitors is positively regulated by cooperative interactions between Gata, Tcf/Lef, Cdx and ER71/Etv2 coregulators.[28] In continuance to these understandings, our molecular biological analyses have identified the *FLK1* promoter to be a direct target of HNF4α in thrombin-challenged ECs. Worthy to note, multiple putative *cis*-regulatory elements have been reported in

the *FLK1* promoter regions. Mixl1 binds to −1384 to −1394 bp upstream of the transcription start site.[27] FOXF1 is recruited onto −362 to −354 bp of the *FLK1* promoter.[26] Additionally, the Notch pathway transcriptional regulator Rbpj negatively regulates *FLK1* transcription, and the binding site of Rbpj on the *FLK1* promoter is more than 3 kb upstream of the transcription start site.[29] In contrast, the promoter region of *FLK1* bound by HNF4α (−447 to −442 bp) was not included in the above-mentioned *cis*-acting elements. These results together help in understanding how *cis*-regulatory diversity permits differential expression outcomes from a limited repertoire of transcriptional regulators in the regulation of one single target gene.

In summary, our studies have identified a compendium of thrombin-regulated HNF4α that provides a resource for further functional and mechanistic studies aimed at dissecting the molecular explanations for microvascular dysfunction in T2DM. Because transcriptional regulation of *FLK1* represents one of the earliest and most important steps in the endothelial differentiation program, the observations that HNF4α directly represses *FLK1* transcription and the recruitment of HNF4α onto *FLK1* chromatin is enhanced by thrombin confirm the position of HNF4α as a key player orchestrating the transcriptional networks that promote hypercoagulant condition-induced angiogenic dysfunction via VEGF resistance.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHORS CONTRIBUTIONS

JJ and XBC designed the study. XBC, JY, and YYG carried out the study. XBC, and JJ participated in the analysis of experimental results. JJ wrote and revised the manuscript. All authors read and approved the final manuscript.

ORCID

Jing Jin http://orcid.org/0000-0002-2435-7165

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SUPPORTING INFORMATION

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