



Endothelial FAM3A positively regulates post-ischaemic angiogenesis

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ABSTRACT

Background: Angiogenesis improves reperfusion to the ischaemic tissue after vascular obstruction. The underlying molecular mechanisms of post-ischaemic angiogenesis are not clear. FAM3A belongs to the family with sequence similarity 3 (FAM3) genes, but its biological function in endothelial cells in regards to vascular diseases is not well understood.

Methods: Gain- and loss-of-function methods by adenovirus or associated-adenovirus (AAV) in different models were applied to investigate the effects of FAM3A on endothelial angiogenesis. Endothelial angiogenesis was analysed by tube formation, migration and proliferation in vitro, and the blood flow and capillary density in a hind limb ischaemic model in vivo.

Findings: Endothelial FAM3A expression is downregulated under hypoxic conditions. Overexpression of FAM3A promotes, but depletion of FAM3A suppresses, endothelial tube formation, proliferation and migration. Utilizing the mouse hind limb ischaemia model, we also observe that FAM3A overexpression can improve blood perfusion and increase capillary density, whereas FAM3A knockdown has the opposite effects. Mechanistically, mitochondrial FAM3A increases adenosine triphosphate (ATP) production and secretion; ATP binds to P2 receptors and then upregulates cytosolic free Ca²⁺ levels. Increased intracellular Ca²⁺ levels enhance phosphorylation of the transcriptional factor cAMP response element binding protein (CREB) and its recruitment to the VEGFA promoter, thus activating VEGFA transcription and the final endothelial angiogenesis.

Interpretation: In summary, our data demonstrate that FAM3A positively regulates angiogenesis through activation of VEGFA transcription, suggesting that FAM3A may constitute a novel molecular therapeutic target for ischaemic vascular disease.

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1. Introduction

Angiogenesis plays important roles in physiological as well as pathological settings such as ischaemia, cancer, inflammation, and diabetes [1,2]. During angiogenesis, vascular endothelial cells are rapidly activated, then migrate to distant ischaemic tissue sites and sprout to form new capillaries, thus improving the distribution and use of the remaining blood flow [3–5]. Accumulating evidence has demonstrated that impairment of the balance between pro- and anti-angiogenic factors facilitates endothelial dysfunction, delays endovascular revascularization, and ultimately leads to tissue necrosis [6].

Various genes and signalling participates in endothelial angiogenesis, while the vascular endothelial growth factor (VEGF) signalling occupies a central position. The VEGF family now consists of seven

members, as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF (placenta growth factor) and snake venom VEGF [7,8]. VEGF-A, produced by alternative splicing, selectively binds to its cell-surface receptors including VEGF receptor 1 (VEGFR1) and VEGFR2 on endothelial cells [9,10]. This binding dimerizes and phosphorylates the receptor, activates the downstream pathways and mediates endothelial growth, proliferation, migration and survival. Because of abnormal vascular development, VEGF-A-knockout mice are lethal embryonically [11,12]. Although VEGF-A is well known to regulate angiogenesis, the detailed mechanisms of this regulation remain poorly understood.

FAM3 gene, a family of cytokine-like genes, has four identified members: FAM3A, FAM3B, FAM3C and FAM3D [13,14]. All members contain a predicted secondary structure of a four-helix bundle, a structure that exists in many other cytokines [14]. Most of the previous research on FAM3 family are focused on FAM3B, which is highly expressed in pancreatic islets and promotes the progression of glucose and lipid metabolism [15]. Recently, the biological role of FAM3A has been gradually revealed. Forced expression of FAM3A significantly alleviates hyperglycaemia and insulin resistance [16], or aggravates neointimal

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Research in context

Evidence before this study

Peripheral arterial disease is a major cause of limb loss and its prevalence is increasing worldwide. As most standard-of-care therapies yield only unsatisfactory outcomes, more options are needed. FAM3A, a highly conserved and broadly expressed protein that is part of the heterologous FAM3 protein family, has been involved in the multiple diseases; like hyperglycaemia, insulin resistance and vascular remodelling.

Added value of this study

In the present study, we demonstrate that overexpression of FAM3A promotes, but depletion of FAM3A suppresses, endothelial tube formation, proliferation, migration, blood perfusion and capillary density. Furthermore, we find that mitochondrial FAM3A increases ATP production and secretion. ATP binds to P2 receptors, which upregulates cytosolic free Ca²⁺ levels, and then activates VEGFA transcription by phosphorylation of CREB, finally leading to endothelial angiogenesis.

Implications of all the available evidence

Our study reveals that FAM3A can accelerate angiogenesis through activation of VEGFA transcription in ischemic tissue, suggesting that FAM3A may constitute a novel molecular therapeutic target for ischaemic vascular disease.

formation after vascular injury [17]. More importantly, the FAM3A protein has been discovered to be a novel mitochondrial protein that promotes ATP production and attenuates mitochondrial dysfunction [16]. However, the role for FAM3A in endothelial dysfunction and mitochondria abnormalities in the vasculature has not been investigated. Here, we present the first data suggesting that ectopic FAM3A induces a protective effect on endothelial angiogenesis by increasing VEGFA transcription.

2. Materials and methods

2.1. Murine model of hind limb ischaemia

Wild-type C57BL/6J male mice (6 weeks old) were purchased from Beijing HFK Bio-technology Co., Ltd., and housed in specific pathogen-free facilities. All animal work used in this study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, and conformed to the Guide for the Care and Use of Laboratory Animals. Mice were anaesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg), and fixed on the back. A 1 cm operative incision, vertical to the inguinal ligament in the right hind limb, was exposed, and the external iliac artery was clearly isolated and ligated with the 8/0 polypropylene suture (Premilene, Melsungen AG). Blood perfusion in the footpad was assessed by a laser Doppler system (Perimed AB, Sweden) preoperatively, postoperatively and on days 3, 7 and 14. The perfusion rate in the right footpad was normalized to the left limb [18].

2.2. Immunofluorescence

Mice were killed 14 days after surgery and the tibial anterior muscles from the ischaemic side were cryosectioned in 4 µm increments for immunostaining with anti-vWF antibody (ab6994; Abcam), CD31 (ab24590; Abcam), NG2 (ab129051; Abcam), Flag (ab1257; Abcam)

and Myosin (ab3481; Abcam), followed by incubation with the indicated secondary antibodies (1:400, ThermoFisher). Assessment of the capillary densities was analysed by counting 10 random (magnification ×100) fields and was expressed as the positive CD31⁺ or NG2⁺ area which were normalized by fiber density (Per field).

2.3. Cell culture

Primary human umbilical vein endothelial cells (HUVECs), purchased from Lonza (Allendale, NJ), were maintained in endothelial basal medium (EBM) with EGM-2 Single Quots (Lonza, Allendale, NJ) as previously described. For cultured cells in defined normoxia (21% O₂) or hypoxic conditions (1% O₂), a modular incubator chamber (Forma Scientific) was used. CoCl₂ was purchased from Sigma-Aldrich and added to the media (200 µM) for different periods of time (0, 6, 12 and 24 h).

C2C12 myoblasts (from ATCC) were grown in DMEM medium plus 10% FBS (Sigma) at subconfluent density. Myogenic differentiation is enhanced in low-passage cells when the medium is supplemented with 2% horse serum (Sigma).

2.4. Protein extracts and Western blot assay

Western blot analysis was performed to detect the related protein levels [19,20]. Tissues or cells were homogenized, boiled, separated by SDS-PAGE electrophoresis and transferred to PVDF membranes. Blots were probed with appropriate primary antibodies against FAM3A (MA5-24107, ThermoFisher; HPA056991, SAB4300968, Sigma), HIF1α (ab51608, Abcam), VEGFA (ab51745, Abcam), CREB (9197, Cell Signalling Technology (CST)), pCREB (Ser133) (9198, CST), COXIV (ab14744, Abcam), PPARγ (ab45036, Abcam), Flag (ab205606, Abcam) and β-actin (3700, Cell Signalling Technology) at 4 °C overnight. After washing, the membrane was incubated with the appropriate secondary antibodies. Image signals were detected using Image Lab software.

2.5. Real-time PCR

Total RNA from mouse tissues or cells was extracted with TRIzol reagent (Takara, China) and transcribed into cDNA using a PrimeScript™ RT Reagent Kit (Takara) following manufacturer's instructions. The reaction mixture was taken as the template to perform real-time PCR (StepOne Plus Real-Time PCR System). The following primers were applied: 18 sRNA: forward (F): 5'-GTAACCCGTTGAACCCATT-3', reverse (R): 5'-CCATCCAATCGGTAGTAGCG-3'. FAM3A: F: 5'-TTCGTGGCCTACGACG-3', R: 5'-GCGGGATACAGCCTTCCATC-3'. VEGFA: F: 5'-AGGGCAGAATCATCACGAAGT-3', R: 5'-AGGGTCTCGA TTGGATGGCA-3'.

2.6. Adenovirus and adeno-associated virus construction

Adenoviruses encoding Null, FAM3A, Scr shRNA and FAM3A shRNA were applied to infect HUVECs in vitro, while the rAAV system (type 2) containing Null, FAM3A, Scr shRNA and FAM3A shRNA was employed to transduce endothelial cells in vivo via tail vein injection. Adenoviruses were provided by Vigene Biosciences, and AAVs were provided by Biowit Technologies.

2.7. Transwell migration assay

Migration assays were performed using a modified Boyden chamber (Corning, NJ) [21]. HUVECs (5 × 10⁴ cells/ml) were placed in the upper chamber with EBM in the lower chamber, and allowed to migrate through the pores of the membrane for 6 h. The residual cells in the upper chamber were wiped, and the migrated cells in the lower surface were stained with 0.2% crystal violet for 15 min. Stained cells were then counted under a light microscope. Cell migration is presented as the number of migrated cells/field.

2.8. Tube formation assay

Tube formation *in vitro* was assayed using the Corning Matrigel [22]. The 24-well culture plates were first coated with Matrigel (400 μ l/well). HUVECs (2×10^4 cells/well) were then seeded in the plates and incubated at 37 °C for 6 h. Tube formation was observed using an inverted microscope (Olympus IX73, Japan). Five randomly selected fields were photographed in each group. Tube length was calculated by drawing a line along each tube and measuring the length of the line in pixels using the ImageJ software. The average of five fields was taken as the value for each treatment.

2.9. EdU staining

For EdU *in vitro* experiment, after treatment, HUVECs were incubated with EdU solution for 2 h, and then subject to the staining using the Cell-Light EdU Proliferation Kit (RiBoBio Co. Ltd). For EdU *in vivo* experiment, each surgical mice was injected intraperitoneally with 50 μ g EdU per gram body weight 2 h before sacrifice.

2.10. siRNA-mediated gene knockdown

HUVECs were transfected with VEGFA siRNA (sc29520, Santa Cruz), CREB siRNA (sc29281, Santa Cruz), or scrambled (Scr) siRNA (sc-37007, Santa Cruz) using Lipofectamine RNAi MAX Reagent (13778150, Thermo Fisher Scientific) according to the protocol.

2.11. Mitochondria isolation

The Mitochondria/Cytosol Fractionation Kit (Pierce) was applied to isolate mitochondria from HUVECs according to the manufacturer's recommendation. Fractionated lysates were then extracted and subject to western blot.

2.12. Oxygen consumption rate (OCR)

An XFe24 extracellular flux analyser (Seahorse Biosciences, MA) was used to measure oxygen consumption rates [23]. Briefly, thirty thousand of treated endothelial cells were seeded in a provided 24-well plate in the CO₂ incubator. One hour before measurements, cells were removed and incubated at 37 °C in a normal atmosphere. The medium was then replaced with FX assay medium. Stock solutions of oligomycin (1 μ M at final concentration), FCCP (0.5 μ M at final concentration) and rotenone/antimycin A (0.5 μ M/0.5 μ M at final concentration) in an XF Cell Mito Stress Test Kit were prepared in FX assay medium and loaded into indicated injection ports respectively. Measurements were obtained at 37 °C. Assay cycles included 3 min of mixing, 2 min of waiting period, and 3 min of measurement. The Wave software provided by Seahorse Biosciences was used for data analysis. Basal respiration (Mean of Stage A – Mean of Non-mitochondria respiration (Stage D)), maximal respiration (Mean of Stage C – Mean of Non-mitochondria respiration (Stage D)) and ATP production (Mean of Stage A – (Mean of Non-mitochondria respiration + proton leak) (Stage B)) were determined by the XF Cell Mito Stress Test Generator that was provided by Seahorse Bioscience.

2.13. ATP content detection

ATP-Lite Assay Kit (Vigorous Biotechnology) was used to detect ATP content from cultured cells and cell medium. The ATP content was measured and normalized against the protein concentration and presented as a percentage of the control group.

2.14. Measurements of calcium concentration

HUVECs were loaded with 1 μ M Fluo-8 AM for 15 min, and then imaged by an Olympus ix73 fluorescence microscope. The ratio of the emission densities (F340/F380) reflects the intracellular free calcium concentration.

2.15. Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed using the Pierce™ Agarose ChIP Kit (Thermo Scientific). Briefly, treated HUVECs were cross-linked, harvested, and sonicated to obtain DNA fragments between 500 and -1000 bp. The supernatants were incubated with antibodies against IgG or CREB and rotated overnight at 4 °C. After washing, DNA was pulled down and purified using the QIAquick PCR purification kit (Qiagen). Purified DNA was analysed by real-time PCR with specific primers for the VEGFA promoter. F: 5'-GCGTGTCTCTGGACAGAGTTTCCGG-3', R: 5'-CAGCGGAGCTCAGCCCTCCACACGC-3'.

2.16. Luciferase activity assay

The pGL3 Luciferase Reporter Vectors (Promega, Madison, WI) were used for constructing luciferase vectors. A series of VEGFA promoter deletions (pGL-VEGFA-200, pGL-VEGFA-400, pGL-VEGFA-600, pGL-VEGFA-800, and pGL-VEGFA-1000) were constructed. The putative binding site of CREB, which is located at -250 bp of the human VEGFA promoter, was deleted by site-directed mutagenesis using a QuikChange II Kit (Stratagene, La Jolla, CA). HUVECs were transfected with the plasmids as indicated. The luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

2.17. Data analysis

Values are shown as the means \pm S.D. of at least three independent experiments. Homogeneity of the variance was assessed by the F test (two groups) or the Brown-Forsythe test (\geq three groups). For comparing two groups, the statistical significance of differences was analysed by Student's *t*-tests. For comparing more than two means, one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis was employed. Values of *P* < .05 were considered statistically significant.

3. Results

3.1. Endothelial FAM3A expression is decreased under hypoxia

In ischaemic tissues, hypoxia is thought as a pathological phenomenon to activate angiogenesis [24]. To explore FAM3A expressions under hypoxia in endothelial cells *in vitro*, HUVECs were exposed to hypoxia conditions (1% O₂). Hypoxia obviously inhibited the protein and mRNA levels of FAM3A in a time-dependent manner, with concomitantly upregulated HIF1 α expression (Fig. 1a–b). Similarly, treatment of HUVECs with cobalt chloride (CoCl₂) also inhibited FAM3A levels in a time-dependent manner (Fig. 1c–d), indicating that hypoxia downregulates FAM3A expression in endothelial cells.

3.2. FAM3A overexpression promotes angiogenesis

Previous data have suggested that hypoxia could downregulate the expression of FAM3A in endothelial cells, so we went on assessing whether manipulating FAM3A level could regulate angiogenesis *in vitro* and *in vivo*. HUVECs were infected with adenovirus containing FAM3A or Null, and the efficiency of FAM3A overexpression was verified by western blot assay (Fig. 2a). In tube formation, as shown in Fig. 2b, ectopic FAM3A significantly enhanced the tube formation compared with that in the Ad-Null group. Since endothelial migration and

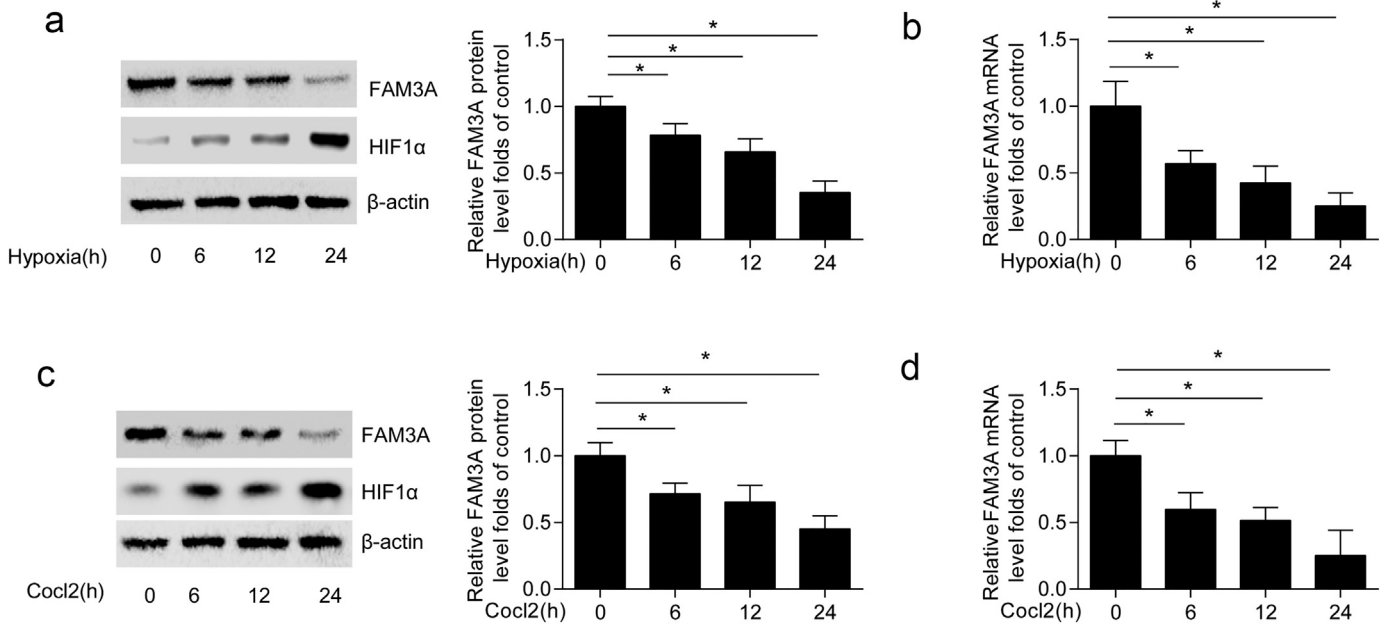


Fig. 1. Hypoxia inhibits FAM3A expression in HUVECs. (a) HUVECs were cultured in normoxia (21% O₂) or hypoxic (1% O₂) conditions for 6 h, 12 h, 24 h, and FAM3A and HIF1 α protein levels were detected by western blot assays. The densitometric analysis of FAM3A level was shown. (n = 5 per group) (b) The FAM3A mRNA level was detected via real-time PCR. (n = 5 per group) (c) HUVECs were treated with CoCl₂ (200 μ M) for 0 h, 6 h, 12 h, 24 h, and FAM3A and HIF1 α protein levels were detected by western blot. (n = 5 per group) The densitometric analysis of FAM3A was shown. (d) The mRNA level of FAM3A expression was tested through real-time PCR. (n = 5 per group). Data were analysed with one-way ANOVA. Data are represented by mean \pm S.D. *P < .05.

proliferation represent important steps in the formation of new blood vessels [25], we next evaluated HUVEC proliferation by an EdU assay. Ad-FAM3A-infected HUVECs incorporated more EdU than the corresponding Ad-Null control (Fig. 2c). To evaluate HUVEC migration, we performed a modified Boyden chamber migration assay. FAM3A overexpression consistently increased the number of HUVECs migrating through the membrane (Fig. 2d). These data indicate that overexpression of FAM3A can accelerate endothelial angiogenesis through enhanced tube formation, migration and proliferation.

To determine whether FAM3A overexpression affected ischaemia-induced angiogenesis *in vivo*, adeno-associated virus 2 (AAV2) carrying FAM3A or Null was transduced into mice by tail vein injection after a murine hind limb ischaemia operation. Endothelial cells from ischaemic adductor tissue were purified using a CD31/PECAM1 antibody and then subjected to real-time PCR assay. AAV2-FAM3A dramatically increased the mRNA level of FAM3A (Fig. 2e). Immunostaining also showed that AAV2-carrying FAM3A heavily accumulated in CD31⁺ endothelial cells or NG2⁺ pericyte cells but not Myosin⁺ skeletal myocytes in ischaemic adductor tissue (Fig. S1a-c). At 14th day after surgery, the blood flow ratio recovered to 0.55 in AAV2-Null-transduced mice, but in AAV2-FAM3A-transduced mice the blood flow ratio returned to 0.9, showing a marked improvement in perfusion recovery in AAV2-FAM3A-transduced mice (Fig. 2f-g). Furthermore, HE staining showed that FAM3A overexpression attenuated tissue degeneration to some degree and maintained the large muscle fibrils (Fig. 3h). Overexpression of FAM3A promoted more CD31⁺ endothelial cells and NG2⁺ pericyte cells to accumulate in ischaemia regions, thus increasing the related capillary density (Fig. 2i-j). We also evaluated EdU/vWF-positive cells in ischaemic tissues by immunostaining. There were more noticeable EdU/vWF-positive capillaries surrounding the skeletal muscle fibres compared with that in control mice (Fig. 2k). Thus, FAM3A promotes endothelial angiogenesis and ischaemic recovery.

3.3. FAM3A deficiency inhibits angiogenesis

Since FAM3A overexpression promoted hypoxia-induced endothelial angiogenesis, we then sought to explore the effects of FAM3A

ablation on endothelial angiogenesis. In HUVECs, FAM3A knockdown markedly reduced the expression of FAM3A (Fig. 3a), thus retarding the formation of tube-like structures compared with the scrambled group (Fig. 3b). FAM3A deficiency by adenovirus also significantly reduced HUVECs proliferation as assessed by the EdU assay. The number of EdU-positive cells was lower than that of Scr shRNA-infected cells (Fig. 3c). Consistent with these findings, the Transwell assay revealed that the number of HUVECs migrated through the barrier in the Ad-FAM3A shRNA group was less than that in the Ad-Scr shRNA group (Fig. 3d). We further investigated the role of FAM3A deficiency in angiogenesis in a hind limb ischaemia model. AAV2 carrying FAM3A shRNA successfully inhibited the expression of FAM3A in CD31-positive endothelial cells from ischaemic tissues (Fig. 3e). The blood flow ratio resumed to 0.6 in the control mice but only returned to 0.2 in the AAV2-FAM3A shRNA-transfected mice (Fig. 3f-g), indicating FAM3A knockdown lead to an obvious impairment in perfusion recovery. HE staining for ischemic limbs showed FAM3A knockdown aggravated tissue degeneration, compared with Scr shRNA group (Fig. 3h). In addition, knockdown of FAM3A resulted in fewer CD31⁺ endothelial cells or NG2⁺ pericyte cells being recruited to ischaemia regions (Fig. 3i-j). We also detected EdU/vWF-positive cells in ischaemic tissues by immunostaining, which showed a significant reduction in EdU/vWF-positive capillaries surrounding the ischaemic muscle (Fig. 3k), all suggesting that endothelial FAM3A deletion disrupts perfusion recovery and angiogenesis.

3.4. FAM3A regulates VEGFA expression

We next explored the effects of FAM3A on angiogenesis-related genes' alterations in endothelial cells. Angiogenesis PCR array showed that the expression of several growth factors and receptor genes were dramatically upregulated in FAM3A-overexpressing HUVECs, especially the gene VEGFA, a major growth factor for the angiogenesis process [26] (Fig. 4a). To confirm that the VEGFA expression pattern was specific to FAM3A in endothelial cells, PCR and western blot were performed. Similar to the PCR array results, the mRNA and protein levels of VEGFA were all suppressed in FAM3A-deficient HUVECs (Fig. 4b-c). Consistently,

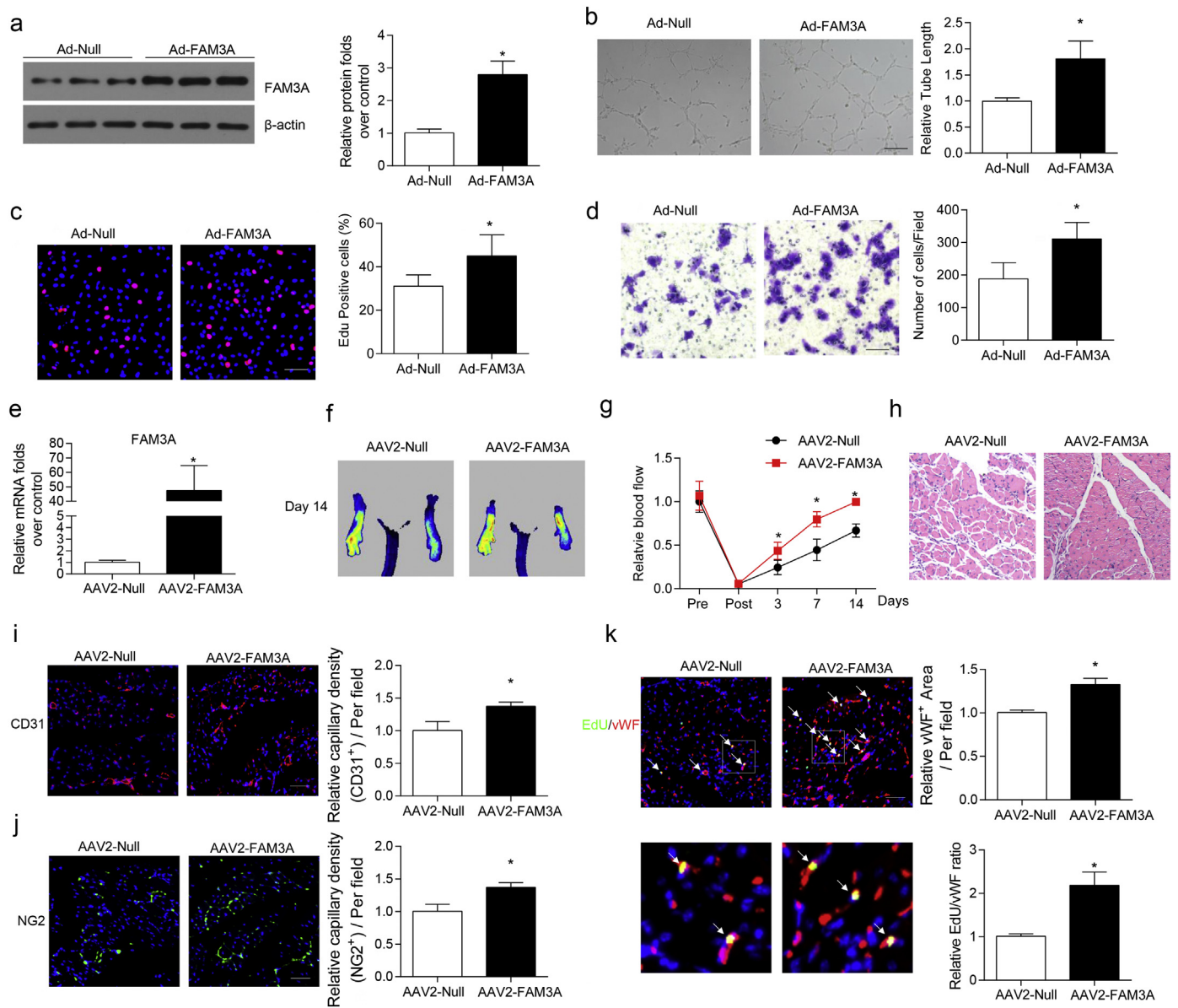


Fig. 2. Endothelial FAM3A promotes endothelial angiogenesis. (a) HUVECs were infected with Ad-Null or Ad-FAM3A for 24 h, and the FAM3A protein level was measured by western blot. (b) Ad-Null- and Ad-FAM3A-infected HUVECs were placed in plates coated with Matrigel and tubular structures were photographed. ($n = 5$ per group) (c) The proliferation of Ad-Null- and Ad-FAM3A- infected HUVECs was measured by an EdU staining assay. ($n = 5$ per group) Scale bar, 50 μm . (d) The migration of Ad-Null- and Ad-FAM3A-infected HUVECs was measured by Transwell assays. ($n = 5$ per group) Scale bar, 25 μm (e) Endothelial cells from ischaemic adductor tissue were purified using a CD31/PECAM1 antibody and then subjected to real-time PCR assays to check the mRNA level of FAM3A. (f) The hind limb blood perfusion in the AAV2-Null or AAV2-FAM3A- transduced group were analysed by Laser Doppler imaging. Normal perfusion is indicated by Yellow, and a reduction in blood flow is indicated by Blue. ($n = 8\text{--}10$ per group). (g) The ischaemic: normal hind limb perfusion ratios in AAV2-Null- and AAV2-FAM3A-treated mice at different time were calculated to analysis blood perfusion. (h) H&E staining showed massive muscle degeneration in the ischemic regions. (i-k) The immunofluorescent staining of CD31, NG2 and EdU/vWF in the ischaemic gastrocnemius muscle was performed and quantitatively analysed ($n = 5$ per group). Scale bar, 50 μm . Data were analysed with two-tailed Student's *t*-tests (a-e, h-j) and two-way ANOVA (g). Data are represented by mean \pm S.D. * $P < .05$.

forced expression of FAM3A enhanced the expression of VEGFA in HUVECs (Fig. 4d–e). To further investigate whether VEGFA mediated the regulation of angiogenesis by FAM3A, the VEGFA level was silenced using siRNA in HUVECs. Tube formation assays showed that the improvement in tube length by FAM3A was largely reversed by VEGFA knockdown (Fig. 4f). All of these data suggest that FAM3A upregulates VEGFA and then initiates angiogenesis.

3.5. Endothelial FAM3A overexpression increases cellular ATP levels

FAM3A has been previously reported to be located in mitochondria and involved in mitochondrial function in the liver [16]. Interestingly, we also found that FAM3A was primarily located in the mitochondria

of HUVECs (Fig. S2a–b). Since mitochondria plays a critical role in ATP production, we then examined if FAM3A could regulate mitochondrial function and cellular ATP content in endothelial cells. FAM3A overexpression elevated intracellular and extracellular ATP levels in HUVECs (Fig. 5a), which was contrarily downregulated after FAM3A shRNA infection (Fig. 5b). We next determined whether FAM3A interfered with mitochondrial respirational capacity due to changed ATP content. Overexpression of FAM3A showed significantly higher basal and maximal respiratory capacity compared with that in Ad-Null-infected HUVECs (Fig. 5c), while the normalized oxygen consumption rate (OCR) was decreased after FAM3A depletion compared with Scr shRNA in endothelial cells (Fig. 5d), all indicating that FAM3A promotes ATP production and mitochondrial function in endothelial cells.

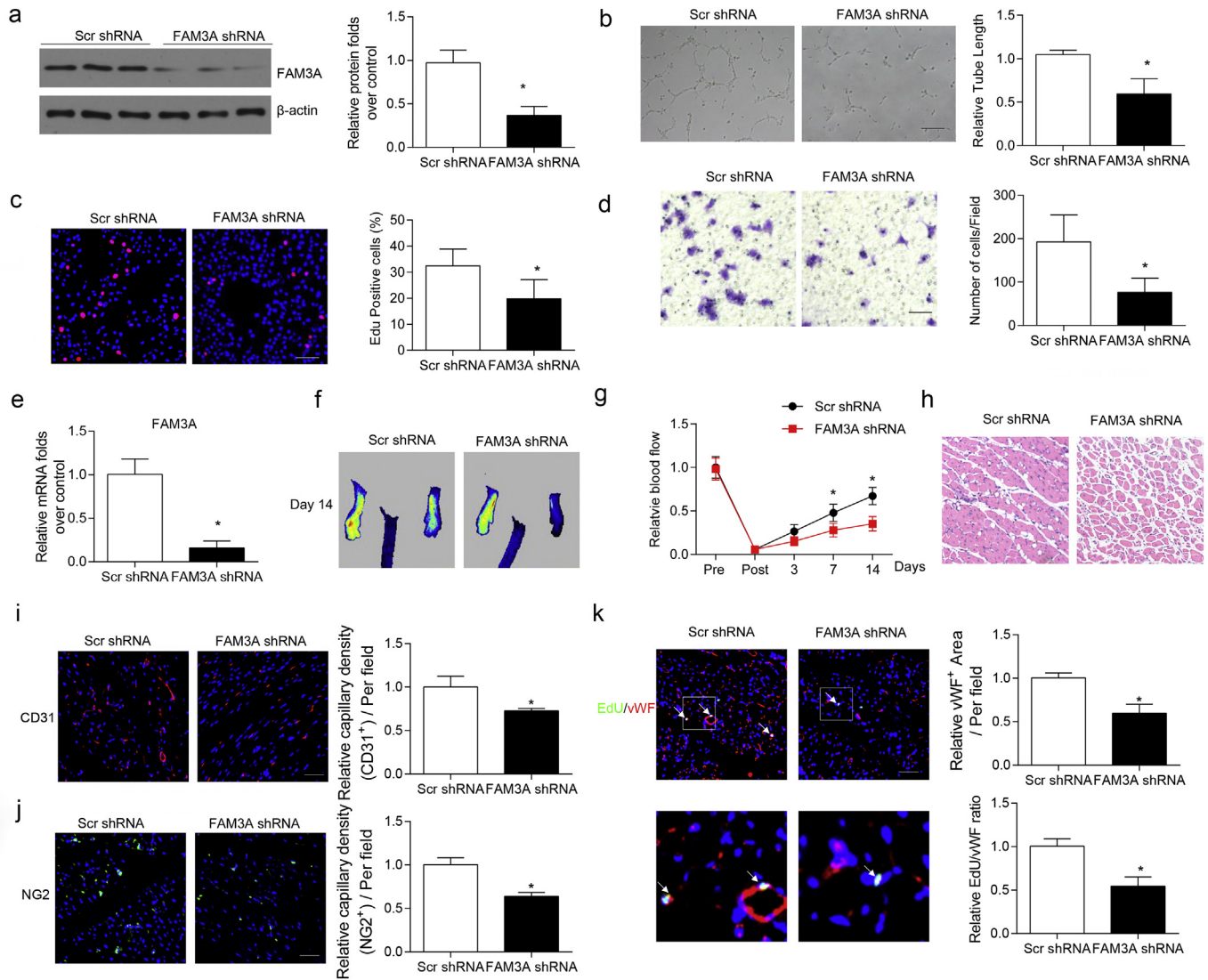


Fig. 3. Endothelial FAM3A knockdown suppresses angiogenesis. (a) HUVECs were infected with adenovirus coding Scr shRNA or FAM3A shRNA for 24 h, and the FAM3A protein level was measured by western blotting. (b) Ad-Scr shRNA- and Ad-FAM3A shRNA-infected HUVECs were placed in plates coated with Matrigel and tubular structures were photographed. ($n = 5$ per group) Scale bar, 50 μ m. (c) The proliferation of HUVECs pre-infected with Ad-Scr shRNA or Ad-FAM3A shRNA was measured by an EdU staining assay. ($n = 5$ per group) Scale bar, 50 μ m. (d) The migration of HUVECs pre-infected with Ad-Scr shRNA or Ad-FAM3A shRNA was measured by Transwell assay. ($n = 5$ per group) Scale bar, 25 μ m. (e) Endothelial cells from ischaemic adductor tissue were purified using a CD31/PECAM1 antibody and then subjected to qRT-PCR assay to check the mRNA expression of FAM3A. (f) The hind limb blood perfusion in the AAV2-Scr shRNA- or AAV2-FAM3A shRNA-transduced group were analysed by Laser Doppler imaging. Normal perfusion is indicated by Yellow, and a reduction in blood flow is indicated by Blue. ($n = 8$ –10 for each group) (g) The ischaemic: normal hind limb perfusion ratios in AAV2-Scr shRNA- or AAV2-FAM3A shRNA-treated mice at indicated time were collected to analysis blood perfusion. (h) H&E staining showed massive muscle degeneration in the ischemic regions. (i–k) The immunofluorescent staining of CD31, NG2 and EdU/vWF in the ischaemic gastrocnemius muscle was performed and quantitatively analysed. ($n = 5$ per group) Scale bar, 50 μ m. Data were analysed with two-tailed Student's *t*-tests (a–e, h–j) and two-way ANOVA (g). Data are represented by mean \pm S.D. * $P < .05$.

3.6. CREB mediates the regulation of VEGFA expression by FAM3A

To address how FAM3A regulates VEGFA expression at both the mRNA and protein level, we constructed a series of VEGFA promoter luciferase reporter plasmids with different lengths, including pGL3 (control), pGL3-VEGFA-200 (–200 to +200 bp), pGL3-VEGFA-400 (–400 to +200 bp), pGL3-VEGFA-600 (–600 to +200 bp), pGL3-VEGFA-800 (–800 to +200 bp), and pGL3-VEGFA-1000 (–1000 to +200 bp), and performed luciferase activity assays to detect which fragment was involved in its transcriptional regulation. Interestingly, FAM3A overexpression markedly enhanced the luciferase activity of four of the plasmids (pGL3-VEGFA-1000, pGL3-VEGFA-800, pGL3-VEGFA-600, and pGL3-VEGFA-400), but not the activity of pGL3 (control) or pGL3-VEGFA-200, indicating that the region essential for VEGFA regulation is located between –400 and –200 bp (Fig. 6a). We screened and

identified one consensus CREB binding element candidate (<http://gene-regulation.com>). CREB, a ubiquitous transcription factor, has been previously reported to promote the expression of VEGFA [26].

To validate whether CREB mediated the effect of FAM3A on VEGFA promoter activity, we deleted the putative CREB binding site in pGL-VEGFA-1000 to generate pGL-VEGFA-1000- Δ , and found the luciferase activity of this plasmid was sharply abolished, regardless of co-infection with FAM3A adenovirus (Fig. 6a). We then tested whether FAM3A promoted CREB binding to this specific region in the VEGFA promoter. ChIP assays showed that the binding affinity of CREB for the VEGFA promoter was dramatically increased by ectopic FAM3A in HUVECs (Fig. 6b). Collectively, these results indicate that CREB indeed mediates FAM3A-regulated VEGFA transactivation.

Next, we assayed the involvement of CREB in the angiogenesis process mediated by FAM3A. CREB phosphorylation is critical for the

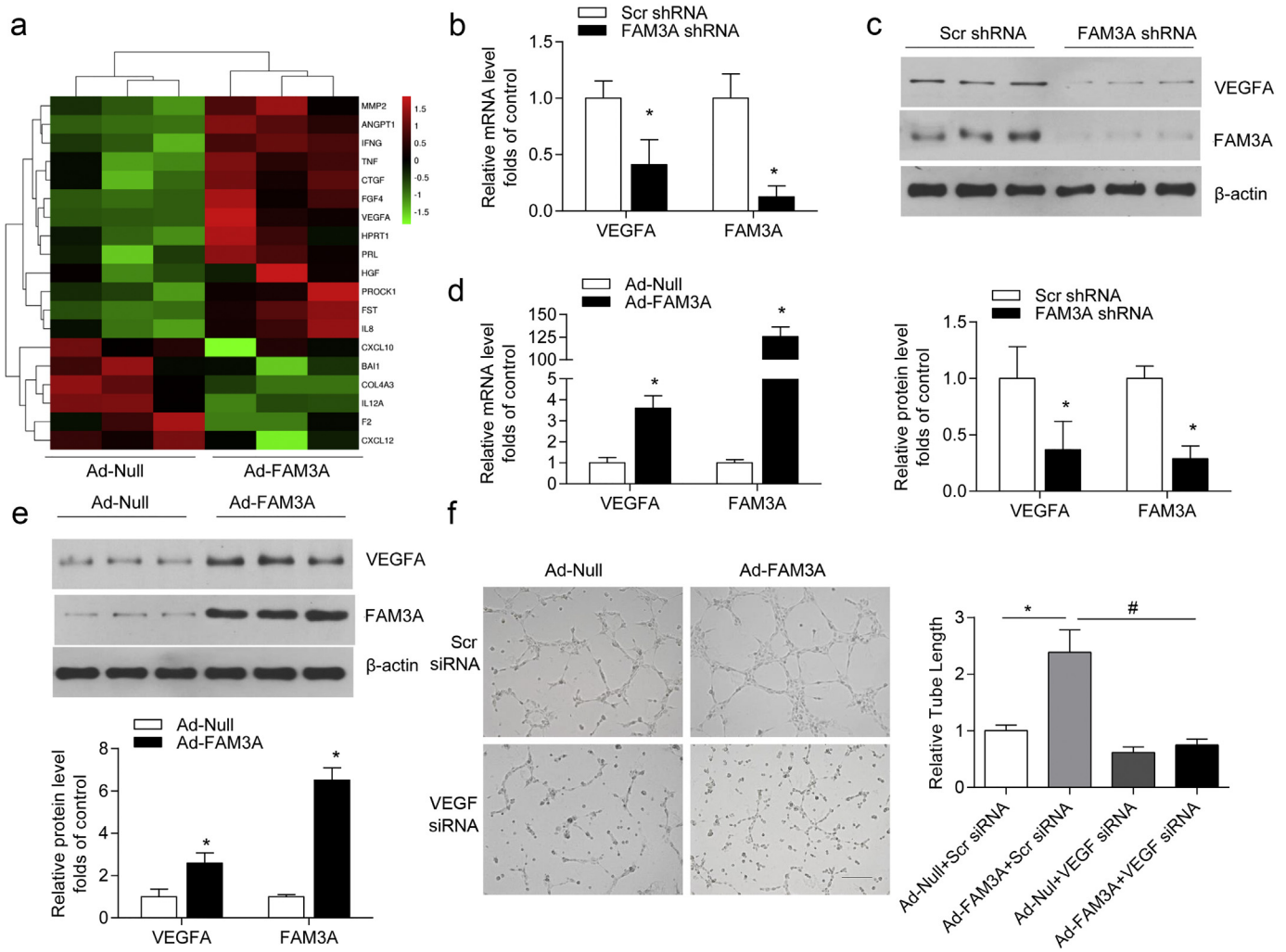


Fig. 4. FAM3A regulates VEGFA expression. (a) Angiogenesis PCR array was performed in HUVECs that were infected with adenovirus containing FAM3A, and a heatmap of related gene expression levels is shown. Unsupervised hierarchical clustering analysis was performed using Ct values. Low expression is shown in green, and red indicates high expression. ($n = 3$ per group) (b–e) HUVECs were infected with adenovirus encoding Scr shRNA, FAM3A shRNA, Null and FAM3A. The VEGFA mRNA and protein levels in HUVECs were determined by real-time PCR ($n = 5$ per group) (b,d) and western blot assays (c, e). (f) HUVECs were first transfected with Scr siRNA or VEGF siRNA for 24 h and then placed in plates coated with Matrigel and tubular structures were photographed. ($n = 5$ per group) Scale bar, 50 μ m. Data were analysed with two-tailed Student's t-tests (a–e) and one-way ANOVA (f). Data are represented by mean \pm S.D. * $P < .05$.

induction of CREB-dependent downstream genes. We observed that overexpression of FAM3A enhanced the phosphorylation of CREB (Ser133) and VEGFA expression (Fig. 6c). When we silenced the CREB level using siRNA in HUVECs, the elevation of VEGFA expression induced by FAM3A was reversed (Fig. 6c). Tube formation assays also suggested that the improvement induced by FAM3A was partially reversed by CREB knockdown (Fig. 6d). Hence, we demonstrated that FAM3A promotes phosphorylation of CREB and recruitment to the VEGFA promoter, thus activating VEGFA transcription and ultimately angiogenesis.

3.7. FAM3A regulates CREB and VEGFA via the ATP/P2 receptor/calcium pathway

We further explored how mitochondrial FAM3A could regulate CREB-dependent VEGFA transcriptional activation. CREB activation is closely related with changes in intracellular Ca^{2+} levels [27]. ATP can function as a signalling messenger that is implicated in multiple biological processes through the ATP/P2 receptor/calcium pathway [28]. We therefore reasoned that the ATP/P2 receptor/calcium pathway might participate in the CREB-mediated VEGFA expression regulation by FAM3A.

To test this hypothesis, we pharmacologically modulated the activity of the P2 receptor in HUVECs that were pre-infected with Ad-Null or

Ad-FAM3A. P2 receptor antagonists, PPADS and suramin, significantly inhibited the FAM3A-induced phosphorylation of CREB and VEGFA expression (Fig. 7a and Fig. S3a). The increased luciferase activity of VEGFA promoter was consistently suppressed by treatment with PPADS (Fig. S4). In addition, PPADS and suramin also abrogated the increased secretion of VEGFA induced by FAM3A in cultured medium (Fig. 7b and Fig. S3b). Both PPADS and suramin could suppress the effect of FAM3A on tube formation (Fig. 7c and Fig. S3c), suggesting that ATP/P2 receptor participates in the regulation of CREB-VEGFA signalling mediated by FAM3A.

Growing evidence indicates that P2X receptors are ligand-gated ion channels, such as the inositol 1,4,5-tri-phosphate receptor (IP3R) that are permeable to calcium, while P2Y receptors are G-protein-coupled receptors that stimulate phospholipase C (PLC) to increase IP3 production, resulting in calcium release from internal stores [29–31]. We observed that forced expression of FAM3A dramatically increased cytosolic calcium levels, which was disrupted by PPADS or suramin (Fig. 7d and Fig. S3d).

We further sought to verify the role of intracellular calcium in the FAM3A-influenced VEGFA expression. IP3R antagonist (2-APB) or PLC inhibitor (U73122) were added to HUVECs pre-infected with Ad-FAM3A. The enhanced phosphorylation of CREB and VEGFA expression

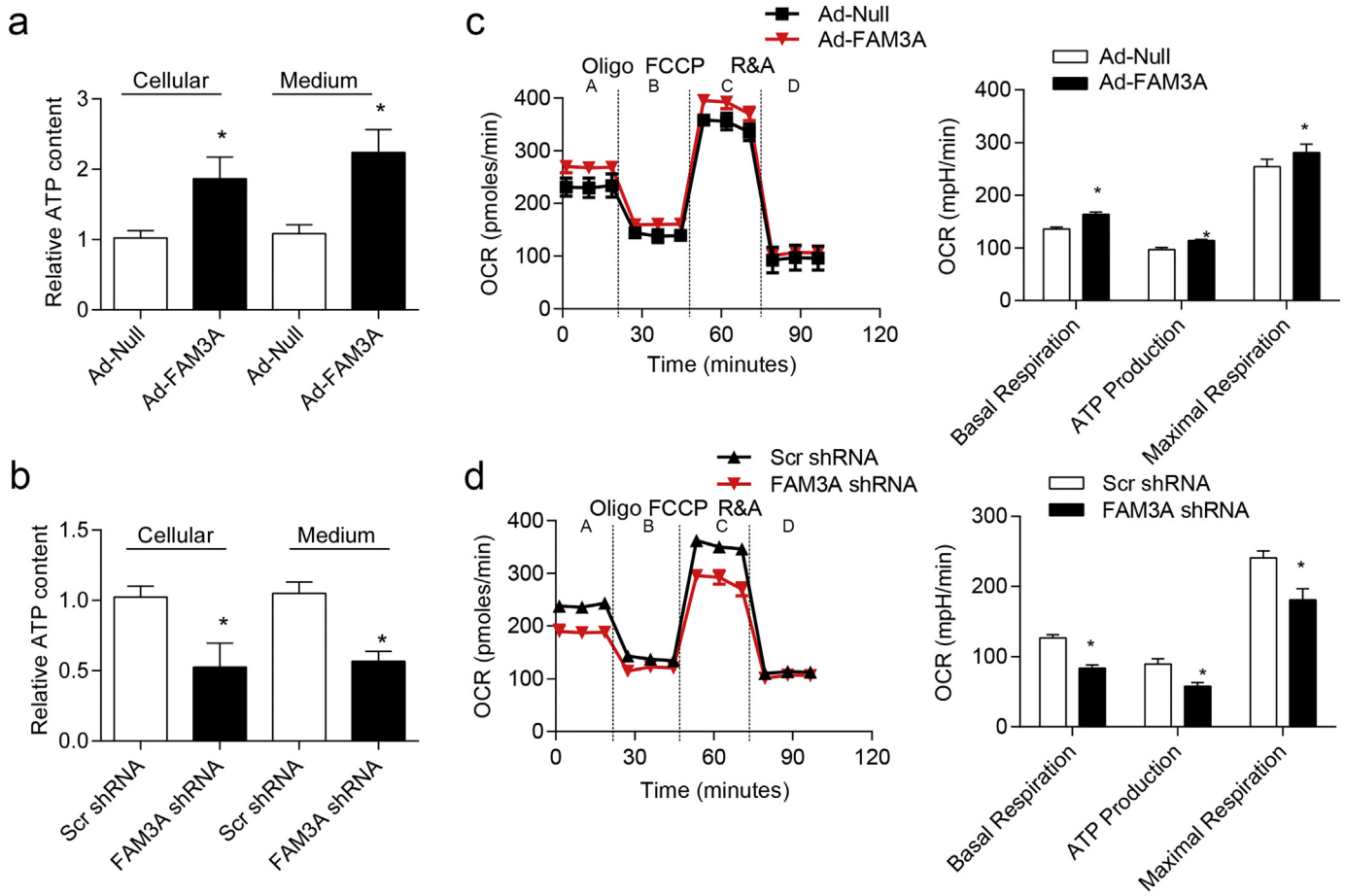


Fig. 5. FAM3A regulates cellular ATP level in HUVECs. (a) HUVECs were firstly infected with adenovirus containing Null or FAM3A for 24 h, and intracellular and extracellular ATP levels were measured by an ATP-Lite Assay Kit. (b) HUVECs were infected with Ad-Scr shRNA or Ad-FAM3A shRNA for 24 h, and cellular ATP levels as indicated were detected by an ATP-Lite Assay Kit. (c, d) HUVECs were infected with the indicated adenoviruses for 24 h. Oxygen consumption ratio (OCR) was measured using a Seahorse extracellular flux analyser. Basic respiration, ATP production and maximal respiration measurements in endothelial cells grown under indicated conditions were evaluated. ($n = 4$ per group). Data were analysed with two-tailed Student's *t*-tests. Data are represented by mean \pm S.D. * $P < .05$.

and secretion by FAM3A were all significantly abolished by exposure to 2-APB or U73122 (Fig. 7e-f and Fig. S5). These results together suggest that FAM3A regulates CREB-dependent VEGFA transcription mainly via the ATP/ P2 receptor/Ca²⁺ pathway.

4. Discussion

In this study, we provided the first evidence that FAM3A expression was decreased in hypoxia-exposed endothelial cells. Elevated FAM3A promoted proper endothelial migration, proliferation and tube formation, and deletion of FAM3A aggravated these aspects of endothelial dysfunction. FAM3A promoted angiogenesis through the positive regulation of VEGFA production and secretion. Mechanistically, FAM3A enhanced mitochondria ATP production, which binds to the P2 receptor and increases intracellular calcium level, then promoted the transcriptional activity of pCREB, and finally accelerated the downstream VEGFA transcriptional activation and expression. These findings thus indicate that endothelial FAM3A has a critical role in regulating VEGFA expression via the ATP/P2 receptor/Ca²⁺/CREB pathway to maintain normal angiogenesis.

FAM3A is a highly conserved and broadly expressed protein that is part of the heterologous FAM3 protein family. Jia et al. reported that in balloon-induced neointimal formation, FAM3A was repressed by upregulation of the prostaglandin E receptor 2 (EP2), leading to the attenuation of excessive migration and proliferation of VSMCs [17]. Wang et al. observed that FAM3A may alleviate high glucose-induced ROS production in HUVECs via the p38 MAPK signalling pathway [32]. Another

research group found that overexpression of FAM3A led to activation of the PI3K-Akt signalling and suppression of hepatic gluconeogenesis and lipogenesis [16]. However, our studies for the first time demonstrated that FAM3A protein played a critical role in angiogenesis. Overexpression of FAM3A increased while knockdown of FAM3A reduced endothelial tube formation, migration and proliferation. Our data strongly support that increased endothelial FAM3A expression could be a new clinical target for ischaemic recovery.

Hypoxia led to decreased levels of FAM3A and concomitantly increased HIF1 α expression, leading us to wonder how hypoxia regulated FAM3A expression. We first targeted HIF1 α and silenced HIF1 α expression using siRNA in HUVECs. The results showed that HIF1 α silencing relieved the suppression of hypoxia on FAM3A expression (Fig. S6a-b). Moreover, the decreased luciferase activity of the FAM3A promoter (-2 kp) in response to hypoxia was also reversed by HIF1 α knockdown (Fig. S6c). Unfortunately, we could not find the consensus HIF1 α -binding motif 5'-(A/G)CGTG-3' in the FAM3A promoter. Zhou Y et al. have reported PPAR γ (peroxisome proliferator-activated receptor- γ) can directly bind to the FAM3A promoter and then trigger its transactivation. (PPAR γ) [16]. In addition, hypoxia represses PPAR gamma 2 promoter activation via the HIF-1/DEC1/Stra13 signalling pathway [33], suggesting that HIF1 α might regulate FAM3A via PPAR γ . Interestingly, our results showed that hypoxia exposure decreased the expression of PPAR γ , which was reverted by HIF1 α knockdown in HUVECs (Fig. S6a-b). We then tested whether hypoxia inhibited PPAR γ binding to the -1258/-1246 bp motif in the FAM3A promoter. CHIP assays showed that under normoxic conditions, PPAR γ

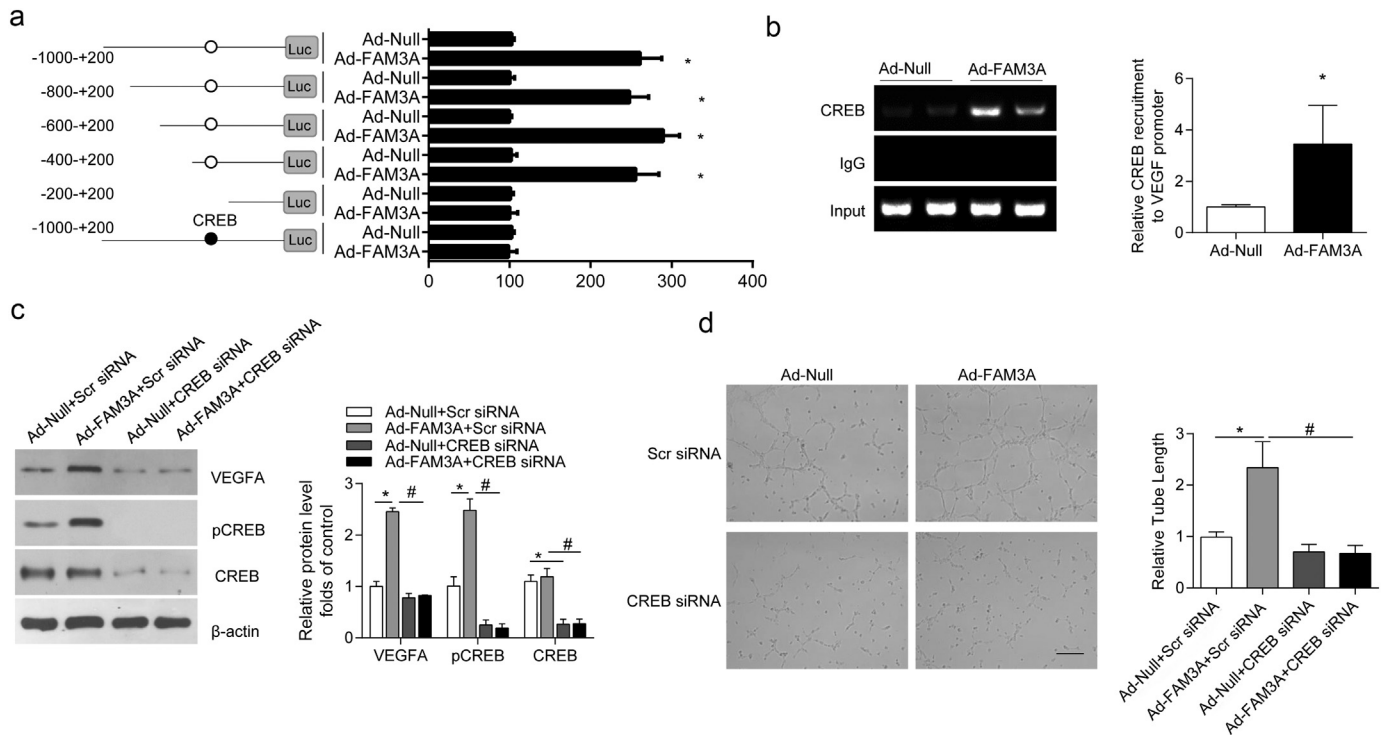


Fig. 6. CREB mediates the effect of FAM3A on VEGFA transcriptional activation. (a) Cartoon depiction of the promoter of VEGFA. The candidates for CREB binding sites in the VEGFA promoter are marked with black circles. For the VEGFA promoter reporter assays, HUVECs, pre-infected with adenovirus encoding Null or FAM3A, were transfected with the indicated plasmids, and luciferase activities were detected 48 h later. (n = 5 per group) (b) The interaction between CREB and the VEGFA promoter under FAM3A overexpression was performed by ChIP assays. (c, d) HUVECs were transfected with Scr siRNA or CREB siRNA for 24 h, and later infected with indicated adenoviruses for another 24 h. (n = 5 per group) (c) Western blot assays were applied to detect VEGFA and pCREB protein levels. (d) HUVECs were placed in plates coated with Matrigel, and tubular structures were photographed. Scale bar, 50 μ m. Data were analysed with two-tailed Student's t-tests (a, b) and one-way ANOVA (c, d). Data are represented by mean \pm SD. *P < .05.

bound to the FAM3A promoter; however, hypoxia dramatically decreased its binding affinity, and this effect was inhibited by HIF1 α silencing (Fig. S6d). Collectively, these results suggest that HIF1 α possibly suppresses FAM3A expression via PPAR γ inactivation under hypoxia in HUVECs.

FAM3A is ubiquitously expressed in all mouse tissues, including the heart, liver and arteries [9,16,34]. It is localized to the mitochondria in cells, not to the nucleus or plasma membrane. A recent study indicated that overexpression of FAM3A alleviated mitochondrial swelling and mitochondrial Ca²⁺ disruption after tunicamycin treatment [34]. We also discovered that ectopic FAM3A increased the intracellular calcium level. More importantly, we further exhibited that FAM3A overexpression released ATP that functioned as a signalling molecule to activate the Ca²⁺-mediated signal transduction by way of ATP receptors. A recent study reported that ATP can bind to VEGF, which then induces a conformational change in the secondary structure [35]. They also emphasized that purinergic signalling by ATP via P2 receptors could be excluded in the ATP-mediated function of VEGF [35]. However, our results showed that FAM3A-induced VEGFA expression was dependent on the ATP and ATP receptors. Inhibition of the ATP receptor could abrogate FAM3A-induced VEGFA expression and tube formation. However, the mechanism how FAM3A promotes mitochondrial ATP synthesis in endothelial cells needs further investigation.

Mitochondrial oxidative respiration is required for VEGF-induced angiogenesis [36]. FAM3A overexpression could enhance mitochondria function and ATP production, which could directly supply enough energy for the migration and proliferation of endothelial cells [37,38]. Here, our data demonstrated that ATP is not only an energy supplier, but it can also function as a messenger that binds to the P2 receptor on the membrane and triggers intracellular signalling activation. When we applied P2 receptor inhibitors, PLC inhibitors or IP3R antagonists, the enhanced tube formation induced by FAM3A was abolished, suggesting that the ATP/ P2 receptor/Ca²⁺/CREB/

VEGFA pathway is indeed involved in the regulation of angiogenesis by FAM3A. This reveals a novel pathway that controls the connection between mitochondrial function and nuclear VEGFA transcription. It has been reported that VEGF could promote mitochondrial ATP production [36], which might possibly form a positive feedback loop. Taken together, these data elucidate that FAM3A can switch on this positive feedback control and combine metabolism with transcriptional regulation, which may generate a sufficient angiogenic output for ischaemic regions.

It has been reported that under ischemic condition, most of the pro-angiogenic factors are also produced from the surrounding ischemic tissue, like skeletal myocytes. These growth factors could affect the VEGFR, AKT signalling in the endothelial cells, and then facilitate process of angiogenesis. We measured VEGF levels in the ischemic muscles and serum, there were no significant difference in serum samples after AAV2-FAM3A infection, but FAM3A overexpression increased the local VEGF levels in ischemic tissues (Fig. S7). Besides, our results demonstrated that ectopic Flag-FAM3A in vivo was majorly located in endothelial cells or pericytes, not skeletal myocytes (Fig. S1). In vitro, FAM3A also did not interfere with the expression of VEGF in muscle cells (Fig. S8c). These data suggested that effect of AAV2-mediated FAM3A on angiogenesis is not directly via skeletal myocytes. Next, we wonder whether the message from ECs to skeletal myocytes could alter the VEGF expression in skeletal myocytes. Co-cultured system was used to investigate the communication between endothelial cells and skeletal myocytes. After co-culture with medium from Ad-Null/Ad-FAM3A-infected endothelial cells, there were no any changes in expression of VEGF from the skeletal myocytes (Fig. S8a-b). We also applied extracellular ATP to hypoxia-exposed C2C12 skeletal muscle cells, and found that the cell viability had no difference compared with vehicle (Data not shown). These data strongly suggested that skeletal myocytes-derived VEGF were not involved in positive regulation of FAM3A on angiogenesis. The effect of FAM3A on angiogenesis may be the local effect

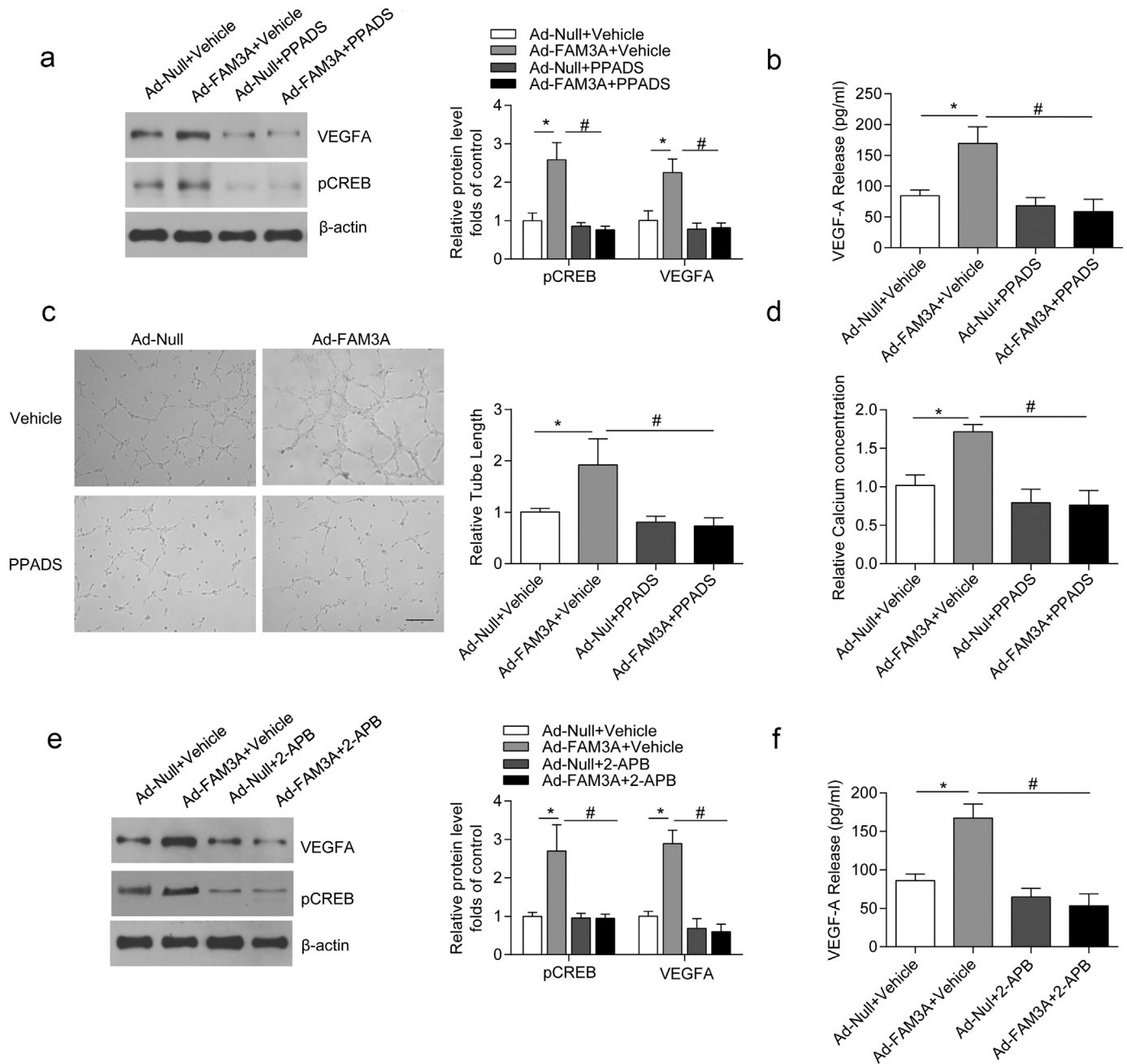


Fig. 7. FAM3A regulates pCREB and VEGFA via the ATP/P2 Receptor signalling pathway. (a–d) HUVECs, pre-infected with adenoviruses containing Null or FAM3A, were exposed with PPADS (50 μ M). (a) Western blot assays were used to determine the expression of pCREB and VEGF. (b) The VEGF level in the supernatant of the HUVECs was determined by ELISA. (c) HUVECs were placed in plates coated with Matrigel, and tubular structures were photographed. Scale bar, 50 μ m. (n = 5 per group) (d) The intracellular calcium level was determined by a calcium sensitive probe Fluo-8 AM. (e, f) HUVECs, pre-infected with indicated adenoviruses, were exposed with 2-APB (10 μ M). (e) Western blot assays were used to determine the pCREB and VEGF protein levels. (f) The VEGF level in the cultured cell medium was tested by ELISA. (n = 5 per group). Data were analysed with one-way ANOVA. Data are represented by mean \pm S.D. *P < .05.

of VEGFA on ischemic region via autocrine and paracrine function of endothelial cells.

CREB is a Ca^{2+} -sensitive transcriptional factor regulating expressions of many genes, such as VEGF [39]. Multiple Ca^{2+} entry pathways may contribute to CREB activation in endothelial cells. The phosphorylation of CREB serine-133 leads to CREB activation, and augments CREB-dependent gene transcription [40]. We discovered here the phosphorylation level of CREB at Ser133 site was significantly upregulated in FAM3A-overexpressed HUVECs. Importantly, the activation of CREB was due to the increased level of intracellular calcium. The chromatin immunoprecipitation and luciferase activity assays further showed that FAM3A enhanced CREB binding to

the VEGFA promoter and promoted its transcriptional activation. Interestingly, previous studies have reported that the binding of VEGF to the VEGFR2 tyrosine kinase can induce phosphorylation of CREB on serine 133, and in turn, this increases CREB DNA binding and transactivation [40]. These results may unveil a reciprocal link between VEGFA and CREB in endothelial cells. This positive feedback control might enhance the sensitivity of the regulation of VEGFA by FAM3A and produce a sufficient angiogenic output under weak VEGF stimulation.

In conclusion, we report a direct role for FAM3A in angiogenesis. The positive effects of FAM3A on angiogenesis appear to be dependent on the increased expression and secretion of VEGFA. Taken together,

these data identify FAM3A might be a potential therapeutic target for vascular complications.

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Declaration of interests

The authors declare no competing interests.

Author contributions

C. Wang and W.J. Xu contributed to the design of research; M.L. Liang also contributed to the design of research and performed the data analysis; C. Wang and W.J. Xu guided the cellular experiments; W.J. Xu, Y.Q. Zhang and C. Wang conducted the animal experiments. C. Wang and W.J. Xu wrote the manuscript; K. Huang edited the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.03.038>.

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