MATERIALS AND METHODS

Mouse breeding and genotyping

The use of experimental animals was approved by the IACUC at Georgia Regents University and Texas A&M University in accordance with NIH guidelines. The floxed PFKFB3 mice were generated by Xenogen Biosciences Corporation (Cranbury, NJ, USA). Briefly, BAC clone containing the designated mouse PFKFB3 gene was first isolated, confirmed and characterized. A conditional gene targeting construct (2 Frt/2loxP construct), as shown in Fig. 3A, was generated. C57BL/6 ES cells were transfected with the targeting construct DNA for the gene target in the presence of G418. After screening, G418 resistant clones were analyzed with independent southern blot analysis with 5', 3' and Neo probes in order to confirm homologous recombination clones. The confirmed ES clones were injected into up to a total of two hundred blastocysts. The blastocysts were then injected into pseudo-pregnant females to generate chimeras. Chimeric mice were bred with wild type mice and F1 generation with germline transmission of mutant allele was confirmed through tail DNA PCR and/or Southern blot analysis.

Cell culture and treatments

HUVECs (ATCC), at passage of 5-8, were cultured in endothelial growth medium 2 (EGM-2; Cambrex).. In some experiments, 10 mM sodium lactate (Sigma) dissolved in PBS, 4 μg/ml AKT activator II (EMD; Millipore) dissolved in DMSO, or 100 ng/ml hVEGF (R & D systems) was added to the culture medium. B16 tumor cells were routinely cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. For the experiments requiring

hypoxia, HUVECs were placed in a modular incubator chamber (Thermo Scientific) and incubated with 0.5% oxygen.

Adenoviral transduction of HUVECs

The GFP-labeled-PFKFB3 shRNA adenovirus targeting the 3' UTR sequence of human PFKFB3 and the control adenovirus were constructed by Vector Biolabs. The adenovirus containing the full-length coding DNA sequence of PFKFB3 and the relative control were constructed by Applied Biological Materials These adenoviruses were expanded inside HEK293 cells, and the virus concentration was determined using an Adeno-X^{TM} rapid titer kit (Clontech). HUVECs at 80% confluence were transduced with the adenovirus (10 pfu/cell) and were used for experiments 36 h after the transduction.

Cell number analysis

For HUVEC counting, HUVECs were seeded in 12-well plates in triplicate at an equal density, and cell numbers were manually counted at indicated days with a hemocytometer.

Quantitative real time RT-PCR (qRT-PCR) analysis

The total RNA from HUVECs or from retinas was extracted with a RNeasy Mini Kit (Qiagen) and qRT-PCR was done as described previously.[1] Briefly, a 0.5-1 μg sample of RNA was utilized as a template for reverse transcription using the iScriptTM cDNA synthesis kit (Bio-Rad). qRT-PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems) with the respective gene-specific primers listed in Supplemental Table 1. All samples were amplified in duplicate, and every experiment was repeated independently twice. Relative gene

expression was converted using the $2^{-\triangle\triangle ct}$ method against the internal control 18S ribosomal RNA for human RNA and hypoxanthine phosphoribosyltransferase 1 (HPRT) for mouse RNA.

Protein extraction and Western blotting

HUVECs were lysed with a RIPA buffer (Fisher) with 1% proteinase inhibitor cocktail (Pierce) and 1% PMSF. Retinas were dissected from P7, P12 and P17 control or mutant mice with or without OIR and then ground with a glass homogenizer in RIPA buffer (Fisher) with 1% proteinase inhibitor cocktail (Pierce) and 1% PMSF. After sonication and centrifugation of the cell and/or tissue lysates, protein was quantified with a BCA assay and then loaded in a 6-9% SDS-PAGE gel at 5-10 μg per lane. Antibodies used in this study were as follows: PFKFB3 (Proteintech; rabbit, 1:1000), pAKT (Cell Signaling Technology; rabbit, 1:2000), AKT (Cell Signaling Technology; rabbit, 1:2000), and actin (Cell Signaling Technology; rabbit, 1:5000). Images were taken with the ChemiDoc MP system (Bio-Rad), and band densities were quantified using Image Lab software (Bio-Rad).

BrdU incorporation analysis

After 16 h serum starvation, HUVECs were treated with 10 μM BrdU (5-bromodeoxyuridine, Sigma) for 16 h. Following BrdU treatment, cells were fixed, heated at 98°C for 8 min in citric acid buffer for antigen retrieval and incubated with a monoclonal anti-BrdU antibody (Sigma; mouse, 1:200) followed by incubation with an Alexa Fluor 594-labeled anti-mouse secondary antibody (Molecular Probes; 1:250). The cells were then immersed in ProLong Gold mounting medium with DAPI (Invitrogen) to visualize the nuclei. Images were obtained using a Zeiss Axio Observer Z1 inverted microscope at 10x magnification.

Cell-cycle analysis with flow cytometry

After trypsinization, HUVECs were washed in PBS containing 1% fetal bovine serum and fixed by adding cold ethanol to a final concentration of 80%. After washing and resuspending the fixed cells in 0.5 ml of PBS containing 1% fetal bovine serum, the DNA was stained by adding 200 μg/ml of propidium iodide and 10 mg/ml of DNAse-free RNAse A. The stained cells were analyzed using a FACSCalibur system (Becton Dickinson). The percentage of cells in each phase of the cell cycle was determined using CellQuest v3.3 software (BD Bioscience).

Flow cytometric analysis of endothelial apoptosis

HUVECs infected with a PFKFB3 shRNA adenovirus or control virus were exposed to normoxia (21% O_2) or hypoxia (0.5% O_2) for 24 h followed by annexin-V and propidium iodide (PI) (Invitrogen) staining. Apoptotic (annexin-V positive) cells were analyzed with flow cytometry. At least 10,000 events were collected. Data were analyzed with CellQuest v3.3 software (BD Bioscience) as instructed.

***In vitro* tube formation analysis**

HUVECs were transfected with a PFKFB3 shRNA-containing adenovirus or control virus 36 h before the assay were performed. To examine tube formation, growth factor-reduced Matrigel (BD Bioscience) was placed in 96-well tissue culture plates (60 μl/well) and allowed to form a gel at 37°C for at least 30 min. The HUVECs infected with virus were resuspended in 0.5% FCS growth medium at a 1×10^5 concentration. Aliquots of 150 μl of the cell suspension were added to each well, and the plates were incubated with 20% or 0.5% oxygen at 37°C for 8 h. The

endothelial tubes were observed using a fluorescent microscope after staining with Calcein AM. Three image fields were selected at random and photographed.[2] The tube formation was analyzed with WimTube quantitative tube formation image analysis program (Ibidi). The results are expressed as the mean fold-change of tube length compared with the control.

Lactate measurements

The levels of secreted and intracellular lactate were determined using the lactate assay kit (Sigma). Data were normalized to final cell counts.

Oxygen-induced retinopathy model

To induce vessel loss, mice were exposed to 75% oxygen from P7 to P12 and returned to room air. Retinal vessel loss (avascular area) was assessed at P12, and retinal neovascularization (termed as neovascular tuft, NVT) was evaluated at P17 when the neovascular response was greatest.[3]

Quantification of the avascular region and retinal neovascularization

For retinal vessel analysis, mice were given lethal doses of ketamine (Boehringer), and the eyes were collected and fixed in 4% paraformaldehyde for 1 h at 4°C. The retinas were isolated and stained overnight at 25°C with Alexa Fluor 594-labeled Isolectin B4 (Molecular Probes) in 1 mM $CaCl_2$ in PBS. Following 2 h of washes, retinas were whole-mounted onto Superfrost/Plus microscope slides (Fisher Scientific) with the photoreceptor side down and embedded in SlowFade Antifade reagent (Invitrogen). Images of each of the four quadrants of the whole-mounted retina were taken at 5x magnification on a Leica SP2 confocal microscope and

imported into Adobe Photoshop. Retinal segments were merged to produce an image of the entire retina. The avascular region and neovascular tuft formation were quantified by comparing the number of pixels in the affected areas with the total number of pixels in the retina[3]. Percentages of the avascular region and neovascularization in mouse retinas were compared with percentages in retinas from age-matched control mice with identical oxygen conditions. The evaluation was conducted blind to the identity of the sample; n is the number of eyes quantified.

Intraperitoneal injection of pharmacological reagents

Intraperitoneal administration of 3PO (Calbiochem) dissolved in DMSO (injection volume 0.07 mg per g body weight per day) was performed daily from P12 to P17 in the ischemic retinopathy model.[4] Control mice were injected with the same amount/type of solution.

Tumor implantation

B16 mouse melanoma cells were cultured in DMEM containing 10% FBS and implanted subcutaneously into the backs of 12-week-old female mice (5×10^5 cells in 0.1 ml per mouse) as described.[5]

Blood flow assessment

Blood perfusion images of the tumor were obtained 15 d post tumor inoculation by laser speckle contrast imaging. Briefly, the mouse was anesthetized using isofluorane, body temperature was maintained at 37 ±0.2 °C using a thermo-regulated heating pad (Harvard Apparatus) and the local fur was shaved. Perfusion images of equal size area including tumor (1.6 cm x 2.0cm) were acquired using PeriCam PSI HD system (Perimed Inc., Sweden) with a 70 mW built-in laser

diode for illumination and 1388 x 61038 pixels CCD camera for image acquisition. Once the perfusion is consistent, images (2 per sec) were acquired for 2 min at a speed of 2 Hz. Acquired images were analyzed for blood flow using PIMSoft, a PeriCam dedicated computer program (Perimed). The mean tumor blood flow was calculated as the ratio of the blood flow in the tumor area to that of the surrounding normal tissue area and was presented as the percent. Six mice were analyzed in each group.[6]

Statistical analysis

The data are presented as the mean ± SD and were analyzed by either a Student's *t*-test (two group comparison) or two-tailed ANOVA (multiple group comparison). Differences were considered significant at the two-tailed $p < 0.05$.

Reference

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