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Endothelial 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase, Isoform 3 Plays a Critical Role in Angiogenesis

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Objective—Vascular cells, particularly endothelial cells, adopt aerobic glycolysis to generate energy to support cellular functions. The effect of endothelial glycolysis on angiogenesis remains unclear. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 (PFKFB3) is a critical enzyme for endothelial glycolysis. By blocking or deleting PFKFB3 in endothelial cells, we investigated the influence of endothelial glycolysis on angiogenesis both in vitro and in vivo.

Approach and Results—Under hypoxic conditions or after treatment with angiogenic factors, endothelial PFKFB3 was upregulated both in vitro and in vivo. The knockdown or overexpression of PFKFB3 suppressed or accelerated endothelial proliferation and migration in vitro, respectively. Neonatal mice from a model of oxygen-induced retinopathy showed suppressed neovascular growth in the retina when endothelial PFKFB3 was genetically deleted or when the mice were treated with a PFKFB3 inhibitor. In addition, tumors implanted in mice deficient in endothelial PFKFB3 grew more slowly and were provided with less blood flow. A lower level of phosphorylated AKT was observed in PFKFB3-knockdown endothelial cells, which was accompanied by a decrease in intracellular lactate. The addition of lactate to PFKFB3-knockdown cells rescued the suppression of endothelial proliferation and migration.

Conclusions—The blockade or deletion of endothelial PFKFB3 decreases angiogenesis both in vitro and in vivo. Thus, PFKFB3 is a promising target for the reduction of endothelial glycolysis and its related pathological angiogenesis. (*Arterioscler Thromb Vasc Biol.* 2014;34:00-00.)

Key Words: angiogenesis ■ anoxia ■ endothelial cells ■ glycolysis

Angiogenesis is the growth of a new blood vessel from the existing vasculature. This capability is critical for many physiological and pathological processes.¹ When the angiogenic process is dysregulated, the formation of new blood vessels can take the form of pathological angiogenesis, leading to the development and progression of various malignant, ischemic, inflammatory, and immune diseases.^{1,2} Endothelial cells are especially critical in the process of angiogenesis. For example, the migration and proliferation of endothelial cells initiate the formation of capillary networks, which provide a frame for further vascular maturation.³ Much attention has been given to investigating the effect of angiogenic growth factors on endothelial activities during angiogenesis. Indeed, several mechanisms have been described for the actions

of these angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor among many others.⁴

Endothelial cells have high glycolytic activity.⁵⁻⁹ The level of glycolysis in endothelial cells is comparable with that of tumor cells and much higher than that of other healthy cells.⁶ In addition, glycolytic flux in endothelial cells is >200-fold higher than glucose oxidation, fatty acid oxidation, and glutamine oxidation, resulting in the generation of >85% of the total cellular ATP content.⁵ In glycolytic flux, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate is 1 of 3 rate-limiting checkpoints. 6-Phosphofructo-1 kinase, the enzyme that catalyzes the above reaction, is activated by its allosteric activator, fructose-2,6-bisphosphate.¹⁰ In endothelial

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Nonstandard Abbreviations and Acronyms	
G6PD	glucose-6-phosphate dehydrogenase
HUVEC	human umbilical vein endothelial cell
OIR	oxygen-induced retinopathy
pAKT	phosphorylated AKT
PFKFB3	6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3
VEGF	vascular endothelial growth factor

cells, fructose-2, 6-bisphosphate is synthesized by 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3).¹¹ A recent study demonstrated that PFKFB3-driven glycolysis is important for the migration of endothelial cells. In response to angiogenic factors, PFKFB3-knockdown endothelial cells exhibit defects in the formation of filopodia and lamellipodia.⁶ However, it remains unclear whether PFKFB3 is important for angiogenesis.

We were interested in determining whether the inhibition of PFKFB3 could suppress angiogenesis, especially pathological angiogenesis. Using PFKFB3 knockdown, overexpression, or inhibition of PFKFB3 activity with an inhibitor, we examined endothelial cell proliferation and tube formation *in vitro*. Furthermore, we generated floxed PFKFB3 (PFKFB3^{fl/fl}) mice. By breeding these mice with endothelial cell-specific Cre (cdh5-Cre) mice, we generated mice with a deficiency of PFKFB3 specifically in endothelial cells. With these mice and their controls, the role of endothelial PFKFB3 in angiogenesis was evaluated by comparing the size of implanted tumors and the blood supply of these tumors and the severity of retinal neovascularization in the retina of mice from an oxygen-induced retinopathy model. Furthermore, the underlying mechanisms contributing to PFKFB3-associated angiogenesis were explored.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Hypoxia and Angiogenic Factors Upregulate Endothelial PFKFB3

To examine the effect of hypoxia on the expression of PFKFB, human umbilical vein endothelial cells (HUVECs) were cultured in an incubator with 0.5% oxygen for 24 hours. The mRNA expression levels of PFKFB isoforms 1 to 4 were analyzed with real-time reverse transcription-polymerase chain reaction. PFKFB isoforms 1 and 3 were increased by 1- and 5-fold, respectively, whereas isoforms 2 and 4 did not show significant changes compared with normoxia controls (Figure 1A). The upregulation of PFKFB3 protein expression in endothelial cells under hypoxic conditions was confirmed using Western blotting (Figure 1C). The expression of endothelial PFKFB3 in response to VEGF treatment was also examined. PFKFB3 mRNA expression was increased at 12 but not 24 hours after VEGF stimulation, and the protein expression was increased at both time points (Figure 1B and 1D), indicating that in addition to the regulation of PFKFB3 at

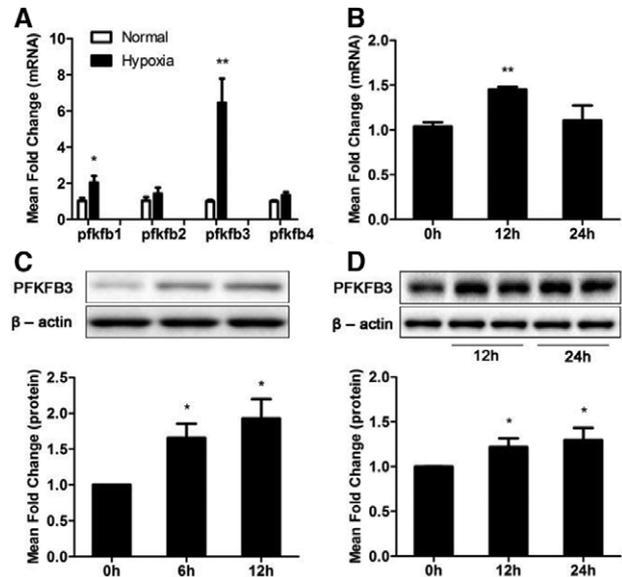


Figure 1. Induction of endothelial 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) by hypoxia and human vascular endothelial growth factor (hVEGF). **A**, Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of PFKFB transcripts from total RNA isolated from human umbilical vein endothelial cells (HUVECs) exposed to hypoxia (0.5% oxygen) for 24 hours. **B**, Real-time RT-PCR analysis of PFKFB3 transcripts in HUVECs treated with or without hVEGF (100 ng/mL) for 24 hours. **C**, Immunoblot analysis of PFKFB3 and β -actin (loading control) expression in lysates of HUVECs exposed to hypoxia for 0, 6, and 12 hours. **D**, Immunoblot analysis of PFKFB3 and β -actin expression in HUVECs treated with hVEGF (100 ng/mL) for 0, 12, or 24 hours. **C** and **D** (top), Representative images. For bar graphs (**A–D**), data are the mean \pm SD; $n=3$. * $P<0.05$ and ** $P<0.01$ for hypoxia (6 or 12 hours) vs normal (0 hours) (in **C**) and for the same gene (in **A**) and 12 or 24 vs 0 hours (in **B** and **D**).

the transcriptional level, VEGF may also regulate endothelial PFKFB3 at the post-transcriptional level.

Endothelial PFKFB3 Is Involved in Endothelial Proliferation and Endothelial Tube Formation *In Vitro*

To examine whether PFKFB3 affects endothelial proliferation, endothelial cells pretreated with control adenovirus (Ad-shctl) or PFKFB3-knockdown adenovirus (Ad-shp_{pfkfb3}) were placed in endothelial growth cell medium under normoxic (21% oxygen, Figure 2A, left) or hypoxic (0.5% oxygen, Figure 2A, middle) conditions. Infection with PFKFB3-knockdown adenovirus resulted in a decreased expression of PFKFB3 in HUVECs (Figure 1A in the online-only Data Supplement) The growth of PFKFB3-knockdown endothelial cells decreased by 50% to 80% compared with control cells during a period of 96 hours (Figure 2A). In contrast, the growth of endothelial cells infected with a PFKFB3-overexpressing adenovirus increased by 35% compared with endothelial cells treated with a control virus (Figure 2A, right). Furthermore, under normoxic conditions, the cell cycle was analyzed and BrdU incorporation was evaluated in endothelial cells cultured in a complete growth medium for 24 hours after the cells were synchronized by total FBS depletion. Flow cytometry showed that the percentage of cells in S phase was 20% to 22% in

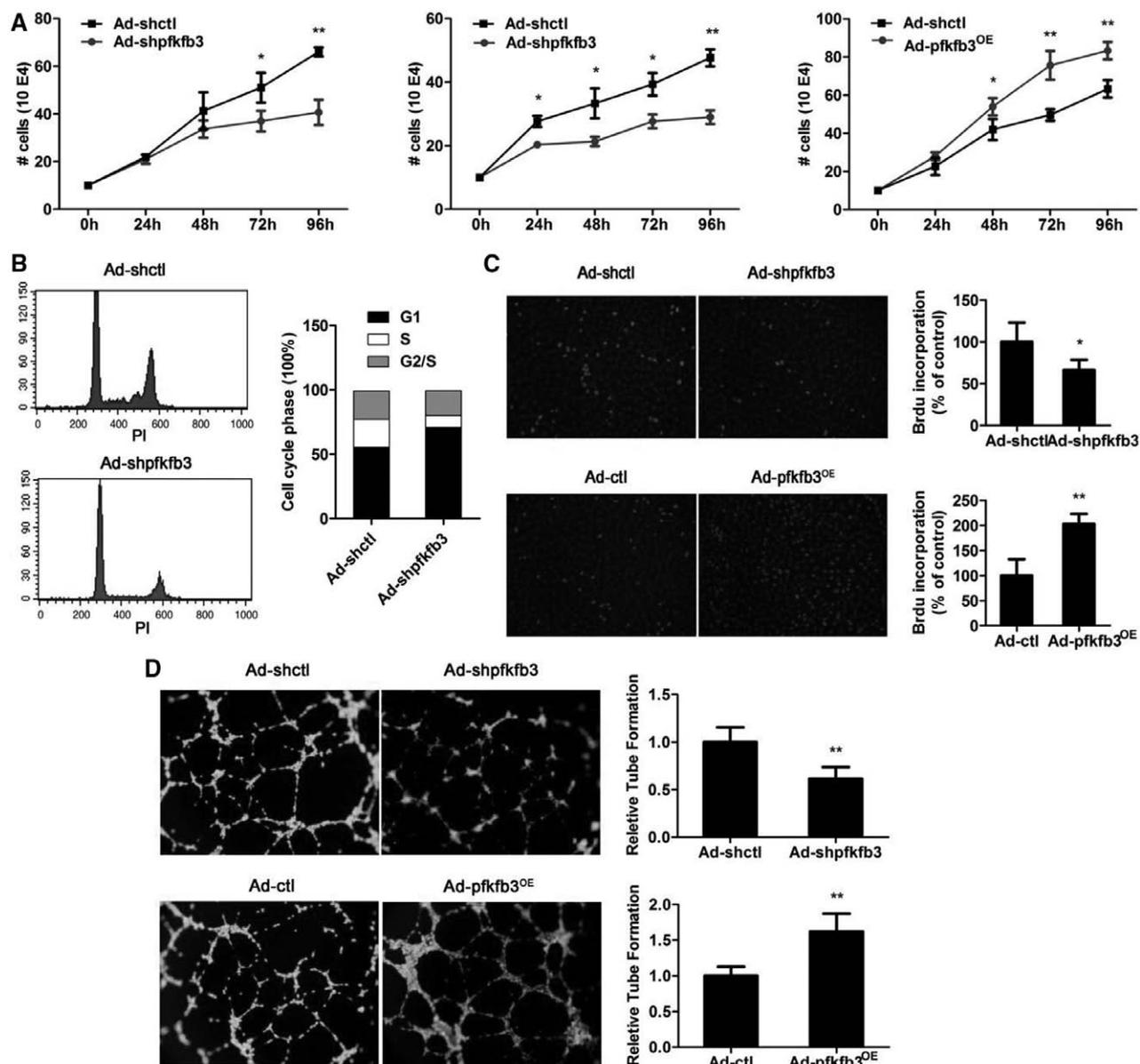


Figure 2. Involvement of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) in endothelial proliferation and tube formation. **A**, Hypoxia-induced cell growth. The number of PFKFB3-knockdown or -overexpressing human umbilical vein endothelial cells (HUVECs) was counted after exposure to hypoxia (0.5% oxygen, **middle**) or normal oxygen (**left and right**) for 0, 24, 48, 72, or 96 hours. **B**, Flow cytometric analysis of the cell cycle. PFKFB3-knockdown HUVECs and control cells were synchronized in G₀/G₁ by total FBS depletion for 16 hours and then exposed to complete medium for an additional 24 hours. **C**, BrdU staining of proliferating HUVECs. PFKFB3-knockdown, PFKFB3-overexpressing, and control cells were synchronized in G₀/G₁ by total FBS depletion for 16 hours and then incubated with complete medium for an additional 24 hours. **D**, Representative images and quantification of tube formation in control, PFKFB3-knockdown, and PFKFB3-overexpressing HUVECs cultured in growth factor–deprived Matrigel. Experiments in **B**, **C**, and **D** were performed under normoxic conditions. For bar graphs (**A–D**), data are mean±SD; n=3. **P*<0.05 and ***P*<0.01 for Ad-shpfkfb3 or Ad-pfkfb3^{OE} vs Ad-shctl or Ad-ctl (in **A**, **C**, and **D**).

control HUVECs, whereas this percentage decreased by 9% to 11% for PFKFB3-knockdown HUVECs (Figure 2B). Immunostaining in the BrdU incorporation assay showed that the percentage of BrdU-positive cells decreased by 30% to 35% in PFKFB3-knockdown cells and increased by 85% to 95% in PFKFB3-overexpressing cells compared with control cells (Figure 2C). To investigate whether PFKFB3 affected endothelial migration, a tube formation assay was conducted, which consisted of placing cells on growth factor–deprived Matrigel. The number of formed tubes was decreased by 40%

to 46% in PFKFB3-knockdown HUVECs compared with control HUVECs (Figure 2D). In PFKFB3-overexpressing HUVECs, the number of tubes was 52% to 60% higher than that of control HUVECs (Figure 2D), indicating that PFKFB3 increases endothelial migration.

Endothelial PFKFB3 Participates in Pathological Angiogenesis In Vivo

To investigate the role of endothelial PFKFB3 in angiogenesis in vivo, floxed PFKFB3 mice (PFKFB3^{WT}) were generated

(Figure 3A–3C) and then bred with *cdh5-Cre* mice to create mice with deficiency in PFKFB3 in endothelial cells only (PFKFB3^{VEC-KO}, Figure 3D), and then both the oxygen-induced retinopathy (OIR) model and the tumor implantation model were used in these mice. For mouse pups in normal room air, the expression of retinal PFKFB3 in 7-day-old (P7), P12, and P17 mice was comparable (data not shown). In contrast, in OIR mice, the mRNA expression level of retinal PFKFB3 was decreased by 25% to 30% at P12 and increased by >2-fold at P17 compared with the expression level at P7 (Figure 4A). This dynamic change in retinal PFKFB3 expression was also observed at the protein level (Figure 4B). Retinal angiogenesis was evaluated by measuring the vascular area in retinal whole mounts after isolectin staining. The retinal density was slightly lower in PFKFB3^{VEC-KO} mice than in PFKFB3^{WT}

mice at P4, and this difference was insignificant at P7 (Figure IIA and IIB in the online-only Data Supplement). After P7, mice were exposed to 75% oxygen for 5 days. PFKFB3^{VEC-KO} mice at P12 showed an increase in avascular retinal area compared with PFKFB3^{WT} mice, although this increase was not statistically significant (Figure 4C). At P17, when the neovascular tuft reaches its maximum, the neovascular tuft area of PFKFB3^{VEC-KO} mice was 50% to 55% smaller than that of PFKFB3^{WT} mice (Figure 4D). Similar decreases in neovascular tuft area were also observed in control mouse pups treated with the PFKFB3 inhibitor 3PO¹² (Figure 4E). In addition, the same OIR models were generated in heterozygotes of PFKFB3 deletion mice,^{13–15} the retinal areas of both avascular and neovascular tuft were the same as those in littermate controls (Figure III in the online-only Data Supplement),

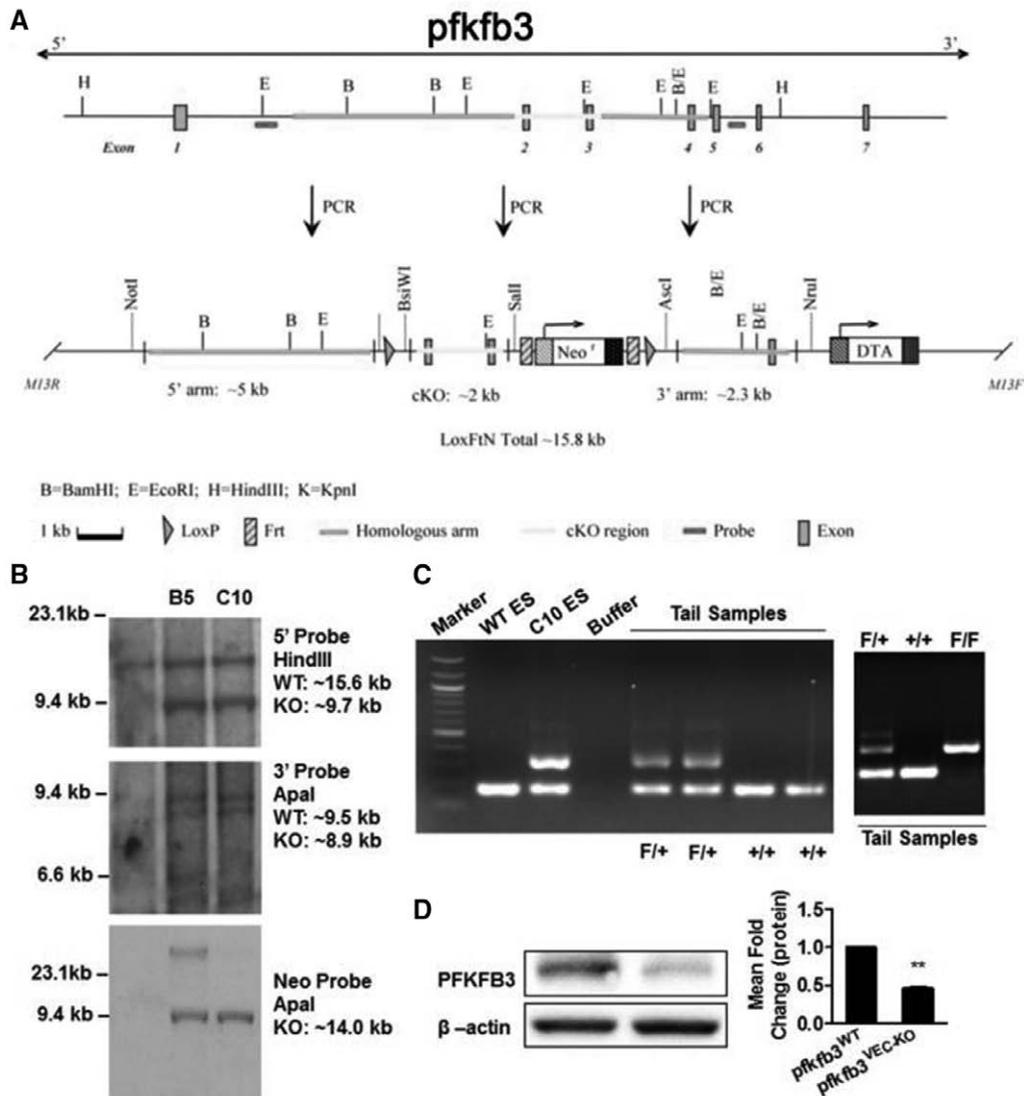


Figure 3. Generation of mice with a 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) deficiency in endothelial cells. **A**, LoxP targeting of PFKFB3. The targeting construct to introduce the LoxP sites into the *PFKFB3* gene. **Top**, Wild-type *PFKFB3* locus. **Bottom**, Targeted *PFKFB3* locus. cKO indicates conditional knockout. **B**, C57BL/6 ES cells were transfected with a PFKFB3-floxed vector and selected with 200 mg/mL G418. Selected positive clones (B5 and C10) were expanded for further analysis. Clone C10 was confirmed for homologous recombination with a single neo integration using the 5', 3' Neo probes. **C**, Heterozygous (F/+, PFKFB3 floxed) mice were obtained by breeding the male chimera with wild-type (WT; +/+) C57BL/6 females. **D**, Floxed mice were bred with *cdh5-Cre* mice to generate mice with a deficiency of PFKFB3 in the endothelial cells. Aortic endothelial cells were isolated from the mice and assayed for the expression of PFKFB3 with Western blotting.

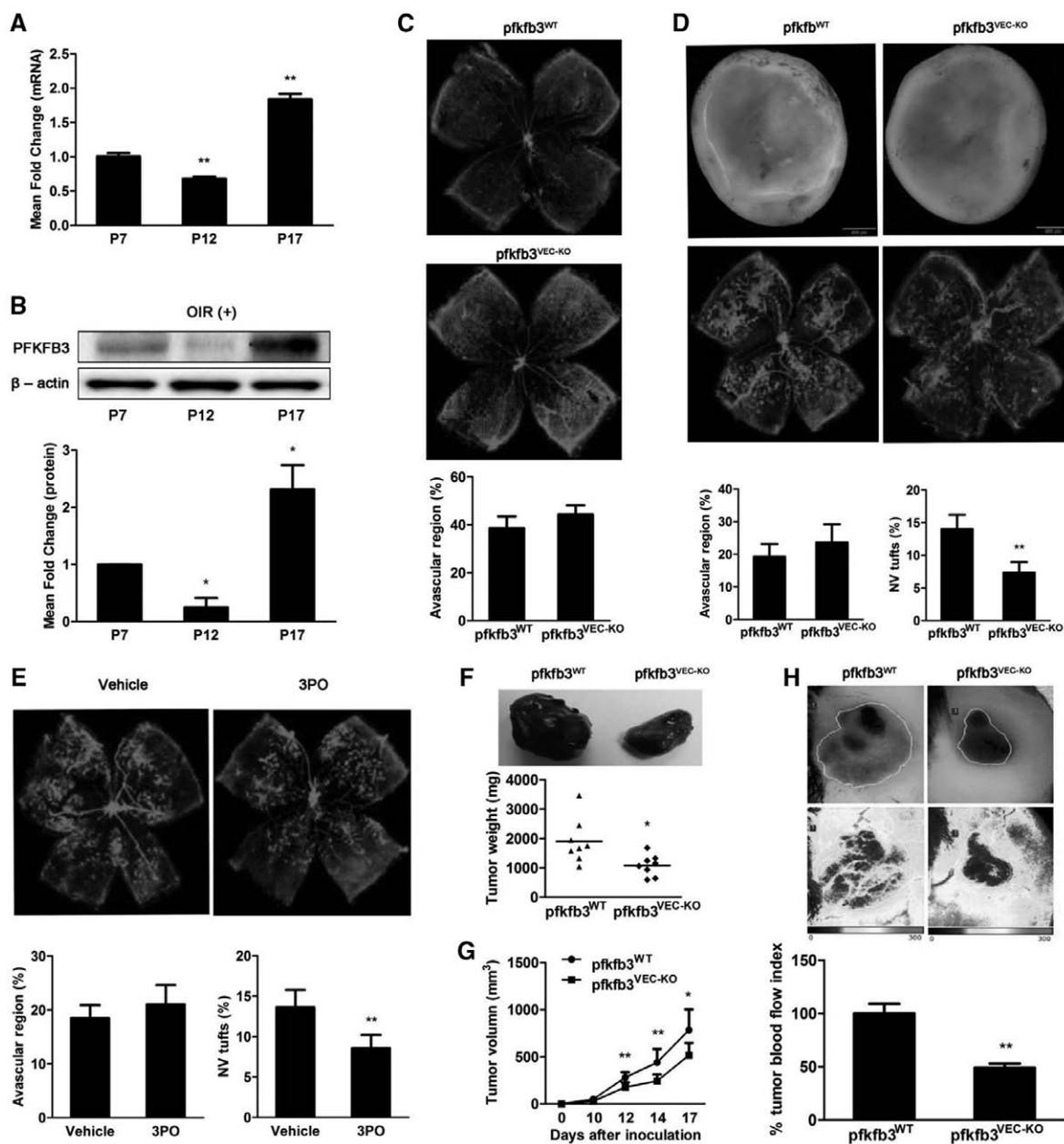


Figure 4. Endothelial 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) participates in pathological angiogenesis in vivo. **A**, Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of PFKFB3 mRNA expression levels and immunoblot analysis of PFKFB3 protein expression levels (**B**) in retinas of oxygen-induced retinopathy (OIR) mice at P7, P12, and P17. **C**, Isolectin staining of whole-mount retinas from *pfkfb3*^{fl^{ox}/fl^{ox}} (*pfkfb3*^{WT}) and *pfkfb3*^{fl^{ox}/fl^{ox}cdh5^{cre}} (*pfkfb3*^{VEC-KO}) OIR mice at P12 and quantification of the avascular areas (n=7 mice per genotype). **D**, Isolectin staining and stereomicroscopy of whole-mount retinas from *pfkfb3*^{WT} and *pfkfb3*^{VEC-KO} OIR mice at P17 and quantification of the neovascular (NV) tuft and avascular areas (n=9 mice per genotype). **E**, Isolectin staining and quantification of whole-mount retinas of C57BL/6 mice treated systemically with 3PO or a vehicle at P17 in OIR mice (n=9 mice per group). **F**, The appearance of B16 tumors under the skin of *pfkfb3*^{WT} and *pfkfb3*^{VEC-KO} mice generated with a dissecting microscope and the quantification of tumor weight (n=8 mice per group). **G**, Quantification of tumor volume in *pfkfb3*^{WT} and *pfkfb3*^{VEC-KO} mice (n=8 mice per group). **H**, Laser Doppler imaging (including reconstituted tumor photographs) and quantification of perfusion in tumors in *pfkfb3*^{WT} and *pfkfb3*^{VEC-KO} mice at 15 days after implantation (n=6 mice per group). For bar graphs (**A–G**), data are the mean±SD. **P*<0.05 and ***P*<0.01 for P12 or P17 vs P7 (in **A** and **B**) 3PO vs vehicle control (in **E**), or *pfkfb3*^{VEC-KO} vs *pfkfb3*^{WT} (in **D**, **F**, **G**, and **H**).

indicating that the deleting 1 allele of *PFKFB3* gene in endothelial cells is not sufficient to alter endothelial angiogenesis.

Tumor implantation models were also used to examine the effect of endothelial PFKFB3 on angiogenesis. Adult female *PFKFB3*^{VEC-KO} and *PFKFB3*^{WT} mice were injected with B16 mouse melanoma under the back skin of the mouse. After tumor implantation, the tumor size and weight increased

during a period of 18 days. However, at the same time points, the tumors in *PFKFB3*^{VEC-KO} were much smaller and lighter compared with those in *PFKFB3*^{WT} (Figure 4F and 4G). More importantly, the laser speckle contrast imaging showed 55% decreased blood flow in *PFKFB3*^{VEC-KO} mice as compared with their wild-type littermates, *PFKFB3*^{WT} mice (Figure 4H).

AKT Acts Downstream of PFKFB3 in Endothelial Cells

AKT and its phosphorylation are directly associated with angiogenesis.^{16,17} To examine whether AKT and phosphorylated AKT (pAKT) are involved in the effect of PFKFB3 on angiogenesis, the expression levels of AKT and pAKT in PFKFB3-knockdown HUVECs and control cells were examined using Western blotting. There were no significant differences in the AKT expression levels between PFKFB3-knockdown HUVECs and control cells. However, the pAKT expression level in PFKFB3-knockdown cells was decreased compared with control cells under both normoxic and hypoxic conditions (Figure 5A) and in response to human VEGF treatment (Figure IV in the online-only Data Supplement). Consistent with the *in vitro* results from cultured HUVECs, the expression level of retinal pAKT was decreased by 50% to 60% in OIR PFKFB3^{VEC-KO} mice compared with OIR PFKFB3^{WT} mice at P17 (Figure 5B). To further examine the correlation between the expression levels of

pAKT and PFKFB3, HUVECs were treated with an adenovirus encoding PFKFB3 (Ad-PFKFB3). Treatment of HUVECs with Ad-PFKFB3 resulted in overexpression of PFKFB3 (Figure IB in the online-only Data Supplement) and increased expression of pAKT in HUVECs (Figure 5C), suggesting that the expression level of PFKFB3 stimulates pAKT expression in endothelial cells. To determine the effect of decreased pAKT on endothelial proliferation and migration in PFKFB3 knockdown, PFKFB3-knockdown HUVECs were treated with an AKT activator to elevate the level of pAKT. During a period of 72 hours, control HUVECs cultured in complete growth cell medium with or without the addition of the AKT activator II exhibited similar levels of proliferation. However, the addition of the AKT activator II increased the proliferation of PFKFB3-knockdown HUVECs to levels similar to those observed in control HUVECs grown in a complete growth cell medium with or without the addition of the AKT activator II (Figure 5D). Compared with control HUVECs, PFKFB3-knockdown HUVECs exhibited a defect in tube formation. However, the addition of the AKT activator II improved tube formation in both control and PFKFB3-knockdown HUVECs (Figure 5E), resulting in no differences in tube formation between control and PFKFB3-knockdown HUVECs. These results indicate that a decrease in pAKT in PFKFB3-knockdown HUVECs is a major cause of the observed defects in angiogenesis (Figure 5E).

Lactate Is Involved in PFKFB3-Mediated Endothelial Proliferation and Tube Formation

Recent studies have indicated the involvement of lactate in angiogenesis. To examine whether lactate plays a role in PFKFB3-associated endothelial proliferation and migration, the levels of lactate were measured. Consistent with the differences observed in the expression level of pAKT in PFKFB3-knockdown, PFKFB3-overexpressing, and control HUVECs (Figure 5A and 5C), the levels of intracellular lactate and lactate in the cell medium were decreased in PFKFB3-knockdown cells and increased in PFKFB3-overexpressing cells compared with their respective levels in control cells under both normoxic (Figure 6A) and hypoxic conditions (Figure V in the online-only Data Supplement). To determine the relationship between the expression level of pAKT and lactate, lactate was added to the cell medium. The levels of pAKT in PFKFB3-knockdown HUVECs cultured in a complete growth cell media were increased to levels similar to those in control HUVECs with the addition of lactate (Figure 6B). In addition, Ad-shpfb3-transduced endothelial cells were pretreated with lactate and then exposed to hypoxic conditions. The responses of pAKT to hypoxia after correcting the basal pAKT by lactate were dramatically inhibited in PFKFB3-knockdown cells compared with control cells (Figure VI in the online-only Data Supplement). The addition of lactate to the complete growth cell medium in which control HUVECs were cultured did not significantly enhance the expression of pAKT (Figure 6B). Similarly, during a period of 72 hours, the culture of control HUVECs in complete growth cell medium with the addition of lactate did not alter proliferation. In contrast, the culture of PFKFB3-knockdown HUVECs in complete growth cell medium with the addition

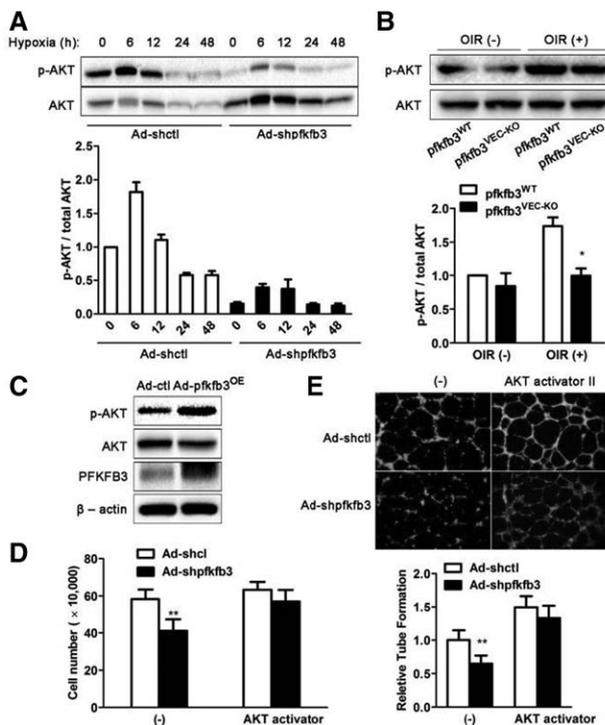


Figure 5. AKT acts downstream of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) in endothelial cells. **A**, Western blot analysis of phosphorylated AKT (pAKT) and AKT protein expression in control and PFKFB3-knockdown human umbilical vein endothelial cells (HUVECs) under hypoxia for 0, 6, 12, 24, or 48 hours. **B**, Western blot analysis of pAKT and AKT protein expression in retinas from pfb3^{WT} and pfb3^{VEC-KO} oxygen-induced retinopathy (OIR) mice at P17. **C**, Western blot analysis of pAKT and AKT protein expression in control and PFKFB3-overexpressing HUVECs. **D**, Quantification of the number of control and PFKFB3-knockdown HUVECs exposed to vehicle or the AKT activator II (4 μ g/mL) for 72 hours. **E**, Representative images and quantification of tube formation in control and PFKFB3-knockdown HUVECs treated with vehicle or the AKT activator II (4 μ g/mL) on growth factor-deprived Matrigel. All images shown are representative, and data are the mean \pm SD (n=3 independent experimental groups). * P <0.05 and ** P <0.01 for Ad-shpfb3 vs Ad-shctl under the time point (in **A**) or same condition (in **D** and **E**) or for pfb3^{VEC-KO} vs. pfb3^{WT} for the same condition (in **B**).

of lactate increased the proliferation to levels similar to that of control HUVECs grown in a complete growth cell medium with or without the addition of lactate (Figure 6C). As shown in Figures 2D and 5E, compared with control HUVECs, PFKFB3-knockdown HUVECs exhibited a defect in tube formation. The addition of lactate to the medium improved tube formation in both PFKFB3-knockdown HUVECs and control cells (Figure 6D). Furthermore, the addition of lactate to PFKFB3-knockdown HUVECs rescued the observed decrease in tube formation, indicating that a decrease in lactate in PFKFB3-knockdown HUVECs is a major cause of the defects in tube formation observed in this group of cells.

Discussion

Angiogenic factors upregulate endothelial PFKFB3. Endothelial cells are highly glycolytic even under resting conditions.^{6,9} A recent study indicated that the glycolysis level in endothelial cells is much higher than in any other healthy cells, including cardiomyocytes, hepatocytes, fibroblasts, and macrophages, and is even similar to that of many tumor cells. The majority of intracellular ATP is generated from glycolysis in cultured endothelial cells. PFKFB3 is critical in endothelial cell glycolysis. The knockdown of PFKFB3 protein expression in endothelial cells (76%–84%) results in a decrease in glycolysis (35%–40%), indicating that PFKFB3 plays a significant role in the physiological

activity of endothelial cells. PFKFB3 is also critical for glycolysis under pathological conditions. A recent study demonstrated that after VEGF stimulation, PFKFB3 protein expression was significantly upregulated.⁶ We have studied the expression of all of the isoforms of PFKFB and found that the PFKFB3 isoform is the most significantly upregulated in stimulated endothelial cells. This upregulation was also observed in vivo, as evidenced by OIR retinal PFKFB3 expression. Compared with the other isoforms of PFKFB, PFKFB encoded by PFKFB3 is an enzyme with a kinase activity >700-fold higher than its phosphatase activity, thereafter signaling to achieve a high level of glycolysis in proliferative endothelial cells.

Endothelial PFKFB3 participates in pathological angiogenesis. In cancers of the breast, colon, lung, pancreas, prostate, and ovary, PFKFB3 exhibited increased protein and mRNA expression as well as increases of its phosphorylated form compared with healthy controls.^{10,11,18} This increased expression leads to high rates of aerobic glycolysis in tumor cells, a phenomenon known as the Warburg effect.¹⁹ In addition to general tumor cells, vascular cells in tumors also exhibit an increased expression and activation of PFKFB3, suggesting that endothelial PFKFB3 is involved in the Warburg effect.^{11,20} In our study, the tumor blood supply and tumor size were decreased in mice deficient in endothelial PFKFB3 compared with control mice, demonstrating that PFKFB3 in endothelial cells is

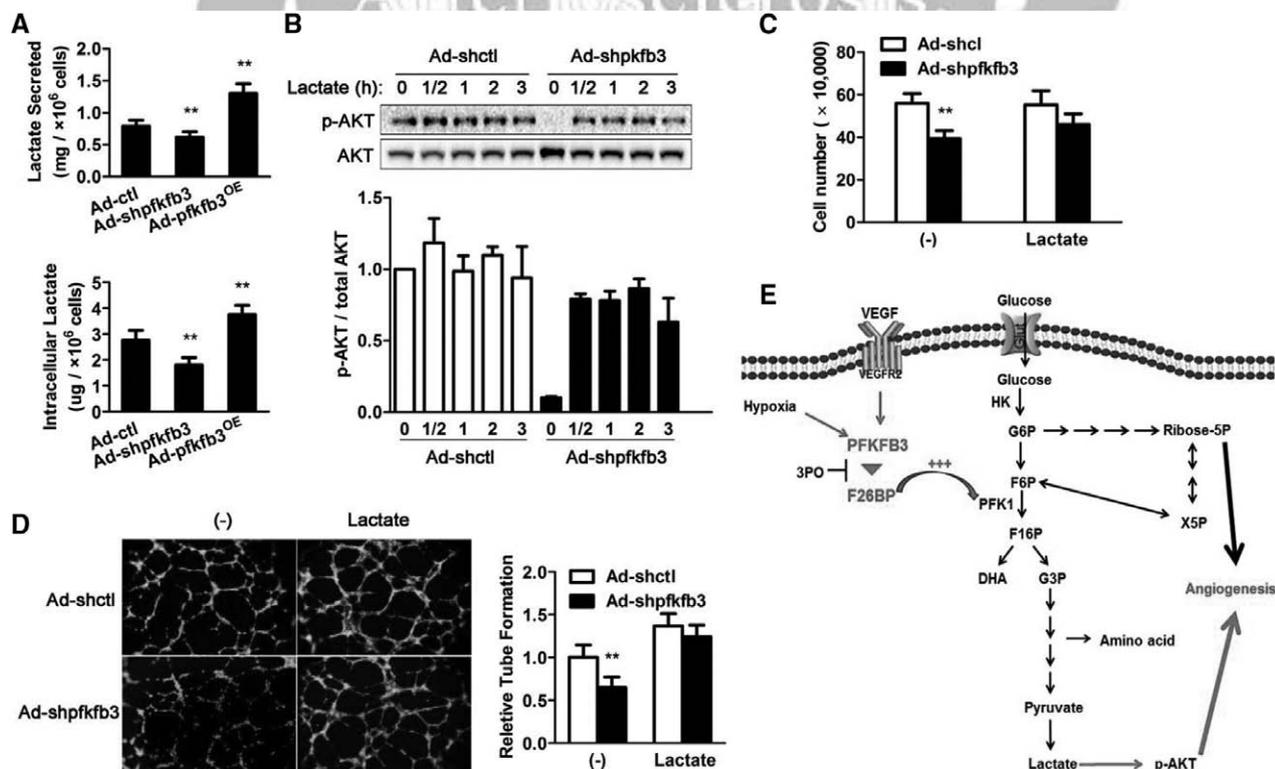


Figure 6. Lactate is involved in 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3)-mediated endothelial proliferation and tube formation. **A**, Quantification of secreted lactate and intracellular lactate in control and PFKFB3-knockdown human umbilical vein endothelial cells (HUVECs) after 72-hour incubation. **B**, Immunoblot analysis of phosphorylated AKT (pAKT) and total AKT in control and PFKFB3-knockdown HUVECs on lactate (10 mmol/L) treatment for 0, 0.5, 1, 2, or 3 hours. **C**, Quantification of the number of control and PFKFB3-knockdown HUVECs treated with lactate (10 mmol/L) for 72 hours. **D**, Representative images and quantification of tube formation in control and PFKFB3-knockdown HUVECs treated with lactate (10 mmol/L) for 8 hours on growth factor–deprived Matrigel. **E**, Schematic of the proposed role of PFKFB3 in angiogenesis. For bar graphs (**A–E**), data are the mean±SD (n=3). *P<0.05 and **P<0.01 for Ad-shpfkfb3 or Ad-pfkfb3^{OE} vs Ad-shctl (in **A**) under the same condition (in **C** and **D**) or for the same time point (in **B**). F6P indicates fructose-6-phosphate; and VEGF vascular endothelial growth factor.

indeed critical for tumor angiogenesis. The role of endothelial PFKFB3 in pathological angiogenesis was further supported by its effect on neovascularization in OIR mouse retinas. In this model, both the selective deletion of endothelial PFKFB3 and the application of a PFKFB3 inhibitor to mice dramatically suppressed retinal neovascularization. In addition, consistent with the data from De Bock et al,⁶ we found that the density of retinal vessels in newborn mouse pups deficient in endothelial PFKFB3 was lower than that in control pups (Figure IIA in the online-only Data Supplement). Furthermore, in OIR mice, the vascular regrowth in the avascular area of the retina was also relatively delayed in mice deficient in endothelial PFKFB3 compared with control mice, implying that endothelial PFKFB3 may play a role in physiological angiogenesis.

The underlying mechanisms for the role of endothelial PFKFB3 in pathological angiogenesis arise from the influence of this gene (enzyme) in endothelial migration and proliferation. PFKFB3 affects endothelial migration by regulating the formation of filopodia and lamellipodia and directional migration. One possible mechanism underlying this effect is that PFKFB3 compartmentalizes with F-actin in motile protrusions to provide ATP.⁶ Thus, deficiency in or knockdown of PFKFB3 impairs tip cell formation. Consistent with these results, we also observed a significant defect in the formation of lamellipodia in PFKFB3-knockdown endothelial cells under both normoxic and hypoxic conditions (Figure VII in the online-only Data Supplement). In addition, an analysis of the endothelial cell tube formation indicated that endothelial tubes are formed much more rapidly in control endothelial cells than in PFKFB3-knockdown cells. The tube formation assay was performed within 6 to 8 hours after the endothelial cells were placed on the Matrigel, indicating that the compromised tube formation is mainly attributable to a defect in migration. PFKFB3 is also important for endothelial proliferation.^{21–23} In both HeLa cells and lymphocytes, a temporal expression pattern of PFKFB3 has been observed during the cell cycle progression. PFKFB3 appears in mid-to-late G1 and is vital for cell division.²² Silencing PFKFB3 dramatically reduced T-lymphocyte proliferation.²¹ Consistent with these findings, in the current study, the knockdown of endothelial PFKFB3 prevented endothelial cells from proceeding to the S phase, resulting in a much lower proliferative rate compared with control endothelial cells under both normoxic and hypoxic conditions (Figure 2B and Figure VIII in the online-only Data Supplement). In addition, we have found that under hypoxic condition, knockdown of PFKFB3 accelerated endothelial apoptosis, indicating that, in addition to endothelial proliferation and migration, a PFKFB3-knockdown-associated decrease in angiogenesis may involve other mechanisms (Figure IX in the online-only Data Supplement).

Lactate-induced AKT phosphorylation is involved in PFKFB3-driven endothelial angiogenesis. De Bock et al⁶ examined PFKFB3-knockdown cells and found no significant differences in ATP levels, cell death, oxidative stress, glucose oxidation, and fatty acid oxidation or respiration between these cells and wild-type controls. Therefore, because of the cellular adaptation to PFKFB3 silencing, cellular energy distress does not arise, and thus, energy distress is not responsible for the compromised angiogenesis observed in PFKFB3-knockdown

endothelial cells. Because of a high glycolytic activity, activated endothelial cells produce a substantial amount of lactate. In endothelial cells, lactate functions as a signaling molecule for angiogenesis.^{24–29} Lactate acts through AxI, Tie2, and VEGF receptor 2 to activate PI3K/AKT.²⁸ Likely, Pim-1 and foxo transcription factors are also involved in the lactate-mediated effect because it has been demonstrated that these molecules are tied to AKT in mediating proliferation and antiapoptotic action of glycolysis.^{30,31} The effect of lactate on angiogenesis has been demonstrated in a few models, including pulmonary microvascular endothelial cell proliferation and the healing of superficial and ischemic wounds. The knockdown or overexpression of PFKFB3 in endothelial cells decreased or increased the levels of lactate, respectively, whereas the latter closely correlated with the levels of pAKT. The addition of lactate to PFKFB3-knockdown endothelial cells increased pAKT levels and rescued impaired endothelial migration. Thus, as summarized in Figure 6E, endothelial PFKFB3 knockdown led to a low level of glycolysis and a low level of intracellular lactate and secreted lactate, which caused a decrease in pAKT and a failure to signal properly for the generation of a strong angiogenic response. In addition, low levels of lactate and glycolysis in PFKFB3-knockdown endothelial cells are not able to provide sufficient substrates for the generation of macromolecules, including DNA, RNA, and relevant lipids, which lead to the impairment of endothelial proliferation^{19,20} (Figure 6E).

As a key enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) critically regulates glucose metabolism during angiogenesis and tissue repair.^{32,33} Deficiency of G6PD decreases endothelial proliferation, migration, and tube formation, whereas activation or overexpression of G6PD increases angiogenesis as well as cardiac repair in diabetes mellitus. The effect of G6PD on angiogenesis and tissue repair involves Flk-1, AKT, and Pim-1.^{32,33} However, pentose phosphate pathway was increased during PFKFB3 knockdown.³⁴ Given this, PFKFB3 seems to not act through G6PD-associated pathway to alter endothelial angiogenesis, although PFKFB3 knockdown and G6PD deficiency share many similarities in the effect and mechanisms.

This study has indicated that the knockdown or inhibition of PFKFB3 leads to significant suppression of pathological angiogenesis. In addition to tumors/cancers, many other diseases are also associated with pathological angiogenesis. For instance, retina angiogenesis is an important cause for vision loss in retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration.³⁵ Also, angiogenesis in atherosclerotic lesion is an important contributor to the formation and the progression of atherosclerosis, whereas some antiangiogenic approaches suppress atherosclerosis.³⁶ Thus, PFKFB3 is a promising target for the treatment of pathological angiogenesis and its associated diseases.

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Disclosures

None.

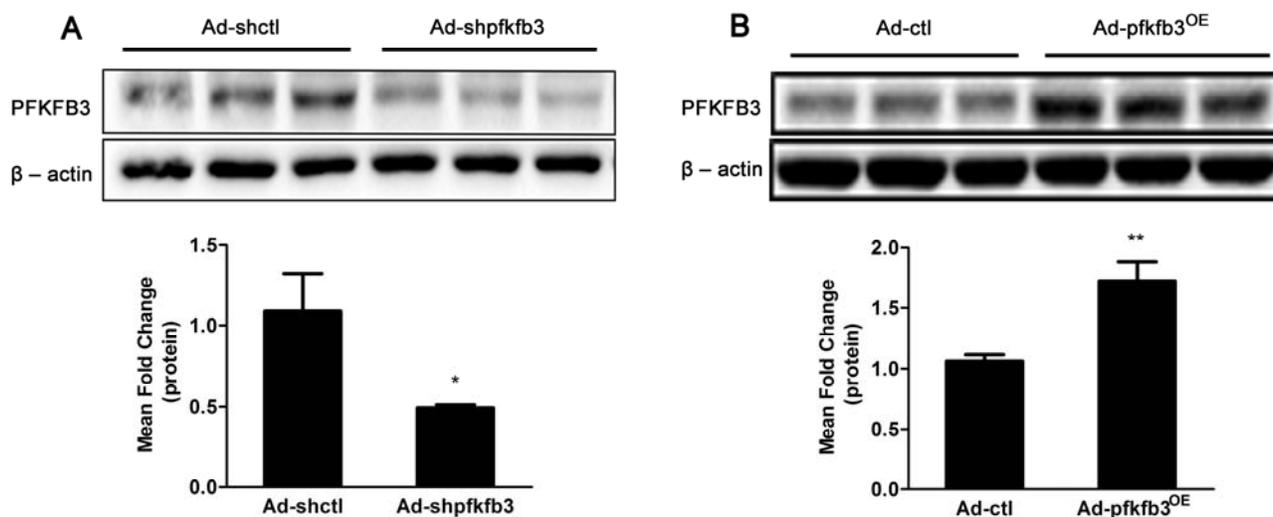
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Significance

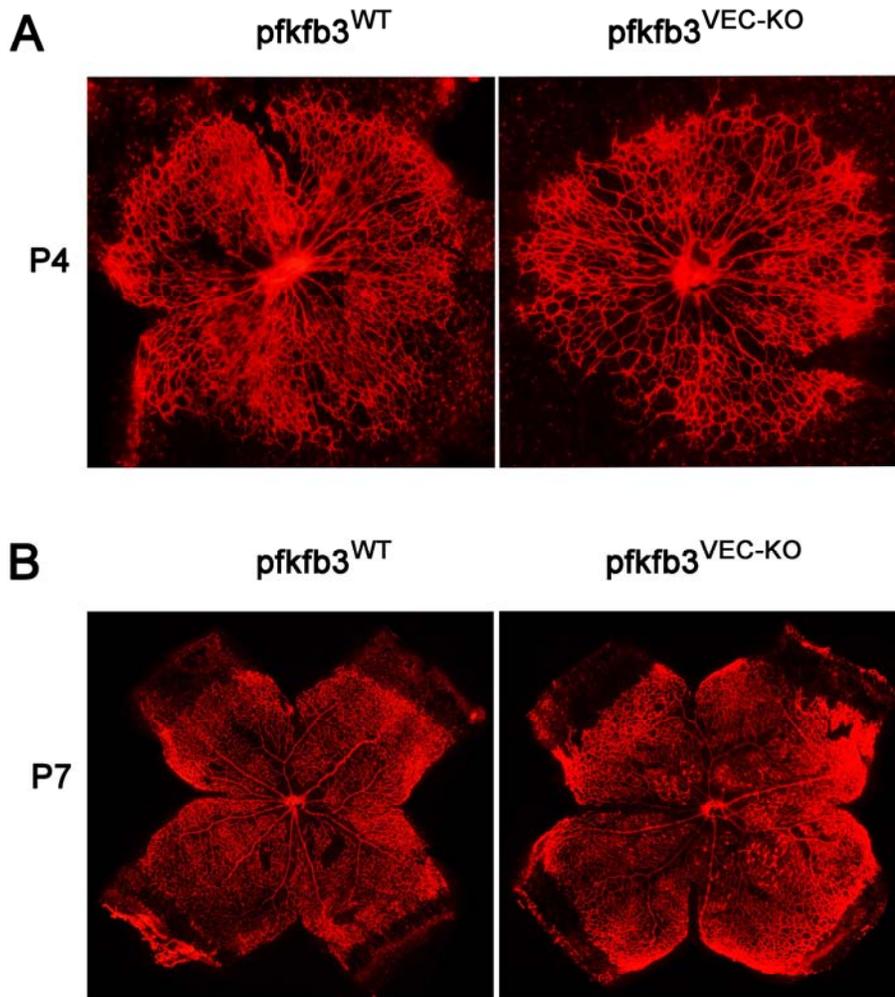
The glycolytic activity of endothelial cells is critical for angiogenesis. Fructose-2, 6-bisphosphate, a product of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3), is an activator of 6-phosphofructo-1 kinase, 1 rate-limiting checkpoint of glycolysis. Using both chemical and genetic approaches, we demonstrated that the knockdown or inhibition of endothelial PFKFB3 reduced endothelial glycolysis, leading to significant suppression of endothelial proliferation *in vitro* and pathological angiogenesis *in vivo* in murine models of tumor growth and oxygen-induced retinopathy. Furthermore, we found that lactate-associated Akt phosphorylation contributed to PFKFB3-modulated angiogenesis. Many diseases are associated with pathological angiogenesis, including tumors/cancers, retinopathies (retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration), and the formation and the progression of atherosclerosis. This study indicates that PFKFB3 is a promising target for the treatment of pathological angiogenesis and its associated diseases.

SUPPLEMENTAL DATA



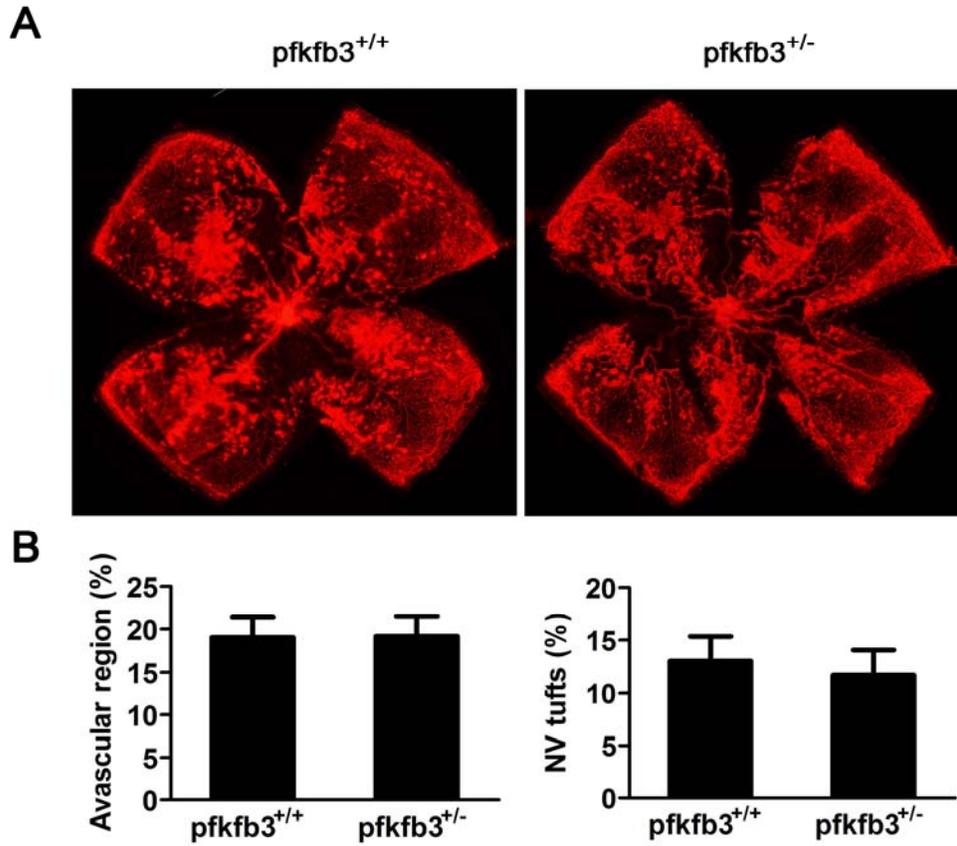
Supplemental figure I. PFKFB3 knockdown or overexpression in HUVECs.

HUVECs were transfected with Ad-shctl, Ad-shpfb3 (A), Ad-ctl, and/or Ad-pfb3 (B) for 36 h and then collected for analysis of PFKFB3 expression using Western blotting. For bar graphs, data are the mean \pm SD, n = 3. *, $P < 0.05$ and **, $P < 0.01$ Ad-shpfb3 vs. vs. Ad-shctl or Ad-pfb3^{OE} vs Ad-ctl.



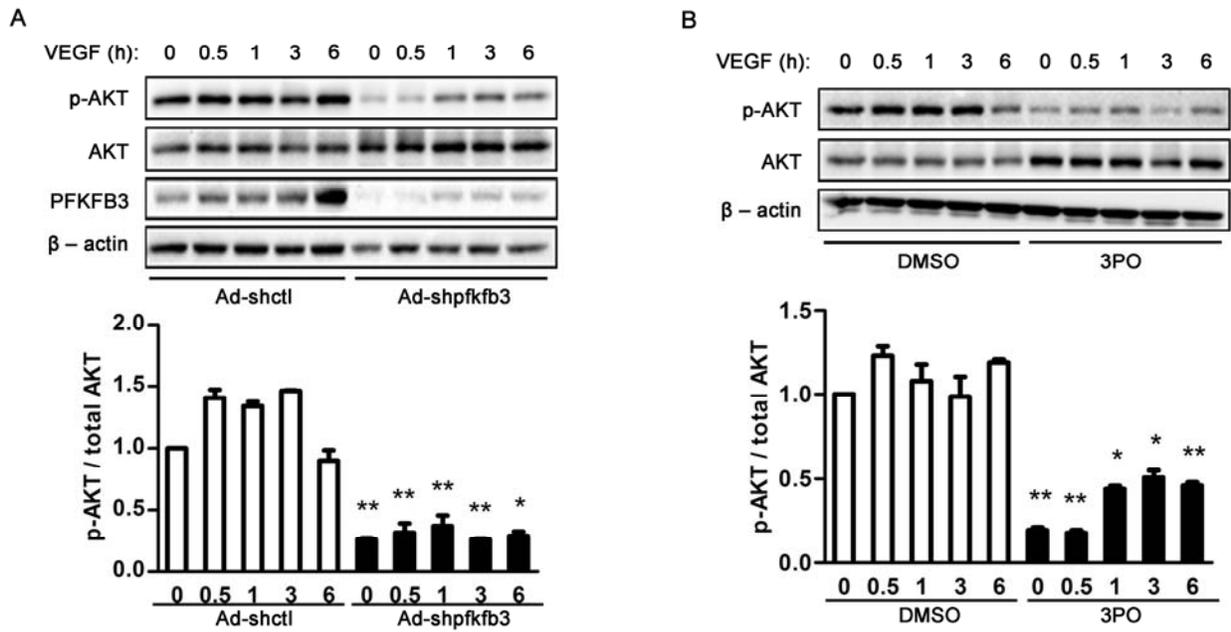
Supplemental figure II. The effects of PFKFB3 deficiency on normal postnatal retinal development.

A, Isolectin staining of whole-mount retinal samples of PFKFB3^{flox/flox} and PFKFB3^{flox/flox}cdh5^{cre} mice at P4 (n = 8 mice per genotype). B, Isolectin staining of whole-mount retinal samples of PFKFB3^{flox/flox} and PFKFB3^{flox/flox}cdh5^{cre} mice at P7 (n = 8 mice per genotype).



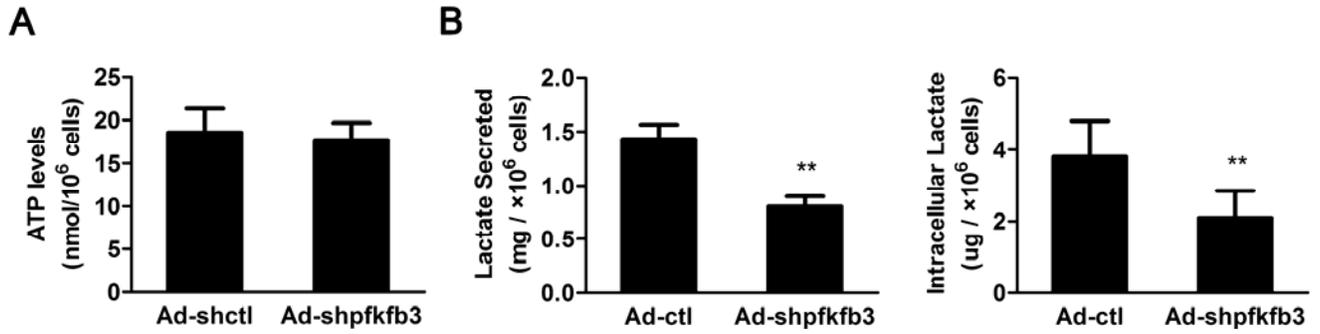
Supplemental figure III. The oxygen-induced retinopathy in $pkfkb3^{+/+}$ and $pkfkb3^{+/-}$ mice.

A, Representative images of isolectin-stained whole-mount retinal samples of PFKFB3^{+/+} and PFKFB3^{+/-} OIR mice at P17. B, Quantification of the neovascular tuft (NVT) and avascular areas (n = 8 mice per genotype).



Supplemental figure IV. AKT as downstream event of PFKFB3 in endothelial cells following VEGF treatment.

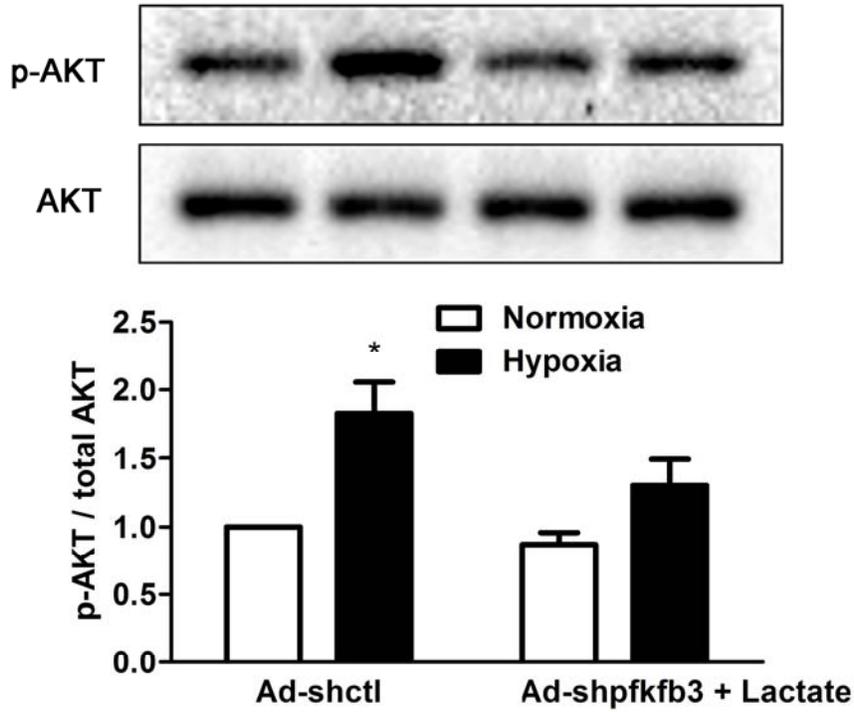
A, Western blot analysis of p-AKT and AKT in control and PFKFB3-knockdown HUVECs upon VEGF treatment for 0, 1/2, 1, 3 or 6 hrs. B, Western blot analysis of p-AKT and AKT in control and 3PO-treated HUVECs exposed to VEGF for 0, 1/2, 1, 3 or 6 hr. All images shown are representative, and data are the mean \pm SD (n = 3 independent experimental groups). *, $P < 0.05$ and **, $P < 0.01$ Ad-shpfkfb3 vs. Ad-shctl or 3PO vs DMSO under the same condition.



Supplemental figure V. Lactate production in PFKFB3-knockdown HUVECs under hypoxic conditions.

A, Quantification of ATP levels in PFKFB3-knockdown HUVECs and control cells after 24 h incubation. B, Quantification of secreted lactate levels and intracellular lactate levels in PFKFB3-knockdown HUVECs and control cells after 72 h hypoxic treatment. Data are the mean \pm SD (n = 3).

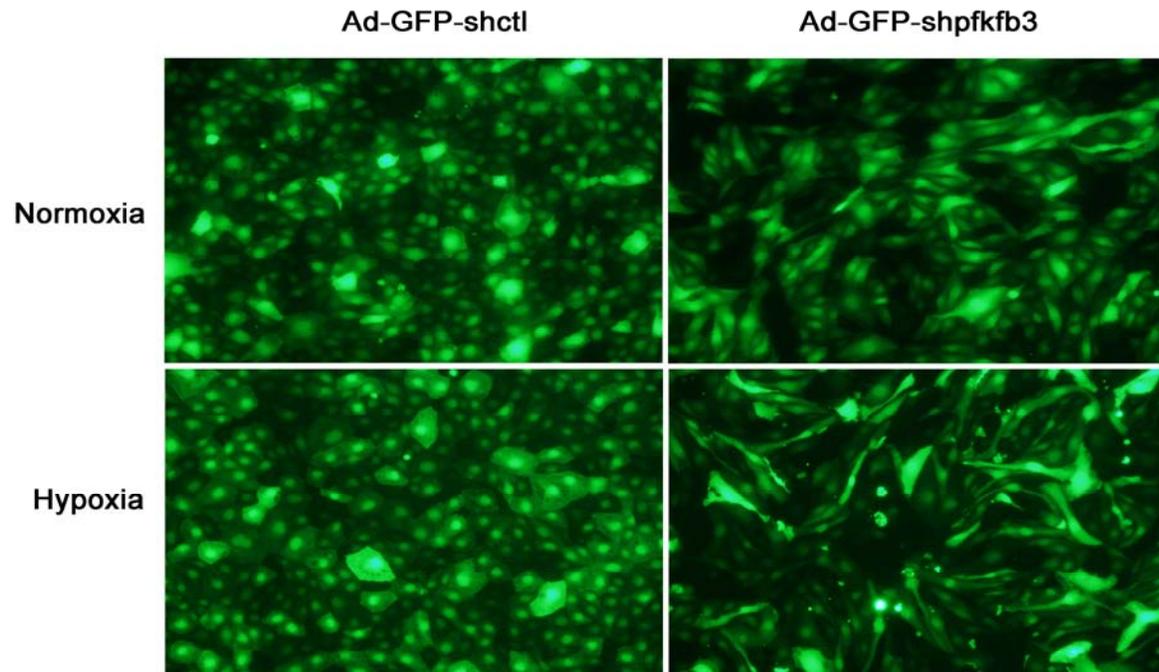
***P* < 0.01 Ad-shpfkfb3 vs. Ad-shctl under the same condition.



Supplemental figure VI. Western blot analysis of p-AKT and AKT in PFKFB3-knockdown HUVECs upon hypoxia.

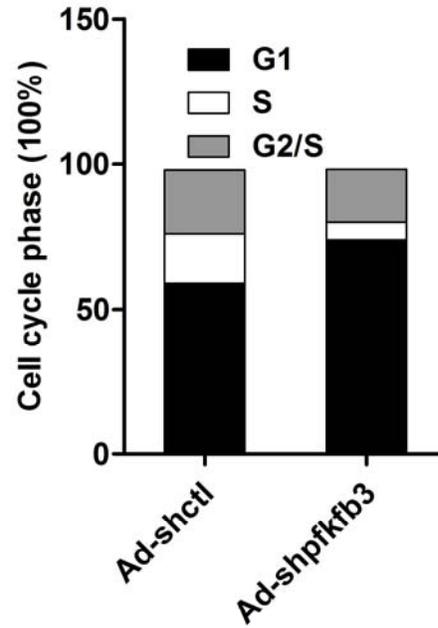
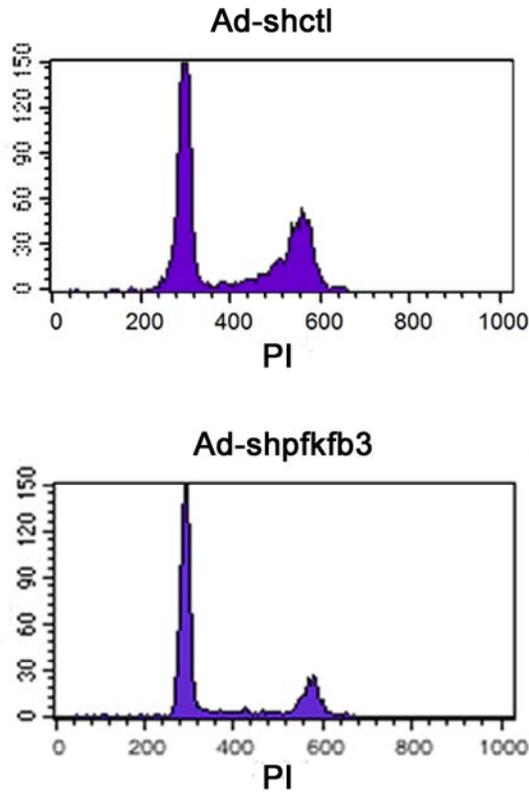
PFKFB3-knockdown HUVECs and control cells were exposed to normoxia and/or hypoxia for 6 h.

For the PFKFB3-knockdown HUVECs, 10 mM sodium lactate was added 1 h before exposed to normoxia and/or hypoxia. Data are the mean \pm SD (n = 3). *, $P < 0.05$ hypoxia vs. normoxia in control HUVECs.



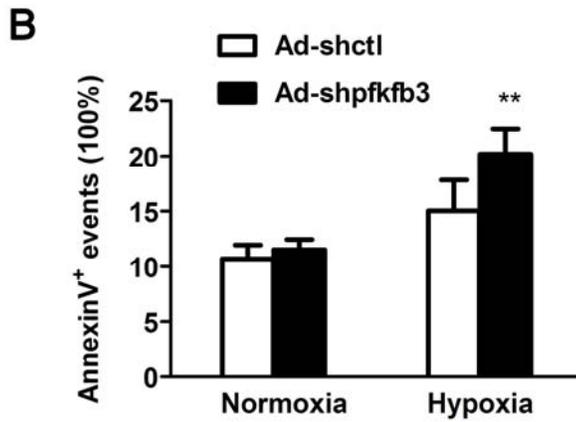
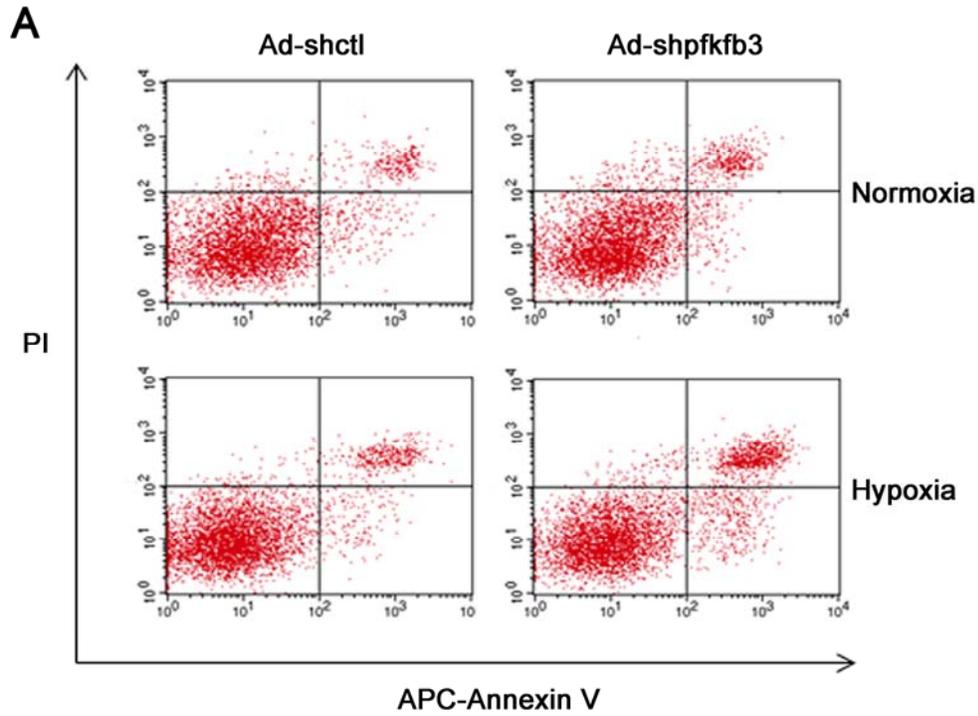
Supplemental figure VII. Morphology of PFKFB3-knockdown HUVECs.

Representative photomicrographs of cultured PFKFB3-knockdown HUVECs and control cells exposed to normoxia or hypoxia (0.5% oxygen) for 24 h.



Supplemental figure VIII. The cell cycle of PFKFB3-knockdown HUVECs under hypoxic conditions.

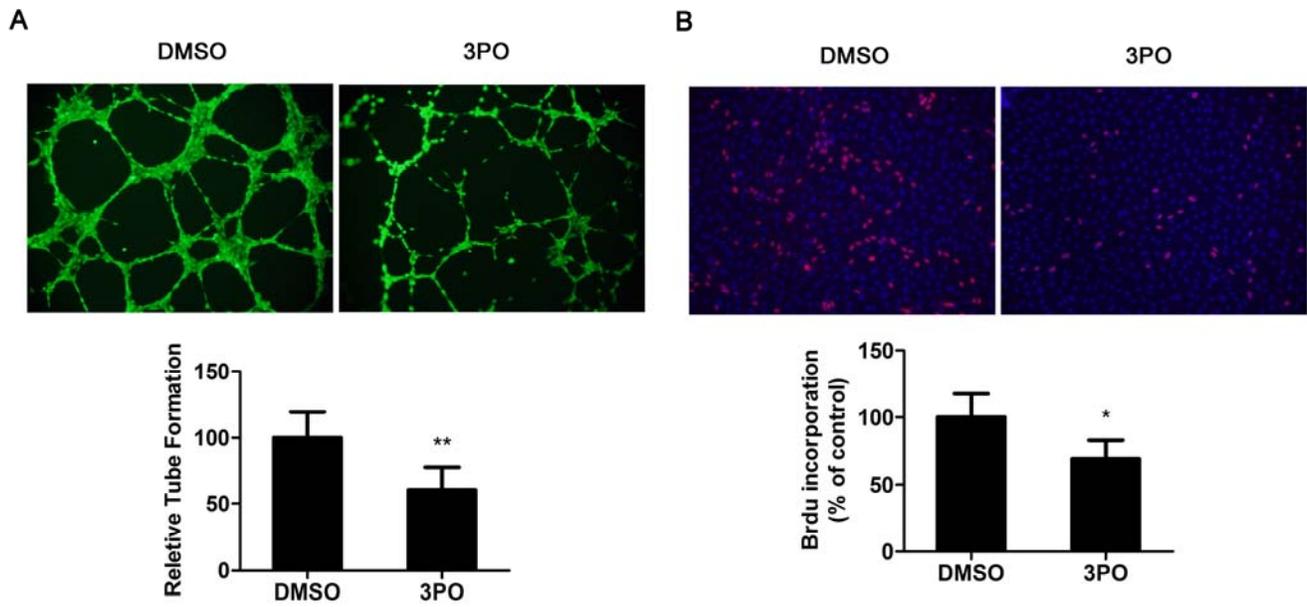
PFKFB3-knockdown HUVECs and control cells were synchronized in G0/G1 by total FBS depletion for 16 h and then exposed to complete medium for an additional 24 h under hypoxic condition (0.5% oxygen).



Supplemental figure IX. Involvement of PFKFB3 in endothelial apoptosis.

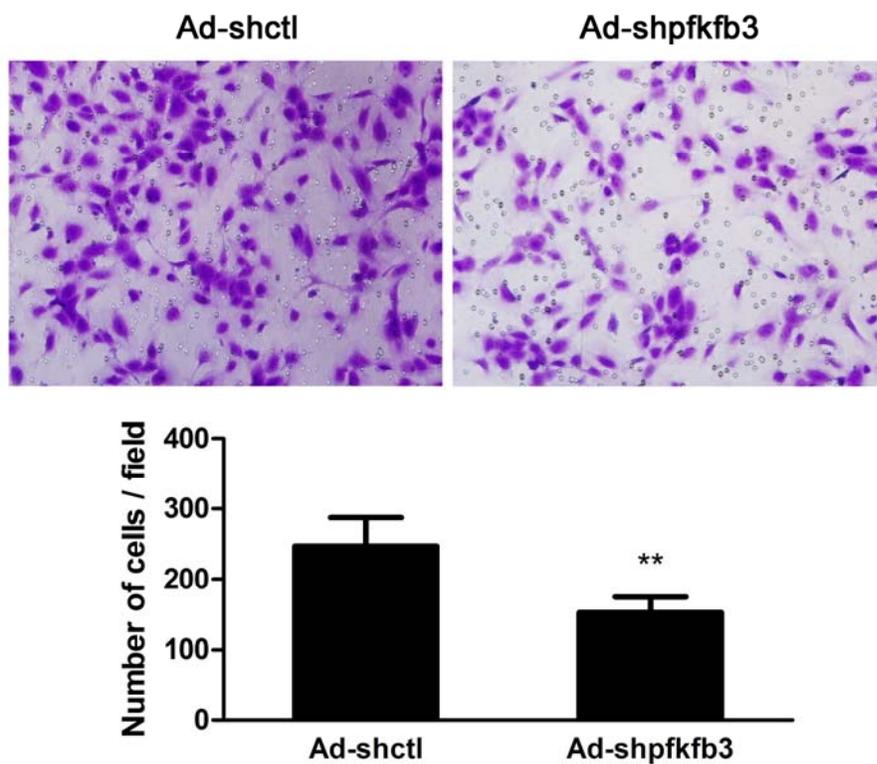
A, Flow cytometric analysis of apoptosis in PFKFB3-knockdown HUVECs and control cells under a normoxic or hypoxic environment. B, Quantification of the annexin V⁺ HUVEC ratio for each group.

***P* < 0.01 Ad-shpfb3 vs. Ad-shctl under the same condition.



Supplemental figure X. Involvement of PFKFB3 in endothelial proliferation and tube formation.

A, Representative images and quantification of tube formation in 3PO-treated HUVECs and control cells cultured in growth factor-deprived Matrigel. B, BrdU staining of proliferating HUVECs. 3PO-treated cells and control cells were synchronized in G0/G1 by total FBS depletion for 16 h and then incubated with complete medium for an additional 24 h. For bar graphs, data are the mean \pm SD, n = 3. *, $P < 0.05$ and **, $P < 0.01$ 3PO vs. control. All images shown are representative.



Supplemental figure XI. Involvement of PFKFB3 in endothelial migration.

Representative images and quantification of endothelial migration. PFKFB3-knockdown HUVECs and control cells cultured in a transwell migrated to cell medium with hVEGF at 20ng/mL. For bar graph, data are the mean \pm SD, n = 3. **, $P < 0.01$ for Ad-shpfkfb3 vs. Ad-shctl.

Supplemental table I. Primer sequences for QRT-PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse pfkfb3	GATCTGGGTGCCCGTCGATCACCG	CAGTTGAGGTAGCGAGTCAGCTTC
Human pfkfb1	CTCCATCTACCT TTGCCGACA	GCCCTGGGACTGAATGAAGTT
Human pfkfb2	CACCAATACAACCCGGGAGA	GCAGCAATGACATCAGGATCA
Human pfkfb3	CTCGCATCAACAGCTTTGAGG	TCAGTGTTTCCTGGAGGAGTC
Human pfkfb4	CCAAGTGCCCAACTCTCATTG	GCGATACTGGCCAACATTGAA
Mouse HPRT	AGTGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT
18S ribosomal RNA	GCCTCACTAAACCATCCAA	TCAGTGTTTCCTGGAGGAGTC

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™ 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 iScript™ 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 $2^{-\Delta\Delta ct}$ 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 μ g 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 μ M 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% O₂) or hypoxia (0.5% O₂) 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 CaCl₂ 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 retina³. 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 isoflurane, 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 \pm 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 $p < 0.05$.

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