CELL BIOLOGY

The requirement of phosphoenolpyruvate carboxykinase 1 for angiogenesis in vitro and in vivo

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We here examined the potential biological function of phosphoenolpyruvate carboxykinase 1 (PCK1) in angiogenesis. shRNA- or CRISPR-Cas9–induced PCK1 depletion potently inhibited endothelial cell proliferation, migration, sprouting, and tube formation, whereas ectopic PCK1 overexpression exerted opposite activity. In HUVECs, $G\alpha_{i3}$ expression and Akt activation were decreased following PCK1 depletion, but were augmented by ectopic PCK1 overexpression. In vivo, retinal expression of PCK1 gradually increased from postnatal day 1 (P1) to P5. The intravitreous injection of endothelial-specific PCK1 shRNA adenovirus at P1 potently inhibited the radial extension of vascular plexus at P5. Conditional endothelial knockdown of PCK1 in adult mouse retina increased vascular leakage and the number of acellular capillaries while decreasing the number of RGCs in murine retinas. In diabetic retinopathy patients, *PCK1* mRNA and protein levels were up-regulated in retinal tissues. Together, PCK1 is essential for angiogenesis possibly by mediating $G\alpha_{i3}$ expression and Akt activation.

INTRODUCTION

Vascular dysfunction is commonly observed in a number of human diseases, including stroke, heart diseases, diabetes, chronic kidney failure, tumor progression, retinal diseases, venous thrombosis, and severe viral infectious diseases (*1*, *2*). It disrupts the delivery of oxygen and nutrients, leading to an imbalance between metabolic demand and supply (*3*, *4*). In most adult organisms, the endothelial cells, located in the lumen of blood vessels (*3*, *4*), rarely proliferate and exist in a quiescent state (*3*, *4*). Nevertheless, these cells can rapidly form new vessels, a programmed process termed angiogenesis (*3*, *4*). Angiogenesis can be initiated under nutrient-deprived conditions (i.e., ischemic and/or hypoxic environments) when oxygen and nutrient supply is strongly needed (*3*–*7*). Stimulation with multiple growth factors, including VEGF (vascular endothelial growth factor) and many others, can also stimulate angiogenesis (*3*–*7*).

Activated endothelial cells are known to secrete multiple matrix metalloproteinases (MMPs), thereby degrading the basement membrane and enabling invasiveness (*8*). Endothelial tip cells migrate to the site of the angiogenic signals (*7*, *9*, *10*). Tip cells are polarized, extending filopodia and lamellipodia that guide the nascent sprout to the angiogenic signals (*7*, *9*, *10*). The following stalk endothelial cells are mostly proliferative and can promote sprout elongation and lumen formation (*7*, *9*, *10*). When tip cells from neighboring sprouts meet, they can form a perfused new vessel (*7*, *9*, *10*). The perfusion will promote vascular maturation by reestablishing pericyte recruitment and basement membrane, thereby stabilizing blood vessels (*6*, *11*).

Angiogenic growth of blood vessels is a tightly controlled process integrating numerous cellular processes, including cell proliferation, differentiation, and migration, as well as matrix adhesion and cell-cell signaling communication (*3*, *4*, *6*, *7*). Signaling by receptor tyrosine kinases (RTKs) and the serine/threonine kinases is essential for the angiogenic response (*3*, *4*, *6*, *7*). Phosphoenolpyruvate carboxykinases (PEPCKs or PCKs), including the cytoplasmic PCK1 and the mitochondrial PCK2, are key enzymes catalyzing the first rate-limiting step in gluconeogenesis (*12*–*14*). *PCK1* gene is located on chromosome 20q13.31, and the protein is mainly located in the cytoplasm under resting condition, but activated PCK1 is reported to translocate into the endoplasmic reticulum (*15*).

Recent studies propose a nongluconeogenic function of PCK1 [i.e., functioning as a protein kinase (*16*)] that promotes growth, migration, and metastasis of different human cancer cells (*15*–*19*). In hepatocellular carcinoma (HCC) cells, silencing of PCK1 suppressed INSIG (insulin-induced gene) 1/2 phosphorylation, thus inhibiting cell proliferation and HCC tumorigenesis (*15*, *16*). Shao *et al.* (*17*) reported that PCK1 promoted nuclear sterol regulatory element-binding transcription factor 1 (SREBF1) activation in non–small cell lung cancer (NSCLC) cells, thereby promoting cancer growth. Under hypoxia conditions, PCK1 promoted pyrimidine nucleotide biosynthesis, which was important for colorectal cancer metastatic growth (*18*). Zhu *et al.* (*20*) recently reported that PCK1 promoted pancreatic cancer cell growth by mediating Akt activation. The biological function and underlying signaling mechanisms of PCK1 in angiogenesis have not been studied. The results of the present study show that PCK1 is essential for angiogenesis in vitro and in vivo, possibly by mediating $G\alpha_{i3}$ expression and Akt activation.

RESULTS

PCK1 shRNA inhibits proliferation, migration, tube formation, and sprouting in cultured endothelial cells

To study the potential effect of PCK1 on angiogenesis in vitro, stable human umbilical vein endothelial cells (HUVECs) expressing lentiviral short hairpin RNA (shRNA) targeting the nonoverlapping sequences of *PCK1*, shPCK1-seq-1 and shPCK1-seq-2 [from M. B. Chen (*20*)], were established. Quantitative real-time polymerase chain reaction (qRT-PCR) assay results showed that the applied PCK1 shRNAs resulted in robust *PCK1* mRNA silencing [$P < 0.05$ versus cells with

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scramble control shRNA (shC)] (Fig. 1A). *PCK2* mRNA expression was, however, unchanged (Fig. 1B). Expression of PCK1 protein, but not PCK2, was down-regulated in shPCK1-expresing HUVECs (Fig. 1C).

As demonstrated, shRNA-induced silencing of PCK1 robustly decreased the EdU (5-ethynyl-2′-deoxyuridine)–positive nuclei ratio in HUVECs, confirming proliferation inhibition (Fig. 1D). Moreover, PCK1 shRNA inhibited HUVEC in vitro migration, tested by "Transwell" (Fig. 1E) assays. A tube formation assay (Fig. 1F) demonstrated that the number of the tube-like structures was decreased in PCK1-silenced HUVECs. Results from an in vitro sprouting assay showed that HUVEC sprouting was inhibited following PCK1 silencing, as the sprouting number and the average length of each sprout were both markedly decreased (Fig. 1G). shC treatment failed to significantly alter PCK1 and PCK2 expression (Fig. 1, A to C) as well as HUVEC functions (Fig. 1, D to G). In contrast, shRNA-induced silencing of PCK2 did not alter *PCK1* mRNA and protein expression in HUVECs (fig. S1, A to C). Moreover, PCK2 silencing failed to inhibit HUVEC proliferation and migration, which were tested by nuclear EdU staining (fig. S1D) and Transwell (fig. S1E) assays, respectively.

In human retinal microvascular endothelial cells (hRMECs) and hCMEC/D3 brain endothelial cells, the application of the shPCK1-seq-1 lentivirus also resulted in *PCK1* mRNA silencing (Fig. 1H). PCK1 silencing potently inhibited proliferation (confirmed by the decreased EdU-positive nuclei ratio; Fig. 1I) in hRMECs and hRMEC/D3 cells. These results support the idea that PCK1 silencing results in an antiangiogenic response in cultured endothelial cells.

PCK1 shRNA induces moderate but notable apoptosis in endothelial cells

Studies have shown that PCK1 silencing or knockout (KO) can provoke apoptosis in human cancer cells (*20*). Silencing of PCK1 in HUVECs (see Fig. 1) increased caspase-3 activity (Fig. 2A) and caspase-7 activity (Fig. 2B) ($P < 0.05$ versus shC-expressing cells). Figure 2C demonstrates that PCK1 silencing induced cleavage of caspase-3, caspase-9, and poly(adenosine diphosphate–ribose) polymerase (PARP). In addition, single-stranded DNA (ssDNA) contents were significantly increased in PCK1-silenced HUVECs (Fig. 2D), suggesting increased DNA breakage. PCK1 silencing induced significant apoptosis activation in HUVECs, increasing TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling)–positive nuclei ratio (Fig. 2E). However, overall, the PCK1 silencing–induced HUVEC apoptosis was relatively weak, and only about 15% of HUVECs were apoptotic after PCK1 silencing (Fig. 2E).

In contrast, PCK2 silencing by the lentiviral shRNA did not induce cleavage of caspase-3, caspase-9, and PARP in HUVECs (fig. S2A). No apoptosis was induced by PCK2 silencing in HUVECs, as the TUNEL-positive nuclei ratio was not significantly increased (fig. S2B). In hRMECs and hCMEC/D3 brain endothelial cells, shRNA-induced silencing of PCK1 (see Fig. 1) also provoked moderate apoptotic activation, as demonstrated by the increased TUNEL-positive nuclei ratio (Fig. 2F) and annexin V staining (Fig. 2G).

CRISPR-Cas9–induced PCK1 KO exerts antiangiogenic function in cultured endothelial cells

To rule out the possible off-target effect of the applied PCK1 shRNAs and to further confirm the role of PCK1 in angiogenesis in vitro, a lentiviral CRISPR-Cas9–PCK1–KO construct [from M. B. Chen (*20*)] was transduced into Cas9-expressing HUVECs. Stable HUVECs, namely, "koPCK1" cells, were established after puromycin selection and *PCK1* KO screening. As demonstrated, the expression of *PCK1* mRNA (Fig. 3A) and protein (Fig. 3B) was depleted in the koPCK1 cells, whereas *PCK2* mRNA (Fig. 3A) and protein (Fig. 3B) expression was unchanged. CRISPR-Cas9–induced PCK1 KO inhibited HUVEC proliferation and migration, which were tested by nuclear EdU staining (Fig. 3C) and Transwell (Fig. 3D) assays, respectively. After PCK1 KO, the number of tube-like structures in HUVECs was remarkably decreased (Fig. 3E). Moreover, results from the in vitro sprouting assays showed that PCK1 KO decreased the sprouting number and reduced the average sprouting length in HUVECs (Fig. 3F). In line with the shRNA data, PCK1 KO also exerted antiangiogenic activity in cultured HUVECs. PCK1 KO also provoked apoptosis, as the TUNEL-positive nuclei ratio was significantly increased in koPCK1 HUVECs (Fig. 3G). The lentiviral CRISPR-Cas9 control construct ("Cas9-C") did not significantly alter PCK1/2 expression (Fig. 3, A and B) or HUVEC functions (Fig. 3, C to G).

Using the same CRISPR-Cas9 gene editing method, PCK2 KO in HUVECs did not affect *PCK1* mRNA and protein expression (fig. S1, A to C). HUVEC proliferation (EdU-positive nuclei ratio; fig. S1D) and migration (Transwell assays; fig. S1E) were unchanged after PCK2 KO. Moreover, CRISPR-Cas9–induced PCK2 KO failed to induce caspase-3, caspase-9, and PARP cleavages (fig. S2A) as well as apoptosis activation (TUNEL assays; fig. S2B) in HUVECs.

Ectopic overexpression of PCK1 exerts proangiogenic functions in cultured endothelial cells

We tested whether ectopic PCK1 overexpression would exert proangiogenic activity in HUVECs. A lentiviral construct encoding the *PCK1* cDNA was transduced to HUVECs, and two stable cell selections, "oePCK1-sL-1" and "oePCK1-sL-2," were established. As shown, the expression of *PCK1* mRNA (Fig. 4A) and protein (Fig. 4B) was significantly augmented in oePCK1 HUVECs ($P < 0.05$ versus vector control cells). Evidenced by the increased EdU-positive nuclei ratio, PCK1 overexpression promoted HUVEC proliferation (Fig. 4C). Moreover, PCK1 overexpression accelerated HUVEC migration (Fig. 4D). The number of tube-like structures was significantly increased in oePCK1 HUVECs (Fig. 4E). The in vitro sprouting assay results showed that the average sprout number and the average sprout length were both significantly increased after PCK1 overexpression in HUVECs (Fig. 4, F and G). In hRMECs and hCMEC/D3 cells, lentiviral PCK1 expression construct transduction similarly led to overexpression of *PCK1* mRNA (Fig. 4H). Ectopic overexpression of PCK1 augmented proliferation (EdU-positive nuclei ratio; Fig. 4I) in hRMECs and hCMEC/D3 cells. These results further support the idea that ectopic overexpression of PCK1 exerts proangiogenic activity in cultured endothelial cells.

PCK1-induced proangiogenic activity in cultured endothelial cells is mediated by promoting Akt activation

Akt activation is essential for angiogenesis (*21*–*23*). A recent study has shown that PCK1-mediated Akt activation promotes cancer cell growth (*20*). We therefore tested whether PCK1 was required for Akt activation in endothelial cells. As shown, PCK1 silencing inhibited Akt activation (Ser^{473} and Thr^{308} phosphorylation) in HUVECs (Fig. 5A). Similarly, Akt activation was largely inhibited in HUVECs

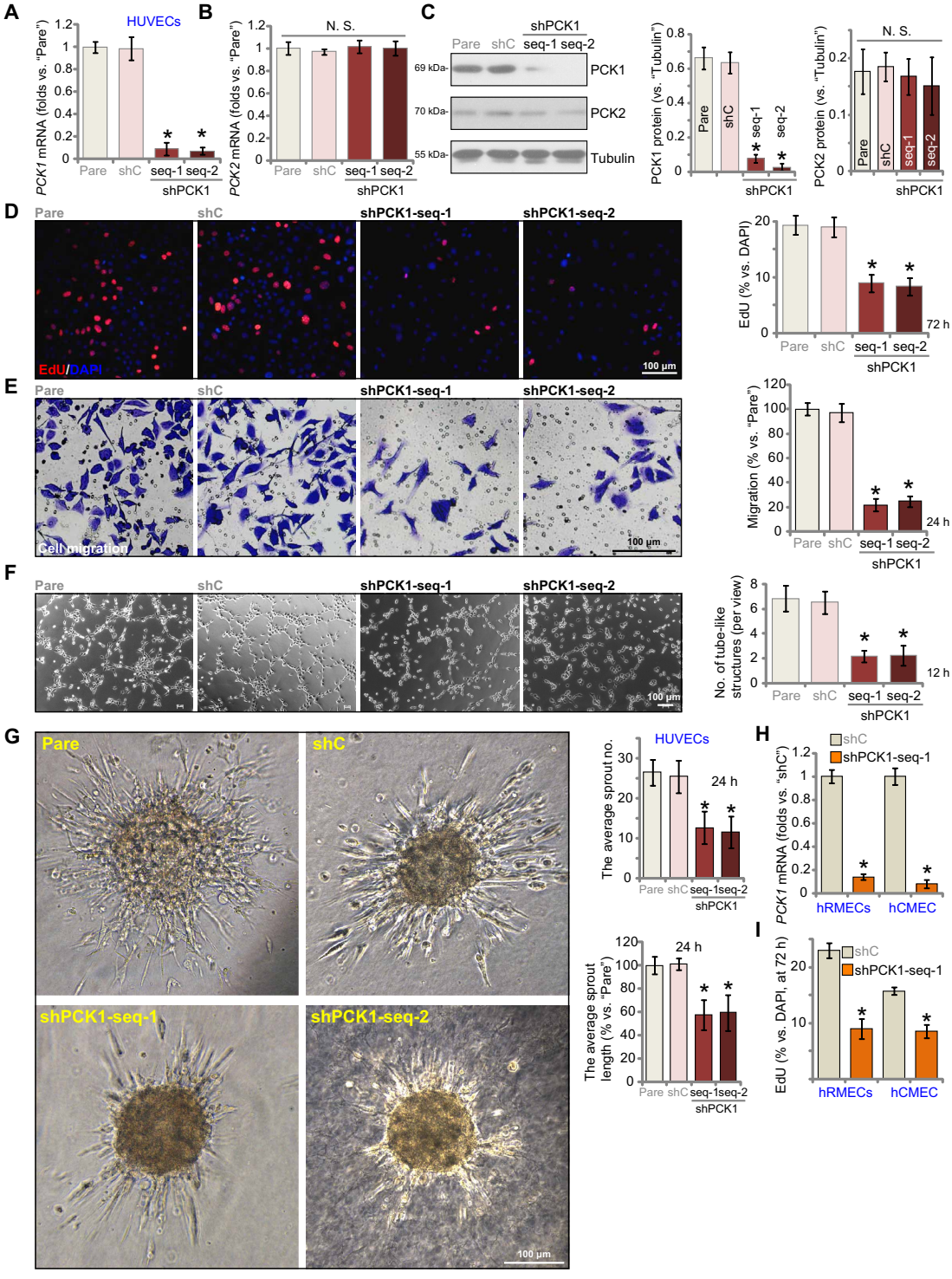

Fig. 1. PCK1 shRNA inhibits proliferation, migration, tube formation, and sprouting in cultured endothelial cells. HUVECs (**A** to **G**), hRMECs (**H** and **I**), or hCMEC/D3 brain endothelial cells (H and I) expressing the applied PCK1 shRNA (shPCK1-seq-1 or shPCK1-seq-2, with different sequences) or the scramble control shRNA (shC) were established, and expression of listed genes was shown (A to C and H). Cells were further cultured for the indicated time periods, and cell proliferation (by testing EdU-positive nuclei ratio) (D and I) and migration (Transwell assays) (E) as well as the tube formation (F) and in vitro sprouting (G) were tested by the listed assays. "Pare" stands for the parental control cells. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Pare/shC cells. "N. S." stands for nonstatistical differences ($P > 0.05$) (B and C). The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (D to G).

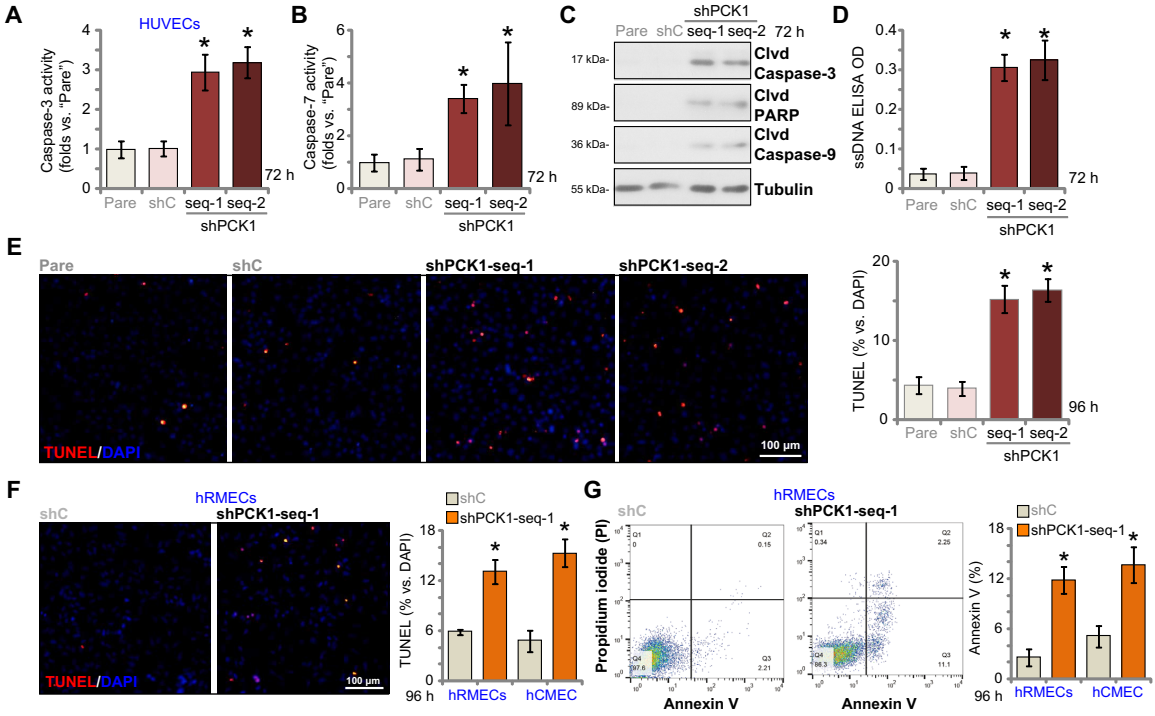

Fig. 2. PCK1 shRNA induces moderate but notable apoptosis activation in endothelial cells. HUVECs (**A** to **E**), hRMECs (**F** and **G**), or hCMEC/D3 brain endothelial cells (F and G) expressing the applied PCK1 shRNA (shPCK1-seq-1/shPCK1-seq-2) or the scramble control shRNA (shC) were established and cultured for the designated time periods. The relative caspase-3 activity (A), relative caspase-7 activity (B), expression of apoptosis-associated proteins (C), and ssDNA contents (ELISAs) (D) were tested. Cell apoptosis was determined by nuclear TUNEL staining (E and F) and annexin V–propidium iodide (PI) FACS (G) assays, with results quantified. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Pare/shC cells. The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (E and F).

with PCK1 KO (by the CRISPR-Cas9 method; Fig. 5B). Conversely, ectopic overexpression of PCK1 augmented Akt Ser^{473} and Thr^{308} phosphorylation in HUVECs (Fig. 5C). These results indicate that PCK1 is important for Akt activation in endothelial cells.

To demonstrate that Akt activation is important for PCK1-driven proangiogenic activity, an adenovirus encoding constitutively active Akt1 (caAkt1, S473D) was transduced into shPCK1-seq-1–expressing HUVECs. caAkt1 restored Akt activation (Ser^{473} phosphorylation) in PCK1-silenced HUVECs (Fig. 5D), without affecting PCK1 expression (Fig. 5D). PCK1 shRNA–induced inhibitions of cell proliferation (Fig. 5E), migration (Fig. 5F), and tube formation (Fig. 5G) were largely mitigated by caAkt1, suggesting that PCK1-induced proangiogenic activity was by promoting Akt activation. Lending further support, MK-2206, an Akt-specific inhibitor (*24*), was used to inhibit Akt activation in PCK1-overexpressed HUVECs (oePCK1-sL-1; see Fig. 4). As shown, the augmented cell proliferation (Fig. 5H), migration (Fig. 5I), and tube formation (Fig. 5J) by PCK1 overexpression were almost completely reversed by MK-2206.These results support the idea that PCK1-induced proangiogenic activity in cultured endothelial cells is due to promoting Akt activation.

PCK1-induced Akt activation and proangiogenic activity are possibly due to its promotion of $G\alpha_{i3}$ expression

Our previous studies have shown that $G\alpha_i$ proteins, including $G\alpha_{i1}$ and $G\alpha_{i3}$, are important in mediating phosphatidylinositol-3 kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) cascade activation by a number of different RTKs (*25*–*31*) and certain non-RTK receptors (*32*). Since PCK1 is essential for Akt activation in endothelial cells, we examined whether PCK1 is important for the expression of $G\alpha_i$ proteins. As shown, in PCK1 shRNA–expressing HUVECs, mRNA and protein expression of $G\alpha_{i3}$, but not $G\alpha_{i1}$ and $G\alpha_{i2}$, was significantly decreased (Fig. 6A). Moreover, PCK1 KO by the CRISPR-Cas9 method decreased $G\alpha_{i3}$ protein expression, leaving $G\alpha_{i1}$ and $G\alpha_{i2}$ protein expression unchanged (Fig. 6B). Conversely, in PCK1-overexpressed HUVECs, oePCK1-sL-1 and oePCK1-sL-2, protein expression of $G\alpha_{i3}$, but not $G\alpha_{i1}$ and $G\alpha_{i2}$, was significantly increased (Fig. 6C). These results show that PCK1 is important for $G\alpha_{i3}$ expression in HUVECs.

To further support a role of PCK1 in $G\alpha_{i3}$ and Akt activation, a kinase-dead PCK1-S90A (*15*, *20*) construct [from M. B. Chen's group (*20*)] was stably transduced to HUVECs. PCK1-S90A downregulated $G\alpha_{i3}$ expression and Akt activation in HUVECs (Fig. 6D). Moreover, HUVEC proliferation and migration were inhibited by PCK1-S90A mutation (Fig. 6, E and F).

We also focused on the underlying mechanisms of PCK1-mediated $G\alpha_{i3}$ expression in endothelial cells. In line with the previous finding (*33*), GATA binding protein 4 (GATA4) could be an important transcription factor for $G\alpha_{i3}$ in the endothelial cells. $G\alpha_{i3}$ mRNA and protein expression was significantly decreased in GATA4 shRNA–expressing HUVECs ("shGATA4") (Fig. 6G), but was increased in HUVECs with GATA4 overexpression ("oeGATA4") (Fig. 6H). The coimmunoprecipitation assay results confirmed a direct binding between PCK1 and GATA4 in HUVECs (Fig. 6I). PCK1 silencing by targeted shRNAs largely inhibited GATA4 serine phosphorylation (Fig. 6I). The chromatin immunoprecipitation (ChIP) assay results further showed that PCK1 silencing inhibited

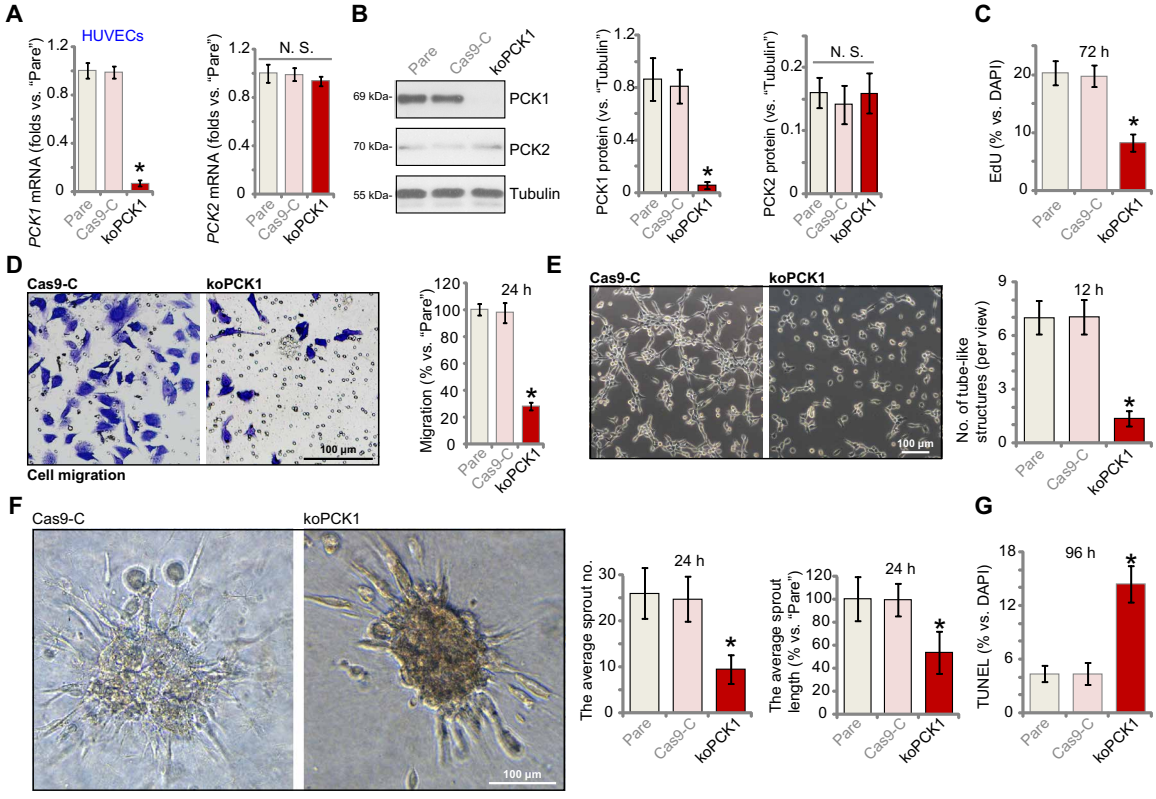

Fig. 3. CRISPR-Cas9–induced PCK1 KO exerts antiangiogenic function in cultured endothelial cells. (**A** and **B**) Stable HUVECs expressing the lentiviral CRISPR-Cas9–PCK1–KO construct ("koPCK1") or the Cas9 control construct ("Cas9-C") were established, and expression of listed genes and proteins was tested. Cells were further cultured for the indicated time periods, and cell proliferation (by testing EdU-positive nuclei ratio) (**C**), migration (**D**), tube formation (**E**), and in vitro cell sprouting (**F**) were tested. (**G**) Cell apoptosis (by measuring the TUNEL-positive nuclei ratio) was tested. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Cas9-C cells. "N. S." stands for nonstatistical differences ($P > 0.05$) (A). "N. S." stands for nonstatistical differences ($P > 0.05$) (A and B). The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (D to F).

GATA4 binding to the $G\alpha_{i3}$ promoter DNA (*33*) in HUVECs (Fig. 6J), without affecting GATA4 expression (Fig. 6I). GATA4 serine phosphorylation is shown to be vital for its transcription activity (*34*–*36*); we propose that PCK1 directly associates with GATA4, which is important for GATA4 phosphorylation and $G\alpha_{i3}$ transcription in endothelial cells.

Notably, adenovirus expression of $G\alpha_{i3}$ ("Ad-$G\alpha_{i3}$") in shPCK1-seq-1–expressing HUVECs restored $G\alpha_{i3}$ mRNA (fig. S3A) and protein (fig. S3B) expression without affecting *PCK1* mRNA expression (fig. S3C). Moreover, Ad-$G\alpha_{i3}$ also restored Akt activation in PCK1-silenced HUVECs (fig. S3B). Ad-$G\alpha_{i3}$ significantly attenuated PCK1 knockdown-induced inhibitions of cell proliferation (fig. S3D), migration (fig. S3E), and tube formation (fig. S3F). These results suggest that PCK1-induced Akt activation and proangiogenic activity involved $G\alpha_{i3}$ expression in endothelial cells.

To further support the hypothesis, $G\alpha_{i3}$ shRNA lentivirus ("sh$G\alpha_{i3}$") was transduced into the PCK1-overexpressed HUVECs (oePCK1-sL-1; see Fig. 4). As shown, sh$G\alpha_{i3}$ silenced $G\alpha_{i3}$ and inhibited Akt activation in the oePCK1-sL-1 HUVECs, without affecting PCK1 expression (fig. S3G). The enhanced cell proliferation (increased EdU-positive nuclei ratio; fig. S3H) and increased tube-like structures (fig. S3I) by PCK1 overexpression were largely inhibited after $G\alpha_{i3}$ silencing.

Endothelial PCK1 knockdown inhibits retinal vasculature development in natal mice and disrupts retinal angiogenesis in adult mice

Retinal vasculature can be viewed directly and noninvasively, offering an accessible window to explore the mechanism behind angiogenesis and vascular dysfunction (*37*). Abnormal retinal angiogenesis can be detected in a broad spectrum of retinal disorders, including retinopathy of prematurity, diabetic retinopathy, neovascular age-related macular degeneration, neovascular glaucoma, and corneal neovascularization (*38*). We next tested whether PCK1 silencing affected normal retinal vasculature development in vivo. In murine retinal tissues, mRNA expression of *PCK1* (Fig. 7A) and $G\alpha_{i3}$ (Fig. 7B) gradually increased from postnatal day 1 (P1) to P5. *PCK2* and $G\alpha_{i2}$ mRNA levels were unchanged (Fig. 7, A and B). In line with the mRNA expression, the protein expression of PCK1 and $G\alpha_{i3}$ as well as Akt phosphorylation gradually increased (Fig. 7C). To test the effect of PCK1 silencing, C57BL/6 mice were intravitreously injected at P1 with AAV5-TIE1-PCK1 shRNA. The adeno-associated virus (AAV) shRNA contained TIE1, the endothelial cell–specific promoter sequence, thereby specifically knocking down endothelial PCK1 ("PCK1-EKD"). The control eye was intravitreously injected with AAV5–TIE1–scramble control shRNA ("shC"). As shown, the expression of PCK1 and $G\alpha_{i3}$ as well as the Akt phosphorylation

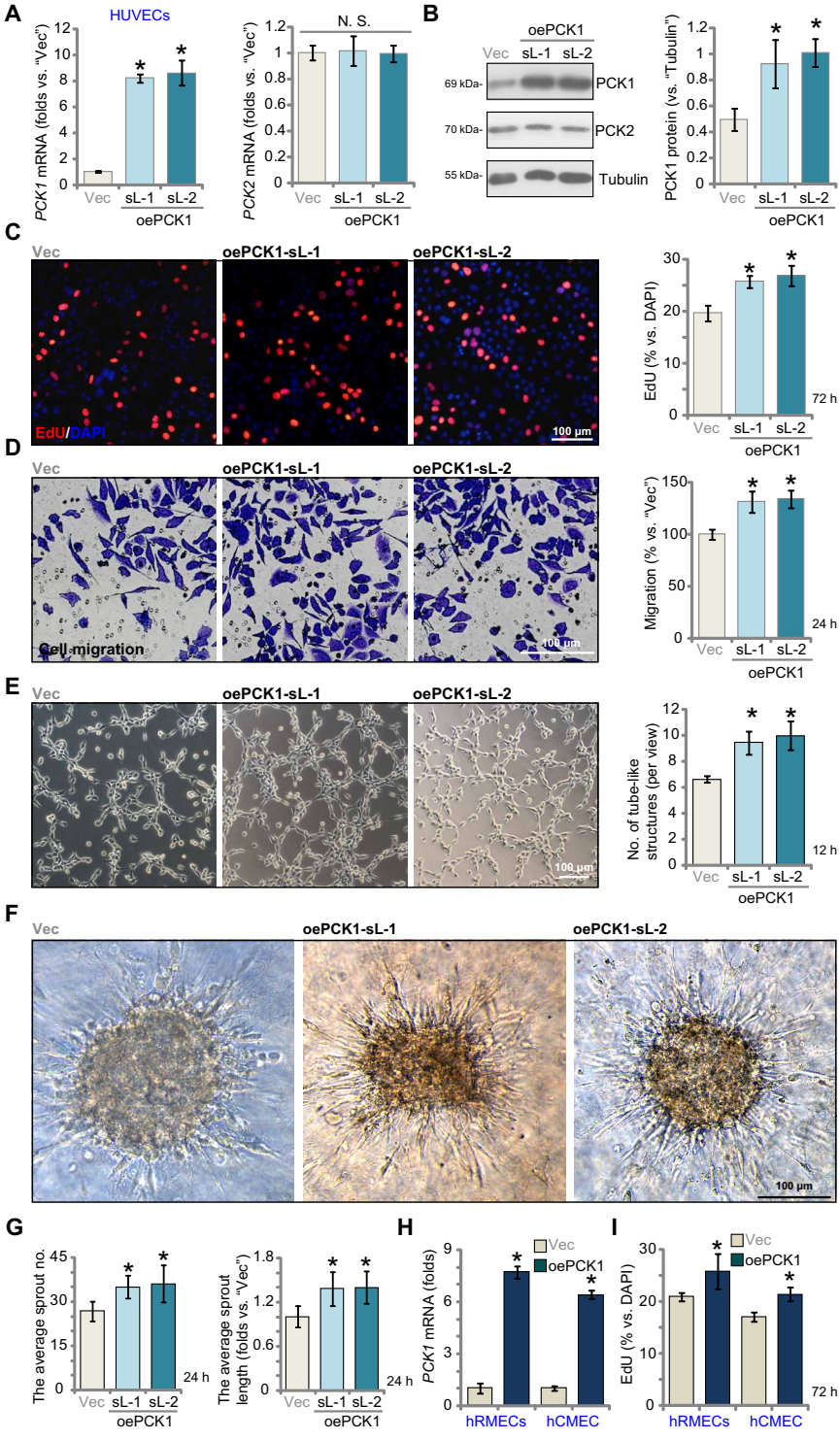

Fig. 4. Ectopic overexpression of PCK1 exerts proangiogenic functions in cultured endothelial cells. HUVECs (**A** to **G**), hRMECs (**H** and **I**), or hCMEC/D3 brain endothelial cells (H and I), with the lentiviral PCK1–expressing construct ("oePCK1") or the empty vector ("Vec") were established, and expression of listed genes and proteins was tested by qRT-PCR (A and H) and Western blotting (B) assays. Cells were further cultured for indicated time periods, and cell proliferation (by testing the EdU-positive nuclei ratio) (C and I), migration (Transwell assays) (D), tube formation (E), and the in vitro cell sprouting (F and G) were tested. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Vec cells. "N. S." stands for nonstatistical differences ($P > 0.05$) (A). The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (C to F).

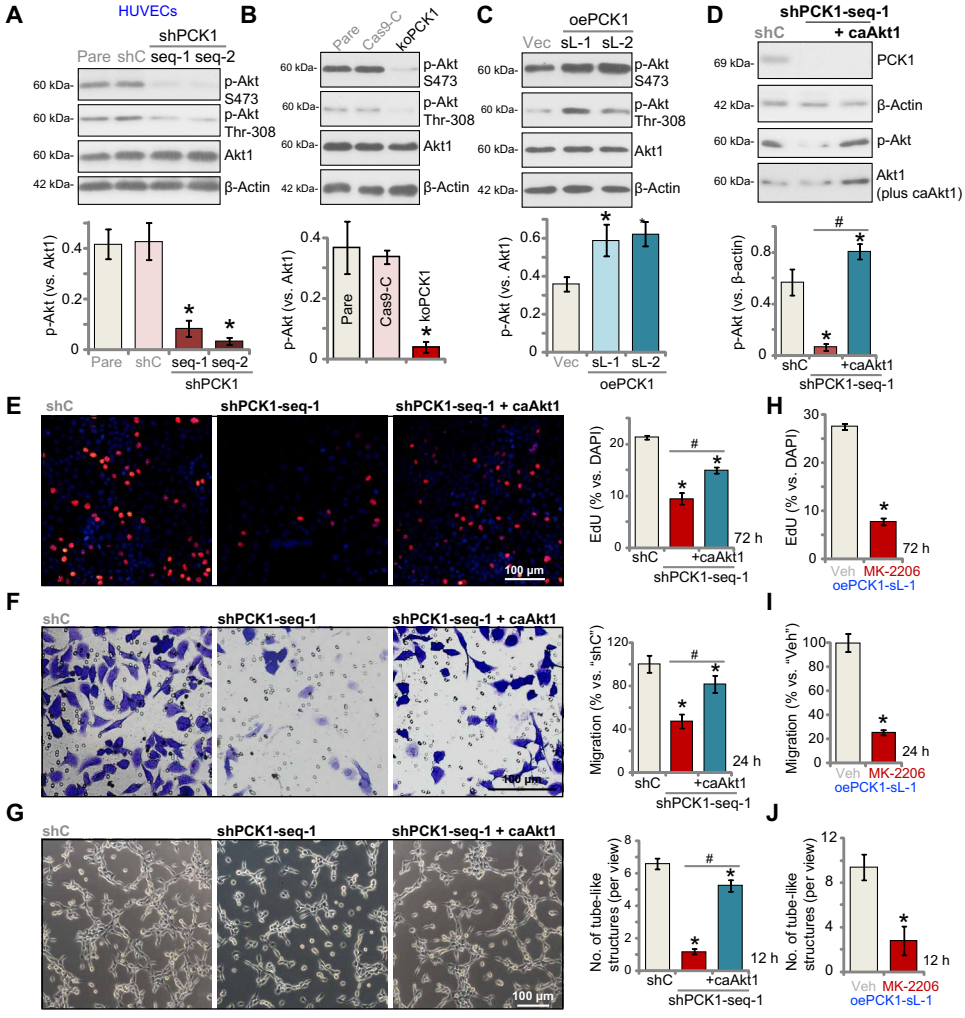

Fig. 5. PCK1-induced proangiogenic activity in cultured endothelial cells is mediated by promoting Akt activation. (**A** to **C**) Expression of listed proteins in the HUVECs with designated genetic modifications. HUVECs expressing the PCK1 shRNA (shPCK1-seq-1) were further infected with or without the adenovirus encoding the constitutively active Akt1 (caAkt1, S473D), with stable cells established after selection. (**D**) Control HUVECs were with scramble control shRNA (shC), and expression of listed proteins was shown. Cells were further cultured for the applied time periods, and cell proliferation (by testing EdU-positive nuclei ratio) (**E**), migration (Transwell assays) (**F**), and tube formation (**G**) were tested, with the results quantified. HUVECs with the lentiviral PCK1–expressing construct ("oePCK1-sL-1") were treated with MK-2206 (10 μM) or the vehicle control (0.1% dimethyl sulfoxide, "Veh") for indicated time periods. Cell proliferation (**H**), migration (**I**), and tube formation (**J**) were tested using the same methods, with results quantified. "Vec" stands for the vector control. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Pare/Vec/shC/Veh group. #$P < 0.05$ (D to G). The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (E to G).

were significantly decreased in retinal tissues with PCK1-EKD (Fig. 7D). The isolectin B4 (IB4) staining was performed at P5, and the retinal vasculature was visualized. As demonstrated, the PCK1-EKD retinas displayed a substantial delay in radial extension of vascular plexus from optic nerve to periphery at P5 (Fig. 7E). Moreover, PCK1-EKD retinas had a significantly fewer tip cells at P5 ($P < 0.05$ versus shC-treated retinas; Fig. 7F). These results show that PCK1 silencing interfered with normal retinal vasculature development. In the primary murine retinal ganglion cells (RGCs; fig. S4A), infection with the AAV5-TIE1-PCK1 shRNA failed to affect PCK1 expression. The regular AAV5-PCK1 shRNA down-regulated PCK1 expression (fig. S4B).

We next tested whether PCK1 silencing could affect retinal angiogenesis in adult mice. C57B/6 mice (4 weeks old, male) were intravitreously injected with the AAV5-TIE1-PCK1 shRNA (PCK1-EKD) or the AAV5–TIE1–scramble control shRNA (shC). Western blotting assay results showed that PCK1 and $G\alpha_{i3}$ expression as well as the Akt phosphorylation decreased in adult retinal tissues after the PCK1-EKD treatment (Fig. 7G). The Evans blue assay confirmed significantly increased vascular leakage in PCK1-EKD retinas (Fig. 7H). Moreover, the retinal trypsin digestion assay demonstrated that there were a significantly increased number of acellular capillaries in the PCK1-EKD murine retinas ($P < 0.05$ versus shC-treated retinas; Fig. 7I).

Recent studies have used NeuN (neuronal nuclei) as a marker for RGC population in the retina. As shown, the number of RGCs (NeuN-positive staining) was significantly decreased in the PCK1-EKD retinas (Fig. 7J). These results suggest that with the endothelial PCK1 silencing in the mouse retina, angiogenesis was disrupted and the nutrient and energy supply was dramatically decreased,

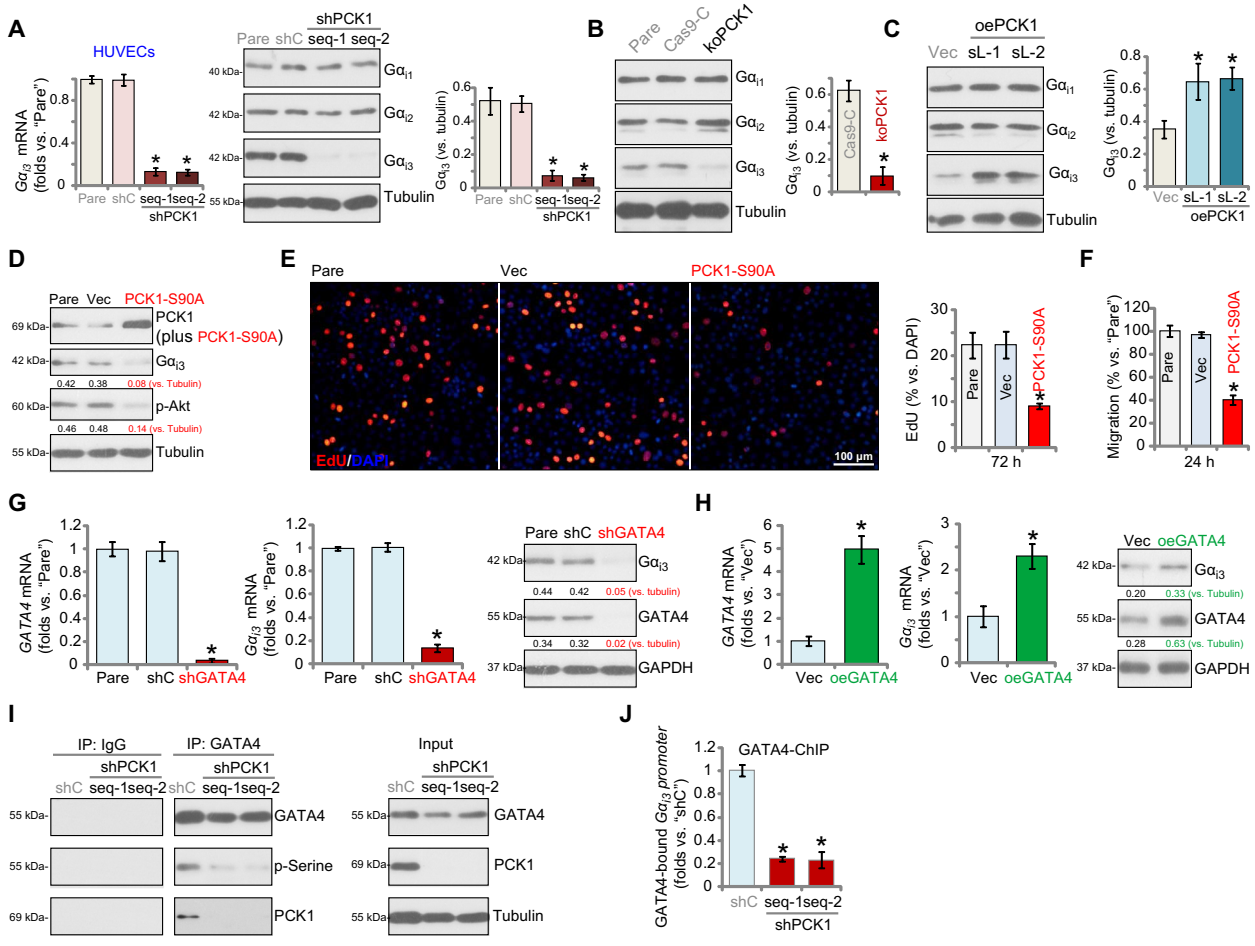

Fig. 6. PCK1-induced Akt activation and proangiogenic activity are possibly due to its promotion of $G\alpha_{i3}$ expression. (**A** to **C**) Expression of listed mRNAs and proteins in the HUVECs with designated genetic modifications. (**D**) Expression of listed proteins in the HUVECs with the kinase-dead PCK1-S90A construct and the empty vector and in the parental control cells. Cells were further cultured for the applied time periods, and cell proliferation (by testing EdU-positive nuclei ratio) (**E**) and migration (Transwell assays) (**F**) were tested, with results quantified. Stable HUVECs expressing the scramble control shRNA (shC), the lentiviral GATA4 shRNA ("shGATA4"), the GATA4-expressing lentiviral construct ("oeGATA4"), or the empty vector (Vec) were established. (**G** and **H**) Expression of listed genes and protein. HUVECs, expressing the applied PCK1 shRNA (shPCK1-seq-1/shPCK1-seq-2) or the scramble control shRNA (shC), were established, and cell lysates were obtained. (**I**) The PCK1-GATA4 association was tested by coimmunoprecipitation (co-IP) assays, and GATA4 phosphorylation and expression of PCK1- GATA4 were also tested. (**J**) ChIP showed the relative levels of *Gαi3* promoter DNA binding to GATA4 in the listed HUVECs. Data are means ± SD ($n = 5$). *$P < 0.05$ versus Pare/Vec/shC. The experiments were repeated five times with similar results obtained. Scale bar, 100 μm (E).

eventually leading to RGC degeneration. Notably, intravitreous coinjection of AAV5-TIE1 shRNA–resistant PCK1 ("shR-PCK1") restored PCK1 and $G\alpha_{i3}$ expression as well as the Akt phosphorylation in PCK1-EKD retinal tissues (Fig. 7G). PCK1-EKD–induced vascular leakage (Fig. 7H), increased acellular capillaries (Fig. 7I), and RGC degeneration (Fig. 7J) were significantly mitigated by shR-PCK1. These results demonstrate that PCK1 silencing disrupts retinal angiogenesis in adult mice.

AAV5-TIE1-$G\alpha_{i3}$ shRNA ("$G\alpha_{i3}$-EKD") was also intravitreously injected to C57B/6 mice (4 weeks old, male). Western blotting assay results showed that $G\alpha_{i3}$ expression and Akt phosphorylation were inhibited in adult retinal tissues after the $G\alpha_{i3}$-EKD treatment (fig. S4C). Evans blue assay results confirmed significantly increased vascular leakage in $G\alpha_{i3}$-EKD retinas (fig. S4D).

Endothelial conditional knockdown of PCK1 disrupts angiogenesis in mouse retina

To further support the essential role of PCK1 in angiogenesis in vivo, AAV5-FLEX-PCK1 shRNA was intravitreously injected into the TIE1-DIO-Cre C57 mice (4 weeks old, male), thereby generating PCK1 endothelial conditional knockdown (PCK1-ECKD) mice (5 days after injection). As shown, expression of PCK1, $G\alpha_{i3}$, and p-Akt was significantly decreased in the retinal tissues of the PCK1-ECKD mice (Fig. 8A). Importantly, in the retinas of PCK1-ECKD mice, significant vascular leakage (Fig. 8B) and an increased number of the acellular capillaries (Fig. 8C) were detected. Conversely, the number of NeuN-positive RGCs was decreased (Fig. 8D). Therefore, endothelial conditional knockdown of PCK1 disrupts angiogenesis and induces RGC degeneration in the retinas of adult mice.

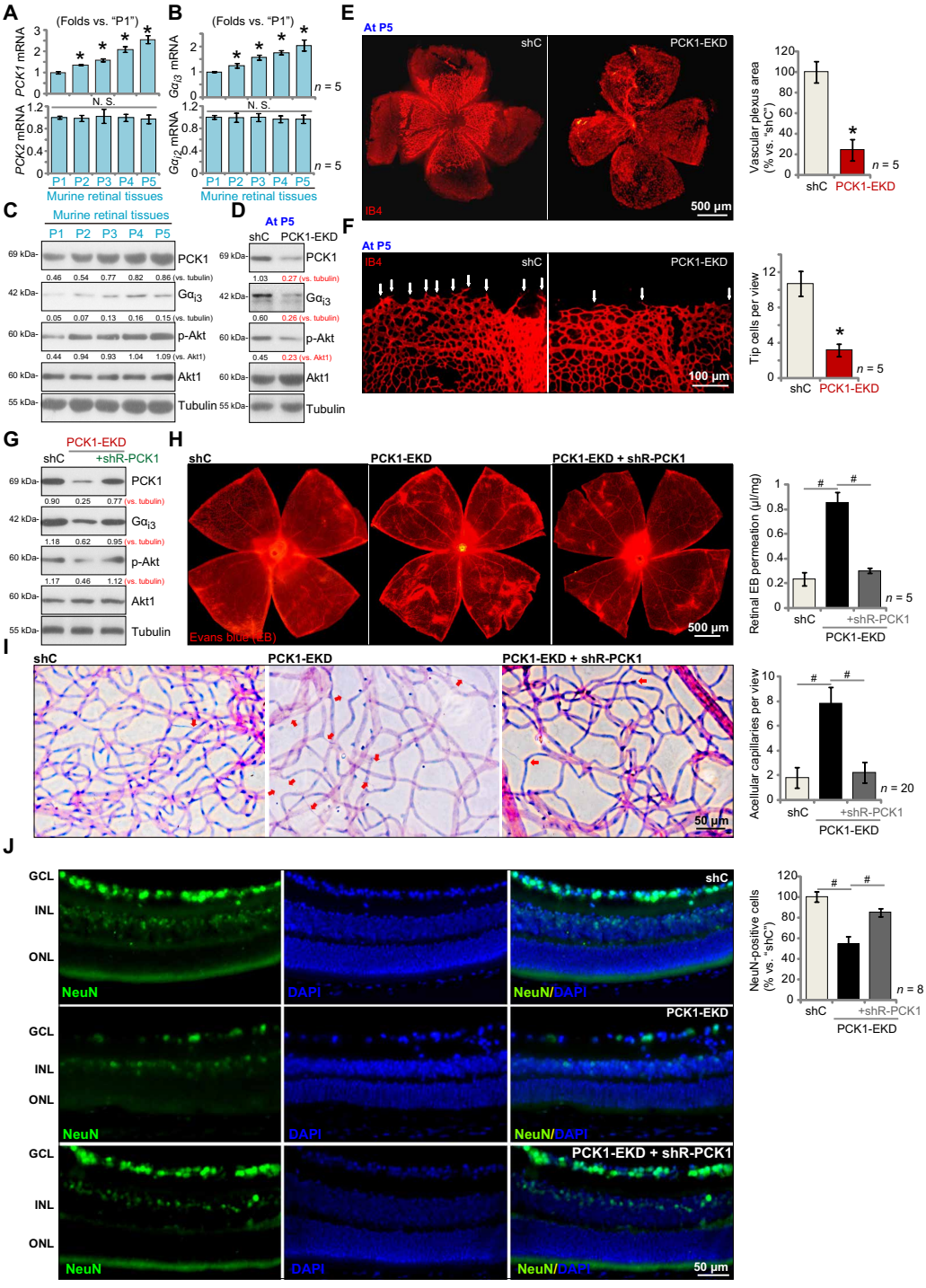

Fig. 7. Endothelial PCK1 knockdown inhibits retinal vasculature development in natal mice and disrupts retinal angiogenesis in adult mice. (**A** to **C**) Expression of listed mRNAs and proteins in the retinal tissues of P1 to P5 mice. (**D**) At P1, the C57BL/6 mice were subjected to intravitreous injection of the AAV5–TIE1 (promoter)–PCK1 shRNA ("PCK1-EKD") or the AAV5–TIE1–scramble control shRNA (shC), and expression of listed proteins in the retinal tissues was tested. (**E**) IB4 staining of the whole retina was conducted at P5. Scale bar, 500 μm. (**F**) IB4 staining of the retinal tip cells at P5. Scale bar, 100 μm. The C57B/6 adult mice (4 weeks old, all male) were intravitreously injected with the AAV5–TIE1–scramble control shRNA (shC), the AAV5-TIE1-PCK1 shRNA (PCK1-EKD), or plus the AAV5-TIE1 shRNA–resistant PCK1 expression construct ("PCK1-EKD + shR-PCK1"). (**G**) After 5 days, expression of listed proteins in the retinal tissues was tested. (**H**) The mice were infused with Evans blue (EB) for 2 hours, and the fluorescence images of flat-mounted retinas and quantification of Evans blue leakage were conducted. Scale bar, 500 μm. (**I**) The retinal trypsin digestion was performed to detect acellular capillaries, and the number of acellular capillaries (red arrows) was recorded from 20 random fields per retina. Scale bar, 50 μm. (**J**) NeuN immunofluorescence staining in the retinal slides of the mice was shown, and the number of NeuN-positive RGCs was recorded from eight retinas per group. Scale bar, 50 μm. Expressions of the listed proteins were quantified using ImageJ software (C, D, and G). Data are means ± SD. GCL, ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer (J). *$P < 0.05$ versus "P1"/shC group. #$P < 0.05$. The experiments were repeated three times with similar results obtained.

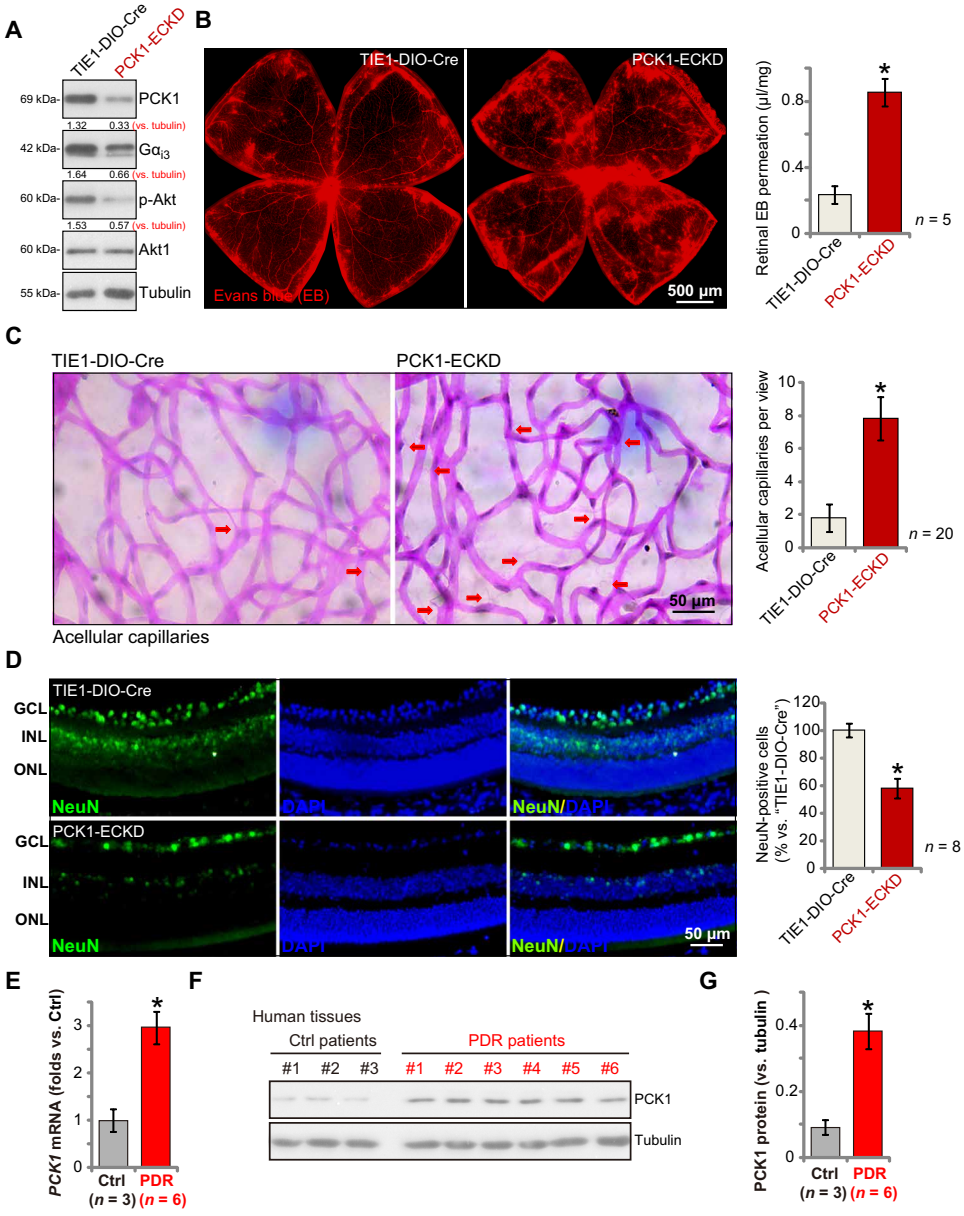

Fig. 8. PCK1 up-regulation in proliferative retinal tissues of PDR patients. The AAV5-FLEX-PCK1 shRNA was intravitreously injected to the TIE1-DIO-Cre C57BL/6 adult mice. After 5 days, PCK1 endothelial conditional knockdown (PCK1-ECKD) mice were established. (**A**) Expression of listed proteins in the retinal tissues in PCK1-ECKD mice or the TIE1-DIO-Cre C57BL/6 control mice was tested by Western blotting assays. (**B**) The mice were infused with Evans blue (EB) dye for 2 hours, and the fluorescence images of flat-mounted retinas and quantification of Evans blue (EB) leakage were conducted. Scale bar, 500 μm. (**C**) The retinal trypsin digestion was performed to detect acellular capillaries, and the number of acellular capillaries (red arrows) was recorded from 20 random fields per retina. Scale bar, 50 μm. (**D**) NeuN immunofluorescence staining in the retinal slides of the PCK1-ECKD or the control mice was shown (scale bar, 50 μm), and the number of NeuN-positive RGCs was recorded from eight retinas per group. The proliferative retinal membrane of six PDR patients and retinas of three age-matched traumatic retinectomy patients were homogenized, and lysates were tested by qRT-PCR and Western blotting assays of *PCK1* mRNA (**E**) and protein (**F** and **G**). Expression of the listed proteins was quantified using ImageJ software. Data are means ± SD. *$P < 0.05$ versus "TIE1-DIO-Cre" group (A to D). *$P < 0.05$ versus "Ctrl" group (E and G). The experiments were repeated three times with similar results obtained.

PCK1 up-regulation in the proliferative retinal tissues of PDR patients

Last, we examined whether PCK1 is dysregulated in the proliferative retinal tissues of human patients. As described in the previous study (*27*), a total of six retinal proliferative membrane tissues from proliferative diabetic retinopathy (PDR) patients, as well as three retinas of age-matched traumatic retinectomy patients were investigated. PCK1 expression in human tissue was tested by qRT-PCR and Western blotting assays. Results in Fig. 8E demonstrated that *PCK1* mRNA levels were up-regulated in the proliferative retinal tissues ($P < 0.05$, as compared to that in the control retina tissues). Moreover, PCK1 protein up-regulation was detected in the retinal tissues of PDR patients (Fig. 8, F and G). Our previous study also demonstrated $G\alpha_{i3}$ up-regulation and Akt overactivation in the proliferative

retinal tissues of PDR patients (*27*). Therefore, PCK1 up-regulation in the proliferative retinal tissues of PDR patients is correlated with $G\alpha_{i3}$ up-regulation and Akt overactivation.

DISCUSSION

Recent studies emphasize the essential roles of metabolic-associated enzymes in angiogenesis. Endothelial deletion of glutamine synthetase (GS) induced a selective impairment of endothelial cell migration, leading to vascular defects during active vessel growth periods. GS inhibition also impaired remodeling of the actin cytoskeleton, which was necessary for endothelial cell motility (*39*). Endothelial loss of fatty acid synthase (FASN) disrupted angiogenesis in vivo, while FASN blockade largely inhibited the pathological ocular neovascularization and endothelial cell proliferation (*40*). In addition, the M2 isoform of pyruvate kinase (PKM2) inhibited nuclear factor κB (NFκB)–p53 signaling, which is essential for maintaining endothelial cell proliferation and vascular barrier function (*41*).

Our results demonstrate that PCK1 plays an essential role in angiogenesis in vitro and in vivo. PCK1 emerges as a key molecule in the normal development of vascular endothelium, and the absence of PCK1 disrupts endothelium function. In cultured endothelial cells, PCK1 shRNA or KO potently inhibited cell proliferation, migration, sprouting, and tube formation while provoking moderate apoptosis. Conversely, PCK1 overexpression accelerated endothelial cell proliferation, migration, cell sprouting, and tube formation. In vivo, endothelial knockdown of PCK1 delayed retinal vasculature development in neonatal mice and disrupted angiogenesis in the retinas of adult mice. Moreover, endothelial conditional knockdown of PCK1 disrupted angiogenesis in mouse retina. PCK1 is up-regulated in proliferative retinal tissues of PDR patients. We propose that the PCK1-driven angiogenic response is mainly due to the Akt signaling pathway.

In a PI3K-dependent manner, Akt is a serine/threonine protein kinase that is activated by a number of proangiogenic stimuli, including growth factors, mechanical stress, and ischemic/hypoxia conditions (*22*, *23*). Akt phosphorylation at two key residues by PDK1 (T308) and mTORC2 (S473) is required for its full activation (*42*, *43*). Activated Akt in endothelial cells regulates several key downstream events essential for angiogenesis, including survival, proliferation, glucose metabolism, and protein synthesis, as well as nitric oxide production, migration, and tube formation (*22*, *23*). Growth factor– and cell attachment–induced endothelial cell survival and tube formation are primarily mediated by PI3K-Akt signaling (*44*). Akt was also shown to phosphorylate sphingosine-1-phosphate (S1P) receptor EDG-1, causing RAC activation and endothelial cell migration (*45*). Akt inactivation, by the pharmacological inhibitors or dominant-negative mutation, will exert potent antiangiogenic activity (*22*, *23*).

In the present study, our results support the idea that PCK1-driven angiogenesis is due to promoting Akt activation. In cultured endothelial cells, Akt activation was significantly decreased after PCK1 silencing or KO but was increased after PCK1 overexpression. Restoring Akt activity alleviated PCK1 shRNA–induced antiangiogenic activity in HUVECs. MK-2206, an Akt-specific inhibitor, blocked PCK1 overexpression–induced proangiogenic response in HUVECs. In vivo, endothelial knockdown of PCK1 led to Akt inhibition in the retinal tissues of mice. Moreover, PCK1 up-regulation in the proliferative retinal tissues of PDR patients was correlated with Akt overactivation. We propose that PCK1-mediated Akt activation in endothelial cells requires $G\alpha_{i3}$ expression.

Our previous studies have shown that $G\alpha_i$ proteins, $G\alpha_{i1}$ and $G\alpha_{i3}$, are essential signaling molecules mediating Akt cascade activation by RTKs (*25*, *27*–*31*) and other receptors (*32*, *46*). $G\alpha_{i1}$ and $G\alpha_{i3}$ are required for epidermal growth factor (EGF)–induced Gab1 (a key adaptor protein) and downstream Akt-mTOR activation (*31*). Conversely, $G\alpha_{i1}$ and $G\alpha_{i3}$ double KO (DKO) in mouse embryonic fibroblasts (MEFs) blocked EGF-induced Akt-mTOR activation (*31*). In addition, brain-derived neurotrophic factor (BDNF)–induced (*28*) and keratinocyte growth factor (KGF)–induced (*30*) Akt-mTOR activation required $G\alpha_{i1}$ and $G\alpha_{i3}$ as well. $G\alpha_{i1}$ and $G\alpha_{i3}$ mediated VEGF-induced VEGF receptor 2 (VEGFR2) endocytosis, downstream Akt-mTOR signaling activation, and angiogenesis response (*27*). Moreover, $G\alpha_{i1/3}$ KO or silencing robustly inhibited Akt-mTOR activation by interleukin-4 (IL-4) (*32*) and the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) (*46*). These results showed an unconventional but essential role of $G\alpha_i$ proteins in mediating Akt activation by a number of different stimuli.

Our results showed that PCK1-mediated Akt activation was possibly due to its promotion of $G\alpha_{i3}$ expression. In cultured endothelial cells, PCK1 shRNA or KO led to substantial $G\alpha_{i3}$ down-regulation, while ectopic overexpression of PCK1 exerted opposite functions and increased $G\alpha_{i3}$ expression. In PCK1-silenced HUVECs, restoring $G\alpha_{i3}$ expression by the Ad-$G\alpha_{i3}$ construct not only restored Akt activation but also recovered the angiogenic response. PCK1 overexpression–induced proangiogenic response in HUVECs was blocked by $G\alpha_{i3}$ silencing. In vivo, $G\alpha_{i3}$ expression was decreased in the mouse retinal tissues after endothelial knockdown of PCK1. Moreover, PCK1 overexpression is correlated with $G\alpha_{i3}$ overexpression [see our previous study (*27*)] in the proliferative retinal tissues of PDR patients.

Here, we propose that PCK1-mediated $G\alpha_{i3}$ expression is possibly due to regulating GATA4 function in endothelial cells. Xu *et al.* (*47*) have shown that GATA4 promotes NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase 4 (NOX4) transcription to protect HUVECs against hyperglycemia-induced endothelial cell dysfunction. GATA4 promoted *Erbb3* transcription and activated *Erbb3-Erk* cascade in endothelial-derived cells (*48*). GATA4 is proposed as a transcription factor for $G\alpha_{i3}$ (*33*). We found that $G\alpha_{i3}$ expression in HUVECs was decreased after GATA4 shRNA but increased after GATA4 overexpression.

GATA4 serine phosphorylation is important for its transcriptional activity (*34*–*36*). In hypertension rats, GATA4 phosphorylation at serine-105 was vital for left ventricular remodeling process (*34*). Phosphorylation of GATA4 at serine-105 in male mice was required for testosterone production (*35*). We found that association of PCK1 with GATA4 was important for GATA4 phosphorylation. shRNA-induced silencing of PCK1 inhibited GATA4 serine phosphorylation and robustly decreased its binding to $G\alpha_{i3}$ promoter DNA. Therefore, PCK1 association with GATA4 is important for GATA4 serine phosphorylation, promoting $G\alpha_{i3}$ transcription in endothelial cells.

Together, we demonstrate that PCK1 is essential for angiogenesis and acts to mediate $G\alpha_{i3}$ expression and Akt activation. Targeting PCK1 is therefore a prospective therapeutic approach for the treatment of vascular dysfunctional diseases. In-depth understanding of PCK1 in angiogenesis may provide new perspectives on the pathogenesis of the vascular diseases, offering therapeutic strategies for the diseases associated with dysfunction of angiogenesis.

MATERIALS AND METHODS

Reagents

Polybrene and puromycin were provided by Sigma-Aldrich (St. Louis, MO). The anti-GATA4 antibody was purchased from Abcam. The phosphorylated serine antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies, as reported previously (*20*, *31*), were provided by Cell Signaling Technology (Beverly, MA) and SignalAntibody (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), antibiotics, and other cell culture reagents were provided by Gibco-BRL (Suzhou, China).

Cell culture and transfection

hRMECs and hCMEC/D3 brain endothelial cells were purchased from the Biological Institute of the National Academy of Science of China (Shanghai, China). HUVECs were reported in our previous studies (*26*, *27*). Cells were cultured in high-glucose (17.51 mM) DMEM/F-12 medium + 10% FBS (Gibco, Suzhou, China), insulin (5 μg/ml), EGF (5 μg/ml), VEGF (5 ng/ml), and adenine (24 μg/ml; all from Sigma-Aldrich) in an incubator with humidified atmosphere of 5% CO_2. Cells maintained proangiogenic state in the basal condition. The culture of primary murine RGCs was described previously (*49*). Cells were routinely subjected to mycoplasma and microbial contamination examination. The short tandem repeat (STR) DNA profiling, population doubling time, and morphology were examined to verify their genotypes.

qRT-PCR and Western blotting

The detailed protocols for qRT-PCR, Western blotting, and co-immunoprecipitation assays were described previously (*27*, *29*, *50*). Primers for *GATA4* were described early (*51*, *52*). Other primers were described previously (*20*, *27*). For Western blotting assays, the parallel gels were used to test different proteins in the same set of lysates when necessary. For data quantification, the ImageJ software (National Institutes of Health, USA) was used.

shRNA and viruses

Two different lentivirus-packed shRNAs in the GV248 vector (hU6-MCS-CBh-IRES-puromycin) targeting nonoverlapping sequences of *PCK1*, shPCK1-seq-1 and shPCK1-seq-2, as well as one lentivirus-packed PCK2 shRNA were provided by M. B. Chen at Jiangsu University (*20*). The GATA4 shRNA (targeted sequence: *AGCCCAAGAACCTGAATAAAT*) was also inserted into the GV248 lentiviral construct [no green fluorescent protein (GFP)]. The lentivirus was then obtained after transducing the construct and lentivirus package constructs to human embryonic kidney (HEK)–293 cells. Endothelial cells were seeded into six-well plates at 50 to 60% confluence in polybrene-containing complete medium and infected with the lentivirus [at a multiplicity of infection (MOI) of 10]. After 24 hours, cells were cultured in puromycin (3.0 μg/ml) containing complete medium for another 72 hours. PCK silencing in the stable cells was verified by Western blotting and qRT-PCR assays. The lentivirus containing the scramble nonsense control shRNA (shC, GeneChem) was added to the control cells. For the in vivo studies, the PCK1 shRNA sequence (shPCK1-seq-1) or $G\alpha_{i3}$ shRNA sequence (*28*, *32*) was inserted into an AAV5-TIE1 construct that contained the sequence of TIE1 (GeneChem, Shanghai, China), the endothelial-specific promoter. For the conditional knockdown experiments, the PCK1 shRNA (shPCK1-seq-1) was inserted into the AAV5-FLEX (the LoxP site–based genetic switch) construct (GeneChem, Shanghai, China). These constructs were transfected to HEK-293 cells, generating adenovirus (AAV). The virus was then intravitreally injected to the mice.

CRISPR-Cas9–induced PCK1/2 KO

The lenti-Cas9-puro construct (GeneChem) was transduced to endothelial cells, and stable cells were established after puromycin selection. Thereafter, a lentiviral CRISPR-Cas9–PCK1–KO construct or a lentiviral CRISPR-Cas9-PCK2-KO construct [from M. B. Chen (*20*)] was transduced to the Cas9-expressing endothelial cells, and stable colonies were established after puromycin selection. Cells were then distributed into 96-well plates and subjected to *PCK1/2* KO screening. Thereafter, the single stable PCK1/2 KO endothelial cells, or koPCK1/"koPCK2" HUVECs, were established.

Overexpression

Endothelial cells were seeded into six-well plates at 50 to 60% confluence (cultivated in polybrene-containing complete medium). The lentivirus-packed *PCK1*-expressing GV492 construct [from M. B. Chen (*20*)] or lentivirus-packed *GATA4* (NM_002052)–expressing GV492 construct (from GeneChem) was added, and stable cells were established after puromycin selection. *PCK1* or *GATA4* overexpression was verified by qRT-PCR and Western blotting assays. Control cells were stably transduced with the GV492 empty vector.

ChIP assay

The detailed protocols of ChIP assay were described previously (*53*). In brief, cell lysates were treated with the Misonix Sonicator 3000 Homogenizer (*54*), and fragmented genomic DNA was obtained. Lysates diluted with ChIP dilution buffer were immunoprecipitated with an anti-GATA4 (Abcam) antibody. GATA4-bound DNA was eluted from the protein A/G agarose, and NaCl was added to eliminate cross-linking between proteins and genomic DNA. DNA containing the proposed conserved $G\alpha_{i3}$ promoter site I (*33*) was analyzed via quantitative PCR (qPCR).

Sprouting assay

HUVECs were detached from cell culture plates by trypsin-EDTA solution and were resuspended in compete medium (with 10% FBS). Thereafter, 4 ml of cell culture medium containing 8×10^3 cells was mixed carefully with 1 ml of methocel stock solution, containing 12 mg of methylcellulose (Sigma-Aldrich). Next, 25 μl of drops of the cell solution was placed onto the lid of a cell culture dish (100 × 20 mm) and incubated upside-down in a humidified cell culture incubator for 24 hours, and the spheroid shape was formed. The hanging drops were then washed off by phosphate-buffered saline, and HUVECs were resuspended with 2 ml of methocel solution containing FBS. Afterward, 2 ml of rat collagen I (R&D system, Shanghai, China) was added softly. The spheroid-collagen solution was added to 12-well plates (1 ml per well) for 24 hours. The sprouting assays were stopped by adding 1 ml of 10% paraformaldehyde. Sprouts were photographed under a Zeiss Axio Observer A1 microscope. The sprout number and the length of each sprout were recorded.

Tube formation assay

The basement membrane matrix (BD Biosciences, Shanghai, China) was placed into 24-well plates for 30 min. HUVECs (1.7×10^5 cells

per well) were seeded onto the precoated plates and incubated in the humidified cell culture incubator set at 37°C and 5% CO_2. After 12 hours, the tube formation was photographed under a Zeiss Axio Observer A1 microscope. For each condition, five random microscopy views (1 × 100 magnification) were included to calculate the average number of formed tubes.

Other assays, including the nuclear EdU staining assay, the Transwell assay, the nuclear TUNEL staining assay, the caspase-3 and caspase-7 activity assays, and the ssDNA enzyme-linked immunosorbent assay (ELISA), as well as annexin V fluorescence-activated cell sorting (FACS), $G\alpha_{i3}$ silencing by lentiviral shRNA, and stable cell selection were described in detail previously (*26*, *27*, *50*, *53*, *55*, *56*).

$G\alpha_{i3}$ overexpression

The recombinant adenovirus containing full-length $G\alpha_{i3}$ cDNA [Ad-$G\alpha_{i3}$, as described previously (*25*, *27*)] was added directly to the cultured endothelial cells (maintained in the polybrene-containing complete medium). After 48 hours, cells were selected by puromycin, and stable cells were established. $G\alpha_{i3}$ mRNA and protein expression was verified by qRT-PCR and Western blotting assays.

Constitutively active mutant Akt1

The constitutively active Akt1 (caAkt1, S473D)–expressing adenovirus (from G. Li at Wenzhou Medical University) (*57*) was added to the cultured endothelial cells (maintained in the polybrene-containing complete medium). After 48 hours, cells were selected by puromycin. The expression of caAkt1 was verified by Western blotting assays.

Intravitreal injection of AAV

The adult C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center, CAS (Shanghai, China). The TIE1-DIO-Cre C57 mice were commercially available and provided by GeneChem (Shanghai, China). Mice were maintained under clean animal facility, with 12-hour dark/12-hour light cycle, 24° ± 1°C temperatures, and free access of water and food. Mice were anesthetized through the intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). A 33-gauge disposable needle was passed through the sclera at the equator and posterior to the limbus into the vitreous cavity. Approximately 0.1 μl of AAV was subsequently injected into the vitreous cavity with the needle directly above the optic nerve head. NeuN immunofluorescence staining of RGCs in the mouse retinal slides was described previously (*49*). The animal protocols were conducted according to the Institutional Animal Care and Use Committee and the Ethics Committee of Soochow University, and complied with the provision of the ARVO (Association for Research in Vision and Ophthalmology) statement.

Retinal vasculature

Retinal vasculature was isolated by trypsin digestion method. Briefly, freshly isolated eyes were fixed with 10% buffered formalin overnight. Retinas were carefully removed and dialyzed in ddH_2O overnight on shaking table. Then, the retinas were digested in 2% trypsin solution for 1 hour at 37°C and washed for 5 min. Retinal vasculature was dried in a ventilated place and stained with periodic acid Schiff and hematoxylin.

Retinal vascular permeability assay

The adult C57BL/6 mice were anesthetized with ketamine and xylazine. Evans blue (30 mg/kg) was injected into the vein, which was followed by bradykinin injection. One hour after Evans blue injection, the chest was opened. The mice were perfused with citrate buffer (0.05 M, pH 3.5). After perfusion, eyes were enucleated and the cornea, sclera, lens, and vitreous humor were removed, with retinas fixed in 4% paraformaldehyde for 30 min at room temperature. The retinas were then incubated with dimethylformamide (Sigma-Aldrich) overnight and then centrifuged at 12,000*g* for 15 min. The supernatant was detected spectrophotometrically at 620 nm (blue) and 740 nm (background).

Human tissues

As reported previously (*27*), a total of six PDR patients undergoing lensectomy combined with vitrectomy surgery and the three age-matched traumatic retinectomy patients were enrolled in the present study. Each participant provided written informed consent. The anterior retinal hyperplastic membrane of PDR patients was stripped, and fresh tissue specimens were obtained. The traumatic normal retinas were preserved as well. The protocols were according to the principles of Declaration of Helsinki and were approved by the Ethics Board of Soochow University.

Statistical analysis

Experimental data with normal distribution were expressed as means ± SD. Differences between two treatment groups were calculated by Student's *t* test, with multiple-group comparisons tested by the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values of $P < 0.05$ were considered as statistically significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abn6928

View/request a protocol for this paper from *Bio-protocol*.

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