VEGFR1–mediated pericyte ablation links VEGF and PlGF to cancer-associated retinopathy

Renhai Cao, Yuan Xue, Eva-Maria Hedlund, Zhaodong Zhong, Katerina Tritsari, Barbara Tondelli, Franco Lucchini, Zhenping Zhu, Steen Dissing, and Yihai Cao

1Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, 171 77 Stockholm, Sweden; 2Department of Cellular and Molecular Medicine, Center for Healthy Aging, Panum Institute, University of Copenhagen, Copenhagen 2200N, Denmark; 3Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, 20090 Segrate, Italy; 4Istituto Clinico Humanitas, 20089 Milano, Italy; 5Centro Ricerche Biotecnologiche and Istituto di Microbiologia, Universita’ Cattolica del Sacro Cuore, 26100 Cremona, Italy; and 6ImClone Systems Incorporated, New York, NY 10014

Edited by Tadamitsu Kishimoto, Osaka University, Graduate School of Frontier Biosciences, Suita, Japan, and approved December 2, 2009 (received for review October 8, 2009)

VEGF coordinates complex regulation of cellular regeneration and interactions between endothelial and perivascular cells; dysfunction of the VEGF signaling system leads to retinopathy. Here, we show that systemic delivery of VEGF and placental growth factor (PlGF) by protein implantation, tumors, and adenoviral vectors ablates pericytes from the mature retinal vasculature through the VEGF receptor 1 (VEGFR1)-mediated signaling pathway, leading to increased vascular leakage. In contrast, we demonstrate VEGF receptor 2 (VEGFR2) is primarily expressed in nonvascular photo-receptors and ganglion cells. Moreover, blockade of VEGFR1 but not VEGFR2 significantly restores pericyte saturation in mature retinal vessels. Our findings link VEGF and PlGF to cancer-associated retinopathy, reveal the molecular mechanisms of VEGFR1 ligand-mediated retinopathy, and define VEGFR1 as an important target of antiangiogenic therapy for treatment of retinopathy.

Although vascular endothelial growth factor (VEGF) significantly contributes to pathological retinal angiogenesis, little is known about its function in nonendothelial vascular cells such as pericytes and vascular smooth muscle cells (VSMCs) (1–3). Pericytes and VSMCs are not only essential for vascular remodeling and stability and maintenance of retinal vasculature integrity, they also significantly prevent vascular leakage, which is one of the key pathological processes of vision impairment in human ocular diseases (4–7). Current anti-VEGF drugs, including pegaptanib and ranibizumab, for the treatment of age-related macular degeneration (AMD) have shown marked beneficial effects in patients (8–11). The mechanisms underlying beneficial effects of anti-VEGF drugs involve suppression of both choriocapillary neovascularization and retinal edema (8–11). In contrast to VEGF, little is known about the physiological and pathological functions of placental growth factor (PlGF), a VEGF-related protein that is often expressed at high levels in pathological tissues (12–15).

Cancer-associated retinopathy (CAR) is a relatively uncommon but severe syndrome presenting with bilateral and progressive loss of vision and/or a restricted field of vision. It occurs in patients with a variety of different types of cancers, including lung cancer, melanoma, and ovarian cancer (16, 17). Pathological changes include attenuation of arterioles with thinning and mottling of the retina, resembling coidstoid macular edema and retinal vascular edema (18). Vision loss is thought to be due to cancer-associated autoimmune retinal degeneration (19). However, a significant number of CAR patients lack detectable autoimmune antibodies and are completely resistant to immunosuppressive therapy, suggesting that alternative mechanisms contribute to the onset and progression of CAR (20, 21). Currently CAR remains an untreatable cancer-associated disease that significantly impairs the quality of life of cancer patients.

In this study, we have uncovered a mechanism by which vascular defects induced by tumor-derived VEGF and PlGF contribute to retinal pathology, leading to ablation of perivascular cells and vascular leakage. Surprisingly, we determined that the VEGF receptor 2 (VEGFR2) is primarily expressed in nonendothelial nerve cells and photoreceptors. Notably, blockade of VEGF receptor 1 (VEGFR1), but not VEGFR2, significantly inhibited VEGF-A-induced retinal damage. Thus, our study also implies that anti-VEGFR1, but not anti-VEGFR2 agents, are preferred for the treatment of VEGF–related ocular diseases including AMD, CAR, and diabetic retinopathy. Current anti-VEGF therapeutic drugs without distinguishing VEGFR1 and VEGFR2 signaling pathways and defined therapeutic targets should be administrated with great caution.

Results

Distribution of VEGFR1 and VEGFR2 in the Retinal Vasculature. To study the role of VEGF and its receptors in maintenance of vascular integrity and function, the distribution of VEGFR1 and VEGFR2 was examined in the retinal vasculature of adult mice. Surprisingly, VEGFR2, primarily known as a signaling receptor in endothelial cells, was mainly expressed in nonvascular cells, including ganglions and photoreceptors (Fig. 1 A–C). Indeed, VEGFR2 and the glia marker, glial fibrillary acidic protein (GFAP), exhibited overlapping staining, validating the distribution of VEGFR2 in neuronal cells (Fig. 1C). Expression of VEGFR1 was restricted in the retinal vasculature, as expected (Fig. 1A). Double immunohistochemical staining did not reveal obvious overlapping positive signals of VEGFR1 and VEGFR2. Similarly, VEGFR2 positive signals did not show overlapping staining with CD31, a pan marker for vascular endothelial cells (Fig. 1A–C). Interestingly, VEGFR1, but not VEGFR2, was also localized to NG2-positive vascular pericytes (Fig. 1B). These findings demonstrate that VEGFR1 is distributed to both vascular endothelial cells and pericytes, whereas VEGFR2 is localized to nonvascular cells, suggesting a nonvascular function of VEGFR2 in the retina.

Expression of VEGFR1 in Isolated Primary Vascular Pericytes. To further investigate the expression pattern and function of VEGFR1, we isolated mouse primary pericytes and VSMCs. As expected, isolated primary NG2-positive cells expressed NG2, whereas only a subpopulation of these cells expressed alpha smooth

Author contributions: R.C. and Y.C. designed research; R.C., Y.X., E.-M.H., Z. Zhong, K.T., B.T., F.L., and S.D. performed research; R.C. and Z. Zhu contributed new reagents/analytic tools; R.C., Y.X., and Y.C. analyzed data; and Y.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Author contributions: R.C. and Y.C. designed research; R.C., Y.X., E.-M.H., Z. Zhong, K.T., B.T., F.L., and S.D. performed research; R.C. and Z. Zhu contributed new reagents/analytic tools; R.C., Y.X., and Y.C. analyzed data; and Y.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed. E-mail: yihai.cao@ki.se.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911661107/DCSupplemental.
the elevated levels of VEGF that occur under pathological con-
maintenance of physiological functions of the adult retina and that
and nonvascular cell types suggests that VEGF is crucial for the

Cao et al. PNAS Retinal Vasculature. VEGF Protein and an Adenovirus VEGF Ablate Pericytes from the
muscle actin (αSMA) (Fig. 2A). Consistent with in vivo data, both
isolated primary pericytes and VSMCs expressed VEGFR1 (Fig.
2B). The identity of these primary perivascular cells was validated
by abundant expression of PDGFR-β, which is known to be highly
expressed in pericytes and VSMCs (Fig. 2B). Using reverse-tran-
scriptase PCR analysis, expression of VEGFR1 mRNA was also
detected in endothelial cells and pericytes (Fig. 2C). Stimulation
of pericytes with VEGF resulted in enhanced activation of Src and
Erk (Fig. 2D). In contrast, phosphorylation of Akt was decreased by
VEGF (Fig. 2D), suggesting signal transductions through the sur-
vival pathway were inhibited. We further showed that VEGF-
induced activation of Erk was significantly inhibited by an anti-
VEGFR1 neutralizing antibody (Fig. 2E). Recently, an Src/myr-
ristoyltransferase inhibitor has been reported to inhibit both activ-
ation of the Src and Erk signaling pathways (22), suggesting its
inhibitory role in VEGF-induced pericyte repelling in the retina.
These findings demonstrate that VEGF directly acts on pericytes
via activation of the VEGFR1 signaling pathway.

**VEGF Protein and an Adenovirus VEGF Ablate Pericytes from the Retinal Vasculature.** The broad distribution of VEGFRs in vascular
and nonvascular cell types suggests that VEGF is crucial for the
maintenance of physiological functions of the adult retina and that
the elevated levels of VEGF that occur under pathological con-
ditions such as tissue hypoxia, inflammation, and tumor growth
likely result in severe impairment of function. To study the impact
of VEGF on vascular integrity in the retina, VEGF was both
locally and systemically administered to the adult mice. Intrigu-
ingly, local delivery of VEGF protein via corneal micropocket
assay resulted in progressive ablation of NG2-positive pericytes
with time (Fig. 3), suggesting that chronic exposure of retinal
vessels to VEGF may significantly impair vascular integrity. At day
25 after VEGF delivery, approximately 60% of pericytes were lost
from retinal vessels (Fig. 3A and D).

To study whether VEGF mediates pericyte ablation, PlGF
protein, a ligand exclusive to VEGFR1, was locally administered
to mouse retinas via micropocket assay. Similar to VEGF, treat-
ment with PlGF protein also led to progressive loss of
pericytes in the retinal vasculature (Fig. 3A and D). It should be
emphasized that PlGF and VEGF displayed a similar potency of
pericyte ablative effect (Fig. 3D). These findings demonstrate
that VEGFR1-mediated signals are primarily responsible for
pericyte loss in the adult retinal vasculature.

To further validate VEGF–mediated ablation of retinal per-
cytes, VEGF was systemically administrated to adult mice using an
adenoviral vector (Ad-VEGF). Systemic delivery of Ad-VEGF
resulted in significant loss of pericytes in the adult retinal vascula-
ture compared to treatment of mice with the empty adenoviral
vector (Fig. 3B). We also performed local administration of
antagonists of PDGFR-β, a known signaling receptor associated
with vascular recruitment of pericytes and VSMCs. The antago-
nists included a neutralizing antibody and a tyrosine kinase
inhibitor (STI571, imatinib). As expected, PDGFR-β antagonists
significantly ablated pericyte coverage, albeit the ablative effect
was less potent than VEGF and PlGF treatment (Fig. 3B–D).

**Xenograft Tumor-Derived VEGF Ablates Pericytes from the Retinal Vasculature.** Retinal vasculature abnormality induced by Ad-
VEGF and by corneal micropocket administration of VEGF and
PlGF suggested that circulating VEGF leads to considerable loss

---

**Fig. 2.** Expression and activation of VEGFR1 in isolated pericytes and
VSMCs. (A) Isolated primary mouse pericytes (Upper) express the pericyte-
specific marker NG2 (green); a subpopulation express αSMA (red). Isolated
VSMCs (Lower) express αSMA (red); a subpopulation express NG (green).
(Merged images are shown at right. (B) Immunocytochemistry indicating that
isolated pericytes and VSMCs exhibit positive staining of VEGFR1 (green) and
PDGFRβ (red). (C) Reverse transcriptase-PCR analysis of VEGFR1 mRNA
expression in isolated mouse pericytes and endothelial cells (SVEC). (D) Western
blot analysis demonstrating activation (increased phosphorylation) of Src and Erk and
down-regulation (decreased phosphorylation) of Akt in isolated pericytes after VEGF
treatment for the indicated time. GAPDH was analyzed as a control. (E) Western blot
analysis demonstrating VEGFR1 blockade inhibits VEGF–induced Erk activation (phosphorylation). GAPDH
was analyzed as a control.
from the retinal vasculature. We therefore tested the hypothesis that pericyte ablation in the retinal vasculature is caused by tumors. In two mouse tumor models, T241 fibrosarcoma and Lewis lung carcinoma (LLC) cells were transfected with a VEGF overexpression plasmid and s.c. injected into mice. The plasma level of VEGF in these tumor-bearing mice was 0.8 ng/mL (Fig. 4F), which is physiologically relevant compared with those of clinical cancer patients (23). Similar to systemic delivery of Ad-VEGF, s.c. implantation of the VEGF-transfected T241 and LLC cells into mice resulted in substantial loss of retinal pericytes (Fig. 4A–D). These findings demonstrate that systemic tumor-derived VEGF induced pericyte ablation in the retinal vasculature and functionally link VEGF to CAR, which occurs among cancer patients with a range of tumor types.

Tumor-Derived PlGF Ablates Vascular Pericytes in the Retina. Because VEGFR1 was shown to mediate pericyte ablation, we examined whether overexpression of PlGF in a tumor would result in similar pericyte loss in the retina. To test this possibility, PlGF was transfected into the same tumor cell lines used in VEGF experiments, i.e., T241-PlGF and LLC-PlGF; s.c. implantation of T241 tumors resulted in a systemically ablative effect on vascular pericytes (Fig. 4F). These findings demonstrate that systemic tumor-derived VEGF induced pericyte ablation in the retinal vasculature and functionally link VEGF to PlGF. (Fig. 4E). After i.v. injection, extravasation of rhodamine-labeled dextran was only detected in the retinas of VEGF-transfected tumor-bearing mice but not in control mice. These findings show that VEGF–induced pericyte ablation significantly impairs the function of the retinal vasculature by increasing leakage.

Retinal Pericyte Ablation in a Spontaneous Tumor Model. To study the physiological relevance of our findings in the xenograft tumor models, we used a spontaneous tumor model, which was not genetically engineered to express high levels of VEGF or PlGF. In this model, mouse mammary tumor virus (MMTV)-neu transgenic mice carrying mammary tumors were analyzed. Similar to the xenograft tumor models, the MMTV-neu transgenic mouse also developed marked pericyte loss in the retinal vessels (Fig. 5A and B). These findings show that spontaneous and xenograft-derived tumors growing in distal tissues induce systemic ablation of retinal pericytes.

Pericyte Rescue by VEGFR Blockade. To further study VEGFR1-mediated pericyte ablation, neutralizing monoclonal antibodies against VEGFR1 or VEGFR2 were systemically administrated into tumor-bearing mice. As expected, VEGFR1 blockade, but not VEGFR2 blockade, led to significant restoration of pericytes in retinal vessels of mice with VEGF–transfected tumors (Fig. 5A and C). Similarly, VEGFR1 blockade in the MMTV-neu tumor model significantly prevented pericyte loss in the retinal vasculature (Fig. 5B and D). Notably, VEGFR1 or VEGFR2 blockade did not affect the retinal vascular density, indicating that the adult retinal vasculature remained quiescent.

Discussion

VEGF displays several vascular activities including vasculogenesis, angiogenesis, vessel survival, and vascular permeability, all of which are thought to be mediated by its functional receptor, VEGFR2. In vascular endothelial cells of most tissues, VEGFR2 exhibits a restricted vascular expression pattern (12, 24). In contrast to VEGFR2, VEGFR1 is widely distributed in vascular
endothelial cells and in a broad spectrum of nonendothelial cells and is suggested to positively and negatively regulate angiogenesis (12, 15). Here we show surprising findings that VEGFR2 is not primarily expressed in endothelial or perivascular cells of the retinal vasculature. Instead, VEGFR2 expression was detected in nonendothelial cell types such as ganglion cells and neuronal photoreceptor cells in the retina. Consistent with our findings, a recent study reported that VEGFR2 is expressed in Müller cells and photoreceptors, indicating nonvascular function of VEGF in the retina (25). In contrast to VEGFR2, VEGFR1 is expressed in both endothelial cells and pericytes in the retinal vasculature. Differential distribution of VEGFR1 and VEGFR2 in the retina is in striking contrast to other tissues where VEGFR2 is almost exclusively expressed in blood vessel endothelial cells, except hematopoietic progenitor cells and nerve tissues (26, 27).

In addition to the intact VEGFR1, a soluble VEGFR1 (sVEGFR1) has been found at high levels in patients with proliferative diabetic retinopathy (28). Although the role of sVEGFR1 in development of retinopathy remains unknown, it has been speculated that this version of VEGFR1 may act as a negative regulator for retinal angiogenesis. The existence and biological functions of sVEGFR1 in CAR patients need to be established.

In the present study, local delivery or systemic expression of VEGF led to marked pericyte loss from the mature retinal vasculature, suggesting that VEGF acts as a negative regulator for maintenance of vascular integrity. In support of these findings, a recent study demonstrated that VEGF antagonizes PDGFRβ-mediated angiogenesis by ablation VSMCs in newly formed vessels (29). The molecular mechanism underlying the reported antagonistic effect involves the formation of a VEGF/VEGF-B/PDGFRβ complex. In contrast to those findings, we showed that VEGF ablates pericytes by directly activating VEGFR1 independent of the PDGFR-mediated signaling system. Although PDGFRβ blockade expectedly affects association between pericytes and the mature retinal vasculature, it is unlikely that VEGFR2 and PDGFRβ form a complex to down-regulate PDGF signaling because these two receptors are distributed in different cell types in the retina. Consistent with this, VEGFR2 blockade did not result in significant pericyte ablation in the retinal vasculature.

CAR is characterized by attenuation of the arterioles with thinning and mottling of the retina, resembling cystoid macular edema and retinal vascular edema. CAR has been proposed to be an autoimmune disorder (19). However, various immunotherapies have resulted in only modest vision recovery in rare cases (21). Other therapeutic approaches including combination of surgery, hormone, and immunotherapy have produced only disappointing results. Thus, the overall prognosis of CAR patients is very poor due to lack of effective therapies. Our present findings uncover the molecular mechanisms of attenuation of retinal arterioles by tumor-derived VEGF and PIGF. In contrast to VEGF, PIGF is considered a pathological angiogenic factor that is often up-regulated in various tumor tissues (12, 15, 30). PIGF likely participates in both positive and negative regulation of tumor angiogenesis depending on its relation to VEGF production (12, 31, 32). When PIGF and VEGF are coexpressed in the same population of cells, they may form heterodimers that bind to VEGFR1. Interestingly, a NADPH inhibitor, fulvene 5, has been reported to down-regulate PIGF expression and might be potentially beneficial for the treatment of retinopathy (33). In this study, we uncovered an unexpected function of PIGF that significantly impairs the integrity and functions of the retinal vasculature by ablation of pericytes. These findings could further extend to another VEGF binding factor, VEGF-B, whose binding function remains largely unknown (34, 35). Various tumor tissues also express VEGF-B at high levels (36), and it is plausible that VEGF-B might cause systemic ablation of pericytes in the retinal vasculature. Consistent with this notion, VEGF-B has been reported to act as potent survival factor for vascular and nonvascular cells (35). The role of VEGF-B in the control of vascular integrity warrants further investigation. Our findings also suggest that circulating levels of VEGF, PIGF, and VEGF-B might serve as surrogate biomarkers to predict occurrence of CAR in cancer patients. Moreover, measurement of circulating levels of these angiogenic factors in combination of analysis of retinal vessels may provide important and dynamic biomarkers to predict therapeutic efficacy of cancer patients in response to anti-VEGF therapy.

Under physiological conditions, retinal arterioles are profusely coated with pericytes and VSMCs (37). VEGF- or PIGF-induced pericyte/VSMC ablation agrees with arteriole attenuation in CAR patients, manifesting in loss and thinning of arteriole vessel walls and retinal edema. In both xenograft and spontaneous tumor models, we have shown that tumor-produced VEGF attenuates the retinal arteriole system by ablating pericytes/VSMCs from the retinal vasculature. The functional consequence of VEGF-induced pericyte loss is increased vascular leakage, which is manifested as retinal edema and progressive vision loss in clinical settings. Thus our animal tumor models recapitulate the retinal pathology of cancer patients with CAR.
Our data also demonstrate that VEGFR1 blockade is crucial for the recovery of VEGF-induced retinal damage. Paradoxically, VEGFR2 blockade may produce nonbeneficial and even harmful effects because VEGFR2 is expressed in the retinal nerve system. These results suggest that anti-VEGFR1 agents that do not affect VEGFR2 signaling need to be developed for the treatment of retinopathy. Currently, available anti-VEGF drugs for treatment of retinopathy do not distinguish signaling systems mediated by VEGFR1 and VEGFR2 (8–11). However, Long-term exposure to VEGFR2 blockade could potentially have serious consequences for the retinal nerve system.

Taken together, our present study reveals the molecular mechanisms underlying VEGF-induced pericyte ablation in the retinal vasculature and links VEGF and PlGF to the development of CAR, a currently mechanistically unknown and untreatable disease that is poorly understood. These findings suggest that anti-VEGFR1 agents need to be developed for the treatment of CAR and other types of retinopathies.

Materials and Methods

Reagents. The antibodies used in our studies included: rat anti-mouse CD31 monoclonal antibody (BD Pharmingen), mouse anti-human α-SMA antibody (Dako), rabbit anti-mouse NG2 antibody (Chemicon), rat anti-mouse VEGFR1 antibody (MF1; ImClone Systems Inc.), rat anti-mouse VEGFR2 antibody (DC101; ImClone Systems Inc.), neutralizing PDGFβR antibody (ImClone Systems Inc.), rabbit anti-mouse VEGFR2 antibody (T014; kindly provided by Dr. Rolf Brekken, University of Texas Southwestern Medical Center, Houston, TX), goat anti-mouse VEGFR2 antibody (R&D Systems), rat anti-mouse PDGFβR antibody (eBioscience), and monoclonal anti-GFAP antibody (Sigma). Recombinant human VEGF165 and PlGF-1 were obtained from R&D Systems. Recombinant human VEGF165 and PlGF-1 were obtained from R&D Systems.

Animals. Male or female 6- to 8-week-old C57BL/6 mice and MMTV-neu transgenic mice generated in a CD1 background were acclimated and caged in groups of six or less. Animals were analyzed for up to 4 months and anesthetized by injection of a mixture of hypnorm and domnicum (1:1) before all procedures. Animals were killed using a lethal dose of CO2, followed by cervical dislocation. All animal studies were reviewed and approved by the animal care and use committees of the Stockholm animal ethical board.

Isolation of Pericytes and Smooth Muscle Cells. Mice were killed in a CO2 chamber and lungs were immediately resected and cut into small pieces. The tissue was digested in collagenase 2/DMEM for 1 h in 37°C. The cells were collected and resuspended in RPMI-1640 medium. The red blood cells were lysed with ammonium chloride lysis reagent (BD Pharmingen). Cells were resuspended in PBS with 0.1% BSA and incubated with antibodies against NG2, followed by a secondary Cy3-labeled goat anti-rabbit antibody. Later, the cells were transferred to FACS tubes, resuspended in PBS with 0.1% goat serum, and sorted with the FACS Vantage/Diva (Becton Dickinson). The sorted cells were cultured in 12 well-plates with EGM-2 containing 10% FCS until further analysis. For isolation of smooth muscle cells, mouse aortas were immediately dissected from C57Bl6 mice after scarification. Aorta tissue was digested in DME medium containing collagenase 2 for 1 h in 37°C. Smooth muscle cells were collected and resuspended in DME medium. The purified cells were cultured in DME medium supplemented with 10% FBS until further analysis.

Western Blot. See SI Text for details.

Reverse Transcription PCR. Reverse transcription was performed by using total RNA from pericytes or SVEC cells and Omniscript Reverse Transcriptase kit (Qiagen). Complementary cDNAs were amplified by PCR to generate a VEGFR fragment of 102 bp, using the following primer pairs: forward:5'-tgccaaagctggcaagtgtggt-3'; reverse: 5'-tgcaaatcttcaccacatg-3'.
Immunocytochemistry. See SI Text for details.

Mouse Corneal Micropocket Assay. The mouse corneal assay was performed as previously described (38). Briefly, micropockets (0.35 x 0.35 mm) of sucrose aluminum sulfate (Bukh Meditec) were coated with hydron polymer type NCC containing 160 ng VEGF or PIGF. Micropockets were surgically implanted into each micropocket in the mouse eyes. Growth factor-implanted mouse eyes were enucleated at day 5 or 25 after micropocket implantation following exposure to a lethal dose of CO2. Retinas were isolated after fixation in 3% PFA for 30 min.

Whole-Mount Immunofluorescence Staining. Whole mount staining was performed according to a previously reported method (39). See SI Text for details.

Vascular Permeability Assay. Mice were anesthetized and received 2 mg lysinated rhodamine-labeled dextran (70 kDa; Invitrogen) by tail vein injection. After 30 min, animals were killed and eyes were enucleated and immediately fixed in 4% PFA for 1 h at 4°C. The retinas were carefully dissected, flat mounted and examined by confocal microscopy.

Mouse Tumor Models. Murine T241 fibrosarcoma and LLC cell lines were used for generation of transfected cell lines overexpressing EGFP and hVEGF, hPlGF. Mouse Tumor Models. Exposure to a lethal dose of CO2. Retinas were isolated after previously described (38). Briefly, mice were killed and eyes were enucleated and immediately fixed in 4% PFA for 1 h at 4°C. The retinas were carefully dissected, flat mounted and examined by confocal microscopy.

Cao et al. PNAS — January 12, 2010 — vol. 107 | no. 2 | 861

10. Steinbrook R (2006) The price of sight—ranibizumab, bevacizumab, and high af–treatment was continued for 10 days. Animals were killed and the eyes were enucleated and immediately fixed in 4% PFA for 30 min.
11. ELISA. Serum levels of human VEGF were quantified by ELISA according to the manufacturer’s instruction (R&D Systems).

Statistical Analysis. Statistical analysis was performed using the student’s t test. Data were presented as means of determinants (±SD) and P < 0.05 were considered statistically significant.

ACKNOWLEDGMENTS. This work was supported by the laboratory of Y.C. through research grants from the Swedish Research Council, the Swedish Cancer Foundation, the Karolinska Institute Foundation, the Swedish Synfrjândets Research Foundation, the Karolinska Gender Foundation, the Torsten and Ragnar Söderberg’s Foundation, and by an European Union Integrated Projects of Angiogenic Targeting Contract 504743 (to Y.C.) and VascuPlug Contract STRP 013811 (to Y.C.).
Supporting Information

Cao et al. 10.1073/pnas.0911661107

SI Materials and Methods

Immunocytochemistry. Primary cells were seeded on 10-mm glass slides in a 12-well plate for staining. When cell confluency reached approximately 50%, cells were fixed in either 4% paraformaldehyde (PFA) for 20 min or 100% methanol for 5 min. The cells were washed three times with PBS and then incubated overnight at 4°C in the presence of primary antibodies. Cells were washed three times in PBS and were incubated for 45 min at room temperature with appropriate secondary antibodies, including an anti-mouse Alexa488, an anti-rat Alexa555 (Invitrogen), or an anti-rat Cy3 antibody (Chemicon). DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining and cells were mounted using Vectashield mounting medium (Vector Laboratories) and images were photographed with a Nikon 90i fluorescent microscope.

Western Blot. Pericytes were grown to 90% confluency in 60-mm dishes, washed with PBS, and incubated for 60 min in serum-free RPMI-1640 medium before stimulation with growth factors. Cells were lysed and total protein samples were collected and separated by 10% SDS/PAGE. Briefly, proteins were transferred to nitrocellulose membranes. Membranes were probed overnight at 4°C with rabbit anti-mouse P-Akt (Ser473) (Cell Signaling), mouse anti-mouse P-Erk 1/2 (Tyr204) (Santa Cruz Biotechnology), rabbit anti-mouse P-Src (Tyr-418) (Biosource) and mouse anti-mouse GAPDH (Millipore), diluted in PBS with 5% BSA and 0.1% Tween, as recommended by the manufacturers. This was followed by incubation for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000), or peroxidase-conjugated goat anti-mouse IgG (1:5,000–1:10,000). Protein bands were visualized by enhanced chemiluminescence using a LAS3000 Lumi-Imager (Fujifilm Life Science).

Whole-Mount Immunofluorescence Staining. Retinal tissues were digested with proteinase K (20 μg/mL), followed by incubation with primary antibody at 4 °C overnight. In some experiments, a mixture of rat anti-mouse CD31 monoclonal antibody and rabbit anti-NG2 polyclonal antibody was used. After rigorous rinsing, blood vessel endothelial cells and pericytes were detected using secondary antibodies coupled to fluorescent, including an anti-mouse Alexa488, an anti-rat Alexa555 (Invitrogen), an anti-rat Cy3 or anti-rabbit Cy5 antibody in different combinations. After washing, retinas were flattened and mounted in Vectashield mounting medium (Vector Laboratories) and analyzed under a Zeiss Confocal LSM510 microscope (Carl Zeiss) or Nikon C1 Confocal microscope (Nikon Corporation). By scanning five to six layers (with 4–5 μm between adjacent layers) for each date collection, 3D images of each date collection were assembled using a confocal microscope software program. The images were further analyzed using Adobe Photoshop CS2 (Adobe).

Fig. S1. Tumor-derived PIGF ablates pericytes from the retina vasculature. (A) VEGF expression vector (T241-VEGF) or PIGF expression vector (T241-PIGF) were s.c. injected in the backs of C57BL mice. After tumor development, retinas were harvested and subjected to whole-mount immunofluorescence staining to detect NG2 (green) and CD31 (red). (B) Quantification of pericyte coverage of vessels from retinas of MMTV-neu tumor-bearing mice treated as indicated. Approximately 10 randomized fields were used for quantification in each group.