GPR124, an orphan G protein-coupled receptor, is required for CNS-specific vascularization and establishment of the blood–brain barrier

Mike Cullen[a,1], Mohammed K. Elzarrad[a,1], Steven Seaman[a,1], Enrique Zudaire[b,1], Janine Stevens[a], Mi Young Yang[a], Xiujie Li[a], Amit Chaudhary[a], Lihong Xu[a], Mary Beth Hilton[a,c], Daniel Logsdon[c], Emily Hsiao[a], Erica V. Stein[a], Frank Cuttitta[b], Diana C. Haines[d], Kunio Nagashima[e], Lino Tessarollo[f], and Brad St. Croix[a,2]

[a]Tumor Angiogenesis Section, Mouse Cancer Genetics Program, National Cancer Institute, National Institutes of Health, Frederick, MD 21702; [b]Angiogenesis Core Facility, National Cancer Institute, National Institutes of Health, Gaithersburg, MD, 20877; [c]Basic Research Program, [d]Veterinary Pathology Section, Pathology/Histotechnology Laboratory, and [e]Image Analysis Laboratory, Advanced Technology Program, Science Applications International Corporation (SAIC), National Cancer Institute, Frederick, MD 21702; and [f]Neural Development Section, Mouse Cancer Genetics Program, National Cancer Institute, National Institutes of Health, Frederick, MD 21702

Edited* by Bert Vogelstein, Johns Hopkins University, Baltimore, MD, and approved February 23, 2011 (received for review November 16, 2010)

Every organ in the body requires blood vessels for efficient delivery of oxygen and nutrients, but independent vascular beds are highly specialized to meet the individual needs of specific organs. The vasculature of the brain is tightly sealed, with blood–brain barrier (BBB) properties developing coincident with neural vascularization. G protein-coupled receptor 124 (GPR124) (tumor endothelial marker 5, TEM5), an orphan member of the adhesion family of G protein-coupled receptors, was previously identified on the basis of its overexpression in tumor vasculature. Here, we show that global deletion or endothelial-specific deletion of GPR124 in mice results in embryonic lethality associated with abnormal angiogenesis of the forebrain and spinal cord. Expression of GPR124 was found to be required for invasion and migration of blood vessels into neuroepithelium, establishment of BBB properties, and expansion of the cerebral cortex. Thus, GPR124 is an important regulator of neurovasculature development and a potential drug target for cerebrovascular diseases.

Several signaling pathways important for widespread stimulation of blood vessel growth in health and disease have been described (e.g., ref. 1). However, less is known about the signaling pathways important for organ-specific vessel growth and function. The majority of CNS vessels are distinct from those of other organs in that they are lined by a tightly sealed layer of specialized endothelial cells (ECs) that play a central role in regulating transport of substances between the blood and brain, i.e., the blood–brain barrier (BBB). Whereas the first evidence for BBB function dates back more than a century, only recently has the unique molecular complexity of brain-specific endothelium been revealed through transcriptomic and proteomic profiling (2, 3). Still, little is known about the vascular receptors functionally required for initiating CNS-specific vascularization and BBB function. However, manipulation of such key signaling receptors would have potential widespread therapeutic application in the treatment of cerebrovascular diseases, the third leading cause of death in the United States (4), and a variety of neurodegenerative disorders including Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis, multiple sclerosis, and others (5). Precise control of the BBB could also aid in the delivery of drugs to the brain, a major challenge in the treatment of brain tumors and other neuropathological diseases (6). Thus, the identification of specific receptors required for CNS-specific angiogenesis and BBB formation has important clinical ramifications.

Vascularization of the CNS during embryonic development occurs by a two-step process that involves both vasculogenesis and angiogenesis. In rodents, vasculogenesis begins at embryonic day 8.5 (E8.5) when mesodermal-derived angioblasts coalesce around the avascular neural tube, forming the perineural vascular plexus (PNVP). At E9.5, angiogenesis, defined as the sprouting of new vessels from preexisting ones, begins as vessels from the PNVP begin to sprout and invade the neural tube. Endothelial tip cells, specialized cells that deploy long filopodia that act as environmental sensors, help guide the new vessel sprouts across the neocortex (7). Sprouting endothelial tip cells are followed by proliferating endothelial stalk cells that are rapidly ensheathed by supporting mural cells, or pericytes. Vascular endothelial growth factor (VEGF), a ubiquitous heparin-binding angiogenesis stimulator, is produced in largest quantity in the subventricular zone (SVZ) of the neocortex (8), forming a gradient across the neuroepithelium and providing a key extracellular cue for VEGF receptor-positive endothelial tip cells (7, 9, 10). In addition to VEGF and its receptors, several other pleiotropic factors including platelet-derived growth factor (PDGF)-B, PDGF receptor-β, neuropilin-1, wnt7, transforming growth factor-β (TGFβ), and integrins alpha-v and beta-8 have been found to play important roles in regulation of CNS vasculature (11–16). Here, we show that G protein-coupled receptor 124 (GPR124) plays a critical, nonredundant role in vessel sprouting, guidance, and maturation in the developing brain with a unique specificity for the CNS.

GPR124 (tumor endothelial marker 5, TEM5), first identified as a gene overexpressed in the vasculature of human colorectal cancer (17), is an orphan seven-pass transmembrane receptor (Fig. S1*A*) that belongs to the G protein-coupled receptor superfamily. GPCRs are important targets for drug development, as this family comprises the targets of about 30% of all clinically marketed drugs (18). In this work, we evaluated GPR124 function in vivo and found that *Gpr124* null mice die in utero with major alterations in vascular development of the CNS.

Results

Disruption of *Gpr124* Results in Embryonic Lethality Associated with Hemorrhaging and Vascular Defects in the CNS. To better understand the normal physiologic function of GPR124, we used homologous recombination to disrupt *Gpr124* in mice by removing exon 1 containing the start codon and signal peptide (Fig. S1). Disruption of a single *Gpr124* allele (heterozygous, $Gpr124^{+/-}$) did not result in any obvious anatomical, behavioral, or histopathological abnormalities. However, genotyping of pups

Author contributions: M.C., M.K.E., L.T., and B.S.C. designed research; M.C., M.K.E., S.S., E.Z., J.S., M.Y.Y., X.L., A.C., L.X., M.B.H., D.L., E.H., E.V.S., F.C., D.C.H., K.N., and B.S.C. performed research; M.C., M.K.E., S.S., E.Z., D.C.H., K.N., and B.S.C. analyzed data; and M.C. and B.S.C. wrote the paper.

Conflict of interest statement: B.S.C. is a co-inventor on technologies related to tumor endothelial markers (TEMs), including GPR124 (TEM5) and is entitled to a share of royalties received from sales of the licensed technologies.

*This Direct Submission article had a prearranged editor.

Data deposition: The sequence referenced in this paper has been previously deposited in the GenBank database (accession no. AF378755).

[1]M.C., M.K.E., S.S., and E.Z. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: stcroix@ncifcrf.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017192108/-/DCSupplemental.

10 d (P10) or older derived from *Gpr124*$^{+/-}$ intercrosses revealed no *Gpr124*$^{-/-}$ (knockout) mice from 194 offspring analyzed. A subsequent analysis of 501 offspring from E9.5 until postnatal day 0.5 (P0.5) revealed that *Gpr124*$^{-/-}$ mice die late in gestation or perinatally (Table S1).

Both macroscopic and microscopic analysis of *Gpr124*$^{-/-}$ embryos revealed hydrocephaly and intracranial hemorrhage in the forebrain, phenotypes that became more pronounced with development (Fig. 1 and Fig. S1). Blood in the spinal cord was also common but varied in severity; and in rare cases, blood was present in the spinal cord but not the brain (Fig. S1 *H* and *I*). A comprehensive histopathological analysis at E17.5 failed to identify defects outside of the CNS.

Hematoxylin and eosin (H&E)-stained *Gpr124*$^{-/-}$ E11.5 to E17.5 brain sections revealed severe hemorrhage in the pallium (outer wall of the telencephalic vesicles) (Fig. 1), with intraventricular blood leakage and enlarged, distorted, lateral ventricles. No abnormalities were noted in the choroid plexus, the epithelial cells responsible for secreting cerebral spinal fluid into the ventricular system. At E13.5, the pallium was notably thinner in the *Gpr124*$^{-/-}$ knockout, and by E15.5, a marked failure of neocortical development was obvious (Fig. S2*A*). In the pallium, *Gpr124*$^{-/-}$ blood vessels formed abnormal "glomeruloid-like" bodies adjacent to the PNVP (Fig. 1*C*). The irregular vascular structures were confirmed using whole mount CD31-vessel staining (Fig. 2*A*) and immunofluorescence staining for endothelium using either isolectin-B4 (ISL, green) or antilaminin antibodies (LN, red) (Fig. 2 *B–E* and Fig. S2 *B–F*). Isolectin staining highlighted the presence of glomeruloids in the ganglionic eminence as early as E10.5 (Fig. 2*B*). Also at E10.5, some tip cells were observed invading into the pallium of *Gpr124*$^{-/-}$ brains and resembled those found in WT mice at E9.5. However, by E10.5, most wild-type vessels had already traversed the neocortex and branched in the subventricular zone (compare Fig. S2 *C* and *D* with *B*). By E11.5, the vessel sprouts in the KO formed small glomeruloids that increased in size over time (Fig. 2*C*). Short brush-like filopodia extended from multiple interconnected ECs on the ventricular surface of large glomeruloids (Fig. S2*E*). Vessels developed normally on either side of the longitudinal cerebral fissure between the lateral ventricles, forming a normal subventricular vascular plexus immediately adjacent to the glomeruloids in the dorsal pallium (Fig. S2*F*). Abnormal glomeruloid bodies were also found in the ventral aspect of the spinal cord (Fig. 2*D*), consistent with the observed spinal cord hemorrhage. Endothelial stalks connected the glomeruloids with the underlying PNVP and in the thicker region of the ganglionic eminence penetrated deeper into the neuroepithelium (Fig. 2*E* and Fig. S2*G*, arrowheads). No vessel defects were observed in the mid- and hindbrain of *Gpr124*$^{-/-}$ mice, the dorsal spinal cord, or in non-CNS tissues such as lung, heart, kidney, and liver.

We reasoned that the abnormal blood vessels could be a result of a defect in pericyte recruitment as vessels invaded the neuroepithelium. However, immunofluorescence staining for the pericyte markers NG2 (Fig. 2*E* and Fig. S2*G*) and PDGFR-β, mRNA in situ hybridization (ISH) for NG2, PDGFR-β, and RGS5, and transmission electron microscopy (Fig. S2 *H–J*) confirmed the presence of pericytes on the surface and throughout the glomeruloids. Ultrastructural analysis revealed that the abnormal glomeruloid structures were typically composed of multiple tufted vascular channels (Fig. S2*H*), although a few contained a single blood-filled cavity (Fig. S2*I*). Nucleated blood cells were present in the vascular channels, which were surrounded by a continuous layer of ECs. Gpr124$^{-/-}$ brains also exhibited a loose packing of neuroepithelial cells with large open spaces immediately surrounding the glomeruloids (Fig. S2*H* and Fig. 1*C*) and had occasional breaks between cortical marginal glia (compare Fig. S2 *J* and *K* with *L*).

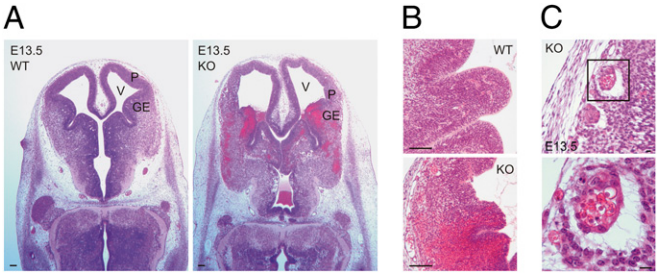

Fig. 1. H&E staining reveals defects in the CNS of *Gpr124*$^{-/-}$ mice. (*A*) E13.5 *Gpr124*$^{-/-}$ H&E-stained brain coronal sections showing bilateral hemorrhage in the pallium (P) extending into the ganglionic eminence (GE). V, lateral ventricle. (*B*) Hemorrhage, hypoplasia, and limited distinction between the lateral and medial lobes of the ganglionic eminence at E12.5. (*C*) Loose attachment of glomeruloids to the underlying neuroepithelium at E13.5. (Scale bar, 100 μm.)

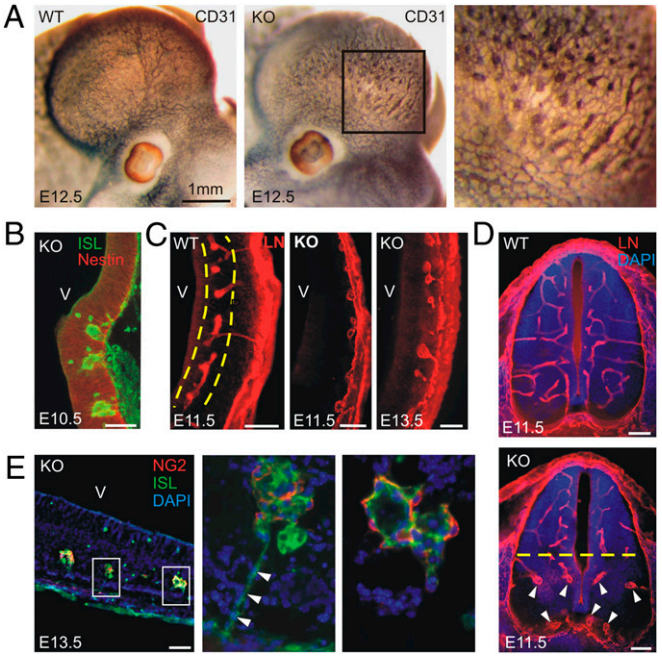

Fig. 2. Histological staining reveals glomeruloid-like bodies in the forebrain and spinal cord of *Gpr124*$^{-/-}$ mice. (*A*) Whole mount CD31-vessel stained surface glomeruloids attached to the PNVP. (*B*) Glomeruloids in the region of the ganglionic eminence at E10.5. Nestin staining (red) highlights the neuroepithelium. (*C*) At E11.5, vessels in WT mice penetrate the neuroepithelium and begin to form a subventricular vascular plexus (SVP), whereas corresponding vessels in *Gpr124*$^{-/-}$ mice form glomeruloids, which increase in size with time. (*D*) Glomeruloids (arrowheads) in the ventral spinal cord of *Gpr124*$^{-/-}$ mice. (*E*) NG2^{+} pericytes (red) closely associated with isolectin-labeled ECs (green) in the pallium. Arrowheads in *E* indicate an endothelial stalk. ISL, isolectin; LN, laminin; V, ventricle. (Scale bars, 100 μm, unless stated otherwise.)

Gpr124 Expression Is Highest in Vascular Cells and Peaks During Angiogenesis of the Developing CNS. The observed defects and previously described up-regulation of *GPR124* in tumor vessels (17, 19) suggested that *Gpr124* expression may be elevated during brain angiogenesis. Real-time quantitative PCR (qPCR) revealed a fourfold increase in *Gpr124* mRNA expression in embryonic brain compared with adult brain, reaching peak levels at E13.5 (Fig. S3*A*). Because the defects observed were localized to the forebrain, we also compared *Gpr124* expression levels in microdissected forebrain versus hindbrain, but no differences were observed (Fig. S3*B*). Similarly, the closest homolog of *Gpr124*, called *Gpr125* (*TEM5-Like*) was also found at similar levels in forebrain and hindbrain (Fig. S3*B*), suggesting that compensation in the hindbrain is unlikely responsible for the localized defect.

To determine which cells express *Gpr124*, we performed ISH on WT embryos from E11.5 to E15.5. At each stage, *Gpr124* was

detected in vessels throughout the brain and spinal cord, in the PNVP, and in vessels outside the CNS (Fig. S3 *D–F*). Combining *Gpr124* mRNA ISH with isolectin histochemical staining revealed clear colocalization of *Gpr124* with isolectin-positive ECs, similar to the pattern observed with *Vegfr2* staining (Fig. S3*G*). However, the *Gpr124* riboprobe also stained lectin-negative vascular cells with a pattern similar to *Pdgfrβ*, suggesting that *Gpr124* may also be expressed by pericytes. Evaluation of *Gpr124* mRNA expression levels in human primary brain-derived pericytes and ECs confirmed the expression of *Gpr124* in both cell types (Fig. S3*H*).

Selective Disruption of *Gpr124* in Endothelial Cells Results in a Phenocopy of the Defects Observed in Global *Gpr124*$^{-/-}$ Knockout Mice. We reasoned that the limited expansion of the neocortex in *Gpr124*$^{-/-}$ mice could be secondary to a defect in the developing brain vasculature. Alternatively, a low level of GPR124 expression in neural stem cells or their progeny may have escaped detection in our mRNA ISH assays and could potentially contribute directly to the lack of cortical growth. To distinguish between these possibilities and further define the cell type responsible for the observed vascular defects in global *Gpr124*-deficient mice, we sought to disrupt *Gpr124* selectively in ECs. For this purpose, conditional KO mice were generated, which carried one *Gpr124* null allele and one "floxed" *Gpr124* allele as well as the Tie2-cre transgene (*Gpr124*$^{-/flox}$; *Tie2-cre*). In this model, one *Gpr124* allele is deleted globally, whereas the other is deleted in Tie2^{+} cells due to the presence of *Cre* recombinase driven by the Tie2 promoter (Fig. S1*E*). We chose this model because immunofluorescent staining using high-resolution confocal microscopy revealed uniform endothelial-specific expression of Tie2 in the developing brain (Fig. S4). A comparison of the conditional KO (*Gpr124*$^{-/flox}$; *Tie2-cre*) with control littermates (*Gpr124*$^{+/flox}$; *Tie2-cre*) revealed a phenotype that was strikingly similar to that observed in the globally deleted *Gpr124*$^{-/-}$ mice and included hemorrhaging, glomeruloid formation, and a failure of the neocortex to expand (Fig. 3 *A–C*). Taken together, these results suggest that loss of GPR124 expression in ECs is responsible for the phenotypes observed in *Gpr124*$^{-/-}$ mice.

GPR124 Is a Cell Surface Glycoprotein That Promotes Endothelial Migration. To further investigate GPR124 function and characterize its subcellular distribution, we generated an anti-human GPR124 monoclonal antibody, which detected an ∼185 kDa product by Western blotting (Fig. S5*A*). Treatment of 293/GPR124 lysates with glycosidase resulted in a smaller product of 140 kDa, suggesting that GPR124 was glycosylated. Because our monoclonal antibody only recognized the denatured protein and not the native protein, a FLAG tag was engineered into the N terminus of GPR124 and anti-FLAG antibodies used to show that the N terminus resides outside the cell (Fig. S5*B*). Direct visualization of a GPR124–GFP fusion protein (Fig. S5*C*) and immunofluorescent staining of cells expressing exogenous FLAG- or myc-tagged GPR124 (Fig. S5 *D–K*) revealed that GPR124 was localized predominantly to the lateral border between cells and was highly expressed in filopodia. Cells expressing the C-terminal myc-tagged GPR124 required cell permeabilization to visualize GPR124 by immunofluorescence staining, consistent with a C-terminal cytosolic tail (Fig. S5 *D–I*). Interestingly, cells mechanically detached from the culture flask left residual "footprints" of GPR124 staining on the flask (Fig. S5*K*), and phalloidin staining revealed colocalization of GPR124 with the underlying actin cytoskeleton (Fig. S5*E*). These results and earlier studies, which showed binding of GPR124 to glycosaminoglycans (20), suggest that GPR124 may promote cell migration by linking the actin cytoskeleton with extracellular matrix. To test this, we stably overexpressed exogenous GPR124 in both mouse (bEnd3) and human (human cerebral microvascular endothelial cell, hCMEC) brain ECs (Fig. 4*A* and Fig. S3*J*) and found that migration was markedly stimulated (Fig. 4*B*). Thus, GPR124 may facilitate angiogenesis by promoting EC migration across the neocortex.

***Gpr124* Disruption Leads to Overexpression of VEGF.** To gain further insight into mechanisms regulating the abnormal angiogenesis observed in *Gpr124*$^{-/-}$ forebrains, we evaluated the expression of 50 genes potentially important for blood vessel formation or maturation at E13.5. Due to the limited amount of microdissected forebrain available, we chose to use qPCR because of its high sensitivity, reproducibility, and need for small amounts of starting material. In total, 9 genes were found to be 2.5-fold or higher in *Gpr124*$^{-/-}$ versus *Gpr124*$^{+/+}$ forebrains, whereas only 1 gene, *Mmp9*, showed less than 50% expression (Fig. 5*A*). The angiogenesis inhibitor *thrombospondin2 (Thbs2)* showed the largest increase with eightfold higher expression.

To examine the onset of these changes, a secondary qPCR screen was performed on each of the genes that were downregulated (below 50%) or up-regulated (above 2.5-fold) using forebrains microdissected at E11.5, E12.5, and E13.5. Of the 10 genes analyzed, only 1 gene, *Vegfa*, displayed a significant alteration in expression by E11.5 (Fig. S6 *A* and *B* and Fig. 5*B*). Thus, an early induction of VEGF in *Gpr124*$^{-/-}$ brains may promote some of the later gene expression changes noted, such as increased *thrombospondin2*, which may participate in a feedback mechanism to limit angiogenesis. VEGFA mRNA expression was readily detected in the pallium of *Gpr124*$^{-/-}$ brains, but only in the SVZ, opposite the glomeruloids (Fig. 5*C*). An analysis of individual VEGF splice variants expressed in *Gpr124*$^{+/+}$ and *Gpr124*$^{-/-}$ brains during embryogenesis revealed that

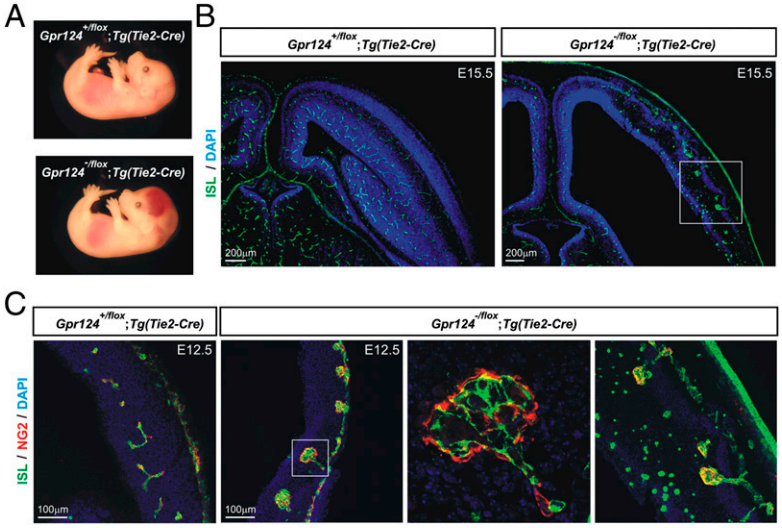

Fig. 3. GPR124 expression in endothelial cells is responsible for the phenotypes observed in Gpr124$^{-/-}$ mice. (*A*) Macroscopic hemorrhaging in the E15.5 embryonic brain was obvious in the *Gpr124*$^{-/flox}$; *Tg(Tie2-Cre)* mice but was absent from the *Gpr124*$^{+/flox}$; *Tg(Tie2-Cre)* mice. (*B*) The pallium was notably thinner in the E15.5 embryonic brain of *Gpr124*$^{-/flox}$; *Tg (Tie2-Cre)* mice. (*C*) Isolectin-B4 and NG2^{+} glomeruloids were found in the lateral ventricular wall in the region adjacent to the ganglionic eminence of E12.5 *Gpr124*$^{-/flox}$; *Tg(Tie2-Cre)* brains. Isolectin positive, NG2^{+} macrophages were frequently observed in the ventricular wall of both the global *Gpr124*$^{-/-}$ and *Gpr124*$^{-/flox}$; *Tg(Tie2-Cre)* brains and are particularly abundant in the E15.5 *Inset* (*C*, far *Right*), which is taken from the region shown in *B*.

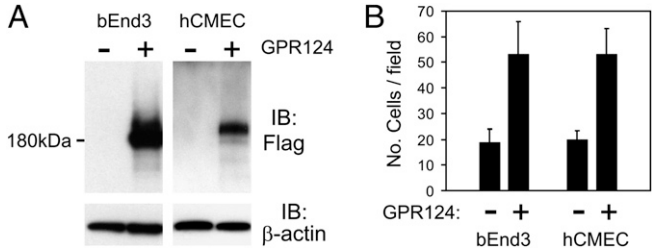

Fig. 4. GPR124 promotes cell migration. (*A*) Exogenous Flag-GPR124 is detected in bEnd3 or hCMEC brain endothelial cells by immunoblotting with anti-Flag antibodies. The total levels of GPR124 (endogenous plus exogenous) in bEnd3/GPR124 cells are about 2.6-fold higher than bEnd3 cells and similar to those in ECs of developing brain (Fig. S3*J*) (*B*) Overexpression of GPR124 enhanced migration in brain endothelial cells. $n = 6$, $P < 0.001$. Results are representative of three independent experiments.

$VEGF_{164}$ was the predominant form, whereas $VEGF_{120}$ and $VEGF_{188}$ were expressed at lower levels, but each of the variants was overexpressed in *Gpr124*$^{-/-}$ brains (Fig. S6*C*). Because *Vegfa* expression is hypoxia responsive, we used Hypoxyprobe staining to assess hypoxia levels and found extensive hypoxia in the SVZ of *Gpr124*$^{-/-}$ brains (Fig. 5*D*) with a hypoxia gradient forming across the pallium by E11.5. Although exogenous overexpression of VEGF in the adult mouse brain using adenoviral vectors was sufficient to stimulate mature quiescent vessels to proliferate (21, 22), at E14.5 endothelial proliferation was not significantly elevated in the embryonic brains of *Gpr124*$^{-/-}$ versus WT controls (Fig. S7), perhaps because the vessels at this developmental stage are already close to a state of maximum proliferation. Thus, a number of molecular alterations were identified in the developing forebrain in response to *Gpr124* disruption and some of the changes, such as increased VEGF, may be regulated by changes in the local microenvironment.

GPR124 Regulates the BBB Properties of Brain Endothelium. On the basis of the subcellular localization of GPR124 at cell–cell borders and the hemorrhaging observed in *Gpr124*$^{-/-}$ brains, we hypothesized that loss of GPR124 may result in a failure of the affected CNS vasculature to establish BBB properties. This prediction was supported by the observation that large deposits of extravascular fibrin surrounded glomeruloids in *Gpr124*$^{-/-}$ brains but were absent from the extravascular compartment of wild-type brains (Fig. S8*A*). We also analyzed the expression of GLUT-1, a glucose transporter that is frequently used as a BBB indicator because it is turned on in new intracranial blood vessels concomitant with BBB development and maturation (23, 24). As previously reported (12), in WT brains GLUT-1 was initially highly expressed in neuroepithelium at E9.5, but its expression in neuroepithelial cells rapidly declined, coincident with its induction on CNS neovasculature such that by E13.5 its expression was exclusively vascular (Fig. 6). In *Gpr124*$^{-/-}$ brains, however, GLUT-1 expression remained high in the neuroepithelium surrounding glomeruloids at E13.5, and expression in glomeruloid endothelium was never observed even in late gestation embryos (Fig. 6 and Fig. S9 *A* and *B*). We also evaluated expression of plasmalemma vesicle-associated protein (PLVAP), a marker of stomatal and fenestral diaphragms that is normally silenced with tightening of the BBB and absent from mature CNS vasculature (25–28). By E13.5, PLVAP was absent from WT vessels but was readily detected in the abnormal vessels of *Gpr124*$^{-/-}$ brains (Fig. S9*C*). The Glut-1 and PVLAP expression patterns observed in the global KO mice were phenocopied by the Tie2-conditional KO (Fig. S9 *D* and *E*).

The aforementioned studies support a role for GPR124 in regulation of BBB but are based on correlative data. To directly evaluate the role of GPR124 in barrier formation, we measured vascular leakage in the CNS following intracardiac injection of live embryos with amine-reactive sulfo-NHS-biotin (443 D), a tracer that covalently cross-links to accessible proteins. In wild-type mice, biotin was localized inside the vasculature of the neocortex and spinal cord. In *Gpr124*$^{-/-}$ embryos, however, biotin was detected in both intra- and extravascular regions in the forebrain and ventral spinal cord (Fig. 7*A* and Fig. S9*F*). Biotin levels were highest immediately surrounding the glomeruloids with a decreasing concentration gradient extending into the neural tissues (Fig. 7*A*, *Right*). In general, CNS vessels from the KO leaked a similar amount of biotin as somatic vessels of non-CNS origin. Although non-CNS vessels were much leakier than CNS vessels, in non-CNS vessels no differences in permeability were observed between WT and KO mice.

GPR124 could potentially play an indirect role in the establishment of BBB properties if, for example, GPR124 loss prevents maturation and differentiation of newly formed vessels. However, GPR124 could also play a more direct role in BBB formation given its location at cell–cell borders. To determine whether GPR124 could directly affect barrier properties, we evaluated the effect of its overexpression on brain ECs in culture, taking advantage of the fact that brain ECs spontaneously adopt a relatively leaky phenotype and express a relatively low level of Gpr124 (Fig. S3*J*) when cultured in vitro. Exogenous overexpression of GPR124 in bEnd3 brain ECs restored levels to those found in ECs of the developing brain (Fig. S3 *I* and *J*) and enhanced barrier properties in both bioelectric impedance and permeability assays (Fig. 7 *B* and *C*). These results suggest that barrier properties may be regulated, at least in part, by the direct action of GPR124.

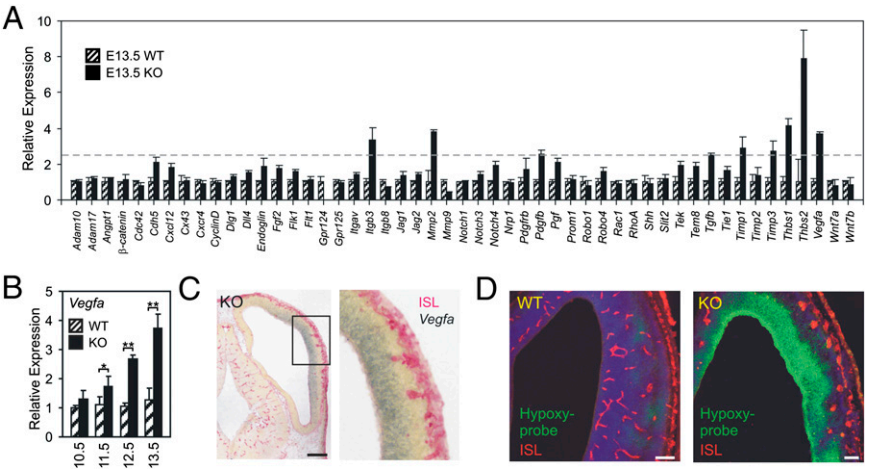

Fig. 5. *Vegfa* is overexpressed by E11.5 in the *Gpr124*$^{-/-}$ forebrain and is associated with hypoxia. (*A*) Real-time qPCR was used in a primary screen to evaluate the expression of *Gpr124* and an additional 50 genes in *Gpr124*$^{+/+}$ (WT) or *Gpr124*$^{-/-}$ (KO) forebrains microdissected at E13.5. Note that a mutant *Gpr124* mRNA transcript is expressed in the KO, but its levels are ~1% that of the WT transcript. (*B*) *Vegfa* is significantly overexpressed in *Gpr124*$^{-/-}$ brains by E11.5. *$P < 0.02$, **$P < 0.005$. (*C* and *D*) VEGF mRNA expression (*C*, black) or hypoxia (*D*, green) is highest in the SVZ of the pallium opposite the isolectin-B4–labeled glomeruloids (red). (Scale bar, 100 μm.)

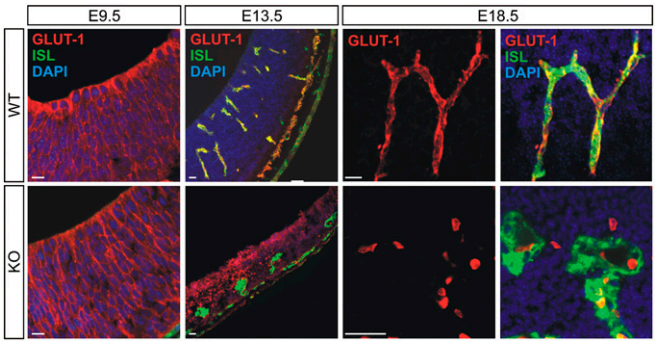

Fig. 6. Silencing of GLUT-1 expression in the abnormal glomeruloid-like bodies of *Gpr124*$^{-/-}$ brains. GLUT-1 (red), an indicator of BBB integrity, is not observed in glomeruloid vessels, even in viable *Gpr124*$^{-/-}$ embryos at E18.5, although expression in the neuroepithelium has declined by that stage. GLUT-1 was constitutively expressed in erythrocytes, which can be seen inside and outside the glomeruloids at E18.5. (Scale bar, 30 μm.) Blue: DAPI.

Discussion

These studies reveal a role for GPR124 as a critical regulator of angiogenesis and barriergenesis of the developing CNS. In vivo, GPR124 was required for proper migration and branching of ECs as they invaded into the neocortex and subsequent acquisition of BBB properties. Consistent with the in vivo defects observed, overexpression of GPR124 in cultured brain ECs promoted their migration and barrier properties. Altered interactions of brain ECs with other cell types such as pericytes or neuroepithelial cells, which are known to influence vessel maturation, stability, and barriergenesis, could potentially contribute to the defects observed in vivo. However, both our endothelial-specific KO and our in vitro studies with cultured brain ECs suggest an autonomous role for endothelial GPR124 in driving the observed phenotypes.

GPR124 was also found to be essential for establishment of BBB properties in vivo, which could be caused, at least in part, by a developmental delay in vessel maturation. However, other factors could potentially contribute to the enhanced vessel permeability in vivo. For example, the overexpression of VEGF, also known as vascular permeability factor, in *Gpr124*$^{-/-}$ mice may enhance vessel leakiness. VEGF has been shown to induce permeability of brain ECs both in vitro and in vivo (29–31). Furthermore, elegant studies by Dvorak et al. showed that localized overexpression of VEGF in adult mice using a nonreplicating adenoviral vector is sufficient to enhance permeability and induce glomeruloid formation (21, 32). Thus, the inability of vessels to migrate and form a normal subventricular plexus may ultimately lead to a build up in hypoxia that stimulates overexpression of VEGF and prevents BBB formation. Our in vitro studies suggest that GPR124 may also play a direct role in regulating barrier permeability. Further studies are required to determine whether GPR124 affects paracellular transport, transcellular transport, or both processes.

The defects in developmental angiogenesis reported here are consistent with the original identification of *Gpr124* as a gene overexpressed during angiogenesis (17, 19). However, the specific localization of the angiogenesis defects to particular regions of the CNS was unexpected. Although *Gpr124* expression levels appeared highest in vessels of the developing brain, a lower level of expression could also be detected in vessels throughout the developing embryo (19), the adult brain (3), and in cultured brain pericytes and ECs derived from non-CNS tissues such as human umbilical vein endothelial cells (HUVECs). One possibility that we considered was that GPR125, the closest homolog of GPR124, might share a redundant function and compensate for loss of its function. However, *Gpr125* and *Gpr124* expression levels were similar in both forebrain and hindbrain, regions found to be afflicted or nonafflicted, respectively, by *Gpr124* loss. Another possibility is that a GPR124 ligand or guidance cue with a relatively restricted expression during embryogenesis is expressed specifically in the afflicted regions. Alternatively, lack of compensatory guidance cues specifically in the afflicted region could be involved. It should be noted that GPR124 could also share similar functions in nonafflicted vessels but with a subtleness that escaped detection here, or at later stages of development that were not appreciated because of the early developmental lethality.

GPR124 was originally identified as a gene overexpressed in tumor vessels of human colorectal cancer. In that context, overexpression of GPR124 in tumor associated vessels may stimulate endothelial migration and facilitate tumor angiogenesis. Upregulation of GPR124 in tumor vessels may also help to limit vessel leakiness caused by VEGF and other tumor-derived permeability factors. Thus, GPR124^{+} tumor vessels may be more "normalized" (i.e., less leaky) than GPR124^{-} tumor vessels. Our *Gpr124* condi-

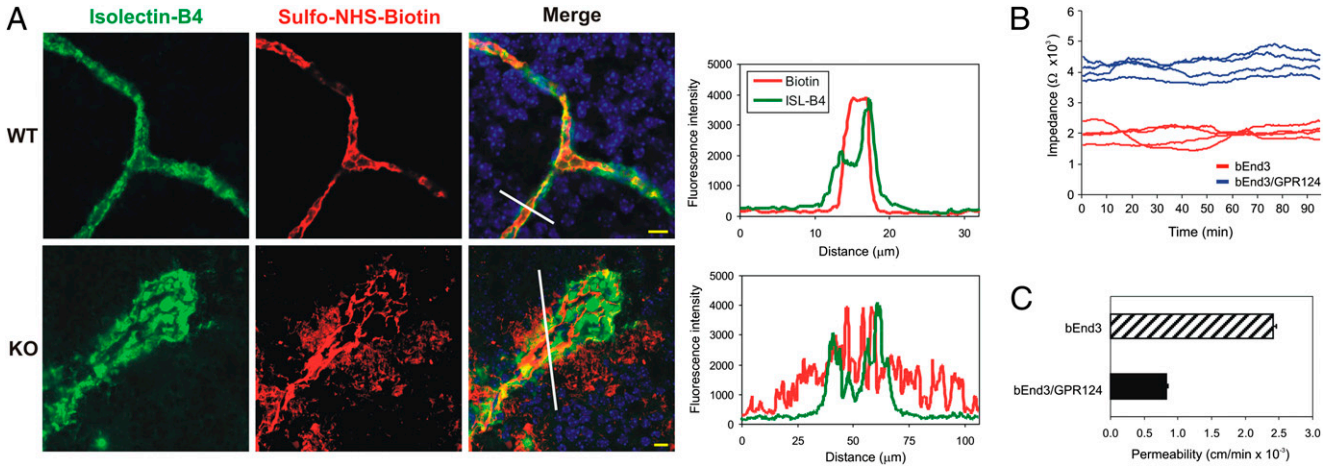

Fig. 7. GPR124 expression regulates the BBB properties of brain endothelium. (*A*) Permeability of CNS vessels in the forebrain of E18.5 wild-type (WT) or *Gpr124*$^{-/-}$ (KO) embryos was measured by coimmunofluorescent staining for biotin (red) and isolectin-B4 (green) on brain sections following an intracardial injection of live embryos with sulfo-NHS-biotin. The relative green and red fluorescence intensity of two representative regions in *A* (white lines, merged images) are shown on the *Right*. Note the leakage of biotin in *Gpr124*$^{-/-}$ embryos with a decreasing gradient extending from the glomeruloid into the neuroepithelium. (Yellow bar, 10 μm.) (*B*) Barrier properties were monitored by electric cell-substrate impedance sensing (ECIS) in bEnd3 or bEnd3/GPR124 cells. Values were collected from four independent wells per group. The small fluctuations are due to movements in the cell monolayer. (*C*) Permeability of FITC-dextran (40,000 Da) through a monolayer of brain ECs is decreased in cells overexpressing GPR124. The stably transfected bEnd3/GPR124 cells used in these experiments are the same as those used in Fig. 4*A* and Fig. S3*J*. Results in *B* and *C* are representative of three independent experiments.

tional knockout strain will provide an important research tool for future studies to assess the function of GPR124 under pathological conditions, for example, during tumor angiogenesis.

The endothelial specific $Gpr124^{-/-}$ strain recapitulated the defects observed in the global $Gpr124^{-/-}$ mouse. Although Tie2-cre is also known to be expressed in cells of the monocytic lineage, we did not detect expression of *Gpr124* in lectin-positive macrophages by ISH (for example, Fig. S3*G*). Thus, loss of GPR124 in ECs is most likely responsible for driving each of the defects observed in the global $Gpr124^{-/-}$ mice. The lack of functional vasculature in the developing forebrain likely leads to an inadequate supply of nutrients which, in turn, may contribute to the inability of the neocortex to expand. Moreover, the vasculature in the subventricular zone is thought to provide a critical niche required for neural stem cell growth (33, 34).

While this manuscript was in preparation, another study was published that reported GPR124 knockout mice with phenotypes strikingly similar to those reported here (35), providing independent validation of the importance of GPR124 in angiogenesis of the developing CNS. Analogous to these genetically engineered rodent models, in humans there is a lethal prenatal condition known as proliferative vasculopathy and hydranencephaly-hydrocephaly (PVHH), where affected fetuses share several striking similarities with $Gpr124^{-/-}$ mice, including vascular glomeruloids in both the pallium and ventral spinal cord, enlarged cerebral ventricles, and a thin underdeveloped pallium (36). Although the mutations responsible for PVHH have not yet been identified, these studies suggest that GPR124 might be involved in this or a myriad of other neurovascular diseases associated with BBB breakdown or in the formation of glomeruloids associated with the most malignant forms of glioblastoma and gastrointestinal carcinomas (37, 38).

Materials and Methods

Animal Experimentation. Mice handling and experimentation were performed in accordance with institutional guidelines and were approved by the National Cancer Institute Animal Care and Use Committee. To disrupt the *Gpr124* gene, a *Gpr124* targeting vector was designed to introduce lox-p sites on either side of exon 1, containing the *Gpr124* signal peptide and start codon. Details regarding the generation of KO mice and animal experimentation can be found in *SI Materials and Methods*.

Histological Examination. Methods used for H&E staining, immunofluorescence staining, whole mount staining, in situ hybridization, and electron microscopy can be found in *SI Materials and Methods*.

Vectors and Western blotting. Western blotting was performed as previously described (3) and details regarding the vectors and antibodies can be found in *SI Materials and Methods*.

Cell-Based Studies. Migration assays, electric cell-substrate impedance sensing (ECIS) assays, and permeability assays are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Babette Weksler for the immortalized hCMEC (HCMEC/D3) cells. We thank members of the Mouse Cancer Genetics Program for helpful discussion and Dr. Neal Copeland for critical reading of the manuscript. This research was supported by the intramural research program of the National Cancer Institute (NCI), National Institutes of Health, Department of Health and Social Services (DHSS), and with federal funds from the NCI under Contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the DHSS nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

1. Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. *Nature* 438:967–974.
2. Pottiez G, Flahaut C, Cecchelli R, Karamanos Y (2009) Understanding the blood-brain barrier using gene and protein expression profiling technologies. *Brain Res Rev* 62: 83–98.
3. Seaman S, et al. (2007) Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell* 11:539–554.
4. Xu J, Kochanek KD, Tejada-Vera B (2009) *Deaths: Preliminary Data for 2007* (National Center for Health Statistics, Hyattsville, MD).
5. Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57:178–201.
6. Pardridge WM (2007) Blood-brain barrier delivery. *Drug Discov Today* 12:54–61.
7. Gerhardt H, et al. (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 161:1163–1177.
8. Breier G, Albrecht U, Sterrer S, Risau W (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114:521–532.
9. Raab S, et al. (2004) Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. *Thromb Haemost* 91:595–605.
10. Ruhrberg C, et al. (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev* 16: 2684–2698.
11. McCarty JH, et al. (2002) Defective associations between blood vessels and brain parenchyma lead to cerebral hemorrhage in mice lacking alphav integrins. *Mol Cell Biol* 22:7667–7677.
12. Stenman JM, et al. (2008) Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* 322:1247–1250.
13. Zhu J, et al. (2002) beta8 integrins are required for vascular morphogenesis in mouse embryos. *Development* 129:2891–2903.
14. Lindahl P, Johansson BR, Levéen P, Betsholtz C (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277:242–245.
15. Gerhardt H, et al. (2004) Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. *Dev Dyn* 231:503–509.
16. Mu Z, Yang Z, Yu D, Zhao Z, Munger JS (2008) TGFbeta1 and TGFbeta3 are partially redundant effectors in brain vascular morphogenesis. *Mech Dev* 125:508–516.
17. St Croix B, et al. (2000) Genes expressed in human tumor endothelium. *Science* 289: 1197–1202.
18. Siehler S (2008) Cell-based assays in GPCR drug discovery. *Biotechnol J* 3:471–483.
19. Carson-Walter EB, et al. (2001) Cell surface tumor endothelial markers are conserved in mice and humans. *Cancer Res* 61:6649–6655.
20. Vallon M, Essler M (2006) Proteolytically processed soluble tumor endothelial marker (TEM) 5 mediates endothelial cell survival during angiogenesis by linking integrin alpha(v)beta3 to glycosaminoglycans. *J Biol Chem* 281:34179–34188.
21. Stiver SI, Tan X, Brown LF, Hedley-Whyte ET, Dvorak HF (2004) VEGF-A angiogenesis induces a stable neovasculature in adult murine brain. *J Neuropathol Exp Neurol* 63: 841–855.
22. Yang GY, et al. (2003) Induction of focal angiogenesis through adenoviral vector mediated vascular endothelial cell growth factor gene transfer in the mature mouse brain. *Angiogenesis* 6:151–158.
23. Pardridge WM, Boado RJ, Farrell CR (1990) Brain-type glucose transporter (GLUT-1) is selectively localized to the blood-brain barrier. Studies with quantitative western blotting and in situ hybridization. *J Biol Chem* 265:18035–18040.
24. Bauer H, et al. (1995) Ontogenic expression of the erythroid-type glucose transporter (Glut 1) in the telencephalon of the mouse: correlation to the tightening of the blood-brain barrier. *Brain Res Dev Brain Res* 86:317–325.
25. Shue EH, et al. (2008) Plasmalemmal vesicle associated protein-1 (PV-1) is a marker of blood-brain barrier disruption in rodent models. *BMC Neurosci* 9:29.
26. Ioannidou S, et al. (2006) An in vitro assay reveals a role for the diaphragm protein PV-1 in endothelial fenestra morphogenesis. *Proc Natl Acad Sci USA* 103: 16770–16775.
27. Stan RV, Kubitza M, Palade GE (1999) PV-1 is a component of the fenestral and stomatal diaphragms in fenestrated endothelia. *Proc Natl Acad Sci USA* 96:13203–13207.
28. Stan RV, Tkachenko E, Niesman IR (2004) PV1 is a key structural component for the formation of the stomatal and fenestral diaphragms. *Mol Biol Cell* 15:3615–3630.
29. Davis B, et al. (2010) Role of vasodilator stimulated phosphoprotein in VEGF induced blood-brain barrier permeability in endothelial cell monolayers. *Int J Dev Neurosci* 28: 423–428.
30. Dobrogowska DH, Lossinsky AS, Tarnawski M, Vorbrodt AW (1998) Increased blood-brain barrier permeability and endothelial abnormalities induced by vascular endothelial growth factor. *J Neurocytol* 27:163–173.
31. Fischer S, et al. (1999) Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO. *Am J Physiol* 276:C812–C820.
32. Sundberg C, et al. (2001) Glomeruloid microvascular proliferation follows adenoviral vascular permeability factor/vascular endothelial growth factor-164 gene delivery. *Am J Pathol* 158:1145–1160.
33. Shen Q, et al. (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 304:1338–1340.
34. Goldberg JS, Hirschi KK (2009) Diverse roles of the vasculature within the neural stem cell niche. *Regen Med* 4:879–897.
35. Kuhnert F, et al. (2010) Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124. *Science* 330:985–989.
36. Fowler M, Dow R, White TA, Greer CH (1972) Congenital hydrocephalus-hydrencephaly in five siblings, with autopsy studies: A new disease. *Dev Med Child Neurol* 14:173–188.
37. Ohtani H (1992) Glomeruloid structures as vascular reaction in human gastrointestinal carcinoma. *Jpn J Cancer Res* 83:1334–1340.
38. Rojiani AM, Dorovini-Zis K (1996) Glomeruloid vascular structures in glioblastoma multiforme: An immunohistochemical and ultrastructural study. *J Neurosurg* 85: 1078–1084.