Endothelial Wnt/β-catenin signaling inhibits glioma angiogenesis and normalizes tumor blood vessels by inducing PDGF-B expression

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Endothelial Wnt/β-catenin signaling is necessary for angiogenesis of the central nervous system and blood–brain barrier (BBB) differentiation, but its relevance for glioma vascularization is unknown. In this study, we show that doxycycline–dependent Wnt1 expression in subcutaneous and intracranial mouse glioma models induced endothelial Wnt/β-catenin signaling and led to diminished tumor growth, reduced vascular density, and normalized vessels with increased mural cell attachment. These findings were corroborated in GL261 glioma cells intracranially transplanted in mice expressing dominant-active β-catenin specifically in the endothelium. Enforced endothelial β-catenin signaling restored BBB characteristics, whereas inhibition by Dkk1 (Dickkopf-1) had opposing effects. By overactivating the Wnt pathway, we induced the Wnt/β-catenin–Dll4/Notch signaling cascade in tumor endothelia, blocking angiogenic and favoring a quiescent vascular phenotype, indicated by induction of stalk cell genes. We show that β-catenin transcriptional activity directly regulated endothelial expression of platelet-derived growth factor B (PDGF-B), leading to mural cell recruitment thereby contributing to vascular quiescence and barrier function. We propose that reinforced Wnt/β-catenin signaling leads to inhibition of angiogenesis with normalized and less permeable vessels, which might prove to be a valuable therapeutic target for antiangiogenic and edema glioma therapy.

Angiogenesis describes the growth of new blood vessels from preexisting ones and occurs during embryonic development and in the female reproductive cycle. In addition to physiological angiogenesis, vessel growth is closely associated with the progression of various tumors (Streit and Detmar, 2003). More specifically, the onset of tumor vascularization, known as angiogenic switch, defines progression to a highly malignant and eventually metastatic state (Bergers and Benjamin, 2003).

The vascular endothelial growth factor (VEGF) is crucial for physiological as well as pathological angiogenesis. VEGF, binding to the tyrosine kinase receptor VEGFR2 together with Nrp1 (neuropilin-1) and VEGFR3, requires the concerted interaction with other modulating pathways such as Notch, angiopoietin/Tie2, and ephrin/Eph to foster functional vessel growth (Adams and Alitalo, 2003).

Abbreviations used: α-SMA, α smooth muscle actin; BBB, blood–brain barrier; CM, conditioned medium; CollIV, collagen IV; DOX, doxycycline; EC, endothelial cell; GBM, glioblastoma; GOF, gain-of-function; HUVEC, human umbilical vein EC; IF, immunofluorescence; ISH, in situ hybridization; MBE, mouse brain endotheloma; mRNA, messenger RNA; PC, pericyte; PDGF-B, platelet-derived growth factor B; Podxl, podocalyxin; qRT-PCR, quantitative RT-PCR; SMC, smooth muscle cell; TJ, tight junction; VEGF, vascular endothelial growth factor.

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however, VEGF overexpression downstream of HIF1α (hypoxia inducible factor 1α) drives abnormal vessel growth and functions as a vascular permeability factor.

The WHO grade IV astrocytoma or glioblastoma (GBM) is one of the most vasculatured and deadliest tumors (Machein and de Miguel, 2009). Because of its brain location, clinicians have to face specific complications such as loss of blood–brain barrier (BBB) characteristics in tumor vessels, leading to edema formation. Targeting glioma angiogenesis by VEGF inhibition had only minor impact on patient survival (Brazier and Batchelor, 2009). Instead, it has been proposed that tumor vessel normalization rather than inhibition of angiogenesis is beneficial for tumor treatment, leading to reduced interstitial tumor pressure and metastasis and increased drug delivery (Carmeliet and Jain, 2011).

Beside VEGF, Notch, and other factors, the Wnt pathway has recently been shown to participate in developmental brain angiogenesis and in vascular differentiation to the BBB phenotype (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009). Wnts are glycosylated growth factors binding to receptors of the frizzled family (Smolich et al., 1993; Willert et al., 2003). Depending on the Wnt growth factor and the receptor context with Lrp5/6 coreceptors, signaling differs between the β-catenin/β-catenin signaling for brain tumor functions as a vascular permeability factor.

RESULTS

The Wnt/β-catenin pathway is active in vessels of human glioma and does not correlate with tumor grade

As Wnt/β-catenin signaling has been shown to be active in vessels of human GBM by staining of nuclear β-catenin (Yano et al., 2000b), we wanted to evaluate whether gene expression of crucial Wnt pathway genes correlates with GBM. Analysis via the Texas Cancer Genome Analysis (TCGA) for selected Wnt pathway genes revealed up-regulated Wnt5a, a noncanonical factor, in human GBM (Fig. 1 A). Canonical Wnt factors, of which Wnt1, Wnt3a, and Wnt7a/b have been implicated in brain angiogenesis and BBB induction, were not regulated compared with normal brain. Interestingly, the soluble Wnt/β-catenin inhibitor Dkk1 showed in mean an elevation with big variability, whereas Dkk2, which has been proposed to have opposing function to Dkk1 in angiogenesis (Min et al., 2011), showed a contrary expression profile.

Staining for β-catenin of normal human brain sections revealed no nuclear staining of the protein in ECs, confirming our own previous results in adult mice (Fig. 1 B; Liebner et al., 2008). Compared with normal brain, several vessels in the tumor proper showed nuclear β-catenin localization but revealed no correlation with tumor grade (Fig. 1 C). However, the function of Wnt/β-catenin signaling in pathological angiogenesis has not been elucidated so far.

Wnt1 reduces subcutaneous glioma growth in NMRI–Foxn1nu (NUDE) mice

To study the role of endothelial Wnt/β-catenin signaling in glioma angiogenesis, we established doxycycline (DOX)-inducible GL261 mouse glioma cell lines that upon DOX removal (~DOX) stably express either the empty vector (control), mouse Wnt1 (Wnt1−/−), or human Dkk1 (Dkk1−/−). The induction of Wnt1 or Dkk1 expression was verified by Western blotting (Fig. 2 A), and the functional activity of the
sized tumors revealed no obvious difference between the control−D and Wnt1−D condition (Fig. 2 E). Notably, necrotic areas with surrounding pseudopalisading cells were observed, suggesting that both tumors suffered from nutrition and oxygen deprivation. However, Dkk1−D tumors were rather compact with small necrotic areas (Fig. 2 E).

Hypoxia is increased in Wnt1− compared with Dkk1-expressing tumors. To evaluate tumor hypoxia, mice bearing similar sized tumors were injected with hypoxyprobe-1, revealing in control−D and Wnt1−D tumors pronounced hypoxia with no significant difference. Dkk1−D tumors showed significantly reduced hypoxic areas (Fig. 2 F), matching the few necrotic areas evaluated by tumor histology (Fig. 2 E). Because cell culture experiments showed no differences in proliferation (Fig. 2 C), cell cycle, and cell death between the three GL261 lines (not depicted), we hypothesized that Wnt1 and Dkk1 affect tumor vascularization, thus leading to differences in tumor growth. Wnt1 and Dkk1 functionally targeted the angiogenic endothelium in vivo, analyzed by nuclear localization of β-catenin in tumor vessels. Compared with controls−D, the Wnt1−D tumor endothelium showed a significant increase in nuclear β-catenin, which is the hallmark of canonical Wnt signaling (Fig. 2 G). In contrast, nuclear β-catenin was decreased in Dkk1−D tumor vessels, suggesting that both Wnt1 and Dkk1 effectively stimulated the tumor endothelium.

Vessel density is decreased, whereas vessel stabilization is increased in Wnt1-expressing subcutaneous GL261 tumors. To exclude variations between the Wnt1− and Dkk1-expressing cell populations and to specifically show the effects of Wnt1 and

Figure 1. Nuclear β-catenin in vessels of human astrocytoma does not correlate with WHO grade. (A) TCGA database analyses for Wnt1, Wnt3a, Wnt5a, Wnt7a, Wnt7b, Dkk1, and Dkk2 mRNA expression as log2-fold expression. Differences in mRNA expression in GBM compared with normal central nervous system tissue (dashed red line) are shown. (B and C) Paraffin sections of three normal human brains (B) and five different human astrocytoma WHO grades I–IV (C) stained for β-catenin and analyzed for its endothelial, nuclear localization. Insets show individual nuclei in higher magnification. Bars: (B) 14 µm; (C) 20 µm.
Dkk1 release on tumor growth, we repeated the initial subcutaneous tumor experiment with Wnt1- or Dkk1-GL261 lines in the presence or absence of DOX (+D, −D). In the presence of Wnt1 expression (Wnt1−D), GL261 tumors grew significantly smaller and mouse survival was increased (Fig. 3A). On Dkk1-GL261 tumors, DOX withdrawal (Dkk1−D) had the opposite effect (Fig. 3B), confirming the finding of the initial comparison between control−D, Wnt1−D, and Dkk1−D

Figure 2. Wnt1 expression decreased subcutaneous GL261 tumor growth and increased animal survival. (A) Western blots showing Wnt1 and Dkk1 expression +/− DOX in GL261 cells. (B) sTOP-FLASH assay on human embryonic kidney (HEK293)/GL261 co-cultures without DOX. Monoculture of transfected HEK293 cells served as baseline. Wnt1 and Dkk1 cells were cultured without (−) or with (+) Wnt3aCM (one experiment in triplicate). (C) In vitro proliferation of the control-, Wnt1-, and Dkk1-GL261 line cultured +/− DOX. (D, left) Representative pictures of NUDE mice with subcutaneous tumors −DOX. (right) Tumor volumes (n = 7/group) of the transplanted glioma cell lines +/− DOX (*, P < 0.05; **, P < 0.01). (E) H&E-stained paraffin sections revealed reduced necrotic areas for Dkk1-expressing tumors (N). (F, left) Pimonidazole (brown) immunohistochemistry staining revealed tumor hypoxia, hematoxylin counterstaining (blue). (right) Hypoxia in Dkk1−D compared with the control−D (*, P < 0.05) and Wnt1−D tumors (**, P < 0.01; n = 4 tumors/group, slices from the center of similar sized tumors). (G, left) Representative vessels, stained for nuclear β-catenin (brown) and hematoxylin (blue) of control−D, Wnt1−D, and Dkk1−D tumors. Arrowheads point to β-catenin nuclei. (right) Quantification of β-catenin nuclei (n = 4 tumors/group, 20 vessels/tumor; ***, P < 0.001). Bars: (D) 1 cm; (E and F) 400 μm; (G) 35 μm. Error bars indicate SEM.
tumors (Fig. 2 D) and arguing for a specific, expression-dependent effect of Wnt1 and Dkk1.

We investigated tumor vessel density and morphology by staining for the endothelial marker CD31/PECAM-1 and α smooth muscle actin (α-SMA; Fig. 3, C and D). Interestingly, Wnt1−/− tumors showed decreased vessel density compared with Wnt1+/+ (Fig. 3 C), whereas in Dkk1−/− tumors vessel density was increased (Fig. 3 D). The vessel reduction in Wnt1−/− tumors was accompanied by increased attachment of α-SMA+ cells (Fig. 3 C). Notably, in Dkk1 tumors in general, α-SMA+ cells were frequently detached from the tumor endothelium and were regularly detected in the tumor stroma, showing no difference upon DOX treatment (Fig. 3 D). From the images in Fig. 3 (C and D) it can be deduced that the size of tumor vessels was increased in the Wnt1−/− group as compared with the Wnt1+/+ and Dkk1+/− groups (see also Fig. 8 B). To determine functionality of tumor blood vessels, mice were perfused with isolectin, revealing that the majority of vessels from control−/−, Wnt1−/−, and Dkk1−/− tumor groups exhibited blood flow (Fig. 3 E). The effects of Wnt1 on tumor vascularization and growth in the subcutaneous transplantation paradigm could be confirmed independently by a C6 rat glioma cell line stably expressing Wnt1 (not depicted).

Figure 3. Tumor-derived Wnt1 reduced and normalized subcutaneous tumor vascularization, whereas Dkk1 caused the opposite effect. (A) Tumor growth in Wnt1−/− compared with Wnt1+/− condition (left; n = 12/group; **, P < 0.01; ***, P < 0.001 and mouse survival (right; n = 12/group). (B) Tumor volume for Dkk1−/− compared with Dkk1+/− condition (left; n = 12/group; *, P < 0.05; **, P < 0.01; ***, P < 0.001) and mouse survival of Dkk1+/− tumors (right; n = 12/group). (C, top) IF staining on subcutaneous Wnt1 tumors +/− DOX (n = 5 tumors/group, 10 pictures/tumor; **, P < 0.01). (bottom left) Vessel density of subcutaneous Wnt1 tumors +/− DOX (n = 5 tumors/group, 10 pictures/tumor; **, P < 0.01). (bottom right) Association of α-SMA+ cells to ECs in Wnt1−/− tumors (n = 5 tumors/group; 10 vessels/tumor; ***, P < 0.001). (D) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (E) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (F) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (G) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (H) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (I) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (J) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (K) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (L) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (M) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (N) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (O) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (P) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (Q) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (R) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (S) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (T) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (U) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (V) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (W) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (X) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (Y) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (Z) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (A) Tumor growth in Wnt1−/− compared with Wnt1+/− condition (left; n = 12/group; **, P < 0.01; ***, P < 0.001 and mouse survival (right; n = 12/group). (B) Tumor volume for Dkk1−/− compared with Dkk1+/− condition (left; n = 12/group; *, P < 0.05; **, P < 0.01; ***, P < 0.001) and mouse survival of Dkk1+/− tumors (right; n = 12/group). (C, top) IF staining on subcutaneous Wnt1 tumors +/− DOX (n = 5 tumors/group, 10 pictures/tumor; **, P < 0.01). (bottom left) Vessel density of subcutaneous Wnt1 tumors +/− DOX (n = 5 tumors/group, 10 pictures/tumor; **, P < 0.01). (bottom right) Association of α-SMA+ cells to ECs in Wnt1−/− tumors (n = 5 tumors/group; 10 vessels/tumor; ***, P < 0.001). (D) Same staining and experimental settings as in C for subcutaneous Dkk1−/− tumors (n = 5 tumors/group, 10 vessels/tumor; ***, P < 0.001). (E) Perfusion with isolectin revealed that vessels from control−/−, Wnt1−/−, and Dkk1−/− tumors exhibited blood flow. Bars: (C and D) 400 µm; (E) 200 µm. Error bars indicate SEM.
plug assays. For this purpose, Matrigel plugs were supplemented with recombinant Wnt1 or Dkk1 protein and injected in C57BL/6 mice, which were sacrificed after 7 days. Vessel density was analyzed by measuring the CD31/PECAM-1-positive (CD31+ area) in relation to the plug area. Compared with controls, the CD31+ area was decreased in Wnt1 and increased in Dkk1 Matrigel plugs, confirming the finding in GL261 tumors that Wnt1 decreased and Dkk1 fostered angiogenesis (Fig. 4A and B).

**Wnt1 expression leads to quiescent vessels and reduces tumor volume and IgG leakage in intracranial transplantations**

To evaluate orthotopic growth, vascularization, and BBB characteristics of glioma vessels, we transplanted the engineered GL261 cell lines into the striatum of NUDE mice (Fig. 5A). Consistent with the subcutaneous tumor experiments, Wnt1−/− tumors grew the slowest and the volume was notably decreased compared with controls−/− and even more pronounced compared with the Dkk1−/− glioma (Fig. 5B).

Vascular density was also significantly reduced in intracranial Wnt1−/− tumors (Fig. 5, A and B). We further characterized GL261 glioma vessels for PC coverage by staining for desmin. Consistent with the subcutaneous paradigm, the vessels of Wnt1−/− showed significantly augmented attachment of desmin+ cells, supporting a normalized vascular morphology (Fig. 5C).

Wnt/β-catenin signaling as well as PCs have been shown to be important for the establishment and maintenance of the BBB (Armulik et al., 2010; Daneman et al., 2010). Therefore, we investigated whether endothelial barrier properties are affected by modulating the Wnt pathway in experimental glioma by staining thick sections of tumor-bearing brains for endogenous mouse IgG, which is frequently used as a marker for BBB disruption. Confocal imaging and large image reconstruction revealed strong IgG leakage into the tumor and the surrounding brain parenchyma in control−/− and Dkk1−/− glioma, which was substantially reduced in Wnt1−/− tumor-bearing brains (Fig. 5D). Notably, in control−/− and Dkk1−/− brains, IgG was detectable around the lateral ventricles and on the contralateral hemisphere. Instead, for Wnt1−/− tumors this was not observed. Higher magnifications confirmed weaker IgG staining also in the immediate circumference of podocalyxin (Podxl)-positive (Podxl+) vessels in Wnt1−/− tumors, suggesting that these vessels retained or regained barrier properties (Fig. 5D).

**Endothelial Wnt/β-catenin signaling partially rescues the loss of BBB junction markers in GL261 glioma vessels**

The TJ protein Cldn3 (claudin-3) has previously been shown to become down-regulated in several brain pathologies such as tumors and inflammation, and it has been identified as a Wnt/β-catenin target in ECs (Wolburg et al., 2003; Liebner et al., 2008). Stainings for Cldn3 together with the endothelial marker CD31/PECAM-1 showed that CD31 was localized at cell–cell junctions of tumor vessels in the three tumor types (Fig. 6A), whereas Cldn3 showed a punctuate staining only occasionally overlapping with the CD31+ areas in control−/− and Dkk1−/− tumors. Instead, in Wnt1−/− tumors more continuous, junctional staining of Cldn3 that colocalized with CD31 was observed, arguing for more elaborate TJs as observed in physiological brain vessels. In tumor vessels of Wnt1−/− glioma, other junctional markers such as Clhn5 and ZO-1 (zonula occludens 1) also showed more restricted junctional staining (Fig. 6B).

**Endothelial-specific activation of β-catenin signaling results in vessel quiescence of intracranial GL261 tumors**

We further asked whether dominant endothelial-specific activation of β-catenin signaling can mimic the effects observed in Wnt1-expressing tumors. Therefore, we generated mice expressing a dominant-active form of β-catenin specifically in the endothelium (GOF) and intracranially transplanted the parental, not engineered GL261 glioma cells. In brief, we made use of the Pdgfb-iCreERT2 mice crossed to the β-catenin Exon3flox/flox line as previously described (Liebner et al., 2008). The mice were sacrificed when the first animals showed symptoms (see Materials and methods). Recombination was induced by subcutaneously transplanted tamoxifen pellets (free base) on the same day of tumor transplantation. Recombination of intracranial tumor vessels was evaluated by crossing Pdgfb-iCreERT2 and Rosa26 STOPfloxedLacZ mice. Staining for Podxl and β-galactosidase revealed endothelial recombination of ~30% (Fig. 7A).

In the GOF (Pdgfb-iCreERT2 × β-catenin Exon3flox/flox + tamoxifen) tumors, we observed reduced vessel density compared...
increased desmin+ cells attached to the endothelium (Fig. 7 D and Videos 3 and 4). We next asked for the mechanism by which endothelial Wnt/β-catenin signaling hampers tumor angiogenesis, leading to the quiescent and normalized vessel phenotype described above.

### Wnt/β-catenin signaling results in Dll4 expression and a stalk cell gene signature

The Notch pathway via Notch1/4 and Dll4 has been shown to be a key regulator in vessel sprouting by inhibiting the tip cell and promoting the stalk cell phenotype (Phng and Gerhardt, 2009). Recently, Corada et al. (2010) could show that β-catenin signaling regulates Dll4 during early embryonic angiogenesis but becomes silenced during late embryonic and postnatal development.

To understand whether endothelial Wnt/β-catenin signaling regulates Dll4 also in the tumor endothelium, we performed Dll4 in situ hybridization (ISH). Hematoxylin counterstaining revealed tumor vessel–specific Dll4 expression in all three tumor conditions (Fig. 8 A). Analysis of vessel diameter revealed significantly decreased small and increased big vessels for Wnt1−/− tumors, as opposed to the vascular phenotype in Dkk1−/− glioma (Fig. 8 B). Interestingly, in all tumor conditions small vessels were positive for Dll4, which is in line with an endothelial tip cell characteristic described in a previous publication (Hellström et al., 2007). In this regard, it was apparent that in Wnt1−/− tumors...
bigger vessels >300 \( \mu m^2 \) were also strongly positive for Dll4, which was not the case in controls and specifically not in Dkk1-D tumors (Fig. 8 B).

To test whether GL261-derived Wnt1 directly induces Dll4 expression in ECs, we established a co-culture system of GL261 cells with human umbilical vein ECs (HUVECs; Fig. 8 C). Using human-specific primers, quantitative RT-PCR (qRT-PCR) analysis revealed significant up-regulation of AXIN2, an endogenous Wnt target gene, and DLL4 in Wnt1-D/HUVEC compared with control-D/HUVEC co-cultures, which was not the case in Dkk1-D/HUVECs (Fig. 8 C). In addition to DLL4, we also observed an up-regulation of JAG1 (jagged-1) and of the Notch target NRARP (Notch-related ankyrin repeat protein), whereas other targets such as HES1 (hairy and enhancer of split homologue 1) and HEY (hairy/ enhancer of split related with YRPW motif) were not significantly regulated. In Dkk1-D co-cultures except for JAG1, none of the tested genes were up-regulated, but NRARP was significantly down-regulated.

One of the key pathways to modulate vascular activation is the angio-poietin-Tie2 system. In particular, ANGPT2 (angiopoietin-2), which is highly expressed in angiogenic ECs, leads to SMC/PC drop out and vascular instability (Thomas and Augustin, 2009). In HUVEC co-cultures, neither Wnt1 nor Dkk1 produced by GL261 cells had a significant effect on endothelial ANGPT2 expression (Fig. 8 C). In turn, GL261-derived, murine Angpt1 was also not regulated in Wnt1-D and Dkk1-D co-culture conditions (not depicted). Analysis of total RNA from subcutaneous GL261 tumors revealed a diminished expression of Angpt2 specifically in Wnt1-D glioma when normalized to the endothelial-specific gene VE-cadherin, supporting the more quiescent phenotype in these tumors (not depicted).

To understand the expression of Notch pathway genes downstream of Wnt/\( \beta \)-catenin in more detail, we stimulated mouse brain endotheliomas (MBEs) with Wnt3a conditioned medium (CM [Wnt3aCM]; Liebner et al., 2008). Wnt3aCM robustly induced Axin2 and DLL4 expression as well as the Notch targets Hes1 and Hey1 after 18 h of stimulation (Fig. 8 D). Jag1, Notch1, and Nar1 were not changed at this time point, whereas Flt-1 (VEGFR1), a putative VEGF-A decoy receptor, showed significant up-regulation. Other Notch targets showed no significant regulation, which was also true for arterial genes such as ephrinB2 (not depicted). However, after 48 h of stimulation (Fig. 8 D), Jag1 and Notch1 were significantly up-regulated, whereas Nr1 and Flt4 (VEGFR3) were down-regulated.

Transcriptionally active \( \beta \)-catenin regulates expression of PDGF-B in ECs in vitro

Finally, we intended to clarify the mechanism leading to increased tumor vessel attachment of vascular SMCs/PCs upon
regulation of PDGFβ as the best known SMC/PC attractant produced by the endothelium and observed a significant up-regulation of PDGFβ messenger RNA (mRNA) in the presence of Wnt1-expressing GL261 cells (Fig. 9A). Comparable results were obtained by Wnt3a stimulation of MBEs after 18 and 48 h (Fig. 9B).

To understand whether the Pdgfb gene is directly regulated by transcriptionally active β-catenin, we generated EC lines derived from β-catenin-deficient endothelioma cells (Cattelino et al., 2003), expressing a chimeric construct of Lef1 and the transactivation domain of β-catenin (LefΔN-βCTA), conferring dominant-active transcription without affecting the membrane function of β-catenin or the corresponding vector control (Vleminckx et al., 1999). LefΔN-βCTA strongly induced Axin2 as an endogenous reporter gene and Pdgfb as indicated by qRT-PCR analysis. Western blot analysis revealed an up-regulation of PDGF-B and the homodimer PDGF-BB by ~60%, suggesting that protein translation is also taking place (Fig. 9C).

To further characterize whether Pdgfb expression downstream of β-catenin transcription is dependent on subsequent endothelial Wnt/β-catenin activation. As ANGPT2 was not significantly regulated upon canonical Wnt pathway activation in the HUVEC co-culture setting, we investigated the
coating, and moreover, blockage of the Notch pathway by Dll4-Fc had no noticeable effect on \( \text{Pdgfb} \), suggesting that its regulation by Lef\( \beta \)-N-CTA is Notch signaling independent.

GL261-derived Wnt1 up-regulates expression of PDGF-B in ECs in subcutaneous xenografts

To confirm the \( \text{Pdgfb} \) mRNA regulation by the Wnt/\( \beta \)-catenin pathway in tumor ECs in vivo, we performed ISH from Wnt1-GL261 tumors \( ^{-/-} \), showing an increased coating, and moreover, blockage of the Notch pathway by Dll4-Fc had no noticeable effect on \( \text{Pdgfb} \), suggesting that its regulation by Lef\( \beta \)-N-CTA is Notch signaling independent.
endothelial-specific signal for \( Pdgfb \) upon \( Wnt1 \) expression (Fig. 10 A). Furthermore, we mechanically isolated tumor vessels from \( Wnt1 \) tumors\(^{+/−}\) (Fig. 10 B). SDS-PAGE and Western blot revealed increased PDGF-B protein expression in vessels of \( Wnt1^{−/−} \) tumors (Fig. 10 B).

**DISCUSSION**

The results of our present manuscript provide the first evidence for an antiangiogenic and vessel-normalizing role of endothelial \( Wnt/β \)-catenin signaling in glioma vascularization. As tumor vascularization is crucial for the progression of various types of malignancies (Saharinen et al., 2011), our findings will consolidate the understanding of pathological angiogenesis in general.

Comparing GL261 mouse glioma cells producing either \( Wnt1 \) or the soluble Wnt inhibitor \( Dkk1 \) in a DOX-inducible manner, we show that \( Wnt1 \) decreased and \( Dkk1 \) promoted tumor vascularization, leading to decreased and increased tumor growth, respectively. These findings were independently validated in a subcutaneous C6 rat glioma model constitutively expressing \( Wnt1 \) (not depicted). We also provide direct evidence that the tumor vasculature is targeted by glioma \( Wnt1 \), as indicated by increased nuclear \( β \)-catenin staining as the hallmark of \( β \)-catenin transcription. This finding is of particular importance as systemic treatments that activate the canonical \( Wnt \) pathway do not specifically target the endothelium. Moreover, we show that \( Wnt1 \)-driven \( β \)-catenin signaling did not lead to increased GL261 and C6 tumor growth. Along this line, canonical \( Wnts \), as opposed to the noncanonical \( Wnt5a \), are not overexpressed in human GBM, suggesting that glioma cell–specific \( β \)-catenin signaling does not contribute to glioma initiation and progression. Interestingly, the canonical \( Wnt \) pathway has been described as anti-tumorigenic for glioma cells, making it, in combination with the vascular effects we report here, an interesting target to be activated for glioma therapy (Kotliarova et al., 2008).

The tumor phenotypes in \( Wnt1^{-} \) and \( Dkk1^{-} \)-expressing tumors were comparable in the subcutaneous and intracranial transplantation models with some differences regarding vessel size. The latter might be explained...
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Figure 10. PDGF-B is up-regulated in the tumor endothelium upon Wnt1 expression. (A) Representative ISH for Pdgfb of subcutaneous Wnt1+/− tumors. Asterisks indicate vessels, and arrowheads point to Pdgfb+ ECs. Insets show representative vessels in higher magnification. Bars: (full images) 50 μm; (insets) 18 μm. (B, left) Representative picture of isolated subcutaneous tumor vessels and dot blot for von Willebrand factor (vWF). (right) Representative Western blot for PDGF-B with pooled subcutaneous tumors vessels (n = 2 tumors/group). Densitometric analysis for PDGF-B of five independent Western blot experiments. Error bars indicate SEM.

by the diverging tumor microenvironment that has been shown by multiple studies comparing subcutaneous versus intracranial glioma growth (Arosarena et al., 1994; Blouw et al., 2003). As hypoxia and subsequent VEGF expression are major driving forces of tumor angiogenesis, we investigated the three different tumor conditions for hypoxic areas. Control−/− and Wnt1−/− subcutaneous tumors showed a similar hypoxic status, whereas Dkk1−/− tumors were almost devoid of hypoxia (Fig. 2 F). The finding that Dkk1−/− tumor vessels were functional regarding perfusion suggests that reduced hypoxia was a consequence of increased vascularity and oxygen supply (Fig. 3 E). As VEGF is a known target of Wnt signaling (Zhang et al., 2001), we tested the release of VEGF by the three GL261 glioma lines in vitro and observed higher levels of VEGF in Wnt1−/− compared with control−/− and Dkk1−/− glioma cells (not depicted).

Surprisingly, Wnt1−/− tumors showed reduced angiogenesis and normalized vessels given that these tumors were as hypoxic as the controls−/−, likely coinciding with high VEGF-A levels. These results suggest that reduced VEGF levels are not the cause of quiescent vessels in Wnt1−/− glioma. Furthermore, high VEGF expression in tumors is known to lead to SMC and PC drop off and to increase vessel permeability (Olsson et al., 2006). However, in Wnt1−/−-GL261 tumors, we observed decreased vascularization and an increased investment of α-SMA+/desmin+ cells, speaking in favor of a diminished responsiveness of the endothelium to VEGF stimulation.

To mechanistically understand how endothelial Wnt/β-catenin signaling influences VEGF responsiveness, we focused on the cross talk of Wnt/β-catenin with Dll4/Notch as a vessel-stabilizing pathway. In this regard, Corada et al. (2010) recently provided evidence for the direct, transcriptional control of Dll4 by β-catenin signaling during early embryonic angiogenesis, leading to a lack of vascular remodeling and of venous specification. Interestingly, the authors point out that no obvious effects of sustained Wnt/β-catenin signaling have been observed after midgestation, despite the fact that angiogenic processes were not complete.

In the present manuscript, we show for the first time that in pathological glioma angiogenesis, ECs become again responsive to the Wnt/β-catenin–Dll4 signaling axis. Specifically, endothelial-specific Dll4 was up-regulated by glioma-derived Wnt1, leading to an inhibition of the angiogenic phenotype and thus to diminished tumor angiogenesis.

In control−/− and in particular in Dkk1−/− tumors, predominantly small, angiogenic vessels were Dll4+, supporting its role as a tip cell marker (Mailhos et al., 2001; Hellström et al., 2007). Interestingly, in Wnt1−/− tumor vessels, almost all ECs showed high Dll4 levels. It is known that Dll4 activates Notch signaling in adjacent ECs, influencing the levels of VEGFR expression (Hellström et al., 2007; Siekmann and Lawson, 2007), namely by lowering levels of VEGFR2 (Williams et al., 2006) and Nrp-1 (Gerhardt et al., 2004), thereby determining stalk cell identity. We show on a molecular level that the tip cell receptors VEGFR2/flk1, VEGFR3/flt4, and Nrp-1 were down-regulated and the stalk cell receptor VEGFR1/flt1 was up-regulated upon Wnt/β-catenin signaling, suggesting the induction of a stalk cell–like gene signature. Along this...
line, Jakobsson et al. (2010) showed that the tip cell identity is controlled by the relative availability of VEGFR2 and VEGFR1, which are downstream of Notch1.

The Notch ligand Jag1 is highly expressed in stalk cells, and its overexpression leads to Dll4/Notch1 inhibition and angiogenic sprouting in a fringe-dependent manner (Benedito et al., 2009). Interestingly, Jag1 was also significantly up-regulated in ECs upon canonical Wnt signaling (Fig. 8, C and D). Although Jag1 is a described Wnt target in the hair follicle (Estrah et al., 2006), our data support the interpretation that Jag1 is not directly regulated by β-catenin, as Jag1 together with Notch1 were only induced after 48 h of Wnt3a stimulation, whereas direct Wnt targets such as Axin2 and Dll4 were already significantly regulated after 18 h (Fig. 8 D). It has also been reported that Dll4 coating leads to up-regulation of JAG1 in HUVECs, strengthening the interpretation that JAG1 is controlled by Notch signaling (Harrington et al., 2008).

Interestingly, we observed a slight but significant up-regulation of JAG1 in the HUVEC co-culture model with Dkk1−/− GL261 cells, whereas other Notch pathway genes remained unchanged. This finding underlines that the relative up-regulation of Jag1 compared with Dll4 might foster the angiogenic phenotype observed in Dkk1−/− tumors (Benedito et al., 2009).

Segarra et al. (2008) have shown that artificial overexpression of Dll4 by BL41 lymphomas leads to reduced angiogenesis and tumor growth. Li et al. (2007) followed a similar approach by overexpressing Dll4 in five different tumor cell lines. They also observed a reduction in vessel density and increased vessel maturation; however, subcutaneous U87 human GBM grew significantly bigger compared with the controls. This suggests that there is a high degree of cell specificity for Dll4 concerning tumor cell response, whereas the endothelial response is apparently independent of the tumor type. Therefore, Dll4 might not be a good primary target in GBM but possibly in other malignancies.

Matrigel plug assays showed that the antiangiogenic effect of Wnt1 and the proangiogenic effect of Dkk1 are a consequence of endothelial-specific Wnt pathway activation and inhibition, respectively. Most relevant, the normalized vascular phenotype could be mimicked by endothelial-specific GOF for β-catenin in intracranial transplanted, parental GL261 glioma cells. Specifically, endothelial activation of β-catenin signaling (a) reduced tumor vessel density, (b) led to smooth deposition of CollIV to the vessel wall, and (c) resulted in thinner, more regular-shaped vessels and (d) increased vessel investment by PCs.

The latter have been shown to be important for vessel stability and barrier function in the brain (Armulik et al., 2010; Daneman et al., 2010). Consistently, we could show that permeability for endogenous mouse IgG was considerably lower in Wnt1−/− compared with Dkk1−/− or control−/− glioma. In accordance with our previously described role of Wnt/β-catenin for BBB development (Liebner et al., 2008), we observed a partial rescue of junction protein localization in Wnt1−expressing tumors. This was even more remarkable, as Cldn3 has been shown to be one of the earliest junction proteins at the BBB that is down-regulated under pathological conditions (Liebner et al., 2000; Wolburg et al., 2003).

However, the central molecular finding of the present manuscript is that we provide the first evidence that the SMC/PC-attracting factor PDGF-B is regulated downstream of Wnt/β-catenin signaling in ECs. The regulation of PDGFB became apparent in HUVEC co-cultures with Wnt1-expressing GL261 cells and in Wnt3a stimulation of MBEs, speaking in favor of a direct regulation. Using the LefΔN-βCTA dominant-active signaling construct in β-catenin-deficient ECs, we establish Pdgfb to be directly regulated by the canonical Wnt pathway. Neither activation nor inhibition of Notch signaling in LefΔN-βCTA–transduced ECs affected Pdgfb up-regulation in vitro. ISH and Western blot analysis of tumor vessels corroborated the regulation of Pdgfb in Wnt1-expressing tumors contributing to SMC/PC recruitment.

The relevance of tumor vessel investment with SMCs/PCs for vessel normalization and tumor growth is strongly supported by the finding that PDGF-B–overexpressing colorectal tumors exhibit increased mural cell investment, normalized vessels, and reduced tumor growth (McCarty et al., 2007). Nevertheless, our results provide the first evidence for the direct regulation of PDGF-B by Wnt/β-catenin in ECs, as endothelial-derived PDGF-B is crucial for the proper recruitment of PCs (Abramsson et al., 2003).

However, PDGF-B has been proposed to be a tip cell marker but at the same time to be important for mural cell recruitment to stalk and phalanx cells (Gerhardt et al., 2003). Given that Wnt/β-catenin signaling has been suggested to be predominantly active in stalk cells (Phng et al., 2009), the regulation of PDGF-B downstream of β-catenin might contribute to the stalk cell behavior and vascular maturation. However, the exact mechanism of the cellular PDGF-B regulation in the context of tip and stalk cell identity needs further investigation.

Regarding mural cell investment and vessel stabilization, we did not observe regulation of human Angpt2 in HUVEC/ GL261 co-cultures (Fig. 8 C). Instead, analysis of total RNA from subcutaneous glioma suggested a relative down-regulation of Angpt2 in Wnt1−/− tumors, supporting the observation of quiescent tumor vessels (not depicted). This might shift the balance toward Angpt1, in turn contributing to the stabilized vascular phenotype, as mural cell–derived Angpt1 seems to potentiate β-catenin signaling in ECs (Zhang et al., 2011).

Regarding the protumorigenic effect of Dkk1 over-expression from GL261 cells, it should be noted that Dkk1 has effects beyond mere inhibition of Wnt/β-catenin. Blocking Dll4 in a subcutaneous C6 rat glioma model has been shown to result in smaller sized tumors with increased vessel density caused by a nonfunctional tumor vascularization (Nogueira-Troise et al., 2006). Interestingly, in the present work, tumor-derived Dkk1 resulted in hypervascularization by functional vessels, as revealed by isoelectin perfusion, leading to reduced hypoxia (Figs. 2 F and 3 E) and thus increased...
tumor growth. There is emerging evidence that Dkk1 drives mobilization and the angiogenic potential of endothelial precursor cells (Aicher et al., 2008; Smadja et al., 2010). Here we corroborate these data in a mouse glioma model, underlining a proangiogenic role of Dkk1. It should be noted that Min et al. (2011) proposed opposing function of Dkk1 and Dkk2, suggesting that Dkk1 confers anti- and Dkk2 proangiogenic properties. The apparent discrepancies between the data described by Min et al. (2011) and our data, which are in line with the data by Aicher et al. (2008) and Smadja et al. (2010), might be a result of the different experimental settings in vivo and in vitro. It should be noted that expression analysis of Dkk1 and Dkk2 in highly angiogenic human GBM supports a proangiogenic function of Dkk1, as it is up-regulated and Dkk2 is down-regulated compared with normal brain tissue (Fig. 1A; Zhou et al., 2010). To conclusively describe the function of Dkk1 in angiogenesis, additional work needs to be done.

In summary, we demonstrate that continuously elevated Wnt/β-catenin signaling leads to the initial induction of Dil4 and Pdgfb, followed by the acquisition of a stalk cell–specific gene signature downstream of elevated Notch signaling. Our findings might have multiple consequences for GBM therapy; (a) physiological levels of Wnt/β-catenin signaling are associated with angiogenesis and stalk cell proliferation, and (b) reinforced and sustained signaling leads to reduced angiogenesis independently of VEGF levels, with concomitant vessel normalization via Dil4 and PDGF-B expression, as well as junctional stabilization. As a consequence of the latter, endothelial Wnt/β-catenin partially rescues BBB disruption and prevents vasogenic edema. Collectively, these findings make the Wnt/β-catenin pathway an interesting target that could be exploited for antiangiogenic and/or antiedema therapy particularly in GBM but potentially also in other angiogenic tumors in the periphery such as melanoma (Lucero et al., 2010).

MATERIALS AND METHODS

Human GBM specimens. Biopsies of human glioma were provided by the University Cancer Center, Goethe University Frankfurt via M. Mittelbronn and P.N. Harter. We investigated five cases for each WHO grade I–IV of human glioma specimens embedded in paraffin. Histological examination was performed by at least two experienced neuropathologists (M. Mittelbronn and P.N. Harter). Gene expression signatures were analyzed in 424 primary GBMs and 11 normal brain samples by assessing the TCGA data portal that used the 244K G4502A microarray (Agilent Technologies) to determine mRNA profiles for Wnt1, Wnt5a, Wnt5b, Wnt7a, Wnt7b, Dkk1, and Dkk2 to correlate the expression to normal brain (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp, accessed 2012 March 30; Cancer Genome Atlas Research Network, 2008). The raw data for the selected genes were downloaded and analyzed in Prism (GraphPad Software) to generate scatter plots. Utilization of all human specimens was in accordance with the ethics commission of the Goethe University Clinic Frankfurt, Germany.

Cells. GL261 cells cultivated in D-MEM Media-GlutaMAX-I (Invitrogen) containing 10% FCS were stably transfected with pTet-off plasmid using TransPass D1 Transfection Reagent (New England Biolabs, Inc.) and selected with 400 µg/ml G418. Tet-Off-responsive single cell clones were selected for stable transfection with the empty vector pTRE2hyg (control) or the vector containing either murine Wnt1 or human Dkk1 cDNA. Cells were selected with 200 µg/ml hygromycin B and cultivated in the presence of 1 µg/ml DOX. HUVECs were grown on Matrigel basement membrane matrix from BD, Heparin-Natrium 25000 from Ratoporph, and bFGF from R&D Systems. Reombinant proteins were as follows: human Wnt1 (PeproTech), mouse Dkk1 as well as human Dil4 (R&D Systems), and Dil4 (mouse)–Fc (human; rec; AdipoGen). BCA Protein Assay kit was purchased from Thermo Fisher Scientific. Plasmids used were as follows: pTet-Off, pTRE2hyg, and pTRE2hyg-Luc (Takara Bio Inc.); pRL-TK (Promega); and pOTB7-human Dkk1 (IRAPU59740447) and pYX-Asc–mouse Dil4 (IRAP596810130D; imaGenes).pcDNA3-mouse Wnt1 was a gift from G. Cosui (DBT San Raffaele Scientific Institute, Milan, Italy). Super(8×)TOP-FLASH and super(8×)FOP-FLASH were provided by R. Moon (University of Washington School of Medicine, Seattle, WA). ChromoMap red was purchased from VENTANA Medical Systems Inc. Monoclonal mouse antibodies used were as follows: anti-β-catenin clone14/β-catenin (BD), anti-α-SMA clone 1A4 Cy3-conjugated and anti–α-tubulin clone DM1A (Sigma-Aldrich), antipimonidazole FITC MAb4 6.3.11.3 (NPI Inc.), anti-FITC MAb40SPs Mx Fluor HRP (EMD Millipore), and rat anti-mouse CD31 (BD and DiaNocea). Polyclonal antibodies used were as follows: rabbit anti-β-galactosidase (MP Biomedicals), rabbit anti-Cldn3 (Invitrogen), rabbit anti-ColIV (AbD Serotec), rabbit anti-PDGF-B (AB Biotec), and goat anti-human Dkk1, goat anti–mouse Podil, and goat anti–mouse Wnt1 (R&D Systems). Secondary antibodies used were as follows: appropriated antibodies, streptavidin conjugates, and Alexa Fluor 488 and 568 conjugates (Invitrogen). For mouse IgG staining, a goat anti–mouse Alexa Fluor 488 antibody was used (Invitrogen). Mouse food containing 100 mg/kg DOX and control food was purchased from suft Spezialdiäten GmbH.

Luciferase reporter assay. To reveal canonical Wnt signaling, HEK293 cells were transfected with super(8×)TOP-FLASH and super(8×)FOP-FLASH. Firefly luciferase activity was normalized to Renilla luciferase by cotransfection with pRL-TK plasmid. All measurements were performed in a Lumat LB 9507 luminometer (Berthold Technologies).

qRT-PCR. Primers used for qRT-PCR are listed in Tables S1 and S2. RNA from cells was isolated with the RNeasy Mini kit from QIAGEN. qRT-PCR was performed as previously described (Schneider et al., 2010). For qRT-PCR analysis on HUVECs, G6pd1 and β-actin were used as human reference genes for normalization. qRT-PCR results are shown from at least three independent experiments (specified in figure legends).

Western blot analysis. For GL261 glioma cell analysis, 4 × 10^5 cells were cultured for 2 d in media supplemented either with or without DOX. Cells were harvested for Western blot analysis. Western blot was performed as previously described (Devraj et al., 2009; Schneider et al., 2010). In brief, ECs were harvested in 10 mM Heps, 1 mM EDTA, and 250 mM sucrose plus protease inhibitor cocktail with final pH 7.4 (HES + PI), followed by mild sonication. Subsequently, BCA assay was performed and samples were stored.
at ~20°C. For Western blot, a buffer containing 2.3 M urea, 1.5% SDS, 15 mM Tris, 100 mM DTT, and 0.01% Brij (final concentrations) was added to the samples, and proteins were solubilized for 2 h at room temperature followed by SDS-PAGE.

The gels were then transferred by standard submerged method, followed by Western blotting for the indicated antibodies. The blots were visualized with the aid of an enhanced chemiluminescence kit, digitized into film images using an AlphaEase FC Imaging System (Alpha Innotech), and the densitometric images were then analyzed using Multi Gauge software 3.0 (FujiFilm). Quantitation was performed from 8-bit linear TIF images by selecting equal area from each sample lane that encompassed the band of interest and gray values obtained after subtracting the background from an empty lane. Densitometric measurement was performed on three separated blots for PDGF-B, normalized to α-tubulin, and shown as relative amounts in percentages.

Tumor experiments. All animal experiments were performed in accordance with the German Legislation on the Protection of Animals and the Guide for the Care and Use of Laboratory Animals with permission of the Regierungspä-
dsium Darmstadt (approval no. PF4/10). Mice were anesthetized by i.p. injection of Ketamine/Xylazine, and 10^6 cells were implanted subcutaneously into the flanks of 6-8-week-old female NUDEx mice. Tumor volume was calculated by the formula \( V = \frac{4}{3} \pi \times (\text{diam}_{\text{max}} \times \text{diam}_{\text{min}})^2 \) (Yoshida-Hoshino et al., 2007). Mice were sacrificed when a tumor diameter of 1.5 cm was reached. For DOX-dependent tumor experiments, mice were fed with DOX-containing food 2 wk before tumor cell transplantations. The DOX-containing diet was continued during tumor growth. Vessel isolation from subcutaneous tumors was performed as previously described for brain vessels, but omitting the dextran separation step (Fisher et al., 2007). For intracranial tumor transplantations, mice were inserted into a stereotactic device, and 10^5 cells (living cell number) in 2 µl PBS were injected into the striatum using the coordinates relative to bregma: 0.5 (anterior–posterior), 2 (medial–lateral), and 3.5 (dorso–ventral). Mice were sacrificed at once when showing symptoms. For β-catenin activation in vivo, Pdgfb-CreERT2 (Claxton et al., 2008) lines were crossed to mice harboring floxed alleles of the β-catenin exon 3 to obtain homozygous β-catenin\(^{\text{Flox/Fl}}\) mice (Harada et al., 1999), of which 50% also carried one Pdgfb-CreERT2 allele. After intracranial transplantation of unmodified (parental) GL261 cells, a tamoxifen pellet (1 mg, BioSolveIT) was administered retrobulbarly after mouse anesthesia 4 min before induction of hypoxia, 60 µg Hypoxyprobe per gram was injected i.p. 30 min before induction of hypoxia. The DOX-containing diet was continued during tumor cell transplantations. The DOX-containing diet was continued until the tumor volume = slice size (40 µm) × step size between the slices (12) × sum of tumor areas from one tube.

Matri
gen plug assay. 1 ml Matri
gen basal membrane matrix was mixed with 30 µl U. Harada et al., 50% also carried one Pdgfb-iCreERT2 allele. After intracranial transplantation of unmodified (parental) GL261 cells, a tamoxifen pellet (1 mg, BioSolveIT) was administered retrobulbarly after mouse anesthesia 4 min before induction of hypoxia, 60 µg Hypoxyprobe per gram was injected i.p. 30 min before induction of hypoxia. The DOX-containing diet was continued during tumor cell transplantations. The DOX-containing diet was continued until the tumor volume = slice size (40 µm) × step size between the slices (12) × sum of tumor areas from one tube.

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