Autocrine VEGFR1 and VEGFR2 signaling promotes survival in human glioblastoma models in vitro and in vivo

Emese Szabo, Hannah Schneider, Katharina Seystahl, Elisabeth Jane Rushing, Frank Herting, K. Michael Weidner, and Michael Weller

Background. Although the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) system has become a prime target for antiangiogenic treatment, its biological role in glioblastoma beyond angiogenesis has remained controversial.

Methods. Using neutralizing antibodies to VEGF or placental growth factor (PIGF) or the tyrosine kinase inhibitor, cediranib, or lentiviral gene silencing, we delineated autocrine signaling in glioma cell lines. The in vivo effects of VEGFR1 and VEGFR2 depletion were evaluated in orthotopic glioma xenograft models.

Results. VEGFR1 and VEGFR2 modulated glioma cell clonogenicity, viability, and invasiveness in vitro in an autocrine, cell–line-specific manner. VEGFR1 silencing promoted mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling, whereas VEGFR2 silencing resulted in cell-type-dependent activation of the protein kinase B (PKB)/AKT and MAPK/ERK pathways. These responses may represent specific escape mechanisms from VEGFR inhibition. The survival of orthotopic glioma-bearing mice was prolonged upon VEGFR1 silencing in the LNT-229, LN-308, and U87MG models and upon VEGFR2 silencing in LN-308 and U87MG. Disruption of VEGFR1 and VEGFR2 signaling was associated with decreased tumor size, increased tumor necrosis, or loss of matrix metalloproteinase 9 (MMP9) immunoreactivity. Neutralizing VEGF and PIGF by specific antibodies was superior to either antibody treatment alone in the VEGFR1-dependent LNT-229 model.

Conclusions. Differential dependence on autocrine signaling through VEGFR1 and VEGFR2 suggests a need for biomarker–stratified VEGF(R)-based therapeutic approaches to glioblastoma.

Keywords: angiogenesis, glioblastoma, PIGF, signaling, VEGF.
establishment of specific biological responses remain incom-
pletely understood. In addition, VEGF heterodimerization and
interactions of VEGFR with coreceptors such as neuropilins
(NRP), heparan sulfate proteoglycans or αβ3 integrin further
expand the complexity of signaling pathways activated by
VEGF and PlGF homo- or heterodimers.11–14 Finally, VEGF/C/D
binding to VEGFR3 (FLT4) TKR is required for lymphangiogenesis
and may play a role in developmental and tumor angiogenesis
by modulating VEGFR2-mediated signals.15

Although VEGF receptors, NRPs, integrins, and their ligands
are expressed in several tumor cell types,6,8,16–18 it is unclear
how distinct biological responses emanate from these recep-
tors, specifically in glioblastoma. Autocrine VEGF effects medi-
ated by VEGFR2 signaling have been proposed to promote
glioblastoma cell invasion, viability, and migration.6,19
In contrast, VEGF binding to VEGFR2 has also been reported
to inhibit invasiveness by suppressing hepatocyte growth
factor-dependent c-MET activity through recruitment of the
phosphatase protein tyrosine phosphatase 1B (PTP1B) to the
VEGFR2/MET heterocomplex.20

These overall conflicting data on autocrine VEGF signaling
led us to propose that responses to VEGF pathway stimulation
or inhibition in glioma are heterogeneous and may, among
others, depend on the differential expression of VEGF family
ligands and receptors. In fact, we report here that VEGFR1 or
VEGFR2 signaling may exhibit distinct survival properties in
human glioma models in vivo and that a thorough characteri-
zation of VEGFR signaling in tumor cells may facilitate patient
enrichment for more successful clinical trials exploring
VEGFR inhibition in the future.

Materials and Methods

In Vitro Studies

Detailed information on reagents, cell lines, cell culture, viabil-
ity, clonogenicity, and spherogenicity assays is summarized in
Supplementary material, Note 1. Details on real-time quantita-
tive reverse transcription-PCR (qRT-PCR) and primers are provid-
ed with Supplementary material, Table S1, and details on immuno-
blotting, flow cytometry, and ELISA are provided in
Supplementary material, Note 2.

The nonsilencing control (#RHS4348) and the silencing
microRNA-adapted shRNA (shRNAmir) pGIPZ lentiviral vectors
(#RHS4531-V3LHS_403557; #RHS4531-V3LHS_302174) containing
either a single VEGFA (TCTGTATCGATCGTTCTGT) or VEGFR1-
targeting hairpin sequence (TGAACCTGAACTAGATCCT) provided
in bacterial stocks of E. coli were purchased from Thermo Scien-
tific Open Biosystems. Lentiviral infectious particles were pro-
duced in HEK 293T cells using pGIPZ shRNAmir lentiviral vector,
pCMV-dr8.91 second-generation packaging, and pMD2.G enve-
lope plasmids. To generate stable VEGFR2 gene-silenced cells,
glioma cells were transduced with VEGFR2-silencing shRNA
lentiviral particles (#sc-29318-V, Santa Cruz Biotechnology) con-
taining 3 target-specific constructs: ACTGTGGTGATTCCATGTCTT
CAAGAGACATGGAAATCACCACAGTTTTTT; ACTTGAAAACCAGA
CCTTACAAGAGATAGGTCGTTTTCAAGTTTTTT; and CACCTGTGT
GCAAGACATTTGCAAGAAAAGTTCTGCAGAAGTGTTTTT. BLAST
analysis showed that the VEGFR1 targeting sequence (TGAA
CCTGAAGACTAGATCCT) may target the HRNR (Hornerin) gene (ex-
pect value (E) of 11); therefore, we performed a quantitative
PCR analysis to exclude this possibility in VEGFR1-silenced cells
(data not shown).

Nonsilencing shRNA virus was used as a negative control
(#sc-108080). In all cases, stable transduced clones were
selected with 4 μg/mL puromycin and used for analysis and
assays after 1–2 passages post selection. A pool of 3 target-
specific PlGF siRNA and control siRNA was purchased from
Santa Cruz and transfected with TransIT-X2 Dynamic Delivery
system (Mirus Bio LLC).

The invasive potential of glioma cells was measured by
spheroid invasion assay. Glioma spheroids were generated
from the respective cell lines by seeding 1–5 × 103 cells in
100 μL of media onto 96-well plate base-coated with 1% nobel
agar/PBS medium substrate. After 2–3 days in culture, spheroids with a mean diameter of 200 μm were transferred to
collagen I matrix-coated wells and covered by complete Dul-
becco’s modified Eagle’s medium. Every 24 hours for 3 days,
the pixel area covered by cells sprouting from these spheroids
was determined after subtraction of the initial spheroid pixel
area at time zero. Image J software (NIH) was used to deter-
mine the invasion area.

Animal Studies

The effects of antiangiogenic treatments or VEGF depletion on
tumor growth and the survival of glioma-bearing mice were
examined in immunodeficient Crl:CD1-Foxn1™ nude mice (Charles River). Mice were xenografted with 75 000 LNT-229 or
100 000 LN-308 or U87MG cells. Cells were stereotactically
implanted into the right striatum of 6- to 12-week-old mice.
Neurological symptoms were assessed daily according to the
Cantonal Veterinary Office Zurich guidelines (grade 0: no visible
impairment; grade 1: reduced activity, slight balance and coordi-
nation impairments; grade 2: reduced activity, 15% weight loss
compared with peak weight, slight paralysis of left legs, moder-
ate signs of pain). Seven animals were used to assess survival,
defined as the timepoint of the onset of symptoms (grade 2).
Data are presented as the number of surviving mice over the
time. For histology, 3 prerrandomized animals per group were
euthanized when the first animal became symptomatic.

Animal experiments were conducted under valid licence and
permission of the Cantonal Veterinary Office Zurich and Federal
Food Safety and Veterinary Office. Mice were anesthetized by
intraperitoneal injection of fentanyl (Sintetica SA, J)midazolam
(Roche Pharma)/medetomidine (Orion Pharma) and combined with
analgesia using carprofen (Pfizer AG). Details on histology and immunohistochemistry are provided in
Supplementary material, Note 3.

Statistical Analyses

Detailed information on statistical analysis is summarized in
Supplementary material, Note 4.

Results

Glioma Cell Lines Show Different Levels of Constitutive
and Inducible VEGFR1 and VEGFR2 Phosphorylation

To select appropriate models, we first screened our glioma cell
line panel for the expression of VEGF and PlGF and their recep-
tors at the mRNA and protein levels (Supplementary material,
Notes 5 and 6; Supplementary material, Fig. S1–S6). Compared with long-term cell (LTC) lines, glioma-initiating cell lines (GIC) expressed lower levels of PlGF mRNA but were more responsive to hypoxia with regard to VEGF release (Supplementary material, Fig. S1D, F and G). Most cell lines exhibited VEGFR1 protein on the cell surface (Supplementary material, Fig. S2C), whereas VEGFR2 was not detected on the surface by flow cytometry (Supplementary material, Fig. S2D). However, immunoblot (Fig. 1A and B) and intracellular flow cytometry (Supplementary material, Fig. S2E and F) revealed significant intracellular levels of both receptors. Immunoblot showed the highest VEGFR2 protein levels in U87MG, LN-308, and T-325 (Fig. 1B). Total protein levels did not correlate to phosphorylation of the major tyrosine site, VEGFR1 (Tyr1213) or VEGFR2 (Tyr1059) (Fig. 1A and B) or the total levels of p-VEGFR1 or p-VEGFR2 measured by capture ELISA (Fig. 1C and D), suggesting multiple effects of interacting mechanisms triggered by various ligands.

Next, we studied the phosphorylation status of VEGFR1 and VEGFR2 (total p-VEGFR) at baseline and after stimulation with recombinant VEGF. p-VEGFR1 was induced by VEGF only in LN-308 and p-VEGFR2 only in human umbilical vein endothelial cells (HUVEC) (Fig. 1C and D). The specificity of the ELISA was supported by stimulation of HUVEC with VEGF and the reduction by cediranib in LN-308. Incomplete correlation between Figs. 1A–D is likely a result of comparing single versus all phosphorylated residues of VEGFR.

Constitutive VEGFR1 and VEGFR2 phosphorylation was sensitive to the pan-VEGFR inhibitor, cediranib, in ZH-161 cells with

**Fig. 1.** Autocrine and induced vascular endothelial growth factor receptor (VEGFR) activation in glioma cells. (A, B) The levels of total VEGFR1 and VEGFR2 as well as of phosphorylated VEGFR1 (Tyr1213) or VEGFR2 (Tyr1059) were assessed by immunoblot, using actin as a loading control. (C, D) Constitutive and VEGF-evoked total phosphorylation levels of VEGFR1 or VEGFR2 were determined by ELISA (*p < .05). (E) VEGFR1 and VEGFR2 phosphorylation in response to increasing concentrations of cediranib (2 h) in ZH-161 cells were detected by immunoblot. (F) Effects of VEGF or PlGF stimulation or neutralizing anti-VEGF (B20) or anti-placental growth factor (PlGF) (TB403) antibodies on VEGFR1 (Tyr1213) in LNT-229 cells were assessed by immunoblot; cells were incubated for 15 minutes with VEGF (500 ng/mL) or PlGF (1 μg/mL) alone or in combination with neutralizing VEGF or PlGF antibodies (100 μg/mL). (G) Effects of lentivirus-mediated VEGFA shRNAmir (left) or PlGF siRNA (right) on constitutive p-VEGFR1 (Tyr1213) in U87MG or LNT-229 cells, respectively, were evaluated by immunoblot.
VEGFR2 as well as of phospho-VEGFR2. Fig. 1.

Fig. 1. Major tyrosine site, VEGFR1 protein levels in U87MG, LN-308, and T-325 (Fig. 1B). Total protein levels of both receptors. Immunoblot showed the highest VEGFR2 (Fig. 1A and B) and intracellular flow cytometry (Supplementary material, Fig. S2D). However, immunoblot analysis, Fig. S1D, F and G). Most cell lines exhibited VEGFR1 protein expression with long-term cell (LTC) lines, glioma-initiating cell lines (GIC) Notes 5 and 6; Supplementary material, Fig. S1–S6). Compared to hypoxia with regard to VEGF release (Supplementary material, Fig. S7A and B). mRNA expression levels were associated with higher tumor grade and worse prognosis (Supplementary material, Fig. S8).

VEGFR1 and VEGFR2 Gene Silencing Affect Major Signaling Pathways in Glioma Cells

Fig. 2. Altered downstream signaling in vascular endothelial growth factor receptor (VEGFR1)- and VEGFR2-depleted glioma cells. (A) Stably VEGFR1 gene-silenced LNT-229 or LN-308 cells or (B) VEGFR2 gene-silenced LN-308, LN-428, ZH-161 or T-325 cells, or corresponding controls were assayed for changes in downstream signaling by immunoblot. After 12 hours of serum starvation, subconfluent cells were untreated or stimulated with VEGF (500 ng/mL) or placental growth factor (PIGF) (1 + 2) (100 + 100 ng/mL) as indicated for 15 minutes.
immunoblot (Fig. 2A; Supplementary material, Fig. S10A), flow cytometry (Supplementary material, Fig. S2E), and total p-VEGFR1 ELISA (data not shown). VEGFR2 gene silencing was verified by qRT-PCR (Supplementary material, Fig. S7D, F, H, I, J, and L), immunoblot (Fig. 2B, Supplementary material, Fig. S10A), flow cytometry (Supplementary material, Fig. S2F) and total p-VEGFR2 ELISA (data not shown). Flow cytometry revealed a minor induction of apoptosis upon VEGFR1 depletion in LN-308 but not LNT-229. VEGFR2 depletion had no such effect in LN-308 but increased the G2/M fraction (Supplementary material, Fig. S9C, D and E). Accordingly, VEGFR1/2 depletion did not affect the doubling time of LNT-229 (25–30 h), where depletion of either receptor prolonged doubling times from ~40–50 hours in LN-308 (data not shown). Further, VEGFR1 gene silencing decreased clonogenicity to 67% in LNT-229 and 14% in LN-308 and spherogenicity to 7% in ZH-161 (Fig. 3A). Similarly, VEGFR2

![Figure 3](image_url)

**Fig. 3.** Biological effects of vascular endothelial growth factor receptor (VEGFR) signaling inhibition in glioma cells. Effects of VEGFR1 gene silencing on (A) clonogenicity or spherogenicity and (B, C) invasion of LNT-229 were studied. (D, E) Effects of VEGFR2 gene silencing on clonogenicity and spherogenicity was evaluated. (F, G). Invasiveness of VEGFR2-depleted LN-308 cells was assessed by spheroid invasion assays. The data represent the average fold change in area of 3 spheroids ± standard deviation (⁎P < .05).
gene silencing reduced clonogenicity of U87MG, LN-308, and LN-428, although not in LNT-229, and spherogenicity in ZH-161 and T-325 (Fig. 3D and E). The specificity of the knockdown effects was confirmed by rescue experiments: clonogenicity was restored by CMV promotor-driven exogenously re-expressed VEGFR1 (P = .01) or VEGFR2 (P = .01) (Supplementary material, Fig. S10). At 72 hours VEGFR1 gene silencing inhibited invasion by 29% (P = .03) in LNT-229 (Fig. 3B and C). The invasiveness of the less invasive LN-308 cells was not suppressed by VEGFR1 depletion. Conversely, VEGFR2 depletion reduced invasiveness of LN-308 cells (Fig. 3F and G) to a similar extent as cediranib, but there was no effect on LNT-229 (data not shown).

**VEGFR1 and VEGFR2 Support Tumor Growth in Orthotopic Rodent Glioma Models**

Finally, we investigated whether genetic or pharmacologic VEGFR inhibition affected tumor growth in vivo. Mice inoculated with VEGFR1-silenced LNT-229 (P = .026), LN-308 (P = .042), or U87MG (P = .003) cells experienced a significant survival benefit (Fig. 4A and B; Supplementary Material, Fig. S11B). In the LN-308 model, all control mice had to be euthanized because of tumor growth, whereas 3 mice in the shVEGFR1mir group were alive and free from major signs at day 190. Analysis of brain sections of these 3 surviving mice showed that only one

![Fig. 4.](image-url)
mouse harbored a small tumor of 0.12 mm². Appropriate control experiments confirmed that p-VEGFR1 levels were strongly suppressed in the VEGFR1-silenced tumors at days 22 (LNT-229), 27 (U87MG) or 44 (LN-308) in all models (Fig. 4C; Supplementary material, Fig. S11F and S12B). Furthermore, immunoreactivity of matrix metalloproteinase (MMP) 9 was strongly reduced in LNT-229 (Fig. 4D). A survival effect similar to VEGFR1 silencing was afforded by treatment of mice carrying LNT-229 control tumors with TB403 but not B20 (Fig. 4A). The latter was in part due to early onset of score 2 adverse events (≥15% weight loss) with B20 treatment. All interventions reduced areas involved by tumor at day 22 (Fig. 4E).

To determine possible synergy by inhibiting both growth factors, we allowed the tumors to establish for 15 days and then initiated treatment with either VEGF or PlGF antibody or both until progression. In this paradigm, compared with the control group, only cotreatment afforded a survival advantage (P = .009) (Fig. 5A). In control tumors, CD31 staining revealed a prominent signal, particularly at the tumor edges, which was associated with the invasion of glioma cells along the vessels. Invasive cells at the tumor periphery, as well as glioma cells in tumor satellites of control tumors, displayed strong p-VEGFR1 staining relative to the tumor core, indicating a role of VEGFR1 in tumor cell invasion in vivo. All interventions resulted in decreased p-VEGFR1 levels, decreased MMP9 immunoreactivity, and a trend towards decreased vessel density determined by CD31 staining and (Fig. 5B and C).

VEGFR2 depletion delayed tumor growth profoundly in LN-308 (P = .003) and U87MG (P = .009) but not in LNT-229 (Fig. 6A, B and E; Supplementary material, Fig. S11C and D), although gene silencing was confirmed to persist in all models (Fig. 6C and D; Supplementary material, Fig. S11F). Compared with the controls, tumor sizes and MMP9 protein levels in the tumor core and invasive area were strongly reduced by VEGFR2 gene silencing in LN-308 (Fig. 6E and F).

Fig. 5. Synergistic growth inhibition by targeting both vascular endothelial growth factor receptor (VEGFR)1 ligands, VEGF and placental growth factor (PlGF) in vivo. (A) A similar experiment as in Fig. 4A was performed, but with the modification that antibody treatment was delayed until day 15 after tumor implantation and that another group of animals treated with both VEGF and PlGF antibody were included. (B) Tumor specimens obtained per randomization list from animals sacrificed on the same day in each group when the first animal(s) became symptomatic were stained for p-VEGFR1 (fig. 9) (upper row, brown color), CD31 (middle row) or MMP9 (lower row). Sections were counterstained with hematoxylin (blue). (C) Quantification of immunoreactivity (n = 3; *P < .05, t test).
Synergistic growth inhibition by targeting both vascular endothelial growth factor receptor (VEGFR)1 ligands, VEGF and placental growth factor (PlGF) in vivo. (A) A similar experiment as in Fig. 4A was performed, but with the modification that antibody treatment was delayed until day 15 after tumor implantation and that another group of animals treated with both VEGF and PlGF antibody were included. (B) Tumor specimens obtained per randomization list from animals sacrificed on the same day in each group when the first animal(s) became symptomatic were stained for p-VEGFR1 and expression of matrix metalloproteinase (MMP)9 was strongly reduced in LNT-229 (Fig. 4D). A survival effect was strongly suppressed in the VEGFR1-silenced tumors at days 22 (LNT-229), 27 (U87MG) or 44 (LN-308) in all models. Control experiments confirmed that p-VEGFR1 levels were prominent signal, particularly at the tumor core and invasive area were strongly reduced by B20 (Fig. 4A). The latter was in part due to early onset of tumor satellites of control tumors, displayed strong p-VEGFR1 immunoreactivity, and a trend towards decreased vessel density determined by CD31 staining and (Fig. 5B and C). To determine possible synergy by inhibiting both growth factors, we allowed the tumors to establish for 15 days and (Fig. 4E).

**Discussion**

The standard of care for patients with newly diagnosed glioblastoma includes resection or biopsy followed by radiation therapy and concomitant maintenance temozolomide.\(^{32,23}\) Many contemporary efforts to improve on this standard have explored the hypothesis that inhibition of angiogenesis will provide a survival benefit. In 2 randomized phase 3 trials, the most advanced antiangiogenic agent (ie, the VEGF antibody bevacizumab) has shown activity defined by an increased radiological response rate and prolonged progression-free survival, although not overall survival.\(^{26,29}\) In contrast, other VEGF-targeting agents such as cediranib or VEGF trap or non-VEGF-targeting antiangiogenic agents such as enzastaurin or cilengitide have failed.\(^{26}\) The search for and clinical validation of biomarkers that help select patients deriving benefit from antiangiogenic treatment continues.\(^{27,28}\)

Of note, VEGF may also assume an angiogenesis-independent tumor-promoting function.\(^{29,30}\) Despite interest in the autocrine effects of VEGF on tumor cells, and specifically glioma cells,\(^{8,20}\) distinct biological functions and signaling pathways mediated by different VEGF-receptors in glioma cells have not been systemically analyzed. Here we have performed a comprehensive expression profiling of human glioma cells, including GIC, for VEGF family ligands and receptors. Most glioma cells coexpress various VEGF and PlGF species and their cognate receptors, however, at different levels (Supplementary Notes 5 and 6).
VEGFR1 expression was identified at the surface of almost all glioma cell lines by flow cytometry. The presence of an intracellular VEGFR1 pool was evidenced by the major shift of the flow cytometry signal in the permeabilized LNT-229 and LN-308 cells (Supplementary material, Fig S2). Only LNT-229 and LN-308 cells expressed soluble VEGFR1 (Supplementary material, Fig S6B). VEGFR2 internalization and intracellular signaling have been described.31 VEGFR2 protein was only revealed by immunoblot and flow cytometry of prepermeabilized cells but not at the surface of nonpermeabilized cells, confirming intracellular localization (Supplementary material, Fig. S2F).

Most glioma cells exhibited autocrine VEGFR1 phosphorylation that is only slightly inducible by recombinant VEGF (Fig. 1A, C and F; Fig. 2A). VEGFR1 is phosphorylated at tyrosine Y1213 in response to both VEGF and PI GF on immunoblots. In contrast to cediranib, neutralizing antibodies to VEGF (B20) or PI GF (TB403) did not inhibit constitutive Tyr1213 phosphorylation. Yet, stimulation of VEGFR11213 by recombinant VEGF or PI GF in LNT-229 was neutralized by B20 and less so by TB403, respectively (Fig. 1E and F).

Phosphorylation of VEGFR2 Tyr1059, Tyr1175 suggestive of autocrine signaling was also detected in some cell lines. Total VEGFR2 phosphorylation was not inducible by exogenously added VEGF in vitro, supporting the absence of VEGFR2 on the cellular surface (Fig. 1B and D; Fig. 2B).

We confirm that exogenous VEGF or PI GF and anti-VEGF or PI GF neutralizing antibodies have little or no effect on glioblastoma cell growth in vitro32 (Supplementary material, Fig. S9A and B). Ligand interaction with VEGFR1 and VEGFR2 may be sterically protected from antibody interference but still targeted by intracellularly acting agents such as cediranib (Fig. 1E and F). Accordingly, VEGFA- or PI GF-deficient glioma cells had reduced basal p-VEGFR1 Tyr1213, confirming endogenous ligand-dependent receptor phosphorylation (Fig. 1G).

To better delineate autocrine signaling and deduce the biological role of VEGF family receptors, cell lines with different levels of VEGFR1 or VEGFR2 expression and activation were subjected to receptor-specific gene silencing by lentivirus-delivered shRNA. VEGFR2 gene silencing had major effects in cell lines with increased intracellular VEGFR2 levels lacking detectable VEGFR2 at the cell surface and indicating that autocrine VEGFR2 signaling is regulated at the level of cytoplasmic intracellular receptor cycling. Silencing of either receptor resulted in distinct changes in downstream signaling that may be interpreted as a stress response and point to potential escape strategies that might be exploited therapeutically: phosphorylation of MAPK in response to VEGFR1 depletion and of AKT and MAPK in response to VEGFR2 depletion (Fig. 2).

VEGFR-depleted glioma cells showed a strong phenotype at the level of clonogenicity, spherogenicity, and invasiveness in a cell line- and receptor type-dependent manner (Fig. 3). For example, clonogenic growth and invasion of LNT-229 cells were unaffected by VEGFR2 depletion, consistent with low-level VEGFR2 expression and phosphorylation, whereas VEGFR1 depletion led to a significant decrease of both clonogenic and motogenic potential demonstrating that VEGFR2 signaling is dispensable in some glioma cell lines. In contrast, the clonoegenic survival of VEGFR1 and VEGFR2 high-expressing LN-308 cells was strongly affected by the silencing of both receptors. Unlike LNT-229, downregulation of VEGFR2 significantly inhibited invasion, whereas VEGFR1 gene silencing hardly affected invasion in LN-308. Rescue experiments further confirmed the specific biological functions of both receptors (Supplementary material, Fig. S10).

These observations were expanded and confirmed by in vivo studies in nude mice. VEGFR1 phosphorylation was unevenly distributed throughout LNT-229 tumors with predominant staining localized to the infiltrating tumor edge. Either shRNA mir-mediated suppression of VEGFR1 or the early exposure of mice to neutralizing antibodies to VEGF or PIGF inhibited VEGFR1 phosphorylation and reduced tumor growth (Fig. 4A). Using a paradigm of pre-established tumors, the combination of both antibodies was superior to administration of either antibody alone and conferred a significant survival benefit in the LNT-229 model (Fig. 5A). The inhibition of VEGFR1 expression or activity was uniformly associated with loss of MMP9 levels in the tumors. Among various MMP, only the transcriptional and enzymatic levels of MMP9 correlated with tumor grade in gliomas.33 Correlation analyses using gene expression data from TCGA-S40 database (http://hgservr1.amc.nl/cgi-bin/r2/main.cgi#) confirmed that MMP9 expression correlated with VEGFR1 expression (r = 0.26, 8e-10) and VEGFR2 expression (r = 0.22, P = 1.4e-7) (data not shown).

A role for VEGFR1 signaling in tumor cells in promoting tumor growth has been reported in different tumor models33,34-36 however, not yet in glioblastoma. Although the addition of TB403 to bevacizumab did not generate a signal of enhanced activity in a phase 1 study in human patients with recurrent glioblastoma,37 the patient number was small, and no effort was made to preselect patients based on VEGFR1 phosphorylation. Thus, a biomarker-driven clinical trial focusing on p-VEGFR1 levels and PI GF expression in glioblastoma might still represent an effective strategy to define a role for PI GF targeting in glioblastoma or other cancers. Similarly, one might speculate that an enrichment of glioblastomas dependent on VEGFR signaling might have helped to define a role for cediranib in subsets of glioblastoma patients.38 VEGFR2 gene silencing resulted in profound growth inhibition associated with reduced MMP9 immunoreactivity in the LN-308 model, further delineating an important tumor-promoting function of VEGFR2 in selected gliomas (Fig. 6). The potential role of VEGFR1/2 in tumor growth was confirmed in the U87MG model (Supplementary material, Fig. S11).

Alternatively, this systematic analysis of VEGF receptors using different glioma models indicates differential biologic functions of VEGFR1 and VEGFR2 that may be context-dependent. Such in-depth studies may also resolve some apparently contradictory research findings (eg, PI GF has been shown to promote tumor growth and local invasiveness in subcutaneous melanoma, orthotopic pancreatic syngeneic tumors and GL-261 rodent glioma models,7,39 to inhibit tumor growth in lung, colon, and U87MG glioma models).40 Similarly, VEGFR2 has been reported to promote glioma cell viability and invasion,6,4,19 however, blocking VEGFR2 may also trigger invasiveness of some glioma cells by activating the c-MET pathway.20

Although VEGF antagonism has been shown to limit glioma growth in rodent models in vivo,41 this effect has commonly been attributed to antiangiogenesis. We now provide firm evidence that intrinsic VEGFR signaling in glioma cells sustains glioma growth at least in certain models: VEGFR1 suppression...
induced a major delay of tumor growth in the LNT-229, LN-308, and U87MG models (Fig. 4 and 5; Supplementary material, Figs. S11 and S12), whereas VEGFR2 decreased growth in LN-308 and U87MG (Fig. 6; Supplementary material, Fig. S11).

Translating VEGFR expression into a prognostic or predictive biomarker may remain challenging and require careful consideration of the type and level of intratumoral VEGFR phosphorylation, tumor versus endothelial expression, intratumoral heterogeneity, alternatively spliced VEGFR variants, and soluble, proteolytically cleaved, truncated VEGFR1 and VEGFR2 variants.

**Supplementary Material**

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

**Funding**

Swiss National Science Foundation (SNF) (to M.W.).

**Acknowledgments**

The authors would like to express their sincere appreciation to Konrad Honold (Roche Innovation Center Penzberg, Pharma Research and Early Development) for his support. The authors thank Silvia Dolski and Julia Friesen for expert technical assistance.

**Conflict of interest statement.** E.S. was a recipient of a Postdoctoral Fellowship from Roche (Basel, Switzerland). H.S. and E.R. report no conflicts of interest. K.S. has received honoraria for advisory board participation from Roche. F.H. and K.M.W. are employed by Roche. M.W. has received research grants from Acceleran, Actelion, Alpinia Institute, Bayer, Isarna, M.S.D., Merck & Co, Novocure, PIQUER and Roche and honoraria for lectures or advisory board participation or consulting from Celldex, Immunocellular Therapeutics, Isarna, Magforce, M.S.D., Merck & Co, Northwest Biotherapeutics, Novocure, Pfizer, Roche, and Teva.

**References**

1. Folkins C, Shked Y, Man S, et al. Gliona tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. Cancer Res. 2009;69(18):7243–7251.

2. Plate KH, Breier G, Weich HA, et al. Vascular endothelial growth-factor and gliona angiogenesis - coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. Int J Cancer. 1994;59(4):520–529.

3. Yao J, Wu XM, Zhuang GL, et al. Expression of a functional VEGF-F1 in tumor cells is a major determinant of anti-PIGF antibodies efficacy. Proc Natl Acad Sci U S A. 2011;108(28):11590–11595.

4. Nomura M, Yamagishi S, Harada S, et al. Placenta growth factor (PIGF) mRNA expression in brain tumors. J Neurooncol. 1998;40(2):123–130.

5. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med. 2001;7(5):575–583.

6. Hamerlik P, Lathia JD, Rasmussen R, et al. Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. J Exp Med. 2012;209(3):507–520.

7. Kerber M, Reiss Y, Wickersheim A, et al. Fit-1 signaling in macrophages promotes glioma growth in vivo. Cancer Res. 2008;68(18):7342–7351.

8. Kiznetova P, Ehrmann J, Hlobilkova A, et al. Autocrine regulation of glioblastoma cell cycle progression, viability and radioresistance through the VEGF-VEGFR2 (KDR) interplay. Cell Cycle. 2008;7(16):2553–2561.

9. Autiero M, Waltenberger J, Communi D, et al. Role of PI GF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flik1. Nat Med. 2003;9(7):936–943.

10. Tchaikovski V, Fellibrich G, Waltenberger J. The molecular basis of VEGFR-1 signal transduction pathways in primary human monocytes. Arterioscl Throm Vas. 2008;28(2):322–328.

11. Cao YH, Chen H, Zhou L, et al. Heterodimers of placenta growth factor vascular endothelial growth factor - Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. J Biol Chem. 1996;271(16):3154–3162.

12. Cudmore MJ, Hewett PW, Ahmad S, et al. The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. Nat Commun. 2012;3:1–12.

13. Dixelius J, Makiinen T, Wirzenius M, et al. Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem. 2003;278(42):40973–40979.

14. Favier B, Alam A, Barron P, et al. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood. 2006;108(4):1243–1250.

15. Zhang LQ, Zhou F, Han WC, et al. VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. Cell Res. 2010;20(12):1319–1331.

16. Gee MFW, Tsuchida R, Eichler-Jonsson C, et al. Vascular endothelial growth factor acts in an autocrine manner in rhematomyosarcoma cell lines and can be inhibited with all-trans-retinoic acid. Oncogene. 2005;24(54):8025–8037.

17. Soker S, Takashima S, Miao HQ, et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1998;92(6):735–745.

18. Tanno S, Ohsaki Y, Nakashiki K, et al. Human small cell lung cancer cells express functional VEGF receptors, VEGFR-2 and VEGFR-3. Lung Cancer. 2004;46(1):11–19.

19. Kil WJ, Tofilon PJ, Camphausen K. Post-radiation increase in VEGF enhances glioma cell motility in vitro. Radiat Oncol. 2012;7:1–9.

20. Lu KV, Chang JP, Parachoniak CA, et al. VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. Cancer Cell. 2012;22(1):21–35.

21. Pan Q, Chathery Y, Wu Y, et al. Neuropilin-1 binds to VEGF(121) and regulates endothelial cell migration and sprouting. J Biol Chem. 2007;282(33):24049–24056.

22. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New Engl J Med. 2005;352(10):987–996.

23. Weller M, van den Bent M, Hopkins K, et al. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. Lancet Oncol. 2014;15(9):E395–E403.
24. Chinot OL, Wick W, Mason W, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. New Engl J Med. 2014;370(8):709–722.

25. Gilbert MR, Dignam JJ, Armstrong TS, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. New Engl J Med. 2014;370(8):699–708.

26. Batchelor TT, Reardon DA, de Groot JF, et al. Antiangiogenic therapy for glioblastoma: current status and future prospects. Clin Cancer Res. 2014;20(22):5612–5619.

27. Sandmann T, Bouron R, Garcia J, et al. Patients with proneural glioblastoma may derive overall survival benefit from the addition of bevacizumab to first-line radiotherapy and temozolomide: retrospective analysis of the AVAglio trial. J Clin Oncol. 2015;33(25):2735–U2737.

28. Tabouret E, Boudouresque F, Farina P, et al. MMP2 and MMP9 as candidate biomarkers to monitor bevacizumab therapy in high-grade glioma. Neuro Oncol. 2015;17(8):1174 –1176.

29. Lee TH, Seng S, Sekine M, et al. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. Plos Med. 2007;4(6):1101–1116.

30. Cao Y, E GQ, Wang EF, et al. VEGF exerts an angiogenesis-independent function in cancer cells to promote their malignant progression. Cancer Res. 2012;72(16):3912 –3918.

31. Lampugnani MG, Orsenigo F, Gagliani MC, et al. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. J Cell Biol. 2006;174(4):593 –604.

32. Bais C, Wu X, Yao J, et al. PI GF blockade does not inhibit angiogenesis during primary tumor growth. Cell. 2010;141(1):166–177.

33. Forsyth PA, Wong H, Loing TD, et al. Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. Brit J Cancer. 1999;79(11–12):1828 –1835.

34. Frank NV, Schatton T, Kim S, et al. VEGF-1 expressed by malignant melanoma-initiating cells is required for tumor growth. Cancer Res. 2011;71(4):1474 –1485.

35. Lesslie DP, Summy JM, Parikh NU, et al. Vascular endothelial growth factor receptor-1 mediates migration of human colorectal carcinoma cells by activation of Src family kinases. Brit J Cancer. 2006;94(11):1710–1717.

36. Mezquita B, Mezquita P, Pau M, et al. Unlocking doors without keys: activation of Src by truncated C-terminal intracellular receptor tyrosine kinases lacking tyrosine kinase activity. Cells. 2014;3(1):92–111.

37. Lassen U, Chinot OL, McBain C, et al. Phase 1 dose-escalation study of the antiplacental growth factor monoclonal antibody RO5323441 combined with bevacizumab in patients with recurrent glioblastoma. Neuro Oncol. 2015;17(7):1007–15.

38. Batchelor TT, Mulholland P, Neyns B, et al. III randomized trial comparing the efficacy of cediranib as monotherapy, and in combination with lomustine, versus lomustine alone in patients with recurrent glioblastoma. J Clin Oncol. 2013;31(26):3212 –3218.

39. Fischer C, Jonckx B, Mazzone M, et al. Anti-PIGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell. 2007;131(3):463–475.

40. Xu L, Cochran DM, Tong RT, et al. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. Cancer Res. 2006;66(8):3971–3977.

41. Cheng SY, Huang HJS, Nagane M, et al. Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc Natl Acad Sci U S A. 1996;93(16):8502–8507.