Angiopoietin-4 Promotes Glioblastoma Progression by Enhancing Tumor Cell Viability and Angiogenesis

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Abstract

Glioblastoma multiforme (GBM) is a highly invasive and vascularized aggressive brain tumor. Less than 10% of GBM patients survive >5 years after diagnosis. Angiogenesis plays an important role in GBM growth, and antiangiogenesis-based therapies have shown clinical efficacy for GBM patients. Unfortunately, therapeutic resistance often develops in these patients, suggesting that GBM cells are capable of switching their dependency on one proangiogenic signaling pathway to an alternative one. Therefore, it is important to identify novel angiogenic factors that play essential roles in tumor angiogenesis and GBM progression. Angiopoietins (Ang-1, Ang-2, and Ang-4) are the ligands of the Tie-2 receptor tyrosine kinase (RTK). The roles of Ang-1 and Ang-2 in tumor angiogenesis have been established. However, little is known about how Ang-4 affects tumor angiogenesis and GBM progression and the mechanism underlying its effects. In our current study, we establish that Ang-4 is upregulated in human GBM tissues and cells. We show that, like endothelial cells, human GBM cells express Tie-2 RTK. We first establish that Ang-4 promotes *in vivo* growth of human GBM cells by promoting tumor angiogenesis and directly activating extracellular signal-regulated kinase 1/2 (Erk1/2) in GBM cells. Our results establish the novel effects of Ang-4 on tumor angiogenesis and GBM progression and suggest that this pro-GBM effect of Ang-4 is mediated by promoting tumor angiogenesis and activating Erk1/2 kinase in GBM cells. Together, our results suggest that the Ang-4–Tie-2 functional axis is an attractive therapeutic target for GBM. *Cancer Res; 70(18); 7283–93. ©2010 AACR.*

Introduction

Despite advances in surgery, radiation, chemotherapy, and targeted therapy, the majority of patients with malignant glioma have poor prognosis and a median survival length of ~1 year. Malignant glioma is highly vascularized, and angiogenesis is known to play an important role in the progression of this deadly disease (1). Therapeutic agents targeting vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) have shown clinical benefits for patients with malignant glioma and other cancer types (2, 3). However, response duration of patients varied, and at least 50% of patients failed to respond to the antiangiogenesis treatments (3, 4). Resistance to anti-VEGF/VEGFR therapy suggests that malignant glioma is capable of switching its dependence or addiction of the VEGF-VEGFR signaling pathway to alternative proangiogenic signaling pathways. To overcome the resistance to current antiangiogenesis therapies and achieve better therapeutic efficacy, it is essential to first identify other important proangiogenic factors that play critical roles in the progression of malignant glioma.

Tumor angiogenesis is regulated by numerous molecules (5–8). Angiopoietins are among the factors that play important roles in the process and are often upregulated in the tumors that have developed resistance to anti-VEGF/VEGFR agents (9, 10), suggesting their potential roles in mediating the resistance to anti-VEGF/VEGFR therapy. Angiopoietins are the ligands of the Tie-2 receptor tyrosine kinase (RTK), which is reportedly expressed primarily by endothelial cells (EC; refs. 11–16). Tie-2 plays an important role in tumor angiogenesis (17, 18). Three angiopoietins have been identified in humans: angiopoietin-1 (Ang-1), Ang-2, and Ang-4 (19–21). All three angiopoietins have a similar protein domain organization, which consists of a signal peptide, an amino terminal coiled-coil domain, a linker peptide region, and a carboxyl terminal fibrinogen homology domain (FHD). The coiled-coil domain is responsible for the dimerization/mulimerization of angiopoietins, whereas the FHD binds to the Tie-2 receptor (19–23). At least tetrameric aggregation of angiopoietins is required to activate Tie-2 (24). The contributions of

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Ang-1 and Ang-2 to tumor angiogenesis are relatively well understood, and they are known to display context-dependent proangiogenic or antiangiogenic effects (11, 12, 16, 25). We have previously shown that cell surface–tethered monomeric mouse Ang-3 inhibits tumor angiogenesis and metastasis (14, 26). However, the role of Ang-4 in tumor angiogenesis and progression has not been established.

In the current study, we investigate how Ang-4 affects tumor angiogenesis and the growth and progression of human glioblastoma or glioblastoma multiforme (GBM; the stage IV and most aggressive form of glioma) and the mechanism underlying Ang-4 bioactivity. We establish for the first time that Ang-4 is upregulated in human GBM tissue and cells and show that human GBM cells express aggregated multimeric Ang-4, which can be dissociated into monomers under reducing conditions. We show that, like ECs, human GBM cells express Tie-2, the angiopoietin receptor, and that Ang-4 overexpression promotes *in vivo* growth of human GBM by promoting tumor angiogenesis and directly activating extracellular signal-regulated kinase 1/2 (Erk1/2) in GBM cells whereas knockdown of Ang-4 expression inhibits GBM growth *in vivo*. Our results establish a novel effect of Ang-4 on tumor angiogenesis and glioma progression and suggest that the proglioma effect of Ang-4 is mediated through enhancing tumor angiogenesis and directly activating Erk1/2 kinases in GBM cells. Together, these results suggest that the Ang-4–Tie-2 functional axis is an attractive therapeutic target for GBM.

Materials and Methods

Patient glioma samples, cells, and reagents

GBM and normal brain tissues were obtained from the Co-operative Human Tissue Network at University of Pennsylvania and Ohio State University. Human umbilical vein ECs (HUVEC) and normal human astrocytes (NHA) were from AllCells, Inc., U87MG was obtained from American Type Culture Collection, and U251 was obtained from Neurosurgery Tissue Bank at University of California, San Francisco (https://gnome.ucsf.edu/btrc/cell_line.php). These cells were tested and authenticated by the providers and maintained according to the providers' instructions. The cumulative culture length of these cells was <6 months after reception.

Antibodies against v5 epitope (Invitrogen); Tie-2 and Ang-2 (Santa Cruz); Ang-1 and Ang-4 (R&D Systems); CD31 (Chemicon, BD Biosciences, ThermoScientific for fluorescence); smooth muscle actin (SMA)–Cy3 (Sigma); and Erk1/2, phosphorylated Erk1/2, Akt, and phosphorylated Akt (Santa Cruz) antibodies and Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) were used in the experiments. Purified Ang-1, Ang-2, and Ang-4 were obtained from R&D Systems.

Reverse transcription-PCR and expression and knockdown constructs

Reverse transcription-PCR was performed, and full-length human Ang-1 and human Ang-4 (hAng-4) cDNAs were generated as described (11, 14, 27). hAng-1 and

hAng-4, along with their COOH terminal v5 epitope tags from the expression vector (pEF6/v5-HisTOPO, Invitrogen), were cloned into the retroviral expression vector pQCXIP (BD Bioscience) as described (28). All expression constructs were verified by DNA sequencing. Retroviruses were generated using these expression constructs and pVSVG in GP2-293 cells following the manufacturer's instructions (BD Bioscience). To knock down expression of human Ang-1 or Ang-4, several hAng-1–specific or hAng-4–specific short hairpin RNA (shRNA)–TRC constructs and a nontargeting control shRNA were obtained from Open Biosystems and Addgene. Lentiviruses carrying these shRNAs were generated following the manufacturer's instructions.

Retrovirus and lentivirus transduction

U87MG-Luc and U251-Luc human GBM cells expressing luciferase were established as described (28, 29). These cells were transduced with the retroviruses carrying the empty retroviral expression vector, hAng-1, or hAng-4. Infected cells were selected for their resistance to puromycin. Anti-v5 monoclonal antibody (mAb; Invitrogen) was used to detect expression of exogenous v5-tagged hAng-1 and hAng-4. To knock down expression of endogenous Ang-1 or Ang-4, these GBM cells were transduced with the lentiviruses carrying shRNAs against hAng-1 or hAng-4. Knockdown expression of Ang-1 or Ang-4 in these GBM cells was validated by Western blotting using anti-hAng-1 or anti-hAng-4 antibodies, respectively.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed as described (28) by using SYBR Green PCR Master Mix (Roche) and Mx3005 Real-Time PCR Machine (Stratagene). The cycling parameters used were 95°C for 10 minutes followed by 42 cycles of 95°C (30 seconds), 60°C (1 minute), and 72°C (1 minute) and a melting curve analysis. Relative quantification of the targets was normalized with an endogenous housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase), and data analyses were performed using a comparative ($\Delta\Delta Ct$) method using the manufacturer's (Stratagene) software and according to manufacturer's instructions.

Western blot analysis

Serum-free cell culture supernatants were collected from the cultured GBM cells. Serum-free medium (SFM; 1 mL) was concentrated by NanoSep Centricon-3K (Life Science) to 100 µL. Cells were extracted with 8× SDS Laemmli sample buffer without the dye. Protein concentrations from all the samples were determined using Bio-Rad DC Protein Assay Reagents. Equal amounts of proteins were analyzed by Western blotting, as described previously (27).

Erk and Akt phosphorylation

U87MG cells were cultured until subconfluence and switched to SFM for 72 hours. Purified Ang-1, Ang-4 (R&D Systems), or 10% fetal bovine serum (FBS) were applied to the serum-starved U87MG cells for 30 minutes or 24 hours,

as detailed in the figure legend. The cells were then lysed, and equal amounts of extracted proteins were analyzed by Western blotting with anti-phosphorylated Erk1/2 or anti-phosphorylated Akt and anti-Erk1/2 or anti-Akt to detect phosphorylated and total amounts of Erk1/2 and Akt proteins, respectively.

Cell viability assay

Cell viability assays were performed using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) following manufacturer's instructions. U87MG cells were plated in triplicate (2×10^5 per well) in 96-well plates and switched to SFM for 48 hours before treatment for 24 hours with purified 200 ng/mL of angiopoietins or 20 ng/mL of VEGF (R&D Systems; refs. 14, 26, 30, 31) and as detailed in the panels. Cell viability was measured by Modulus Microplate Multimode Reader (Turner Biosystems).

EC proliferation, survival, migration, and tube formation assay

EC proliferation assay was performed by seeding HUVECs at 2.5×10^4 per well in six-well plates in 1:1 HUVEC medium/Ham's F-12 medium (Invitrogen). After 12 hours, the cells were switched to fresh 1:1 HUVEC medium/Ham's F-12 medium (Invitrogen) in the absence or presence of 200 ng/mL of angiopoietins (14, 26, 30, 31), and the numbers of the cells were counted every day for 4 days. EC survival assays were performed by switching confluent HUVECs to SFM in the presence or absence of 200 ng/mL Ang-1, Ang-2, or Ang-4 or 20 ng/mL of VEGF (14, 26, 30, 31). The cells were cultured for an additional 48 hours, and viable cells were detected using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) following manufacturer's instructions.

To assess the effects of angiopoietins on tube formation, Matrigel tubulogenesis assays were performed. Matrigel (300 μL, ~10.5 mg/mL, BD Biosciences) was coated onto each well of a 24-well plate and allowed to polymerize at 37°C for 30 min. HUVECs (5×10^4) in 200 μL of 1:1 HUVEC medium/Ham's F-12 medium (Invitrogen) with or without 200 ng/mL of angiopoietins (14, 26, 30, 31) were seeded on Matrigel and cultured for 12 hours to allow tubes to form. Photographs of five randomly selected 100× microscopic fields were taken for each type of treatment. The tube length and the number of branch points of tubes were counted in these fields to assess the extent of tubulogenesis.

Subcutaneous and intracranial tumor growth experiments and bioluminescence imaging analysis of intracranial gliomas

Pooled populations of transduced U87MG and U251 cells were used for subcutaneous tumor growth experiments, as described previously (28, 29). Briefly, 1×10^6 GBM cells were injected s.c. into each immunocompromised B6.129S7-Rag1[tmMom] mouse (Rag1; The Jackson Laboratory). Six mice were used for each type of the infected GBM cell line. After solid tumors became visible, the longest and shortest dia-

meters of the solid tumors were measured using a digital caliper every other day for 4 to 6 weeks. Tumor volumes were calculated using the following formula: tumor volume = $1/2 \times$ (shortest diameter)^2 × longest diameter (mm^3). At the end of the experiments, tumors were fixed and sectioned for histologic and immunologic analyses.

For intracranial tumor growth, U87MG or U251 cells (2×10^5 in 10 μL HBSS/Rag1 mouse) were injected stereotactically as described (28, 29). Following injection, mice were closely monitored, and the duration of their survival was recorded. Mice that showed signs of morbidity were euthanized and considered as if they had died on that day, and the number of surviving mice was recorded. The survival rates were calculated as follows: survival rate (%) = (number of mice still alive/total number of experimental mice) × 100%. At the end of the experiments, mouse brains were removed, fixed, and sectioned for further analysis. Bioluminescence imaging was used to monitor the growth of intracranial gliomas in live animals as described (28, 29). Images were acquired at 7, 12, and 15 days after the intracranial injections of the GBM cells using IVIS-200 imaging system (Xenogen) at the *In vivo* Molecular Imaging Shared Facility (Mount Sinai School of Medicine).

Histology, immunohistochemistry, and scoring of immunohistochemistry results

Histology was performed as described (28, 29). Paraffin sections derived from GBM patients and normal brains were reacted with anti-Ang-1, anti-Ang-2, or anti-Ang-4 antibody (Santa Cruz). The intensity of immunoreactivity in six randomly selected 200× microscopic fields for each case was scored by two people independently as follows: 0, negative; 1, weak; 2, intermediate; 3, strong staining (32). The scores were averaged, and SDs and P values were calculated.

The frozen and paraffin sections derived from gliomas grown in experimental mice were reacted with antimouse CD31 (Chemicon, BD Biosciences, ThermoScientific), FITC-conjugated antihamster secondary antibody, and then Cy3-conjugated anti-SMA (Sigma) antibody to assess tumor angiogenesis and pericyte coverage and with anti-phosphorylated Erk1/2 antibody to assess phosphorylated Erk1/2 levels in GBM cells *in vivo*. To determine microvessel density (MVD), CD31$^+$ vessels within five randomly selected 400× microscopic fields from each of the four vascular hotspots were counted per group. A blood vessel was counted as positive for pericyte coverage when at least 50% of the CD31$^+$ vessel was covered by SMA-positive pericytes (33). Perivascular coverage was assessed in 10 randomly selected 200× microscopic fields per group.

Statistics

Other than survival experiments, one-tailed Student's t test was used to analyze statistical differences between the control and experimental groups. For mouse survival experiments, log-rank statistical analysis (SigmaPlot) was used. Differences were considered statistically significant at $P < 0.05$.

Results

### Ang-4 is upregulated in GBM tissues and GBM cells

Analyses of the available public data sets (www.oncomine. org) indicated that, compared with normal brain, Ang-1 (Beroukhim, Sun, and TCGA Brain; refs. 34, 35) and Ang-2 (Shai and Bredel Brain; refs. 36, 37) mRNAs are upregulated in GBM, whereas Ang-4 mRNA is either unchanged (Beroukhim Brain; ref. 34) or slightly upregulated in GBM (TCGA Brain 2). To further determine the expression levels of endogenous angiopoietin proteins in GBM tissues, we performed an immunohistochemistry analysis of paraffin sections derived from 14 GBM tissues and 6 normal brain tissues. Intensity of immunoreactivity was scored as described in

Materials and Methods. Our results showed that protein levels of Ang-4, but not Ang-1 or Ang-2, are significantly upregulated in GBM tissues compared with normal human brain (Fig. 1A).

We then compared the levels of angiopoietins, Tie-2, and Tie-1 transcripts in U87MG and U251 GBM cells to that of NHAs and found that the GBM cells express higher levels of Ang-2, Ang-4, and Tie-2 than NHAs (Fig. 1B). The real-time qPCR results reveal only the relative levels of mRNAs. To further confirm that human GBM cells produce endogenous angiopoietin proteins, we collected and concentrated serum-free cell culture supernatants derived from U87MG cells and showed that U87MG cells readily secrete detectable levels of multimeric forms of Ang-1 and Ang-4 but a
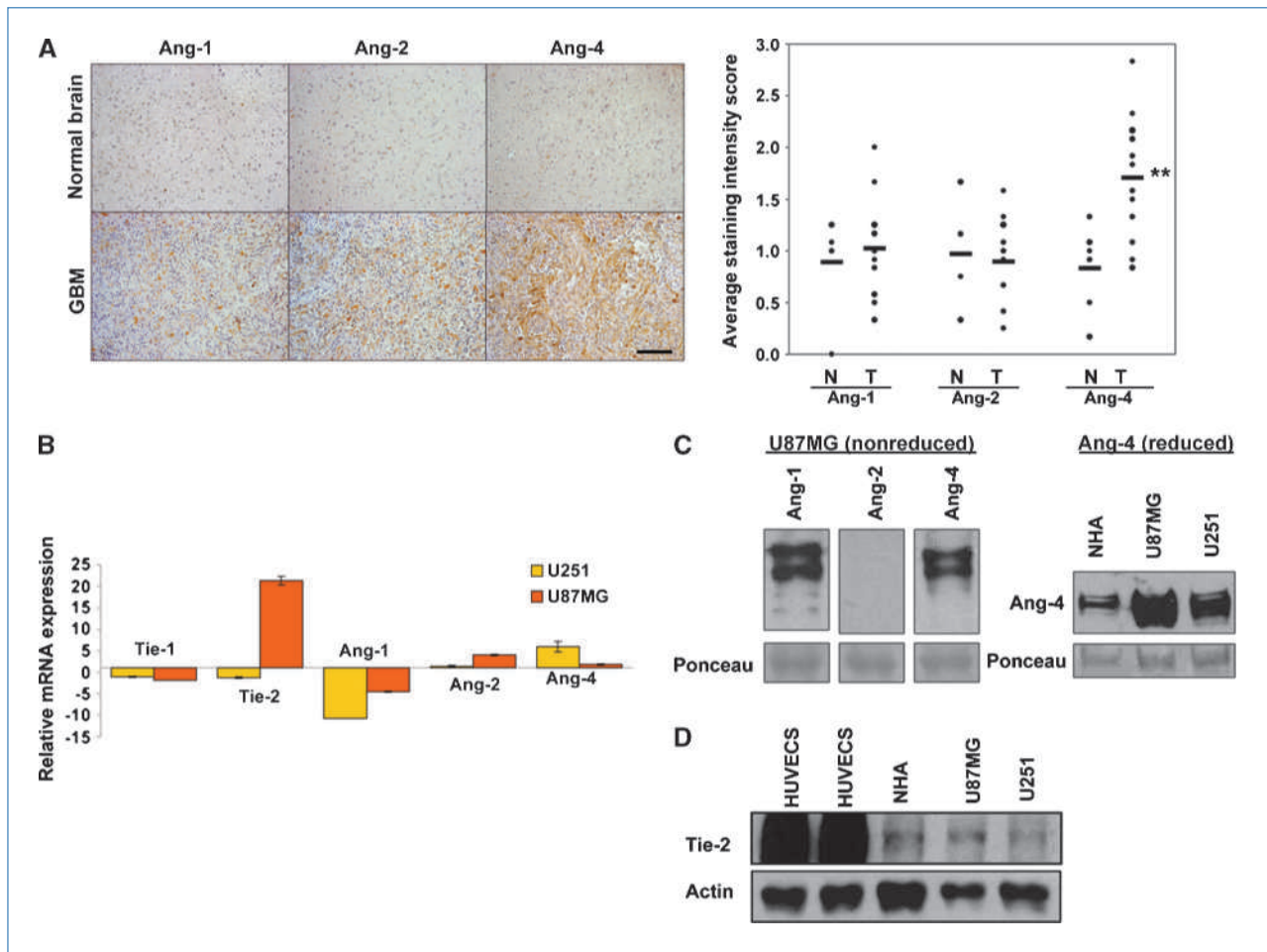
Figure 1. Expression levels of angiopoietins and Tie-2 in GBM tissues and cells. A, angiopoietin expression was assessed by immunohistochemistry using antihuman Ang-1, Ang-2, or Ang-4 antibodies (Santa Cruz). Left, representative pictures of 14 GBM samples and 6 normal human brain samples. Bar, 200 μm. Right, the plots of intensity scores of the immunohistochemistry results as determined following the description in Materials and Methods. N, normal; T, tumor. B, real-time qPCR assessing relative transcript levels of human Ang-1, Ang-2, Ang-4, Tie-1, and Tie-2 in U87MG and U251 GBM cells compared with NHAs. C, endogenous Ang-1, Ang-2, and Ang-4 secreted by U87MG cells (left) and endogenous Ang-4 produced by NHAs, U251, and U87MG (right) were detected by Western blotting with anti-Ang-1 (R&D Systems), Ang-2 (Santa Cruz), or Ang-4 (R&D Systems) antibodies. The SDS-PAGEs were run either under nonreducing conditions (left) or reducing conditions (right). Proteins (160 μg) from concentrated serum-free cell culture media were loaded in each lane. The intensities of ~50-kDa ponceau-stained bands on the transferred membranes were used as the controls for protein loading and transferring efficiency. D, expression of endogenous Tie-2 RTK was determined by Western blotting using anti-Tie-2 antibody (Santa Cruz), and the cell lysates were derived from HUVECs, NHAs, and U87MG and U251 GBM cells (top). Total protein (25 μg) was loaded in each lane. Actin was used as a control for protein loading (bottom).
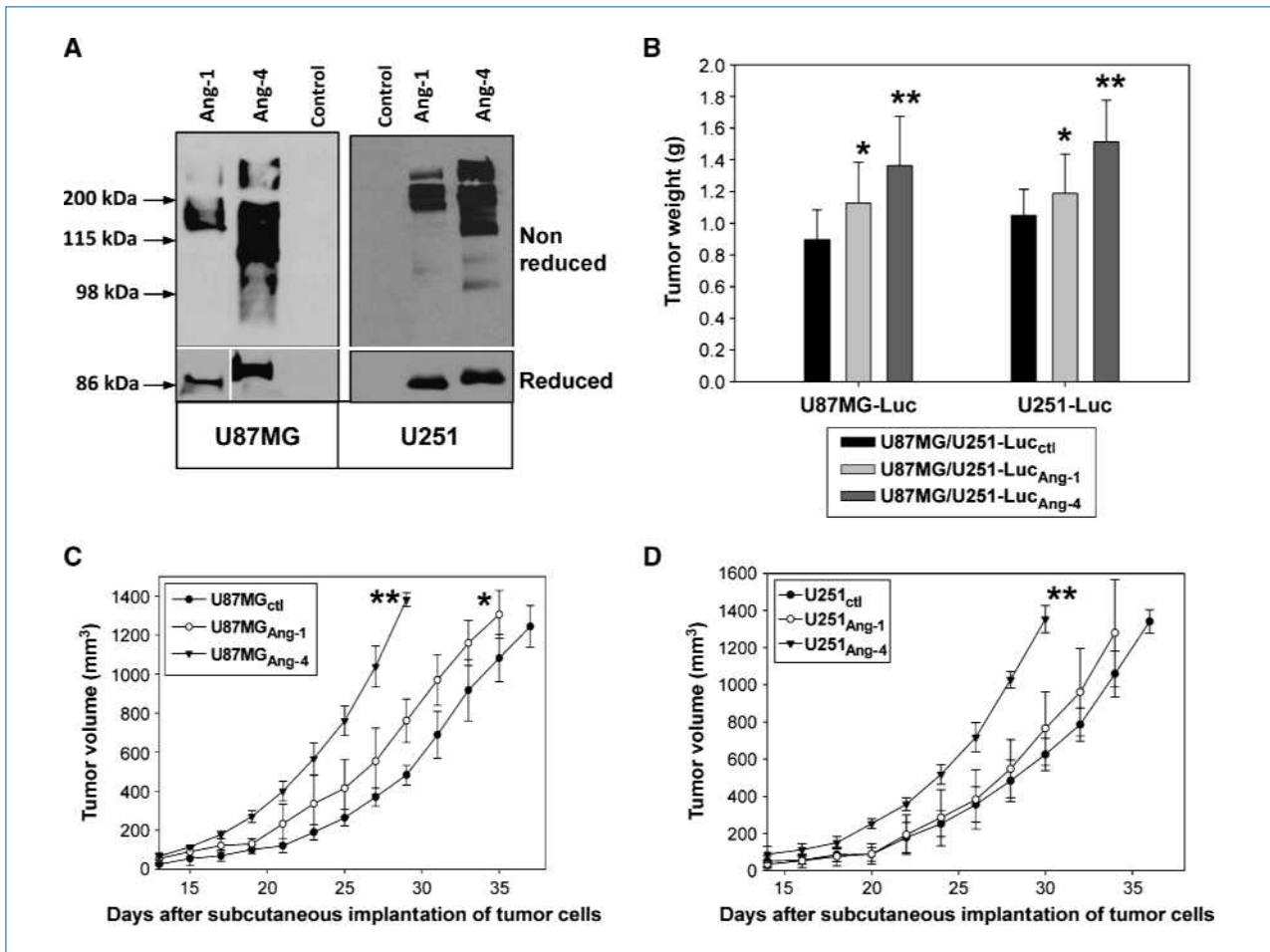
Figure 2. Ang-4 promotes subcutaneous growth of GBM cells. A, establishment of U87MG-Luc and U251-Luc GBM cells expressing v5 epitope tagged Ang-1 (Ang-1$_{v5}$) or Ang-4 (Ang-4$_{v5}$). Secreted Ang-1$_{v5}$ and Ang-4$_{v5}$ was detected by anti-v5 mAb (Invitrogen) under nonreducing (top) and reducing conditions (bottom). B, the effects of Ang-1 and Ang-4 on subcutaneous growth of U87MG and U251 cells were assessed by tumor weight 5 wk after tumor cell implantation. Six mice were used to implant each type of transduced GBM cells. C and D, growth rates of the subcutaneous tumors derived from U87MG-Luc (C) and U251-Luc (D) cells expressing Ang-1 or Ang-4 or transduced with empty expression vector. The growth rates are expressed as the mean of tumor volumes (mm^3) ± SD. Six mice were used for each type of transduced GBM cells.

low level of Ang-2 into the culture media (Fig. 1C, left), which can be dissociated into monomers under reducing conditions (Supplementary Fig. S1A). To assess Ang-4 levels in U87MG and U251 GBM cells and NHAs, we performed Western blotting using the proteins derived from the concentrated serum-free culture supernatants. Our result showed that U87MG and U251 GBM cells express higher levels of Ang-4 than NHAs (Fig. 1C, right; Supplementary Fig. S1B). Slight differences in the molecular weight of Ang-4 produced by different cells may reflect differences in the glycosylation status of angiopoietins.

Although Tie-2 is thought to be expressed largely by ECs and bone marrow progenitor cells (38, 39), our real-time qPCR results indicated that the Tie-2 transcript is present in the tested GBM cells (Fig. 1B). To establish that Tie-2 protein is expressed by these GBM cells, we performed Western blotting using cell lysates derived from U87MG and U251 cells. We found that U87MG and U251 clearly express endog-

enous Tie-2, albeit at levels much lower than that expressed by ECs (Fig. 1D).

Ang-4 promotes subcutaneous and intracranial growth of human GBM

To determine how Ang-4 affects tumor angiogenesis and GBM growth and progression, we first transduced U87MG-Luc and U251-Luc cells that express luciferase (28) with retroviruses carrying v5 epitope–tagged human Ang-1 (U87MG-Luc/U251-Luc$_{Ang-1}$) or human Ang-4 (U87MG-Luc/U251-Luc$_{Ang-4}$) or with empty expression vector (U87MG-Luc/U251-Luc$_{ctl}$). The latter cells were used as the controls. U87MG-Luc/U251-Luc$_{Ang-1}$ and U87MG-Luc/U251-Luc$_{Ang-4}$, but not U87MG-Luc/U251-Luc$_{ctl}$, cells express high levels of v5-tagged aggregated Ang-1 and Ang-4, respectively (Fig. 2A, top), which can be dissociated into monomers under the reducing condition (Fig. 2A, bottom).

The pooled populations of transduced U87MG-Luc and U251-Luc cells were assessed for their capacity to grow subcutaneously and intracranially. Our results showed that similar to Ang-1, Ang-4 promotes subcutaneous growth of U87MG and U251 GBM cells (Fig. 2B–D). In addition, Ang-4 and, to a lesser extent, Ang-1 promoted intracranial growth of the GBM cells, and Ang-4 significantly reduced survival length of the mice bearing these intracranial gliomas (Fig. 3A and B).

To further investigate the potential mechanism underlying the pro-GBM growth effect of Ang-4, we assessed the extent of angiogenesis in the gliomas derived from U87MG-Luc$_{Ang-1}$, U87MG-Luc$_{Ang-4}$, or U87MG-Luc$_{ctl}$ cells. To achieve that, the GBM sections were analyzed for the presence of CD31$^+$ blood vessels in 400× microscopic fields, as detailed in Materials and Methods. The MVD values in the control GBM are consistent with the published glioma MVD data (40, 41). Our results showed that the intracranial and subcutaneous tumors derived

from the GBM cells expressing Ang-4 are more angiogenic than the control tumors and the tumors expressing Ang-1 as determined by MVD (Figs. 3C and D and 4A and B), suggesting that Ang-4 is the predominant angiopoietin that promotes tumor angiogenesis during GBM progression. We also assessed coverage of these blood vessels by perivascular smooth muscle cells by costaining the FITC-CD31$^+$ blood vessels with Cy3-conjugated anti-SMA antibody. We found that Ang-1 promotes pericyte coverage of blood vessels whereas Ang-4 reduces the coverage in the gliomas expressing exogenous Ang-1 or Ang-4, respectively, when compared with the controls (Fig. 4C and D).

Knocking down Ang-4 expression inhibits GBM growth *in vivo*

Because the GBM cells express sufficient amounts of endogenous Ang-1 and Ang-4 (Fig. 1C), we investigated how knockdown of Ang-1 or Ang-4 expression affects GBM
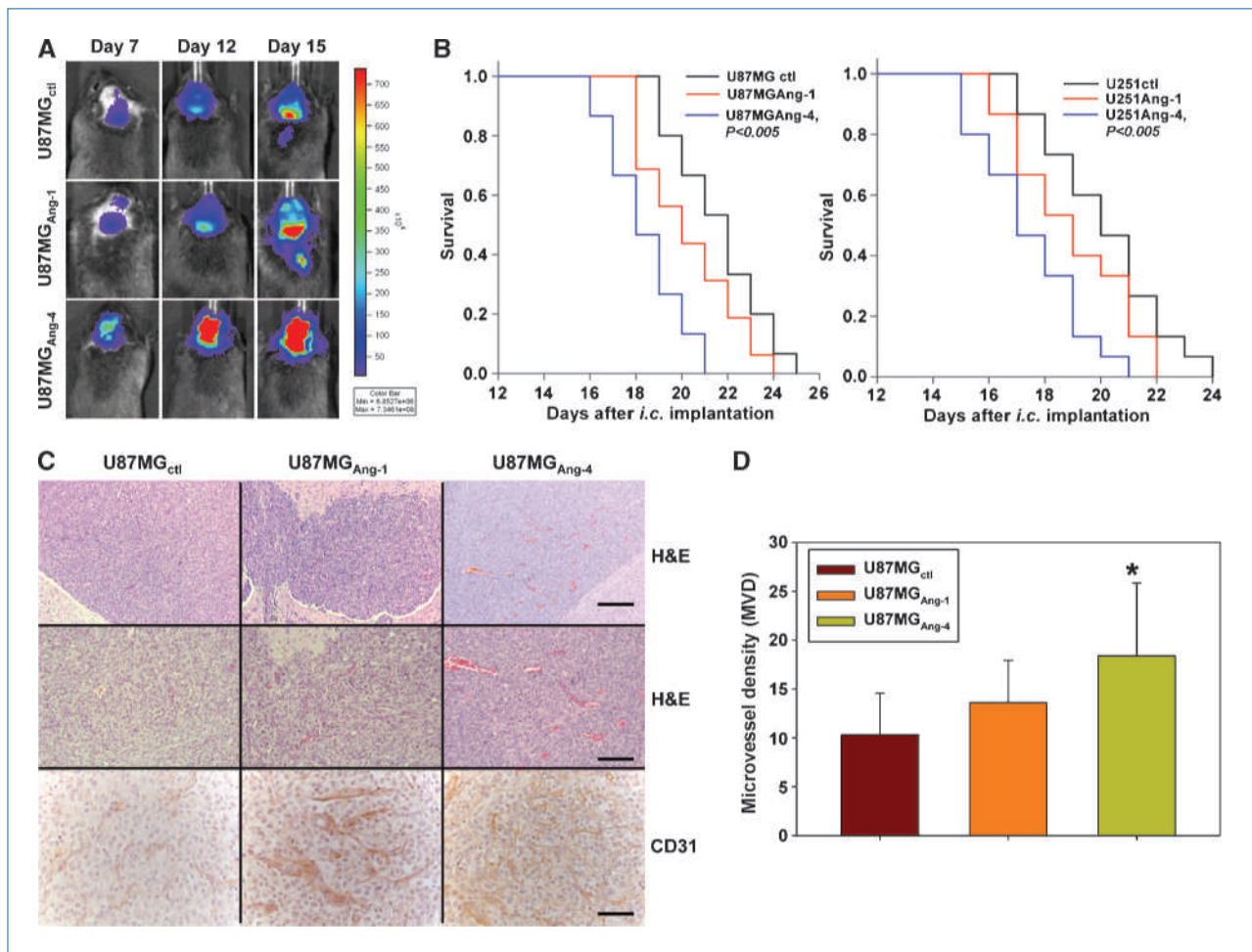
Figure 3. Ang-4 promotes intracranial growth of U87MG-Luc and U251-Luc gliomas. A, progression of intracranial GBMs was monitored through bioluminescence imaging at 7, 12, and 15 d after the intracranial injection of the transduced U87MG cells. The images were obtained 12 min after injection of D-luciferin using the same intensity scale. B, survival rates of mice following intracranial injections of the transduced U87MG-Luc (left) and U251-Luc (right) cells. Fifteen mice were used for each type of transduced GBM cells. C, histologic (H&E) and immunologic (anti-CD31) analyses of the GBM sections derived from U87MG-Luc$_{ctl}$, U87MG-Luc$_{Ang-1}$, and U87MG-Luc$_{Ang-4}$ cells were performed. Bar, 100 μm (top), 50 μm (middle), and 25 μm (bottom). D, MVD in each type of intracranial gliomas was determined by counting the CD31$^+$ blood vessels in 12 randomly selected 400× microscopic fields within four vascular hotspots.
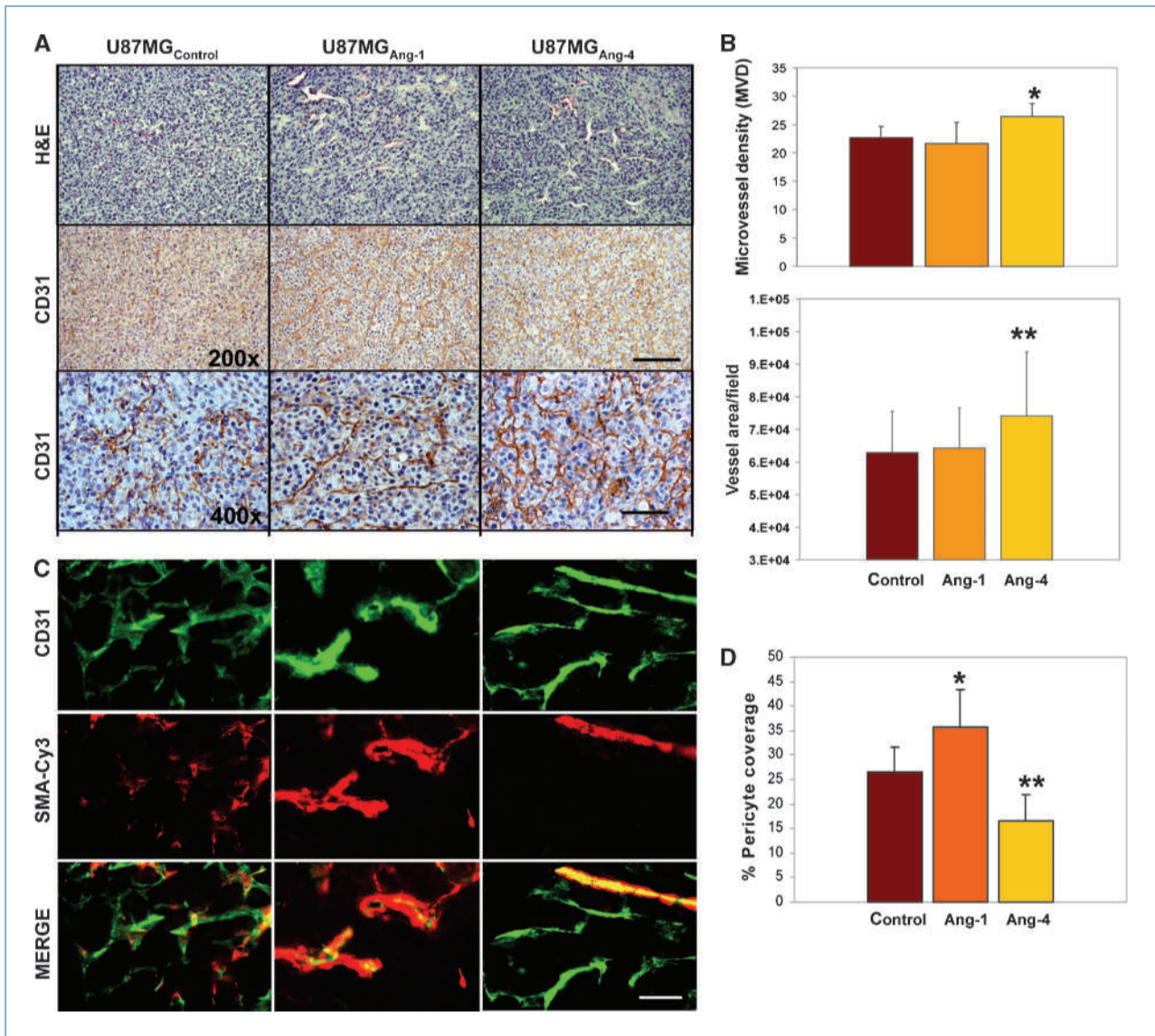
Figure 4. Ang-4 promotes GBM progression by stimulating tumor angiogenesis. A, representative H&E and anti-CD31 immunoreactivity pictures of the GBM sections derived from subcutaneous tumors of U87MG-Luc$_{Ang-1}$, U87MG-Luc$_{Ang-4}$, or U87MG-Luc$_{ctl}$. Bar, 50 μm (top and middle) and 25 μm (bottom). B, assessment of tumor angiogenesis. Top, to determine MVD, CD31$^+$ blood vessels within five randomly selected 400× microscopic fields in each of four vascular hotspots of each type glioma were counted. Bottom, to assess blood vessel area per microscopic field, the perimeter of CD31$^+$ blood vessels from four randomly selected 400× microscopic fields in each of three vascular hotspots of each type glioma were outlined, and the area was calculated using Image-Pro Plus software. C, the representative pictures of the GBM sections derived from subcutaneous gliomas of U87MG-Luc$_{Ang-1}$, U87MG-Luc$_{Ang-4}$, or U87MG-Luc$_{ctl}$ that show CD31$^+$ ECs and Cy3-SMA + perivascular cells. Merged pictures of the top and the middle are shown in the bottom panel. Bar, 20 μm. D, perivascular pericyte coverage was determined by assessing numbers of CD31$^+$ blood vessels that are covered by Cy3-SMA-positive pericytes in 10 randomly selected 400× microscopic fields of each glioma type. A blood vessel was counted as positive for pericyte coverage when at least 50% of a CD31-positive vessel was covered by SMA-positive pericytes.

growth *in vivo*. We established that knockdown of Ang-4 expression and, to a lesser extent, knockdown of Ang-1 inhibited intracranial growth of U87MG glioma (Fig. 5). We did not obtain sufficient Ang-4 knockdown in U251 cells to assess the effect of Ang-4 knockdown on U251 cell growth *in vivo*; however, we obtained the similar growth inhibitory result when Ang-1 was knocked down in U251 cells (data not shown). Together, these results suggest that Ang-4

constitutes an essential proangiogenic factor that promotes tumor angiogenesis during GBM progression and is a potential target for GBM therapy.

Ang-4 directly affects GBM cell viability and activates Erk1/2 kinase in GBM cells *in vivo*

To better understand the cellular mechanisms that mediate Ang-4 bioactivity, we first assessed the effects of Ang-4
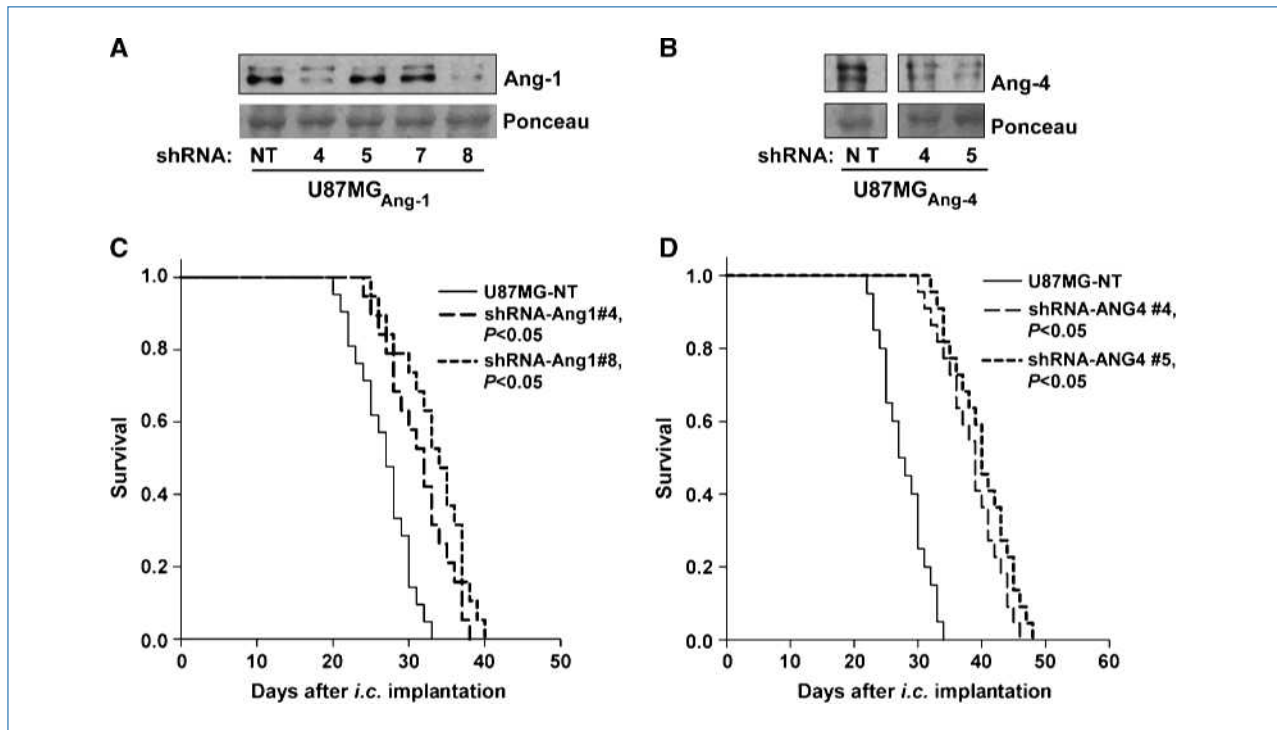
Figure 5. Knocking down the expression of Ang-4 and, to a lesser extent, Ang-1 inhibits GBM growth *in vivo*. A and B, Western blots using anti-Ang-1 (Santa Cruz) or Ang-4 (R&D Systems) antibody were performed to assess the effectiveness of Ang-1 (A) or Ang-4 (B) knockdown by a panel of TRC-shRNAs (4, 5, 7, and 8) against human Ang-1 and two TRC-shRNAs (4 and 5) against human Ang-4. A nontargeting (NT) TRC-shRNA was used as a negative control. Proteins (120 μg) in concentrated serum-free culture supernatants were loaded in each lane. The intensities of ~50-kDa ponceau-stained bands were used as the controls for protein loading and transferring efficiency (bottom). C and D, survival rates of mice following intracranial injections of the transduced U87MG-Luc with Ang-1 knockdown or NT shRNA (C) or with Ang-4 knockdown or NT shRNA (D). Fifteen mice were used for each type of transduced GBM cells.

on EC functions by performing endothelial proliferation, survival, migration, and tubulogenesis assays in the presence or absence of Ang-4. Angiopoietins and VEGF are often used ranging from 100 to 400 ng/mL and from 10 to 40 ng/mL, respectively, for *in vitro* assays (14, 26, 30, 31). We have found that 200 ng/mL of angiopoietins sufficiently affect Erk1/2 activation and EC proliferation and survival (14). This dose of angiopoietin (200 ng/mL) was therefore used in the following assays. We found that Ang-1 and Ang-4 weakly enhance EC proliferation (Supplementary Fig. S2A) and significantly promote EC survival, migration, and formation of branches in the tubulogenesis assay (Supplementary Fig. S2B–D).

Like ECs, GBM cells express Tie-2; we therefore investigated whether GBM cells can respond to angiopoietins. It is well appreciated in the research field that it is much easier to assess the activation of the Tie-2 downstream signaling molecules (Erk1/2 and AKT kinase) than to directly assess the phosphorylation status of Tie-2 (42–45). To determine whether and how angiopoietins affect the Tie-2 signaling pathway in the GBM cells, we treated serum-starved U87MG cells with Ang-1, Ang-2, or Ang-4 for various lengths of time as detailed in Fig. 6A and B. Our results showed that Ang-4, as well as Ang-1 and Ang-2, activates Erk1/2 kinases (Fig. 6A), but not Akt kinase (data not

shown), and that the activation occurred at 30 minutes and was sustained throughout 24 hours after administrating different angiopoietins (Fig. 6A and B). We then assessed GBM cell viability in the absence (control) or presence of Ang-1, Ang-4, or 10% FBS under serum-withdrawn conditions and found that Ang-4, but not Ang-1, significantly promoted U87MG viability (Fig. 6C), suggesting an important and direct prosurvival effect of Ang-4 on the GBM cells.

To further establish that Ang-4 enhances Erk1/2 kinase activity in GBM cells *in vivo*, we assessed phosphorylated Erk1/2 levels in the GBM tumors derived from U87MG-Luc$_{Ang-1}$, U87MG-Luc$_{Ang-4}$, or U87MG-Luc$_{ctl}$ cells. We found that the phosphorylated Erk1/2 levels and the numbers of phosphorylated Erk1/2–positive GBM cells were significantly increased when Ang-4, but not Ang-1, was expressed by these cells (Fig. 6D), suggesting that Ang-4–induced Erk1/2 activation occurs in growing GBM tumors *in situ*, further implying that this novel effect of Ang-4 contributes to the pro-GBM activity of Ang-4.

Discussion

Tumor angiogenesis is regulated by proangiogenic and antiangiogenic factors produced by tumor cells and the

surrounding and infiltrating host cells (8, 46). Sufficient angiogenesis is essential for GBM growth and progression. Antiangiogenesis-based therapies, such as anti-VEGF/VEGFR therapies, have shown clinical benefits for GBM patients. However, the resistance to anti-VEGF/VEGFR therapies often occurs or develops in a large fraction of patients (1–3), which demands better understanding about other angiogenic factors and their downstream signaling pathways that play essential roles and/or overlapping roles with VEGF/VEGFR in regulating tumor angiogenesis during progression of this deadly disease. In our present study, we establish the novel proangiogenic and pro-GBM growth effects of Ang-4 (Figs. 2 and 3). We show that Ang-4 is upregulated in human GBM tissues and cells compared with their normal counterparts (Fig. 1) and that increased expression of Ang-4 promotes, whereas knockdown of Ang-4 inhibits, GBM growth *in vivo*. We further show that GBM cells express Tie-2 RTK and that Ang-4 induces activation of Erk1/2 kinases in GBM cells *in vitro* and *in vivo*. Together, these results suggest for the first time that the Ang-4–Tie-2 functional axis plays an important

role in promoting tumor angiogenesis and GBM growth and is an attractive therapeutic target for GBM.

The effect of Ang-4 on tumor angiogenesis: the tales of monomeric and aggregated forms of Ang-4

Little has been done to determine the role of Ang-4 in tumor growth and angiogenesis. One published study showed that Ang-4 inhibited angiogenesis in tumors derived from a small cell lung cancer cell line, GLC19 (47). Although the authors did not describe in their paper the aggregation status of Ang-4 expressed by GLC19 tumor cells, the migration pattern of the produced Ang-4 resembles monomeric Ang-4 (ref. 47; see Fig. 2A for the patterns of monomeric and oligomeric Ang-4). It has been established that tetrameric or higher orders of aggregation of angiopoietins are required to induce Tie-2 activation (24), suggesting that monomeric or dimeric angiopoietins may bind to their receptor but are incapable of inducing its activation and therefore serve as dominant negative inhibitors of Tie-2. We have shown in our previous studies that Ang-3, the mouse orthologue of human Ang-4, is tethered on tumor
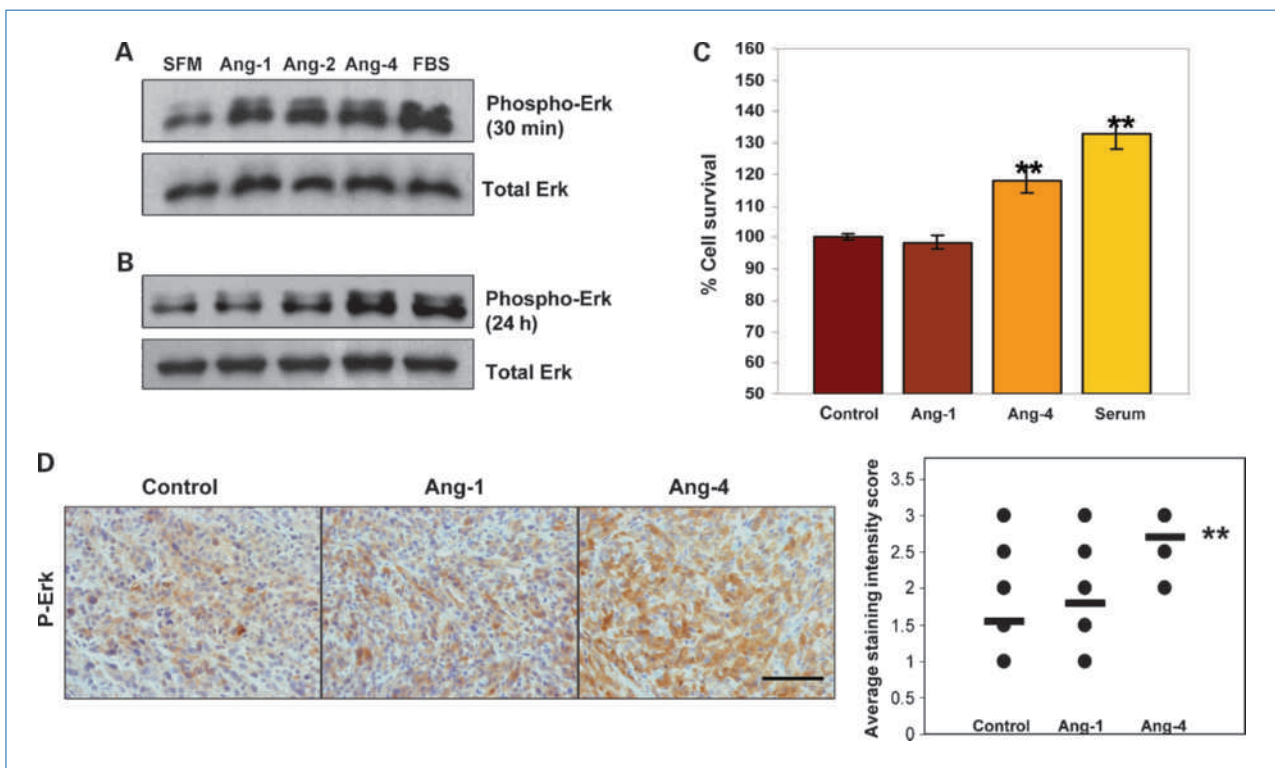
Figure 6. Ang-4 enhances GBM cell viability and activates Erk1/2 kinase *in vitro* and *in vivo*. A and B, serum-starved U87MG cells were supplied with SFM, 10%FBS containing medium, or SFM containing 200 ng/mL of Ang-1 or Ang-4 for 30 min (A) and 24 h (B). The cells were lysed, and proteins were analyzed by Western blotting with anti-phosphorylated Erk1/2 (top) or with anti-Erk1/2 (bottom) antibodies. C, cell viability assays were performed. U87MG cells were plated at 2×10^4 per well of 96-well plates in triplicates and allowed to grow overnight. The cells were switched to SFM, cultured for an additional 48 h, and then treated with 200 ng/mL of Ang-1 or Ang-4, 10%FBS, or SFM for another 24 h before cell viability was measured using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) following the manufacturer's instructions. D, levels of phosphorylated Erk1/2 in the GBM sections derived from subcutaneous tumors of U87MG-Luc$_{Ang-1}$, U87MG-Luc$_{Ang-4}$, or U87MG-Luc$_{ctl}$ were detected using anti-phosphorylated Erk1/2 antibody (Santa Cruz). The immunoreactivity intensity was scored in 10 randomly selected 400× microscopic fields using a 0 to 3 scoring system described in Materials and Methods.

cell surface by heparan sulfate proteoglycans, which maintains Ang-3 in a monomeric form (26) to exert antiangiogenic and anticancer activity (14). Both endogenous and exogenous Ang-4 expressed by the GBM cells is in the aggregated forms (Figs. 1C and 2A). We showed that the aggregated Ang-4 promoted tumor angiogenesis and GBM progression and significantly reduced survival of the mice bearing glioblastomas. Together, these results suggest that apparent discrepant results derived from different studies may reflect different aggregation statuses of Ang-4 expressed by different tumor cells and/or modified by the different tumor microenvironments and that monomeric/dimeric Ang-4 inhibits tumor angiogenesis whereas oligomeric Ang-4 promotes tumor angiogenesis.

The effects of Ang-4 on tumor angiogenesis and GBM cell viability

We showed for the first time that Ang-4 promotes GBM progression by promoting tumor angiogenesis and that Ang-4 seems to display a more potent proangiogenic activity than Ang-1 (Figs. 2–4). There were higher levels of exogenous Ang-4 than Ang-1 secreted into the culture medium by the GBM cells used in our *in vivo* experiments (Fig. 2A), which may account for the more potent proangiogenic and pro-GBM growth activity of Ang-4 observed. However, it has been established that a significant proportion of Ang-1 is incorporated into the insoluble extracellular matrix (ECM; ref. 23), which was not accounted for in the Western blot analyses outlined in Fig. 2A. Furthermore, knockdown of Ang-4 in these GBM cells resulted in more dramatic anti-GBM and antiangiogenic effects than knockdown of Ang-1 (Fig. 5 and data not shown). These *in vivo* angiopoietin knockdown results may reflect the fact that Ang-1 is predominantly produced by host cells, wherein Ang-1 expression was not eliminated by the knockdown strategy used in our study, or support a potentially more predominant role of Ang-4 in GBM angiogenesis, growth, and progression. Further studies are required to distinguish these possibilities and to reach a definitive conclusion.

We established that GBM cells express Tie-2 and showed a novel role of Ang-4 in promoting Erk1/2 kinase activation in GBM cells and in enhancing GBM cell viability. These results suggest that Ang-4 not only can stimulate tumor angiogenesis but also can promote GBM cell survival directly through Tie-2 RTK expressed by these cells. This dual function of Ang-4 makes it an ideal target for anti-GBM therapy.

Erk1/2 kinases are well-established downstream effectors of Tie-2. It has been well appreciated in the research field that Tie-2 phosphorylation is difficult to detect and that activation of the downstream effectors of Tie-2 has often been used as the indicator of activation of the Tie-2 signaling pathway (42–45). We have tried to determine whether Ang-4 directly induces Tie-2 phosphorylation in GBM cells using several different strategies and antibodies but had difficulty obtaining positive results, which may merely reflect a technical difficulty or suggest that the effect of Ang-4 on the GBM cells is Tie-2 independent. Studies have shown that Ang-1 induces PC12 neurite outgrowth in a Tie2-independent and β1-integrin–dependent manner (48) and that Ang-1 monomers reduce cardiac hypertrophy through integrins, but not Tie-2 (49). Furthermore, Ang-2 can stimulate breast cancer metastasis through the $\alpha(5)\beta(1)$ integrin, but not Tie-2–mediated pathway (50). The precise mechanism underlying the effect of Ang-4 on the viability of GBM cells requires further study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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