EXTENDED MATERIALS AND METHODS

Ethical statement

All animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees of the Lerner Research Institute (LRI), Cleveland Clinic and of Memorial Sloan-Kettering Cancer Center. The approved protocol numbers are 2013-1029 (LRI, last approved June 25, 2013), MSKCC-00-11-189 and Emory University 2003253 (EU last approved on September 15th 2015).

Cell cultures and transfections

DF-1 cells were purchased from ATCC (Manassas, VA). Cells were grown at 39°C in accordance with the manufacturer's instructions. Transfections with RCAS-PDGFB-HA and RCAS-sh-p53 were performed using a Fugene 6 transfection kit (# 11814443001; Roche, Mannheim, Germany).

Generation of RCAS/Tva system-based PDGFB-driven

Ntv-a/ink4a-arf-/- and *Gli-luc;Ntv-a;Ink4a-Arf*$^{-/-}$ mice (6-8 weeks old) were sedated with intraperitoneal (i.p.) injections of ketamine (0.1 mg/g) and xylazine (0.02 mg/g). One microliter of 5 x 10^4 RCAS-PDGFB-HA-transfected DF-1 cell suspension was delivered from a Hamilton syringe with a 30-gauge needle attached to a stereotactic fixation device (Stoelting, Wood Dale, IL). For the *N-tva/*Ef-Luc, we injected 1 μL of a 1:1 mixture of 10^5 cells containing an equal mixture of RCAS-PDGFB-HA and RACS-shp53. Coordinates in the right frontal striatum were determined according to a mouse brain atlas[1] (coordinates from bregma: A/P 1.7 mm, Right- 0.5 mm, and depth 1.5 mm). Mice were sacrificed 4-6 weeks after injections, a time point after they had developed tumors. Mice were monitored and sacrificed when they displayed signs of tumor development (lethargy, weight loss).

Dexamethasone (DEX) treatment

Glioma-bearing mice received intraperitoneal (i.p.) injections of 10 mg/kg DEX (DexaJect, NDC #11695-4017-1, Butler Schein) and Sigma (D4902-25MG). Either upon becoming symptomatic

(Figure 4A,B,C) or based on MRI scans (Figure 6H,I), mice were treated with 10 mg/kg of DEX via i.p. injection for 3, 7 or 6 days. The DEX concentration in brain tissue is expected to be ~1 μM when mice are treated with 10 mg/kg [2,3].

B20-4.1.1 treatment

B20-4.1.1 (Genentech, CA) is an anti-VEGF-A monoclonal antibody (mAb) that inhibits VEGF-A interactions with VEGF receptors (VEGFR1 and VEGFR2) *in vitro*[4] and has been shown to inhibit VEGF-A-mediated angiogenesis *in vivo*[5,6]. For *in vivo* experiments, dilutions were made in sterile saline. For most mouse experiments, we used 5 mg/kg 2x/week dosing, or alternatively for short experiments we used 5 mg/kg for 1 week (only 2 doses). Vehicle-treated mice received the same volume of saline in 10 μl volume per g/body weight. For the survival experiment in Figure 5, B20-4.1.1 was used at a dose of 7.5 mg/kg.

For Figure 4D, four different treatment groups were included: vehicle-treated, B20-4.1.1 treatment alone (5 mg/kg, 2x/week), RT alone (20 Gy treatment, radiation was dosed at 2 Gy/day for five days followed by 2 days off and again dosed for another 5 days) and B20-4.1.1 treatment combined with RT simultaneously. During treatment, mice were weighed daily to assess toxicity from a single treatment or a combination of treatments, followed by weighing every other day to track the development of tumor-associated neurological signs (severe weight loss >15%, seizures, lethargy, lack of grooming), which was the end point of survival.

MRI scans

For T2-weighted MRI scans, each animal was first anesthetized with 1-2% isoflurane in oxygen and positioned at the isocenter of a 9.4T Bruker Biospec small animal MRI scanner (Bruker Inc., Billerica, MA) at the Center for Imaging Research at Case Western Reserve University (CWRU), Cleveland, OH for Figure 6G and H and 9.4T Bruker Biospec at Emory University small animal imaging core (Figure 6H,I) (Atlanta, GA). After initial localizer scans, a high-resolution coronal proton-density RARE (Rapid Acquisition with Relaxation Enhancement) acquisition was obtained for each animal (TR/TE = 5000/12 ms, resolution = 700 x 270 x 270 μm), slice thickness 0.7mm, 12 slices total . To acquire T1-contrast MRI scans, the animals were then removed from the scanner and injected with 100 μl of the contrast agent gadolinium (Magnevist, Bayer, #NDC 50419-188-01) intravenously (i.v.). Animals were then repositioned

within the scanner for a post-contrast T1-weighted spin echo scan (TR/TE = 1000/14 ms, resolution = 700 x 270 x 270 μm). The average total examination time for each animal, including both T2 and T1, was 30 to 35 min. Tumor boundaries were manually determined based on hypo-intense regions in T2-weighted images. Total volume was determined from multi-slice T2-weighted MR data. In this model, T2-weighted hypo-intensities have been previously confirmed by histology to correlate with tumor tissue.[7] Volumes of interest were contoured around the enhancing rim of tumors on the contrast-enhancing T1 images for volume measurements as described. [8] Tumor volume and gender were used to randomize mice into treatment and vehicle groups.

Hoechst dye assay

Tumor-bearing mice were treated with B20-4.1.1 or vehicle for 6 days (two dose of 5 mg/kg, 3 days apart). At post-treatment day 6, animals were i.v. injected with 50 μl of DyLight488-labeled Lectin (Vector, #DL-1174, 2 mg/ml). After 5 min, mice were i.v. injected with a 2 mg/ml (50 μl) aqueous solution of Hoechst 33342 (Sigma, #H6024). After 5 min, mice were euthanized and perfused with fresh 4% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, #15714). The brain was extracted and fixed in 4% PFA overnight and then transferred to a 30% sucrose solution in PBS for at least 72 h. Brains were cut into 40 μm slices using a Leica SM2010 R microtome. For confocal microscopy (Leica CTR6500), slices were washed in PBS and mounted onto a glass slide with anti-fade mounting medium and further analyzed. For quantification, at least 5 representative 10X images per tumor were taken to obtain the average number of positive cells per tumor and areas positive for Hoechst were quantified using ImageJ. At least 3 tumors were included in the B20-4.1.1 and vehicle groups.

TUNEL assay

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays for optical microscopy were performed on 5 μm sections using a Terminal Transferase recombinant kit (Roche, #3333566). Fluorescent TUNEL assays were performed using the In Situ Cell Detection Kit, TMR red (Roche, #12156792910).

Tissue processing

Animals were sedated with i.p. injections of ketamine (0.1 mg/g) and xylazine (0.02 mg/g), perfused with ice-cold Ringer's solution, and sacrificed. Brains were fixed in 10% neutral-buffered formalin for 72 h at room temperature, processed in a tissue processer (Leica TP1050), embedded in paraffin, sectioned (5 μm), and slide-mounted for immunohistochemistry (IHC) and immunofluorescence staining. Sections were deparaffinized in Histo-Clear (Richard-Allan Scientific, Walldorf, Germany) and were passed through graded alcohols before staining with a hematoxylin and eosin (H&E) reagent.

Immunohistochemistry

IHC staining was performed using an automated staining processor (Ventana Discovery XT Roche Inc., Tucson, AZ). The following primary antibodies were used at the following dilutions: rabbit polyclonal anti-Iba1, 1:250 (Wako, #019-19741); rabbit polyclonal anti-Olig2, 1:250 (Millipore, #AB9610); mouse monoclonal anti-PCNA, 1:2000 (DAKO, #M0879); rabbit polyclonal anti-phospho-Histone H3, 1:500 (Millipore, #06-570); rat monoclonal anti-CD31, 1:50 (Dianova, #DIA 310); rabbit polyclonal anti-KI67, 1:100 (Vector Laboratories, #VP-K451) and rabbit polyclonal anti-cleaved caspase 3 1:100 (Cell Signaling, #9661). Nuclei were counterstained with hematoxylin. Slides were visualized using a Leica DM5500 B widefield microscope. For quantification, 5 representative 20X images were taken for each tumor to obtain the average number of positive cells per tumor and were quantified using ImageJ. For some quantification, slides were scanned using a Spectrum system from Aperio and analyzed using ImageScop v11.1.2.760 (Aperio). Tumor regions were selected based on H&E staining and percent positivity was based on the percentage of total nuclei that were deemed positive by pixel density.

Immunofluorescence

Paraffin-embedded sections (5 μm) were deparaffinized in Histo-Clear (Richard-Allan Scientific # 6901) and passed through graded alcohols. Next, we performed antigen retrieval with citric acid (vector lab #H-3300) in a boiling water bath for 15 min. After two washes in PBS and permeabilization in 0.3% triton in 0.1 M PBS for 45 min, sections were incubated in 0.1 M PBS containing 2% BSA, 5% NDS, and 0.1% Triton for 1 h at room temperature. For staining, sections were incubated with the following antibodies overnight at 4°C in PBS plus 1% BSA: rabbit polyclonal anti-Iba1, 1:200 (Wako Pure Chemicals, #019-19741); rabbit polyclonal anti-

Olig2, 1:200 (Millipore, #AB9610); rat monoclonal anti-CD31, 1:50 (Dianova, #DIA 310); mouse monoclonal anti-GFAP, 1:1000 (Merck Millipore, #MAB360);. Secondary antibodies were conjugated to different Alexa-Fluor dyes (488, 555, 568 and 647) and used at a dilution of 1:500 in PBS. Nuclear counterstaining was performed with DAPI (Sigma, #D9542). Slides were visualized using a Leica CTR6500 microscope. For quantification, at least 5 representative 20X images for each tumor were taken to obtain the average number of positive cells per tumor and were quantified using ImageJ.

Bioluminescence imaging (BLI)

Mice were anesthetized with 3% isoflurane. The hair covering the head was shaved before retro-orbital injection with 75 mg/kg body weight of D-luciferin (30 mg/ml dH_2O at 2.5 µl per gram mouse weight). One min after injection of D-luciferin, images were acquired for 5 min for *Gli-luc;Ntv-a;Ink4a-Arf*$^{-/-}$ and 15 min for *Nestin-tv-a;E2F1-Luc* mice with an IVIS 100 (Xenogen) imaging system. A photographic image was taken, onto which the pseudocolor image representing the spatial distribution of photon count was projected. A circular region (1.5 cm diameter) of interest (ROI) covering the tumor region was defined and used to quantify the bioluminescent signals in all experiments. All representative images were formatted using the same maximum and minimum threshold parameters. DEX-treated mice were treated immediately after imaging on Day 0, and subsequently were treated 1 h before imaging on days 1 and 2. BLI was also used for monitoring the response to treatment. Tumor growth was monitored every 5 days using BLI. As soon as tumor-bearing mice produced a signal equal to at least 10^3 photons/sec, they were randomized into different treatment or vehicle groups.

Quantification of total vessel area and average vessel size

CD31+ vessels were quantified by counting vessel numbers and using the freehand tool with ImageJ and Fiji software to determine total area covered by vessels-total vessel area (excluding lumens). Average vessel size was calculated from total area and vessel density per 20X field. At least five and up to 15 20X images were analyzed per tumor to obtain representative values per tumor sample.

Quantification of necrotic areas

Paraffin-embedded tumor sections were stained with H&E. Subsequently, slides were scanned using a Spectrum system from Aperio and analyzed using ImageScop v11.1.2.760 (Aperio). The tumor region, as well as regions of necrosis, were selected and the necrotic index was calculated according to the following formula; Necrotic index = Area of necrosis (μm^2)/Total area of tumor section (μm^2)[9].

Radiation treatment (RT)

Mice were lightly sedated with ketamine (0.1 mg/g) and xylazine (0.02 mg/g) and were irradiated using a Pantak X-RAD 320 x-ray unit (PXI, North Branford, CT). Only the head was irradiated; the rest of the mouse was shielded with a specialized lead jig. The total dose and schedules are described in detail in Figures 5.

Primary glioma cell cultures

Glioma-bearing mice were euthanized and transcardially perfused with ice-cold PBS. The tumor was grossly dissected and enzymatically digested in Hanks balanced salt solution containing 12% papain and 10 μg/ml DNase at 37˚C for 15 min, with subsequent inactivation using ovomucoid (1 mg/mL) (Worthington, Lakewood, NJ). The resulting single cell suspension was resuspended in DMEM media containing 10% FBS, 2mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. In order to mimic *in vivo* conditions, the primary cultures were treated with physiologically relevant, low and high concentrations (i.e. 0.1, 1 and 10 μM = 39.5 ng/ml, 395 ng/ml and 3950 ng/ml) of DEX. The cultures used in these experiments were freshly isolated from tumors and passaged in serum a maximum of 5 times.

MTT assays

Cell viability was measured using MTT assays (Sigma No. M5655). Primary glioma cultures were seeded at a density of 1000 per well in 96-well plates. A 50 mM stock solution of DEX (Sigma D4902) was prepared by dissolving the drug in DMSO. Cells were allowed to attach overnight and DEX or vehicle control was added to the appropriate wells. After 3 days of drug exposure, MTT was added and allowed to incubate for 3 h. Afterwards, the media was aspirated and the purple formazan crystals were dissolved in 100 μl DMSO (Fisher). Absorbance was

measured at wavelengths of 570/690 nm using a FLx800 fluorescence microplate reader (BioTek Instruments, Inc.).

Cell cycle analysis

Primary glioma cultures were seeded at a density of 4000 cells/mL in 10 cm plates. Cells were allowed to attach to the plates overnight and then treated with DMSO or 0.1, 1 and 10 μM DEX for 3 days. After trypsinization and washing in ice-cold PBS, cells were fixed in ice-cold 70% ethanol for at least 1 h. Subsequently, cells were washed with PBS and stained with 0.05 mg/ml propidium iodide (PI) in the presence of 0.1 mg/ml RNase and then analyzed using a BD Accuri C6 flow cytometer. Quantification of different phases of the cell cycle (G0/G1, S, G2/M) was performed using Modfit LT.

Microarray analysis

Gliomas were grossly dissected from four untreated and four DEX-treated (3 days @ 10 mg/kg) mice. Upon dissection, tissue was flash-frozen in liquid nitrogen, homogenized, and dissolved in Trizol (Invitrogen, Carlsbad, CA). RNA was extracted and processed for Illumina mouse-ref 8 array platform. Array data were analyzed using Partek analysis software. ANOVA statistics were run to determine significantly altered genes. The DEX gene signature was generated using an unadjusted p-value of $p > 5x10^{-4}$. Gene lists were uploaded into Ingenuity Pathway Analysis (www.ingenuity.com) for analysis of relevant pathways.

qRT-PCR

Total RNA was extracted from control and DEX-treated mouse tumor samples using Trizol reagent (Invitrogen). The yield and purity of the RNA was confirmed using Nanodrop; ND-1000 V3, spectrophotometer. cDNA was synthetized using transcriptor first strand cDNA synthesis kit using 1 μg of RNA (Roche Applied Sciences, Indianapolis, MN). Primers were designed using the Roche Universal Probe Library. The following primers were used: *Aurka* 5′-TTGCAGACTTCGGGTGGT-3′ (forward) and 5′-TCCAGGGTGCCACACATT-3′ (reverse), *Bub1b* 5′-TTACGCCGTACGTGGAAGA-3′ (forward) and 5′-GCTCAATCTTGCATGGTGTC-3′ (reverse), *Ccnb1* 5′-TGCATTTTGCTCCTTCTCAA-3′ (forward) and 5′-CAGGAAGCAGGGAGTCTTCA-3′ (reverse), *cdc20* 5′-ACATCAAGGGCGCTGTCAAG-3′

(forward) and 5′-AATGTGCCGGTCACTGGT-3′ (reverse), *Cenpa* 5′-CAAGGAGGAGACCCTCCAG -3′ (forward) and 5′-TTCTGTCTTCTGCGCAGTGT-3′ (reverse), *Kif2c* 5′-CGAAGGAGGTACCACAAAAGG-3′ (forward) and 5′-TTCGGTCGTAAGGGAAGAAG-3′ (reverse), *Plk1* 5′-TTGTAGTTTTGGAGCTCTGTCG-3′ (forward) and 5′-AGTGCCTTCCTCCTCTTGTG-3′ (reverse), *TBP* 5′-GGCCTCTCAGAAGCATCACTA-3′ (forward) and 5′-GCCAAGCCCTGAGCATAA-3′ (reverse),

The housekeeping gene, TATA-binding protein (TBP), was used for normalization. The qRT-PCR was performed using a LC480 light cycler under the following conditions; activation cycle for 5 min at 95°C, followed by 50 cycles of amplification. Each amplification cycle started with an activation step for 5 min at 95°C, an annealing step for 30 sec at 60°C, and finally an extension step at 72°C for 10 sec.

TCGA analysis

Expression values for each gene of interest were obtained from the MSKCC computational biology cancer genomics portal (http://www.cbioportal.org/cgx/index.do) which has annotated TCGA data [10,11]. Survival data were obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/).

Retrospective clinical analyses

A retrospective clinical analysis was performed with permission from the Institutional Review Board of MSKCC. Adult patients (>18 years old) at the time of histologic diagnosis of a glioblastoma who underwent external beam radiotherapy between 1998 and 2008 were eligible for the study. From initial 677 pathologically confirmed glioblastomas, 55 were excluded (17 patients ≤18 years of age, 1 patient with brainstem glioma, 10 patients missing decisive RPA information and 27 patients with unknown DEX status at start of RT).Patients were not eligible for the study if more than one of the clinical or treatment characteristics listed were missing from the medical records. Clinical characteristics of patients were recorded, including: age at diagnosis, Karnofsky performance score (KPS), mental status (mini-mental status from initial encounter or description of patient interaction from first encounter), neurologic functional

status (defined as "working" or "not working" at time of diagnosis, as described in the original RTOG RPA analysis), and duration of symptoms before diagnosis. Treatment characteristics were recorded, including: extent of surgery (biopsy, subtotal resection, or gross total resection), RT dose and fractionation, use of corticosteroids at the start of RT, and use of temozolomide during RT. Clinical and treatment characteristics were used to group patients according the Radiation Therapy Oncology Group recursive partitioning analysis (RTOG RPA) classification system[12,13]. Categorical multivariate cox regression models were constructed correlating RTOG RPA class, initial chemotherapy use, and baseline corticosteroid use with clinical outcomes.

In the retrospective clinical analysis of 573 patients from the EORTC 26981/22981 trial, correlation analyses of baseline steroid use and dose with outcomes were performed by univariate and multivariate Cox regression models. In the whole population, all models were stratified by treatment and all multivariate models were adjusted for main prognostic factors (age, extent of surgery, performance status). In this subgroup study, all analyses were performed at an exploratory 5% significance level. P-values higher than 5% but lower than 10% were considered as "borderline non-significant".

We also studied 832 patients with glioblastoma enrolled in the German Glioma Network (GGN) (www.gliomnetzwerk.de). Diagnosis for all patients was confirmed by central pathology review according to the WHO classification of tumors of the central nervous system. Inclusion criteria for this analysis were RT alone or RT plus chemotherapy (TMZ in all but 17 patients) as the first-line therapy as well as documentation about medication with steroids during radiotherapy (yes or no). Progression-free survival (PFS) was calculated from the day of first surgery until progression, death or the end of follow-up. Overall survival (OS) was calculated from the day of first surgery until death or the end of follow-up. Logrank tests were used to compare outcome data. Cox regression models were built to assess the independent association of steroid use with OS adjusted for relevant clinical parameters.

Statistical analysis

Graphs were created using GraphPad Prism 6 (GraphPad Software, San Diego, CA) and were

analyzed as noted in the figure legends. Cox proportional hazards analysis was performed in the survival package 'survival' version 2.36-10[14] in the R studio software[15]. In the retrospective clinical analysis of EORTC 26981/22981 trial, SAS 9.4 was used, in particular the PHREG procedure for Cox modeling. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, no asterisks = not significant.

References

1. Keith B, Franklin G. The Mouse Brain in Steriotaxic Coordinates. San Diego 1997;Academic Press.
2. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2008;22:659-61.
3. Nestler U, Winking M, Boker DK. The tissue level of dexamethasone in human brain tumors is about 1000 times lower than the cytotoxic concentration in cell culture. Neurological research 2002;24:479-82.
4. Fuh G, Wu P, Liang WC, et al. Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab. The Journal of biological chemistry 2006;281:6625-31.
5. Liang WC, Wu X, Peale FV, et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. The Journal of biological chemistry 2006;281:951-61.
6. Bagri A, Kouros-Mehr H, Leong KG, Plowman GD. Use of anti-VEGF adjuvant therapy in cancer: challenges and rationale. Trends Mol Med 2010;16:122-32.
7. Koutcher JA, Hu X, Xu S, et al. MRI of mouse models for gliomas shows similarities to humans and can be used to identify mice for preclinical trials. Neoplasia 2002;4:480-5.
8. McConville P, Hambardzumyan D, Moody JB, et al. Magnetic resonance imaging determination of tumor grade and early response to temozolomide in a genetically engineered mouse model of glioma. Clinical cancer research : an official journal of the American Association for Cancer Research 2007;13:2897-904.
9. Li L, Lin X, Staver M, et al. Evaluating hypoxia-inducible factor-1alpha as a cancer therapeutic target via inducible RNA interference in vivo. Cancer Res 2005;65:7249-58.
10. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer discovery 2012;2:401-4.
11. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Science signaling 2013;6:pl1.
12. Curran WJ, Jr., Scott CB, Horton J, et al. Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. Journal of the National Cancer Institute 1993;85:704-10.
13. Scott CB, Scarantino C, Urtasun R, et al. Validation and predictive power of Radiation Therapy Oncology Group (RTOG) recursive partitioning analysis classes for malignant glioma patients: a report using RTOG 90-06. Int J Radiat Oncol Biol Phys 1998;40:51-5.

14. Borgan Ø. Modeling Survival Data: Extending the Cox Model. Terry M. Therneau and Patricia M. Grambsch, Springer-Verlag, New York, 2000. No. of pages: xiii + 350. ISBN 0-387-98784-3. Stat Med 2001;20:2053-4.
15. RStudio. RStudio: Integrated development environment for R Version 0.96.122 ed. Boston, MA.2012:Retrieved May 11, 2013.