STAT3 Blockade Inhibits Radiation-Induced Malignant Progression in Glioma

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Abstract

High grade gliomas (HGG) are classified into four subgroups based on transcriptional signatures and phenotypic characteristics. In particular, the proneural-to-mesenchymal transition (PMT) is associated with increased malignancy, poor prognosis, and disease recurrence, but the underlying causes of PMT are still unclear. In this study, we investigated whether radiotherapy promotes PMT using a genetically engineered mouse model of proneural HGG. We found that cranial ionizing radiation induced robust and durable PMT in tumors. Additionally, we isolated primary proneural HGG cells from mouse and human tumors and demonstrate that radiation induced a sustained cell-intrinsic mesenchymal transition associated with increased invasiveness and resistance to the alkylating agent temozolomide. Expectedly, irradiation-induced PMT was also associated with activation of the STAT3 transcription factor, and the combination of STAT3 blockade using JAK2 inhibitors with radiation abrogated the mesenchymal transition and extended survival of mice. Taken together, our data suggest that clinical JAK2 inhibitors should be tested in conjunction with radiation in patients with proneural HGG as a new strategy for blocking the emergence of therapy-resistant mesenchymal tumors at relapse. Cancer Res; 75(20); 4302-11. ©2015 AACR.

Introduction

Malignant high-grade gliomas (HGG) are highly lethal brain tumors that include anaplastic oligodendroglioma, anaplastic astrocytoma, and glioblastoma (GBM). The introduction of radiotherapy improved median survival of HGG patients from 6 months to one year (1, 2). In addition to radiation, multimodal therapy improved median survival of HGG patients from 6 months to one year (1, 2). In addition to radiation, multimodal therapy includes surgical resection and systemic treatment with alkylating agents (2–4). Despite treatment, however, patients with HGGs invariably relapse and recurrent tumors are typically refractory to further therapies.

Transcriptional profiling has categorized HGGs into distinct groups, represented as a continuum between proneural and mesenchymal subclasses (5–9). The mesenchymal signature correlates with poor prognosis when compared with proneural tumors (10), and proneural tumors tend to shift to a mesenchymal signature at recurrence (5, 11–13). The proneural-to-mesenchymal transition (PMT) is enriched for genes associated with an epithelial-to-mesenchymal transition (EMT; refs. 14, 15), and likely represents an analogous process. Genes important in the mesenchymal phenotype in HGGs include transcription factors CEBPβ, STAT3 (16), TAZ (17), NF-kB (10), and ID proteins (18). Signals from the microenvironment also activate the mesenchymal phenotype, which is enriched in genes associated with inflammation, necrosis, and angiogenesis (6, 10, 19).

Radiation may contribute to a PMT in glioma. Transcriptional profiling of PDGFB-driven murine glioma cells isolated 6 hours after irradiation revealed a mesenchymal shift in gene expression (20). Although durability of radiation-induced PMT in glioma has not been addressed in vivo, radiation of cultured human proneural GBM cells induced a PMT that was sustained for at least 5 days (21). If radiation therapy in patients induces a sustained mesenchymal switch in the remaining radioresistant cells, blocking this transition may prevent relapse, or block therapy resistance in relapsed tumors.

Here, we radiated proneural HGG in a genetically engineered glioma mouse model, demonstrating a durable PMT. Radiation-induced PMT occurred in a cell-intrinsic manner, resulting in increased cell motility and invasion, and was associated with resistance to the alkylating agent temozolomide. Importantly, STAT3 signaling was induced in irradiated cells, and pharmacologic inhibition of STAT3 using clinical agents effectively prevented PMT. Our results suggest that radiotherapy as part of standard of care for patients with proneural HGG may contribute to a PMT in recurrent tumors, and that blockade of JAK2–STAT3 signaling may prevent PMT and recurrence, increasing the survival of HGG patients.
Materials and Methods

Mice

All mouse experiments were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of California, San Francisco (San Francisco, CA). Mice were genotyped by Transnetyx, Inc. ERBB2 (measuring 0.5%), H3 K4me3, H3 K27me3, and H3 K56Ac by ChIP-qPCR. All samples were prepared by a previously published protocol as previously published (22, 24). GFAP-HA-V12-Ras-IRESCreER+/C14 mice were genotyped as previously published (23).

Tissue preparation

Mice were perfused transcardially using PBS followed by 4% paraformaldehyde (PFA). Brains were harvested and incubated overnight in 4% PFA at 4°C, and transferred to 30% sucrose at 4°C. Brains were sectioned using a cryostat (Leica) as 30 μm free-floating sections. Sections were stored in cryoprotectant solution (25% glycerol, 25% ethylene glycol, 50% 0.1 mol/L phosphate buffer) at −20°C until immunostaining procedure.

Immunofluorescence

Free-floating sections were retrieved from cryoprotectant solution and washed 3 × 10 minutes in TBS (pH 7.4). The sections were then incubated in blocking solution (5% donkey serum, 1% BSA, 0.3% TritonX-100) for 1 hour at room temperature. Primary antibodies were diluted in blocking solution and incubated on sections overnight at 4°C. Antibodies used were SOX10 (1:200; Santa Cruz Biotechnology), PDGFRα (1:200; Cell Signaling Technology), and CD44 (1:400; BD Pharmingen). Sections were washed in TBS, and incubated with the appropriate Alexa488 (1:400), 555 (1:400), 647 (1:200) secondary antibodies (Life Technologies). Sections were then incubated in blocking solution (5% donkey serum, 1% BSA, 0.3% TritonX-100) for 1 hour at room temperature. The sections were washed in TBS, and incubated with the appropriate Alexa488 (1:400), 555 (1:400), 647 (1:200) secondary antibodies (Life Technologies) in blocking solution for 2 hours at room temperature. The sections were washed in TBS at 3 × 10 minutes (DAPI was included in the second wash), followed bymounting with Pro-Long Gold antifade reagent (Life Technologies).

Imaging and quantification

Mosaic images were acquired and tiled using the Zeiss M1 AxioImager and the Zen software. For quantification, tumor regions (DAPI bright) were identified, and region of interest was determined, avoiding areas at the edge of tumor/section and necrotic regions. The mean fluorescence intensity was determined using the image analysis tool.

Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>mSox10</td>
<td>GTGCCAGCAAGAGCAAGGCCG</td>
<td>CTGCCTCCGTCCTTCGCTG</td>
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<td>mPdgfra</td>
<td>ACAAGCCGAGGCACCCCTCTC</td>
<td>CTCACGCGGCAACAGGGTGA</td>
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<td>mCd44</td>
<td>TCAGTTGCGCTTTCACCACTCAG</td>
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<td>mChi31</td>
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<tr>
<td>hGAPDH</td>
<td>CAATGACCGGGCTCGTACC</td>
<td>GACAAGCTCCGCTTCTGAC</td>
</tr>
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Western blotting

For tissue specimens, samples were frozen in dry ice and stored at −80°C until processed. Samples were homogenized and sonicated in cell lysis buffer (Cell Signaling Technology). For cells, samples were washed in ice-cold PBS before lysis with cell lysis buffer (Cell Signaling Technology). All samples were clarified by centrifugation at 16,000 rpm for 10 minutes at 4°C and quantified using the Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of total protein lysates were loaded and resolved on a 4% to 12% Bis-Tris Gel with MOPS Running buffer and transferred to PVDF membranes. The membranes were blotted with antibodies against PDGFRα, pSTAT3 (Tyr705), STAT3 (all from Cell Signaling Technology), CD44 (Thermo Scientific), SOX10 (Santa Cruz Technologies), and GAPDH (Millipore). Bound antibodies were detected with horseradish peroxidase-linked antibody against mouse (Calbiochem), rabbit (Calbiochem), or goat (Santa Cruz Technology) IgG, followed by ECL (Amersham).

Quantitative PCR analysis

Dissected tissue samples or cells were homogenized using the QiAShredder spin column (Qiagen). Total RNA was extracted from specimens using RNeasy Mini Kit (Qiagen) and contaminating DNA was digested using RNase-free DNase Kit (Qiagen). cDNA was synthesized using 500 ng total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or SuperScript VILO (Life Technologies). Real-time PCR was performed with the intron-spanning primers (5 μmol/L) listed in Table 1 and KAPA SYBR FAST ABI Prism qPCR Master Mix (KAPA Biosystems) on a 7900 HT Fast Real-Time PCR system (Applied Biosystems).

Allograft experiments

FVB/N recipient mice were anesthetized using Ketamine and Xylazine. Oligodendroglioma tumor cells were dissociated with accutase and intracranially grafted into mice using a Hamilton syringe and stereotactic equipment. Injections were done at coordinates 2 mm anterior and 1.5 mm lateral of the right hemisphere relative to Bregma, at a depth of 3 mm.

Ionizing radiation

Mice and cells were placed in a cesium-137 source (J.L. Shepherd & Associates) irradiator. Mice were shielded with iron collimators to focus the beam to the brain. Mice were given cranial...
irradiation of single dose of 10 Gy, or 10 fractions of 2 Gy, delivered at a rate of 5 fractions per week. 1 fraction per day for 2 weeks (total 10 Gy). Cells were irradiated using a single dose of 10 Gy.

Drug treatment

AZD1480 powder was purchased from ChemieTek and resuspended before each administration in water supplemented with 0.5% hypromellose (Sigma #H3785) and 0.1% Tween-80 (Sigma #P4780). Brief sonication ensured a uniform suspension before delivery to mice. Mice were given 30 mg/kg drug treatment or vehicle by oral gavage daily for 8 days.

Flow cytometry

Cells were dissociated briefly with accutase to obtain single cell suspensions. Samples were incubated for 20 minutes on ice with DyLight 800 viability dye (Thermo Scientific) and then incubated for 30 minutes on ice in autoMACS buffer with 1:100 Brilliant Violet 421 anti-mouse CD140A (BD Biosciences) and 1:100 APC anti-mouse/human CD44 (Biolegend). Cells were washed once, fixed with BD cytofix on 30 minutes on ice, and washed again until analysis with an LSRII flow cytometer (BD Biosciences). For intracellular p-STAT3 staining, cells were permeabilized with cold Perm Buffer III (BD Biosciences) for 30 minutes after fixation, and then incubated with 1:10 PE anti-pSTAT3 (pY705) for 30 minutes. Cells were washed with autoMACS buffer once, before flow analysis.

CyQuant proliferation assay

Five-day post-irradiated (or control) cells were plated at 3,000 cells per well in 96-well polyornithine/laminin coated plates in NB media. Temozolomide (100µmol/L) was added a day after plating, and DNA content was analyzed 4 days later, using the CyQuant NF proliferation assay (Life Technologies), as previously described (24). Fluorescent dye labels DNA that gives an indirect measure of the number of cells.

2D motility

Cells were seeded on laminin-coated glass coverslips and subjected to ionizing radiation (IR). Four days following IR, the coverslips were then mounted on custom-built microscope-compatible imaging chambers and cells were imaged on a Nikon Eclipse Ti-E base microscope equipped with the Yokogawa CSU-X1 confocal scanner and Andor’s iXon3 EMCCD camera. Brightfield images were captured every 15 minutes over 8 hours. Cell migration time course images were compiled and cell speed was analyzed on a single cell basis with Imagel Software (NIH) and Manual Tracking plugin (Fabrice P. Cordelières, Institut Curie, Orsay, France).

3D neurosphere invasion

Cells were seeded on nonadherent plates and subjected to IR. Four days following IR, tumorspheres were embedded in 3D Matrigel gels (BD Biosciences) and mounted on custom-built microscope-compatible imaging chambers and imaged on a Nikon Eclipse Ti-E base microscope equipped with the Yokogawa CSU-X1 confocal scanner and Andor’s iXon3 EMCCD camera. Brightfield images were captured every 30 minutes over 24 hours. Invasion areas were identified and quantified using Imagel Software (NIH).

Cellular rheology

Cells were sparsely seeded on laminin-coated glass coverslips and subjected to IR. Four days following IR, atomic force microscopy measurements were performed directly on single cells using an Asylum MFP 3D BIO inverted optical atomic force microscope mounted on a Nikon TE2000-U inverted fluorescent microscope (Asylum Research). Cellular rheology (Young’s modulus) was determined by nano-indenting single cells with low force (below 2 nN) to ensure shallow cell indentation using calibrated Si3N4 cantilevers (60 µm/nm) modified with a 5-µm borosilicate microsphere (Novascan Tech.). The resulting force-distance curves were used to derive the elastic modulus of cells by fitting Hertz model to the curves with the Igor Pro AFM Software.

Statistical analysis

Statistical tests were performed using GraphPad Prism v6.0 software. Statistical analyses for experiments with two groups were performed using Student t test. Statistical analyses for experiments with more than two groups were performed using one-way ANOVA with Tukey multiple comparisons test. Kaplan–Meier survival analysis was conducted and the P value of the comparison between survival curves was determined to be significant by the log-rank (Mantel–Cox) test.

Results

Oligodendroglial progenitor cell-derived HGG has proneural character

To study the effects of radiation on murine gliomas, we first compared protein and gene expression in tumors arising in two genetically engineered mouse models for glioma (22, 23). We chose a mouse model (ERB/p53<sup>−/−</sup>) predisposed to oligodendroglioma (22), the most proneural of gliomas (8), that we previously showed is derived from oligodendroglial progenitor cells (OPC; ref. 24). We compared the ERB/p53<sup>−/−</sup> tumors to those of an astrocyte-derived model for glioma driven by GFAP-HRAS<sup>120V</sup>-G(RAS; refs. 23, 25). Glioma mutant for isocitrate dehydrogenase 1/2 (IDH1/2) do not undergo a mesenchymal transition (11), and ERB/p53<sup>−/−</sup> tumors are wild-type for IDH1 (data not shown). Immunofluorescent staining of ERB/p53<sup>−/−</sup> tumors showed high levels of SOX10 and PDGFRa proteins, known markers of OPCs and human proneural HGGs (6, 26), compared with the low levels observed in the G-RAS model (Fig. 1A and B). Levels of the cell surface glycoprotein CD44, a known marker of OPCs and human proneural HGGs (6, 26), were lower in ERB/p53<sup>−/−</sup> versus G-RAS gliomas (Fig. 1A and B). Preparation of whole brain protein lysates from tumor-bearing animals demonstrated that CD44-expressing mesenchymal (G-RAS) gliomas also showed increased abundance of phosphorylated STAT3, a known driver of PMT (16), relative to PDGFRa-expressing proneural HGGs (Fig. 1C).

RT-PCR of RNA from tumors confirmed higher expression of Sox10 and Pdgfra mRNAs in proneural HGGs compared with mesenchymal tumors and normal wild-type (WT) brain (Fig. 1D). Relative mRNA expression for known markers of human mesenchymal tumors (CD44, vimentin (Vim), and Tgfb1, Chitinase-3-like protein 1 (Chit3l1 – mouse homologue for the human Ykl-40 gene) and Podoplanin (Pdpn; ref. 6) was elevated in mesenchymal murine gliomas compared with both proneural tumors and WT mouse brain (Fig. 1D and...
Supplementary Fig. S1A). Our data demonstrate that OPC- and astrocyte-derived murine gliomas display proneural and mesenchymal phenotypes, respectively.

In vivo radiation of proneural murine HGG induces PMT

To study the effect of cranial radiation in mice with proneural HGGs (ERB/p53<sup>C0/C0</sup>/C0; PN), we analyzed proneural and mesenchymal markers in tumors from symptomatic animals, 7 days after a single 10 Gy dose of IR. Radiation reduced levels of proneural proteins (SOX10, PDGFRα) and increased abundance of the mesenchymal marker CD44 (Fig. 2A and B). Immunoblotting of protein lysates from tumors demonstrated reduced levels of PDGFRα following radiation (Supplementary Fig. S2A). Gene expression analysis confirmed that radiation reduced mRNA levels of proneural genes Sox10 and Pdgfra, and mesenchymal genes CD44, Vim, and Tgfb1 in isolated proneural (purple) and mesenchymal (red) tumors compared with normal wild-type (WT) mouse brain.

*, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 1.

OPC- and astrocyte-derived transgenic murine glioma models display proneural and mesenchymal characteristics, respectively. A, immunofluorescence of proneural markers (SOX10, PDGFRα) and mesenchymal marker (CD44) in OPC-derived HGG model (ERB/p53<sup>C0/C0</sup>; PN) and astrocyte-derived glioma model (G-RAS; MES). Red region of interest excludes necrotic regions and tumor edges for relative fluorescence quantification. Yellow boxes indicate regions shown in higher magnification images in A. Scale bars, 1,000 and 50 μm. B, fluorescent intensity quantification of SOX10, PDGFRα, and CD44 in tumors (region of interest marked red in A) in proneural (purple) and mesenchymal (red) murine glioma models. C, immunoblotting for PDGFRα, CD44, and phosphorylated STAT3 of whole brain lysates from mice with proneural and mesenchymal gliomas. D, relative mRNA expression by RT-PCR of proneural genes Sox10 and Pdgfra, and mesenchymal genes CD44, Vim, and Tgfb1 in isolated proneural (purple) and mesenchymal (red) tumors compared with normal wild-type (WT) mouse brain.

www.aacrjournals.org Cancer Res; 75(20) October 15, 2015 4305

Published OnlineFirst August 17, 2015; DOI: 10.1158/0008-5472.CAN-14-3331

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Sox10 and CD44, and increased expression of mesenchymal genes CD44, Vim, and Fibronectin1 (Fn1; Fig. 2C).

To better model the radiation regimen of patients, we also irradiated symptomatic mice with 5 consecutive daily doses of fractionated radiation (2 Gy) each week for 2 weeks. Fractionated radiation led to improvement in median survival (81.5 vs. 60 days; Supplementary Fig. S2B). Similar to a single 10 Gy dose of radiation, tumors treated with fractionated radiation showed increased levels of SOX10 and PDGFRα proteins, and decreased levels of CD44 (Supplementary Fig. S2C).

**Irradiation induces a sustained cell-intrinsic PMT in HGG Cells**

Both cell-intrinsic changes as well as extrinsic cues from the tumor microenvironment can induce a PMT in glioma (10, 21). To determine whether radiation-induced PMT occurs in a cell-intrinsic manner, we isolated tumor cells from mice with proneural HGGs, and irradiated low-passage tumor cells in vitro. Immunoblotting and real-time PCR analyses revealed a PMT starting 3 days after irradiation (Supplementary Fig. S3A and S3B). On the basis of these results, we subsequently analyzed tumor cells at 5 days after irradiation. RT-PCR analysis showed significant upregulation of a panel of mesenchymal genes (CD44, Tgfβ1, Fnl1, Chi3l1, Vim, Slug, Snail, Twist) in irradiated murine proneural HGG cells 5 days following radiation (10 Gy), compared with nonirradiated controls (Fig. 3A). Consistent with observations by others (21), we demonstrated that radiation of proneural human GBM cells led to reduced expression of SOX10 and increased expression of CD44 mRNAs (Supplementary Fig. S3C). To confirm that a homogenous population of PDGFRα+ tumor cells shows a similar response to irradiation, we acutely sorted PDGFRα+ cells isolated from a murine proneural HGG tumor and subjected these cells to irradiation. Immunoblotting results verified that, similar to heterogeneous proneural tumor cultures, we observed increased STAT3 activation and reduced levels of PDGFRα in irradiated cells compared with nonirradiated cells (Supplementary Fig. S3D). These data demonstrate that radiation induces a cell-intrinsic PMT in mouse and human proneural HGG cells and that PDGFRα+ tumor cells alone can undergo a PMT.

We next conducted a 2-week time course analysis by flow cytometry, using PDGFRα and CD44 as markers of proneural and mesenchymal phenotypes, respectively. Irradiated murine HGG cells maintained a high fraction of CD44+ cells and a low
fraction of PDGFRα+ cells 2 weeks after irradiation, compared with control nonirradiated cells (Fig. 3B). Hence, the cell-intrinsic radiation-induced PMT is maintained durably over time.

**Radiation-induced PMT is associated with therapy resistance**

To determine whether proneural HGG cells undergoing PMT display increased invasion, we measured migration and 3D invasion of cells following radiation. Mouse and human proneural HGG cells were exposed to a single dose of radiation, and mounted onto chambers for time-lapse imaging 4 days following irradiation. Irradiated proneural HGG cells undergoing PMT showed higher motility than nonirradiated cells (Fig. 4A). Similarly, irradiated cells were allowed to form tumorspheres, and embedded in Matrigel 4 days after irradiation to measure invasiveness. Proneural HGG tumorspheres exposed to radiation were more invasive than nonirradiated tumorspheres (Fig. 4B). Using atomic force microscopy, we found that irradiated mouse and human HGG cells displayed reduced cellular stiffness 4 days following radiation, compared with nonirradiated control cells (Supplementary Fig S4A).

To study temozolomide resistance following radiation, proneural murine and human HGG cells were subjected to a single dose of radiation, equal numbers of live cells were replated 4 days after irradiation, incubated with temozolomide (100 μmol/L) and proliferation was measured using a CyQuant proliferation assay. As expected, irradiated cells demonstrated reduced proliferation compared with nonirradiated control cells (Supplementary Fig S4B). Importantly, mouse and human HGG cells post-irradiation displayed temozolomide resistance compared with nonirradiated control cells (Fig. 4C). These results suggest that resistance to temozolomide and reduced cellular stiffness in post-irradiated mesenchymal tumor cells enable survival and migration in the tumor microenvironment, promoting malignant progression and recurrence.

**STAT3 as a driver of radiation-induced PMT in HGG**

STAT3 is a master regulator of the glioma mesenchymal transcriptional network in human HGGs (16), and activated STAT3 drives tumorigenicity and self-renewal of GBM cancer stem cells (27, 28). Primary cultures from three murine HGGs and a human proneural GBM showed increased levels of phosphorylated STAT3 (Fig. 5A) at 5 days following radiation. Levels of proneural proteins PDGFRα and SOX10 showed a corresponding decrease, consistent with a PMT (Fig. 5A).

We next investigated whether the activation of STAT3 was durable. Phospho-flow analysis of cells 5 days after irradiation showed increased levels of phosphorylated STAT3, consistent with immunoblotting results. The increased level of phosphorylated
STAT3 was lost at later time points (Supplementary Fig. S5A), suggesting that radiation induces a transient activation of STAT3 that is sufficient to trigger durable activation of downstream mesenchymal transcriptional targets.

**Pharmacologic inhibition of JAK2–STAT3 signaling prevents PMT in HGG cultures**

The transient nature of STAT3 phosphorylation in response to radiation raises questions as to whether STAT3 is a driver of PMT in response to radiation. JAK2 phosphorylates and activates STAT3 (29). The JAK2 inhibitor AZD1480 and the dual JAK1/2 inhibitor ruxolitinib (INC8018424) have been tested in clinical trials and shown to block downstream activation of STAT3 in irradiated human GBM cultures (30–32). To assess pharmacologic blockade of STAT3 phosphorylation, we first incubated murine proneural HGG cells with increasing doses of AZD1480 (0.5–2.0 μmol/L) or ruxolitinib (0.25–2.0 μmol/L), stimulating STAT3 signaling with 10 ng/mL oncostatin M 15 minutes before cell harvest (Supplementary Fig. S5B). AZD1480 and ruxolitinib significantly inhibited STAT3 phosphorylation at 1.0 and 0.5 μmol/L, respectively. A similar experiment conducted by incubating murine proneural HGG cells with each inhibitor for 5 days demonstrated a sustained inhibition of STAT3 activation for 5 days after treatment (Supplementary Fig. S5C).

Treatment of cells with either JAK inhibitor, in combination with radiation, prevented PMT 5 days after irradiation. Immunoblotting showed that irradiated cells treated with AZD1480 or ruxolitinib had a corresponding rescue of SOX10 protein levels (Fig. 5B and Supplementary Fig. S5D). Using the more specific JAK2 inhibitor AZD1480, we found that cells treated with AZD1480 in combination with radiation failed to increase mesenchymal gene expression compared with control irradiated cells not treated with AZD1480 (Fig. 5C). Combination treatment of AZD1480 with radiation also abrogated the invasiveness of irradiated human GBM5 tumourspheres (Fig. 5D). To study the in vivo survival benefit of combination treatment of AZD1480 and radiation, we allografted PDGFRα+ proneural HGG cells into cohorts of FVB/N mice. Irradiated mice showed a survival benefit compared with nonirradiated mice (P < 0.001). Furthermore, an additional 8-day treatment regimen with AZD1480 (30 mg/kg/d) resulted in a significant extension of survival compared with irradiation alone (P < 0.01; Fig. 5E). These results show that pharmacologic inhibition of JAK2 effectively reduces phosphorylation of STAT3, associated with blockade of radiation-induced PMT in murine proneural HGG cells.

**Discussion**

Despite aggressive treatment, patients with HGGs invariably relapse, and prognosis remains dismal. Recurrent tumors are often refractory to further therapy, showing both a high degree of invasiveness and a shift to a mesenchymal gene signature. Radiation remains an integral part of standard of care in HGG
patients. Does radiation as a treatment contribute to recurrence and increased aggressiveness in tumors at relapse?

Using a genetically engineered model for proneural HGG, we show that radiation induces a robust and durable PMT in vivo. Oligodendroglioma represents the most proneural of gliomas (8), hence we chose a mouse model for oligodendroglioma for these studies. Nevertheless, to extend our findings to human proneural glioma, we show similar results in a primary culture from a human proneural glioma (GBM5 cells), also confirming results of a previous study (21).

Components of the tumor microenvironment have been suggested to promote a mesenchymal signature (6). Areas of higher necrosis are associated with more mesenchymal character (19), and microglia/macrophages have been proposed as a source of TNFα paracrine signaling to tumor cells (10). In previous studies, tumor cells isolated after irradiation in vivo showed a PMT on a transcriptional level (20), but it remains unclear whether PMT induction was due to the tumor microenvironment interactions or was cell-intrinsic. Although our results demonstrate that exposing isolated primary tumor cells to radiation induces a cell-intrinsic PMT, they do not exclude the possibility that tumor microenvironment components can modulate the mesenchymal phenotype.

Prior analyses of the radiation-induced PMT were limited to 6 hours for mouse HGGs in vivo (20) and 5 days for human GBM cells in vitro (21). We demonstrate a durable PMT at 2 weeks following radiation of proneural murine HGG in vivo and in vitro. Furthermore, we demonstrated that the mesenchymal transition was associated with temozolomide resistance, increased invasiveness, and reduced cell stiffness, findings also observed in human proneural GBM5 cells. Thus, radiation treatment may drive a mesenchymal signature associated with treatment resistance and increased invasiveness at relapse.

We also identified STAT3 as a driver for radiation-induced PMT, supporting previous findings that STAT3 is a master regulator of the mesenchymal transcriptional network (16). Interestingly,
phosphorylation of STAT3 occurred transiently in irradiated tumor cells that nevertheless showed sustained expression of the mesenchymal transcriptional network. Because HGG patients receive 6 weeks of fractionated radiation, this transient activation of STAT3 may be repeated at each cycle, conceivably expanding a population of radioresistant cells with a therapy-resistant mesenchymal phenotype. Aldehyde dehydrogenase has also been implicated in PMT in glioma cells (21); however, there are currently no selective ALDH antagonists available for clinical use (33).

To assess STAT3 as a driver of radiation-induced PMT, we tested pharmacologic inhibition of JAK2 to determine whether blockade of downstream STAT3 signaling could prevent radiation-induced PMT. We showed that blockade of STAT3 activation before, and concurrent with radiotherapy was effective in blocking the mesenchymal transition in glioma cells, and conferred survival benefit in mice with proneural HGG tumors. Therefore, our study suggests that inhibitors of JAK2 may block radiation-induced PMT in patients receiving radiation therapy for HGG.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Davide Ruggero, Lisa Cossens, and Gabriele Bergers for helpful discussions and critical review, and Fong Ming Koh for generously providing Snail and Slug mouse primers.

Grant Support
This work was supported by US4CA163155 (W.A. Weiss, A.I. Persson, and V.M. Weaver), the Samuel Waxman Cancer Research Foundation (W.A. Weiss), the National Brain Tumor Society 58112-01 (A.I. Persson), R21NS088114 (A.I. Persson), the TDC Foundation (A.I. Persson), and the Guggenheim Endowment Fund (A.I. Persson). J. Lau was supported by the National Science Scholarship by the Agency for Science, Technology and Research of Singapore and the GEMS-CTSI Graduate Student Scholar Award. S. Ilkhanizadeh was supported by the Swedish Pediatric Brain Tumor Foundation and the Swedish Childhood Cancer Foundation. Y.A. Miroshnikova was supported by NSF GRFP and NIH F31CA180422.

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Received November 11, 2014; revised May 14, 2015; accepted July 5, 2015; published OnlineFirst August 17, 2015.

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STAT3 Blockade Inhibits Radiation-Induced Malignant Progression in Glioma

Jasmine Lau, Shirin Ilkhanizadeh, Susan Wang, et al.

Cancer Res 2015;75:4302-4311. Published OnlineFirst August 17, 2015.

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