Heparan sulfate sulfatase SULF2 regulates PDGFRα signaling and growth in human and mouse malignant glioma

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Glioblastoma (GBM), a uniformly lethal brain cancer, is characterized by diffuse invasion and abnormal activation of multiple receptor tyrosine kinase (RTK) signaling pathways, presenting a major challenge to effective therapy. The activation of many RTK pathways is regulated by extracellular heparan sulfate proteoglycans (HSPG), suggesting these molecules may be effective targets in the tumor microenvironment. In this study, we demonstrated that the extracellular sulfatase, SULF2, an enzyme that regulates multiple HSPG-dependent RTK signaling pathways, was expressed in primary human GBM tumors and cell lines. Knockdown of SULF2 in human GBM cell lines and generation of gliomas from Sulf2–/– tumorigenic neurospheres resulted in decreased growth in vivo. We found a striking SULF2 dependence in activity of PDGFRα, a major signaling pathway in GBM. Ablation of SULF2 resulted in decreased PDGFRα phosphorylation and decreased downstream MAPK signaling activity. Interestingly, in a survey of SULF2 levels in different subtypes of GBM, the proneural subtype, characterized by aberrations in PDGFRα, demonstrated the strongest SULF2 expression. Therefore, in addition to its potential as an upstream target for therapy of GBM, SULF2 may help identify a subset of GBMs that are more dependent on exogenous growth factor–mediated signaling. Our results suggest the bioavailability of growth factors from the microenvironment is a significant contributor to tumor growth in a major subset of human GBM.

Introduction

Glioblastoma (GBM) is the most common malignant brain tumor of adults, with a median survival of less than 1 year (1). The disease is characterized by invasion of the tumor into the adjacent brain parenchyma and by the abnormal activation of receptor tyrosine kinase (RTK) signaling pathways.

However, despite the testing of a number of chemotherapeutic modalities targeting known GBM signaling pathways, only limited clinical success has been achieved. One explanation for the limited efficacy of targeted therapeutics may be that GBM is driven by the summation of multiple signaling inputs (2). Thus, effective therapeutic strategies may require a more comprehensive understanding of tumor signaling, including modulation by its microenvironment (3), a known regulator of lethal characteristics of other cancers (4). The identification of distinct GBM subtypes, based on expression and genomic and proteomic data (3, 5–8), supports the notion that GBM is a heterogeneous disease with different patterns of abnormal signaling.

RTK signaling pathways regulate many aspects of tumorigenesis, including cell growth and proliferation. In GBM, abnormal activation of these pathways can be driven by altered ligand availability and altered receptor levels. Indeed, the second most commonly amplified gene in GBM is PDGFRα (8–11). Overexpression of its ligand, PDGF-B, mediates the oncogenic influence of TGF-β in human GBM (12) and can drive tumorigenesis in murine models for glioma (13–15). Once released from the cell, growth factors can be sequestered by the extracellular microenvironment. Mechanisms regulating their post-synthetic availability are just beginning to be elucidated, but include enzymatic release from the extracellular matrix (ECM), thus allowing the growth factors to be available for signaling or alternatively to diffuse away. Because GBMs diffusely invade into the surrounding brain parenchyma, ligand availability in the tumor microenvironment, an underappreciated factor, may be a critical determinant of RTK signaling pathway activity and tumor growth.

Heparan sulfate proteoglycans (HSPGs) are ubiquitously produced by most animal cells and are a major component of the extracellular environment in normal brain and GBM (16, 17). Present on the cell surface and in the ECM, HSPGs play a key role in a number of biological processes based on their ability to bind and regulate the activity of diverse molecules including chemokines and growth factors, such as PDGF, VEGF, and FGF, in many tissues, including the brain (18–22). Indeed, HSPG binding can sequester ligands and decrease signaling, such as with the Wnts, or it can act as a coreceptor and actually promote receptor signaling, such as with FGF2 and VEGF (23–25). HSPGs consist of a protein core and heparan sulfate (HS) chains containing repeating disaccharide units of glucuronic/iduronic acid and glucosamine. The fine structure of HSPG is highly modified through a combination of posttranslational modifications, including sulfation on the N, 3-O, and 6-O positions of glucosamine and the 2-O position of the uronic acid units (26). The sulfation pattern of the HS chains is a major determinant of the specificity and the affinity of ligand interactions (27, 28). Changes in this pattern can alter growth factor bioavailability and thus influence cell signaling during development and disease. Pertinent to the present study, 6-O–sulfation is pivotal for the binding of many growth factors to HSPGs and is critical for normal development (29–31).
Although a number of enzymes control HSPG biosynthesis, the extracellular sulfatases, or SULFs, are unique because they modify the sulfation status of HSPGs post-synthetically in the cellular microenvironment. By removing internal 6-O–sulfates of heparin-binding EGF-like growth factor (HB-EGF) signaling (46). SULF-negative human ovarian adenocarcinoma cell lines, overexpression of SULF1 results in decreased FGF2 and heparin-binding EGF-like growth factor (HB-EGF) signaling (46).

GBM is driven by the abnormal activation of RTK signaling pathways. We hypothesized that GBM uses the extracellular SULFs to manipulate the tumor microenvironment and affect tumorigenesis. We tested this hypothesis in human GBM cell lines and in an orthotopic murine model for high-grade glioma (47, 48) by altering SULF2 expression and examining the effects on tumor growth and activation of critical growth factor signaling pathways. In this study, we also explored SULF expression levels in human GBM. Our findings indicate SULF2 expression may contribute to the pathogenesis of an important subset of human GBM.

Results

SULF2 protein is expressed in 50% of human GBM. By in silico analysis of human expression data (49), we found elevated expression of SULF2 in GBM (Figure 1A). Using a stringent cutoff of a 10-fold increase in SULF2 SAGE expression in 197/424 (46%) primary human GBM tumors by immunohistochemistry on an independent set of 57 primary human GBM tumors. The percentage of SULF2-positive tumor cells was scored from no positive cells (score 0) to more than 75% of tumor cells positive (score 3) (see Methods). (F–I) Representative images from 2 SULF2-positive tumors (F, G, and I) and a SULF2-negative tumor (H). SULF2-positive (brown) tumor cells (F) were widely distributed except in occasional tumors that displayed a more prominent perivascular distribution (G). Many SULF2-positive (brown) tumor cells were also OLIG2-positive (red). See also Supplemental Figure 2. (I) Examples of SULF2-positive tumor cells (arrowheads) and microvascular proliferation characteristic of GBM (arrow). Scale bars: 50 μm (F–H); 10 μm (I).

Figure 1
SULF2 expression in human GBM. (A and B) In silico analysis of SULF2 and SULF1 expression in 16 human GBM tumor samples (49). Each bar represents normalized expression (y axis), as number of SAGE tags per million tags, for each patient tumor listed on the x axis. Expression in normal (N) brain is shown in each graph. (C) Increased SULF2 expression in 197/424 (46%) primary GBM tumors. log₂(tumor/normal) greater than 1.0 (fold change of tumor versus normal greater than or equal to 2.0). See also Supplemental Figure 1 and Supplemental Table 1. (D) Western blot analysis of 6 human high-grade astrocytoma cell lines for SULF2 (Figure 1D). Furthermore, in an independent set of 424 primary human GBM tumors, log₂(NL) brain is shown in each graph. (E) Distribution of SULF2 protein expression in 57 primary human GBM tumors by immunohistochemistry.
human GBM tumors demonstrated SULF2 protein in tumor cells in 50% of tumors (29/57 tumors) (Figure 1E). In a majority of the tumors, SULF2-positive tumor cells were widely distributed throughout the tumors and many coexpressed OLIG2 (Figure 1, F–I).

The SULF2 gene resides within a region of chromosomal amplification in GBM on chromosome 20q13 (50). In 18 of 372 primary human GBM tumors analyzed, the region containing SULF2 was amplified, and in 12 of these tumors (67%), there was increased SULF2 expression (Supplemental Figure 1A and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI8215DS1). Furthermore, in the human glioma cell lines, the line with the most abundant SULF2 protein also had amplification of SULF2 (Supplemental Table 2). We found no uniform trend between expression levels of SULF2 and TP53 or WT1, 2 transcription factors implicated in regulating SULF2 (51, 52) in glioma cell lines and primary human GBMs (Supplemental Figure 1, B–E).

SULF2 confers a growth advantage to human GBM cells. We transduced U251 cells, which contain moderate levels of SULF2 protein (Figure 1D), with either 1 of 2 validated SULF2 shRNAs (43) or a scrambled control shRNA (Figure 2A). Transduced cells also expressed EGFP, allowing for enrichment by flow cytometry. Over time in culture, however, the SULF2-positive cells outgrew the SULF2-knockdown cells, as reflected by a decrease in the ratio of EGFP-positive to total cells (Figure 2B). Selective growth of EGFP-negative cells was not observed in scrambled control shRNA cultures. Thus, cells were sorted for EGFP and used immediately for in vitro and in vivo assays. SULF2 knockdown resulted in a significant decrease in cell viability (Figure 2, C and D). This decrease was largely rescued by overexpression of murine Sulf2 (mSulf2) (Figure 2, E and F), as demonstrated by increased SULF2 protein (Figure 2E). A similar decrease in cell viability with SULF2 knockdown was observed in SF295 and SNB75 cells (Supplemental Figure 7, A and B).

Likewise, we observed a growth advantage of SULF2-positive U251 cells in vivo following subcutaneous transplant of SULF2-knockdown cells into nude mice (Figure 2G). SULF2-knockdown resulted in smaller tumor volumes in subcutaneous tumors. Decreased tumor cell growth in vitro and in vivo with SULF2 knockdown suggested a role for SULF2 in human GBM growth.

Sulf2 is expressed in a murine model for high-grade glioma. To model invasive aspects of the adult disease further, we adapted a murine model for high-grade glioma, based on the genetic manipulation of embryonic neural progenitor/stem cells (47, 48). We cultured adult neural progenitor cells from the subventricular zone (SVZ)
of 11-week-old Ink4a/Arf−/− mice, transduced them with EGFRvIII (EGFR*), a constitutively active variant of EGFR derived from a human GBM (53), cultured them as tumorigenic neurospheres, and transplanted these cells orthotopically into host mice (Figure 3A). Both of these genetic alterations are common in adult human GBM. Within 7 weeks, 100% of mice developed highly invasive high-grade glial tumors (Figure 3, B–D).

We collected the tumors at the time of sacrifice for biochemical analysis or we cultured the tumor cells as tumor neurospheres (tumor-NS), as this enriches for tumor-initiating cells and retains the molecular properties of the parental tumor (54). Indeed, we observed that the tumorigenicity of tumor-NS increased as compared with the parental tumorigenic neurospheres, as reflected by a 38% decrease in median survival following orthotopic transplant of tumor-NS (median survival 23 days vs. 37 days; P < 0.0001).

Similar to human GBM, Sulf2 expression was readily detected in the murine tumor cells in vivo by in situ hybridization and immunohistochemistry (Figure 3, E–G, and Supplemental Figure 2). Tumor cells were identified by morphology and expression of hEGFR. Sulf2 protein was present in a subpopulation of the tumor cells have abundant expression of Sulf2 and are a useful model to study Sulf2 function.

Sulf2 expression increased tumorigenicity and proliferation. To establish Sulf2 function in glioma, we generated tumorigenic neurospheres from double-transgenic mice that were Ink4a/Arf−/− and either wild type, heterozygous, or null for Sulf2 (55). As expected, the extent of HSPG sulfation was greater in Sulf2−/− versus Sulf2+/- neurospheres, as measured by a phage-display antibody whose binding to HSPGs depends on O-sulfation (ref. 56, Supplemental Figure 4, A and B; relative MFI 2.3 versus 1.0; P < 0.05, n = 2). When tumor-NS were grown in minimal medium with only EGF and FGF2, however, Sulf2−/− and Sulf2+/- cells had similar in vitro growth (Figure 4A). Strikingly, following orthotopic transplant, we observed a significant delay in tumor development from Sulf2−/− tumorigenic neurospheres versus those that were Sulf2−/− or Sulf2+/- (55). The median survival of mice transplanted with 3 independent Sulf2−/− lines was 48 days (n = 14) compared with the median survival of mice transplanted with Sulf2+/- cells (37 days; n = 9) or Sulf2−/− cells (38 days; n = 4; P < 0.001) (Figure 4B). In addition to prolonged mouse survival, Sulf2−/− tumors were 23% smaller than Sulf2+/- tumors (mean ratio of tumor weight to body weight was 0.014 ± 0.00034 versus 0.018 ± 0.0014; ± SEM, P < 0.05; Supplemental Figure 4C). Although Sulf2−/− and Sulf2+/- tumors had similar histologic appearance, only tumor-NS from Sulf2−/− tumors expressed Sulf2 protein, and this was associated with decreased HSPG sulfation relative to Sulf2+/- tumors (Figure 4C and Supplemental Figure 4). These data support a functional requirement for Sulf2 in optimal gliomagenesis in the context of the brain microenvironment.

Because Sulf2−/− tumor progenitor cells exhibited a marked delay in tumor growth relative to controls, we wondered whether these late-forming Sulf2−/− tumors had escaped Sulf2 dependence. However, when we isolated tumor-NS from mouse tumors and retransplanted them, the Sulf2−/− cells maintained their dependence on Sulf2 and exhibited significantly (P < 0.001) delayed growth (median survival, 35 days; n = 11) relative to Sulf2+/- cells (median survival, 23 days; n = 7) (Figure 4D), thus indicating that the growth disadvantage conferred by genetic ablation of Sulf2 is durable in vivo.

Sulf2−/− tumors had a greater than 2-fold decrease in the number of proliferating tumor cells as compared with Sulf2+/- tumors (n = 4 for Sulf2−/− and n = 6 for Sulf2+/-; P < 0.05) (Figure 5, A–C). There was no difference in the number of tumor cells undergoing apoptosis, as determined by cleaved caspase-3 immunostaining (data not shown). These data demonstrate that ablation of Sulf2 function in vivo results in decreased tumor cell proliferation, decreased tumor growth, and prolonged survival.
We found no significant differences in tumor cell morphology or differentiation between Sulf2+/− and Sulf2−/− tumors. Both tumor types contained similar proportions of GFAP-positive and Nestin-positive tumor cells (Figure 5, D–I, and Supplemental Figure 5, A–D), and they were negative for NG2, a marker of oligodendrocyte or differentiation between tumor types contained similar proportions of GFAP-positive cells, we hypothesized that SULF2 may act to alter the activity of important signaling pathways in human GBM. In carcinoma, SULF2 modulates the activity of several RTKs in GBM. Overexpression of mSulf2 protein rescued the decreased activity of PDGFRα in cells with knockdown of SULF2, a finding consistent with the ability of SULFs to decrease FGF2-mediated signaling (61).

The Journal of Clinical Investigation

We found no significant differences in tumor cell morphology or differentiation between Sulf2+/− and Sulf2−/− tumors. Both tumor types contained similar proportions of GFAP-positive and Nestin-positive tumor cells (Figure 5, D–I, and Supplemental Figure 5, A–D), and they were negative for NG2, a marker of oligodendrocyte differentiation (Supplemental Figure 5, E and F). In addition, although VEGF binds HSPG and is sensitive to SULF action (57, 58), we did not observe a phenotype resembling altered VEGF signaling (59), including differences in vascular morphology or tumor cell invasion, when we ablated Sulf2 (Supplemental Figure 5, G and H).

SULF2 alters the activity of multiple RTKs in human GBM. Since we observed a SULF2-mediated growth phenotype in brain tumor cells, we hypothesized that SULF2 may act to alter the activity of important signaling pathways in human GBM. In carcinoma, SULFs have been implicated in abnormal Wnt signaling (43–45). Wnt signaling has previously been implicated in U251 growth (60). However, we observed no SULF2-dependent increase in canonical β-catenin-dependent transcriptional activity, as detected by a TCF/LEF reporter assay (Supplemental Figure 6E).

Instead, we observed that several RTKs were influenced by knockdown of SULF2 in U251 cells (Figure 6A), with a greater than 50% reduction in the activity of 3 RTKs of significance in GBM, PDGFRα, IGF1Rβ, and EPHA2 (Figure 6B). Moreover, EGFR activity was decreased by 30%. This did not reflect a global decrease in RTK activation, but a selective decrease in the activity of specific RTKs. Indeed, the closely related PDGFRβ and EPHB2 exhibited similar degrees of phosphorylation in cells with or without SULF2 knockdown. Furthermore, FGFR3 showed increased phosphorylation with SULF2 knockdown, a finding consistent with the ability of the SULFs to decrease FGF2-mediated signaling (61).

To determine whether these effects were generally true, we knocked down SULF2 in another high-grade astrocytoma cell line, SNB75, and again performed human phospho-RTK antibody arrays. As with U251 cells, SULF2 knockdown resulted in alterations in RTK activity, including decreased phosphorylation of PDGFRα and decreased cell viability (Supplemental Figure 6, A–C, and Supplemental Figure 7B). Together, these data suggest that SULF2 modulates the activity of several RTKs in GBM.

Because PDGFR is a major signaling pathway in human GBM (3, 8), we sought to validate the regulation of this RTK pathway by SULF2. Consistent with our array results, cells with knockdown of SULF2 exhibited a 43% decrease in phosphorylated PDGFRα (Figure 6C) and a modest decrease of 19% in total PDGFRα phosphorylation with SULF2 knockdown, a finding consistent with the ability of SULF2 to decrease PDGFRα in cells with knockdown of SULF2 in an imatinib mesylate.
Ablation of Sulf2 confers decreased activation of PDGFRα and downstream signaling pathways in murine high-grade glioma. Similar to our results in 2 human astrocytoma cell lines, Sulf2-regulated PDGFRα activity in our murine tumors. In Sulf2−/− tumor-NS, we observed a marked reduction in activation of PDGFRα, with a slight reduction in total PDGFRα levels (Figure 8, A and B). Sulf2−/− tumor-NS stimulated with PDGF-BB also had reduced activation of PDGFRα relative to Sulf2+/+ tumor-NS (Supplemental Figure 8, A and B). In contrast, there was no decrease in activation of EGFR in Sulf2−/− tumor-NS stimulated with EGF relative to Sulf2+/+ tumor-NS (Supplemental Figure 8C). Although the tumor-prone cells expressed EGFRvIII, they still responded to growth factor stimulation in vitro (Supplemental Figure 8D).

Phosphorylation of PDGFRα results in the activation of downstream signaling pathways, including the MAPK pathway, also known to be important in human GBM. Accordingly, we observed that Sulf2−/− tumor-NS had decreased activation of the MAPK family members Erk1/2 (p44/p42) (Figure 8, C and D). Furthermore, in Sulf2−/− tumors, we observed greater p-Erk immunostaining (2.5 ± 0.2, n = 10) than in Sulf2+/+ tumors (1.6 ± 0.3, n = 11; P < 0.05) (Figure 8E). Together these data demonstrate a role for Sulf2 in modulating the activity of RTKs and downstream signaling pathways in high-grade glioma.

Sulf2 expression is associated with the proneural subtype of GBM characterized by abnormalities in the PDGFRα-signaling pathway. Since we found that SULF2 alters ligand-mediated RTK activity in GBM, we hypothesized that SULF2 may be enriched in a specific molecular GBM subtype. In human tumors of different subtypes, we observed a striking difference in SULF2 expression levels using data from The Cancer Genome Atlas (TCGA) (9A). Interestingly, SULF2 was most highly expressed in the proneural subtype of GBM (n = 173, P < 0.005), which is characterized by alterations in PDGFRα signaling. Indeed, SULF2 expression was associated with the expression of signature genes for the proneural GBM subtype (Figure 9B; Supplemental Table 3). There was also a less robust but positive association between expression of SULF2 and genes that are characteristic of the mesenchymal GBM subtype. Consistent with SULF2 expression, immunohistochemistry for SULF2 in 28 subtyped GBMs demonstrated that proneural and mesenchymal subtypes had the most abundant SULF2 protein (Figure 9C).

Interestingly, the mesenchymal subtype, including the designated signature genes, is characterized by the upregulation of genes associated with the ECM and angiogenesis (7, 8). These data suggest SULF2 may help identify functional subsets of GBM.

Discussion

GBM pathogenesis depends on abnormal activation of RTK signaling pathways, in common with many cancers. Our data demonstrate that GBM utilizes SULF2 for optimal ligand-mediated RTK activation. We observed, first, that SULF2 protein is expressed in 50% of human GBM and in a murine model for high-grade glioma. Second, tumors generated from Sulf2−/− progenitor cells were smaller with decreased proliferation, resulting in prolonged survival. Third, we establish that SULF2 alters the activity of several RTKs, including PDGFRα, which may explain the SULF2 dependency for optimal tumor growth. Finally, high SULF2 expression was strongly associated with the proneural GBM subtype. Together, these data support what we believe is a novel role for SULF2 and identify a potential mechanism of regulating GBM growth.

In view of its regulation of multiple RTK signaling pathways in GBM, SULF2 is likely upstream of many of the currently targeted cell signaling pathways. Therefore, in patients with ligand-dependant tumors, inhibition of SULF2 may be a useful therapeutic strategy in combination with other treatments in GBM, such as...
as inhibition of PDGFRα. Interestingly, the HS mimetic, PI-88, inhibits SULF function (56) and has been tested in a number of cancer clinical trials (62). Originally designed for their ability to inhibit heparanase, PI-88 and other HS mimetics have antiproliferative and antiangiogenic properties (63–65). These activities and our findings in human GBM make these potentially promising new therapeutic agents.

**SULF2 regulates RTK signaling in high-grade glioma.** Our RTK activity profiling suggests SULF2 expression might have multiple and diverse effects on GBM. The 3 RTKs most affected by SULF2 knockdown, PDGFRα, IGF1Rβ, and EPHA2, each may play important roles in GBM. Increased PDGFRα signaling can drive tumor initiation, is important for tumor maintenance, and increases tumor cell proliferation (3, 8, 9, 13, 14, 66). Abnormal IGF1R signaling in GBM is associated with increased proliferation and tumor cell invasion (67, 68). In addition, activation of IGF1R may confer resistance to EGFR inhibitors in glioma (69). Finally, EPHA2 is an RTK commonly expressed in GBM and is thought to play important roles in tumor cell invasiveness (70–72). Although both WNT and VEGF signaling can be influenced by SULF2, these factors did not appear to play a major role in our SULF2-dependent phenotype.

Multiple lines of evidence suggest a key role for SULF2 in PDGFRα signaling. First, we found that SULF2 mediated differences in PDGFRα activity in multiple human glioma cell lines and in a murine model for high-grade glioma. Second, we observed that SULF2 is highly expressed in human tumors of the proneural GBM subtype, characterized by alterations in PDGFRα signaling, supporting a functional role for SULF2 in PDGFRα signaling in vivo. Our murine brain tumor model is based on the genetic manipulation of neural progenitor cells, and the PDGFRα pathway is implicated in progenitor cell gliomagenesis. PDGF stimulation of progenitor cells in the neonatal and adult brain can generate tumors or tumor-like growth (13, 14, 73–75). In addition, Sulf2 is one of a number of candidate genes identified in a screen for genes that, when activated by insertional mutagenesis, promote PDGFB-driven gliomas (76). Furthermore, Sulf2 expression is increased in many PDGFB-driven tumors regardless of the insertion site (77). Finally, PDGFs interact with HSPGs, and this interaction is influenced by 6-O-sulfation (18, 78, 79).

In addition to potential effects on PDGF bioavailability, SULF2 may alter PDGFRα activation via other mechanisms. Indeed, VEGF-A, a factor known to bind heparin/HSPGs in a SULF-dependent manner (18, 78, 79), can directly activate PDGFRα (80). Furthermore, we observed a slight but consistent decrease in the total levels of PDGFRα. This decrease could be due to alterations in the activity of other RTK signaling pathways or in the composition of the ECM, given that PDGF receptor levels are affected by alterations in hedgehog pathway activity (81) and alterations in the composition or amount of HSPGs (82). Thus, there may be multiple mechanisms by which SULF2 alters the activity of critical RTK signaling pathways in GBM.

**Tumor interactions with the microenvironment in GBM.** There is accumulating evidence that the complex interplay between brain tumor cells and their microenvironment can have profound implications on tumorigenesis and invasion (58, 83). SULF modifies HSPGs in the tumor microenvironment and influences exogenous ligand availability (23, 38, 57). As GBM is a heterogeneous disease, SULF2 expression may help identify tumor subtypes with a greater dependence on ligand-mediated signaling, such as with PDGF. Consistent with this idea, we found that SULF2 affected ligand-mediated activation of PDGFRα and that SULF2 expression in human GBM

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Figure 6

SULF2 alters activity of several RTKs in human GBM. (A) RTK phosphorylation in U251 cells expressing SULF2-A shRNA or scrambled control shRNA. Individual RTKs are spotted in duplicate, and the identities of specific receptors are indicated. Positive control spots are located at the corners. See also Supplemental Figure 6. (B) Relative levels of phosphorylated RTKs in cells with knockdown of SULF2 normalized to cells with scrambled shRNA control. Duplicate spots were averaged. Data are representative of 2 independent experiments. (C) Phosphorylated and total PDGFRα levels in cells with SULF2 knockdown (S2) and scrambled shRNA control (C). Western blots were probed for GAPDH as a loading control. Results are means normalized to levels in scrambled shRNA control cells ± SEM (n = 4 independent experiments). *P < 0.01. (D) Knockdown of SULF2 decreases PDGFRα activation in response to PDGF-BB (10, 100, 200 ng/ml) stimulation. Relative levels of phosphorylated to total PDGFRα normalized to levels in unstimulated scrambled shRNA control cells. Data are representative of 2 independent experiments.
most strongly correlates with the proneural GBM subtype, which is primarily ligand driven (3). As SULF2 expression was not exclusive to the proneural subtype, it will be interesting to determine whether SULF2 function differs between subtypes.

Identification of the specific pathways and mechanisms of pathway activation critical in individual tumors is anticipated to lead to improved therapy for GBM. We have identified SULF2 as a mechanism by which GBM increases the activity of several RTKs, including PDGFRα, to promote tumorigenesis. In addition to its potential as a therapeutic target in GBM, SULF2 may help to identify tumors that are more dependent on exogenous ligand–mediated signaling.

**Methods**

**Antibodies and reagents.** We purchased antibodies against the following: p44/42 MAPK, phospho-p44/42 MAPK (Thr202, Tyr204), GAPDH, and β-actin (Cell Signaling); PDGFRα (Millipore); EGFR (Santa Cruz Biotechnology Inc.); phospho-EGFR (R&D Systems); GAP and hEGFR (Dako); Nestin, Olig2, and NG2 (Chemicon/Millipore International); and phospho-PDGFRα (Tyr742), phospho-EGFR (Tyr1068), and human RTK Proteome Profiler (R&D Systems). The monoclonal antibody against Sulf2 (2B4) has been previously described (44). RB4CD12 was a gift of Toin van Kuppevelt (Nijmegen Medical Center, Nijmegen, Netherlands). Imatinib mesylate was purchased from United States Biological. DMEM, Neurobasal Medium, B-27 supplement, N2 supplement, recombinant human EGF, and recombinant human PDGF-BB were purchased from Invitrogen. Recombinant human PDGF-AA (R&D Systems), puromycin (Sigma-Aldrich), AG1478 (Calbiochem; EMD Bioscience), and FGF2 (Chemicon/Millipore Corp.) were also used.

U251 and U87 cells were obtained from the UCSF Brain Tumor Research Center Tissue Bank. Retroviral vectors were used to transduce cells with EGFRvIII (EGFR*) (47) and the Sulf2 shRNAs and scrambled control shRNA (43). Mouse Sulf2 cDNA (mSulf2), as previously described (32), was subcloned into a lentiviral expression vector expressing a fluorescent marker (84). SNB-75, SF295, and SF268 cells, obtained from the DCTD Tumor/Cell Line Repository, were maintained in DMEM plus 10% FCS, and protein lysates were harvested after 4 days of growth in 0.05% FCS.

![Figure 7](http://www.jci.org) Decreased tumor cell viability conferred by knockdown of SULF2 and inhibition of PDGFR signaling. (A) PDGFRα phosphorylation is decreased by imatinib mesylate (9 μM) in both scrambled shRNA control and SULF2-A shRNA–containing cells by Western blot. (B) Knockdown of SULF2 in combination with inhibition of PDGFRα by imatinib mesylate (9 μM) results in decreased cell viability. This effect was not observed with inhibition of EGFR signaling by AG1478 (10 μM). *P < 0.005. (C) Overexpression of mouse SULF2 (mSULF2) in control and SULF2-A shRNA–containing cells by Western blot. (D) Overexpression of mSULF2 restores PDGFRα activity in cells with knockdown of human SULF2 in an imatinib mesylate–dependent manner. All data are representative of 2 independent experiments done in quadruplicate, and data are presented as mean ± SEM. C, scrambled shRNA control; S2, SULF2-A shRNA.

![Figure 8](http://www.jci.org) Sulf2 alters the activity of PDGFRα in murine tumor-NS. (A) Phosphorylated and total PDGFRα levels in Sulf2+/+ and Sulf2–/– tumor-NS. Western blots were probed for GAPDH as a loading control. (B) Quantification of p-PDGFRα and total PDGFRα levels in tumor-NS from Sulf2+/+ and Sulf2–/– cells normalized to mean ± SEM (n = 3 independent experiments). *P < 0.05. (C) SULF2 also affected the activity of downstream signaling pathways. Phosphorylated and total Erk1/2 (p44/p42) levels in Sulf2+/+ and Sulf2–/– tumor-NS. (D) The relative mean ratio of phosphorylated Erk to total Erk levels in Sulf2+/+ and Sulf2–/– tumor-NS normalized to Sulf2+/+ levels ± SEM (n = 3 independent experiments). *P < 0.005. (E) Sulf2+/+ tumors had more prominent phosphorylated Erk immunostaining relative to Sulf2–/– tumors. Scale bars: 50 μm.
Figure 9

**SULF2** expression is associated with the proneural GBM subtype. (A) **SULF2** expression by human GBM subtype. Box plots show the median (range) normalized **SULF2** expression levels were 8.1 (6.9–9.5), 8.9 (7.0–10.2), 7.8 (6.6–9.2), and 8.5 (7.1–9.9) for the classical, proneural, neural, and mesenchymal (mesench) subtypes, respectively (n = 173 tumors); **P < 0.005. The values within the box represent the lower quartile (Q1), median, and the upper quartile (Q3) of the distribution. The horizontal bars at the 2 ends are the smallest and largest nonoutlier observations. The circles beyond the horizontal bars represent outlying cases, defined as 1.5 times the interquartile range (Q3–Q1), below Q1 or above Q3. (B) Similarity (Pearson correlation, r) between **SULF2** expression and the expression of 50 genes characterized as signature genes for each of the previously defined GBM subtypes (8). A positive coefficient denotes a positive relationship between **SULF2** expression and expression of the gene of interest on the x axis (n = 202 tumors). In silico analysis for A and B was performed on expression data from the TCGA Data Portal. (C) **SULF2** protein expression in primary human GBM samples of different subtypes. Tissue microarrays of previously subtyped human tumors were immunostained for **SULF2** and scored. Data are represented as mean ± SEM for the classical, proneural, neural, and mesenchymal GBM subtypes, respectively (n = 28 tumors total). **P < 0.05.

**Cell culture and virus production.** Murine neurospheres were cultured as previously described (48). Briefly, neural progenitor cells were isolated from the SVZ of 11-week-old male Sulf2+/−; Inka4a/Arf−/− mice and Sulf2−/− and Sulf2−/−; Inka4a/Arf−/− male littermates. Cells were cultured on nonadherent plates (Ultralow Attachment Plates; Corning Inc.) in Neurobasal Medium plus B27 plus N2 with 20 ng/ml EGF and 20 ng/ml FGF2. Cells were passaged once per week and plated at equivalent densities (2 × 10^5 cells per well of a 6-well plate). Following transduction, neurospheres were maintained in puromycin (2 μg/ml). Transduced cells were passaged at least 2 to 3 times, but fewer than 7 times prior to transplant.

Neurosphere growth rates were determined by averaging the total number of viable cells per well at various times after plating (n = 3 wells per time point). For analysis of PDGF-Rtx activation, tumors were cultured as neurospheres, and 12–15 hours prior to collection, cells were either stimulated with 2% FCS or PDGF-BB (100 ng/ml).

Human glioma cell lines were cultured in DMEM plus 10% FCS. Growth rates of human glioma cell lines were determined by CellTiter 96 NonRadioactive Cell Proliferation Assay (Promega) or by averaging the total number of viable cells per well and the total number of viable GFP-positive cells per well at various times after plating (n = 3–5 wells per cell line per time point). For experiments involving addition of imatinib mesylate (9 μM) or AG1478 (5 μM), 4 × 10^5 cells were plated per well of a 48-well plate, drug was added on day 2, and cells were collected on day 5. Counting was done using a hemocytometer and trypan blue to exclude dead cells. Transduced cells expressing mSULF2 and a fluorescent marker were sorted by flow cytometry prior to use. For protein analysis, cells were grown for a minimum of 4 days prior to stimulating or harvesting total cell lysates.

**Quantitative RT-PCR.** Real-time PCR with SYBR green detection was performed using a Mastercycler ep realplex real-time PCR system (Eppendorf) and Faststart Universal SYBR Green Master Mix (Roche Applied Science) as follows: 10 minutes at 95°C for initial denaturation, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. After each cycle, fluorescence was measured. Each run included a melting curve for each primer set to determine the correct response of the primers, and only values with the correct melting curves were used.

**Mouse procedures.** Sulf2 mutant mice containing a gene trap insertion in the sixth intron of the Sulf2 locus were generated as previously described (55). Inka4a/Arf−/− mice were obtained from the National Cancer Institute Mouse Repository and bred to the FVB/n background. Immunocompromised 6-week-old male lcrSCID homozygous mice obtained from Taconic Inc. were used for neurosphere transplants. Neurospheres were dissociated and resuspended in HBSS at a concentration of 200,000 viable cells/μl, and 2 μl were injected into the striatum (48). Mice were monitored daily and were sacrificed at the onset of neurological signs or, if no overt signs developed, upon loss of 20% or more of their peak body weight. The brain to body weight ratio was determined at 35 days after transplant (n = 3 Sulf2−/− and n = 4 Sulf2−/− mice). For subcutaneous transplant of U251, cells were sorted for EGFP, briefly cultured, and resuspended in HBSS at a concentration of 250,000 viable cell/μl; 2 μl were injected. Tumors were measured (length/width/height) every other day using a caliper (0.1 cm), and mice were weighed 3 times a week. Tumor volume was calculated based on the equation (n/6) (length × width × height).

To account for potential loss of EGFP-expressing cells during the brief in vitro passage, a cohort of mice were transplanted with equal percentages of EGFP-positive cells (n = 3 mice per group) with similar results.

**Histology, immunohistochemistry, and immunoblotting.** To collect tissue for immunohistochemical analysis, mice were perfused with 4% PFA, and the tissue was fixed overnight in 4% PFA, rinsed in PBS, and stored in 70% ethanol until further processing. Histological analysis of tumor tissue was performed on H&E-stained sections. Formalin-fixed paraffin-embedded sections of human GBM were obtained from the UCSF Brain Tumor Research Center Tissue Bank. Immunohistochemistry was performed according to standard methods, and immunostaining for SULF2 was performed both by hand (44) and on the Ventana Medical Systems Benchmark XT using the iView detection system. To score for human SULF2 and p-ERK1/2 immunostaining, the percentage of tumor cells that stained positive was noted: 0 denoted no positive tumor cells; 1+ indicated 1–25%; 2+ indicated 26–75%; and 3+ indicated more than 75%. To evaluate murine phospho-histone H3 immunostaining, 3 to 9 200X fields were examined per mouse, and the mean number of positive cells per field was calculated per mouse. All histologic and immunohistochemical analyses were performed without knowledge of the Sulf2 status of the tumor.

Human phospho-RTK arrays (Proteme Profiler; R&D Systems) were incubated with 200 μg of protein lysate from human glioma cells with and
Deidentified formalin-fixed paraffin embedded sections of human GBM were obtained from the UCSF Brain Tumor Research Center Tissue Bank.

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