Genetic Validation of the Protein Arginine Methyltransferase PRMT5 as a Candidate Therapeutic Target in Glioblastoma

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Abstract
Glioblastoma is the most common and aggressive histologic subtype of brain cancer with poor outcomes and limited treatment options. Here, we report the selective overexpression of the protein arginine methyltransferase PRMT5 as a novel candidate theranostic target in this disease. PRMT5 silences the transcription of regulatory genes by catalyzing symmetric dimethylation of arginine residues on histone tails. PRMT5 overexpression in patient-derived primary tumors and cell lines correlated with cell line growth rate and inversely with overall patient survival. Genetic attenuation of PRMT5 led to cell-cycle arrest, apoptosis, and loss of cell migratory behavior. Cell death was p53-independent but caspase-dependent and enhanced with temozolomide, a chemotherapeutic agent used as a present standard of care. Global gene profiling and chromatin immunoprecipitation identified the tumor suppressor ST7 as a key gene silenced by PRMT5. Diminished ST7 expression was associated with reduced patient survival. PRMT5 attenuation limited PRMT5 recruitment to the ST7 promoter, led to restored expression of ST7 and cell growth inhibition. Finally, PRMT5 attenuation enhanced glioblastoma cell survival in a mouse xenograft model of aggressive glioblastoma. Together, our findings defined PRMT5 as a candidate prognostic factor and therapeutic target in glioblastoma, offering a preclinical justification for targeting PRMT5-driven oncogenic pathways in this deadly disease. Cancer Res; 74(6); 1–14. ©2014 AACR.

Introduction
High-grade astrocytomas are the most common primary central nervous system (CNS) malignancy accounting for nearly 14,000 new cases per year in the United States. Although surgery remains the mainstay for the treatment of patients with these CNS tumors, grade 3 (anaplastic astrocytoma) and grade 4 (glioblastoma multiforme) astrocytomas exhibit a highly invasive clinical behavior that, in most cases, precludes complete surgical resection (1). In contrast with many cancers, the outcome of patients diagnosed with glioblastoma has shown only marginal improvement over the past several decades with a median survival of only 15 months despite multimodal therapy (2). Recent work has contributed to an improved understanding of the pathophysiology of these high-grade malignancies; however, discovery of effective therapies has been limited by the lack of novel targets that are selectively involved in the complex pathogenesis of this disease. Numerous genome-wide studies have demonstrated that glioblastoma possesses a remarkable degree of heterogeneity with regard to genetic mutation, gene expression profiles, and epigenetic modifications (3, 4). This degree of biologic heterogeneity has contributed to the challenge of identifying targets that are critical to the underlying pathogenesis of this aggressive disease.

Posttranslational modifications of proteins is a common activity involved at virtually all levels of cellular regulation. Enzymes in the protein arginine methyltransferase (PRMT) family represent a group of proteins that are evolutionarily conserved among a wide variety of organisms. PRMT enzymes covalently modify both histone and nonhistone proteins that are critical to the maintenance of numerous cellular regulatory networks (5–7). The PRMT5 enzyme is a type II arginine methyltransferase that uses the cofactor molecule S-adenosyl-L-methionine to catalyze the transfer of a methyl group to
two of three guanidino nitrogen atoms within the arginine molecule. PRMT5 drives the formation of both Ω-NG-mono-
methyl and Ω-NG,N'-symmetric dimethyl arginine residues to affect protein function (8–12). PRMT5-driven methylation of arginine residues leads to symmetric dimethylation of histone proteins H3 (S2Me-H4R3) and H4 (S2Me-H3R8), which in turn alters chromatin structure to promote transcriptional repression (13, 14). A growing number of nonhistone proteins involved in the control of multiple regulatory networks have also been identified as targets of PRMT5 (6, 15).

We have previously reported that PRMT5 overexpression is involved in the pathogenesis of mantle cell lymphoma and knockdown of PRMT5 expression interfered with growth of transformed B cells (16). Although this work contributed information about the mechanism of PRMT5 overexpression, the functional consequences of PRMT5 inhibition on maintenance of the malignant phenotype of the transformed cell remain poorly characterized. Here, we show that overexpression of PRMT5 is associated with more aggressive disease in patients with glioblastoma. Because this enzyme is intimately involved with numerous processes that are frequently dysregulated in cancer, we sought to determine the consequences of PRMT5 silencing in glioblastoma. We demonstrate that inhibition of PRMT5 overexpression in glioblastoma cells leads to restoration of critical regulatory pathways affecting cell growth, survival, and tumor suppressor activity. Our findings provide new information about methods to directly and indirectly affect tumor progression, whereas providing further justification to explore novel approaches to target PRMT5 overexpression in this incurable cancer.

Materials and Methods

Cell lines and culture

The human glioblastoma cell lines used in this study are summarized in Supplementary Table S1. The tet-on–inducible p53 cell line, 2024 (17), was kindly provided by Dr. Erwin Van Meir, Emory University, Atlanta GA.

Evaluation of PRMT5 protein expression in primary astrocytoma tumors

Under an Institutional Review Board–approved protocol, 60 patients with astrocytomas treated at The Ohio State University (Columbus, OH) from January 2003 to October 2007 were identified. Age, gender, race, and previous history of astrocytomas were assessed by review of the medical records of these patients. Reports were reviewed to determine tumor grade, Ki67 proliferation index, as well as clinical characteristics of disease. Immunohistochemistry was performed using a Ventana Benchmark System (Ventana Medical Systems) and the Ultraview Universal Fast Red Kit, following the manufacturer’s recommendations. Optimal conditions for PRMT5 were determined to be 1:125 with antigen retrieval for 30 minutes using mantle cell lymphoma primary tumor tissues and benign, reactive lymph nodes as the positive and negative controls, respectively.Slides were counterstained with hematoxylin II for 4 minutes. See Supplementary Materials for details on calculating the PRMT5 expression index.

siRNA transfection

siRNA and scramble (scr) RNA were constructed by silencer the siRNA Construction Kit by Ambion. si-PRMT5 or scrRNA were transfected into glioblastoma cells by Lipofectamine 2000 according to the manufacturer’s instructions. See Supplementary Materials for sequences of inhibitory RNAs used in this article.

Cell cycle, apoptosis, and migration analysis

Cells were harvested after treatment and fixed in 75% EtOH. After digestion with RNase, DNA was stained with propidium iodide and analyzed with a Beckman Coulter flow cytometer and ModFit software (Verity). For apoptosis assays, treated cells were stained with Annexin V and propidium iodide and evaluated by flow cytometry as described previously (18). Caspase activation followed methods previously described (18). Glioblastoma migration was evaluated as previously described (19).

Real-time quantitative reverse transcription PCR, protein detection, and chromatin immunoprecipitation assay

Total RNA was prepared from untreated and treated glioblastoma cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The cDNA was prepared with the MMLV Reverse Transcription Kit (Invitrogen) following the manufacturer’s recommendations. Real-time PCR was performed using a TaqMan 2 × Universal PCR Master Mix Kit per the manufacturer’s instructions on an Applied Biosystems 7900HT Fast Sequence Detection System. Immunofluorescence and Western blot analysis used antibody reagents (Supplementary Materials) and were performed as described previously (16). Chromatin immunoprecipitation (ChIP) assays were done using the EZ-Magna ChIP Kit (Upstate) according to the manufacturer’s instructions.

Results

PRMT5 protein is overexpressed in glioblastoma cell lines and correlates with proliferation

Aberrant expression of PRMT5 protein has been identified in a number of malignancies, including mantle cell lymphoma (16) and germ cell tumors (20). To determine whether PRMT5 was overexpressed in astrocytomas, we evaluated a panel of cell lines (Supplementary Table S1) derived from primary glioblastoma biopsy specimens for PRMT5 protein expression by Western blot analysis (Supplementary Fig. S1). Compared with normal brain tissue and normal human astrocytes (NHA) that did not express measurable PRMT5 protein, eight astrocytoma-derived cell lines demonstrated abundant levels of PRMT5 protein expression (Supplementary Fig. S1). Because the various cell lines displayed different growth rates, we examined whether the degree of PRMT5 protein overexpression correlated with proliferative index (Fig. 1A). Determination of absolute cell numbers was performed in parallel with Western blot analysis probing for PRMT5 and β-actin proteins at three different time points (representative blot in Supplementary Fig. S1). PRMT5 expression (normalized to β-actin control) correlated in a linear fashion with the proliferation of glioblastoma cell lines ($r = 0.81; P < 0.0001$).
PRMT5 overexpression is associated with primary glioblastoma tumors and correlates with more aggressive disease.

The direct correlation between glioblastoma cell line growth and PRMT5 expression suggested that this enzyme may contribute toward the malignant phenotype (Fig. 1A). Thus, we next examined PRMT5 expression in primary astrocytoma tumors. High-grade astrocytomas that spontaneously develop in a preclinical in vivo murine model are associated with PRMT5 expression (Fig. 1B). The PRMT5 expression index by glioma grade (Fig. 1I) is associated with overall survival of patients with glioblastoma (Fig. 1J). PRMT5 level is continuously associated with time to death (Spearman r = –0.57; P = 0.0001).

Figure 1. PRMT5 overexpression correlates with human glioblastoma (GBM) cell proliferation and inversely correlates with patient survival. A, PRMT5 expression correlates with cell growth (r = 0.81 with P < 0.0001). B, hematoxylin and eosin staining of glioblastoma. D–G, PRMT5 is selectively expressed in high-grade (grade 3 and 4) astrocytomas (F and G), whereas normal brain (C) and low-, or intermediate-grade astrocytomas (D and E) do not. H, Kaplan–Meier plots by glioma grade. I, the PRMT5 expression index by glioma grade. J, Kaplan–Meier plots of overall survival of patients with glioblastoma as a function of the PRMT5 protein expression index. K, time to death and PRMT5 level in patients who died with glioblastoma. PRMT5 level is continuously associated with time to death (Spearman r = –0.57; P = 0.0001).
induction of allelic haploinsufficiency at \textit{Nf1}, \textit{p53}, and \textit{PTEN} tumor suppressor gene loci (21) were examined for PRMT5 and \textit{Ki67} expression (Supplementary Fig. S2). Although control brain tissue from wild-type mice failed to demonstrate any detectable PRMT5 protein expression, all tumor samples taken from tumor-bearing \textit{Nf1-} and \textit{p53}-deficient mice (Mut3 mice, GFAP-cre; cis\textit{Mf1}\textsuperscript{−/−}; \textit{p53}\textsuperscript{−/−}) showed overexpression of PRMT5 that coincided with the cellular proliferation signal \textit{Ki67}. PRMT5 expression was higher in tumor tissue from tumor-bearing Mut3 mice compared with uninvolved brain tissue proximal to tumor or from brain tissue from control mice without CNS tumors of the same genetic background (Supplementary Table S3; data not shown).

We next examined PRMT5 protein expression profiles in primary tumor specimens from 60 patients diagnosed with astrocytomas (Supplementary Table S2 and Fig. 1B–G). To verify that our patient cohort demonstrated clinical behavior consistent with the natural history of this disease (22), we evaluated survival in the context of tumor grade (Fig. 1H) and found median survival to correlate with tumor grade as described previously (23). PRMT5 was overexpressed in grade 3 and 4 tumors with the most prominent expression in a nuclear distribution (Fig. 1F, G and I). Normal brain tissue or tumors from patients with grade 1 (\(N = 10\)) and grade 2 astrocytomas (\(N = 7\)) did not demonstrate any detectable PRMT5 protein (Fig. 1C–E, and I). The median PRMT5 expression index (definition provided in Supplementary Materials) seen in grade 3 tumors was 0.15 (\(N = 7\); interquartile range = 0.11–0.25) and for grade 4 tumors was 0.53 (\(N = 43\); interquartile range = 0.19–0.78). The difference in PRMT5 expression indices between grade 3 and 4 tumors was statistically significant (\(P = 0.01\), by the standard two-sample Wilcoxon rank-sum test). The differences in the PRMT5 expression index observed in grades 1/2 versus grades 3 versus 4 tumors were statistically significant (\(P = 0.0001\), the Kruskal–Wallis equality-of-populations rank test).

Because PRMT5 overexpression was confined to grade 3 and 4 tumors, we excluded patients with grade 1 and 2 tumors from our evaluation of other clinical variables related to PRMT5 expression. In patients with grade 3 and 4 tumors, the PRMT5 expression index was not significantly associated with gender, race, or prior diagnosis with astrocytoma. In patients with grade 4 tumors (glioblastoma), the degree of nuclear PRMT5 expression was associated with age, though the result was of borderline significance (Spearman correlation coefficient = 0.29; \(P = 0.057\)).

The heterogeneity in the degree of PRMT5 expression seen in grade 4 tumors (Fig. 1I) prompted us to examine whether the PRMT5 expression index correlated with glioblastoma patient survival. We stratified grade 4 tumors into three categories of the PRMT5 expression index: (i) \(\leq 0.75\); (ii) \(0.25 \leq \leq 0.74\); and (iii) \(<0.25\). Patients with grade 4 tumors showing a PRMT5 expression index \(\geq 0.75\) demonstrated shorter overall survival compared with patients with lower PRMT5 expression indices. Patients with the PRMT5 expression index \(<0.25\) had a median survival of 726 days (Fig. 1J; 95% confidence interval (CI), 176–1083 days; \(N = 14\)) compared with 277 days (95% CI, 156–604 days; \(N = 14\)) for those with the PRMT5 expression index = 0.25 to 0.74, and 108 days (95% CI, 17–173 days; \(N = 15\)) for those with the PRMT5 expression index \(>0.75\) (Fig. 1J; log-rank \(P < 0.0001\)). Cox proportional hazards were used to model mortality in patients with glioblastoma. In a multivariable model (with age, gender, \textit{Ki67}, and PRMT5 as effects), the PRMT5 expression index (represented as a single continuous variable) was significantly associated with decreased overall survival. In the model a 10% increase in the PRMT5 expression index on average was associated with an unadjusted HR, 1.37 (95% CI, 1.18–1.57; \(P < 0.001\)) and an adjusted HR, 1.40 (95% CI, 1.18–1.66; \(P < 0.001\)) for mortality. In the model, age, gender, and \textit{Ki67} were not significantly associated with mortality. We evaluated the association of PRMT5 expression with time to death graphically in patients with glioblastoma who died over follow-up (only 4 of 43 individuals, 9.3%, were alive at the end of follow-up). We found that PRMT5 expression was strongly and continuously associated with time to death in patients with glioblastoma who died during follow-up (Fig. 1K; Spearman rank correlation coefficient = \(-0.57\); \(P < 0.0001\)).

Previous glioblastoma tumor history was not significantly associated with the PRMT5 expression index or survival in individuals with glioblastoma. Within the 43 patients diagnosed with glioblastoma, 11 had a previous glioblastoma history. We did not observe any difference in survival over follow-up for primary glioblastoma or recurrent glioblastoma (median survival = 259 days in primary glioblastoma and 254 days in recurrent glioblastoma, the log-rank test = 0.6 on the survival curves). The PRMT5 expression index was not significantly associated with prior glioblastoma history, the median PRMT5 expression index = 0.36 in primary glioblastoma and 0.31 in recurrent glioblastoma (Wilcoxon rank-sum \(P = 0.5\)).

\textit{PRMT5} mRNA in glioblastoma cell lines did not differ from NHAs (not shown); however, the amount of protein differed markedly between glioblastoma primary tumors, cell lines, NHA, and normal brain (Fig. 1 and Supplementary Fig. S1), suggesting that \textit{PRMT5} dysregulation occurred at the level of mRNA (\textit{mRNA}), similar to that observed with mantle cell lymphoma (16). We, therefore, evaluated \textit{PRMT5} expression as a function of survival and found that in the Rembrandt dataset for glioblastoma cases (total \(n = 181\) cases), no significant correlation between \textit{PRMT5} transcript levels and survival (Supplementary Fig. S1B). We, therefore, evaluated two miRNAs, \textit{miR}-96 and \textit{miR}-92b, previously shown to be underexpressed in mantle cell lymphoma allowing for efficient \textit{PRMT5} translation and upregulation of \textit{PRMT5} protein levels (16). We used nanostring methodology to quantify \textit{miR}-96 (average = 44 counts) and \textit{miR}-92b (average = 108 counts) and found both miRs to be undetected compared with the top 50 detectable miRs (average counts ranging from 601–123,068) for 11 primary glioblastoma RNA samples (Supplementary Fig. S1C). These observations indicate that \textit{miR} dysregulation may contribute toward more efficient \textit{PRMT5} translation and elevated protein levels.

**Consequences of \textit{PRMT5} knockdown in glioblastoma cell lines \textit{in vitro}**

Because expression of \textit{PRMT5} correlated with proliferation rate of glioblastoma cells and survival of glioblastoma patients,
we next determined the consequences of PRMT5 silencing in vitro. We developed a panel of nine small inhibitor RNA molecules (siRNA, and control scrambled: scrRNA) designed to knockdown PRMT5 transcript and protein expression. Significant decreases in miRNA levels (90% and 80% in U1242 and U251, respectively) and in PRMT5 protein expression were achieved with three lead siRNAs (Fig. 2A and B and Supplementary Fig. S3A). Because PRMT5 and PRMT7 have each been shown to possess type II methyltransferase activity, we examined the specificity of our si-PRMT5 reagent. As shown in Supplementary Fig. S3B, si-PRMT5 did not affect the expression of the other type II PRMT enzyme, PRMT7. More importantly, PRMT5 knockdown by si-PRMT5 led to demethylation of target histone protein arginine residues (H4R3) as determined by immunofluorescence using an antibody specific for symmetric dimethyl arginine of histone 4 (S2Me-H4R3, Fig. 2B). Twenty-four hours following siRNA treatment, cytoplasmic and nuclear PRMT5 protein levels were undetectable and this correlated with the loss of the PRMT5-associated epigenetic mark, S2Me-H4R3 (14).

We next determined the effects of PRMT5 depletion on growth properties of glioblastoma cells. As shown in Fig. 3A, si-PRMT5 elicited a significant antiproliferative effect on cell growth (P < 0.0001). Invasiveness is a characteristic feature of glioblastoma and a major contributing factor that prevents successful surgical resection leading to the low progression-free survival observed in patients with glioblastoma. siRNA induced loss of PRMT5 expression led to cell morphologic changes in glioblastoma cell lines demonstrating a transition from a cellular morphology with elongated cell shape and dendritic-like projections to a more rounded cellular shape (Fig. 3B). To determine whether this change in cellular morphology correlated with decreased invasiveness of glioblastoma cells, we evaluated cellular migration by both wound-healing and Transwell migration assays. Treatment of glioblastoma cell lines with the pan-caspase inhibitors Z-VAD-FMK (100 μmol/L) and Q-VD-OPh (20 μmol/L) led to blockade of caspase-3 activation (Supplementary Fig. S4A) and prevented apoptosis (Fig. 4D and E; P < 0.0001), thus, consistent with a caspase-dependent mechanism of cell death. The addition of temozolomide to radiotherapy has improved the survival of patients with glioblastoma and is now considered standard in the management of these patients (27). Glioblastoma cells were preincubated with 100 μmol/L of temozolomide for 72 hours and transfected with scrRNA or si-PRMT5 (Fig. 4F and Supplementary Fig. S4B). PRMT5 knockdown resulted in the induction of apoptosis in all cell lines compared with scrRNA control (P = 0.002 across cell lines and time points).

The BCL2 family of proteins is known to play a central role in the regulation of apoptosis (25). As shown in Fig. 4C, si-PRMT5 significantly led to an increase in the BAX/BCL2 ratio associated with activation of downstream caspase enzymes and apoptosis (26). ChIP experiments were performed and failed to show PRMT5 recruitment to the BAX gene promoter (data not shown), suggesting other pathways are involved in the change of BAX/BCL2 following PRMT5 depletion. As caspase-3 is a key protease associated with DNA fragmentation, we investigated whether apoptosis induced by PRMT5 knockdown was caspase-3 dependent. Flow cytometry analysis of glioblastoma cells stained with an antiactivated caspase-3 antibody showed increased caspase-3 activation following PRMT5 silencing (Supplementary Fig. S4A). Pretreatment of glioblastoma cell lines with the pan-caspase inhibitors Z-VAD-FMK (100 μmol/L) and Q-VD-OPh (20 μmol/L) led to apoptosis (26).

A172 (29), was cotransfected with siRNA targeting p53 (si-p53) and si-PRMT5. As shown in Fig. 5A and B, PRMT5 knockdown led to greater than additive induction of programmed cell death when combined with temozolomide.

**Knockdown of PRMT5 leads glioblastoma cells to undergo caspase-dependent apoptosis**

PRMT5 silencing can induce apoptosis in different types of cancer (6, 24). To investigate the effect of PRMT5 knockdown on glioblastoma cell viability, three different cell lines (A172, U1242, and U251) were transfected with either scrRNA or si-PRMT5, and cell viability was determined by Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide staining and flow cytometry. As shown in Fig. 4A and B, PRMT5 knockdown resulted in the induction of apoptosis in all cell lines compared with scrRNA control (P = 0.002 across cell lines and time points).

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**Apoptosis induced by PRMT5 depletion occurs independent of p53 mutational status**

The p53 pathway is commonly deleted or mutated in glioblastoma and associated with more aggressive disease and poor prognosis (28). Our initial observations suggested that induction of glioblastoma cell line cell death following PRMT5 deletion occurred independent of p53 mutational status (Fig. 5, A172 p53wt vs. U1242 and U251 p53 mut and Supplementary Table S1). Recent work has described the mechanism by which PRMT5 modulates the activity of p53 (6, 7). The p53 wild-type cell line, A172 (29), was cotransfected with siRNA targeting p53 (si-p53) and si-PRMT5. As shown in Fig. 5A and B, changes in cell viability due to PRMT5 depletion remained unchanged regardless of p53 expression level. We confirmed these studies using pitthrin-α, an inhibitor of p53 transcriptional activity (Supplementary Fig. S5). In addition, in p53-null human glioblastoma cells with a doxycycline-inducible p53 expression system (2024 cell line), the loss of cell viability due to PRMT5 depletion was similar despite
absence or presence of p53 expression, even after inducing DNA
damage with ionizing radiation (Fig. 5C and D). Furthermore,
the cells seemed to be sensitized to radiation-induced cell death
upon depletion of PRMT5, regardless of p53 expression (Fig. 5C
and D). We found all cell lines, regardless of p53 mutational
status, underwent G2–M cell-cycle arrest (Fig. 3D and data not
available).
shown) and apoptosis following PRMT5 depletion, an important finding when taken in the context that p53 defects are associated with chemo- and radioresistance and portend a poor prognosis in patients with glioblastoma.

**PRMT5 inhibition promotes restoration of regulatory gene expression**

Several reports have shown that PRMT5 contributes to transcriptional repression of tumor suppressor genes ST7 (16), NM23 (14), RBL2 (p130; ref. 30), and DOC-1 (31). To investigate potential genes targeted by PRMT5 in glioblastoma, we treated cell lines with PRMT5 siRNA or scrRNA control, and carried out transcriptome analysis using Affymetrix cDNA expression arrays (Supplementary Table S4, genes showing >1.5-fold expression with PRMT5 knockdown). Consistent with the findings of Pal and colleagues (16) who reported a similar experiment using a murine cell line, we found 84 genes to become upregulated >1.5-fold compared with only four
genes downregulated after PRMT5 knockdown, verifying PRMT5 as a global transcriptional repressor in human glioblastoma cells. We observed a wide variety of genes with functions ranging from tumor suppressor (ST7) to immune modulatory activity (CXCL10, CXCL11, and IL6). Similar to data with mantle cell lymphoma cell lines and tumors (16) in which...
Figure 5. si-PRMT5-induced cell death is p53-independent. A, p53 wild-type cell line, A172, treated as indicated and cell death assessed in triplicate by flow cytometry. B, A172 cells were treated as in A and p53 detected by Western blot analysis. C, the 2024 cell line was treated as indicated and irradiated with an X-ray ionizing radiation (8 Gy) to introduce DNA damage signal. Cell death was assessed by flow cytometry. D, Western blot analysis for PRMT5, p53, and β-actin.
we characterized a PRMT5-dependent repressive complex (PRMT5, MBD2, and HDAC2), we noted a 1.4-fold increase in ST7 mRNA expression in glioblastoma cell lines treated with si-PRMT5.

On the basis of this cell line in vitro data suggesting that ST7 is a functional downstream target of PRMT5, we sought to further examine ST7 expression levels as they related to clinical outcomes in glioblastoma. We used the Rembrandt dataset to analyze microarray gene expression data to associated survival data. Kaplan–Meier analysis was performed on all patients with glioma (grade 1–4; Supplementary Fig, S6) and glioblastoma only (Fig. 6A). Both analyses demonstrated that ST7 expression correlated with statistically significant differences in overall survival in patients with glioma. Specifically, 2-fold upregulation of ST7 was associated with a statistically significant increase in overall survival. This supports our hypothesis that lower ST7 expression levels attributed to increased levels of PRMT5 and transcriptional silencing of ST7 correlate with a more aggressive and worse clinical outcome. It also supports the notion that ST7 contributes to the pathogenesis in supporting the malignant behavior of glioblastoma.

Given that ST7 repression in glioblastoma tumors associated with significant reduction in survival (Fig. 6A) and the role played by ST7 in maintenance of extracellular matrix and modulation of genes that prevent metastasis (32), we examined the functional consequences of PRMT5 inhibition and modulation of this anticancer target gene. To verify that PRMT5 was physically recruited to the promoters of these genes and associated with a corepressor complex, we isolated chromatin preparations and performed ChIP studies. Glioblastoma cell lines were grown in the presence of si-PRMT5 (validated with three separate siRNA constructs as shown in Supplementary Fig. S3), scrRNA or left untreated, and 24 hours later cross-linked chromatin was prepared for ChIP assays. Using antibodies specific for PRMT5 and nonreactive antibody control and primers specific for regions within target gene promoters, we confirmed PRMT5 recruitment to the ST7 promoter (Fig. 6B). When PRMT5 levels were knocked down, enrichment of PRMT5, MBD2, DNMT3a, and HDAC2 on the ST7 promoter was markedly diminished consistent with our prior findings (14, 16, 32; Fig. 6B, and data not shown). Loss of PRMT5 recruitment on target promoters led to enhanced transcriptional (Fig. 6C) and translational activity (Fig. 6D). Furthermore, we noted that overexpression of ST7 in three glioblastoma cell lines (A172, U1242, and U251) was able to induce cell death and reduce cell proliferation (Supplementary Fig. S7), indicating that ST7 played a role as a downstream effector of PRMT5 dysregulation.

**PRMT5 inhibition leads to enhanced survival in a preclinical glioblastoma xenograft model**

To further examine whether PRMT5 protein overexpression is relevant to glioblastoma pathogenesis, we studied the consequences of PRMT5 inhibition in an aggressive in vivo glioblastoma xenograft model. Nude mice underwent intracranial implantation of Gli36 delta cells that were treated either with scrRNA or si-PRMT5 (equivalent cell number and viability was validated before engraftment, n = 8/group). Mice engrafted with Gli36 cells treated with a single exposure to the PRMT5 short hairpin RNA (shRNA) group were shown to have prolonged survival (P = 0.0004; Fig. 7B) compared with mice in the control shRNA group. Tumor burden was tracked by clinical assessment and by MRI at day 28 (Fig. 7A). These in vivo data provide further support of the relevance PRMT5 plays in supporting the malignant phenotype of glioblastoma.

**Discussion**

High-grade astrocytomas are the most common primary brain tumors and are associated with a poor prognosis despite aggressive surgical and medical management (23). Over the last several decades, we have observed improved outcome and survival for patients with a wide variety of cancers; however, the minimal improvement in survival of patients with glioblastoma illustrates the need to discover novel targets to develop new therapeutic approaches. In addition to classic gene mutations, inactivation of regulatory and tumor suppressor genes by epigenetic repression is frequently observed in these tumors. Histone deacetylase inhibitors have been shown to possess antitumor activity in malignant astrocytomas (33), clinical trials investigating these agents have been disappointing, highlighting the need to identify other promising epigenetic targets.

The PRMT family of enzymes represents a group of proteins that are highly conserved in nature and involved with a wide variety of developmental and biologic processes (34). PRMT5, a type II arginine methyltransferase, silences the transcription of genes by symmetric dimethylation of arginine residues on histone proteins and works more efficiently when associated with other corepressor enzymes such as HDAC and MBD proteins (13, 35). Recent studies have shown PRMT5 to be a major prosurvival factor regulating eIF4E expression and p53 function (7). Furthermore, PRMT5 depletion sensitizes human colon cancer, lung cancer, and fibrosarcoma cells to TRAIL without affecting TRAIL resistance in nontransformed cells (24). Here, we show that PRMT5 is selectively overexpressed in high-grade astrocytomas and that the degree of PRMT5 expression correlated with cell growth and patient survival. Our work examining potential mechanisms of PRMT5 overexpression point toward miRNA dysregulation, and we are currently investigating how PRMT5 translation is efficiently supported in glioblastoma.

Our findings showing a correlation between PRMT5 overexpression and poor clinical outcome of patients with glioblastoma are intriguing with regard to potential utility as a prognostic factor to identify patients with more aggressive disease. Although several abnormalities affecting epigenetic processes have been described in glioblastoma tumors, including expression of DNMT enzymes (36), and methyl-CpG-binding domain proteins (37), the prognostic relevance of these markers remains unclear. Identifying biomarkers like PRMT5 that are of prognostic and biologic significance will assist in developing novel approaches to this disease. Future prospective analysis will be essential to determine the true prognostic value of PRMT5 overexpression in patients with glioblastoma.
Apart from epigenetic modifications, PRMT5 has been shown to mediate arginine methylation of p53 to regulate its function (7). Because p53 has been shown to be highly relevant as a critical tumor suppressor for both primary and secondary subtypes of glioblastoma (38), we explored the role of p53 in cell death triggered by PRMT5 knockdown. We observed equivalent degrees of cell death induced by PRMT5 silencing in both p53 wild-type, mutant, and deleted glioblastoma cells. Work by several groups has demonstrated that PRMT5 directly targets p53 function by methylating R333, R335, and R337, modifications that negatively affects p53 recruitment to target gene promoters during genotoxic stress and cell death. Scoumanne and colleagues (7) showed that PRMT5 knockdown led to decreased p53 stability, reduced expression of the p53 target gene CDKN1A, and decrease of breast cancer cell proliferation. Other work by Jansson and colleagues (6) demonstrated that the effect of PRMT5 depletion with siRNA on DNA damage-induced apoptosis was more significant in p53 wild-type compared with p53-null colorectal carcinoma cell lines. Our results suggest that cell death induced by PRMT5 silencing occurs independent of p53 mutational status, indicating that the relevance of arginine methylation of p53 may differ between tumor types and, perhaps, PRMT5 might serve as an equally

Figure 6. ST7 is a PRMT5 target gene. A, Kaplan-Meier plot for glioblastoma cases with differential ST7 gene expression (low ST7 n = 169; high ST7 n = 12). B, ChIP assays using IgG control or anti-PRMT5 antibody performed on chromatin from U251 or U1242 treated as indicated. Fold enrichment with each antibody was calculated relative to the IgG control (in triplicate). The data were normalized by the input DNA \( \Delta C_t = C_t(\text{IP}) - C_t(\text{input}) \) and one-way ANOVA was used to analyze the normalized data for each cell line. C and D, expression of ST7 is upregulated by si-PRMT5 determined by real-time PCR (C) and Western blot analysis (D).

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attractive target in glioblastoma tumors in which p53 is often mutated or deleted and associated with a poor prognosis.

Changes observed in the glioblastoma transcriptome following PRMT5 depletion helped us identify PRMT5 as a master transcriptional repressor in this disease. We identified the tumor suppressor gene ST7 as a potential target as it became derepressed with PRMT5 inhibition. Consistent with these results, ChIP experiments identified the ST7 promoter to be direct targets of PRMT5. The human ST7 gene was first recognized as a candidate tumor suppressor based on its chromosomal location (7q31.1), a site of frequent loss of heterozygosity in transformed cells (39) and its reduced expression in cancer (40). Here, we show that reduced ST7 expression correlates with poor outcome in patients with glioblastoma. ST7 protein activity is also involved with extracellular matrix remodeling in several solid tumor models and may mediate tumor suppression by altering the tumor microenvironment and affecting tumor invasion (32). The invasive nature of glioblastoma tumors precludes complete surgical resection in the majority of patients with this disease. Thus, development of experimental therapeutic strategies to target PRMT5 overexpression promoting restoration of tumor suppressors that negatively regulate malignant cell invasion and microenvironment may affect metastatic potential of this disease in vivo. We are currently examining the direct role ST7 plays in glioblastoma invasiveness.

Previous work has shown that PRMT5 associates with SWI/SNF chromatin remodeling complexes along with other corepressor molecules like HDAC2 (35), DNMT3a (13), and MBD proteins (31). Biochemical assays have demonstrated that PRMT5 activity on target H4R3 and H3R8 histone arginine residues is markedly enhanced when lysine residues become deacetylated by HDAC2 (35). Our results showed that inhibition of PRMT5 leads to loss of recruitment at target gene promoters (ST7, CXCL10, and CXCL11, data not shown). Importantly, our prior work and current data suggest that by inhibiting PRMT5 overexpression alone, additional corepressor proteins contributing to anticancer gene silencing may also be affected, providing rationale to further investigate the
antitumor activity of combination strategies incorporating agents that target the epigenome (HDAC inhibitors, hypomethylating agents) with PRMT5 inhibitors.

Finally, PRMT inhibition was shown to prolong survival in an aggressive preclinical mouse glioblastoma model. Collectively, our data suggest that PRMT5 overexpression represents an unfavorable prognostic marker and an attractive therapeutic target for glioblastoma. Experimental therapeutic strategies aimed at inhibiting the effects of PRMT5 overexpression in cancer might lead to a better understanding of how to directly and indirectly affect glioblastoma tumor progression. These findings further justify our current efforts to explore novel approaches to target PRMT5 activity in glioblastoma and other cancers in which this enzyme is dysregulated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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References


Genetic Validation of the Protein Arginine Methyltransferase PRMT5 as a Candidate Therapeutic Target in Glioblastoma

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