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PRMT5–PTEN molecular pathway regulates senescence and self-renewal of primary glioblastoma neurosphere cells

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Abstract

Glioblastoma (GBM) represents the most common and aggressive histologic subtype among malignant astrocytoma and is associated with poor outcomes because of heterogeneous tumour cell population including mature non-stem-like cell and immature stem-like cells within the tumour. Thus, it is critical to find new target-specific therapeutic modalities. Protein arginine methyltransferase enzyme 5 (PRMT5) regulates many cellular processes through its methylation activity and its overexpression in GBM is associated with more aggressive disease. Previously, we have shown that silencing of PRMT5 expression in differentiated GBM cell lines results in apoptosis and reduced tumour growth in mice. Here, we report the critical role of PRMT5 in GBM differentiated cells (GBMDC) grown in serum and GBM neurospheres (GBMNS) grown as neurospheres *in vitro*. Our results uncover a very significant role for PRMT5 in GBMNS self-renewal capacity and proliferation. PRMT5 knockdown in GBMDC led to apoptosis, knockdown in GBMNS led to G1 cell cycle arrest through upregulation of p27 and hypophosphorylation of

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception and design: YK Banasavadi-Siddegowda, B Kaur and R Baiocchi. Development of methodology: YK Banasavadi-Siddegowda. Acquisition of data: YK Banasavadi-Siddegowda, L Russell, E Frair, Vrajesh A Karkhanis, T Relation and J Yoo. Analysis and interpretation of data: YK Banasavadi-Siddegowda, B Kaur, R Baiocchi, J Zhang and J Imitola. Writing, review and/or revision of manuscript: YK Banasavadi-Siddegowda, B Kaur and R Baiocchi.

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retinoblastoma protein, leading to senescence. Comparison of impact of PRMT5 on cellular signalling by the Human Phospho-Kinase Array and chromatin immunoprecipitation-PCR revealed that unlike GBMDC, PRMT5 regulates PTEN expression and controls Akt and ERK activity in GBMNS. *In vivo* transient depletion of PRMT5 decreased intracranial tumour size and growth rate in mice implanted with both primary tumour-derived GBMNS and GBMDC. This is the first study to identify PTEN as a potential downstream target of PRMT5 and PRMT5 is vital to support both mature and immature GBM tumour cell populations.

INTRODUCTION

Glioblastoma (GBM) is the most common and lethal malignant astrocytoma¹ and despite decades of research, prognosis for patients remains dismal.² The highly invasive nature of GBM tumours into adjacent normal brain tissue makes it impossible to achieve 100% surgical resection post therapy, and despite concurrent radiation and chemotherapy almost all will recur within 12 months.^{1,3} The poor outcome despite the current multimodal combination therapy underscores the need to identify novel target-specific therapeutic modalities.⁴

Protein arginine methyltransferase (PRMT) represents a family of enzymes that covalently modifies histone and non-histone proteins to regulate gene transcription and cellular signalling.⁵ PRMT5 catalyses the symmetric dimethylation of two of three guanidine nitrogen atoms in the arginine molecule using S-adenosyl-L-methionine as a cofactor. PRMT5-guided symmetric dimethylation of histone proteins H3 (S2Me-H4R3) and H4 (S2Me-H3R8) regulates chromatin structure to promote transcriptional repression.^{6,7} PRMT5 expression positively correlates with the grade of malignancy in astrocytomas and inversely correlates with survival.^{8,9} In concordance with this role, depletion of PRMT5 in glioma cell lines failed to form tumours in mice engrafted intracranially with GBM xenografts.⁸

GBM consists of heterogeneous types of tumour cells with both differentiated mature non-stem-like and undifferentiated immature stem-like cells. Although the cell type of origin (neural stem cells vs differentiated astrocytes, neurons and so on) remains controversial,¹⁰ genome-wide studies have attributed variation in treatment response to the heterogeneity with respect to genetic mutations, gene expression profiles and epigenetic modifications.^{11,12} Unlike traditional cancer cell lines grown in serum, patient-derived GBM neurospheres recapitulate the genotype, gene expression patterns and *in vivo* tumour biology of human GBM.¹³ The biology of the GBM stem-like cells is emerging as an important translational target to prevent recurrences. Discovery of pathways with distinct roles on GBM stem-like cells and non-stem-like cells holds great potential to change the landscape of GBM treatment. Here, we compared the significance of PRMT5 in primary patient-derived GBM cells grown as undifferentiated neurospheres in neurobasal media that are enriched for stem-like cells (GBMNS) and differentiated cells grown in serum (GBMDC).

Herein we report, that in contrast to GBMDC, PRMT5 regulates the self-renewal capacity of GBMNS via modulation of the PTEN–AKT axis. PRMT5 depletion leads to senescence in GBMNS, decreases the tumour-forming ability and enhances the survival in a GBM

xenograft model. Our work shows that PRMT5 is required for the proliferation and self-renewal of GBMNS, whereas in GBMDC, it is necessary for survival. This study is the first to identify the PTEN as a novel target of PRMT5 and underscores the significance of targeting arginine methylation as a promising therapeutic strategy affecting both differentiated and undifferentiated tumour cell populations via non-overlapping mechanisms.

RESULTS

PRMT5 differentially regulates self-renewal, survival and proliferation of GBMNS

To study the role of PRMT5 in patient-derived GBMNS, we silenced PRMT5 expression using pooled-PRMT5 siRNA (P5i) or single siRNA (P5i3) and validated PRMT5 knockdown by western blot (Figure 1a and Supplementary Figure S1a). Growth of PRMT5 depleted and control GBMNS was examined using an MTT assays (Figure 1b; Supplementary Figures S1b, S2a and b). Although control GBMNS showed atleast twofold increases in the proliferation; the proliferation of PRMT5-depleted GBMNS did not increase significantly for 3 days. Data was analysed using the linear mixed model to account for the covariance structure due to repeated measures at different time points. Data shown are the individual (dots) and mean (line) proliferation for PRMT5 intact and depleted GBMNS ($n = 3/\text{time point}$). Overall, in P5i GBMNS, proliferation was significantly lower than control ($P < 0.001$). We also tested the role of PRMT5 in regulating the self-renewal capacity of GBMNS. Knockdown of PRMT5 decreased both size and number of neurospheres significantly (Figure 1c; Supplementary Figures S1c, S2c and e). To compare the frequency of neurosphere formation, we performed the linear regression analysis and observed a significant reduction in the number of neurospheres with PRMT5 depletion (Figure 1d; Supplementary Figures S1d, S2d and f). These results suggest that PRMT5 is required for the proliferation and self-renewal of primary GBMNS.

PRMT5 knockdown has been shown to cause apoptosis in serum-grown glioma cell lines.⁸ Interestingly Annexin V and propidium iodide (PI) staining did not show a similar increase in the apoptosis of GBMNS after PRMT5 knockdown (Figures 1e and g; Supplementary Figure S3). To see if this effect was related to the differentiation status of the cells, GBMNS were differentiated by growing them in the serum-containing media for atleast 10 days.¹⁴ Increase in gene expression of differentiation markers with a concurrent decrease in 'stem cell-like' markers was tested to confirm the differentiation of these cells after exposure to serum-containing culture conditions (Supplementary Figure S5). Consistent with previous findings in glioma cell lines, differentiation of GBMNS restored their sensitivity to apoptosis upon PRMT5 knockdown (Figure 1f; Supplementary Figures S6 and S7). We further measured the viability of GBMNS and GBMDC with or without PRMT5 depletion by trypan blue dye exclusion. PRMT5 knockdown decreased the viability of GBMDC by 40%; but viability of GBMNS was not affected (Supplementary Figure S8). Overall, these results suggest that PRMT5 regulates GBMNS and GBMDC differentially and although PRMT5 is required for self-renewal in GBMNS, it is essential for survival of GBMDC.

PRMT5 regulates G1 cell cycle progression in GBMNS

Next, we tested impact of PRMT5 on cell cycle progression of GBMNS and GBMDC (Figures 2a and b; Supplementary Figure S9). PRMT5 depletion increased the G1 population of GBMNS by atleast 30% implicating G1/S cell cycle arrest. This arrest is consistent with observations of decreased proliferation and self-renewal as shown in Figure 1. Interestingly, PRMT5 depletion did not have significant impact on the cell cycle progression of GBMDC suggesting a cell cycle independent role for PRMT5 in regulating apoptosis of differentiated cells.

Active, hypophosphorylated-retinoblastoma (Rb) protein is a major regulator of transit of cells from G1 to S phase.^{15–20} As progression of the cells through G1 to S phase requires inactivation (or phosphorylation) of the Rb protein, we tested its phosphorylation status in GBMNS (Figure 2c). Depletion of PRMT5 decreased the levels of phosphorylated-Rb in GBMNS but not in GBMDC suggesting that PRMT5 regulates cell cycle in GBMNS by controlling Rb activation.

Cyclin E/CDK2 causes hyperphosphorylation of Rb proteins.^{21–23} p27 is a member of the cyclin-dependent kinase inhibitors and is capable of associating and with and inhibiting Cyclin E activity.^{24,25} Hence, we tested for the expression of p27 in the PRMT5 intact and depleted GBMNS and GBMDC (Figure 2d). Western blot analysis indicates PRMT5 depletion increased the expression of p27 in GBMNS. This suggested that PRMT5 mediates repression of p27 in GBMNS.

PRMT5 regulates AKT and ERK pathways in GBMNS

To further understand the differential role of PRMT5 in GBMNS and GBMDC, we utilized Human Phospho-Kinase Array kit (R&D Systems, Minneapolis, MN, USA) to analyse changes in phosphorylated proteins involved in cell signalling. This array showed differential expression of many phospho proteins (Figures 3a and b) including downregulation of both phospho-AKT (pAKT) and phospho-ERK (pERK) in GBMNS with PRMT5 depletion. Validation of the antibody array data by western blot confirmed the reduction in pAKT and pERK in GBMNS after PRMT5 knockdown (Figure 3c and Supplementary Figure S10). Interestingly in GBMDC, pERK and pAKT were unaffected with PRMT5 knockdown (Figures 3d and e; Supplementary Figure S10). These results together suggest that in GBMNS, PRMT5 positively regulates the activity of both AKT and ERK.

AKT activity is regulated by PTEN in GBMNS

One of the major upstream targets of AKT is tumour suppressor PTEN.^{26,27} We tested the expression of PTEN in GBMNS and GBMDC. Depletion of PRMT5 led to significant increase in PTEN transcript and protein expression in GBMNS but not in GBMDC (Figures 4a and b). To probe if the regulation of PTEN expression by PRMT5 was at a transcriptional level, we monitored the recruitment of PRMT5 to the PTEN promoter in GBMNS and GBMDC by chromatin immuno precipitation (ChIP) assay (Figure 4c). PRMT5 binding to the PTEN promoter was more enriched in GBMNS but not in GBMDC, suggesting that the expression of PTEN is repressed by PRMT5 in GBMNS.

To further understand the relevance of PRMT5-mediated silencing of PTEN, we overexpressed wild-type PTEN in GBMNS (Figure 4d). Wild-type PTEN led to increased expression of both the long secreted and regular forms of PTEN recently reported (Figure 4d).²⁸ Overexpression of PTEN recapitulated the phenotype of decreased neurosphere renewal observed with PRMT5 knockdown (Figures 4e and f; Supplementary Figures S11a and S11b). PRMT5 depletion in GBMNS overexpressing PTEN did not lead to enhanced reduction in neurosphere formation (Figures 4g and h) suggesting PTEN to be downstream of PRMT5 and that PTEN overexpression was the critical factor leading to decreased proliferation in GBMNS (Figure 4h). Next, we assessed the effect of PTEN overexpression on apoptosis of GBMNS and GBMDC by Annexin V and PI staining (Supplementary Figure S11c). Similar to PRMT5 depletion (Figure 1e), PTEN overexpression did not increase the apoptosis of GBMNS. Consistent with low-basal AKT activity in GBMDC (Figure 3) PTEN overexpression in these cells did not affect their viability. These results indicate regulation of PTEN by PRMT5 in GBMNS has a key role in their ability to self-renew and proliferate.

PTEN knockdown rescues the PRMT5 knockdown effects in GBMNS

To further validate the regulation of PTEN by PRMT5, we performed rescue experiments using pooled-PTEN siRNA (PTENi) and single PTEN siRNA (PTENi3). PRMT5-intact control and depleted GBMNS were transfected with Cntrl or PTENi/PTENi3. PTEN knockdown was validated by western blot (Figure 5a and Supplementary Figure S12a). PTEN depletion did not affect the expression of PRMT5, and PRMT5 knockdown increased the expression of PTEN (Figure 5a and Supplementary Figure S12a) indicating that PTEN is a downstream target of PRMT5. Using same transfection condition, we conducted proliferation and neurosphere formation assay (Figures 5b–d and Supplementary Figures S12b–d). Proliferation and neurosphere self-renewal assays revealed that knockdown of PTEN in PRMT5-depleted GBMNS partially rescued the proliferation, size and number of neurospheres and did not affect cellular proliferation and self-renewal in PRMT5-intact neurospheres (Figure 5c and Supplementary Figure S12c). Collectively these results indicate that PTEN gene expression is one of the key molecules by which PRMT5 maintains undifferentiated GBMNS cells self-renewal and proliferation.

PRMT5 knockdown causes senescence in GBMNS

PRMT5 knockdown caused apoptosis in GBMDC, but not in GBMNS (Figure 1 and Supplementary Figure S3). Decreased proliferation, cell cycle arrest (Figures 1, 2a and b; Supplementary Figures S1a, b and S9) and increased cell size in PRMT5-depleted GBMNS are salient features of senescent cells.^{29–31} Thus, we evaluated senescence with PRMT5 knockdown. We utilized the β -galactosidase (β -gal) staining assay to identify senescent cells.³² Although control cells had undetectable β -gal-positive cells, there were more than 100 β -gal-positive cells per view field in the PRMT5-depleted GBMNS (Figures 6a and b).

Senescence can be mediated by multiple pathways that converge at p53-p21 and Rb induction.³³ To confirm the senescence phenotype, we measured the expression of p53 and p21 mRNA (Figure 6c). PRMT5 knockdown increased the relative mRNA expression of p21 and p53. Further we also probed for the expression of Rb, p53 and p21 proteins with or without PRMT5 knockdown (Figures 3c and 6d). Consistent with senescence phenotype, we

found decreased pRb and increased expression of p53 and its target gene *p21*. These results confirm the senescence in PRMT5-depleted GBMNS.

Next, we tested the impact of PTEN overexpression in GBMNS on senescence (Figures 6e and f). Similar to the results obtained with PRMT5 knockdown, PTEN overexpressing cells showed a significant increase in β -gal-positive GBMNS.

PRMT5 inhibition prolongs survival and stunts tumour growth rate in a preclinical GBM mouse model

To test the relevance of our *in vitro* findings in undifferentiated GBMNS cells *in vivo*, we evaluated the survival of mice implanted with GBMNS and GBMDC transiently knocked down for PRMT5. Luciferase and magnetic resonance imaging (MRI) imaging of tumour bearing mice revealed significant differences in the tumour growth as measured by total flux of the tumours. At individual time points, GBMDC–Cntrl tumours were significantly brighter than GBMDC–P5i (day 7, 11, 14; $P=0.0057$, $P=0.0011$, $P=0.0032$, respectively), and GBMNS–Cntrl were similarly significantly brighter than GBMNS–P5i (day 7, 11, 14; $P=0.0068$, $P=0.0013$, $P<0.0001$, respectively). Overall, the analysis of tumour growth curves as measured by total flux was significantly different between non-GBMNS–Cntrl vs –P5i; $P<0.0001$ and GBMNS–Cntrl vs –P5i; $P=0.0045$, respectively (Figure 7a).

Consistent with luciferase imaging an obvious difference in tumour volume was observed by MRI imaging (day 15 post tumour implantation; Figure 7b). Importantly, PRMT5 knockdown improved survival in both GBMNS and GBMDC tumour-bearing mice (Figures 7c and d). GBMDC bearing mice showed a significant improvement in median survival from 23 (Cntrl) to 26 (P5i) days ($P=0.0083$) (Figure 7c) and GBMNS-bearing mice also showed increased benefit from 21 (Cntrl) to 29 (P5i) days median survival ($P=0.0005$) (Figure 7c).

DISCUSSION

GBM is composed of heterogeneous types of tumour cells that include therapy-resistant immature GBMNS and therapy sensitive mature GBMDC.^{34–36} Genome-wide studies have attributed this variation in treatment response to the heterogeneity with respect to genetic mutation, gene expression profiles and epigenetic modifications.^{11,12} From the clinical therapeutic point of view, targeting both GBMNS and GBMDC is required mainly for two reasons. (1) GBMDC comprises the bulk cellularity of the tumour and (2) existence of dynamic equilibrium between GBMNS and GBMDC; selective targeting of GBMNS alone, will allow surviving GBMDC to give rise to GBMNS. Conversely, targeting only GBMDC can still lead to therapy resistance and tumour recurrence as therapy-resistant GBMNS will survive and give rise to mature GBM.

We have recently reported that the expression of PRMT5 is most pronounced in high-grade gliomas and degree of overexpression correlates inversely with survival. Furthermore, PRMT5 expression directly correlates with the grade of malignancy in astrocytomas.^{8,9} Using traditional GBM cell lines, we had shown that PRMT5 depletion causes regression of intracranial tumours implanted in mice by killing the tumour cells through apoptosis.⁸ Given the impact of undifferentiated primary cells on glioma biology, here we compared the effect

of PRMT5 in both GBMNS and GBMDC. Utilizing primary GBM cells grown as undifferentiated neurospheres or differentiated in serum-culture conditions, we found that PRMT5 is necessary for the proliferation and self-renewal of GBMNS but not GBMDC. PRMT5 regulates cell cycle progression by modulating p27 expression and Rb protein activity in GBMNS. Furthermore, we identified that PTEN is a direct molecular target of PRMT5 and has a key role in controlling senescence of GBMNS. PRMT5 inhibition in both GBMNS and GBMDC prolongs survival and stunts tumour growth. Collectively, our data show that PRMT5 is a relevant therapeutic target in both differentiated and undifferentiated GBM tumour cell populations through very different mechanisms.

PRMT5 inhibition induces senescence in GBMNS

Most chemotherapeutics and radiation therapy rely on extensive induction of DNA damage, resulting in destruction of proliferating mitotic population within solid tumours.³⁷ Although radiation and chemotherapy are standard of care for GBM patients,³⁷ numerous reports have implicated an undifferentiated GBMNS population to be resistant to such therapies.^{34–36} The induction of senescence has been shown to be an effective treatment in tumour cells resistant to apoptosis.³⁸ Although the concept of senescence therapy has been considered a possibility,³⁷ more recent evidence such as identification of SKP2 as a pro-senescence target for therapeutic intervention³⁹ and importance of CD4⁺ T cell lymphocyte activation in the clearance of senescent cells has confirmed the relevance of this approach. Consequently, several senescence-inducing therapeutic approaches have been developed, some of which are either currently being explored (MLN4924; NCT02122770) or have completed (R7112; NCT01164033) clinical trials (ClinicalTrials.gov). In the current study, we show that PRMT5 knockdown leads to pro-senescence effect in GBMNS that are resistant to apoptosis.

PTEN as a target of PRMT5 in inducing senescence

It is well established that senescence can be induced through either the activation of oncogenes or through tumour suppressor genes such as PTEN.³⁷ In spite of extensive studies the role of PTEN in inducing or suppressing senescence is controversial. Although the PTEN loss is known to induce cellular senescence through p53 upregulation resulting from mTOR hyperactivation,^{37,40} increased PTEN expression is known to drive the cells towards senescence through inactivation of AKT and upregulation of p27 pathway.⁴¹ Further it has been reported that when the GBM cell lines were exposed to treatment conditions, PTEN-deficient cells undergo senescence, whereas the PTEN-intact GBM tumour cells undergo apoptosis.⁴² Collectively, even though the reports of these studies are not consistent, they bolster our findings that PTEN can induce senescence in GBMNSs that are resistant to apoptosis.

PTEN is a tumour suppressor gene that negatively regulates function of AKT,^{27,28,43,44} a downstream serine/threonine kinase frequently activated in the majority of GBM.⁴⁵ The prominent role played by AKT in cell growth and survival in cancer has made it an important therapeutic target and led to development of AKT inhibitors for the treatment.⁴⁵ Given the increased expression of PRMT5 in GBM, targeting PRMT5-mediated AKT activity in GBMNS would reduce AKT-imparted therapy resistance in GBM tumour. Further

studies are required to bolster the concept of PRMT5-mediated AKT inhibition to alleviate therapy resistance in GBM.

Overall, our study underlines the significance of PRMT5 in targeting both mature and immature tumour cells and underscores the importance of developing small-molecule PRMT5 inhibitors as a promising therapeutic approach for patients with GBM.

MATERIALS AND METHODS

Tissue culture

The human GBM tumour samples were collected from the consenting patients as per the protocol approved by the Institutional Review Board (IRB) at The Ohio State University. In accordance with the approved guidelines of The Ohio State University's IRB protocol, tumours were washed and dissociated using the TrypLE Express (Invitrogen, Carlsbad, CA, USA) and the resulting dissociated single cells were grown as GBMNSs. Patient-derived GBMNSs were cultured in Neurobasal Medium (Gibco, Grand Island, NY, USA) containing B27 without vitamin A (2%), human epidermal growth factor (50 ng/ml), basic fibroblast growth factor (50 ng/ml) and penicillin–streptomycin (1%) in low-attachment cell culture flasks as described.⁴⁶ Cells were authenticated by short tandem repeat profiling and mycoplasma contamination was tested in October 2015. Cells were negative for mycoplasma.

Western blot analysis

Cell pellets were lysed using RIPA buffer (Sigma-Aldrich, St Louis, MO, USA) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), and phosphatase inhibitor cocktail (Roche). Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) was used to determine the protein concentration. Equal amounts of protein were loaded on to a 4–20% Tris–HCl gel (Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Antibodies PRMT5, H4R3 and GAPDH procured from abcam (Cambridge, MA, USA), Rb, AKT, pAKT, ERK, pERK, p53, PTEN and p21 obtained from (Cell Signaling, Danvers, MA, USA), p27 (R&D, Minneapolis, MN, USA) were used at the dilution of 1:000–1:2000.

RT–PCR

To measure relative mRNA expression, RNA was purified from cell pellets (QIAGEN RNeasy, Qiagen, Hilden, Germany), per the manufacturer's instructions. cDNA was generated (SuperScript Reverse Transcriptase, Life Technologies, Carlsbad, CA, USA) and mRNA expression was measured via quantitative RT–PCR (Mastercycler ep realplex, Eppendorf, Westbury, NY, USA) using SYBR Green (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. Primer sequences: *gapdh*, forward, 5'-GGAGTCAACGGATTGGTTCG-3'; reverse, 5'-GGAATCATATTGGAACATGTAAACC-3'. *p53*, forward, 5'-CCCCCTCCTGGCCCCCTGTCATCTTC-3'; reverse, 5'-GCAGCGCCTCACAACCTCCGTCAT-3'. *p21*, forward, 5'-GAGGCCGGGATGAGTTGGGAGGAG-3'; reverse, 5'-

CAGCCGGCGTTTGGAGT;GGTAGAA-3'. *pten*, forward, 5'-
TGCAGAAAGACTTGAAGGCGTA-3'; reverse, 5'-GCTGTGGTGGGTTATGGTCTT-3'.

siRNA

Control and PRMT5-specific siRNAs were purchased from GE Dharmacon (Lafayette, CO, USA). siRNA were transfected using RNAi Max lipofectamine (Invitrogen, Grand Island, NY, USA) per the manufacturer's protocol.

Cell viability assays

Viability of the cells was measured using trypan blue dye exclusion assay (Life Technologies).

Cell cycle analysis

Post-treatment cells were washed with PBS, fixed with ethanol (80%) and stained with 50 µg/ml PI (Sigma-Aldrich, Saint Louis, MO, USA). Stained cells were analysed in a BD LSRII (Becton Dickinson, San Jose, CA, USA).

Beta-galactosidase (β-gal) assay

GBMNS were transfected every 3 days with scrambled or PRMT5 siRNA for 20 days. Twenty days post transfection cells were collected and senescent cells were identified by probing for β-gal using Senescence cells Histochemical Staining Kit (Sigma-Aldrich).

Antibody array

Antibody array was conducted using human phospho-kinase array (R&D), antibody array was conducted. GBMNS and GBMDC cells were transfected with Cntrl or PRMT5 siRNA. Seventy-two hours post transfection cells were pelleted and lysed with lysis buffer 6. Membrane sets pre-coated with antibodies were incubated with lysates overnight at 4 °C. After overnight incubation membrane were processed as per the protocol. Relative expression of the proteins was measured with respect to reference spots of the membrane.

ChIP assay

ChIP assays were performed using cross-linked chromatin from either GBMNS or GBMDC. Briefly, 10×10^6 cells were treated with 1% formaldehyde for 10 min at room temperature followed by addition of glycine to a final concentration of 0.1 M. Cells were washed twice with PBS and collected in 100 µl of lysis buffer (50 mM Tris-HCl (pH 8.1), 100 mM NaCl, 5 mM EDTA, 0.5% SDS, protease inhibitors). Chromatin was then collected by centrifugation and resuspended in 250 µl of immunoprecipitation buffer (100 mM Tris-HCl (pH 8.6), 5 mM EDTA, 0.3% SDS, 1.7% Triton X-100, protease inhibitors) before it was sonicated and digested with micrococcal nuclease (1 U/ml) at 37 °C for 20 min. Reaction was stopped by adding 200 µl of stop buffer (100 mM Tris-HCl (pH 8.6), 0.45% SDS, 2.5% Triton X-100, 5 mM EDTA (pH 8.0), protease inhibitors) and chromatin was analysed by agarose gel electrophoresis to ensure that DNA fragment sizes did not exceed 500 bp.

To monitor recruitment of PRMT5 and its epigenetic marks to PTEN promoter, preimmune (PIM) and immune antibodies generated against PRMT5, H3(Me2)R8, and H4(Me2)R3 were used to immune-precipitate cross-linked chromatin from control undifferentiated and differentiate neurospheres. After extensive washing, nucleoprotein complexes were eluted in 200 µl of elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS), and cross-links were reversed by incubating samples at 65 °C for 12 h. Immunoprecipitated DNA was purified by phenol and chloroform extraction and resuspended in 40 µl of Tris-EDTA (pH 8.0). To amplify promoter sequences of PTEN, a 3 µl aliquot of eluted DNA was used in a 10-µl RT-PCR in the presence of following primer pair and specific Roche Applied Science universal probe: pTEN-1 (forward, 5'-GTCTCTGAGAACCGAGCTTGA-3'; reverse, 5'-CAAGAGAGTCGAGCATCTTTCC-3'; probe 1).

Intracranial implantation

All experiments involving mice were performed under the approved protocol (Banasavadi-Siddegowda 2012A00000068) of the Committee on Animal Care at The Ohio State University. All methods were carried out in accordance with the approved guidelines. Six-week-old athymic Nu/Nu mice were intracranially implanted with 100 000 GBM30 tumour cells that stably express luciferase (GBM30-Luc). We used 10 mice for each treatment group. As each group were injected with cells transfected with specific siRNA, there was no room for the randomization. Tumour cells were implanted 2 mm lateral and 1 mm rostral to the bregma at a depth of 3 mm. Mice were anaesthetized with ketamine/xylazine solution intraperitoneally and were stereotactically stabilized using Kopf murine stereotaxics. Scalps were cleaned and parted and a 1-mm-wide injection hole was drilled using a 1mm burr bit. Tumour cells were implanted intracranially in a volume of 2 µl Hank's buffered salt solution over 5 min with kd Scientific auto-injectors at a rate of 0.4 µl/min. Mice were implanted with GBM30-Luc GBMDC or GBMNS cells, pre-treated with either si-Scrambled (Cntrl) or si-PRMT5 (P5i). Groups are labelled GBMNS-Cntrl or -P5i, and GBMDC-Cntrl or -P5i. For the survival study, as specified in the protocol (Banasavadi-Siddegowda 2012A00000068), mice were weighted and observed every day; mice losing 10% of their body weight (compared to pre-implantation weight) and/or showing the signs of tumour burdens such as lethargy, hunched back and so on, were considered as the end-point of the survival study.

MRI and tumour size measurement

Twelve mice (three per group) were randomly selected for MRI imaging on day 15 post tumour implantation. T2-weighted MRI images were captured without gadolinium enhancement, and 1-mm width brain slices were analysed for tumour volume calculations.

In vivo luciferase imaging

All mice were monitored for tumour growth via luciferase imaging of the GBM30-luciferase expressing tumours 7, 11 and 14 days post tumour implantation using an IVIS Lumia II imager and accompanying IVIS software (Perkin Elmer, Waltham, MA, USA). Five mice at a time were injected intraperitoneally with 200 µl firefly D-luciferin at 150 µg/ml and were sedated using isoflurane. Sedated mice were placed inside the IVIS imager with isoflurane flowing to a nose cone for each mouse. Mice were then imaged for 5, 10, 30, 60 and 180 s

using an XFOV lens with stage at position A, starting 6–7 min after initial injection of D-luciferin.

Flux analysis of luciferase images was performed on 30 s images for all time points. This was the longest exposure time that did not saturate the system at day 14, and was therefore used for day 7 and 11 analysis for consistency. Images being directly compared were adjusted to identical radiance scales and regions of interest were created surrounding the bright brain tumours. Total flux of regions of interest was quantified using IVIS software (Perkin Elmer).

Sample size justification

The sample size for each experiment was justified to achieve 80% power for the detection of an expected difference between groups based on corresponding two-sided tests. Type I error was controlled at 0.05. Specifically, for *in vitro* studies, $n = 3$ cell line replicates per group was justified to detect a fold change of 2.5, assuming a coefficient of variation of 30%. For *in vivo* survival studies, $n = 10$ per group was justified to detect a hazard ratio of 4 between two groups (that is, P5i vs Control).

Statistical analysis

Results are presented as mean $\pm$ s.d. The Student's *t*-test or ANOVA model was used to compare two or more independent groups. For proliferation experiment, linear mixed model was used to compare groups by accounting for the covariance structure due to repeated measures at different time points. The Kaplan–Meier method was used to estimate survival curves and the log-rank test was used to compare overall survival between groups. *P*-values were adjusted for multiple comparisons and a *P*-value < 0.05 was considered statistically significant. All tests were two-sided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PRMT5 is required for self-renewal in GBMNS and survival in GBMDC: **(a)** GBMNS OG2, OG34 and GBM169 transfected with scrambled (Cntrl) or pooled-PRMT5 siRNA (P5i) were subjected to western blot to probe the expression of PRMT5 using specific antibody. The PRMT5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blots were cropped from the main western blot membrane. **(b)** GBMNS OG2 cells were transfected with Cntrl or P5i and subjected to MTT assay at the specified time points to follow the rate of proliferation. MTT assay read-out were analysed by the linear mixed model to account for the covariance structure due to repeated measures at different time points. Data shown are the individual (dots) and mean (line) proliferation of Cntrl and P5i-transfected GBMNS ($n = 3/\text{time point}$) ($P < 0.001$). **(c)** GBMNS OG2 were transfected with Cntrl or P5i. 7 days post transfection, representative images were taken to show the size and number of neurospheres. **(d)** 1000, 500, 250, 100, 50, 25 and 10 cells of GBMNS OG2 were transfected with Cntrl or P5i. Seven days post transfection, number of neurospheres formed in each well was counted. The thin black line represents the linear trend. Inner panel: graph showing the number of neurospheres formed at lower cell number (10, 50, 100 cells). To compare the frequency of neurosphere formation, linear regression was performed as shown. **(e)** GBMNS OG2 was transfected with Cntrl or P5i. 72 h later, cells were subjected to apoptosis assay using Annexin V and PI staining. **(f)** GBMDC OG2 cells were transfected with Cntrl or P5i. 72 h

later, cells were subjected to apoptosis assay using Annexin V and PI staining. (g)
Quantification of the number of Annexin V-positive cells in the GBMNS and GBMDC with or without PRMT5 depletion (based on the results of **e** and **f**. Results are presented as mean values $\pm$ s.d. of the mean. Statistical analysis was carried out by the unpaired Student's *t*-test using GraphPadPrism software (GraphPad Software, Inc, La Jolla, CA, USA). ***P* < 0.01.

Figure 2.

PRMT5 depletion causes G1 cell cycle arrest in GBMNS: **(a, b)** GBMNS OG2 (left panel) and GBMDC OG2 (right panel) transfected with Cntrl or P5i were subjected to the cell cycle analysis. **(a)** Representative histogram indicating the percent of cells in each stages of cell cycle is shown in the figure. **(b)** Graph showing the quantification of cell cycle analysis. **(c)** GBMNS and GBMDC OG2 were transfected with Cntrl or P5i probed for pRb and total Rb by western blot analysis using appropriate antibodies. GAPDH was used as control. The blots were cropped from the main western blot membrane **(d)** GBMNS and GBMDC

transfected with Cntrl or P5i were probed for p27 expression by western blot. The p27 and GAPDH blots were cropped from the main western blot membrane.

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Figure 3.

PRMT5 regulates both AKT and ERK activity in GBMNS: (a) Images of antibody array for GBMNS and GBMDC transfected with Cntrl or P5i. Red and green boxes indicate the expression of pAKT and pERK, respectively, in duplicates. (b) Heat map showing the quantification of the antibody array of a. (c) Western blot for the specified proteins in GBMNS OG2, OG34 and GBM169 transfected with Cntrl or P5i. The blots were cropped from the main western blot membrane. (d, e) GBMNS and GBMDC OG2 transfected with

Cntrl or P5i were probed for the expression ERK (**d**) and AKT (**e**) by western blot. The blots were cropped from the main western blot membrane.

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Figure 4.

PRMT5 negatively regulates the expression of PTEN in GBMNS: (a) Western blot showing the expression of PTEN in GBMNS and GBMDC transfected with Cntrl or P5i. The PTEN and GAPDH blots were cropped from the main western blot membrane. (b) Relative PTEN mRNA expression in the Cntrl or P5i-transfected GBMNS. (c) ChIP assays on GBMNS or GBMDC showing fold enrichment relative to the PI sample. (d) GBMNS OG2 was transfected with empty pcDNA (Cntrl) or pcDNA containing wild-type PTEN (PTEN) plasmids showing overexpression of both PTEN-long (arrow) and PTEN (asterisk) was

confirmed by western blot. GAPDH was used as control. The PTEN and GAPDH blots were cropped from the main western blot membrane. (e) Representative microscopic image (10 ×) showing the size and number of neurospheres formed in GBMNS OG2 7 days post transfection with Cntrl of PTEN overexpressing plasmid. (f) Quantification of the number of neurospheres formed with Cntrl or PTEN expression in GBMNS OG2 cells. $P = 0.001$. (g) GBMNS was transfected with Cntrl (scrambled) or P5i were re-transfected with Cntrl or PTEN plasmid. Seven days later, representative image showing the size and number of neurospheres formed were taken. (h) Graph showing the quantification of number of neurospheres formed when 1000 GBMNS cells were transfected with Cntrl, P5i or PTEN or combination of PTEN overexpression and PRMT5 knockdown. * $P = 0.05$, ** $P = 0.01$.

Figure 5.

PTEN depletion rescues PRMT5 knockdown phenotype in GBMNS: (a) GBMNS OG2 transfected with Cntrl or P5i or PTEN siRNA (PTENi) were subjected to western blot to probe the expression of PRMT5 and PTEN. The blots were cropped from the main western blot membrane. (b) Using the conditions similar to panel a, proliferation of cells were measured by MTT assay and quantified as shown in the graph. $P = 0.001$. (c) 7 days post-transfection with Cntrl, PRMT5, PTENi or the combination, number and size of the neurospheres were observed. Upper panel: Representative images are shown in the figure.

Lower panel: Graph showing the quantification of the neurosphere formation assay. **(d)**
Upper panel: Representative images of neurosphere formation assay at 20 ×. Lower panel:
Graph showing the quantification of number of neurospheres formed. **** P 0.01.**

Figure 6.

PRMT5 knockdown causes senescence in GBMNS: (a) GBMNS were transfected with Cntrl or P5i every 3 days for 20 days. Twenty days post transfection, cells were stained for β -gal. Representative images showing the β -gal-positive and -negative cells are shown in the figure. (b) Quantification showing number of β -gal-positive cells per field in Cntrl and P5i. (c) Relative expression of p53 and p21 mRNA in GBMNS OG2 transfected with Cntrl or P5i. (d) GBMNS OG2 and OG34 transfected with Cntrl or P5i were probed for the expression of proteins p21 and p53 by western blot using appropriate antibodies. The blots

were cropped from the main western blot membrane. (e) Representative images of GBMNS transfected with Cntrl or PTEN stained for β -gal. (f) Quantification of the number of β -gal-positive cells per field in Cntrl or PTEN transfected GBMNS OG2. $**P = 0.01$.

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Figure 7.

PRMT5 is required for *in vivo* tumour formation: (a) Representative luciferase images of three mice per group at 7, 11 and 14 days post tumour implantation. (b) T2-weighted MRI slices depicting largest tumour section at day 15 post tumour implantation. (c) Kaplan–Meier survival plots and Mantel–Cox analysis of GBMNS–Cntrl ($n = 10$) vs –P5i ($n = 10$), $P = 0.0005$ and GBMDC–Cntrl ($n = 9$) vs –P5i ($n = 10$), $P = 0.0083$, respectively. (d) Shown is the current working model for the regulation of PTEN–AKT pathway by PRMT5. PRMT5 negatively regulates PTEN and helps in activating AKT. Increased AKT activity keeps the

Rb protein in the inactive form through negative regulation of p27 and assists in proliferation. Depletion of PRMT5 increases the expression of p27 through PTEN–AKT pathway and activates Rb protein and causes cell cycle arrest eventually making them to undergo senescence.