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# The role of protein arginine-methyltransferase 1 in gliomagenesis

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**Protein arginine methyltransferase 1 (PRMT1), a type-I arginine methyltransferase, has been implicated in diverse cellular events. We have focused on the role of PRMT1 in gliomagenesis. In this study, we showed that PRMT1 expression was up-regulated in glioma tissues and cell lines compared with normal brain tissues. The knock-down of PRMT1 resulted in an arrest in the G1-S phase of the cell cycle, proliferation inhibition and apoptosis induction in four glioma cell lines (T98G, U87MG, U251, and A172). Moreover, an *in vivo* study confirmed that the tumor growth in nude mouse xenografts was significantly decreased in the RNAi-PRMT1 group. Additionally, we found that the level of the asymmetric dimethylated modification of H4R3, a substrate of PRMT1, was higher in glioma cells than in normal brain tissues and decreased after PRMT1 knock-down. Our data suggest a potential role for PRMT1 as a novel biomarker of and therapeutic target in gliomas. [BMB Reports 2012; 45(8): 470-475]**

## INTRODUCTION

Protein arginine methylation, a type of posttranslational modification (PTM), has either activating or repressing effects on protein activity, which greatly depends on the substrates modified. Arginine methylation is accomplished by a family of protein arginine methyltransferases (PRMTs) that can transfer a methyl group from S-adenosylmethionine to the guanido nitrogens of arginine residues (1). PRMTs are classified into three distinct types (type I–III) on the basis of the location and the type of monomethylarginine or dimethylarginine they introduce. Protein arginine methyltransferase 1 (PRMT1) is a member of the type-I methyltransferases and results in asymmetric dimethylarginine (ADMA) (2). As reported, PRMTs have important roles in DNA repair, signal transduction and gene expression regulation (3, 4). PRMT1 is the most predominant methyltransferase in mammalian cells and accounts for approximately 85% of cellular PRMT activities (5, 6). PRMT1-knockout mice, generated through gene entrapment, fail to develop beyond E6.5, when the cells of the epiblast begin to differentiate. These results suggest that PRMT1 has a fundamental and pre-requisite role in embryonic development (7). Subsequently, a study on conditional PRMT1- knockout mice showed that several proteins were hypomethylated, and the lack of PRMT1 compromised cell proliferation and DNA damage response signaling (8). Because of the importance of PRMT1, many research groups have studied PRMT1 substrates, the major one being histone H4. PRMT1 specifically dimethylates histone H4 arginine 3 (H4R3diMe) and establishes a scaffold for subsequent histone modifications and chromatin remodeling, which have been implicated in transcriptional regulation (9, 10).

Recently, dozens of reports have confirmed that methylation events implicated in disease emergence and progression, including cancers (11). Based on the prevalence of PRMT1 substrates in cells and its predominant enzymatic activity, it is highly probable that PRMT1 is connected with many diseases (12). PRMT1 has been reported to have key roles in breast cancer cell apoptosis and osteosarcoma cell proliferation, and it might be a potential therapeutic target for many types of cancer (8, 13, 14). Additionally, some substrates methylated by PRMT1 may be directly implicated in many diseases. For example, the level of H4R3diMe was higher in prostate cancer compared to normal tissues and could predict the risk of prostate cancer recurrence (15). Cheung discovered that in mixed-lineage leukemia (MLL), PRMT1 was involved in the expression of critical MLL downstream target genes through regulating H4R3 methylation (16).

Gliomas are the most common type of primary tumor in the brain and are rarely curable (17). However, the functional role of PRMT1 in gliomas has not been reported. In our work, we show that PRMT1 is up-regulated in human glioma tissues and cell lines. To elucidate the role of PRMT1 in gliomas, we transfected the Stealth$^{TM}$ RNAi of PRMT1 *in vitro* and *in vivo* and detected the role of PRMT1 in glioma cell processes. Furthermore, we examined the effect of knocking down PRMT1 on the dimethylation of H4R3 in glioma cells.

## RESULTS

### PRMT1 is up-regulated in human glioma tissues and cell lines

To detect the expression pattern of PRMT1 in gliomas, western

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blotting was performed in 2 normal brain tissues and 17 primary glioma samples. In addition, the total RNA and protein were extracted from two normal brain tissues and four glioma cell lines, and real-time PCR and western blotting were performed to analyze the expression profile of *PRMT1*. As shown in Fig. 1A, the expression of PRMT1 was increased in more than 76% of glioma samples compared with the two normal brain tissues. In the glioma cell lines, *PRMT1* expression was also upregulated (Fig. 1B, 1C). Furthermore, one grade II glioma patient sample was analyzed with HE staining and immunohistochemistry with a specific PRMT1 antibody (Fig. 1D). The tumor area showed a strong positive signal for *PRMT1*. By contrast, the normal tissue area showed weak staining. These data indicated that *PRMT1* expression was elevated in glioma tissues and cells compared with normal brain tissues.

**The knockdown of PRMT1 with Stealth™ RNAi inhibited the proliferation of and promoted apoptosis in glioma cells**

To examine the role of PRMT1 in glioma cells, we synthesized Stealth™ RNAi duplexes of *PRMT1*. On the third day after transfection with the RNAi, PRMT1 expression was examined in T98G, U87MG, U251 and A172 cell lines with western blotting. The results showed that the protein level of PRMT1 in the RNAi-PRMT1 group was reduced by at least 70% compared with the RNAi-NC group (Fig. 2A). To investigate whether the knockdown of PRMT1 affected the cell cycle in glioma cells, flow cytometry analysis was performed. The results showed a different cell cycle profile in the RNAi-PRMT1 group compared with the RNAi-NC group. As shown in Fig. 2B, knocking down PRMT1 with RNAi significantly decreased the cell population in S phase in the four glioma cell lines (U87MG: 18.5 ± 1.03% vs. 24.15 ± 1.28%; T98G: 22.03 ± 4.37% vs. 27.93 ± 3.06%; A172: 15.55 ± 0.78% vs. 21.9 ± 0.99%; and U251: 19.32 ± 0.62% vs. 23.12 ± 0.82%). Additionally, the percentage of cells in G1 phase was higher in glioma cells transfected with PRMT1 RNAi than in the control (U87MG: 69.7 ± 1.62% vs. 64.35 ± 2.10%; T98G: 69.87 ± 1.09% vs. 62.6 ± 0.79%; A172: 78.45 ± 0.62% vs. 71.17 ± 1.89%; and U251: 76.08 ± 1.03% vs. 71.87 ± 1.19%). These results showed that the cell cycle of glioma cells was arrested

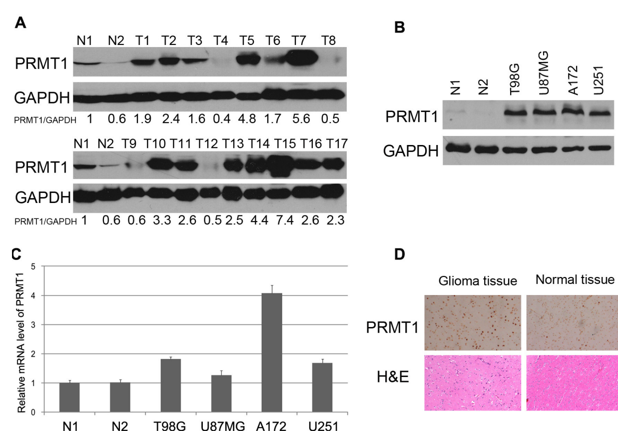


**Fig. 1.** High levels of PRMT1 expression in glioma cell lines and glioma tissues compared with normal brain tissues. (A) Western blotting was performed to detect the expression of PRMT1 in two normal brain tissues (N1 and N2) and seventeen glioma tissues (T1-T5 grade II, T6-T12 grade III and T13-T17 grade IV). The quantification of western blot bands was calculated with Quantity One software, and the values of each band were normalized to corresponding values of GAPDH. The normalized values of each sample were compared with the normalized value of N1, which was the reference value, and calculated as shown. (B) The western blotting of PRMT1 and GAPDH in two normal brain tissues (N1 and N2) and four glioma cell lines (T98G, U87MG, U251, and A172). (C) The real-time PCR analysis of *PRMT1* in normal tissues and glioma cell lines. The RNA input was normalized to GAPDH. The data are presented as the means ± standard deviation (SD) of four wells. (D) The immunohistochemistry of PRMT1 and HE staining in normal tissue adjacent to tumors and glioma tissue in one grade II glioma patient (magnification ×100).

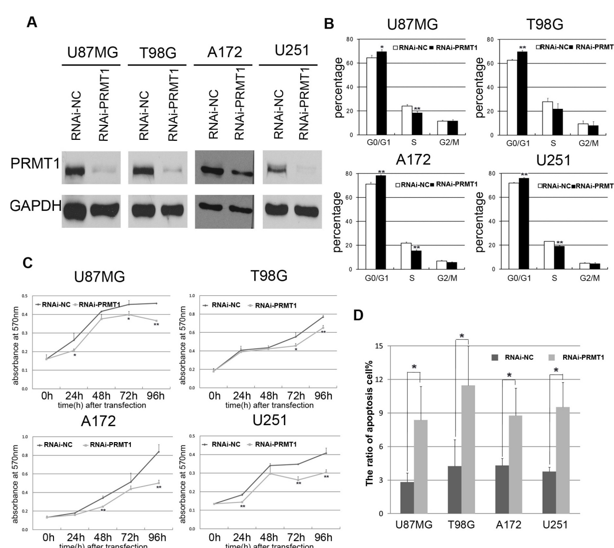


**Fig. 2.** The suppression of PRMT1 inhibited the proliferation and promoted the apoptosis of glioma cells. (A) At 72 h post-transfection with the RNAi of PRMT1 or RNAi-NC, glioma cells were collected, and the total protein was extracted with lysis buffer for western blotting to determine the expression of PRMT1 in four glioma cell lines. (B) Flow cytometry analysis was performed for the RNAi-NC group and the RNAi-PRMT1 group 72 h after transfection. The percentage of the cell population in G0/G1, S and G2/M phase is shown in the histograms. (C) MTT assays were performed in glioma cells after treatment with RNAi-NC or RNAi-PRMT1. The viable cell number was measured at an absorbance of 570 nm with a reference wavelength of 630 nm. (D) After transfection with RNAi-NC or RNAi-PRMT1 for 72 h, TUNEL assays were performed in four glioma cell lines. The ratio of apoptotic cells was calculated and plotted on the histograms. (All of the values represent the means ± standard deviation (SD) of four parallel wells. The asterisks indicate $*P < 0.05$ and $**P < 0.01$, all compared to the negative control).

in G1-S phase after knocking down PRMT1. MTT assays showed that the proliferation rates were significantly decreased in glioma cells after transfection with RNAi-PRMT1 (Fig. 2C). To examine the role of PRMT1 in apoptosis, TUNEL assays were performed. The results showed that after transfection for 72 h, there was more apoptotic programmed cell death in glioma cells transfected with RNAi-PRMT1 compared with cells transfected with RNAi-NC. The apoptotic cells were counted, and the data showed that there was a more than 2-fold increase in apoptotic glioma cells when PRMT1 was knocked down (Fig. 2D). In conclusion, these data revealed that a decrease in PRMT1 inhibited glioma cell cycle progression and induced cell apoptosis, thus suppressing glioma cell growth.

## The suppression of PRMT1 repressed U87MG cell growth *in vivo*

To determine the effect of the suppression of PRMT1 on glioma cell growth *in vivo*, HPLC *in vivo* purity Stealth$^{TM}$ RNAi-NC or Stealth$^{TM}$ RNAi-PRMT1 was transfected into U87MG cells. The next day, each group of cells was injected into either side of nude mouse oxters. We confirmed the inhibition of PRMT1 expression in U87MG cells after transfection for 96 h with western blotting (Fig. 3B). The tumor growth was recorded beginning on the day of injection (0 day). The results showed that on days 25, 30 and 33, the side with RNAi-PRMT1 U87MG cells had a significantly smaller tumor than the RNAi-NC side (n = 9, P < 0.05, Student's t test) (Fig. 3C). At 33 days, two mice had died, and the tumor masses in all of the mice were collected and weighed. As shown in Fig. 3D and Fig. 3E, the average weight of the tumors on the side with RNAi-PRMT1 was much lower than the RNAi-NC side (P < 0.01). These results indicated that the suppression of PRMT1 expression inhibited glioma formation and growth *in vivo*.

## The dimethylation of H4R3 was inhibited by knocking down PRMT1

It has been reported that PRMT1 can asymmetrically dimethylate H4R3 and influence downstream gene expression in MLL (16). However, there are no studies on the relationship between PRMT1 and H4R3 in gliomas. To detect the expression of dimethylated H4R3, we performed western blotting of glioma cells with an H4R3diMe-specific antibody. As shown in Fig. 4A, the level of the asymmetric dimethylated modification of H4R3 was prominently increased in glioma cells. Moreover, after PRMT1 was knocked down in U87MG cells, the level of dimethylated H4R3 was significantly decreased, but there was no change in the expression of histone H4 or the levels of H4K20 monomethylation or dimethylation between the RNAi-NC and RNAi-PRMT1 cells (Fig. 4B). In general, our western blotting data indicated that PRMT1 could also influence the dimethylation of H4R3 in glioma cells.

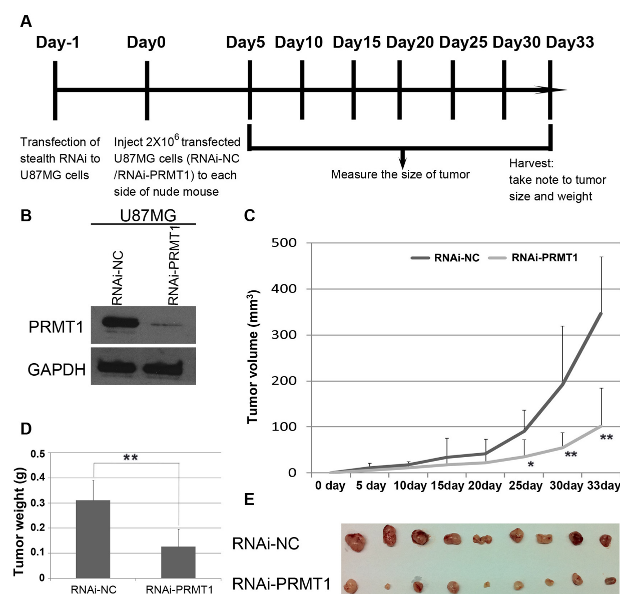


**Fig. 3.** Knocking down PRMT1 affected glioma growth *in vivo*. (A) The *in vivo* experimental procedure is shown in the diagram. (B) The expression of PRMT1 was decreased in U87MG cells after transfection with RNAi-PRMT1 (HPLC *in vivo* purity). (C) The U87MG tumor volumes of the RNAi-NC group and the RNAi-PRMT1 group were measured and are depicted in the line chart. (D) On day 33 after the injection of transfected U87MG cells, the tumors were collected for weighing and (E) imaging. (All of the values represent the means ± standard deviation (SD) of nine tumor samples. The asterisks indicate *P < 0.05 and **P < 0.01, all compared to the negative control).

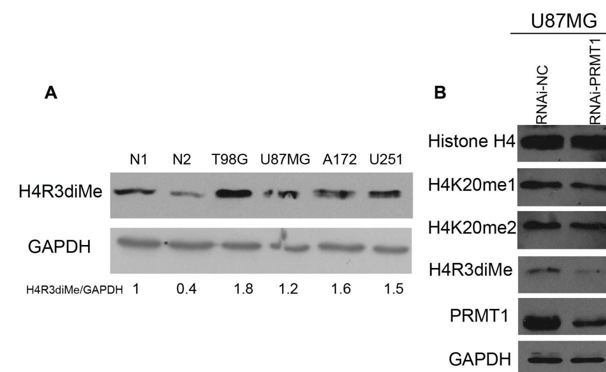


**Fig. 4.** The level of the asymmetric dimethylated modification of H4R3 is consistent with PRMT1 expression in glioma cells. (A) The expression of H4R3diMe was detected in normal brain tissues (N1 and N2) and four glioma cell lines with western blotting. The relative expression of PRMT1 was calculated as described in Fig. 1. (B) At 72 h post-transfection with RNAi-NC or RNAi-PRMT1, western blotting was performed to detect the expression of Histone H4, H4K20me1, H4K20me2, H4R3diMe, PRMT1 and GAPDH.

## DISCUSSION

Protein arginine methylation plays key regulatory roles in DNA damage signaling and epigenetic gene expression (12). In the past, the study of arginine methylation has mostly been focused on identifying abundant substrates. However, recently, it has been discovered that arginine methylation may be implicated in some diseases and biochemical or biological processes, such as signal transduction and proliferation (18). Here, in our work, we report for the first time that the protein and mRNA levels of *PRMT1* are up-regulated in glioma tissues and glioma cell lines compared with normal brain tissues. Moreover, we performed immunohistochemistry to detect the expression of PRMT1 in the glioma or normal tissue of one grade II patient, and the results showed that the glioma tissue had a stronger PRMT1 signal than normal tissue. As previously reported, the expression of PRMT1 has been associated with breast tumor grade. Thus, PRMT1 may be useful as a biomarker of breast cancer (19). In our data, the expression of PRMT1 was different between normal and glioma tissues, which indicated that PRMT1 might be a potential biomarker in the pathological examination of human gliomas.

In our study, after knocking down PRMT1, the proliferation of glioma cells was markedly suppressed through arrest in the G1-S phase of the cell cycle. These results suggested that PRMT1 might be induced in post-mitotic cellular process regulation. Previously, it was discovered that PRMT1 was essential for early development and cellular metabolism (7). In addition, Yu *et al*. discovered that the loss of PRMT1 in MEFs through a conditional allele in mice or U2OS cells led to DNA damage, cell cycle progression delay and checkpoint defects (8). Additionally, in our study, we found that after the transfection of PRMT1-RNAi, the number of apoptotic glioma cells increased notably. Our *in vivo* study also proved that decreased PRMT1 expression resulted in the inhibition of gliomagenesis. Therefore, we conclude that PRMT1 can regulate glioma cell cycle progression and influence glioma cell proliferation and apoptosis.

Additionally, there are dozens of substrates of PRMT1, and their methylation may ultimately affect numerous cellular events and tumorigenesis. For example, PRMT1 can methylate apoptosis signal-regulating kinase1 (ASK1), which has been implicated in the regulation of stress-induced apoptotic signaling (14). Additionally, estrogen receptor α (ERα), which regulates many physiological pathways, can be methylated by PRMT1. In breast cancer, methylated ERα would interact with Src/FAK and p85 to induce downstream kinase activation and activate the Akt pathway, which could promote cell proliferation and inhibit apoptosis (20). Among the substrates of PRMT1, H4R3 is the most popular and of great concern (9). In prostate cancer research, H4R3diMe was positively correlated with tumor grade progression (15). The relationship between PRMT1 and H4R3diMe was also investigated in an erythroid cell line, and H4R3 methylation was almost completely inhibited after PRMT1 knockdown (21). Our data showed that the level of the asymmetric dimethylated modification of H4R3 was up-regulated in glioma cells consistent with PRMT1 expression. Inhibiting the expression of PRMT1 resulted in a dramatic decrease in H4R3 dimethylation. Therefore, we hypothesize that PRMT1 is implicated in gliomagenesis by regulating the hypermethylation of H4R3.

Taken together, our data suggest that PRMT1 is up-regulated in human glioma tissues and glioma cells. Knocking down PRMT1 induces glioma cell G1-S phase arrest, growth inhibition and apoptosis. Additionally, the level of the asymmetric dimethylated modification of H4R3 is consistent with PRMT1 expression in glioma cells. PRMT1 has the potential to be an accessory biomarker of and a therapeutic target in glioma. However, further studies on PRMT1 function should be performed to provide and confirm novel insights into the diagnosis and clinical therapy of gliomas.

## MATERIALS AND METHODS

### Tissue samples

All of our research was approved by the institutional ethics committee. The human glioma tissue and normal brain tissue samples were provided by the Department of Neurosurgery, Beijing Tiantan Hospital. On the basis of the third edition of the histological typing of tumors of the nervous system published by WHO, all of the samples were classified as follows: 5 samples (T1-T5) were grade II, 7 samples (T6-T12) were grade III and 5 samples (T13-T17) were grade IV. The immunohistochemical tissue sample was grade II.

### Glioma cell lines and cell culture

The human glioma cell lines used in our study were from the American Type Culture Collection (ATCC) (T98G, U87MG, and A172) and the Cell Center of Peking Union Medical College (U251). All of the cell lines were confirmed with species restriction to human and cultured according to the guidelines recommended by the ATCC. The glioma cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$-95% air.

### Western blotting analysis

The protocol for western blotting analysis was described previously (22). The GAPDH antibody was purchased from Abmart Company (USA); the PRMT1 antibody was purchased from Millipore Company (USA); the Histone H4 dimethyl Arg3 (asymmetric) antibody was purchased from Active Motif Company (USA); and the Histone H4, Histone H4K20me1 and Histone H4K20me2 antibodies were purchased from Millipore Company (USA).

### RNA extraction and real-time PCR

We used the Trizol reagent (Invitrogen, USA) to extract total RNA from treated cells and the first-strand cDNA kit (TransGen

Biotech, China) to perform reverse transcription according to the manufacturer's instructions. Real-time PCR was performed using a SYBR Green PCR Master Mix kit (Takara, Japan) and the ABI Prism 7500 sequence detection system. The real-time PCR forward primer sequence for GAPDH was 5′-GGTC ATCCATGACAACTTTGG-3′, and the reverse primer sequence was 5′-GGCCATCACGCCACAG-3′. The real-time PCR forward primer sequence for PRMT1 was 5′-ACAAAGACTACA AGATCCACTGGTG-3′, and the reverse primer sequence was 5′-CGGTATAGATGTCCACCTCCTTTATG-3′. The RNA input was normalized to the level of human GAPDH to detect the relative expression of PRMT1.

**Immunohistochemistry**

The immunohistochemical analysis of PRMT1 was performed using formalin-fixed, paraffin-embedded sections of one glioma grade II patient. The section was dipped in antigen-retrieval citra solution (1.8 mM citric acid and 8.2 mM natrium citricum, pH 6.0) at 95°C for 10 min and then incubated with the anti-PRMT1 antibody (Cell Signaling Technology Company, USA) at 4°C overnight. Staining was performed using a diaminobenzidine staining kit (Zhongshan Goldenbridge Biotechnology Co., China).

**Stealth™ RNAi duplex transfection**

The Stealth™ RNAi duplex of *PRMT1* was synthesized by Invitrogen. The sequence of Stealth™ RNAi-*PRMT1* was 5′-GCCUGCAAGUGAAGCGGAAUGACUA-3′, and it bound to the coding region of the human *PRMT1* mRNA sequence (GenBank accession no. NM_001536). The Stealth™ RNAi Negative Control duplex (Cat. No. 12935-300, Invitrogen) was also synthesized by Invitrogen. The cells were seeded in DMEM containing 10% fetal bovine serum without antibiotics. At 24 hours later, the cells were transfected with Stealth™ RNAi with Lipofectamine™ 2000. The final concentration was 100 nM. All of the procedures were performed according to the manufacturer's protocol for Lipofectamine™ 2000- based transfections (Invitrogen Life Technology Inc., USA).

**Cell proliferation and apoptosis assays**

The dimethyl thiazolyl diphenyl tetrazolium (MTT) assay was performed as described previously (22).

For flow cytometry assays, glioma cells were seeded in a 6-well plate for 24 h and then transfected with RNAi. After transfection for 72 h, the cells were fixed in cold 75% alcohol at −20°C overnight. Then, the cells were collected and suspended in 0.5 ml propidium iodide (PI) solution (0.05 mg/ml PI (Sigma-Aldrich, USA), 0.02 mg/ml DNase-free RNase A, 1 mg/ml sodium citrate and 0.1% Triton X-100 in PBS, pH 7.5) for 10 min at 37°C. The samples were then measured with an EPICS® XL (COULTER®) and analyzed using an EXPO 32ADCXL (COULTER®). The percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined using Multicycle for Windows 32 bit.

After transfection with RNAi (RNA interference) for 72 h, a terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) assay was performed with a FragEL™ DNA Fragmentation Detection Kit (Merck, USA) according to the manufacturer's instructions.

**Xenograft model in nude mice**

Nine 4- to 5-week-old male BALB/c athymic nu/nu mice were purchased from China Vital River Company. All of the animal experiments were performed according to ethical approval. First, U87MG cells were cultured in 10 cm cell culture dishes, and 100 nM Stealth™ RNAi-NC or Stealth™ RNAi-PRMT1 (HPLC *in vivo* purity, Invitrogen Life Technologies Inc., USA) was transfected into U87MG cells with Lipofectamine™ 2000. After 24 h, the cells were collected and resuspended at a density of $2 \times 10^6$ cells/100 μl, and $2 \times 10^6$ cells of the Stealth™ RNAi NC group or the Stealth™ RNAi-PRMT1 group were subcutaneously implanted in either side of mouse oxters. The tumors were measured with digital calipers at different time points after injection as indicated in Fig. 3A. The tumor volume was calculated with the formula $V = L \times W^2 \times \pi / 6$ (*V*, volume; *L*, length; *W*, width of tumor). On day 33 after implantation, the tumors were collected and weighed.

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