Department of Pharmacology[1], School of Basic Medical Sciences, Lanzhou University; Key Lab of Preclinical Study for New Drugs of Gansu Province, Lanzhou; Lanzhou University Second Hospital[2], Lanzhou University School of Medicine, Lanzhou, China

Arginine methyltransferase inhibitor 1 exhibits antitumor effects against cervical cancer *in vitro* and *in vivo*

Shu-Hong Dong[1,#], Xing Wang[2,#], San-Chun Tian[1], Neng-Qian Ma[2], Xin-Yang Zhang[2], Yapeng Liu[1], Bao-Lai Zhang[1,*], Yong-Jie Wu[1,*]

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**Corresponding authors: Bao-lai Zhang, Yong-jie Wu, Department of Pharmacology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China*
zhangbl@lzu.edu.cn; wuyj@lzu.edu.cn
[#]These authors contributed equally to this work.

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Protein arginine methyltransferase 5 (PRMT5), a type II PRMT, is highly expressed in several types of tumors including cervical cancer. Arginine methyltransferase inhibitor 1 (AMI-1) inhibits solid tumors by targeting PRMT5. However, the effect of AMI-1 on cervical cancer is still unknown. In this study, we provided the first evidence that AMI-1 reduced cervical cancer cell proliferation, colony formation and promoted cell apoptosis *in vitro*. Suppression of tumorigenicity was also confirmed *in vivo*. Mechanistic studies revealed that AMI-1 significantly reduced PRMT5 level in cells and mice xenografts model of cervical cancer. These results suggest that AMI-1 inhibits cervical cancer by type II PRMT5.

1. Introduction

Protein arginine methyltransferases (PRMTs) are a family of enzymes that transfer the methyl group from *S*-adenosylmethionine to the guanidine nitrogen atoms of arginine reside (Wolf 2009). Among PRMTs, PRMT5 is the predominant type II PRMTs (PRMT5 and PRMT9) that catalyze monomethylation and symmetric dimethylation (Zurita-Lopez et al. 2012). PRMT5 has been extensively characterized as a histone methyltransferase that modifies H2AR3, H4R3 and H3R8. PRMT5 also methylates other proteins, thereby potentially affecting multiple signaling pathways (Karkhanis et al. 2011). Several reports stress the importance of PRMT5 in tumorigenesis, but the mechanism of PRMT5 driving tumorigenesis is still unknown. PRMT5 is upregulated in several types of solid tumors including cervical cancer, and it promotes cancer cell proliferation and survival (Wang et al. 2008; Wang et al. 2009; Powers et al. 2011; Cho et al. 2012; Wei et al. 2012; Yan et al. 2014; Zhang et al. 2015a; Zhang et al. 2015b). Lowering the level of PRMT5 is associated with attenuated cell viability in TRAIL-treated Hela cells, whereas PRMT5 overexpression causes celluar hyperproliferation (Tanaka et al. 2009).

Although numerous efforts have been made to develop PRMT5 inhibitors, there are only three types of PRMT5 inhibitors reported. Arginine methyltransferase inhibitor 1 (AMI-1), a symmetrical sulfonated urea, is mainly used to inhibit type I PRMTs (PRMT1-PRMT4, PRMT6 and PRMT8) *in vitro* (Cheng et al. 2004). However, our previous studies found that AMI-1 inhibited some solid tumors including colorectal cancer (CRC), hepatocellular carcinoma (HCC) and gastric cancer by targeting type II PRMT5 (Zhang et al. 2015a; Zhang et al. 2015b; Zhang et al. 2017). AMI-1 is clearly inhibited in tumor growth and survival, but its role in cervical cancer is unknown. In this study, we set out to investigate the effect of AMI-1 on cervical cancer in vitro and in vivo. We found that AMI-1 significantly inhibited the growth of cervical cancer cell by type II PRMT5.

2. Investigations and results

2.1. AMI-1 inhibits Hela cell proliferation in vitro

We detected cell proliferation via CCK-8 assay. The AMI-1 concentration gradient ranged from 0.6 mM to 2.4 mM. As shown in Fig. 1A, AMI-1 significantly inhibited the proliferation of Hela cells in *vitro*. We next performed colony formation assay to determine the effects of AMI-1 on cervical cancer cell colony formation ability, the results showed that Hela cells treated with AMI-1 formed fewer colonies than control cells (Fig. 1B).

2.2. AMI-1 inhibits growth of Hela tumor in vivo

To evaluate the effect of AMI-1 on tumor growth in *vivo*, BALB/c nude mice bearing Hela xenografts were treated with AMI-1 or vehicle for 18 days. As shown in Fig. 2A and 2B, administration of AMI-1 reduced the tumor volume by 51.9 %, from 930.98±328.73 mm^3 of control to 447.76±286.33 mm^3 ($P < 0.05$). And likewise, administration of AMI-1 reduced the tumor weight by 53.2 %, from 0.79±0.31 g of control to 0.37±0.23 g ($P < 0.01$). In addition, we found that the weight of body in mice treated with this agent showed no significant difference compared to control (data not shown), indicating that AMI-1 treatment was well tolerated.

2.3. AMI-1 induces Hela cell apoptosis in vitro

To determine whether AMI-1 inhibited cell proliferation by inducing cell apoptosis, Hela cells were treated with AMI-1, then apoptosis was evaluated using flow cytometry. As shown in Fig. 3, the percentage of apoptotic cells was low in Hela cells. However, the percentage of early apoptotic cells was 0.3 % and 1.3 % after 1.2 mM AMI-1 treatment for 48 h or 72 h, respectively. The percentage of early apoptotic cells was 5.8% and 10.2% after 2.4 mM AMI-1 treatment for 48 h or 72 h, respectively. Meanwhile, the percentage of late apoptotic and dead cells was 10.1 % and 8.9 % after 1.2 mM

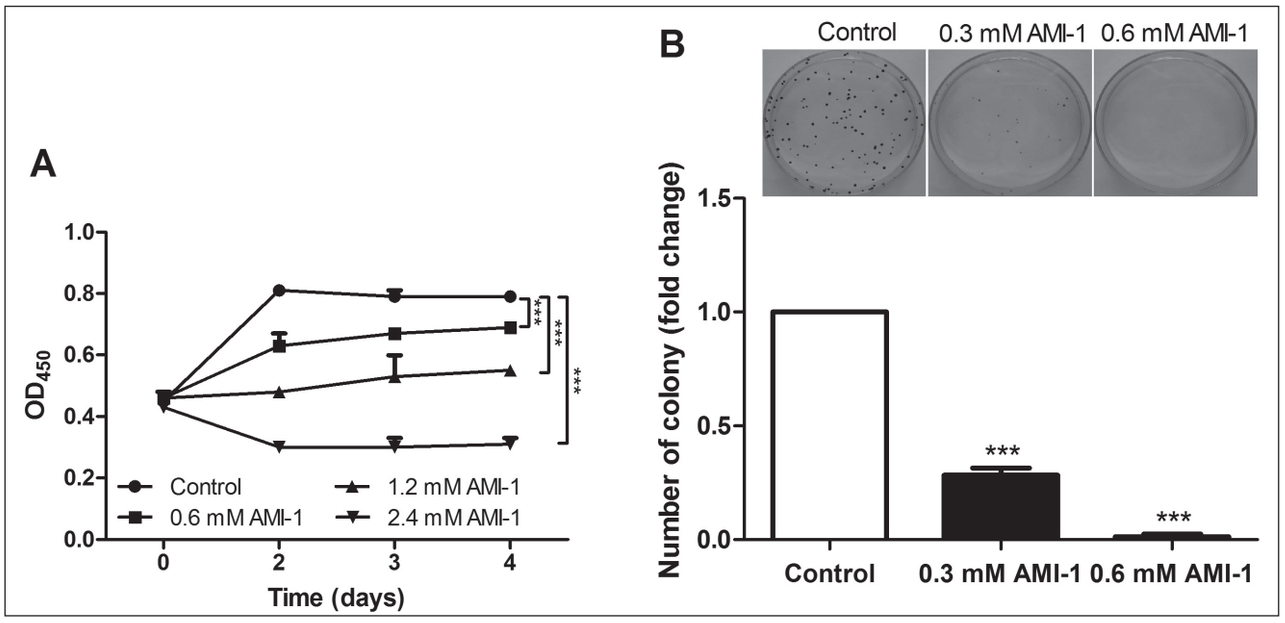

Fig. 1: Effect of AMI-1 on cell proliferation and colony formation in Hela cells. (A) The growth curves of the cells treated with AMI-1 or vehicle *in vitro* (n=3). (B) Representative pictures of colony-formation assay and number of cell colony (fold change). After 16 d exposure to AMI-1 or vehicle, the colony formation ability of Hela cells was tested. Shown were representative graphs from three independent experiments.

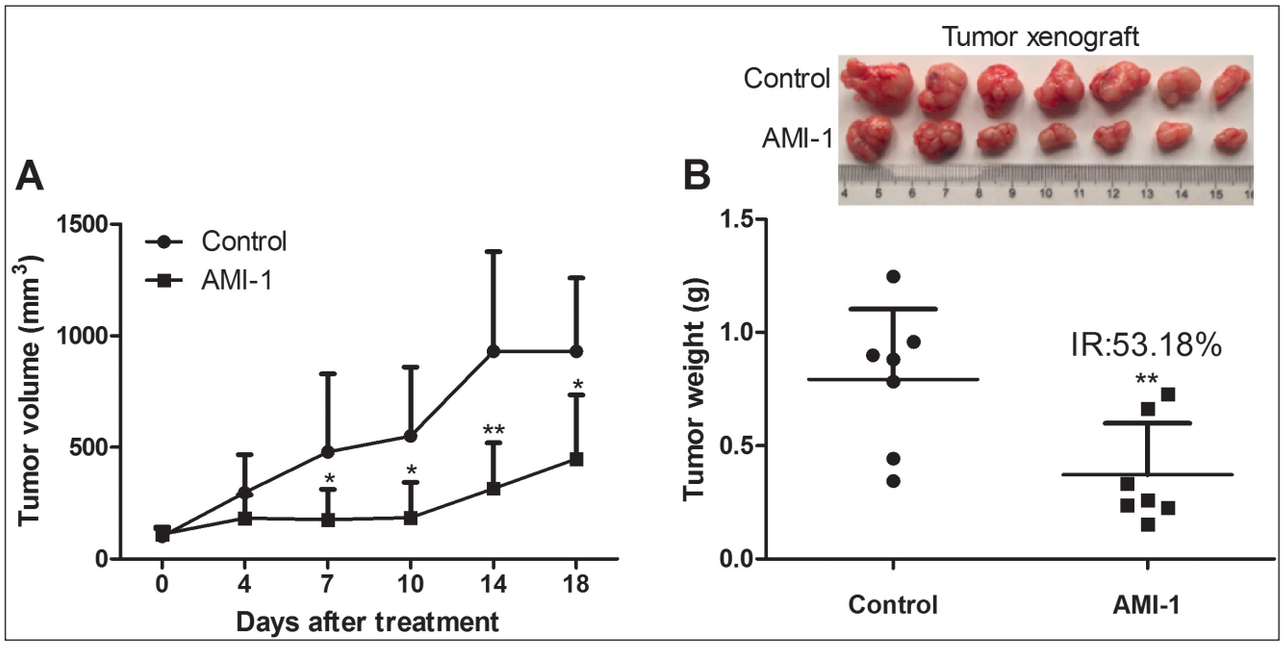

Fig. 2: Effect of AMI-1 on nude mice bearing with Hela xenografts. (A) The growth curves of the tumors of the subcutaneous tumors formed by Hela cells in nude mice. (B) The images and the weight of the tumors of the subcutaneous tumors formed by Hela cells in nude mice. Results are represented as the mean ± S.D. of 7 mice per group.

AMI-1 treatment for 48 h or 72 h, respectively. The percentage of late apoptotic and dead cells was 12.6 % and 13.9 % after 2.4 mM AMI-1 treatment for 48 h or 72 h, respectively.

2.4. AMI-1 downregulates PRMT5 level but does not affect PRMT7 in vitro and in vivo

To further understand the mechanism by which AMI-1 inhibits cervical cancer cell growth, we performed Western blot analysis to assess the effect of AMI-1 on PRMT5 and eIF4E in Hela cells. The results showed that AMI-1 decreased both PRMT5 and eIF4E level (Fig. 4A). Meanwhile, we performed Western blot analysis on tumor-derived protein extracts from mice bearing Hela tumor, AMI-1 significantly reduced PRMT5 level, but did not affect PRMT7 level, in the AMI-1-treated groups compared with control groups (Fig. 4B).

3. Discussion

Many studies have shown that PRMT5 level is significantly increased in multiple human cancers (Pal et al. 2007; Wang et al. 2008; Powers et al. 2011; Cho et al. 2012; Gu et al. 2012; Zhang

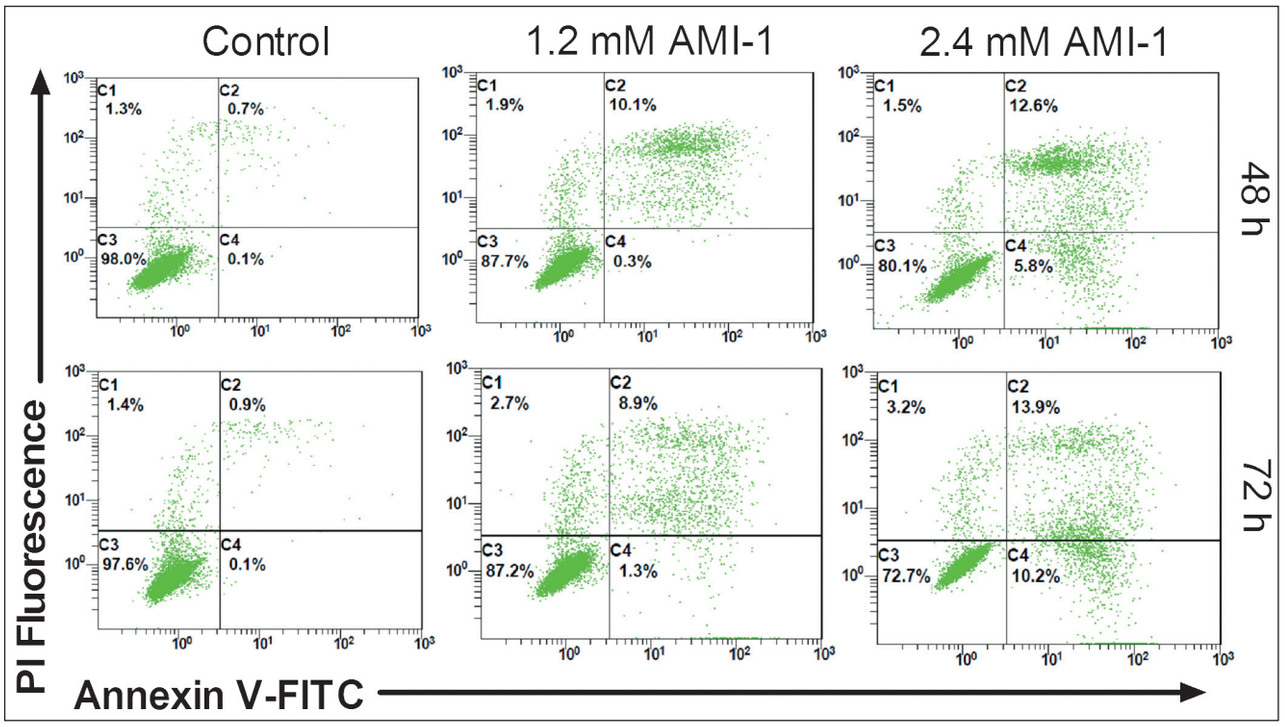

Fig. 3: AMI-1 induces apoptosis of Hela cells. Cells were treated with vehicle or AMI-1 and then stained by Annexin V-FITC and PI, followed by flow cytometry analysis. C2 (the upper right quadrant) represents the percentages of late apoptotic and dead cells, whereas C4 (the lower right quadrant) represents the percentages of early apoptotic cells. Shown were representative graphs from three independent experiments.

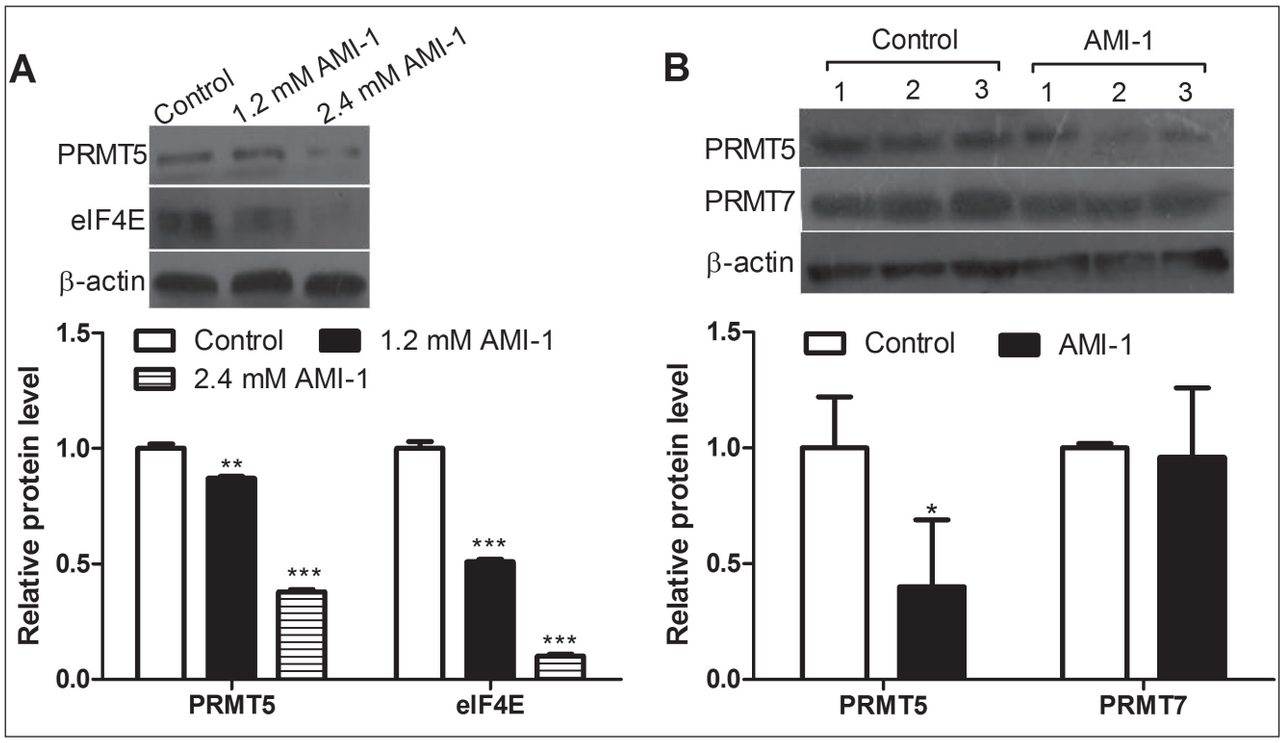

Fig. 4: AMI-1 down-regulates PRMT5 and eIF4E level in Hela cells. (A) Representative blots of the detection of PRMT5 and eIF4E in Hela cells treated with AMI-1 72 h. (B) Representative blots of the detection of PRMT5 and PRMT7 in Hela tumor xenografts treated with AMI-1 or vehicle for 16 days. Relative levels were normalized to β-actin (n=3).

et al. 2015a; Zhang et al. 2015b). Therefore, PRMT5 might be a tumor-promoting factor, and targeting PRMT5 would provide the therapeutic benefits. The multitude of reports implicating PRMT5 in cancer reflects the need for potent and selective inhibitors of PRMT5, so that its pathobiology can be further explored and validated (Mao et al. 2017).

We have found that AMI-1 inhibits the tumor growth in *vitro* and in *vivo* by targeting PRMT5, but its mechanism of action is still

not clear in cervical cancer. Identification of new biomarkers for cervical cancer is important for disease diagnosis and treatment. In this study, utilizing CCK-8 and colony formation assays, we demonstrated that AMI-1 inhibited cell proliferation and colony formation while induced the apoptosis in Hela cells. Similar effects were also confirmed in Hela xenograft tumor model.

eIF4E plays an important role in cell proliferation and promote tumorigenesis, while PRMT5 exerts its function by regulating eIF4E level (Scoumanne et al. 2009; Yoshizawa et al. 2010; Powers et al. 2011; Liu et al. 2015). These findings suggest that eIF4E may represent an attractive cancer target. In this study, we found that AMI-1 decreased the level of eIF4E expression in cervical cancer cells. Both PRMT5 and PRMT7 driven methylation of arginine residues leads to symmetric dimethylation of histon H4 (Bedford and Clarke 2009). Thus by Western blot analysis we demonstrated that AMI-1 significantly decreased PRMT5 level but did not PRMT7 in Hela xenograft tumors model, indicating that AMI-1 exhibits anti-cancer effects against cervical cancer mainly through inhibiting PRMT5.

In summary, our study demonstrated that AMI-1 inhibited cervical cancer growth in *vitro* and in *vivo*, induced cell apoptosis and reduced the level of PRMT5 and eIF4E in cervical cells.

4. Experimental

4.1. Cell lines and reagents

Human cervical cancer Hela cells (Chinese Academy of Science, Shanghai, China) were maintained in RPMI 1640 medium (GIBCO, California, USA) supplemented with 10% fetal bovine serum (GIBCO, California, USA) at 37 °C with 5% CO_2. AMI-1 was synthesized in house and the purity of AMI-1 was higher than 98%.

4.2. Cell proliferation assay

Cell proliferation was analyzed by CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan), as described previously (Zhang et al. 2017). Briefly, a total of 2×10^3 cells were seeded in 96-well plates with 100 µl medium each well. After 24 h cultivation, cells were treated with different doses of AMI-1 or vehicle. At the indicated time point, 10 µl of CCK-8 reagent was added to the wells. The cells were subsequently incubated for 2 h at 37 °C. The absorbance was measured at a wavelength of 450 nm.

4.3. Colony formation assay

Colony formation assay was performed as described previously (Zhang et al. 2017). Briefly, Hela cells were seeded in 60 mm dishes at 250 cells per dish and maintained in the absence or presence of AMI-1 for 16 days. Cells were fixed with fixative (7 parts methanol:1 part glacial acetic acid) and stained with 0.1% crystal violet. The number of colonies was counted for analysis.

4.4. Apoptosis analysis

Hela cells were seeded at 1×10^5 cells/well in 6-well plates and incubated with AMI-1 at different concentration for 48 h or 72 h. About 1×10^5 cells were harvested by EDTA-free trypsinization and washed twice with cold PBS. After being resuspended in 500 µl binding buffer, cells were incubated with 5 µl of annexin V-FITC and 5 µl of propidium iodide (KeyGEN BioTECH, Jiangsu, China) for 15 min at room temperature in dark. Then cell apoptosis was measured by FACS (Navios, Beckman Coulter, CA, USA).

4.5. Tumorigenicity in nude mice

Female BALB/c nude mice aged 6-7 weeks were purchases from Hunan Slac Jingda Laboratory Animal Co., Ltd. (Hunan, China) and maintained under standard conditions. All the animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Treatment Committee of Lanzhou University. Briefly, Hela cells (2.5×10^6) were subcutaneously injected into the right flank of the mice. When the tumors reached an average volume of about 100 mm^3, the mice bearing too large or too small tumors were eliminated and the left were divided randomly into two groups for treatment: AMI-1 (0.6 mg in 100 µl of 0.9% NaCl) or 100 µl 0.9% NaCl were administered intratumorly once every two days for 18 days. Tumor size was measured with calipers every three or four days, and tumor volume was calculated using the following formula: $V = \text{length} \times \text{width}^2 \times 0.52$. All animals were sacrificed at the end of the study, and their tumors were collected for further analysis.

4.6. Western blot analysis

The cell pellets were suspended in RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and incubated on ice for 5 min. The cell lysates were subsequently centrifuged at 12000 x *g* for 15 min at 4 °C. The supernatant was collected and the total protein content was determined using the Quick Start™ Bradford Protein assay (Bio-Rad, CA, USA). Equal amounts of protein (20-40 µg) were loaded on 12% sodium dodecyl sulfate polyacrylamide gels and then transferred to polyvinylidene fluoride membranes (Bio-Rad, CA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline-Tween 20 and incubated with the appropriate primary antibodies at 4 °C overnight, followed by the corresponding horseradish peroxidase-labeled secondary antibodies (1:5000, ZSGB-BIO, Beijing, China). Immunoreactivity was visualized using BeyoECL Plus kit (Beyotime Institute of Biotechnology, Jiangsu, China) and then analyzed by densitometry. Antibodies against the following proteins were used for the experiment: PRMT5 and eIF4E (1:500, Santa Cruz Biotech, CA, USA), PRMT7 (1:1000, Cell Signaling, MA, USA), or β-actin (1:2000, Cell Signaling, MA, USA).

4.7. Statistical analysis

All data were expressed as means±standard deviation (S.D.). Statistical analysis was performed by the Student's *t* test for two groups and by ANOVA for multiple groups. P values < 0.05 were considered statistically significant, $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ level.

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Conflict of interest: None declared.

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