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PRMT6 overexpression upregulates TSP-1 and downregulates MMPs: Its implication in motility and invasion

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ABSTRACT

In this study, we investigate the molecular mechanism by which protein arginine methyltransferase 6 (PRMT6) exerts anti-invasiveness effect against breast cancer cells and prostate cancer cells. PRMT6 has been known to be responsible for asymmetric dimethylation of histone H3 at R2 (H3R2me2a). To investigate the biological role of PRMT6, we first established stable cell lines expressing GFP-PRMT6 with MCF7 and PC3 cells. Growth rates and colony forming abilities of PRMT6-overexpressing cells were significantly retarded compared to control GFP expressing cells. This growth retardation seems to be associated with $p21^{WAF1}$ induction. In addition, our data show that migration and invasion of prostate cancer cells was strongly suppressed by PRMT6 overexpression. In parallel, the levels of thrombospondin-1 (TSP-1), a potent natural inhibitor of angiogenesis, were highly up-regulated in both PRMT6-overexpressing cells. Furthermore, this suppression of migration and invasion by PRMT6 overexpression was significantly rescued by specific knock-down of TSP-1. Concomitantly, down-regulations of MMP-2 and -9 were observed in PRMT6-overexpressing cells. Taken together, our data demonstrate that PRMT6 overexpression is associated with regulation of motility and invasion through up-regulation of TSP-1 and down-regulation of MMPs in human cancer cells.

1. Introduction

The protein arginine methyltransferases (PRMTs) catalyze the mono- and dimethylation of arginine residues in a variety of substrates which are essential to maintain cellular processes including cellular growth signaling, nuclear–cytoplasmic protein shuttling, cell differentiation, embryogenesis, transcriptional regulation, and chromatin remodeling [1–4]. There are three types of PRMTs. The type I isozymes (PRMT1–4, PRMT6, and PRMT8) generate monomethylarginine and asymmetric dimethylarginine; the type II isozyme (PRMT5) generates monomethylarginine and symmetric dimethylarginine; and the type III isozyme (PRMT7) generates only monomethylarginine [5,6]. The PRMTs have emerged as an enzyme family whose activity is dysregulated in human disease [5,7–9].

Arginine methylation has been implicated in the positive and negative regulation of transcription. PRMT1 and PRMT4 have been associated with transcriptional activation. PRMT1 plays as a diverse regulator of protein function, responsible for the bulk of total arginine methyltransferase activity, and generates H4R3me2a. And, PRMT4 was first identified as a steroid receptor coactivator and has an ability to methylate H3R2, H2R17, and H3R26 residues. Certain transcription factors including nuclear receptor, p53, YY1, and NF-κB recruit these enzymes to specific promoters, which lead to a transcriptional activation. In contrast, PRMT5 methylates H3R8 and H4R3 which are strong repressive marks of numerous genes. For example, PRMT5 negatively regulates the expression of tumor suppressor genes ST7 and NM23 [10]. In addition, PRMT6 catalyzes the methylation of histones H3 (H3R2me2a) and H4 (H4R3me2a), and this activity has been shown to play a key role in controlling the expression of the HOX genes as well as Myc-dependent genes [11,12]. Thus, H3R2me2a is known as a transcriptional repressive mark.

PRMT6 is predominantly in nucleus [13], but its function is still poorly understood. PRMT6 has been shown to methylate the HMGA1a protein in the second AT hook and likely regulates chromatin structure organization [14,15]. The fact that PRMT6 methylates DNA polymerase beta indicates a role in DNA repair [11].

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Recent report shows that H2AR29me2a is specifically enriched at genes repressed by PRMT6, establishing H2AR29me2a as a novel repressive player involved in PRMT6 function [16]. In addition, PRMT6 has been reported to be overexpressed in, and to be required for, the proliferation of bladder and lung cancer cells [17]. More interestingly, siRNA knockdown of PRMT6 in U2OS osteosarcoma cells led to the up-regulation of thrombospondin-1 (TSP-1), a natural inhibitor of angiogenesis and cell migration [18]. All these reports imply a potential function of PRMT6 in tumor progression and metastasis.

In this study, we tried to more clarify the biological role of PRMT6 in tumor progression and metastasis. To achieve this goal, we established stable cell lines overexpressing PRMT6 in the human breast cancer cell MCF7 and prostate cancer cell PC3 and then analyzed their phenotypes. However, unlike the previous observations [18], our findings show the negative function of PRMT6 on invasion and migration of cancer cells through up-regulation of TSP-1 and down-regulation of MMP-2 and -9.

2. Materials and methods

2.1. Cell culture and cytotoxicity test

Human breast cancer cell line MCF7 and human prostate cancer cell PC3 were grown in MEM and RPMI 1640 medium (Invitrogen, Carlsbad, CA), respectively, with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Cytotoxicity was determined by the MTS assay (Promega, Madison, WI) according to the manufacturer's instruction.

2.2. Immunoblot analysis

Cell lysates were prepared, clarified, and subjected to immunoblot analysis. In some experiments, histone fraction was prepared as previously described [19]. The following antibodies were used: GFP, Histone H3, E-cadherin (Cell Signaling, Beverly, MA), $p21^{WAF1}$ (Santa Cruz Biotechnology, Santa Cruz, CA), PRMT6 (Bethyl Laboratories, Montgomery, TX), Anti-Histone H3R2me2a (Upstate, Lake Placid, NY), TSP-1 (Abcam Inc., Cambridge, MA), Actin (Sigma Chemical Co., St. Louis, MO).

2.3. RT-PCR and quantitative real-time PCR

Total RNA was extracted using the easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology, Sungnam, Korea), and the integrity of the RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) reaction using the ImProm-II Reverse Transcription System (Promega) and Taq polymerase (Solgent, Daejeon, Korea). The primer sets for TSP-1 were 5′-ATCTCCTGTGATGAGCTGTCCA-3′ and 5′-GCTATCAACAGTCCATTCCTCG-3′; GAPDH, 5′-CTCATG ACCACAGTCCATGCCATC-3′ and 5′-CTGCTTCACCACCTTCTTGAT GTC-3′. Quantitative real-time PCR analysis was performed using the Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) with SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA). The Primer sets for MMP-2 were 5′-ATCCTGGCTTTCCCAA GCTC-3′ and 5′-CACCCTTGAAGTAGCTGTG -3′; MMP-9, 5′-GGG CTTAGATCATTCCTCAGTG-3′ and 5′-GCCATTCACGTCGTCCTTAT-3′; GAPDH, 5′-CTCATGACCACAGTCCATGCCA TC-3′ and 5′-CTGCTTCAC-CACCTTCTTGATGTC-3′.

2.4. Wound healing assay

PC3/GFP, and PC3/PRMT6#16, #24 cells were grown to confluence in six-well plates, and the cells were maintained until they reached confluence. The cross-shaped wound was made on the monolayer using a sterile 200 μl pipette tip. After washing with warmed PBS, the cells were incubated in fresh culture medium, and wound healing was surveyed at 24 h or 36 h after. The cross-shaped wounds were photographed.

2.5. shRNA transfection

The shRNA (Thermo Scientific, Huntsville, AL) transfection was performed using pLKO.1 empty vector (RHS4080), pLKO.1-shPRMT6 (RHS3979-9602092), and pLKO.1-shTSP-1 (RHS3979-9568584). The cells were transfected with 8 μg shRNAs using 20 μl Lipofectamine 2000 (Invitrogen).

2.6. Matrigel invasion assay

Matrigel invasion assay was performed by using 6.4 mm-diameter Transwell inserts with the 8 μm pore membranes coated with Matrigel matrix (BD Biosciences, San Diego, CA) as previously described [19].

2.7. MMP activity

An assay to measure MMP activity was performed using gelatin zymography. Briefly, a 20 μl of medium was loaded under non-denaturing conditions into a zymogram gel supplemented with 0.1% gelatin to detect the presence of MMP-2 and MMP-9. After electrophoresis, the gels were washed in a renaturing buffer and incubated in an incubation buffer at 37 °C for 24 h, stained with coomassie brilliant blue R-250 (Sigma), and then destained with gel-clear destaining solution. Areas of gelatinolytic degradation appeared as transparent bands on the blue background.

2.8. Colony formation assay

For colony formation assay, 400 cells were plated in duplicates on six-well plates. After incubation for 10 days, colonies were stained with crystal violet [0.1% (w/v) in 20% ethanol], photographed and counted.

3. Results

3.1. Overexpression of PRMT6 suppresses the proliferation of cancer cells

To investigate the biological function of PRMT6, we first established stable cell lines expressing PRMT6 in human breast cancer cell line MCF7 and human prostate cancer cell line PC3. Each cell was transfected with pEGFP control or pEGFP-PRMT6 vector. After 48 h, PRMT6 expression cell lines were selected with G-418. MCF7/PRMT6#1, MCF7/PRMT6#7, PC3/PRMT6#16, and PC3/PRMT6#24 were used for this study. PRMT6 expressions in these cells were confirmed by fluorescence microscopy and were predominantly in nucleus (Supplementary Fig. 1). And, we examined an increase in H3R2me2a in these cells which is primary target of PRMT6. As shown in Fig. 1B, a globally increased level of H3R2me2a was detected in these cell clones (MCF7/PRMT6#1, #7, and PC3/PRMT6#24 cells). In addition, an increased expression of $p21^{WAF1}$ was observed in these PRMT6-overexpressing cancer cells (Fig. 1A), indicating that PRMT6 may regulate cell proliferation. As expected, proliferation of PRMT6-overexpressing MCF7 cells

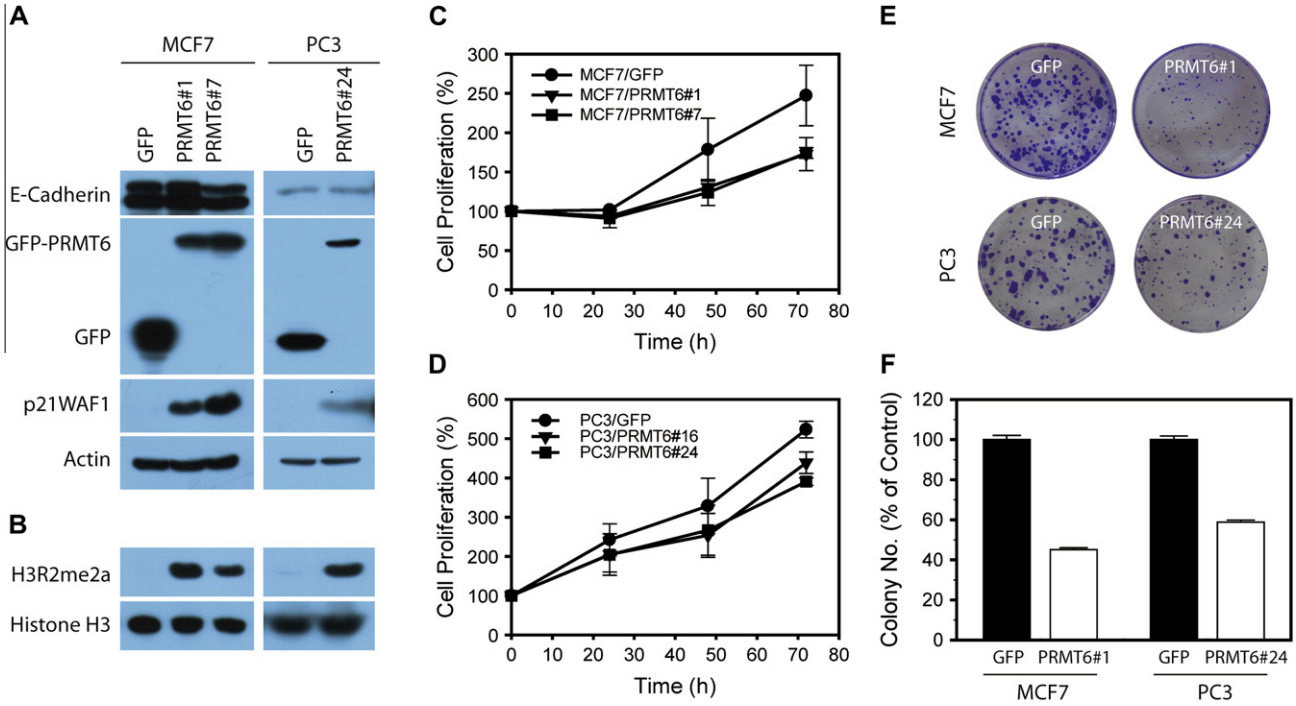

Fig. 1. Overexpression of PRMT6 suppresses the proliferation of cancer cells. (A) PRMT6-overexpressing stable cell lines were established with MCF7 and PC3 cells. Two clones of each cell were chosen for this study (MCF7/PRMT6#1, #7; PC3/PRMT6 #16, #24). The expression levels of E-cadherin, GFP-PRMT6, and $p21^{WAF1}$ were analyzed by immunoblot analysis. (B) The levels of H3R2me2a were analyzed with histone fraction. (C) and (D) Each cell was cultured for 72 h, and cell proliferation was determined by MTS assay. Data were expressed as the mean ± s.d. from three independent experiments. (E) and (F) The colony formation assay was evaluated after 10 days. Representative dishes of crystal violet-stained colonies were photographed. The number of colonies was counted and the quantification is presented as colony number. Data were expressed as the mean ± s.d. from three independent experiments.

was significantly retarded, compared to the control GFP-expressing cells (Fig. 1C). The similar results were observed in PRMT6-overexpressing PC3 cells (Fig. 1D). Furthermore, colony forming abilities of these cells were significantly inhibited (Fig. 1E and F). All these results indicate that PRMT6 suppresses the growth of cancer cells *via* $p21^{WAF1}$ expression. It has been well demonstrated that a salient feature in the progression of prostate cancer is a dysregulation of E-cadherin expression which plays a pivotal role in epithelial cell–cell adhesion. However, our data shows that E-cadherin expression levels are not affected by PRMT6 overexpression in both cells (Fig. 1A).

3.2. PRMT6 attenuates the cell invasion and motility of human cancer cells

We next examined the effect of PRMT6 on the cell invasion and motility of human cancer cells. To further measure the impact of PRMT6 on the cell movement, the invasive capacity of PRMT6-overexpressing cells was assessed using transwell filters coated with matrigel matrix. The invasiveness of PRMT6-overexpressing cells was significantly inhibited up to ~30%, compared to their control GFP counterpart (Fig. 2A). Next, wound-healing assay was performed to further confirm the migratory capabilities of the PRMT6-overexpressing cancer cells. These cells were grown to reach confluence and then a cross-shaped wound was created in monolayer. The cells were cultured for additional 24 h or 36 h. As shown in Fig. 2B, PC3/GFP cells spontaneously migrated and filled about 80% of wounded region for 24 h, and after 36 h the wounded region was completely rescued. However, the migration ability of PRMT6-overexpressing cells was significantly suppressed, compared to the control cells (Fig. 2B), which is in a good accordance with the results from invasion assay with matrigel transwell filters (Fig. 2A). Taken together, these data suggest that PRMT6 leads to a change in gene expression through histone modifications including H3R2me2a, which might contribute to the suppression of migration and invasion of cancer cells.

3.3. TSP-1 expression is responsible for anti-migration effect of PRMT6 in cancer cells

It has been well demonstrated that TSP-1 is a known inhibitor of cell migration. To examine the possible involvement of TSP-1 in anti-migration effect of PRMT6 in cancer cells, we determined the expression levels of TSP-1 in PRMT6-overexpressing cells. Increased levels of both mRNA and protein of TSP-1 were observed in PRMT6-overexpressing MCF7 and PC3 cells (Fig. 3A and B), which was accompanied by the increased secretion of TSP-1 into the culture media (Fig. 3C). These results imply that TSP-1 expression could be up-regulated by PRMT6, which might be responsible for its ability to inhibit cell migration. To investigate the possible involvement of TSP-1 in PRMT6 suppression of cell migration, PRMT6 shRNA was introduced into MCF7/PRMT6 cells. The PRMT6 shRNA completely blocked the expression of PRMT6, which was followed by down-regulation of TSP-1 expression (Fig. 3D). Interestingly, the TSP-1 shRNA only blocked the expression of TSP-1 without affecting PRMT6 expression in MCF7 and PC3 cells (Fig. 3E). All these results strongly suggest that TSP-1 expression is regulated by PRMT6 activity. To confirm the involvement of TSP-1 in anti-migration effect of PRMT6 in cancer cells, we determined whether TSP-1 knocked-down could rescue PRMT6-mediated inhibition of cell invasion and migration. As shown in Fig. 4A, knock-down of TSP-1 dramatically reversed the inhibition of cell migration by PRMT6. These results strongly support that anti-migration effect of PRMT6 is mainly caused by an increase in TSP-1 expression.

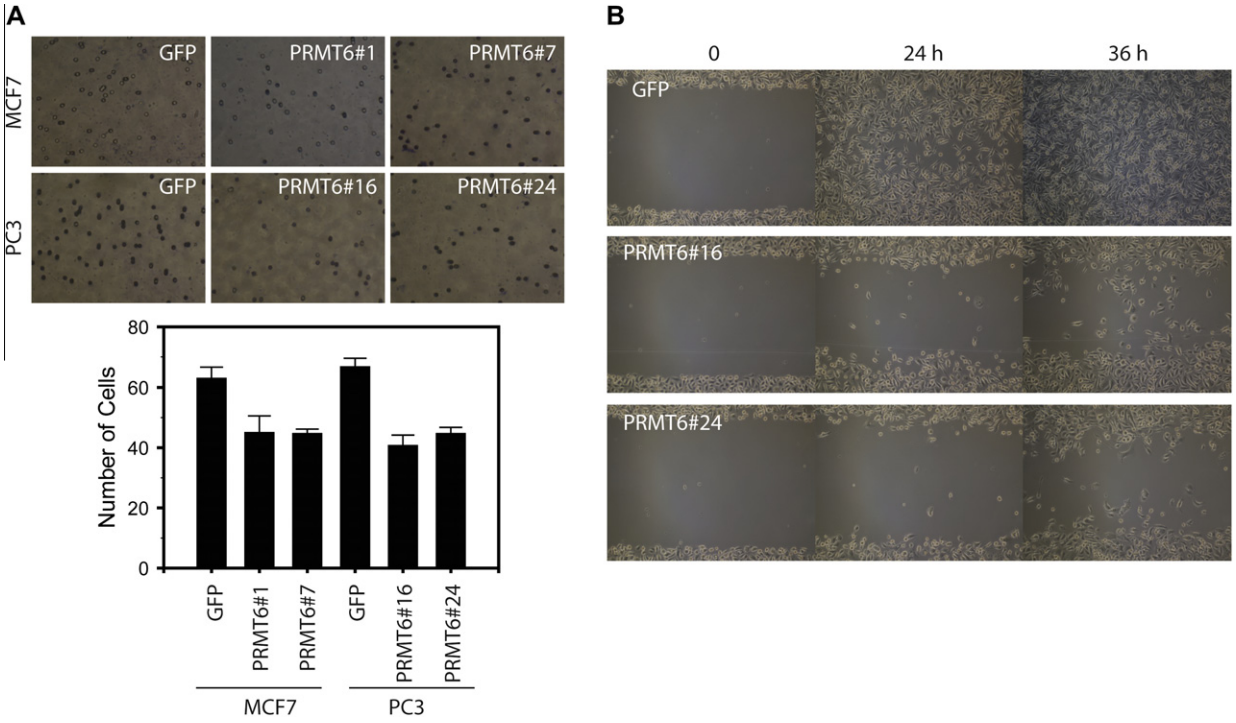

Fig. 2. PRMT6 attenuates the cell invasion and motility of human cancer cells. (A) Invasion potentials of MCF7 and PC3 cells were assessed using BD transwell chamber assays. Cells that migrated to the filter lower side were stained crystal violet, photographed, and counted. Data were expressed as the mean ± s.d. from three independent experiments. (B) PC3 cells (PC3/GFP, PC3/PRMT6#16, PC3/PRMT6#24) were grown to reach confluence. A cross-shaped wound was created in monolayer, and cells were cultured for additional 24 h or 36 h. The representative digital images of wounded region were visualized.

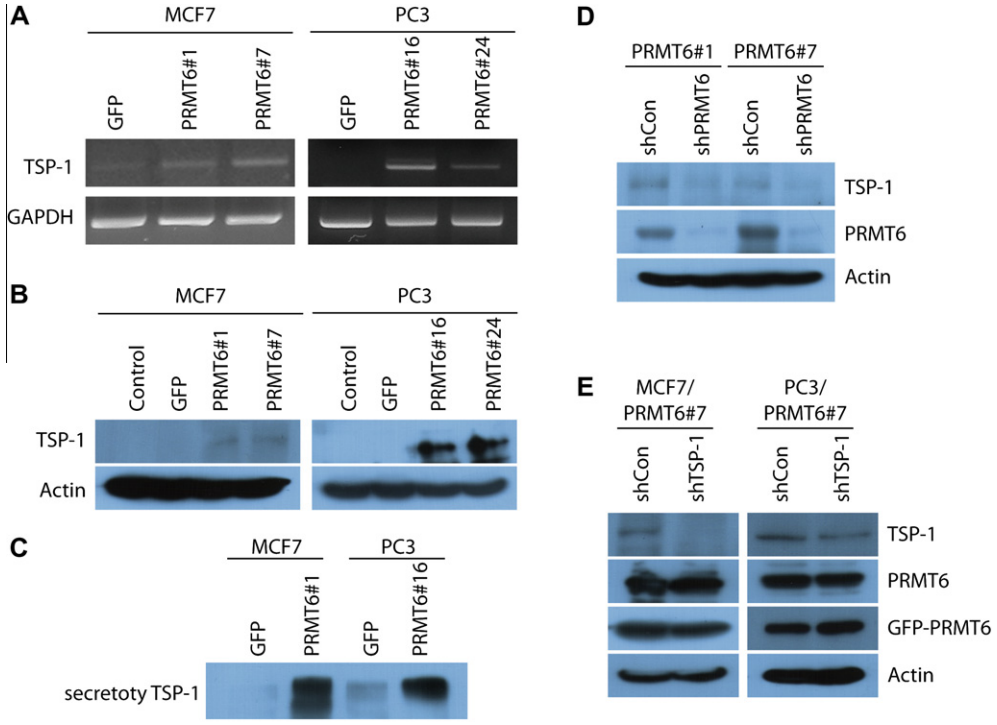

Fig. 3. TSP-1 is PRMT6 target in cancer cells. (A) and (B) The expression levels of TSP-1 mRNA and protein were analyzed by reverse transcription-PCR and Western blot in MCF7/GFP, PRMT6#1, #7 and PC3/GFP, PRMT6#16, #24 cells. (C) The conditioned media from control (MCF7/GFP, PC3/GFP) and PRMT6 stable cells (MCF7/PRMT6#1, PC3/PRMT6#24) were immunoblotted with anti-TSP-1. (D) MCF7/PRMT6#1, #7 cells were transfected with shPRMT6 or non-targeting shCon as a negative control. The expression of PRMT6 and TSP-1 were analyzed by Western blot. When the PRMT6 expression was diminished, TSP-1 expression was decreased as well. (E) MCF7/PRMT6#7, PC3/PRMT6#24 cells were transfected with shTSP-1 or shCon. The expression of PRMT6 and TSP-1 were analyzed by Western blot. The TSP-1 expression was decreased without changing PRMT6 expression.

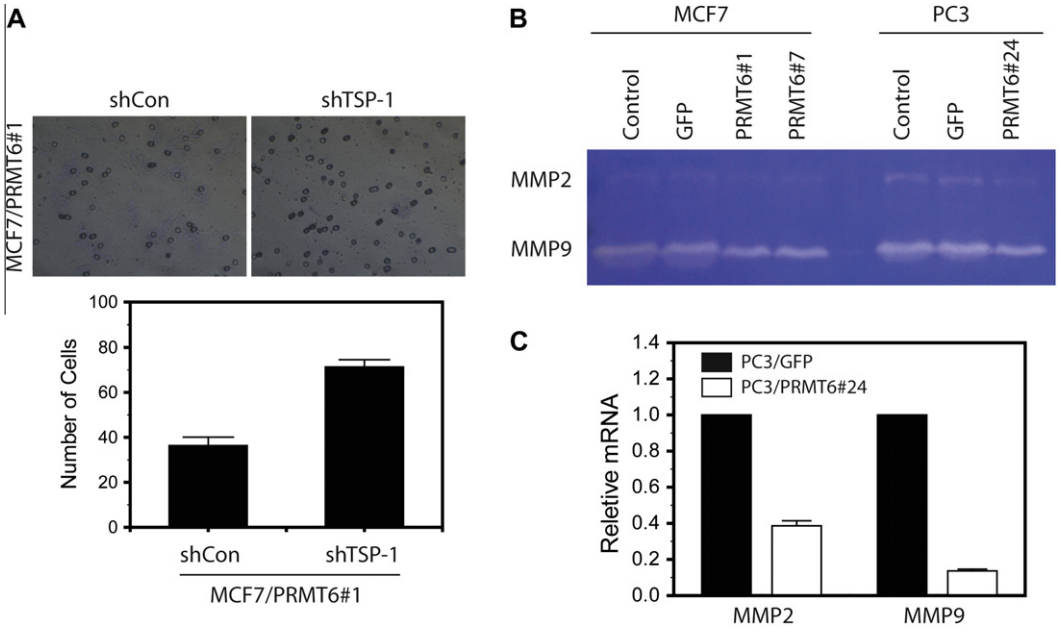

Fig. 4. Up-regulation of TSP-1 and down-regulation of MMP-2 and -9 are associated with PRMT6 function on invasion and migration. (A) MCF7/PRMT6#1 cells were transient transfected with shTSP-1 or non-targeting shCon as a negative control. Invasion potentials of each cell were assessed using BD transwell chamber assays. Cells that migrated to the filter lower side were stained crystal violet, photographed, and counted. Data were expressed as the mean ± s.d. from three independent experiments. (B) Zymograms for the determination of activities of MMP-2 and -9 in both MCF7 and PC3. Gelatinolytic activities of MMP-2 and -9 were detected by electrophoresis of soluble protein on a gelatin-containing 10% polyacrylamide gel. (C) The mRNA expression levels of MMP-2 and -9 were analyzed by real-time PCR in PC3 and PC3/PRMT6#24 cells.

3.4. The expressions of MMP-2 and -9 were down-regulated in PRMT6-overexpressing cells

Although our data show that TSP-1 expression is responsible for anti-migration effect of PRMT6, we next tried to check the involvement of MMP family members which are closely associated with tumor invasion. Among the MMP family members, MMP-2 and -9 are most relevant to tumor invasion. Both MMPs can degrade collagen of the basement membrane, which induces cell migration and is required for cells to break up the extracellular matrix and invade into the surrounding tissues [20,21]. These are synthesized as pro-enzymes in the cell. When secreted, they are cleaved and become catalytically activated [22]. Therefore, we examined whether the overexpression of PRMT6 cells also produce more secreted and active forms of MMP-2 and MMP-9. As shown in Fig. 4B, mature and active forms of MMP-2 and MMP-9 were significantly decreased in PRMT6-overexpressing cells (MCF7/PRMT6#1, #7 and PC3/PRMT6#24), as measured by gelatin zymography. In addition, qPCR analysis revealed that MMP-2 and MMP-9 mRNA levels are 2.5 and five-fold lower, respectively, in clones of PC3/PRMT6#24 cell lines (Fig. 4B). The results suggest that the decreased MMP-2 and MMP-9 expression are responsible for, at least in part, PRMT6 suppression of cell migration and invasion.

4. Discussion

We here demonstrate the negative role of PRMT6 on invasion and migration of cancer cells through up-regulation of TSP-1 and down-regulation of MMP-2 and -9. Tumor invasion and metastasis are the major characteristics of aggressive phenotypes of various human cancers as well as the major causes of cancer deaths [23]. Invasion is one of the critical steps in the metastatic cascade and is a strong indicator of tumor progression. It has been demonstrated that TSP-1 is a potent natural inhibitor of angiogenesis and endothelial cell migration [24,25]. TSP-1 is synthesized by many cell types and secreted into the extracellular matrix [26,27]. TSP-1 inhibits cell migration by binding cell surface receptors, including CD36 with its type 1 sequence repeat domains [28,29]. Our data strongly support that TSP-1 is one of targets of PRMT6 for its anti-invasion effect. The expression levels of TSP-1 in PRMT6-overexpressing cells were highly elevated (Fig. 3 and Supplementary Fig. 2B), however, specific knock-down of PRMT6 led to a dramatic decrease in TSP-1 expression (Fig. 3D). In addition, even though H3R2me2a levels on the TSP-1 promoter region are accumulated in PRMT6 overexpressing cells, Pol II and active gene marker H3K9ac, H3 lysine 9 acetylation, are highly enriched on the promoter region (Supplementary Fig. 2A), strongly supporting PRMT6-mediated TSP-1 expression. However, these results are not consistent with previous observation that TSP-1 is a transcriptional repression target of PRMT6, and blocking of secreted TSP-1 restored cell migration in PRMT6-deficient osteosarcoma cells (U2OS) [18]. Although we cannot provide the exact reason for this discrepancy right now, one of possible explanation might be due to different cellular systems (MCF7/PC3 *vs* U2OS) between these two studies. In some case TSP-1 regulation might be regulated in cell type specific manner through different cellular pathways. The other explanation is difference between PRMT6 overexpression system and PRMT6 knock-down system. In knock-down system, deficient of PRMT6 could not write repressive epigenetic mark H3R2me2a on the TSP-1 promoter region, which leads to an increase expression of TSP-1. However, in overexpression system, the overexpressed PRMT6 may suppress the expression of some repressive complexes, such as txr-1 and id-1 [30,31], for TSP-1 transcription repression through generating H3R2me2a on their promoter region, which turns into the TSP-1 transcriptional activation. In our preliminary data, mRNA levels of both txr-1 and id-1 in PC3/PRMT6#24 were dramatically decreased (Supplementary Fig. 2C). This possibility needs to be elucidated in the future works.

In addition to PRMT6 induction of TSP-1, overexpressed PRMT6 suppresses MMP-2 and -9 expressions in both MCF7 and PC3 cells, which accounts, at least in part, for anti-migration effect of overexpressed PRMT6. We could exclude the possible involvement of E-cadherin on anti-migration effect of overexpressed PRMT6,

because E-cadherin levels were not affected by PRMT6 overexpression (Fig. 1). Furthermore, our data show that overexpression of PRMT6 suppresses the growth of cancer cells *via* $p21^{WAF1}$ expression (Fig. 1). It is still possible that the attenuated cell invasion and motility by PRMT6-overexpression might be due to their retarded growth rate. However, our data are absolutely opposed to the recent observations that PRMT6 regulates negatively the expression of cyclin-dependent kinase inhibitors, $p21^{WAF1}$ and $p27^{Kip1}$, which might contribute to the oncogenic capacity of PRMT6 [32–35]. It is possible to assume that a similar mechanism may be involved in this discrepancy, as in the case of TSP-1 induction by PRMT6 overexpression. In conclusion, our results demonstrate that overexpression of PRMT6 regulates cell migration and invasion by regulating the expression of TSP-1 and MMPs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.085.

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