Protein arginine methyltransferase 6 suppresses adipogenic differentiation by repressing peroxisome proliferator-activated receptor γ activity

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Abstract. The present study demonstrated that protein arginine methyltransferase 6 (PRMT6) negatively regulates the activity of peroxisome proliferator-activated receptor γ (PPARγ). The results indicated that the overexpression of PRMT6 inhibited the transactivity of PPARγ and subsequently decreased the expression levels of PPARγ target genes. Contrarily, the depletion or inhibition of PRMT6 increased PPARγ reporter activity and the expression of its target genes. It was also confirmed that PRMT6 was involved in the process of adipocyte differentiation. In addition, PRMT6 interacted with, but did not methylate, PPARγ. PRMT6 bound to the PPAR-responsive regulatory element of the adipocyte Protein 2 (aP2) promoter in conjunction with PPARγ and generated the repressive epigenetic mark arginine 2 on histone H3 asymmetric di-methylation, which suppressed aP2 gene expression. Therefore, PRMT6 may serve as an important regulator of PPARγ activity in adipogenic differentiation and may be an attractive therapeutic target for human metabolic diseases.

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Abbreviations: PRMT, protein arginine methyltransferase; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, PPAR-responsive regulatory element; RXRα, retinoid X receptor α; TZD, thiazolidinedione; PTM, post-translational modification; ChIP, chromatin immunoprecipitation; CARM1, coactivator-associated arginine methyltransferase 1

Key words: protein arginine methyltransferase 6, peroxisome proliferator-activated receptor γ, adipogenic differentiation, epigenetics, arginine 2 on histone H3 asymmetric di-methylation

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-inducible transcription factors that control the expression of numerous genes involved in adipogenesis, lipid metabolism, inflammation and maintenance of metabolic homeostasis (1-3). Each of the 3 PPAR isoforms, namely, PPARα, PPARβ/δ and PPARγ, organizes to form a heterodimer with retinoid X receptor α (RXRα) and binds to the PPAR-responsive regulatory element (PPRE) present in the target gene promoters (4,5). Despite high structural homology, these 3 PPAR isoforms differ in their tissue distributions, ligand specificity, and physiological roles *in vivo* (6,7). PPARγ is highly expressed in white and brown adipose tissues and overexpressed in several types of human cancer, including prostate cancer (8,9). Due to its vital role in the regulation of insulin sensitivity and glucose metabolism, PPARγ has been studied as the target molecule for the development of therapeutic drugs for the treatment of type 2 diabetes (10-12). In addition, PPARγ agonists are being used as adjuvants in the treatment of prostate cancer (13,14).

Although several potent endogenous ligands with relatively low affinity for PPARγ, including free fatty acids and eicosanoids, have been identified (15-18), a physiologically active endogenous ligand is yet unknown. A number of synthetic ligands including thiazolidinediones (TZDs) exhibit high affinity for PPARγ and exhibit robust insulin-sensitizing activities (10-12). Upon binding to selective ligands, PPARγ undergoes a conformational change that facilitates the dissociation of co-repressors and recruitment of co-activators, including steroid receptor co-activator, cAMP response element binding protein-binding protein, and PPARγ co-activator-1α, leading to the transcriptional activation of target genes (19-21). However, the complete understanding of the dynamics of PPARγ necessitates the study of the detailed mechanisms underlying the recruitment of tissue-specific co-activators and co-repressors.

Protein arginine methylation is a common post-translational modification (PTM) in various proteins and is catalyzed by enzymes called protein arginine methyltransferases (PRMTs) (22,23). In mammals, PRMTs that have been

characterized have been demonstrated to produce 3 types of methylarginine, namely, mono-methylarginine, asymmetric di-methylarginine and symmetric di-methylarginine (22,24). In epigenetic gene regulation, PRMTs are recruited to promoters via interaction with transcription factors as co-activators or co-repressors, followed by methylate arginine residues in histones and other chromatin proteins (23,25). PRMT6 is a type I PRMT enzyme located predominantly in the nucleus that exhibits a high affinity for arginine 2 on histone H3 (H3R2) and catalyzes H3R2 asymmetric di-methylation (H3R2me2a) (26,27). As H3R2me2 is a repressive mark, PRMT6 activity is primarily associated with transcriptional silencing (28). However, the functions of PRMT6 in PPARγ regulation and adipogenesis have not been completely identified, although it is hypothesized to regulate numerous biological process including transcription (28,29), DNA replication (30) and signal transduction. The present study identified that PRMT6 co-repressed PPARγ-dependent transcription through H3R2me2 in PPRE and served as a key regulator of adipocyte differentiation.

Materials and methods

Constructs, reagents and antibodies. Green fluorescent protein (GFP)-PRMT1, GFP-PRMT4, GFP-PRMT5 and GFP-PRMT6 plasmids were obtained from Dr M T Bedford (MD Anderson Cancer Center, Smithville, TX, USA). MS023, pioglitazone, GW7647, GW501516, retinoic acid, dexamethasone, methyl isobutyl xanthine (IBMX) and insulin were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against PRMT5 (cat. no. sc-376937), GFP (cat. no. sc-9996), GAPDH (cat. no. sc-25778) and β-actin (cat. no. sc-47778) were procured from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA) and those against histone H3 (cat. no. 9715) and PPARγ (cat. no. 2430) were procured from Cell Signaling Technology, Inc., (Danvers, MA, USA). Anti-H3R2me2a (cat. no. 07-585) and Asym24 (cat. no. 07-414) were obtained from EMD Millipore (Billerica, MA, USA) and anti-PRMT1 (cat. no. A300-722A), anti-PRMT4 (cat. no. A300-421A) and anti-PRMT6 (cat. no. A300-929A) were from Bethyl Laboratories (Montgomery, TX, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse, cat. no. 315-035-003; and anti-rabbit, cat. no. 211-035-109) were purchased from Jackson ImmunoResearch Laboratories, Inc., (West Grove, PA, USA).

Cell culture and transfection. The human cell line 293T, human prostate cancer PC3 cell line, African green monkey kidney fibroblast CV1 cell line and mouse embryonic fibroblast 3T3-L1 cell line were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) and 100 units/ml penicillin/streptomycin (HyClone Laboratories) at 37˚C and 5% CO_2 in a humidified chamber. For overexpression from mammalian expression plasmids, TransIT-2020™ (Mirus Bio, LLC, Madison, WI, USA) was used according to the manufacturer's protocol. For small-interfering RNA (siRNA) transfection, TransIT-X2™ (Mirus Bio, LLC) was used. All siRNA duplexes were synthesized by Integrated DNA Technologies Pte. Ltd., (Singapore). The sequences of PRMT6-targeting siRNAs used were as follows: Human, 5'-GACAAGACACGGACGUUU-3' and mouse, 5'-GCUACG GACUUCUGCACGA-3'.

3T3-L1 adipocyte differentiation and Oil Red O staining. 3T3-L1 cells were differentiated into adipocytes according to the ATCC protocol. Briefly, cells were grown for 48 h until 100% confluence and maintained in the growth medium (DMEM supplemented with 10% FBS and 100 units/ml penicillin/streptomycin). Cells were incubated in a differentiation medium (growth medium supplemented with 1 μM dexamethasone, 0.5 mM IBMX and 1 μg/ml insulin) for 48 h. Following incubation, the medium was replaced with an adipocyte maintenance medium (growth medium with 1 μg/ml insulin) for an additional 48 h and cells were then fed every other day with growth medium. For Oil Red O staining, cells were washed with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature, followed by staining with Oil Red O (Sigma-Aldrich; Merck KGaA) for 1 h, as previously described (31).

Luciferase gene reporter assay. The transactivation assay for PPARs was measured by reporter gene (PPRE-luciferase plasmid) analysis as described previously (31). CV1 cells were transfected with PPRE-Luc firefly luciferase constructs (Addgene, Inc., Cambridge, MA, USA) and SV40-*Renilla* luciferase plasmids (Addgene, Inc.) using TransIT-2020™ (Mirus Bio, LLC). Following incubation for 24 h at 37˚C, cells were treated with pioglitazone, GW7647 or GW501516 for additional 24 h. The dual luciferase reporter assay kit (Promega Corporation, Madison, WI, USA) was used according to the manufacturer's protocol and the luciferase activities were quantified using GloMax® 20/20 (Promega Corporation). The data are presented as the mean ± standard deviation of three independent experiments.

Immunoblotting and immunoprecipitation (IP). Whole cell extracts were obtained using a lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride (NaCl), 10% glycerol, 1% NP-40 and 2 mM ethylenediaminetetraacetic acid (EDTA)] supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Basel, Switzerland). Following centrifugation at 16,000 x g for 10 min at 4˚C, protein concentration was determined by Bradford assay according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequent to boiling in SDS loading buffer for 5 min, equal amounts (10-30 μg) of protein were resolved through 10% SDS-PAGE and were transferred onto polyvinylidene fluoride (PVDF) membranes. The blots were blocked with 5% skim milk/0.1% Tween 20/TBS for 1 h at room temperature and incubated overnight with primary antibodies, followed by treatment with an HRP-linked secondary antibody for 1 h at room temperature. All primary antibodies were used at a dilution of 1:1,000 and secondary antibodies at a dilution of 1:10,000. Blots were developed with the WesternBright ECL HRP substrate (Advansta, Inc., San Jose, CA, USA), according to the manufacturer's protocols. For the

IP assay, equal amounts (~1 mg) of lysates were incubated with the appropriate antibodies (1 μg of antibody to each IP reaction) overnight at 4˚C, and the antibody-protein complex was captured using Protein A/G Sepharose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4˚C. Following washing twice with NP-40 lysis buffer, the complexes were eluted and analyzed by 10% SDS-PAGE and immunoblotting, as aforementioned.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted using the TRIsure™ RNA isolation kit (Bioline, London, UK), and cDNA was synthesized using the SensiFAST™ cDNA synthesis kit (Bioline). qPCR amplification was performed using 0.5 μl cDNA as the template, 10 μl SensiFAST SYBR™ No-ROX premix (Bioline), 1 μl each of the forward and reverse primers and 7.5 μl RNase-free water, in an Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). Reaction parameters were as follows: cDNA synthesis at 37˚C for 60 min, transcriptase inactivation at 95˚C for 5 min, then PCR cycling at 95˚C for 10 sec, 58˚C for 30 sec and 72˚C for 20 sec for 40 cycles. Data analyses were performed using the Eco software version 3.1 (Illumina, Inc) based on the ΔΔCq method (32). The primer sets for CCAAT-enhancer-binding protein α (C/EBPα) were forward, 5'-AGGTGCTGGAGTTGACCAGT-3' and reverse, 5'-CAGCCTAGAGATCCAGCGAC-3'; the primer sets for adipocyte Protein 2 (aP2) were forward, 5'-ATGTGTGATGCC TTTGTGGGA-3' and reverse, 5'-TGCCCTTTCATAAACTCT TGT-3'. GAPDH was used as control gene (forward, 5'-CTC ATGACCACAGTCCATGCCATC-3', and reverse, 5'-CTG CTTCACCACCTTCTTGATGTC-3').

In vitro methylation assay. GFP-PRMT6 protein was purified from the transfected 293T cells (~1 mg protein) using an anti-GFP IP method. Immobilized GFP-PRMT6 protein was incubated with 50 μl reaction buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethane sulfonyl fluoride and 1 mM dithiothreitol] supplemented with 1 μg recombinant histone (mixture of H2A, H2B, H3, and H4; New England Biolabs, Inc., Ipswich, MA, USA) or recombinant human PPARγ (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) and 1 μCi ^{3}[H]-labeled AdoMet (specific activity: 55-85 Ci/mmol; PerkinElmer, Inc., Waltham, MA, USA) at 37˚C for 1 h. The reaction was stopped by the addition of SDS loading buffer, and the proteins were resolved on 12% SDS-PAGE gels. Proteins were transferred onto PVDF membranes, and the tritium signal was amplified by spraying with EN3HANCE spray (PerkinElmer, Inc.) at room temperature. Membranes were exposed to autoradiography film for at least 1 week at 80˚C.

Chromatin immunoprecipitation (ChIP) assay. Chromatin from the differentiated 3T3-L1 cells ($1x10^6$ cells) was used for the ChIP experiment with each antibody (anti-PPARγ, anti-PRMT6 and anti-H3R2me2a) at 1:100 dilutions. A ChIP assay kit (EMD Millipore) was used according to the manufacturer's protocol. Briefly, samples were crosslinked with 1% formaldehyde and quenched with 0.125 M glycine. Samples were then lysed in an SDS lysis buffer [50 mM Tris-HCl (pH 8.0), 1% SDS and 10 mM EDTA] containing protease inhibitors and were sonicated at 20% amplitude to shear DNA samples to 200-1,000 base pair lengths for five cycles of 30 sec each, resting for 1 min between cycles on ice (Vibra-cell VCX750; Sonics & Materials Inc., Newtown, CT, USA). The lysates were diluted in an IP dilution buffer [16.7 mM Tris-HCl (pH 8.0), 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 150 mM NaCl] containing protease inhibitors. Following agarose-bead clearing, antibodies were added to lysates at a dilution of 1:100 overnight at 4˚C, and the combined antibody/DNA complexes were incubated with protein A/G beads for 1 h at 4˚C. Beads were sequentially washed with a low salt buffer [20 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 2 mM EDTA and 150 mM NaCl], high salt buffer (same buffer containing 500 mM NaCl), and a lithium chloride (LiCl) buffer [10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate and 1 mM EDTA]. The final washing step was performed twice with Tris-EDTA buffer. Complexes were eluted from the beads using an elution buffer [1% SDS and 0.1 M sodium bicarbonate ($NaHCO_3$)] and reverse-crosslinked with proteinase K for 2 h at 65˚C. The samples were purified with DNA spin columns and then analyzed by RT-qPCR as aforementioned. The primer sets used were as follows: aP2 PPRE forward, 5'-GAGCCATGCGGATTCTTG-3' and reverse, 5'-CCAGGAGCGGCTTGATTGTTA-3'; aP2 non-PPRE forward, 5'-CAGCCCCACATCCCCACAGC-3' and reverse, 5'-GGATGCCCAACAACAGCCACAC-3'.

Statistical analysis. Data are presented as mean ± standard deviation of three independent experiments. Comparisons between groups were performed using a two-tailed Student's t-test (SigmaPlot ver. 10.0; Systat Software, Inc., San Jose, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PRMT6 suppresses PPARγ transcriptional activity. To investigate the regulation mechanism underlying PPARγ transactivity by arginine methylation, a PPARγ reporter gene assay was performed using GFP-PRMT1, GFP-PRMT4, GFP-PRMT5 or GFP-PRMT6 plasmids. Overexpression of PRMT6 significantly suppressed PPARγ transactivity, but GFP-PRMT1, GFP-PRMT4 or GFP-PRMT5 had little or no effect on PPARγ activity (Fig. 1A). In addition, the protein expression patterns of PRMT1, PRMT4 and PRMT5 during 3T3-L1 adipogenic differentiation were not significantly altered from visual observation; however, the PRMT6 level was increased (Fig. 1B). To confirm the suppression of PPARγ by PRMT6, the PPARγ transactivity in PC3 human prostate cancer cells that exhibited stable expression of PPARγ protein was examined. Basal and pioglitazone-induced PPARγ activities in PC3 cells were suppressed upon PRMT6 overexpression (Fig. 1C) but increased following PRMT6 depletion (Fig. 1D). However, the transactivities of PPARα, PPARβ/δ and RXRα were unaffected by PRMT6 overexpression or depletion (Fig. 1E-L). Taken together, these results indicate that PPARγ transactivity was negatively regulated by PRMT6.

PRMT6 regulates 3T3-L1 adipogenic differentiation. As PPARγ is a major regulator of adipogenic differentiation, the

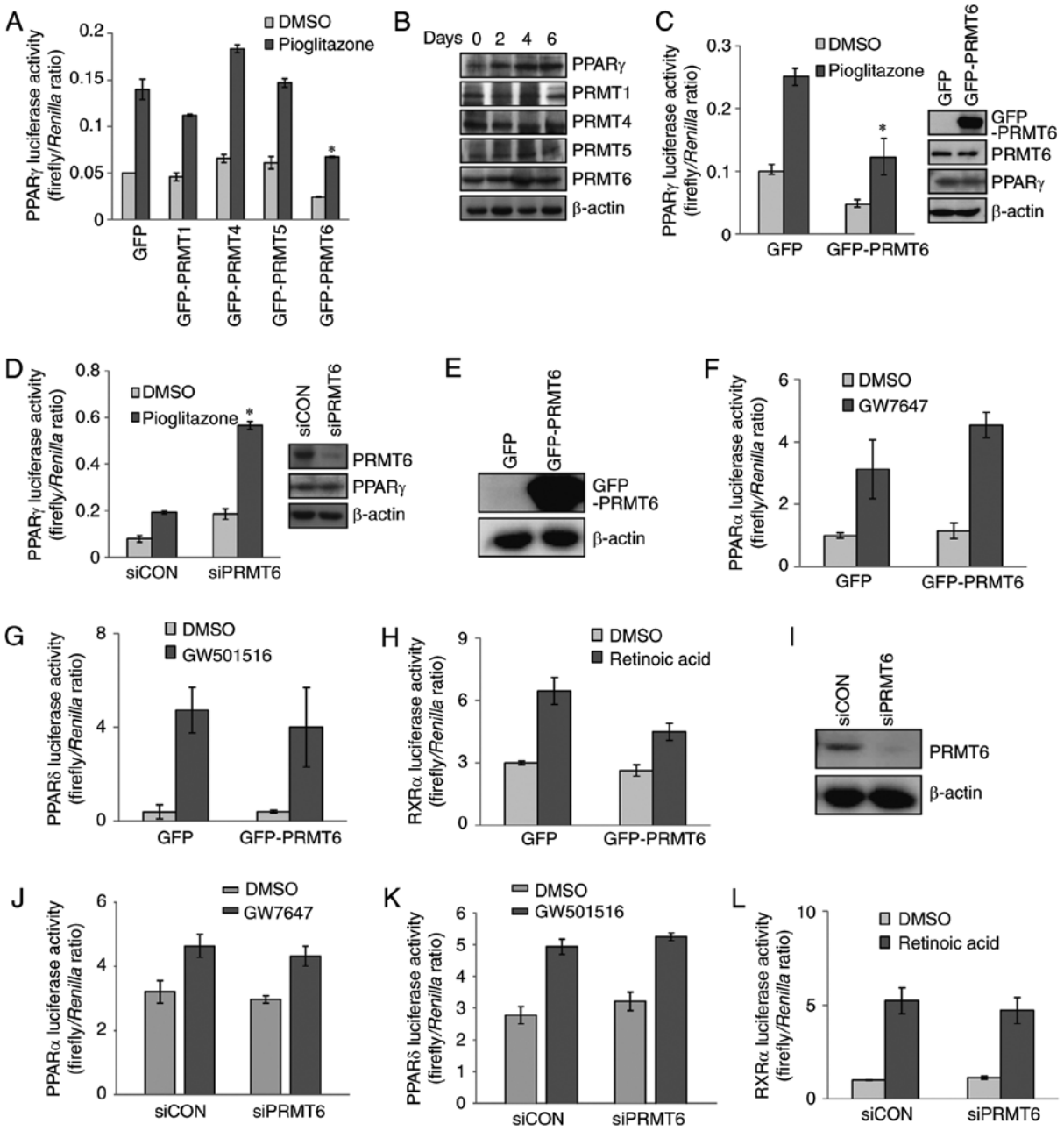

Figure 1. PRMT6 suppresses PPARγ transactivity. (A) CV1 cells were transiently co-transfected with PPARγ, PPRE-firefly luciferase and SV40-*Renilla* luciferase constructs combined with GFP-PRMTs plasmids. After 24 h, cells were treated with 2 μM pioglitazone for an additional 24 h. Firefly/*Renilla* luciferase activities were measured by a Dual luciferase assay kit. (B) The expression levels of PRMTs during adipogenic differentiation of 3T3-L1 cells were measured by western blot analysis. (C and D) PC3 human prostate cancer cells were transiently transfected with PPRE-firefly luciferase and SV40-*Renilla* luciferase constructs combined with (C) GFP-PRMT6 plasmids or (D) siPRMT6 duplex RNA. PRMT6 and PPARγ protein levels were confirmed by western blot analysis. (E-H) Luciferase reporter gene assay following transfection with GFP-PRMT6 plasmids. (E) GFP-PRMT6 level in cells transfected with GFP-PRMT6 plasmids was determined by western blot analysis. (F-H) The cells were then treated with 1 μM (F) GW7647, (G) GW501516 or (H) retinoic acid for 24 h, to examine the levels of PPARα, PPARβ/δ and RXRα activities, respectively, in GFP-PRMT6-overexpressing CV1 cells. (I-L) CV1 cells were transfected with PRMT6-targeting duplex siRNA. (J-L) The cells were then treated with 1 μM (J) GW7647, (K) GW501516 or (L) retinoic acid for 24 h, to examine the levels of PPARα, PPARβ/δ and RXRα activities, respectively. Error bars represent standard deviation (n=3; *P<0.05). PRMT6, protein arginine methyltransferase 6; PPARγ, peroxisome proliferator-activated receptor γ; GFP, green fluorescent protein; PPRE, PPAR-responsive regulatory element; RXRα, retinoid X receptor α; si, small interfering; siCON, scrambled control.

present study examined whether PRMT6 regulated adipocyte differentiation. Differentiated adipocytes were obtained following the transfection of 3T3-L1 cells with GFP-PRMT6 plasmid or siPRMT6 duplex RNA. In concordance with the previous results of PPARγ transactivation (Fig. 1), PRMT6 overexpression decreased intracellular lipid accumulation as compared with the control (data not shown), and the knockdown of PRMT6 expression increased the size and number of intracellular lipid droplets (Fig. 2A). The mRNA levels of C/EBPα and aP2, adipogenic marker genes, were significantly increased in PRMT6-depleted cells (Fig. 2B and C). MS023 is a potent selective inhibitor of type I PRMTs, including PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8, and has high specificity for PRMT6 (33). The present study

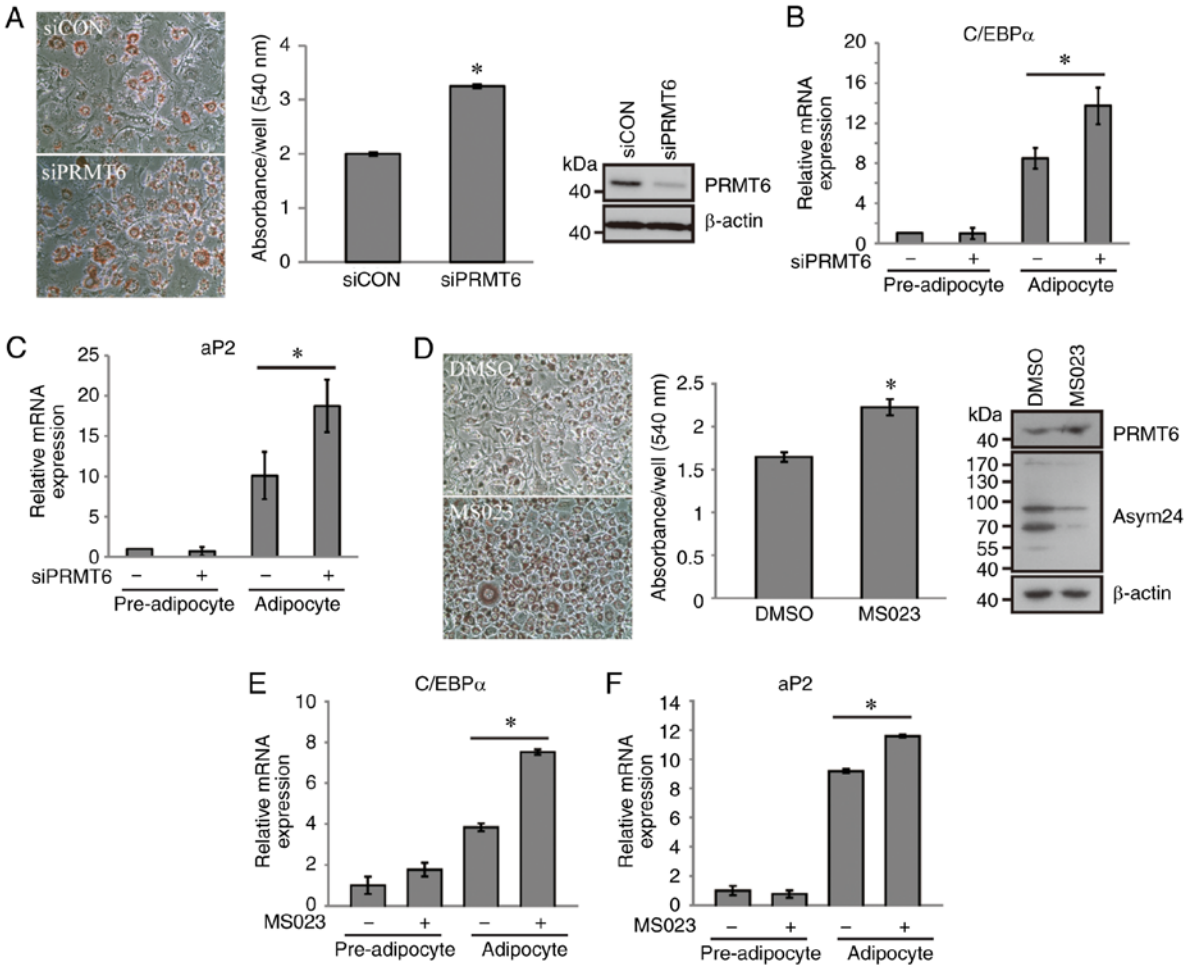

Figure 2. PRMT6 inhibits the adipogenic differentiation of 3T3-L1. (A and B) Prior to differentiation, 3T3-L1 cells were transfected with scrambled or PRMT6-targeting siRNA and then differentiated using a hormonal cocktail for 8 days. (A) The intracellular lipids were stained using Oil Red O (original magnification, x400). (B and C) Relative mRNA expression of (B) C/EBPα and (C) aP2 in 3T3-L1 cells was determined using quantitative polymerase chain reaction. (D) During differentiation, cells were treated with 5 μM MS023 and stained using Oil Red O. (E and F) Following treatment with MS023, relative mRNA expression of (E) C/EBPα and (F) aP2 in 3T3-L1 cells was determined using quantitative polymerase chain reaction Error bars represent standard deviation (n=3; *P<0.05). si, small interfering; siCON, scrambled control; PRMT6, protein arginine methyltransferase 6; C/EBPα, CCAAT-enhancer-binding protein α; aP2, adipocyte Protein 2; DMSO, dimethyl sulfoxide.

identified that the treatment of cells with MS023 resulted in 3T3-L1 adipocyte differentiation (Fig. 2D-F), indicating that PRMT6 controlled adipogenic differentiation processes via PPARγ regulation.

PRMT6 interacts with, but does not methylate, PPARγ. To elucidate the mechanism underlying PRMT6-mediated PPARγ regulation, the physical interaction between PRMT6 and PPARγ was first examined. Ectopically-overexpressed PPARγ protein was co-immunoprecipitated with PRMT6 in 293T cells from visual observation (Fig. 3A). To confirm this result, endogenous PPARγ and PRMT6 were co-immunoprecipitated in differentiated adipocytes. In mature adipocytes, the protein PPARγ exhibited marked interaction with PRMT6, as evident in the reciprocal co-IP experiments (Fig. 3B and C; lane 2). The treatment with pioglitazone, a PPARγ agonist, significantly decreased the interaction between PPARγ and PRMT6 (Fig. 3B; lane 3). In addition, the inhibition of PRMT6 with MS023 treatment led to the disruption of this complex (Fig. 3C; lane 3). These results suggested that the complete activation of PPARγ required the dissociation with, or inhibition of, the negative regulator PRMT6.

Based on these results, we hypothesized that PPARγ served as a substrate for PRMT6. However, no asymmetric di-methyl arginine was detected in the immunoprecipitated PPARγ protein (Fig. 3C). To additionally confirm this result, *in vitro* methylation assays were performed using purified PRMT6 and recombinant PPARγ protein. No ^{3}[H]-radioactive methylation signals were observed following treatment with recombinant PPARγ (Fig. 3D; lane 4), while recombinant histones, used as positive controls, were highly methylated by PRMT6 (Fig. 3D; lane 2). These results suggested that PPARγ protein was not a substrate for PRMT6.

PRMT6 represses PPARγ target gene expression by generating repressive mark H3R2me2a. As PRMT6 serves the role of an epigenetic regulator through the methylation of the histone H3R2me2a (28,29), the present study examined whether PRMT6 was recruited to the promoter region of PPARγ target genes in 3T3-L1 cells. Using a ChIP assay, it was verified that PPARγ and PRMT6 were recruited to the PPRE, but not the

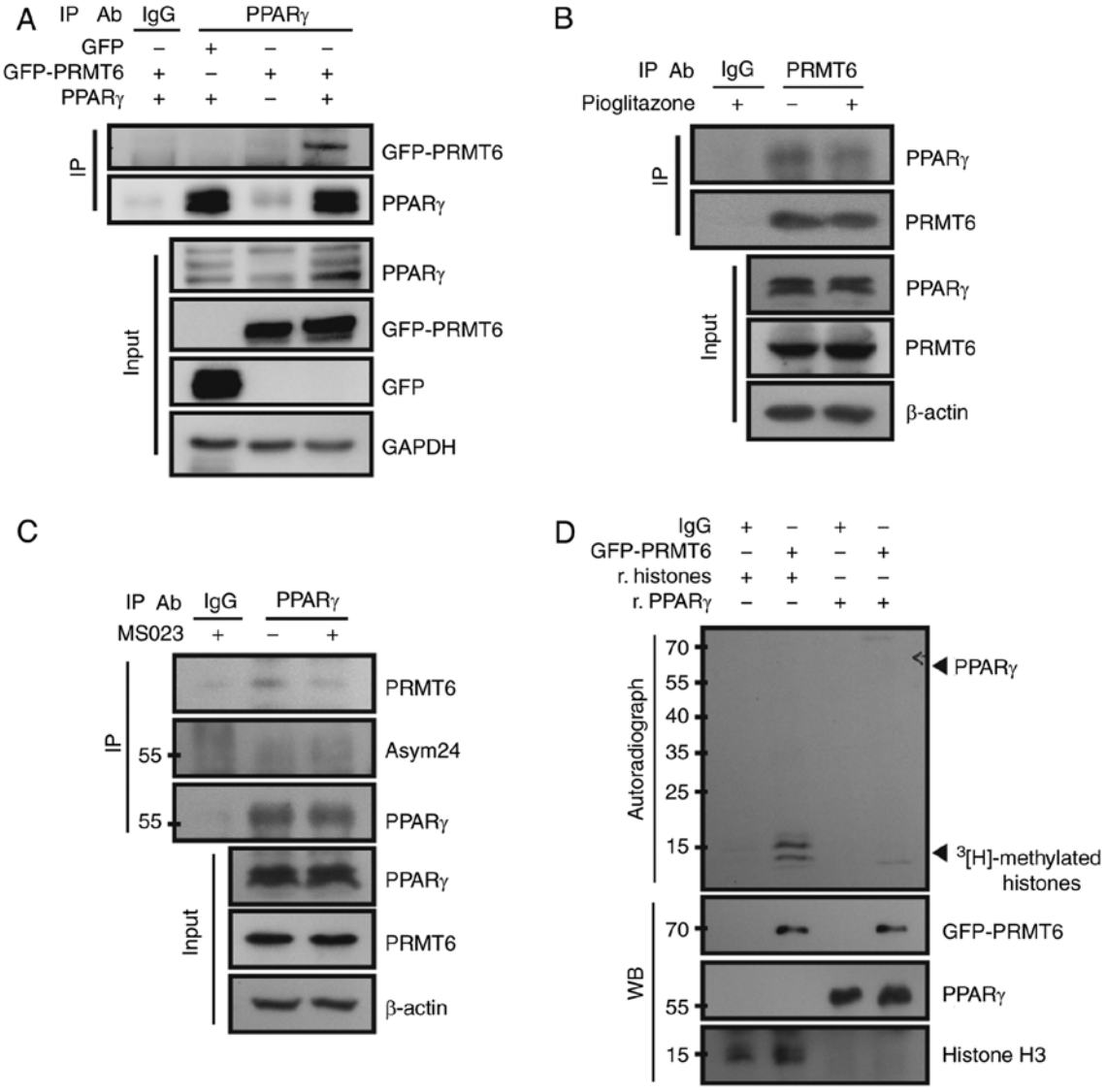

Figure 3. PRMT6 binds to, but does not methylate, PPARγ. (A) 293T cells were co-transfected with GFP-PRMT6 and PPARγ for 24 h and then immunoprecipitated using the anti-PPARγ antibody. (B) 3T3-L1 cells were differentiated with 2 μM pioglitazone for 8 days, and then endogenous PR<T6 was immunoprecipitated. (C) 3T3-L1 cells were differentiated with 5 μM MS023 for 8 days, and then endogenous PPARγ was immunoprecipitated. (D) *In vitro* PRMT6 methylation assay using recombinant PPARγ protein. PRMT6, protein arginine methyltransferase 6; PPARγ, peroxisome proliferator-activated receptor γ; GFP, green fluorescent protein; IP, immunoprecipitation; IgG, immunoglobulin G; r, recombinant; WB, western blotting; Asym24, asymmetric di-methyl arginine.

non-PPRE region, of the aP2 gene upon complete differentiation of the cells (Fig. 4A). In addition, the H3R2me2a level was increased in the PPRE region in response to PRMT6 recruitment, which was suggestive of the mechanism that inhibits the PRMT6-mediated transcriptional activity of PPARγ. It was confirmed that treatment with MS023 markedly decreased the level of H3R2me2a in the PPRE region (Fig. 4B), presumably leading to an increase in adipogenesis (Fig. 2D-F). Taken together, these data demonstrated that PRMT6 served as a co-repressor that generated the H3R2me2a repressive mark in the PPRE region, resulting in the suppression of PPARγ functions during adipogenic differentiation (Fig. 4C).

Discussion

PRMT6 is one of the type I PRMTs present in the nucleus, and the diverse physiological functions of PRMT6 are suggestive of its importance (23,34). Epigenetically, it serves the role of a transcriptional repressor by methylating the R2 residue of histone H3 within chromatin (28,29). However, to the best of our knowledge, the role of PRMT6 in the regulation of PPARγ, one of the nuclear receptors, has not been identified previously. The present study demonstrated that PRMT6 negatively regulated PPARγ transactivity without affecting the activity of other isoforms (PPARα and PPARβ/δ) and RXRα. Using a 3T3-L1 adipocyte cell differentiation model, the inhibitory role of PRMT6 in adipogenesis and differentiation that was predominantly controlled by PPARγ was confirmed. The siRNA-mediated depletion of PRMT6 or the inhibition of PRMT6 by MS023 promoted adipogenic differentiation, indicating that the enzymatic activity of PRMT6 is essential for the regulation of PPARγ function.

In the absence of the ligand, PPARγ is associated with several corepressor molecules, including nuclear receptor

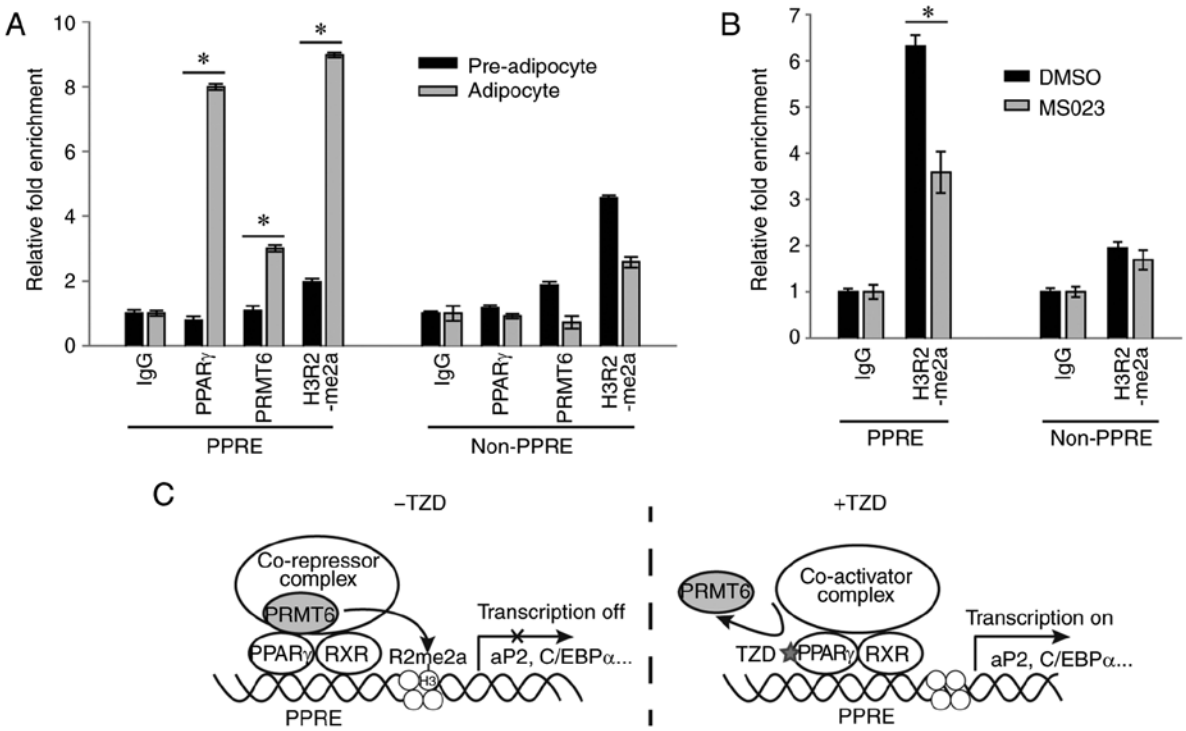

Figure 4. PRMT6 serves as a co-repressor of PPARγ by generating the repressive mark H3R2me2a. (A) ChIP analysis for PPARγ, PRMT6, or H3R2me2a in PPRE or adjacent non-PPRE of aP2 gene. Pre-adipocyte and differentiated (6 days) 3T3-L1 cells were fixed and subjected to ChIP assay. (B) During differentiation, 3T3-L1 cells were treated with MS023 (5 μM) for 6 days, and the presence of H3R2me2a at the aP2 promoter site was measured by ChIP assay. Error bars represent standard deviation (n=3; *P<0.05). (C) A graphic model for the regulation of PPARγ-mediated adipogenic gene expression by PRMT6. PRMT6 binds to PPARγ in the absence of a ligand, which generates an H3R2me2a repressive mark. When ligands, including TZD, bind to PPARγ, the interaction between PRMT6 and PPARγ is weakened and the repressive mark is decreased, resulting in the activation of adipogenic gene transcription. PRMT6, protein arginine methyltransferase 6; PPARγ, peroxisome proliferator-activated receptor γ; ChIP, chromatin immunoprecipitation assay; H3R2me2a, arginine 2 on histone H3 asymmetric di-methylation; PPRE, PPAR-responsive regulatory element; aP2, adipocyte Protein 2; C/EBPα, CCAAT-enhancer-binding protein α; TZD, thiazolidinediones.

corepressor, silencing mediator of retinoid and thyroid hormone receptor, paired amphipathic helix protein Sin3 and receptor-interacting protein 140 (5,19,20,35). Subsequent to binding with the ligand, PPARγ undergoes conformational change, resulting in a decrease in its binding affinity to the co-repressors. In the subsequent steps, a series of co-activators combine together to form an active complex (19,21). The present study demonstrated that PPARγ stably associated with PRMT6 and that this binding was decreased in the presence of the PPARγ agonist pioglitazone, suggesting that PRMT6 may serve as a typical co-repressor to regulate PPARγ functions. This interaction was also disrupted upon PRMT6 inhibition, indicative of the importance of PRMT6 activity for the binding between these two proteins.

Several previous studies have supported the regulation of PPARγ mediated by various post-translational modifications, including phosphorylation, SUMOylation, ubiquitination and acetylation (36,37). The present study failed to detect any evidence of the direct arginine methylation of PPARγ by PRMT6. Instead, it was confirmed that PRMT6 performed H3R2me2a methylation in the promoter region of PPARγ target genes during the adipocyte differentiation process. Taken together, the data from the present study suggested that there are two methods through which PRMT6 represses PPARγ: i) PRMT6 directly interacts PPARγ; and ii) PRMT6 generates the H3R2me2a repressive mark. Additional studies are warranted to evaluate the formation of PPARγ-PRMT6-H3R2me2a and other co-repressor complexes. At present, 2 previous studies have suggested that PRMT(s) may be involved in the regulation of PPARγ and adipogenesis. Brunmeir and Xu (37) suggested that coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4 served as a coactivator of PPARγ and promoted adipocyte differentiation. Quinn *et al* (38) demonstrated that PRMT5 promoted the expression of PPARγ target genes by binding to, and subsequently methylating, chromatin histones at adipogenic promoters. Therefore, PRMT6 as a co-repressor and CARM1/PRMT4 and PRMT5 as co-activators of PPARγ may suggest that a series of arginine methylations by PRMTs optimize the activation process of PPARγ.

The activation of PPARγ by TZDs results in the improvement in insulin sensitivity through the promotion of fatty acid uptake into the adipose tissue, leading to an increase in the production of adiponectin and decrease in the levels of inflammatory mediators including tumor necrosis factor-α, plasminogen activator inhibitor-1 and interleukin-6 (38,39). However, chronic activation PPARγ by TZD causes severe side-effects, including weight gain, fluid retention and osteoporosis, thereby increasing the risk of congestive heart failure, myocardial infarction, cardiovascular diseases and all-cause mortality in patients (40,41). The present study demonstrated that the functional effects of PPARγ were enhanced by the PRMT6 selective inhibitor MS023, suggesting that PRMT6 inhibitors may serve potential roles to synergize the effects of TZD in PPARγ-associated metabolic diseases.

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Availability of data and materials

All data used and/or analyzed during the present study are available from the corresponding author on reasonable request. All materials used are included in Materials and methods.

Authors' contributions

JWH, YSS, and YKK conceived and designed the experiments. JWH and YSS performed experiments. GUB, and SNK provided experimental assistance and conceptual advice. JWH and YKK supervised the experiments and wrote the manuscript. All authors read and commented on the manuscript and agreed to the publication of the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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