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Title: Inhibition of protein arginine methyltransferase 5 enhances hepatic mitochondrial biogenesis

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Running title: *PRMT5 inhibition enhances mitochondrial biogenesis*

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Abstract

        Protein arginine methyltransferase 5 (PRMT5) regulates gene expression either transcriptionallyly by symmetric dimethylation of arginine residues on histones H4R3, H3R8 and H2AR3, or at the post-translational level by methylation of non-histone target proteins. While emerging evidence suggests that PRMT5 functions as an oncogene, its role in metabolic diseases is not well defined. We investigated the role of PRMT5 in promoting high fat-induced hepatic steatosis. High fat diet up-regulated PRMT5 levels in the liver, but not in other metabolically relevant tissues such as skeletal muscle or white and brown adipose tissue. This was associated with repression of master transcription regulators involved in mitochondrial biogenesis. In contrast, lentiviral shRNA-mediated reduction of PRMT5 significantly decreased PI3K/AKT signaling in mouse AML12 liver cells. PRMT5 knockdown or knockout decreased basal AKT phosphorylation, but boosted the expression of PPARα and PGC-1α with a concomitant increase of mitochondrial biogenesis. Moreover, by overexpressing an exogenous wild-type or enzyme-dead mutant PRMT5, or by inhibiting PRMT5 enzymatic activity with a small molecule inhibitor, we demonstrated that the enzymatic activity of PRMT5 is required for regulation of PPARα and PGC-1α expression and mitochondrial biogenesis. Our results suggest that targeting PRMT5 may have therapeutic potential for treatment of fatty liver.

Introduction

        The phenotypic spectrum of non-alcoholic fatty liver disease (NAFLD) is broad and may range from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis. NAFLD is commonly associated with Type 2 diabetes, visceral obesity and hyperlipidemia (1). There is evidence that increased delivery of non-esterified fatty acid (NEFA) to the liver [mainly from white adipose tissue (WAT)] is fundamental to the development of NAFLD (2,3). The role of diet in the pathogenesis of NAFLD has been investigated both in humans and in animal models (4-6). Subjects on a high-fat diet develop fatty liver (5,6); those on a low-fat/high-carbohydrate diet develop fatty liver via increased *de novo* fatty acid synthesis (4). In addition, high dietary fat intake is associated with obesity and insulin resistance. As a consequence of insulin resistance, suppression of lipolysis by insulin is impaired, leading to

increased NEFA delivery to the liver (7,8). Thus high dietary fatty acids have profound effects on insulin resistance, NAFLD and cardiovascular disease (9).

Phosphatidylinositol-3 kinase (PI3K), a lipid kinase, generates the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) (10). The accumulation of PIP3 recruits protein kinase B (AKT) to the cell membrane where it is phosphorylated and activated by phosphoinositide dependent kinase (PDK) 1 and 2 (11,12). Downstream of this PI3K/AKT pathway is the mammalian target of rapamycin (mTOR), a major effector that is specifically implicated in the regulation of cell growth via nutrient availability and cellular bioenergetics (13). Activation of the PI3K-AKT-mTOR pathway is accompanied by aberrant lipid metabolism in cancers (14), where it increases protein levels of sterol regulatory element-binding proteins (SREBP), and its target, fatty acid synthase (FASN) (15,16). Inhibition of the mTOR pathway is associated with impaired induction of transcription factors, i.e. peroxisome proliferator activated receptor-α (PPARα) and its target genes, such as carnitine palmitoyltransferase 1 (CPT1). Under conditions favoring re-esterification over β-oxidation in the liver, newly synthesized triglycerides are carried on very low density lipoproteins (VLDL) to be redistributed to WAT for storage, or to skeletal muscle for utilization (under exercise conditions). Inhibition of PI3K/AKT can abolish insulin-induced suppression of VLDL assembly and secretion by regulating the degradation of Apolipoprotein B (ApoB) and the gene expression of hepatic microsomal triglyceride transfer protein (MTTP) (17). Thus, the PI3K/AKT/mTOR signaling pathway plays an integral role in regulating several pathways contributing to lipid homeostasis in the liver, including *de novo* lipid biosynthesis, fatty acid β-oxidation (18,19), and VLDL assembly and secretion.

The protein arginine methyltransferase family consists of three subfamilies that differ in their ability to carry out mono- or asymmetric di-methylation (type I), mono- or symmetric di-methylation (type II), or exclusively monomethylation (type III) (20,21). PRMT5 functions as the major type II arginine

methyltransferase that symmetrically di-methylates arginine residues on histones as well as non-histone proteins including RNA splicing factors and cell cycle regulators (22,23). The PRMT5 mediated methylation of histones H2AR3 (24), H4R3 (25), and H3R8 (26) regulates chromatin structure and influences transcription. We have shown that PRMT5 is required for activation of adipogenic gene expression by promoting adipogenic master transcriptional factor PPARγ2 expression during pre-adipocyte differentiation (27,28). PRMT5 has been shown to methylate SREBP1a on R321 and increases SREBP1a transcriptional activity, thereby promoting *de novo* lipogenesis and tumor growth(29). Overexpressing exogenous GFP-PRMT5 resulted in elevated levels of phospho-AKT in 293T cells(30). However, little is known about the role of endogenous PRMT5 in regulating lipid homeostasis in the context of NAFLD.

In this study, we investigated the function of PRMT5 in a diet-induced obesity mouse model. We found that high-fat diet increased PRMT5 expression in the liver but not in other metabolically relevant tissues. Hepatic PRMT5 expression, however, was not altered in the liver of the obese Agouti mouse, suggesting that high-fat diet consumption, but not obesity per se, induced PRMT5 expression. Global signaling pathway analysis showed that knockdown of PRMT5 attenuated PI3K/AKT signaling. In the non-transformed mouse liver cell line model alpha mouse liver 12 (AML12), reducing PRMT5 levels by shRNA or inhibiting its enzymatic activity with a small molecule inhibitor led to an increase in PPARα and PGC-1α levels and mitochondrial biogenesis. Our results suggest that inhibiting PRMT5 favors β-oxidation which would contribute to the reversal of hepatic steatosis.

Results
PRMT5 is ubiquitously expressed in mice

While PRMT5 is ubiquitously expressed at varying levels in all mouse tissues examined, its expression is most abundant in the spleen and kidney and it is expressed in tissues important for metabolic control such as liver, muscle, brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) (Figure 1A). Similarly,

PRMT5 was detected in the cytoplasm as well as in the nucleus of the non-transformed AML12 murine hepatocyte cell line (Figure 1B).

High-fat diet increases hepatic PRMT5 expression

PRMT5 mRNA and protein levels were significantly increased in the livers of mice fed a high-fat diet (HFD) compared to those fed a normal diet (RD), as assessed by Western blot analysis (Figure 2A), quantitative PCR (Figure 2B) and immunofluorescent staining of liver sections (Figure 2C). In contrast, high-fat diet did not modify PRMT5 expression in other metabolic relevant tissues such as muscle, brown and inguinal white adipose tissue (Figure 2D). To further elucidate the role of PRMT5 in hepatic steatosis, we asked if PRMT5 up-regulation in hepatocytes was associated with obesity by examining PRMT5 levels in the liver of Agouti mice. Yellow Agouti mice with dominant mutations at the Agouti locus develop obesity, hyperinsulinemia, insulin resistance, hyperglycemia, hyperleptinemia and hepatic steatosis in adulthood (31-33). Young Agouti mice are lean, but adult animals are obese even when they are fed on regular chow (32). Therefore, the obesity in adult Agouti mice is independent of high fat diet. Unlike the diet induced obesity model, PRMT5 protein levels were not different in the liver of obese 5-month versus normal weight 1-month old Agouti mice (Figure 2E). This suggested that the increase of PRMT5 was not associated with obesity per se but with high-fat diet-induced obesity.

PRMT5 gene silencing decreases PI3K/AKT signaling

We sought to dissect the molecular mechanism underlying the regulation of hepatic lipid homeostasis by PRMT5. Because PRMT5 is overexpressed in many cancer cell lines (29,34-36), we generated PRMT5 knockdown in the non-transformed mouse liver cell line AML12 to avoid confounding metabolic effects of cancer-related factors in transformed cell lines. As shown in Figure 3A, reduction of PRMT5 expression was achieved by shRNA mediated gene silencing. A global signaling network analysis measuring 70 key proteins involved in more than 20 signaling pathways

showed that PRMT5 knockdown decreased the PI3K/AKT pathway (Figure 3B). The reduction of phospho-AKT Ser473 was detected in the array (Supplemental Figure 1), which was confirmed by Western blot analysis that also showed decreased Thr308 phosphorylation on AKT in PRMT5 knockdown cells (Figure 3C). To confirm this finding, we utilized the inducible PRMT5 knockout mouse embryonic fibroblast (MEF) cell line which harbors an ER-Cre directed PRMT5$^{flox/flox}$ allele (37). When treated with 4-hydroxytamoxifen (4-OHT), the Cre recombinase translocates to the nucleus and excises an exon of PRMT5 to disrupt its expression. The PRMT5$^{flox/flox}$ MEF cells were cultured in the presence of 2 μM 4-OHT for 10 days followed by DMEM for 3 days without 4-OHT. The efficiency of PRMT5 knockout was confirmed by Western blot analysis (Figure 3D). In this PRMT5 knockout model, complete loss of PRMT5 led to a decrease in phospho-AKT Thr308 and Ser473. In contrast, up-regulation of phospho-AKT Ser473 and Thr308 were detected in the liver of mice fed a high-fat diet (Figure 3E).

PRMT5 regulates the expression of master transcription factors of mitochondrial biogenesis

Peroxisome proliferator-activated receptor alpha (PPARα) has a central role in fatty acid β-oxidation, lipid and lipoprotein metabolism, inflammatory response, and oxidative stress in the liver. Activation of PPARα induces the expression of mitochondrial genes in the oxidation pathways (38). Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) is a transcriptional co-activator that regulates the expression of genes involved in energy metabolism. It is the master regulator of mitochondrial biogenesis (39-41). We surveyed the expression of these transcription factors in PRMT5 knockdown cells. Silencing PRMT5 significantly increased the expression of PPARα and PGC-1α (Figure 4 A and B). These results were confirmed in PRMT5 knockout MEF cells (Figure 4C). Consistent with increased expression of PPARα and PGC-1α, the expression of mitochondrial biogenesis genes such as CytoC and Cox7a-1 was elevated in

PRMT5 knockdown cells (Figure 4D). These results were replicated in PRMT5 knockout MEF cells where the expression of these genes were up-regulated (Figure 4E) and mitochondrial staining was significantly increased (Figure 4F). Activation of PI3K/AKT signaling has been shown to induce the expression of SREBP1(42,43). Liu *et al* showed that in the liver cancer cell line HepG2, silencing PRMT5 decreased SREBP1a translation, but not transcription (29). We examined in the PRMT5 knockdown cells the expression of acetyl-coA carboxylase (ACC) and fatty acid synthase (FASN), the direct downstream targets of SREBP1 in *de novo* fatty acid synthesis. The levels of these proteins were not affected upon silencing PRMT5 (Supplemental Figure 2), which suggested that the *de novo* fatty acid synthesis is not perturbed by PRMT5 silencing. It has been shown that insulin regulates lipid deposition in goose liver by activating PI3K/AKT/mTOR signaling which suppresses VLDL assembly and secretion (44). VLDL assembly involves transfer of lipid by the microsomal triglyceride transfer protein (MTP) to ApoB and fusion of ApoB-containing precursor particles with triglyceride droplets to form mature VLDL (45). Although PRMT5 knockdown in mouse liver cells reduced PI3K/AKT signaling, it did not affect phospho-mTOR, or ApoB and MTP gene expression (Supplemental Figure 2). In contrast, in PRMT5 knockout MEF cells, the loss of PRMT5 significantly decreased the levels of phospho-AKT, but also had no effect on the expression of phospho-mTOR (Supplemental Figure 3).

PRMT5 enzymatic activity is required for the regulation of PGC-1α and PPARα expression
Next, we asked if the enzymatic activity of PRMT5 is required for its regulation of PGC-1α and PPARα expression. To address this question, a wild type PRMT5 or a catalytically-dead PRMT5 (G367A, R368A) double mutant (46) was introduced to AML12 cells by transient transfection (Figure 5A). As opposed to the enzyme-dead mutant, the expression of exogenous wild-type PRMT5 elevated phospho-AKT Thr308 and Ser473 and markedly reduced PPARα (Figure 5B). EPZ015666 (GSK3235025) is a potent and selective small molecule inhibitor

targeting the enzymatic activity of PRMT5 with a half-maximal inhibitory concentration (IC50) of 22 nM in biochemical assays (47). Although EPZ015666 decreases cell proliferation in various cancer lines (48,49), it did not inhibit proliferation in AML12 cells even in the presence of high dose EPZ015666 for 8 weeks (Supplemental Figure 4). On the contrary, EPZ015666 treatment markedly decreased phospho-AKT Thr308 and Ser473, but increased PPARα in a dose-dependent manner (Figure 5C). The expression of PPARα and PGC-1α, the master regulators of mitochondrial biogenesis, was elevated in the presence of EPZ015666 in a dose-dependent manner (Figure 5D) with concomitant increased density in mitochondrial staining (Figure 5E). Inhibition of PRMT5 enzymatic activity did not change the phosphor-mTOR levels in AML12 cells (Supplemental Figure 5). As a biological consequence of increasing mitochondrial biogenesis and promoting fatty acid β-oxidation, lipid accumulation in cells treated with PRMT5 inhibitor was significantly reduced (Figure 5F). Collectively, these results suggest that the enzymatic activity of PRMT5 is required to execute its function in metabolic pathways.

PRMT5 regulates PGC-1α and PPARα through phospho-AKT signaling
We sought to dissect whether PRMT5 directly regulates the expression of PPARα and PGC-1α or indirectly mediates this through phospho-AKT signaling. By analyzing the published PRMT5 ChIP-seq data (GEO: GSE75739)(50), we failed to identify significant PRMT5 binding above the IgG control at these gene loci (Supplemental Figure 6). Introducing an exogenous activated AKT (Myr-AKT) to PRMT5 wild type or knockout cells attenuated the increased expression of PPARα and PGC-1α due to the loss of PRMT5 (Figure 6A). This observation was confirmed in AML 12 hepatocytes, where the up-regulation of PPARα and PGC-1α in PRMT5 knockdown cells was blocked by the overexpression of Myr-AKT (Figure 6B). These results sugguested that the regulation of PPARα and PGC-1α by PRMT5 was mediated through phospho-AKT signaling, although we could not completely rule out the

possibility that PRMT5 might directly regulate these genes.

Discussion

We showed in this study that high fat diet induced obese mice have increased levels of PRMT5 in the liver but not in other metabolically active organs such as adipose tissues and muscle. We found that this elevated hepatic PRMT5 is associated with high fat diet rather than obesity *per se*. It has been shown that HFD dramatically changed chromatin structure, transcription factor binding and the gene expression landscape in mouse liver. Many genes associated with lipid, steroid, cholesterol and amino acid metabolism, gluconeogenesis, inflammatory response, oxidative stress and oxidoreductase activity were altered. It is not clear whether the up-regulation of PRMT5 is one of the early events which further shapes the chromatin environment and impacts gene expression under HFD or if it is regulated by other key transcription factors involved in this process.

PI3K/AKT signaling plays a critical role in regulating diverse cellular functions including metabolism, growth, proliferation, cell survival, transcriptional activity and protein synthesis. Dysregulation of the PI3K/AKT pathway is implicated in cancer, type 2 diabetes, insulin resistance, inflammatory and autoimmune disorders, cardiovascular and neurological diseases (12). The comprehensive understanding of AKT signaling network has been expanded with the identification of numerous downstream pathways (54-56). In contrast, our knowledge of the upstream regulator or activtor of AKT is limited. Wei *et al* reported that overexpressing GFP-PRMT5 led to an increase in phospho-AKT levels in 293T cells (30). However, this observation was not further validated with the endogenous protein (30). In this study, we identified endogenous PRMT5, an epigenetic modifier, as the upstream regulator of AKT activation in the context of high fat diet-induced fatty liver. Wei and co-workers also showed that overexpressing GFP-PRMT5 increased mTOR S2448 phosphorylation, which might be the mechanism by which PRMT5 regulates phospho-AKT (30). We did not detect any change in phospho-mTOR S2448 levels in

PRMT5 knockout MEF cells nor in AML12 cells with PRM5 knockdown or treated with EPZ015666 (Supplemental Figure 2, 3 and 5). In the present study, PRMT5 regulation of phospho-AKT appears to be independent of mTOR signaling. AKT activation involves the phosphorylation of two residues, Thr308 in the activation loop and Ser473 in the C-terminal hydrophobic motif. Phosphorylation of Ser473 has been extensively studied in cancers as a marker for full AKT activity (57-59). Other studies also showed that Thr308 is predominantly phosphorylated in activated AKT kinase in tumor cells (60-62). In our study, knockdown of PRMT5 or treatment with PRMT5 enzymatic inhibitor EPZ015666 in hepatocytes attenuated both phospho-AKT Thr308 and Ser473. Our results indicate that targeting PRMT5 blocks the activation of AKT, which may have a profound effect on its downstream signaling networks.

Mitochondrial dysfunction is associated with many diseases, such as type 2 diabetes and neurodegenerative diseases (63,64). PGC-1α is a master regulator of mitochondrial biogenesis by activating transcription factors nuclear respiratory factor 1 and nuclear respiratory factor 2. These induce mitochondrial transcription factor A which drives the transcription and replication of mitochondrial DNA (64,65). PGC-1α expression levels are regulated in response to a plethora of stimuli, such as exercise, feeding, fasting, and cold exposure and modulated by numerous factors, such as Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) and calcineurin A, AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (p38 MAPK) (66,67). We herein report that PRMT5 repressed the expression of PGC-1α in hepatocytes. Silencing or complete loss of PRMT5 increased the expression of PGC-1α and mitochondrial biogenesis. AKT was found to phosphorylate and inhibit PGC-1α (68). PRMT5 ChIP-seq in MDA-MB-231 cells did not identify PRMT5 binding sites at the gene loci of PPARα and PGC-1α (50). With no PRMT5 ChIP-seq data from hepatocytes, we cannot completely dismiss the possibility that PRMT5 might have a direct role in regulating these genes. Introducing activated Myr-AKT to PRMT5 knockout MEF

or knockdown AML12 cells decreased the up-regulation PPAR α and PGC-1α. These results suggest that it is more likely that PRMT5 regulates the expression of PPAR α and PGC-1α via AKT signaling.

The overexpression of PRMT5 in various cancers has been linked to cancer progression and poor prognosis (34,69). Therefore, PRMT5 is well recognized as a viable therapeutic target in cancer treatment and a number of inhibitors have been developed within the past couple of years to target cancer, β-thalassemia, or sickle cell anemia. EPZ015666 is a potent PRMT5 inhibitor that has been shown to be active *in vivo* (47). A first time in human, open-label, dose escalation study of GSK3326595, a potent and selective PRMT5 inhibitor similar to EPZ015666, in subjects with advanced or recurrent solid tumors, as well as subjects with a subset of solid tumors and non-Hodgkin's lymphoma was approved by the FDA and started in August 2016 (ClinicalTrials.gov Identifier: NCT02783300). We now report that inhibiting PRMT5 by EPZ015666 markedly decreased both phospho-AKT Thr308 and phospho-AKT Ser473, up-regulated PPARα and PGC-1α expression and significantly increased mitochondrial biogenesis. Excessive intake of dietary lipids was shown to decrease hepatic PPARα and PGC-1α expression with a reduced number in the numerical density of hepatic mitochondria (70). A number of PPARα agonists have been shown to enhance hepatic mitochondrial biogenesis and increase fatty acid β-oxidation in diet-induced obese mice (71,72). As an upstream regulator of AKT, PPARα and PGC-1α, PRMT5 might be a valuable target to treat fatty liver via multiple mechanisms, or even more broadly applied to other diseases that are associated with mitochondrial dysfunction. Chen *et al* reported that PRMT5 methylates GATA 4 and inhibits its transcriptional activity in cardiomyocytes which is important in prevention of cardiac hypertrophy and heart failure (73). Thus the balance of PRMT5 levels in the context of different diseases needs to be considered in developing PRMT5 inhibition based therapies.

Experimental procedures

Animals

C57BL/6 (BL6) mice were housed in a temperature-controlled environment with a 12-hour light, 12-hour dark cycle and fed a standard chow ad libitum. Starting at 8 weeks of age, male mice were fed *ad libitum* either a regular diet (RD; 12% fat content) or a high fat diet (HF; 45% fat content, catalog no. D12451; Research Diets, New Brunswick, NJ) for 3 months (74). Animals were then euthanized and tissue samples were collected to be either snap frozen in liquid nitrogen or embedded in OCT blocks. The Agouti mice were housed in a temperature-controlled environment with a 12-hour light, 12-hour dark cycle and fed a standard chow *ad libitum*. All procedures were approved by the Institutional Animal Care and Utilization Committee (IACUC) at the University of Toledo.

Cell culture

Nontransformed alpha mouse liver 12 (AML12) cell line was provided by Dr. Anthony Imbalzano (UMass Medical School). This cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha(75). AML12 cells retain the capacity to express high levels of serum and liver specific proteins(75). These cells were maintained in monolayers, as described (75). They were cultured in DMEM/F12 medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 μg/ml streptomysin, and ITS (Invitrogen). HEK293T cells were cultured in DMEM medium (Invitrogen) with 10% FBS and 100 μg/ml streptomysin. PRMT5$^{flox/flox}$ MEFs were immortalized with PRMT5$^{flox/flox}$ mouse embryo fibroblasts and obtained from Dr. Mark Bedford (at the The University of Texas MD Anderson Cancer Center).

Lentiviral shRNA construction and transduction

PRMT5 shRNA knockdown lentiviral constructs were prepared using psp-108 vector (Addgene), and the lentivirus cDNAs were produced by co-transfecting with plasmids pMD2.G (Addgene) and psPAX2 (Addgene) into HEK293T cells. Titers were determined and the same number of virus was used, as indicated.

PRMT5 knockdown targeting sequences are: shRNA15'GCACAGTTTGAGATGCCTTAT3'; shRNA2 5'CCTCTTGTGAATGCGTCTCTT3'. The Flag-PRMT5 and Flag-PRMT5 PRMT5 (G367A, R368A) double mutant were from Dr. Said Sif (at Qatar University) (46).

Transcient Transfection

Cells were plated in 6-well plates or 60 mm dishes to 60-70% confluency, then transfected with 6 or 15 μg plasmid DNA respectively using Lipofectamine 2000 transfection reagent following the manufacturer's protocol. Cells were harvested 48 hours post transfection for protein analysis by western blotting.

Immunofluorescence

Cells were seeded on glass cover slips and incubated overnight. For mitochondria staining, cells were incubated with Mitotracker™ Red CMXRos (250nM, ThermoFisher, cat#M7512) at 37°C for 30 min, before being fixed with 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization in ice-cold methanol for 10 min at −20°C. Cells were then incubated in blocking buffer (5% bovine serum albumin in PBS) for 1h at room temperature followed by incubation with anti-PRMT5 antibody (Santa Cruz Biotechnology, cat# sc-376937) and anti-β-tubulin antibody (Cell Signaling Technology, cat# 15115) at 4°C overnight. Cells were washed three times for 5 min in PBS, and then incubated for 2h with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody against PRMT5 and Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody for β-tubulin (diluted 1:100 in blocking buffer; Invitrogen) at room temperature. Finally, the nuclei were stained with DAPI for 30 min at room temperature before visualization.

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized and rehydrated through graded ethanol solutions. The slides were incubated with blocking buffer (PBS containing 5% normal goat serum and 0.3% Triton X-100) for 60 min and then the slides were incubated with PRMT5 antibody at 4°C overnight. Subsequently, the slides were washed with PBS, and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and DAPI for 2h before observation using Leica fluorescent microscope.

Real time quantitative RT-qPCR

Total RNA was extracted from AML12 cells or MEF cells using Trizol regent (Invitrogen) and cDNA was synthesized by SuperScript Reverse Transcriptase III kit (Invitrogen) according to the manufacturer's instruction. The cDNA was amplified in 96-well reaction plates with a SYBR green PCR Master Mix (Applied Biosystems) on an ABI 7500 real-time PCR thermocycler. The sequences of forward and reverse primers are listed in Supplementary Table 1. The relative level of target transcripts were calculated from duplicate samples after normalization against U36 housekeeping gene. Dissociation curve analysis was performed after PCR amplification to confirm primers' specificity. Relative mRNA expression was calculated using the $\Delta\Delta C_T$ method.

Western blot analysis

Tissue or cells were washed with cold PBS and lyzed in 1% NP40 lysis buffer containing a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Proteins were analyzed by SDS-PAGE followed by immunoprobing with the following antibodies: PPARα: sc-398394; PRMT5: sc-376937; ACCα: sc-137104; FASN: sc-48357; SREBP1: sc-365513; mTOR: sc-293089; p-mTOR: sc-293133. For normalization, monoclonal antibodies against β-tubulin (DSHB#E7-S) was used. Blots were incubated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology), anti-mouse or anti-rabbit IgG (GE Healthcare Life Science, Pittsburgh, PA, USA) antibodies, and proteins detected by enhanced chemiluminescence (ECL; GE Healthcare Life Science).

Fatty acid Treatments

Cells were pretreated with increasing doses of EPZ015666 for 6 days and seeded at density 10,000 cells/well in 24-well plates and cultured for 24 hours. Palmitic acid (PA) (Sigma-Aldrich, cat#P0500) and oleic acid (OA)

(Sigma-Aldrich, cat#O1008) were prepared by conjugation with non-fat bovine serum albumin (Sigma-Aldrich, cat#A8806). Cells at 50% confluency were exposure to 1 μM PA and 100 μM OA in the presence of EPZ015666 for 5 days. Lipid droplets were stained with Oil Red O.

Oil Red O staining

To assess intracellular neutral lipid, FA treated cells were fixed using 10% formalin and stained using Oil-Red-O (Sigma Aldrich, St. Louis, MO) for 30 minutes at room temperature. Stained cells were washed with water and rinsed with 60% isopropanol. Lipid accumulation were visualized using light microscopy.

MTT assay

Cell proliferation was measured using the MTT assay. Three thousand cells from each treated group were plated in a 96-well plate. Cell proliferation was measured at 24 hour intervals by adding MTT solution (final concentration 5 μg/mL) to each well and incubating for 4 hours. After removal of the media, the plate was air-dried and 100 μL DMSO added. The plate was incubated at room temperature for 30 min with gentle shaking. Absorbance was measured at OD540 in a Synergy H4 Hybrid microplate reader (Bio Tek, Winooski, VT).

ActivSignal IPAD assay

Cells were lysed in PBS + 1%NP40 lysis buffer, and submitted to ActivSignal for further processing (http://www.activsignal.com). The expression or protein phosphorylation of 70 key proteins involved in more than 20 signaling pathways was analyzed by antibodies with high specificity and sensitivity in combination of two distinct antibodies per each target. The signal was normalized to the expression of housekeeping genes. Each pathway is covered by multiple targets. The experiment was performed twice.

KEGG pathway enrichment analysis

Proteins with more than 1.5 fold change after PRMT5 knockdown in IPAD assay were fetched to perform pathway enrichment analysis using DAVID (76). Pathway annotations from KEGG pathway database with a p-value smaller than 0.05 were defined as significantly enriched (77).

Conflict of interest statement-All the authors declare that they have no conflict of interest.

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Figure legend

Figure 1. PRMT5 is ubiquitously expressed in mouse tissues. **A**. PRMT5 protein levels in mouse tissues were determined by Western blot analysis. **B**. Localization of PRMT5 in mouse AML12 liver cell line. PRMT5 expression was visualized by immunofluorescent staining (green), cytoskeleton was labeled by immunofluorescent staining of tubulin (red) and nuclei were stained by DAPI (blue), size bar: 200 µm. All images represent 3 independent experiments.

Figure 2. High-fat diet increases hepatic PRMT5 expression. PRMT5 protein (**A**) and mRNA (**B**) levels in mouse livers fed a normal regular chow (RD) or high-fat diet (HFD) were examined by Western blot analysis or qRT-PCR. Total protein or RNA was extracted from livers of 4 months-old male mice after 12 weeks of feeding with RD or HFD, respectively. **C**. PRMT5 expression in liver sections of above samples were analyzed by immunofluorescent staining of PRMT5 (green), and DAPI nuclear staining (blue), size bar: 200 µm. **D**. PRMT5 levels in muscle, brown or white adipose tissues from mice fed on normal diet or high fat diet were analyzed by Western blot. **E**. PRMT5 expression in the livers of lean or obese Agouti mice was measured by Western blot. Total protein was extracted from livers of RD-fed 1- or 5 month-old Agouti mice. *** $p<0.001$, two-tailed Student's *t* test.

Figure 3. Silencing PRMT5 decreases PI3K/AKT signaling. **A**. Western blot analysis of PRMT5 in AML12 cells following lentiviral shRNA knockdown of PRMT5. **B**. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differential expressed proteins with PRMT5 knockdown. Total protein extracted from scramble or PRMT5 knockdown cells was analyzed for the expression of key proteins involved in more than 20 signaling pathways. Signals were normalized to alpha and beta tubulin. Results were obtained from 2 independent experiments performed in duplicate. Reduction of phospho-AKT Thr308 and Ser473 was observed in PRMT5 knockdown liver cells (**C**) as well as PRMT5 knockout MEF cells (**D**). **E**. High fat diet up-regulated the hepatic expression of phospho-AKT Thr308 and Ser473. Total protein was extracted from livers of 4 month old male mice fed on either normal or high fat diet for 12 weeks.

Figure 4. PRMT5 regulates master transcription factors of fatty acid metabolism and mitochondrial biogenesis. **A**. RT-qPCR analysis of the mRNA levels of PRMT5, PPARα and PGC-1α in AML12 cells expressing either a scrambled sequence shRNA or shRNA-PRMT5. **B**. Western blot analysis of PPARα in scramble and shRNA-PRMT5 AML12 cells. **C**. Western blot analysis of PRMT5 and PPARα in wild-type MEF and PRMT5 knockout MEF cells. **D**. Expression of mitochondrial biosynthesis genes by qRT-PCR in scramble or shRNA-PRMT5 cells. **E**. Relative mRNA expression of mitochondrial biosynthesis genes measured by qRT-PCR in wild-type MEF and PRMT5 knockout MEF cells. Mitochondria in PRMT5 [flox/flox]

wild type or knockout MEF (**F**) and AML12 cells with scramble or PRMT5 knockdown (**G**) were stained by mitochondrion selective dye Mitotracker™ Red CMXRos, PRMT5 expression was visualized by immunofluorescent staining (green), and nuclei were labeled by DAPI (blue), size bar: 200 µm. Each bar presents the mean of 3 independent experiments performed in triplicates; error bars are standard deviations. ** $p < 0.01$, ***$p < 0.001$, two-tailed Student's t test.

Figure 5. PRMT5 enzymatic activity is required for its regulation of PPARα expression. **A**. A flag-tagged wild-type or enzyme-dead (G367A, R368A) double PRMT5 mutant was introduced to AML12 wells by transient transfection. The expression of exogenous proteins was examined by Western blot analysis using anti-flag antibody. The total levels of PRMT5 were measured by Western blot against anti-PRMT5 antibody. **B**. AKT phosphorylation and expression of PPARα in wild type or mutant PRMT expressing cells were analyzed by Western blot. **C**. Protein levels of phospho-AKT and PPARα were determined by Western blot in AML12 cells treated with DMSO or increasing doses of the PRMT5 inhibitor EPZ015666. **D**. The relative mRNA of PPARα and PGC-1α was measured by qRT-PCR in AML12 cells treated with DMSO or increasing doses of EPZ015666. **E**. AML12 cells treated with DMSO or 100nM EPZ01666 were stained by Mitotracker™ Red CMXRos (red) and nuclei labeled by DAPI (blue), size bar: 200 µm. ***$p < 0.001$, two-tailed Student's t test. **F**. AML12 cells pre-treated with DMSO or 100nM EPZ01666 were exposed to media containing 1 µM palmitic acid and 100 µM oleic acid for 5 days. Intracellular lipids were stained with Oil Red O.

Figure 6. PRMT5 regulates PPARα and PGC-1α expression via AKT signaling. A. PRMT5 ^flox/flox^ wild type or knockout MEF cells were transfected with vector or Myr-AKT and harvested 48 hours post-transfection. Protein samples were analyzed by Western Blot for PPARα and PGC-1α expression. B. AML12 cells were transduced with scramble or shPRMT5 viral supernatant for 24 hours then selected in media containing 2 µg/mL puromycin for 48 hours. Then cells were transfected with vector or Myr-AKT and harvested 48 hours post-transfection. Protein samples were analyzed by Western blot for PPARα and PGC-1α expression.

Figure 1

A

PRMT5

Tubulin

Liver Muscle Spleen Heart Kidney Stomach Intestine Brain Lung Pancreas BAT iWAT

82
64
64
49

B

AML12 cells

PRMT5 / **MitoTrackerRed** / **DAPI**

PRMT5 / **Tubulin** / **DAPI**

Figure 2

A

PRMT5

Tubulin

RD　　　　HFD

— 82

— 64

— 64

— 49

B

C

PRMT5 / DAPI

RD　　　　HFD

E

Agouti mice

lean　　　obese

PRMT5

Tubulin

— 82

— 64

— 64

— 49

D

PRMT5

Tubulin

RD　　HFD　　RD　　HFD　　RD　　HFD

Muscle　　　　BAT　　　　iWAT

— 82

— 64

— 64

— 49

Figure 3

A

PRMT5 — scramble / shRNA1 / shRNA2 — 82, 64

Tubulin — 64, 49

C

p-AKT Thr308 — scramble / shRNA1 / shRNA2 — 64, 49

p-AKT Ser473 — 64, 49

Total AKT — 64, 49

Tubulin — 64, 49

B

mmu05220:Chrinic myeloid leukemia
mmu05200:Pathways in cancer
mmu05215:Prostate cancer
mmu05205:Proteoglycans in cancer
mmu04380:Osteoclast differentiation
mmu05161:Hepatitis B
mmu04662:B cell receptor signaling pathway
mmu04012:ErbB signaling pathway
mmu04660:T cell receptor signaling pathway
mmu04151:PI3K-Akt signaling pathway
mmu04668:TNF signaling pathway
mmu04910:Insulin signaling pathway
mmi05221:Acute myeloid leukemia
mmu04210:Apoptosis
mmu05214:Glioma

P value (-log10)

D

PRMT5 — WT / KO — 82, 64

Tubulin — 64, 49

p-AKT Thr308 — 64, 49

p-AKT Ser473 — 64, 49

Total AKT — 64, 49

E

p-AKT Thr308 — RD / HFD — 64, 49

p-AKT Ser473 — 64, 49

Total AKT — 64, 49

Tubulin — 64, 49

Figure 4

A

Relative Gene Expression (Fold Chang over Scram)

Legend: Scramble, PRMT5 sh-1, PRMT5 sh-2

X-axis: PRMT5, PPARα, PGC-1α

B

scramble, shRNA1, shRNA2

PPARα — 64 / 49

Tubulin — 64 / 49

C

WT, KO

PRMT5 — 82 / 64

PPARα — 64 / 49

Tubulin — 64 / 49

E

Relative Gene Expression (Fold Chang over WT)

Legend: WT, KO

X-axis: PPARα, PGC-1α, Cox7a-1, CytoC

D

Relative Gene Expression (Fold Chang over Scram)

Legend: Scramble, PRMT5 sh-1, PRMT5 sh-2

X-axis: Cox7a-1, CytoC

F

PRMT5 flox/flox MEF

WT

KO

MitoTracker Red/ DAPI

G

AML12

Scramble

PRMT5 sh-1

PRMT5 sh-2

MitoTracker Red/ DAPI

Figure 5

A

Flag-PRMT5

PRMT5

Tubulin

B

p-AKT Thr308

p-AKT Ser473

Total AKT

PPARα

Tubulin

C

p-AKT Thr308

p-AKT Ser473

Total AKT

PPARα

Tubulin

D

E

AML12

MitoTracker Red/DAPI

F

Figure 6

A

Vector | Myr-AKT

PGC-1α ——115 / —82

PPARα ——64 / —49

PRMT5 ——82 / ——64

Total AKT ——64 / —49

Tubulin ——64 / —49

WT KO WT KO

PRMT5$^{flox/flox}$ MEF

B

Vector | Myr-AKT

PGC-1α ——115 / —82

PPARα ——64 / —49

PRMT5 ——82 / —64

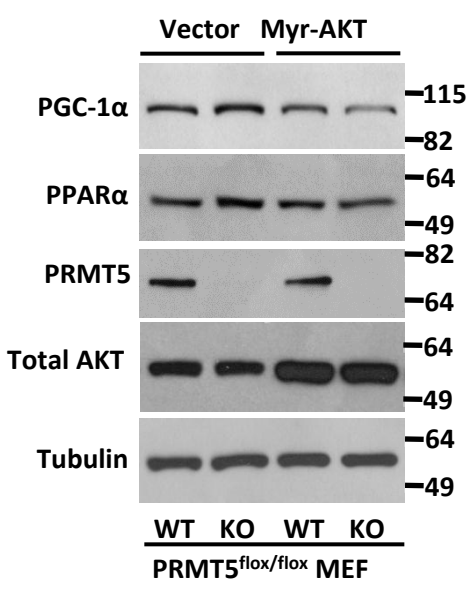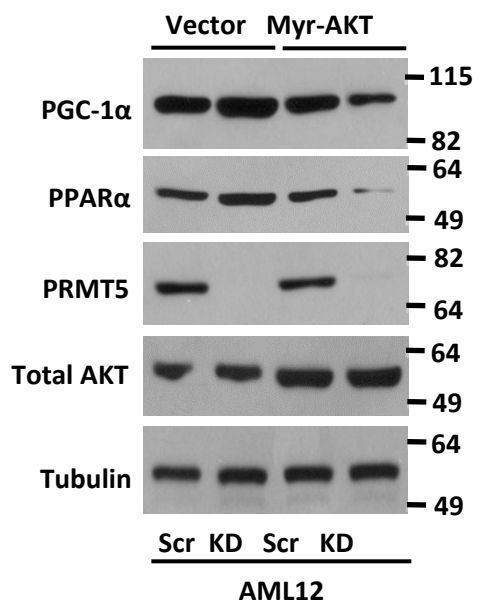Total AKT ——64 / —49

Tubulin ——64 / —49

Scr KD Scr KD

AML12

Inhibition of protein arginine methyltransferase 5 enhances hepatic mitochondrial biogenesis

Lei Huang, Jehnan Liu, Xiao-ou Zhang, Katelyn Sibley, Sonia M. Najjar, Mary M. Lee and Joae Qiong Wu

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