Prmt7 is dispensable in tissue culture models for adipogenic differentiation [version 1; peer review: 2 approved]

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Abstract
Protein arginine methylation is a common posttranslational modification that has been implicated in numerous biological processes including gene expression. The mammalian genome encodes nine protein arginine methyltransferases (Prmts) that catalyze monomethylation, asymmetric dimethylation, and symmetric dimethylation on arginine residues. Protein arginine methyltransferase 7 (Prmt7) is categorized as a type II and type III enzyme that produces symmetric dimethylated arginine and monomethylated arginine, respectively. However, the biological role of Prmt7 is not well characterized. We previously showed that Prmt5, a type II Prmt that associates with Brg1-based SWI/SNF chromatin remodeling complex, is required for adipocyte differentiation. Since Prmt7 also associates with Brg1-based SWI/SNF complex and modifies core histones, we hypothesized that Prmt7 might play a role in transcriptional regulation of adipogenesis. In the present study, we determined that the expression of Prmt7 did not change throughout adipogenic differentiation of C3H10T1/2 mesenchymal cells. Knockdown or over-expression of Prmt7 had no effect on lipid accumulation or adipogenic gene expression in differentiating C3H10T1/2 cells or in C/EBPα-reprogrammed NIH3T3 fibroblasts. Based on these results, we conclude that Prmt7, unlike Prmt5, is dispensable for adipogenic differentiation in tissue culture models.
Introduction
Research over the past 15 years has demonstrated the importance of protein arginine methylation in various biological processes including transcriptional regulation, DNA repair, RNA processing, and signal transduction\(^1\). The fact that hundreds of cellular proteins have been identified as the targets of protein arginine methylation supports the idea that arginine methylation regulates diverse cellular processes\(^6\). Arginine methylation is catalyzed by protein arginine methyltransferases (Prmts) that transfer a methyl group from S-adenosyl methionine (AdoMet) to protein substrates. To date, nine protein arginine methyltransferases have been identified in the mammalian genome and have been classified as type I, type II and type III enzymes by the activity of producing Ω-N\(^6\),N\(^\omega\)-asymmetric dimethylarginine (ADMA), Ω-N\(^6\),N\(^\omega\)-symmetric dimethylarginine (SDMA), and Ω-N\(^\omega\)-monomethylarginine (MMA), respectively\(^1^,^2^,^4^,^7\).

Among the family of Prmts, Prmt7 is unique in that it possesses two AdoMet-binding domains, which may have resulted from a gene duplication event\(^8\). As for many of the Prmts, histones are among the identified substrate molecules, suggesting a functional role for Prmt7 in regulating gene expression as a chromatin modifi-

cer. Initial work indicated that H2A and H4 were symmetrically dimethylated by Prmt7 \textit{in vitro}\(^9\), whereas several studies have reported that Prmt7 only produces MMA on histones and other substrates\(^10^,\,\,\,11\). A recent study indicated that Prmt7 symmetrically dimethylates H4R3 and H2AR3 in a manner that contributes to the repression of expression of genes involved in DNA repair\(^12\). Another study revealed that Prmt7-mediated H4R3 symmetric dimethylation antagonizes MLL4-catalyzed H3K4 methylation on neuron-specific gene promoters during differentiation\(^11\), suggesting that Prmt7 might negatively regulate tissue differentiation by its chromatin-modifying activity. Aside from the role in neuronal differentiation, the regulatory function of Prmt7 in the differentiation of other lineages has not been reported.

Adipocyte differentiation is one of the most intensively studied differ-

tentiation processes. Both human and mouse mesenchymal stem cells (MSCs) that reside in fat pads and bone marrow undergo lineage commitment and terminal differentiation to become mature adipocytes\(^13^-^16\). The adipogenic differentiation process is controlled by a number of tissue-specific transcription factors, such as the CCAAT enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), and numerous chromatin remodeling and modifying enzymes including the ATP-dependent SWI/SNF complex and Prmts\(^17^-^20\). It has been shown that Prrmt5 interacts with Brg1-based SWI/SNF complex\(^21\) and facilitates the binding of Brg1 to the PPAR\(\gamma\) and to PPAR\(\gamma\) target promoters to facilitate the activation of adipogenic genes\(^9\). Furthermore, Prrmt4, which also has been shown to interact with Brg1-based SWI/SNF complex\(^22\), acts as a co-activator of PPAR\(\gamma\) to promote adipocyte gene expression\(^9\). Prmt7 had been recently shown to interact with Brg1-based SWI/SNF complex\(^12\), but whether Prmt7 has functional roles in adipocyte differentiation remains unclear.

In the present study, we examined the role of Prmt7 in adipocyte differentiation in mouse C3H10T1/2 embryonic mesenchymal cells and in C/EBP\(\alpha\)-reprogrammed murine NIH3T3 fibroblasts. By knocking down and over-expressing Prmt7, we showed that Prmt7 has no effect on lipid accumulation and adipogenic gene expression in differentiating cells. Based on the results, we concluded that Prmt7 is not required for differentiation in tissue culture models of adipogenesis.

Methods

Cell culture

Mouse C3H10T1/2 and NIH3T3 cells were obtained from the ATCC. C3H10T1/2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) high glucose (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Sigma) and 100μM of penicillin/streptomycin (Invitrogen). NIH3T3 cells were maintained in DMEM high glucose with 10% calf serum (Sigma). 293T and BOSC23 cells were obtained from S.N. Jones (UMass Medical School) and R.E. Kingston (Massachusetts General Hospital), respectively, and were grown in the same medium as C3H10T1/2 cells. For adipogenic differentiation, two-day postconfluent cells were differentiated with DMEM medium containing 10% FCS, 0.5 mM 3-isobutyl-1-methyloxanthine, 1μM dexamethasone, and 10μM troglitazone (Sigma). After 48 hours incubation, media on the differentiating cells was replaced with media containing 5μg/ml insulin. Subsequently, the media was changed every other day until harvest. To evaluate cell proliferation, 1×10\(^6\) cells were seeded in 6-well plates (Corning Inc.), and the number of viable cells was counted under a microscope (CK2, Olympus) each day from day 1 to day 4 with a hemocytometer (Hauser Scientific).

Plasmid DNA constructs

pENTR/pTER+ vector and pLentiX2 Dest vector were gifts from Dr. Eric Campeau (UMass Medical School). The preparation of lentiviral small hairpin RNA (shRNA) constructs was done as previously described\(^21^-^24\). Briefly, shPrmt7-1, shPrmt7-2 and scrambled control oligonucleotides were cloned into a pENTR/pTER+ vector. These constructs were individually incubated with the pLentiX2 DEST vector and LR clonase II enzyme mix (Invitrogen) to generate pLentiX2 DEST/shPrmt7-1, pLentiX2 DEST/shPrmt7-2 and pLentiX2 DEST/shCtrl constructs. These lentiviral constructs were amplified in Sbi3 competent cells (Invitrogen) for generating lentiviruses. The pBABE puromycin empty vector (pBABE vector), FLAG-tagged Prmt7 construct (pBABE PRMT7) and pBABE CEBP\(\alpha\) construct were previously described\(^21^-^24\) and were individually amplified in TOP10 competent cells (Invitrogen) for generating retroviral DNA as previously described\(^21^-^24\).

Virus production and infection

The preparation of viruses was performed as previously described\(^21^-^24\). Briefly, for lentiviruses, the packaging vectors pLIP, pLIP2, pVSVG (Invitrogen) and pLentiX2 DEST/shRNA constructs were co-transfected into 293T cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. BOSC23 cells were used for pBABE-based retrovirus production. After 48 hours incubation, the supernatant was harvested and filtered through 0.45μm syringe filter (Millipore). For viral infection, 1ml of the filtered supernatant and 4μg/ml of polybrene (Sigma) were used to infect one million cells. After 48 hours incubation, virus infected cells were selected in 2.5μg/ml puromycin (Invitrogen).

Protein expression analysis

Cells were washed twice with cold PBS and were harvested in RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA,
1% Nonidet P-40 (Thermo Scientific) and 0.25% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche). The samples were sonicated at high intensity setting for 3 minutes with 30 sec on/off cycle in a Bioruptor (UCD-200, Diagenode). After quantifying the protein concentration by means of a Bio-Rad protein assay, the protein samples were then mixed with 4x SDS loading buffer (240 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol) and boiled at 95°C for 10 min. 30 μg protein samples were separated on 10% SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). The blots were blocked overnight in 3% non-fat milk (Essential Everyday). The next day, proteins were detected using specific antibodies (1:1000 dilution) and HRP-conjugated secondary antibodies (1:2000 dilution). The rabbit polyclonal antibodies against human PRMT7 (sc-98882) and rat C/EBPα (sc-61) were purchased from Santa Cruz Biotechnology. The mouse monoclonal antibody against mouse PPARγ (sc-7273) and the goat polyclonal antibody against human PRMT5 (sc-22132) were also purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-PI3K (ABS233) antibody was from Millipore. The secondary antibodies (NA9340 and NA9310) were purchased from GE Healthcare Life Sciences. The detection reagents (GE Healthcare Life Sciences) were blocked overnight in 3% non-fat milk (Essential Everyday). The blots were developed on X-ray films with ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences). The signal intensity was quantified by ImageJ.

**Gene expression analysis**

Total RNA was isolated from samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was prepared from 1 μg of total RNA by Superscript III reverse transcriptase kit (Invitrogen). Quantitative PCR was performed on StepOne Plus real-time PCR machine with Fast SYBR Green Master mix (Applied Biosystems). The specific primers for gene expression analysis were:

- Fasn forward 5′-CGTGTGGCCCTACCCAGAGCT-3′
- Fasn reverse 5′-GGCAGCAGGGCTCCACCCCTT-3′
- AdipoQ forward 5′-CAGTGGATCTGACGACACCA-3′
- AdipoQ reverse 5′-CGAATGGGTACATTGGGAAC-3′
- Fabp4 forward 5′-GCCTGGAATTGGATGAAATCA-3′
- Fabp4 reverse 5′-CCCCGCACTCTAGGGATATGA-3′
- 5S rRNA forward 5′-GTCTACGGACATACCACCAGT-3′
- 5S rRNA reverse 5′-TACAGCCCCGGGATTCCAG-3′

Relative expression levels were determined by the comparative Ct method \(^{(5)}\).

**Oil Red O staining**

Differentiating cells were washed once with PBS and fixed in 10% phosphate-buffered formalin (Fisher Scientific) overnight. The next day, the fixed cells were washed with 60% isopropanol and air-dried completely. The cells were then stained with 60% Oil Red O (AMRESCO) for 10 minutes and washed repeatedly with tap water to remove excess stain. To quantify staining, Oil Red O was extracted from the cells with 100% isopropanol, and the optical density was measured at 500 nm (OD\(_{500}\)).

**Results**

The protein levels of Prmt7 remain constant during adipogenic differentiation of C3H10T1/2 cells

The C3H10T1/2 cell line was established from C3H mouse embryos and has served as a faithful cell culture model for mesenchymal lineage differentiation \(^{(27-28)}\). C3H10T1/2 cells can be differentiated into mature adipocytes by treating the confluent cells with a cocktail that contains insulin, dexamethasone, 3-isobutyl-1-methyloxanthine (IBMX) and PPARγ ligands \(^{(29)}\). Using this model, we first examined Prmt7 protein levels during adipogenic differentiation by Western blot analysis. We found that Prmt7 protein levels are relatively constant from the onset of differentiation (day 0) through the day 6 post-differentiation (Figure 1A and Figure 1B) (Dataset 1). We concluded that Prmt7 protein levels were not altered in differentiating C3H10T1/2 cells.

**Virus-mediated knockdown and over-expression of Prmt7 in C3H10T1/2 cells**

To study the function of Prmt7, we used viral vectors to knock down or over-express Prmt7 in C3H10T1/2 cells. Two lentiviral constructs (pLentX2 DEST/shPrmt7-1 and pLentX2 DEST/shPrmt7-2) that encode shRNAs against Prmt7 mRNA were used for knocking down endogenous Prmt7 in proliferating C3H10T1/2 cells. A pBABE retroviral construct (pBABE-PRMT7) encoding FLAG-tagged Prmt7 was used to over-express PRMT7. The virus-infected cells were selected with puromycin and the levels of Prmt7 in the selected cells were examined by Western blot analysis (Figure 2A). Endogenous Prmt7 levels were reduced 10-fold or more in the knockdown

![Figure 1. Prmt7 protein levels are constant in differentiating C3H10T1/2 cells.](image-url)
Figure 2. Prmt7 was specifically knocked down or over-expressed in C3H10T1/2 cells. (A) A representative Western blot analysis from proliferating C3H10T1/2 cells with Prmt7 knockdown and over-expression. Endogenous Prmt7 was specifically depleted by two different lentiviral shRNA constructs (shPrmt7-1 and shPrmt7-2). The scrambled shRNA lentiviral construct (shCtrl) was used as a control. The pBABE retroviral construct encoding FLAG-tagged PRMT7 (pBABE - PRMT7;\textsuperscript{12}) was used to ectopically express PRMT7, and the pBABE empty vector (pBABE vector) was used as a control. Duplicate blots were probed with anti-Prmt7 and anti-Prmt5 antibodies and with anti-PI3K antibody as a loading control. (B) The quantification of Prmt7 in (A) by ImageJ. The levels of Prmt7 were normalized to the PI3K loading control and are presented as expression levels relative to the scrambled shRNA control or pBABE empty vector control. The data represent the average of two independent experiments (n=2).

Figure 3. Knockdown or over-expression of Prmt7 does not affect C3H10T1/2 cell proliferation. The growth rates of control (shCtrl and pBABE vector), Prmt7 knockdown (shPrmt7-1 and shPrmt7-2) and Prmt7 over-expression (pBABE PRMT7) C3H10T1/2 cells were measured by seeding 1×10^5 cells in 6-well plates, and the viable cells were counted each day for 4 days after seeding. The data represent the average of two independent experiments (n=2) counted in duplicate. Error bars show the standard deviation.
Knockdown or over-expression of Prmt7 has no effect on C3H10T1/2 adipogenic differentiation. (A) Representative Oil-Red O staining images of day 6 post-differentiated C3H10T1/2 cells in which Prmt7 was either knocked down (shPrmt7-1 and shPrmt7-2) or over-expressed (pBABE PRMT7). The Prmt7 knockdown and over-expression C3H10T1/2 cells were grown to confluence and differentiated 48 h later. At day 6 post-differentiation, the cells were fixed with 10% formalin and stained with Oil-Red O. (B) The quantification of (A). The Oil-Red O stain was extracted with 100% isopropanol and the optical density at 500nm (OD$_{500}$) was determined. The data represent the average of two independent experiments (n=2) assayed in duplicate. Error bars show standard deviation. (C) A representative Western blot analysis of endogenous Prmt7, PPARγ and C/EBPα expression in day 6 post-differentiated C3H10T1/2 cells. The levels of PI3K are presented as a loading control. (D) Gene expression analysis on day 6 post-differentiated C3H10T1/2 cells. The mRNA levels of fatty acid synthase (Fasn), adiponectin (AdipoQ) and fatty acid binding protein 4 (Fabp4) were measured by RT-qPCR. The individual mRNA levels were normalized to 5S rRNA. The normalized expression levels of the control cells in one of the experiments were set as 1. The data are presented as the average of relative expression levels from two independent experiments (n=2) assayed in duplicate. Error bars show standard deviation.

Oil Red O staining. The Oil Red O staining showed similar levels of lipid accumulation in Prmt7 knockdown as well as Prmt7 over-expression cells as compared to the control cells. (Figure 4A and Figure 4B) (Dataset 4). This result suggests that the changes in Prmt7 levels did not affect lipid accumulation in differentiating C3H10T1/2 cells.

Adipogenic gene and protein expression in C3H10T1/2 cells was not affected by Prmt7 knockdown or over-expression PPARγ and C/EBPα are the key transcription factors for adipogenic differentiation and for the maintenance of the adipocyte phenotype. We examined the protein levels of PPARγ and C/EBPα in day 6 post-differentiation cells by Western blot analysis and found no significant difference in either Prmt7 knockdown or Prmt7 over-expression cells compared to the corresponding controls (Figure 4C). In addition, to rule out the possibility that Prmt7 functions as a cofactor of PPARγ and C/EBPα, we measured the mRNA expression levels of PPARγ and C/EBPα target genes in day 6 post-differentiation samples by real-time quantitative PCR (Figure 4D) (Dataset 5). We found that Prmt7 had no significant impact on fatty acid synthase (Fasn), adiponectin (AdipoQ), and fatty acid binding protein 4 (Fabp4) gene expression in the differentiating cells. These results suggest that Prmt7 is dispensable for adipogenic gene expression.

Prmt7 has no effect on C/EBPα-reprogrammed NIH3T3 fibroblasts

Previous studies had shown that ectopic expression of PPARγ or C/EBPα alone in non-adipogenic NIH3T3 cell line is able to reprogram NIH3T3 fibroblasts into adipocyte-like cells. To test if Prmt7 is required for the reprogramming of NIH3T3 fibroblasts, we first knocked down and over-expressed Prmt7 in NIH3T3 fibroblasts by using the same viral constructs that we used in C3H10T1/2 cells, and confirmed the knockdown and over-expression of Prmt7 by Western blot analysis (Figure 5A). These cells were further infected with retroviruses encoding C/EBPα at 70% confluence. After the
Discussion

Changes in gene expression during cell differentiation require alterations in higher-order chromatin organization as well as in local chromatin structure. Cells possess histone modifying enzymes and ATP-dependent chromatin remodeling enzymes to facilitate chromatin changes. The interplay between these two families of enzymes has been shown to be crucial for both transcription activation and repression[11,31,33]. Prmt7 was identified as a histone arginine methylating enzyme[24–26] and was shown to associate with Brg1-based SWI/SNF ATP-dependent chromatin remodeling complex[27]. These findings led us to investigate the possible roles of Prmt7 in adipogenic differentiation, which is a process that requires the function of Brg1-based SWI/SNF complex[28]. Our data clearly showed that Prmt7 levels were significantly changed in the knockdown or over-expression cells, but manipulation of Prmt7 levels did not cause a differentiation deficiency. It is established that Brg1-based SWI/SNF complex is recruited to the adipogenic promoters upon differentiation[29]. However, whether Prmt7 associates with Brg1-based SWI/SNF complex at adipogenic promoters is still unknown. Since Prmt7 has no effect on adipogenic gene expression, we expect that Prmt7 is not recruited to adipogenic promoters. Alternatively, even if there is binding, the function of Prmt7 is dispensable at these loci.

Functional redundancy within the Prmt family has not been characterized. Prmt7 was classified as a type II and a type III arginine methyltransferase by characterization of its in vitro catalytic activity[30–33]. Whether other Prmts functionally compensate for Prmt7 is still unknown. The predominant type II arginine methyltransferase Prmt5 catalyzes the formation of MMA and SDMA in a nonprocessive fashion[35,36]. Type I arginine methyltransferases also produce MMA[37–39]. It is possible that Prmt5 or type I Prmts partially or fully compensate for the loss of Prmt7 in the cells. Our data showed that Prmt7 knockdown or over-expression has no effect on Prmt5 protein levels in C3H10T1/2 cells. This observation is consistent with the results from the previous study in HeLa cells[35]. However, we still cannot rule out the possibility that Prmt5 compensates for Prmt7 enzyme activity, even though Prmt5 protein levels remain constant. Further investigation is needed to address the possible crosstalk between Prmt5 and Prmt7 in chromatin regulation.

To our knowledge, Prmt7 knock-out or transgenic mice have not been reported. Whether changes in Prmt7 levels cause any developmental deficiencies in vivo remains unknown. However, several studies using cell lines or tissues have revealed regulatory roles for Prmt7 in tissue-specific gene expression. For example, PRMT7 negatively regulates neuronal differentiation of a human embryonal carcinoma cell line by repressing the expression of differentiation-specific genes[38]. In mouse germ cells, Prmt7 was recruited to the imprinting control region through physical interaction with CTCFL, a testis-specific nuclear protein, and repressed imprinted gene expression[39]. Furthermore, mouse embryonic stem cells and germ cells have relative high levels of Prmt7 compared with mouse embryonic fibroblasts[40–42]. This evidence suggests that Prmt7 might have important functions in the maintenance of stem cell pluripotency and that the down-regulation of Prmt7 might be required for early cell fate.
determination. Taken together, these data suggest that Prmt7 might have a role during early development.

Author contributions
ANI and SS conceived the study. Y-JH, SS and ANI designed the experiments and reviewed the data. Y-JH and ANI wrote the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests
No competing interests were disclosed.

References


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In this manuscript, Hu et al. performed a series of high-quality, well-designed and properly controlled experiments in search of potential functions of the protein arginine methylase Prmt7 in adipogenesis of C3H10T1/2 cell cultures. The motivation for the experiment was the group’s previous work that identified central functions of the closely related Prmt5 in the process, which led them to hypothesize guilt-by-homology. Moreover, hints of developmental functions of Prmt7 in other systems, such as neuronal differentiation, warranted testing a broader effect on differentiation in other system.

The conclusion that Prmt7 has no bearing on adipogenesis is fully justified by the clean data, which showed no effect in both loss and gain-of-function approaches. It is reassuring that the experiment was conducted by the same group that identified the roles of Prmt5 in the process, and thus the negative data are likely not due to experimental system variability.

In the Discussion, the authors allow that this lack of effect may be the result of compensation by redundant methylases; this is probably an overly cautious, “just-in-case” argument, considering that even in most cases of true redundancy there are at least some quantitative effects, which is not the case here whatsoever. It is more likely that arginine methylases are target-specific and have a clear partition of labor among them.

With heaps of negative data buried in the archives of many laboratories which never see the light of day, the team has to be commended for publishing their own negative data to seal the issue and allow other research groups that might be drawn to the question to steer away and focus on more productive hypotheses.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
Jeffrey Gimble
Center for Stem Cell Research and Regenerative Medicine, Tulane University, New Orleans, LA, USA

This is a well designed, well conducted, and well described study from a leading lab in the evaluation of histone mediated transcriptional regulation of adipogenesis. The authors provide compelling evidence that Prmt-7 is not directly involved in adipogenic regulation using loss of function/gain of function approaches in two independent pre-adipocyte models \textit{in vitro}. The authors have evaluated their findings objectively and placed it appropriately in the context of the existing literature. The manuscript and figures are of top quality and require no further modification. The findings provide information that will benefit others in the scientific community by documenting the absence of a direct relationship between Prmt-7 and adipogenic mechanisms.

\textbf{Competing Interests:} I am the co-founder and CSO of LaCell LLC, a biotech company focusing on pre-adipocyte cells isolated from adipose tissue. I have not collaborated with Dr. Imbalzano in any past publications.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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