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Histone methyltransferase PRDM8 regulates mouse testis steroidogenesis

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ABSTRACT

A family of PRDM proteins are similar to histone methyltransferases (HMTases) with SET domain in that they modulate different cellular processes, including transcriptional regulation, through chromatin modifying activities. By applying a bioinformatic approach, we searched for proteins containing the SET domain and identified a double zinc-finger domain containing PRDM8 with HMTase activity. *In vitro* HMTase assay and immunoblot analysis revealed that PRDM8 specifically methylates H3K9 of histones which indicates transcriptional repression activity of PRDM8. Direct recruitment of PRDM8 to the promoter mediated transcriptional repression and indicated no involvement of HDAC. Tissue blot analyses identified PRDM8 transcripts from brain and testis in adult mouse. Consistent with these observations, we demonstrate that PRDM8 repressed the expression of steroidogenic markers, *p450c17c* and *LHR*, which indicates its regulatory role in mouse testis development.

Introduction

The members of the PRDM family are characterized by the presence of the PR (PRDI-BF1 and RIZ homology) domain at the N-terminus, followed by a variable number of zinc finger repeats [1]. The PR domain has similarity (20–30% identical in amino acids) to the SET (Su(var)3–9, Enhancer-of-zeste, and Trithorax) domain, a histone methyltransferase (HMTase) catalytic module [2]. However, domains containing HMTases are in two distinct classes of enzymes which differ in many aspects, such as the location of HMTase catalytic domain, the presence of zinc finger DNA-binding domain, and species preference during evolution [3].

Numerous studies indicate that histone methylation is linked to both transcriptional activation and repression depending on which lysine/arginine residue(s) of histones it is targeting [4]. Several studies suggest that PRDM proteins interact with a series of chromatin modifying proteins and act predominantly as negative regulators of transcription [5–8]. Increasing evidence indicates that PRDMs are involved in a broad spectrum of cancers and differentiation of certain cell types.

Testis development and spermatogenesis are governed by endocrine hormones such as pituitary gonadotropin luteinizing hormone (LH) that stimulates the Leydig cell steroidogenesis, and follicle stimulating hormone (FSH) that stimulates Sertoli cell differentiation [9]. Steroidogenesis in Leydig cells starts with the transfer of cholesterol into the mitochondria and is regulated by steroidogenic enzymes, including steroidogenic acute regulatory protein (StAR), cytochrome P450 family, 3β-HSD, and LH receptor (LHR) [10]. The expression of steroidogenic-enzyme genes is regulated mainly at the transcriptional level by histone-associated enzymes.

Here we report that PRDM8 is a novel SET domain containing protein with specific HMTase activity and cellular localization is predominantly in the nucleus. PRDM8 is localized in adult mouse testis and brain and has general transcriptional repression activity in transfection reporter assays. The possible role of PRDM8 in HMTase activity in mouse testis development was indicated by the repression of steroidogenesis markers *p450c17* and *LHR* expression when overexpressed.

Materials and methods

Bioinformatics. To identify a novel SET domain containing protein, EnsMart, a protein mining program (http://www.ensemble.org/), was used. We utilized the filter of Pfam ID (00865) and the novel protein option. Candidate genes were blasted to the Pfam

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site (http://pfam.sanger.ac.uk) to calculate the probability of the SET domain and other structures.

Plasmid construction. The full-length open reading frame of PRDM8 and truncated PRDM8 constructs were amplified by PCR from a mouse cDNA library (Clontech). The PCR products were subcloned into pCRII-TOPO (Invitrogen), pCMX-GAL4 [11], or amino-terminally hemagglutinin (HA)-tagged pcDNA3.1 (Invitrogen) expression vectors.

Immunohistochemistry. Immunohistochemistry was used to examine the tissue distribution of PRDM8 in postnatal 1 day mouse. Mice were obtained 1 day after birth. Sagittal sections were obtained and utilized for immunohistochemistry as described [12]. The protocol for fluorescent immunocytochemistry was described previously [13].

In vitro histone methyltransferase (HMTase) assays. The HMTase assay was conducted as reported, with some minor modifications. In brief, 35 μl of reaction mixture containing 1 μg/μl core histones from calf thymus (Roche) or histone subunits (H3, H2A, H2B, H4) as a substrate for enzyme activity, 100 nCi of S-adenosyl-[methyl-14C]-L-methionine (Perkin Elmer), GST-PRDM8 and GST

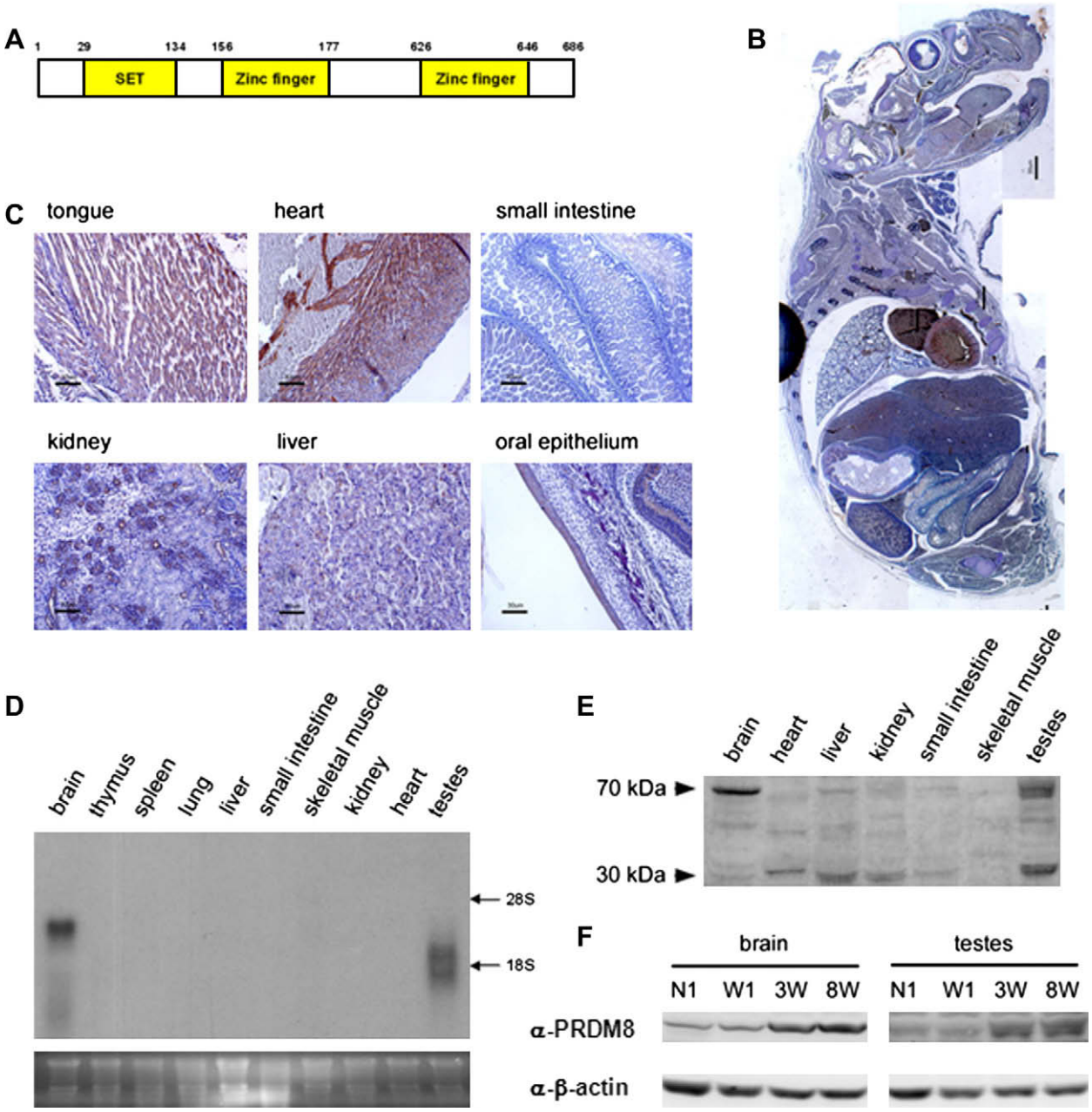

Fig. 1. Cloning of PRDM8. (A) Structure of PRDM8. After investigation to find SET domain-containing proteins, PRDM8 was amplified with specific oligomers from a mouse cDNA library. SET domain was localized in the N-terminal of the protein and two zinc fingers followed. (B) Immunohistochemistry analysis showing the distribution of PRDM8. Sagittal section of postnatal 1 day mouse was used for analysis. (C) PRDM8 was highly expressed in striated muscles, such as in heart, tongue, and back muscles, but was barely detected in intestinal smooth muscles. Note that PRDM8 was not abundant in brain and testis in postnatal 1 day mouse. (D) Northern blot analysis results for PRDM8. A mouse adult tissue blot was hybridized to a ^{32}P-labaled PRDM8 cDNA probe. Note that PRDM8 was most abundant in brain and testis. (E) Western blot analysis to observe PRDM8 expression. Adult mouse tissue blot was used for immunoblot with anti-PRDM8 antibody. Note that the 70 kDa PRDM8 was most abundant in brain and testis, whereas the 30 kDa PRDM8 was most abundant in testis, heart, liver, and kidney. (F) The expression of 70 kDa PRDM8 in brain and testis abruptly increased 3 week after birth.

in 5× HMTase assay buffer (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM MgCl2, 10 mM beta-mercaptoethanol, 1.25 M sucrose) was incubated at 30 °C for overnight. Proteins were filtered using p81 filter paper (Upstate Biotechnology), and washed three times with cold 10% TCA and 70% ethanol for 5 min in RT. The filters were allowed to air dry and added 1 ml of Ultima Gold (Perkin Elmer) and [14C]-SAM was quantified using a scintillation counter.

Western blot analysis. Western blot analysis was performed as described [14] using PRDB8 antibody (Abcam 3796, 1:500). PRDM8 constructs were transfected into COS-7 and HEK 293T cells. Cells were harvested and cell lysates were subjected to Western blot analysis using anti-H3K4-me2 (1:2000), anti-H3K9-me2 (1:2000), anti-H3K27-me2 (1:2000), anti-H3K36-me2 (1:2000), and anti-H4K20-me2 (1:2000) antibodies (Upstate Biotechnology Inc.).

Northern blot analysis and RT-PCR. Northern blot analysis was performed as previously described [15], and the 3′-portion of PRDM8 spanning amino acids 519–686 was utilized as a probe. Total RNA from the each construct-transfected TM3 cells was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instruction. Total RNA (5 μg) was used to synthesize the cDNA. The cDNA synthesis was primed with oligo-dT primer (Maxime RT premix kit, Intron Biotech) and quantified cDNA was applied in *p450c17* and *LHR* mRNA expression pattern analysis.

Immunofluorescence. COS-7 cells were seeded in Lab-Tek II Chamber slides (Nalge Nunc Inter) at 1×10^4 cells/well/4-chamber slide and transfected with HA-tagged PRDM8 constructs. Transfected cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 1% bovine serum albumin in PBS. Cells were incubated overnight with 5 μg/ml anti-HA antibody in 1% BSA, followed by a 2 h incubation with 4 μg/ml Alexa Fluor 568-conjugated goat anti-mouse IgG at room temperature. The nuclei were stained with DAPI and cells were observed using a Zeiss laser-scanning confocal microscope.

Transfection and luciferase assay. For transfection of reporter plasmids, 293T cells (1×10^5 cells/well/24-well plate) were transfected with GAL4-DNA-binding domain (GAL4-DBD) or GAL4-DBD-PRDM8 using FuGENE 6. After 48 h, cells were harvested and subjected to the luciferase assay (Promega) in accordance with the manufacturer's instructions.

Results

Bioinformatics screening and cloning

PRDM8 is predicted to be located in the 4th chromosome of humans (gene ID: ENSG00000152784) and in the 5th chromosome of mice (gene ID: ENSMUSG00000035456). The molecular weight of full-length PRDM8 is approximately 70 kDa and a shorter transcript isoform of 34 kDa was also found. Both variants have intact SET domains in the N-terminal (Fig. 1A). The 34 kDa short form has deletion between amino acids 215 and 602. We cloned 70 kDa full-length form which is expressed in most of the species and we mainly targeted this transcript for the following studies.

Tissue distribution and intracellular localization

We checked expression of PRDM8 in mouse tissues. First, we used sagittal sections of postnatal day 1 mice for immunohistochemistry (Fig. 1B). PRDM8 was expressed most prominently in cardiomyocytes from the heart and in striated skeletal muscle from the tongue. PRDM8 was also expressed in renal tubules and in oral epithelium. PRDM8 was not expressed in intestinal smooth muscles (Fig. 1C).

To determine the expression of PRDM8 in mouse tissues, Northern blot and Western blot analyses were carried out. When we used the 3′-portion of PRDM8 spanning amino acids 519–686, ma-

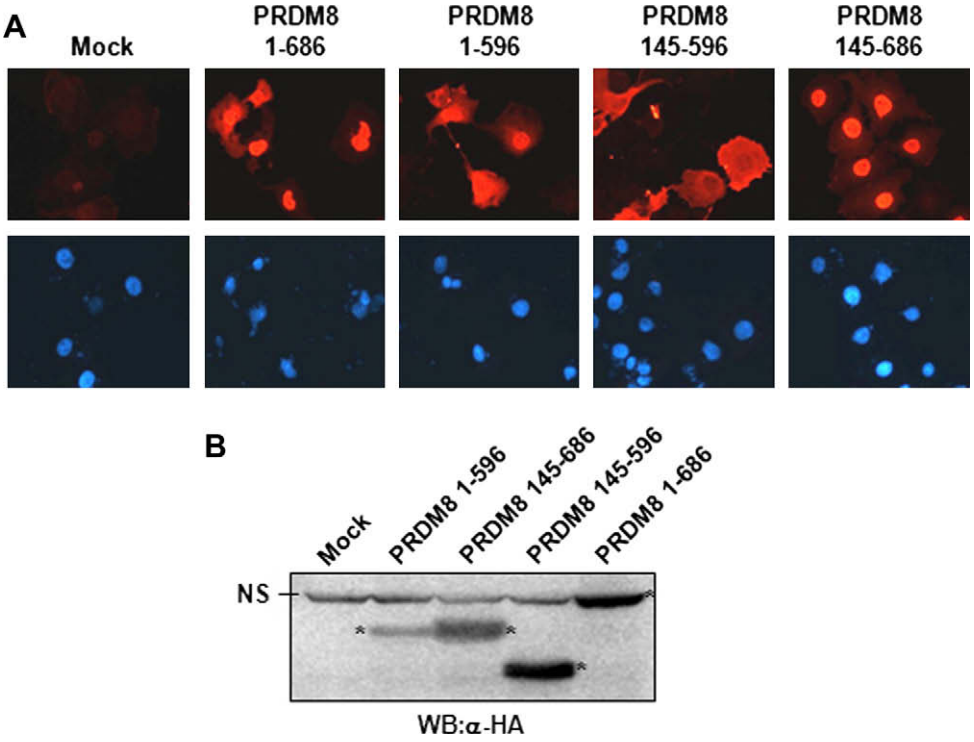

Fig. 2. Nuclear localization of PRDM8. COS-7 cells transiently transfected with HA-tagged PRDM8 constructs. (A) Fluorescent immunocytochemistry was performed. Localization of PRDM8 (upper panel) and the DAPI staining (lower panel) was seen. Images are representative of three independent experiments. (B) Cells were harvested 72 h post transfection and lysates were analyzed by Western blot with an anti-HA antibody. The amino acid positions of the deletion constructs are indicated. The asterisk indicates HA-tagged PRDM8 bands. NS, IgG band.

jor PRDM8 transcripts were detected in brain and testis (Fig. 1D). The different sizes of PRDM8 transcripts in brain and testis might be due to alternative splicing. When we performed Western blot analysis, PRDM8 of 70 kDa was detected in brain, liver, and testis (Fig. 1E). PRDM8 of 30 kDa was prominent in heart, liver, and kidney (Fig. 1E). The expression of PRDM8 in brain and testis from postnatal 1 day to 8-week-old mice was further checked. It increased abruptly in 3-week and 8-week-old mice, suggesting that the PRDM8 may play a role in adult brain or testis (Fig. 1F).

We examined the subcellular localization of HA-tagged PRDM8 in the transfected COS-7 cells. The full-length of PRDM8 was localized exclusively in the nuclear region (Fig. 2A). The domain of PRDM8 responsible for nuclear localization was mapped by deletion mutagenesis (Fig. 2B). The carboxyl terminal zinc-finger domain was necessary for directing PRDM8 to the nucleus.

PRDM8 is a novel HMTase with transcriptional repression activity

In order to determine whether PRDM8 has HMTase activity, we performed *in vitro* HMTase assay with purified GST-PRDM8 protein. Core histones and [14C]-SAM were utilized as the substrate and methyl donor. As shown in Fig. 3A, we detected intrinsic HMTase activity of PRDM8. Next, we performed HMTase assay with individual histone subunits to identify individual histone specificity. The HMTase activity of PRDM8 was very specific for histone H3 compare to those of other histone subunits (Fig. 3B). To further analyze lysine specificity of PRDM8, 293T cells were transiently transfected and total cell lysates were immunoblotted against antibodies specific to H3K4-me2, H3K9-me2, H3K27-me2, H3K36-me2, and H4K20-me2. As expected, transfection of the SET domain lacking PRDM deletion mutant (145–686) failed to show H3K9 methylating activity (Fig. 3C). These results suggest that PRDM8 exhibited H3K9 HMTase activity in a SET domain dependent manner.

To investigate the *in vivo* effects of H3K9 methylation on transcription, full-length PRDM8 was fused to the GAL4-DNA-binding domain, and the ability of the GAL4-PRDM8 chimeras to regulate the expression of GAL-UAS reporter in HEK 293 cells was examined. The GAL4-PRDM8 chimera encoding PRDM8 repressed transcriptional activity significantly in a dose-dependent manner, whereas the control vector (GAL4-DBD) reduced transactivation slightly (Fig. 4A).

Next, we tested whether PRDM8 requires HDAC activity to mediate transcriptional repression. The HDAC inhibitor, trichostatin A (TSA), did not alleviate transcriptional repression mediated by PRDM8, although it relieved a basal level of transcriptional repression on the reporter plasmid (Fig. 4B). These results suggest that HDAC activity is not required for PRDM8-mediated transcriptional repression.

PRDM8 down-regulates p450c17 and LHR expression

The strong signal in testis tissue in Northern blot analysis suggests a role of PRDM8 in mouse testis development and steroidogenesis. Luteinizing hormone (LH) treatment induces testosterone secretion in TM3 cells via induction of steroidogenesis. To investigate the regulatory role of PRDM8 in mouse testis steroidogenesis, we examined *p450c17* and *LHR* expression patterns in TM3 cells with or without LH treatment. RT-PCR analysis showed that the expression of PRDM8 drastically down-regulated

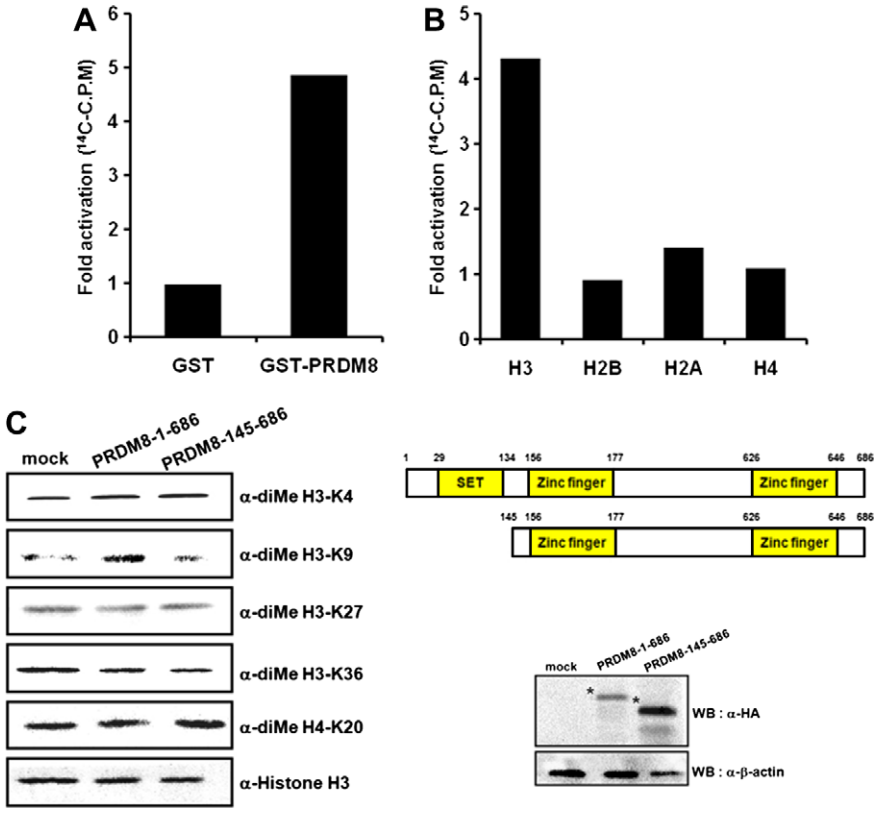

Fig. 3. PRDM8 is a novel histone H3-specific methyltransferase and selectively transferred methyl groups to H3K9. (A) Core histones were used as substrates in the HMTase assay with GST-PRDM8 and GST. Methylation levels were quantified via filter binding assay and represented as raw counts per minute (C.P.M.) incorporated. (B) Similar HMTase assay as (A) was performed with individual histone subunits as substrates. (C) Transiently transfected cell extracts were immunoblotted against anti-H3K4-me2, anti-H3K9-me2, anti-H3K27-me2, anti-H3K36-me2, and anti-H4K20-me2. PRDM8 deletion mutants mapping (upper right) and detection of expressed and purified proteins were detected by Western blot analysis (lower right).

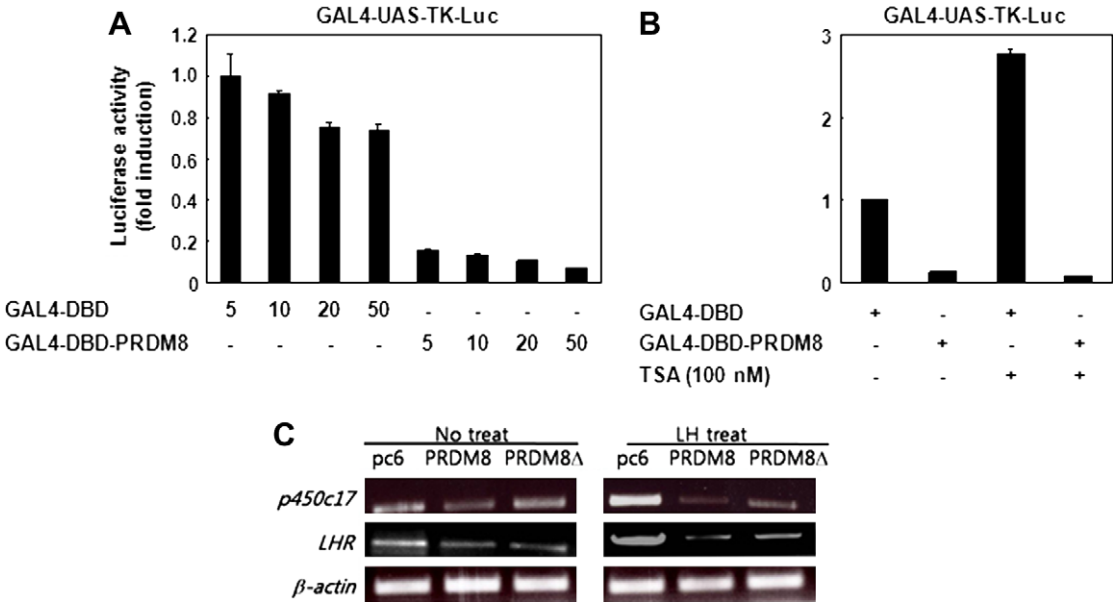

Fig. 4. PRDM8 represses transcription and expression of steroidogenesis marker genes. (A) HEK 293T cells were cotransfected with GAL4-UAS reporter and increasing amounts (5, 10, 20, and 50 ng) of GAL4-DBD or GAL4-DBD-PRDM8 constructs as indicated. The data represent the means and the S.D. of triplicate samples. (B) HEK 293T cells were cotransfected with GAL4-UAS reporter and GAL4-DBD or GAL4-DBD-PRDM8 constructs in the absence or presence of trichostatin A (TSA). The data represent the means and the S.D. of triplicate samples. (C) Total RNA samples from the each construct-transfected TM3 cells were extracted and cDNA was synthesized using oligo-dT primer. RT-PCR analysis of *p450c17* and *LHR* in each construct-transfected cell with or without LH treatment. Anti β-actin indicates loading control.

p450c17 and *LHR* expression upon LH treatment compared to the control. The HMTase domain deleted PRDM8 mutant also down-regulated the expression of *p450c17* significantly and *LHR* relieved the expression of steroidogenesis markers only slightly (Fig. 4C). These results suggest that the PRDM8 might have a negative regulatory role in mouse steroidogenesis through the repression of steroidogenic markers such as *p450c17* and *LHR*.

Discussion

Previously, we performed a homology search using bioinformatic tools and found a series of SET domain-containing proteins with unknown functions. In this study, we describe the cloning and characterization of PRDM8, a novel PR/SET domain containing protein with histone H3K9 methylating activity. It is highly expressed in brain and testis which indicates possible roles in these organs. PRDM8 is distributed ubiquitously from embryo until postnatal 1 day and is further localized in the brain and testis in adult mice. We also detected 34 kDa alternatively spliced form in testis, liver, heart, and skeletal muscle. Although we did not investigate the 34 kDa form further in this study, we expect it has the similar biological functions as full-length PRDM8 considering the same SET domain it contains. However, it would be interesting to further investigate the small variant form which is dominantly expressed in heart.

The results of transient transfection reporter assay led us to conclude that PRDM8 has transcriptional repressive activity by methylating histone H3K9. However, treatment of TSA indicated that no HDAC is involved in this process. A possible regulatory role of PRDM8 in testis development was suggested when we overexpressed PRDM8 before and after LH treatment. The significant level of *p450* and *LHR* expressions were repressed by PRDM8 when steroidogenesis was induced in TM3 cells. The fact that overexpression of deletion mutant PRDM8, which is defective in its HMTase activity, did not repress *p450* and *LHR* expression strongly suggests that the HMTase activity of PRDM8 is important for regulatory activity. Based on the above results, we conclude that PRDM8 regulates testis development by repressing the transcription of steroidogenic markers *p450* and *LHR* through its HMTase activity.

Acknowledgments

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