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PRMT5 is required for human embryonic stem cell proliferation but not pluripotency

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Summary

Human pluripotent stem cells (PSCs) are critical *in vitro* tools for understanding mechanisms that regulate lineage differentiation in the human embryo as well as a potentially unlimited supply of stem cells for regenerative medicine. Pluripotent human and mouse embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts share a similar transcription factor network to maintain pluripotency and self-renewal, yet there are considerable molecular differences reflecting the diverse environments in which mouse and human ESCs are derived. In the current study we evaluated the role of Protein arginine methyltransferase 5 (PRMT5) in human ESC (hESC) self-renewal and pluripotency given its critical role in safeguarding mouse ESC pluripotency. Unlike the mouse, we discovered that PRMT5 has no role in hESC pluripotency. Using microarray analysis we discovered that a significant depletion in PRMT5 RNA and protein from hESCs changed the expression of only 78 genes, with the majority being repressed. Functionally, we discovered that depletion of PRMT5 had no effect on expression of OCT4, NANOG or SOX2, and did not prevent teratoma formation. Instead, we show that PRMT5 functions in hESCs to regulate proliferation in the self-renewing state by regulating the fraction of cells in Gap 1 (G1) of the cell cycle and increasing expression of the G1 cell cycle inhibitor P57. Taken together our data unveils a distinct role for PRMT5 in hESCs and identifies *P57* as new target.

Introduction

Self-renewal refers to the ability to proliferate while retaining the potential to differentiate. Pluripotency, refers to the potential to differentiate into all cell lineages of a mature organism. These two properties, self-renewal and pluripotency are the defining features of pluripotent stem cells (PSCs), which are *in vitro* cell types critical to the field of regenerative medicine. PSCs are generated from a number of sources, including embryonic stem cells (ESCs) derived from pre-implantation embryos [1–3] and by induced

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reprogramming to convert somatic cells to induced PSC (iPSC) [4–6]. Once derived and cultured under self-renewing (undifferentiated) conditions, PSCs are thought to have unlimited potential for cell division. Therefore PSCs represent powerful genetically malleable models to understand lineage decision events in the embryo, as well as an unlimited supply of stem cells that can be used to differentiate clinically relevant cell types to treat disease or injury.

Protein arginine methyltranferases (PRMTs) are a large family of arginine methyltransferase enzymes responsible for catalyzing the formation of mono methylarginine (MMA), asymmetric dimethylarginine ADMA and symmetric dimethylarginine (SMDA) in proteins of mammalian cells [7]. PRMT5 is the most well characterized family member with SMDA activity and catalyzes the formation of SMDA in glycine and arginine-rich motifs of proteins [8]. In the mouse, Prmt5 is critical for mouse ESC derivation, and a knockdown of Prmt5 in the undifferentiated state leads to up-regulation of genes associated with embryonic lineage differentiation together with a modest down-regulation of pluripotency transcription factors such as *Oct4*, *Nanog* and *Sox2* [9]. One mechanism by which Prmt5 functions in mouse ESCs is to SMDA histone H2A in the cytoplasm to generate H2AR3me2s. The modified histone is subsequently incorporated into the nucleus where it regulates expression of differentiation genes [9]. Given the importance of Prmt5 in regulating mouse ESC pluripotency, and the unique SDMA modification of H2A performed by Prmt5 in the cytoplasm of mouse ESCs, we set out to uncover the role of PRMT5 in regulating self-renewal and pluripotency in hESCs. In the current study we examined the role of PRMT5 in hESC self-renewal and pluripotency in the presence of KSR/FGF2 and unexpectedly discovered that unlike mouse ESCs, PRMT5 functions in hESCs to regulate proliferation and not pluripotency. Therefore, we have uncovered a different role for PRMT5 in hESCs and highlight the diverse functions of this protein in alternate cellular states.

Materials and Methods

Cell culture

The hESC lines HSF-1 (UC01, 46XY), H1 (WA01, 46XY), H9 (WA09, 46XX) and UCLA1 (46XX) were maintained under self –renewal conditions on mouse embryonic fibroblast (MEF) layer in DMEM:F12 (Gibco BRL), 20% KnockOut Serum (Gibco BRL), 1% nonessential amino acids (NEAA, Gibco BRL), 1 mM L-glutamine (Gibco BRL), 0.1 mM β-mercaptoethanol (Gibco BRL), and 10ng/ml of basic fibroblast growth factor (FGF) from R&D. Undifferentiated hESC colonies were maintained as previously described [10]. Differentiation was performed on plates coated with growth factor reduced matrigel (BD Pharmigen) in DMEM:F12 supplemented with 20% FBS (Gibco BRL), 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 1 mM L-glutamine. Media was changed every 2 days during differentiation. For all experiments, hESCs were used between passages 35 and 50. All hESC experiments were conducted with prior approval from the UCLA Embryonic Stem Cell Research Oversight Committee. BJ fibroblast somatic cells were cultured in minimum essential medium (MEM) with Earle’s salt (Gibco BRL) and 1 mM L-glutamine, 10% FBS (Gibco BRL), 1% NEAA and 1 mM sodium pyruvate (Gibco BRL). Cells were passaged using 0.25% trypsin (Gibco BRL) every 7 days. HEK 293 FT cells were grown in DMEM High Glucose (Gibco BRL) supplemented with 10% FBS, 1× Pen-Strep (Gibco BRL), 1 mM L-glutamine and 1 mM sodium pyruvate.

Western Blot

Proteins were extracted using M-PER (ThermoScientific) for whole-cell lysate and QProteome cell compartment kit (Qiagen) for nuclear and cytoplasmic fractions. Protein was quantified using the BCA Kit (Thermo), analyzed by electrophoreses on 12% NuPAGE

Novex Bis-Tris gels (Invitrogen) and transferred to Hybond ECL Nitrocellulose Membrane (GE Healthcare) according to standard procedures. Primary antibodies: PRMT5 (Abcam, Cat#12191) at 1:1000, OCT4 (Santa Cruz, Cat#sc-8628) at 1:1000, NANOG (Abcam, Cat#ab21624) at 1:300, H2A (Abcam, Cat#18255) at 1:1000, H2AR3me2s (Abcam, Cat# ab22397) at1:1000, H3 (Abcam, Cat#ab1791) at 1:1000 and β-actin (Abcam, Cat#ab8227) at 1:1000. Secondary HRP-conjugate antibodies were from Santa Cruz, all used at 1:5000 dilution. Blots were developed using ECL Western Blotting Detection Kit (GE Healthcare) according to manufacturer's instructions. Ponseau S (Sigma) staining was performed according to standard procedures.

Flow cytometry and Fluorescence Activated Cell Sorting (FACS)

Cells dissociated with TrypLE (Gibco BRL) at 37° C for 5 min and collected by centrifugation at 1.000 rpm for 5 min, were incubated in 1% BSA in PBS containing primary antibodies on ice for 20 min. Primary antibodies: SSEA 4 (Jackson ImmunoResearch, Cat#MC-813-70), TRA-1-60 (eBioscience, Cat#14-8863), TRA-1-81 (eBioscience, Cat#14-8883), TRA-1-85 (Jackson ImmunoResearch), all at 1:100 dilution. Cells were then washed and incubated in FITC- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) on ice for another 20 min. Before flow or FACS cells were passaged through a 40uM filter (BD). For the Annexin V assay, cells were collected, washed and incubated with 5 μl PE-conjugated Annexin V per 1×10^5 cells at room temperature in the dark for 15 min according to manufacturer's instructions (BD Pharmingen). 7-AAD (BD Pharmigen) was used as a viability dye at 1:50 dilution. Analysis was performed using LSR II (Becton Dickinson) and FlowJo software (Tree Star Inc). FACS was performed on a BD FACSAria with BD FACSDiva software.

EdU analysis

EdU was added at a 30 mM concentration and cells were incubated for 1 hour before EdU analysis was performed according to manufacturers' instructions using the Click-it EdU Flow cytometry Assay Kit (Invitrogen, Cat# C10425). EdU labeled cells were then incubated with DAPI at a 1 mg/ml concentration on ice for 30 min. EdU/DAPI labeled cells were analyzed by flow cytometry on LSR II (BD Biosciences). Graphs were generated using FlowJo software (Tree Star Inc).

Immunofluorescene

Immunofluorescence was performed on cells grown in 4 well chamber slides (BD Pharmigen), fixed with 4% paraformaldehyde for 10 min. Permeabilization was performed in PBS with 0.5% Triton X-100 (Sigma) for 10 minutes and blocking in PBS with 0.05% Tween20, 10% FBS for 10 min. Primary antibodies: PRMT5 (1:500, Millipore; Cat#07-405), OCT4A (1:100, Santa Cruz; Cat#sc-8628), incubated overnight at 4° C. Sections were washed, incubated with FITC/TRITC conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min and mounted in Prolong Antifade Reagent with DAPI (Invitrogen). Imaging performed on Zeiss Axio Imager (Zeiss) using Axio Vision 4.7 Software (Zeiss) or on Zeiss confocal LSM780 using Zeiss Microspore software Zen 2011.

Vectors

Sense and antisense oligonucleotides of the PRMT5 shRNA duplex were as follows: sense: 5′-GGTGCATATTTGGGTCTTC ttcaagaga GAAGACCCAAATATGCACC ttttttgt-3′, antisense: 5′-ctagacaaaaaa GGTGCATATTTGGGTCTTC tctcttgaa GAAGACCCAAATATGCACC-3′. The oligonucleotides were HPLC purified (Invitrogen). After annealing, the duplexes were cloned into the H1P lentiviral vector as already described

[11]. GFP was replaced by mCherry in H1P levtiviral vector by cloning the appropriate fragment in the AsiSI and KPNI unique restriction sites according to standard procedures.

Lentivirus production and hESC transduction

Lentiviral vectors were packaged, concentrated by ultracentrifugation at 50 000 g for 90 min, resuspended in hESC media and stored at −80° C as previously described [12]. Tittering was performed on Human Embryonic Kidney 293 FT cells. Transduction in hESCs line was performed at MOI 1 as previously described [12].

Limited dilution assay

H1 hESCs were transduced with either control or PRMT5 KD virus as previously described [12]. Cells were allowed to recover for 24 hrs after lentiviral transduction before selection in with hygromycin for 3 days. On the fourth day after transduction, cells were dissociated using TrypLE (0.25%, Gibco BRL) and plated at a density of 2000 cells/well on matrigel (BD Pharmingen) coated 24-well plates in mTeSR1 media (Stem Cell Technologies). Media was replaced every other day and colonies were counted 7 days after plating.

Competition assay

Hygromycin selected shRNA-transduced or control transduced hESCs cells were mixed with non-transduced hESCs 4 days after transduction at a ratio of 4:1 and plated on matrigel (BD Pharmigen) with MEF conditioned media. The GFP ratio was analyzed every 3–4 days by flow cytometry on LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc).

RNA extraction and semi qRT-PCR

RNA was extracted using the RNeasy Micro Kit (Qiagen) or the miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was reverse-transcribed using Superscript RT II (Invitrogen). For miRNAs, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Semi qRT- PCR was performed using Taqman Gene Expression Assays (Applied Biosystems) according to manufacturers instructions with TaqMan Gene Expression Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) for miRNAs. Results were normalized against GAPDH or RNU48 for miRNAs.

Microarray Analysis

Microarray targets were prepared using Nu-GEN WT-Ovation FFPE RNA Amplification System and FL-Ovation cDNA Biotin Module V2, and then hybridized to the Human Genome U133 Plus 2.0 Array. Data analyses involved the model-based expression and invariant set probe normalization using D-chip software. Differentially expressed genes between Control and PRMT5 KD were selected at ≥1.5 fold and $p<0.05$. Gene ontology (GO) terms were identified using DAVID (http://david.abcc.ncifcrf.gov/; [13, 14]).

Generation of teratomas

Surgery was performed following Institutional Approval for Appropriate Care and use of Laboratory animals by the UCLA Institutional Animal Care and Use Committee (Chancellor's Animal Research Committee (ARC)), Animal Welfare assurance number A3196-01. Briefly, for testicular tumors, a single incision was made in the peritoneal cavity and the testis was pulled through the incision site. Using a 27-gauge needle, two confluent wells of hESCs were harvested using collagenase and re-suspended in a volume of 50 μl 1X

cold Matrigel (BD) were transplanted into the testis of adult SCID-Beige mice. Six weeks after surgery, mice were euthanized and the tumors removed for histology.

Results

Given that the transcription rate and stability of *PRMT5* mRNA is reported to be low in cell lines *in vitro* [15] we assayed the mRNA levels of *PRMT5* in four independently derived lines of hESC (H1, HSF-1, H9 and UCLA1) to determine if there was any variability in expression between cell lines in the undifferentiated state (Fig. 1a). Using semi-quantitative PCR (qPCR) across multiple replicates between passage 35 and 50 we show no difference in the mRNA levels of *PRMT5* between independent hESC lines (Fig 1a). We also compared *PRMT5* mRNA expression to human somatic cell lines and found that *PRMT5* expression in hESCs was lower than transformed HEK 293 cells, but higher when compared to primary human foreskin fibroblast (BJ) cells (Fig. 1a). Next we examined the intracellular localization of PRMT5 in each hESC line using Western blot of nuclear and cytoplasmic fractions (Fig. 1b). Our data demonstrate that PRMT5 protein is enriched in the cytoplasmic fraction of all hESCs lines and is below the level of detection in the nucleus. Localization of PRMT5 to the cytoplasm is similar to what has been reported previously for mouse ESCs cultured in the presence of LIF [9]. In order to address whether there is heterogeneity of PRMT5 protein expression in hESCs we co-stained PRMT5 with OCT4A and found that PRMT5 expression was cytoplasmic and uniformly expressed by all OCT4A cells in a colony (Fig. 1c). Next, we designed a shRNA construct to knock down *PRMT5* using lentiviral transduction under self-renewing conditions in the presence of FGF2 and KSR (Fig. 1d). By day 4 of selection in hygromycin we observed a significant depletion in *PRMT5* mRNA in two independent lines of hESCs transduced with knockdown (KD) vector relative to cells transduced with control vector. In order to evaluate the effect of *PRMT5* KD on SMDA in H2A (H2AR3me2s), we transduced H1 hESCs with control and PRMT5 KD vectors and evaluated cytoplasmic and nuclear histones after 4 and 11 days. Our data reveals that PRMT5 KD causes a significant depletion in cytoplasmic PRMT5 at day 4 and 11 post transduction. This is associated with a reduction in the cytoplasmic pool of the 25–40 kDa species of H2AR3me2s at day 11. In contrast, the levels of the processed 17 kDa species of H2AR3me2s found in the nucleus at day 11 were unaffected. Interestingly there was a similar reduction in the cytoplasmic pool of histone H2A together with H2AR3me2s, suggesting that the majority of H2A in the cytoplasm is modified by SDMA (Fig. 1e). Notably β-actin protein levels in the cytoplam were unaffected indicating this reduction in protein was specific to PRMT5, H2A and H2AR3me2s in our experiment. To determine whether global gene expression was altered with PRMT5 KD, we performed microarray analysis (Fig. 1f). HSF-1 hESCs were transduced with control or PRMT5 KD lentiviral vectors on day 0. Transduced HSF-1 cells were allowed to recover for 24 hours and then selected with hygromycin for an additional 3 days. In order to avoid contamination with the supporting mouse fibroblast cell layer in the down stream microarray analysis we sorted hESCs for enhanced Green Fluorescent Protein (eGFP) which was expressed from the Ubiquitin promoter in the shRNA construct, and TRA-1-85 (a pan human surface antigen). Microarray was performed on mRNA from three independent biological replicates of sorted PRMT5 KD cells and biological duplicates of control cells four days after transduction (Fig. 1f). Surprisingly we observed very little change in gene expression in hESCs with a KD of *PRMT5* ($p<0.05$ 1.5 fold cut-off for differential expression). Specifically 84 accession numbers corresponding to 78 unique genes were differentially expressed after four days of PRMT5 repression. The most down-regulated gene was *PRMT5*, and of the additional down-regulated genes, two were known developmental genes such as *LEFTY2* and *KLF7*, whereas the others were associated with basic cell biological processes including cell adhesion, for example junctional adhesion molecule 3 (*JAM3*) or integral membrane proteins, such as transmembrane protein 85 (*TMEM85*). Of the sixteen unique genes that

were significantly up-regulated in *PRMT5* KD cells, none were associated with embryonic lineage differentiation, and instead many were associated with cell cycle regulation, cell motility and morphology such as *CDKN1C* (also known as *P57 or KIP2)*, P21-activating kinase 1 (*PAK1*) and tumor protein p53 inducible nuclear protein 1 (*TP53INP1*), as well as an RNA editing gene *APOBEC3B*, the kinase Testis-specific kinase 1 (*TESK1*) and the homeodomain interacting protein kinase 2 (*HIPK2*). For a complete list of differentially expressed genes please refer to Supplemental Table 1. Taken together, the depletion of *PRMT5* mRNA from undifferentiated hESCs caused a reduction in the cytoplasmic fraction of H2AR3me2s, as reported for mouse ESCs [9], however unlike mouse we observed a very modest change in the expression levels of a limited number of genes, and depletion of PRMT5 did not affect the expression of pluripotent transcription factor mRNAs such as *OCT4, NANOG* or *SOX2*.

Next, in order to address whether PRMT5 KD affects the protein levels of OCT4 and NANOG, we evaluated PRMT5 KD and control hESCs at day 4 and day 11-post transduction by Western blot (Fig. 2a). As expected, we find that PRMT5 protein is significantly down regulated at both time points relative to control. In contrast, NANOG and OCT4 protein levels were unaffected. This data supports the microarray analysis and strongly suggests that PRMT5 has a limited role in regulating pluripotent gene expression in hESCs in the undifferentiated state. To further confirm our hypothesis we examined the localization of OCT4A protein in hESCs at day 4 and day 11-post transduction (Fig. 2b). OCT4A functions in the nucleus to regulate pluripotency, however there are reported cases where OCT4 protein has been observed in cytoplasm where it would be incapable of binding to its transcriptional targets [16, 17]. Our data reveals that OCT4A protein localization is indistinguishable in PRMT5 KD relative to control cells (Fig. 2b). We also evaluated the expression of self-renewing surface markers Stage Specific Embryonic Antigen 4 (SSEA 4) and TRA-1-60 by flow cytometry (Fig. 2c). We find that SSEA 4 fluorescence intensity is indistinguishable between all samples. With regard to TRA-1-60, our data reveals a spread in the fluorescence intensity at day 11-post transduction in the PRMT5 KD cells. However, we did not see an increase in cells that were negative for TRA-1-60 (Fig. 2c).

To further ensure that self-renewal was not affected in PRMT5 KD hESCs relative to control we performed a limited dilution assay where, H1 hESCs transduced with either control or PRMT5 KD lentivirus were trypsinized and plated as single cells at a density of 2000 cells/well on matrigel coated 24-well plates. Colony forming potential was quantified 7 days after plating and was similar when comparing PRMT5 KD transduced H1 hESCs to cells transduced with control vector (Fig. 2d). Next, to address whether a PRMT5 KD affects pluripotency we transduced the PRMT5 shRNA KD lentiviral vector into H1 cells and performed qRT-PCR to monitor the relative expression of lineage specific differentiation genes either under self-renewal conditions or with hESC differentiation. Our results indicate that PRMT5 is not involved in regulating pluripotency in the undifferentiated state or upon induction of differentiation for 4 days, as expression of *GATA6*; a marker for mesoderm, *CXCR4*; a marker for endoderm and *NCAM1* an ectodermal marker were not statistically different between PRMT5 KD and control cells in either self-renewal or differentiation conditions (Fig. 2e–f). Finally to further validate that loss of PRMT5 has no effect on hESC pluripotency we performed a teratoma assay. For this, we transduced the PRMT5 shRNA KD lentiviral vector into hESCs under self-renewing conditions and after four days the PRMT5 KD hESCs were transplanted under the testis capsule of immunocompromized SCID Beige mice (Fig. 2g). If a knockdown of PRMT5 affects the potential for differentiation we would anticipate no teratoma after 6 weeks. In contrast, if PRMT5 has no role in hESC pluripotency, we would anticipate that PRMT5 KD cells should form teratomas with examples of differentiation from all three lineages. Our data reveals that PRMT5 KD does not affect the pluripotent potential of hESCs when

depleted in the undifferentiated state, and teratomas were acquired containing examples of all three embryonic linages including mesoderm, endoderm and ectoderm (Fig. 2g). Taken together, our data demonstrates that human PRMT5 does not have a role in regulating human pluripotency or the expression of self-renewal markers such as SSEA 4 or TRA-1-60 in undifferentiated hESCs.

Given that our microarray analysis revealed a potential role for PRMT5 in cell cycle progression we established a competition assay to determine whether PRMT5 KD hESCs exhibit alterations in cell growth over time (Fig. 3a). Given that the lentiviral vector in these experiments contained an eGFP expression cassette under control of the Ubiquitin promoter, the competition assay assessed the competition between transduced (eGFP+) cells with non-transduced (GFP-) cells over fourteen days. Since this experiment is performed in the absence of antibiotic selection, cells transduced with the control vector are evaluated simultaneously and the ratio of GFP+ cells in the PRMT5 KD cells is normalized to the ratio of GFP+ cells transduced with control vector which is set to 1 at each time point. Using two independent lines of hESCs and evaluating each hESC line in biological duplicate we show that PRMT5 KD cells are outcompeted by non-transduced cells starting at day 8-post transduction (Fig. 3a). This result demonstrates that PRMT5 repression reduces hESC proliferation and/or survival *in vitro* under self-renewing conditions.

In order to determine whether a knockdown of PRMT5 affects survival we examined live cells, Annexin V (apoptotic) and 7AAD/Annexin V double positive (dead) cells by flow cytometry. We found no statistically significant difference between the fractions of live, apoptotic or dead cells when comparing PRMT5 KD to control (Fig. 3b). Next we evaluated cell cycle dynamics of hESCs based on EdU incorporation (Fig. 3c) and show that depletion of PRMT5 results in a significant increase in the fraction of hESC in the G1/G0 phase of the cell cycle with a concomitant small yet significant decrease in cells in G2/M. To confirm whether this delay is associated with the G1/S cyclin-dependent kinase regulator *P57* which was significantly up-regulated by microarray (Fig. 1f), we performed qPCR in n=6 replicates of *P57* and compared our results to a second G1/S cell cycle regulator P21, which was unaffected by microarray (Fig. 3d). Our results show that as anticipated, *P57* was significantly up-regulated in PRMT5 KD hESCs whereas *P21* mRNA levels were unchanged relative to control. Given that the PRMT5 KD cells had reduced cells in G2/M we also examined *WEE1*, a critical regulator for mitotic entry in hESCs [18]. Our data shows that similar to *P21*, the levels of *WEE1* mRNA are also not affected. We also examined two microRNAs, *miR-92b* and *miR-93* that are highly expressed in hESCs. The miRNA *miR-92b* has been shown to target *P57* for degradation and augment PRMT5 translation [15, 19], whereas *miR-93* targets is predicted to target *P57* mRNA for degradation together with *P21*, *TP53INP1* [20–22]. Our microarray reveals that both *P57* and *TP53INP*1 were de-repressed in PRMT5 KD cells. Our data shows that neither microRNA is affected in PRMT5 KD hESCs in n=4 biological replicates and opens up a new line of investigation into the regulation of *P57* and G1 cell cycle control by PRMT5 in hESCs (Fig. 3d).

Discussion

In this study, we sought to determine the role of PRMT5 in self-renewal and pluripotency of hESCs. Recent work has highlighted the importance of Prmt5 in safeguarding pluripotency in mouse ESCs using knockdown, and also as a facilitator of murine reprogramming when expressed together with Oct4 and Klf4 during reprogramming [9, 23, 24] yet the importance of PRMT5 in hESC biology was unknown. Our data reveals that human PRMT5 does not play a role in hESC pluripotency, and instead regulates hESC proliferation under self-renewing conditions by acting upstream of the G1/S checkpoint protein P57. Our work

shows that PRMT5 has a unique role in human ESC proliferation *in vitro*, which is critical for enabling the expansion of hESC populations under self-renewing conditions.

One of the most important observations in the current study is the finding that PRMT5 does not have a conserved role in regulating hESC pluripotency when comparing our data to previous reports with mESCs [9]. Human ESCs are similar to mESCs in that they are derived from pre-implantation blastocysts, however the derivation conditions for the two species are distinct. Specifically, hESCs are derived and maintained in the presence of Fibroblast growth factor 2 (FGF2) and Knockout Serum Replacement (KSR) and unlike mouse ESCs do not require LIF to maintain pluripotency. These culture conditions are very similar to the conditions used to derive epiblast stem cells (EpiSCs) from post-implantation mouse embryos [25, 26]. Given the similarities in growth properties and transcriptional profile of hESCs relative to mouse EpiSCs it is speculated that hESCs represent an alternate (poised) pluripotent state similar to mouse EpiSCs and distinct from the naïve pluripotency observed when mESCs are cultured in LIF. In future studies it will be important to examine the role of Prmt5 in mouse EpiSCs to determine whether the differences between mouse and human are due to either derivation and culture differences or species-specific differences (Fig. 4). Similarly, further investigation is also required to determine the role of Prmt5 in mESC ground state pluripotency which involves culturing mESCs in LIF plus a serum free inhibitor-based chemically defined medium, termed 2i (Fig. 4).

The ability of undifferentiated hESCs to proliferate is critical for generating sufficient numbers of cells for use in regenerative medicine. Cell cycle regulation in hESCs is distinct from somatic cells because hESCs exhibit a highly abbreviated G1 which is reversible upon induction of differentiation [12]. Given the tight coupling of a short G1 phase to the undifferentiated pluripotent state, it has been postulated that the unique G1 cell cycle control in hESCs is one mechanism by which hESCs remain undifferentiated. In support of this, depleting the G1/S checkpoint protein cyclin dependent kinase 2 (CDK2) from hESCs causes a massive G1 arrest, repression of OCT4, NANOG and SOX2 and spontaneous differentiation [27]. More recently the heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1) were also shown to regulate the G1/S transition and a knockdown of hnRNP A2/B1 resulted in a phenotype that closely resembled repression of CDK2 [28]. In both cases, induction of G1 arrest was associated with a significant increase in P21. In the current study we show that modulating the G1/S checkpoint downstream of PRMT5 does not affect *P21* and also does not cause repression of *OCT4 or NANOG* mRNA or protein. Critically, depletion of PRMT5 in the self-renewing state also did not cause loss of pluripotency. Therefore we speculate that the difference between PRMT5's effect on increasing the fraction of cells in G1 while not affecting differentiation is due to the unique cell cycle target downstream of PRMT5 in hESCs.

It is well known that PRMT5 functions as a negative regulator of the G1/S transition in somatic cells in culture. For example, an antisense knockdown of Prmt5 in the mouse fibroblast cell line NIH 3T3 results in slower growth, slower transition from G1 to S and an increase in transcription of tumor suppressors [29]. In human embryonic kidney 293T cells, a PRMT5 knockdown or over expression modulates levels of G1 cyclins-cdk complexes including CDK4 and CDK6 [30]. In the breast cancer cell line MCF7 a knockdown of PRMT5 induces cell cycle arrest in G1 by modulating expression of eukaryotic translation initiation factor 4E [31]. Thus the effect of PRMT5 on the G1/S transition in somatic cells is diverse and cell-type dependent, yet the link between PRMT5 and P57 has never been reported.

P57 is a cell cycle protein that negatively regulates the G1 cyclin-cdk complexes. It was recently shown that hESCs under wild type conditions express low levels of *P57* on account

of a hESC enriched miRNA called *miR-92b* which targets P57 for degradation [19]. By analyzing *miR-92b* expression we ruled out the hypothesis that PRMT5 modulates *P57* expression by depleting the levels of *miR-92b* in hESCs. As an alternate hypothesis, we speculated that PRMT5 acted upstream of a different microRNA called *miR-93* given that *TP53INP1* (the target of this miRNA), as well as P57, a predicted target were both de-repressed in PRMT5 KD cells. However, similar to *miR-92b*, the levels of *miR-93* remained unaffected in the PRMT5 KD cells. Therefore, although our data demonstrate that *P57* mRNA is negatively regulated by PRMT5 in hESCs, the major miRNA that regulates *P57* remains unperturbed. Therefore, future studies should be aimed at understanding the mechanism by which PRMT5 regulates *P57* in hESCs and this could be through SDMA of histones at the *P57* promoter and/or enhancer. An example of PRMT5s role in regulating discreet transcriptional targets was recently found for *E-cadherin* during epithelial to mesenchymal transition [32]. In this scenario, PRMT5 was shown to translocate to the nucleus in a SNAIL and AJUBA dependent manner to repress *E-cadherin* expression by inducing SDMA of histone H4 [32]. Alternatively, PRMT5 may also be inducing post-translational modifications on proteins that affect *P57* transcription or RNA stability.

Towards this, our data reveals that the cytoplasmic localization of PRMT5 and the unique SDMA of H2A in the cytoplasm, first discovered in mouse ESCs is also conserved in hESCs. However, globally by Western blot our data reveals that the levels of H2AR3me2s are not altered in the nucleus after 11 days of knockdown despite considerable depletion of the cytoplasmic pool. This result may reflect the slow global turnover of H2AR3me2s in the nucleus, or alternatively the presence of a second PRMT that can compensate specifically in the nucleus to catalyze SMDA of H2A in chromatin. We speculate that the large molecular weight of histone H2A and H2AR3me2s in the cytoplasm may be due to additional post-translational modifications to H2A that are removed upon entrance into the nucleus to generate the 17Kda species of H2A that is incorporated into chromatin. Our *PRMT5* knockdown data demonstrates that the majority of high molecular weight cytoplasmic H2A in hESCs is modified with SDMA and that the absence of SMDA may target H2A for destruction.

In conclusion, hESCs represent an ideal model to study the mechanisms that regulate human pluripotency, self-renewal and embryonic lineage differentiation. Here, we show that unlike mouse ESCs, hESCs do not utilize PRMT5 to safeguard pluripotency, and instead PRMT5 acts to regulate the fraction of hESCs in the G1 phase of the cell cycle. Our data also confirms the unique enzymatic activity of PRMT5 on the histone H2A in the cytoplasm of undifferentiated hESCs, however this does not translate to changing the global levels of H2AR3me2s in the nucleus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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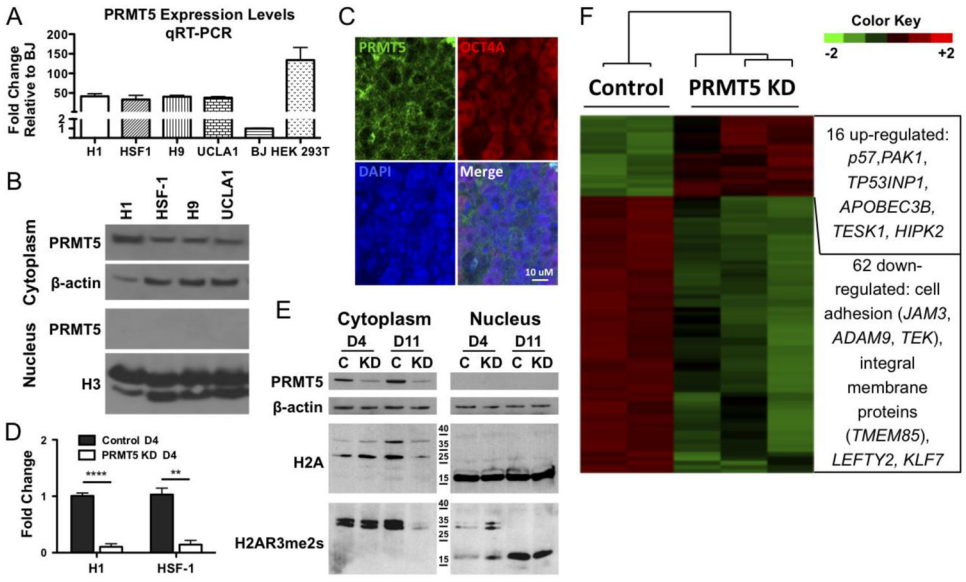


Fig. 1. PRMT5 expression and KD in hESCs

(**a**) qRT-PCR of *PRMT5* in hESC lines and two human somatic lines; BJ foreskin fibroblasts and HEK 293T cells. Expression is shown as fold change relative to BJ fibroblasts. (n=2 biological replicates performed in technical duplicate). (**b**) Western blot for PRMT5 in cytoplasmic and nuclear fraction of indicated cell lines. β-actin was used as loading control for cytoplasmic fraction, histone 3 for nuclear fraction. (**c**) Immunofluorescence staining of PRMT5 in green with OCT4A in red in H1 hESCs. Nuclei are counterstained with DAPI. (**d**) qRT-PCR of *PRMT5* in H1 and HSF-1 cells, 4 days after transduction and hygromycin selection with control and PRMT5 KD lentivirus. (n=3 biological replicates performed in technical duplicate). *t* test was used to calculate statistical significance. ** is $p < 0.005$ and **** is $p<0.0001$. (**e**) Western blot for PRMT5, β-actin and histones H2A, H2AR3me2s, in cytoplasmic and nuclear fraction of H1 hESCs transduced with control or PRMT5 KD lentivirus for 4 and 11 days. Molecular sizes indicated in Kilodalton (kDa). (**f**) Microarray analysis of FACS sorted TRA-1-85 expressing HSF-1 hESCs, 4 days after transduction and hygromycin selection with control and PRMT5 KD lentivirus. Shown is a heatmap of differentially expressed genes between control (in biological duplicate) and PRMT5 KD (in biological triplicate) at $p<0.05$ and 1.5 fold cut-off. Error bars indicate Standard Error of the Mean (SEM). Abbreviations: qRT-PCR, quantitative Real-Time PCR; HEK, Human Embryonic Kidney; PRMT5, Protein Arginine Methyltransferase 5; KD, Knock-Down; D4, day 4; D11, day 11.

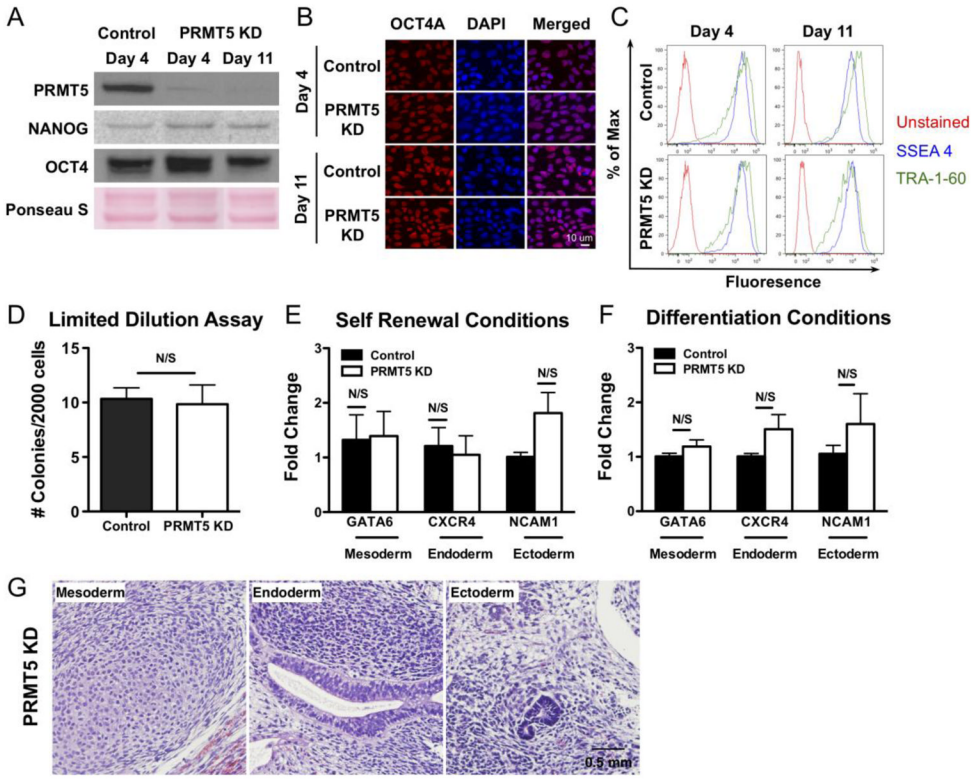


Fig. 2. PRMT5 KD does not affect self-renewal or pluripotency in hESCs

(**a**) Western blot of HSF-1 comparing PRMT5, OCT4 and NANOG protein levels, 4 and 11 days after lentiviral transduction in control and PRMT5 KD cells. Note that we detect two protein bands for OCT4. We speculate that the lower band corresponds to a phosphorylated form that is occasionally observed. Ponseau S staining was used as loading control. (**b**) Immunofluorescence of OCT4A in HSF-1 cells, 4 and 11 days after transduction with control or PRMT5 KD lentivirus. Nuclei were counterstained with DAPI (blue). (**c**) Flow cytometry analysis of HSF-1 cells transduced with control and PRMT5 KD lentivirus at day 4 and 11 after transduction and hygromycin selection. SSEA 4 and TRA-1-60 are surface markers found on undifferentiated pluripotent stem cells (PSCs). (**d**) Limited dilution assay comparing the colony formation potential of H1 hESCs transduced with Control or PRMT5 KD virus. 4. Colonies are counted 7 days after plating. Data are collected from n=4 biological replicates. (**e**) qRT-PCR on FACS sorted GFP/TRA 1-81 expressing H1 cells cultured under self-renewal conditions depicting the expression levels of differentiation-associated genes in PRMT5 KD compared to control transduced H1 cells, (**f**) qRT-PCR on FACS sorted GFP expressing H1 cells upon induction of differentiation for 4 days, depicting the expression levels of differentiation-associated genes in PRMT5 KD compared to control transduced H1 cells. (For (**e**–**f**), n=4 biological replicates performed in technical duplicate). Significance in each case was calculated using *t* test and error bars indicate SEM. (**g**) Hematoxylin and eosin staining of teratomas formed in the testis of SCID beige mice after injection of HSF-1 hESCs transduced with PRMT5 KD lentivirus. Testes were collected and sectioned 6 weeks after injection. Shown are representative images of all three germ layers formed within the teratoma; mesoderm, endoderm and ectoderm. Abbreviations: PRMT5 Protein Arginine Methyltransferase 5; KD, Knock-Down; SSEA 4, Stage Specific Embryonic Antigen 4; N/S, not significant.

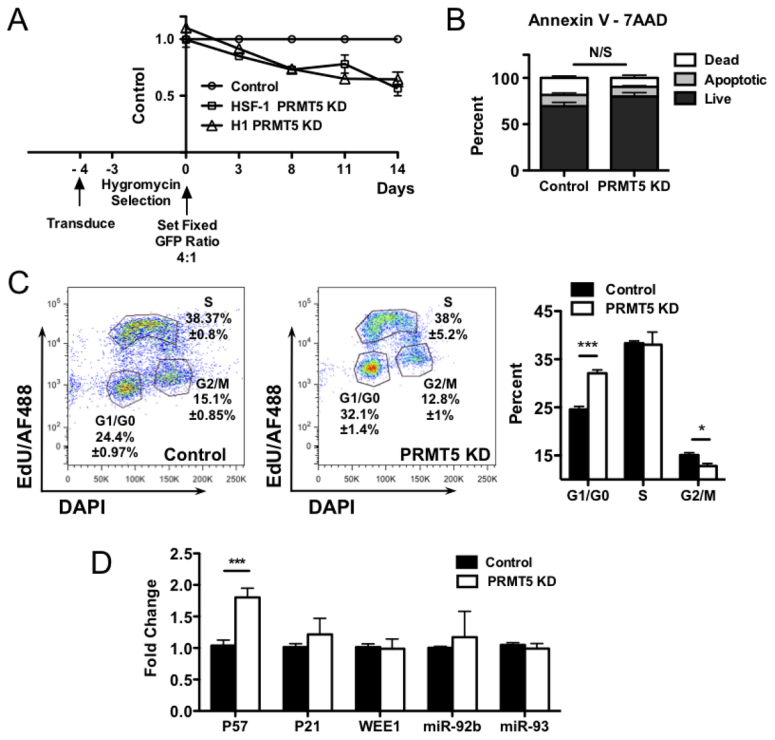


Fig. 3. PRMT5 KD results in a proliferation defect, cell-cycle deregulation and increased expression of *P57*

(**a**) Competition assay of HSF-1 and H1 hESCs transduced with either control or PRMT5 KD lentivirus and non-transduced cells of the same genetic background. Lines correspond to GFP levels detected by flow cytometry. For each time point the ratio between control GFP^+ and GFP^- cells is set to 1 while GFP ratio in HSF-1 and H1 PRMT5 KD cells for each time point is relative to control (n=2 biological replicates performed in technical duplicate for each line). (**b**) Histogram of Annexin V-7AAD staining showing the percent of live, apoptotic and dead H1 hESCs in PRMT5 KD relative to control. Live cells are negative for both Annexin V and 7AAD staining, apoptotic cells are Annexin V positive and 7AAD negative while dead cells stain positive for both Annexin V and 7AAD. Data are collected from n=3 biological replicates. (**c**) Cell cycle analysis of H1 hESCs transduced with control and PRMT5 KD lentivirus based on EdU incorporation. Data are collected from n=4 biological replicates and ± values correspond to standard deviation. (**d**) qRT-PCR analysis for *p57*, *p21*, *WEE1*, *miR-92b* and *miR-93*. For *p57*, *p21* and *WEE1* n=6 biological replicates performed in technical duplicate, for *miR-92b* and *miR-93*, n=4 biological replicates performed in technical duplicate. * is $p< 0.05$ and *** is $p<0.0005$. Significance in each case was calculated using *t* test and error bars indicate SEM. Abbreviations: PRMT5 Protein Arginine Methyltransferase 5; KD, Knockdown; GFP, Green Fluorescence Protein; N/S, not significant.

Role of PRMT5 in Pluripotent Stem Cells

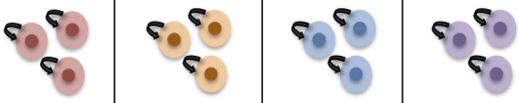
	mESCs		mEpiSCs	hESCs
	2i	FBS+LIF	KSR+FGF2	KSR+FGF2
				
Pluripotency	?	Yes	?	No
Differentiation	?	Yes	?	No
Cell cycle	?	No	?	Yes

Fig. 4. Diverse roles for PRMT5 in Pluripotent Stem Cells

Naïve mESCs maintained in media containing Fetal bovine serum (FBS) + leukemia inhibitory factor (LIF) require Prmt5 to safeguard pluripotency. In the current study we show that hESCs cultured in media containing knockout serum replacement (KSR) + fibroblast growth factor 2 (FGF2) require PRMT5 to regulate the fraction of cells in the G1 phase of the cell cycle with no role in pluripotency. The role of Prmt5 in ground state mESCs cultured in 2i + LIF, a chemically defined inhibitor-based media, or poised mEpiSCs cultured in KSR+FGF2 are currently unknown.