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PRMT5 is an essential survival factor for ground state pluripotency and primordial germ cells: From primordial germ cell differentiation in vitro to mammalian germline in vivo

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PRMT5 is an Essential Survival Factor for Ground State Pluripotency and Primordial Germ Cells: From Primordial Germ Cell Differentiation \textit{in vitro} to Mammalian Germline \textit{in vivo}

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cell & Developmental Biology

By

Ziwei Li

2014
ABSTRACT OF THE DISSERTATION

PRMT5 is an Essential Survival Factor for Ground State Pluripotency and Primordial Germ Cells: From Primordial Germ Cell Differentiation in vitro to Mammalian Germline in vivo

By
Ziwei Li

Doctor of Philosophy in Molecular, Cell & Developmental Biology
University of California, Los Angeles, 2014
Professor Amander Therese Clark, Chair

Our ancestors and our children are linked by a single, special cell lineage called the germline. Germ cells are tasked with the role to accurately pass DNA from one generation to the next. In today’s society infertility is an important health concern as it is estimated to affect 10% of the reproductive age population. In many cases infertility can be traced to abnormal germline cell development. The early events of germline formation are difficult to study because of limited materials, especially low germ cell numbers in the process of embryogenesis.

To overcome the challenge of limited materials, an in vitro method to derive cell types faithfully reporting in vivo phenotypes needs to be devised to facilitate the research process. The key results from the in vitro model should then be
evaluated carefully in vivo to show relevance in development. My thesis first focused on establishing an in vitro model to recapitulate early development of germline, by differentiating embryonic stem cells (ESCs) to form in vitro primordial germ cells (iPGCs), in a dish. This method allows good number of germ cells to be produced for molecular and biochemical studies. Using the in vitro model, key germline modifiers for ESC maintenance and germ cell formation were identified, one of which is protein arginine methyltransferase 5 (PRMT5).

PRMT5 is a type II arginine methyltransferase (PRMT) that modifies symmetrical dimethylated arginines (SDMAs) on proteins, which substrates include histone H2A and H4, as well as the Sm proteins involved in RNA splicing. However, the molecular function of PRMT5 in ground state ESCs remains unknown. In our study, for the first time, we generated an inducible knock out of Prmt5 in ESCs cultured in ground state pluripotency (cultured with 2i inhibitors and leukemia inhibitory factor). We characterized PRMT5’s role in ESCs through the method of paired-end RNA sequencing to show that PRMT5 affects cell survival, proliferation and chromatin organization etc., by affecting RNA splicing. By generating a conditional knock out to specifically deplete PRMT5 in the germline in vivo, we showed that PRMT5 is necessary for mammalian PGC formation.

Taken together, we developed a differentiation system to ask questions about key factors regulating ESC and germline development. One of the key factors we identified is PRMT5. The role of PRMT5 was scrutinized using paired-end RNA
sequencing in the inducible knock out ESCs, suggesting that PRMT5 is necessary to ensure normal splicing in “RNA processing”, “DNA damage response” and “chromatin modification” for cell to survive. Finally, we utilized in vivo mouse model to validate events that happen in vitro, showing that PRMT5 is indeed a survival factor both for ground state pluripotency and mammalian germline.
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2014
DEDICATION

In memory of my grandfather, Shaochuan Li
## Table of Contents

Abstract .......................................................................................................................................................... ii

Committee Page ........................................................................................................................................ v

Dedication Page .......................................................................................................................................... vi

Table of Contents ....................................................................................................................................... vii

List of Figures ............................................................................................................................................. viii

Acknowledgements ..................................................................................................................................... x

Vita ............................................................................................................................................................ xiii

Chapter 1: Introduction .............................................................................................................................. 1

Reference ................................................................................................................................................... 27

Chapter 2: Identifying the potential role of PRMT5 in mammalian germline differentiation using an in vitro tool that stages primordial germ cells derived from mouse embryonic stem cells ................................................................................................................................. 42

Reference ................................................................................................................................................... 74

Chapter 3: PRMT5 is an essential survival factor for ground state pluripotency and primordial germ cells .................................................................................................................................................................................... 80

Reference ................................................................................................................................................... 106

Chapter 4: Conclusion and Preface .......................................................................................................... 134

Reference ................................................................................................................................................... 144
List of Figures

Chapter 1

Figure 1-1 ................................................................................................. 8
Figure 1-2 ................................................................................................. 10
Figure 1-3 ................................................................................................. 12
Figure 1-4 ................................................................................................. 13

Chapter 2

Figure 2-1 ................................................................................................. 64
Figure 2-2 ................................................................................................. 66
Figure 2-3 ................................................................................................. 68
Figure 2-4 ................................................................................................. 69
Figure 2-5 ................................................................................................. 70
Figure 2-6 ................................................................................................. 71
Figure 2-S1 ................................................................................................. 72
Table 2-1 ................................................................................................. 73

Chapter 3

Figure 3-1 ................................................................................................. 116
Figure 3-2 ................................................................................................. 117
Figure 3-3 ................................................................................................. 118
Figure 3-4 ................................................................................................. 119
Figure 3-5 ................................................................................................. 120
Figure 3-6 ................................................................................................. 121
Figure 3-7 ................................................................................................. 122
Figure 3-S2 ................................................................................................. 124
Figure 3-S5 ................................................................................................. 125

Chapter 4
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CHAPTER 1

INTRODUCTION
Our ancestors and our children are linked by a single, special cell lineage called the germline, “oocytes and sperm”, which are terminally differentiated and haploid cells necessary to fertilize, in order to form offspring. Germ cells are tasked with the role of accurately passing DNA from one generation to the next. In today’s society infertility is an important health concern as it is estimated to affect 10% of the reproductive age population\(^1\). In many cases infertility can be traced to abnormal germ cell development. Therefore, study of the mechanisms within germ cell development is of utmost importance to guide clinical treatment of infertility\(^2-5\).

Due to material limitations, the study of the very early events of human germline formation are inaccessible since germ cells start to form even before pregnancy is realized. Also, the fetal materials for study can only be obtained from elected terminations with approved IRB protocols. Therefore, the majority of studies about early mammalian germline formation are performed using the mouse model, given its is genetically malleable through transgenics or embryonic stem cells and these genetic changes can be passed through the germline\(^6-10\).

However, in vivo studies of newly specified mouse germ cells are challenging due to the fact that the onset of germline happens at the time of implantation (Embryonic day (E) 5.5-E6.25) in mouse embryos\(^11-19\) and approximately 40 germ cells are first specified at E7.5\(^12,20-22\). Germ cell number does not significantly increase until after the germ cells have entered the gonad at around
E11.5\textsuperscript{23-26}. Given this small number, large-scale biochemical studies that require a significant number of cells are impossible. The germ cells during this period from specification at E7.5 to complete colonization of the genital ridges and developmental arrest at E13.5 are referred to as \textbf{primordial germ cells (PGCs)}. 

In order to overcome this challenge, there are many studies investigating methods of generating PGC like cells or gamete like cells from a pluripotent cell type: \textbf{embryonic stem cells (ESCs)} and \textbf{induced pluripotent stem cells (iPSCs)}\textsuperscript{27-37}.

The efforts for generating functional gametes from ESCs started as early as 2003 by independent investigators from Dr. Hans Scholer's group\textsuperscript{29} and Dr. George Daley’s group\textsuperscript{38}. Hubner et al. from Scholer group identified a 2-D differentiation method using attached cell cultures with a \textit{gcOct4-GFP} reporter ESC line, resulting in oocyte-like structure as early as \textbf{Day (D) 26} and blastocyst-like structure at D 43\textsuperscript{29}. At around the same time, Geijsen et al. from Dr. Daley’s group identified a method of differentiating germ cell like cells by formation of \textbf{embryoid bodies (EBs)} using the hanging drop method\textsuperscript{38}. 30\textmu l droplets containing 200 cells were plated on the inverted petri-dish lid as hanging drops and collected into non-attachment plates at Day 3. After 3-4 more days of differentiation, SSEA1\textsuperscript{+} cells were sorted from EBs and further differentiated to form haploid male cells, which are able to fertilize oocytes following microinjections, with 50% of injected oocytes progressing to 2-cell stage and
20% progressing to blastocyst stage. These studies indicate that *in vitro* cell types sharing the feature of gametes could be achieved. However, the ability of germ cell like cells to generate live young has not yet been shown.

Following these studies, a majority of experiments have been performed in many groups around the world to form germ-cell like cells, either using the 2-D differentiation method, or using the hanging drop method to form EBs\textsuperscript{27,28,30-37}. Firstly, in order to understand germline development *in vitro*, investigators studied the timing of when known germline-specific genes become expressed *in vivo*. Some typical germline markers for gonadal stage differentiation included *Tex14, Piwil2, Dazl* and *Vasa*. In Geijsen et al., gonadal germline genes such as *Tex14, Piwil2* and *Dazl* were evaluated in SSEA1\textsuperscript{+} cells derived from EBs\textsuperscript{38}. However in Hubner et al., a pregonadal germline marker *ckit* and a gonadal marker *Vasa* were both used for sorted gcOct4-GFP\textsuperscript{+}/cKit\textsuperscript{+} populations, and oocyte markers *ZP 1-3* were used for the oocyte-like structures from 2-D differentiation\textsuperscript{29}. More recently, Wei et al. examined germline specification genes, such as *Blimp1* (expression starts at E6.25) and *Stella/Dppa3* (expression starts at E7.25), in Stella-GFP\textsuperscript{+} cells from both adherent differentiation and EB differentiation\textsuperscript{37}. In conclusion, absent in these studies is a thorough characterization of germline markers which are able to distinguish PGCs by specific stages of development, such as specification, migration and gonadal colonization, to investigate the molecular events within each particular developmental window (mammalian germline development discussed in page 7).
Secondly, most of these studies require ESC lines with a transgenic reporter that is often hard to obtain and create, such as gcOct4-GFP\textsuperscript{29}, Oct4\Delta PE-GFP\textsuperscript{31,39}, Dazl-GFP\textsuperscript{35}, Mvh-RFP\textsuperscript{30} and Stella-GFP\textsuperscript{34,37}, or rely on a single surface marker such as SSEA1\textsuperscript{38}, which is not exclusively expressed in the germline\textsuperscript{40}. The limitations of using the transporter lines are: 1-Changing the genetic background of a transporter line to another is difficult, involving derivation of new ESC lines in a different genetic background which usually takes 5 generations of mouse breeding. 2-PGC like cells isolating from a particular transporter line are referring to a developmental stage expressing the promoter driving the GFP or RFP, therefore overlooking other stages of PGCs. 3-To pinpoint the accurate stage of PGC like cells to endogenous equivalents, more than one reporter is often required. Therefore, a differentiation method without the use of transgenic reporters, but with more than one marker to pinpoint the correct stage of \textit{in vitro} PGCs is highly favorable.

In addition, staging \textit{in vitro} PGCs requires the evaluation of DNA methylation in the whole genome, or at imprinted loci, because DNA methylation is dynamically regulated in endogenous PGCs. Only a few studies analyzed the methylation status of the \textbf{differentially methylated regions (DMRs)} in imprinted genes including \textit{H19}\textsuperscript{32,38}, \textit{Igf2r}\textsuperscript{37,38}, \textit{Peg3}\textsuperscript{37} and \textit{KvDMR1}\textsuperscript{34} and no studies had quantified the global level of DNA methylation in derived \textit{in vitro} PGCs before our study initiated. Absent in all the studies is a well characterized method of
differentiating PGC like cells from a pluripotent stem cell source, with correct staging correspondent to the endogenous germline. Therefore, a systematic differentiation method, starting with defined cell numbers, with reporter free cell lines of different genetic backgrounds, should be invented to derive into germ-cell like cells correspondent to a defined developmental stage. Defined markers for a germ-cell population should be used to isolate germ-cell like cells, and the following aspects should be carefully examined to verify the true germ-cell characteristics: Germline gene expression relative to developmental equivalents, global DNA methylation level, specific methylation at **imprinting control center (ICC)** of imprinted genes and epigenetic states. Then we could utilize this *in vitro* method to derive PGCs from a dish and model endogenous PGC development with *in vitro induced PGCs (iPGCs)* to facilitate the process of screening for important germline modifiers, validate molecular events in the germline, as well as provide insights for creating germ cells in a dish.

The *in vitro* model provides large amounts of cell number to enable molecular and biochemical studies that are impossible using the endogenous germline. After the initial identification of crucial targets by the *in vitro* model, we could then validate roles of key germline modifiers *in vivo* using a transgenic mouse. Combining both *in vivo* and *in vitro* data together, we are able to unveil the role of essential germline genes in a complete and thorough way, which will provide guide and reference for future studies to generate functional gametes *in vitro*, ultimately leading to cure of infertility. The first step of achieving this goal is to
explore the knowledge of mammalian germline which provides guidance for iPGC differentiation in a dish.

1. Study of the mammalian germ line using mouse models

The study of human germline is limited due to human samples being rare, especially for the earliest stages of PGC development prior to 5 weeks post fertilization. Therefore, mouse models serve as a favorable tool to study mammalian germline, which allows different genetic manipulation and shares similar traits as human germline.

From the mouse model, we understand that PGC specification is initiated early in embryogenesis at around E6.0 by the expression of the germline master regulator Blimp1/Prdm1 in the proximal epiblast induced by paracrine signals from adjacent extraembryonic ectoderm and visceral endoderm, such as Bone Morphogenic Protein (BMP) 2, BMP4 and BMP8b\textsuperscript{13-19,41,42}. By E7.25, PGCs start to express Stella/Dppa3. Together with Blimp1, these are the earliest known markers of founder PGCs\textsuperscript{12,43,44}. After specification, PGCs undergo a migratory phase starting at around E8.0 from the allantois through the developing hindgut to finally reach the genital ridge at around E10.5\textsuperscript{22,45,46}. During this migratory phase, epigenetic reprogramming also happens in the germline. After PGCs enter and reside in the gonads, PGCs undergo sex determination as early as E12.5. Later, female germ cells will undergo meiotic arrest at Prophase I, while
male germ cells will undergo mitotic arrest. The time line of endogenous murine PGC development is summarized in Figure 1-1.

![Germ cell specification](image)

**Figure 1-1. Critical events of mammalian germline development.**

**Germ cell specification**

The inductive signals for germ cell specification have been identified using knock out studies\(^4^7\) and more recently culture experiments with cytokines\(^3\). Shown by genetic knock out studies using the mouse model, BMP signals play essential roles in the generation of **tissue-nonspecific alkaline phosphate (TNAP)** positive PGCs from the epiblast. BMP4 is expressed in the **inner cell mass (ICM)**, **extraembryonic ectoderm (ExE)** from E5.5, and **extraembryonic mesoderm (ExM)** during gastrulation. \(Bmp4^{+/−}\) embryos completely lacked PGCs and \(Bmp4^{+/−}\) embryos had reduced PGC number. Loss of BMP4 in the ExM caused aberrant PGC localization and impaired survival\(^1^6,4^8,4^9\). BMP8b is
expressed in ExE from E5.5 and the phenotype with loss of BMP8b resembled loss of BMP4\textsuperscript{19,50}. BMP2 is expressed in \textit{visceral endoderm (VE)} at E6.0-E6.75, with stronger expression in the boundary between ExE and epiblast. Loss of BMP2 resulted in significantly reduced PGC number in both heterozygotes and homozygotes\textsuperscript{51,52}. Since the downstream part of BMP signaling is mediated and amplified through the \textit{mothers against decapentaplegic (SMAD)} proteins, similar phenotypes were observed in certain SMAD mutants such as \textit{Smad1}\textsuperscript{15,17,53}, \textit{Smad4}\textsuperscript{54,55} and \textit{Smad5}\textsuperscript{13,14,56}. \textit{Smad1} and \textit{Smad5} are ubiquitously expressed in the epiblast and \textit{Smad4} is ubiquitously expressed during gastrulation. \textit{Smad1}\textsuperscript{-/-} and \textit{Smad5}\textsuperscript{-/-} embryos completely lacked PGCs. \textit{Smad4} mutants exhibits severely reduced PGC number. Therefore, the BMP induced cell signaling pathway mediated by the SMADs proteins are of absolute importance to the initiation of the mammalian germline.

In addition to the presence of BMP signaling from neighboring tissue, the proximal epiblast needs to be competent to receive the BMP signaling. Through the more recent embryo culture experiments\textsuperscript{3}, it is shown that \textit{wingless-related MMTV integration site (WNT) 3} is required in the epiblast to become BMP4 responsive and germ cell fate is a direct consequence of BMP4 induction from ExE. BMP4 signal is antagonized by signaling from the \textit{anterior visceral endoderm (AVE)}, which development is restricted by BMP8b at E5.5. With the correct gradient of BMP signaling, WNT signaling and inhibitory signaling, the
founder germline is initiated in the proximal epiblast by expressing *Blimp1*. The summary of signaling principle for PGC fate is in Figure 1-2.

**Figure 1-2. The signaling principle for the formation of germ cell fate.** The competent epiblast received the BMP4 signaling expressed from extraembryonic ectoderm (ExE), which is antagonized by inhibitory signaling from the anterior visceral endoderm (AVE) to form the signaling gradient to induce the PGC fate in the proximal epiblast.

To understand PGC specification at a single cell level, Saitou et al. (2002)\textsuperscript{44} performed single cell gene expression profiling on individual TNAP positive cells.
and the neighboring cells in E7.5 embryos. An interferon inducible transmembrane protein, *Fragilis* (also known as *interferon-induced transmembrane protein 3-Ifitm3*), was found to mark the onset of germ cell competence induced by BMP4 signaling. From this cluster of cells highly expressing *Fragilis*, *Stella* was identified to express by E7.2 and therefore restricting these cells into germ cells. From this screen, the PGC specific gene B-lymphocyte-induced maturation protein-1 (*Blimp1* also known as *Prdm1* (PRDI-BF1 and RIZ) domain-containing 1), was identified to enable the visualization of germline as early as E6.25 and is required for germline development before E7.5\textsuperscript{12}. *Blimp1\textsuperscript{−/−}* embryos produced a cluster of TNAP positive PGC-like cells with high expression of Homeobox genes *HoxB1* and *HoxA1*, in contrast to control *Stella* positive PGCs that repressed the somatic Hox genes. Moreover, PGCs were completely absent at E8.5 resulting in infertility of the animals. Another PR domain containing transcriptional regulator, PRDM14 starts to express as early as E6.75, which is downstream of BLIMP1. Loss of PRDM14 lead to reduced PGC number as early as E7.25 and *Prdm14\textsuperscript{−/−}* male/female animals were both sterile\textsuperscript{57}. More recently, a transcriptional factor *Tcfap2c* (also known as AP2, gamma), is shown as another down-stream target of BLIMP1 and starts to express in PGCs from E7.25 to E12.5\textsuperscript{58}. In *Tcfap2c* mutants, PGCs were specified but lost around E8.0. *In vitro* EB formation using *Tcfap2c* deficient ESCs indicated that *Tcfap2c* is required for the expression of germ cell markers such as *Nanos3* and *Dazl*, as well as repression of the somatic genes *HoxB1* and *HoxA1*. Given that Blimp1, Prdm14 and Tcfap2c are
among the earliest genes enriched in the germline and loss of either results in a loss of germline in early embryogenesis, these three genes are considered the top three master regulators of the mammalian germline. This is further proved by recent study that overexpressing the three factors in a cell type derived from pluripotent stem cells results in generation of functional germline (discussed in Chapter 2). The onset of germline genes is summarized in Figure 1-3.

![Figure 1-3. The onset of germline genes before gonadal colonization.](image)

**Germ cell epigenetics**

A dramatic drop of genome-wide DNA methylation (with the DNA methylation at imprinted loci not affected) and remodeling of histone modifications happen in the window between E8.0 to approximately E10.5. At around the same time, PGCs decrease histone H3 lysine 9 dimethylation (H3K9me2) and gain levels of histone H3 lysine 27 tri-methylation (H3K27me3)\(^\text{24,25}\). These pre-gonadal epigenetic changes in concomitant with PGC migration are termed **Reprogramming Phase I**\(^\text{24,59-63}\). During the early stages of reprogramming phase I, there is a G2 pause in PGCs from E8.0-E9.0, which is also associated with a transcriptional pause\(^\text{25}\).
Notably, methylation is maintained at the **imprinting control centers (ICCs)** of imprinted genes, single copy genes and **intracisternal A particle (IAP)** elements, despite global loss of **methylated cytosine (5mC)** by immunofluorescence. The germ cell epigenetics is summarized in Figure 1-4.

![Figure 1-4. Genome-wide epigenetic reprogramming in PGCs.](image)

From E10.5-E13.5, DNA methylation at ICCs, single copy genes and IAPs are further removed and the global DNA methylation is further reduced, nearly depleted from the PGC genome. These events are termed **Reprogramming Phase II**\(^{59,60,63-67}\). Recent studies suggest that the **ten-eleven translocation family proteins (TET)** play critical roles in mediating active demethylation of the imprinted genes\(^{68,69}\). Yamaguchi et al. (2013) used paternal $\text{Tet1}$ knock out mice (paternal$^{\text{KO}}$; progenies from $\text{Tet1}^{\text{-/-}}$ male X wild-type female) to analyze the effect of the paternal TET1 loss on the offspring\(^{68}\), because $\text{Tet1}^{\text{-/-}}$ females had meiotic defects. In E9.5 paternal$^{\text{KO}}$ embryos, 11-46 out of 81 expressed imprinted genes were dysregulated and the $\text{Peg10}$ **differentially methylated region (DMR)**
remained fully methylated compared to the controls. By performing reduced representative bisulphite sequencing (RRBS) on E13.5 male PGCs and sperm, 7 out of 12 commonly covered DMRs showed significantly enriched hypermethylation in paternal KO relative to control. Besides, Dawalty et al. (2003) found that Tet1/Tet2 deficiency partially compromised imprinting because in some of the double knock out (DKO) embryos, the imprinting control regions (ICRs) showed aberrant hypermethylation and associated down-regulation of mRNA levels, such as Mest and Peg3. However, the defects of increased DNA methylation are dynamic among Tet1/Tet2 deficient embryos and one possible explanation is that TET3 is compensating for the loss of TET1 and 2. Therefore, demethylation events in Reprogramming Phase II greatly rely on TET family mediated active demethylation, although more detailed experiments need to be performed to investigate the compensation among TET family proteins or whether other mechanism is possible at the same time.

2. Different states of ESCs in culture

In recent years three pluripotent stem cell states have been described: primed, naïve and ground state. Primed pluripotent stem cells are traditionally cultured in medium supplemented with basic fibroblast growth factor (bFGF) and knock out replacer on mouse embryonic fibroblast (MEF) feeder layers and are poised with low expression of lineage differentiation genes. Two examples are human embryonic stem cells (hESCs) and mouse epiblast stem cells (EpiSCs). Naïve pluripotent stem cells refer to mESCs or iPSCs cultured in LIF with
Fetal Bovine Serum (FBS) on MEFs. They have the potential to derive into all four germ layers and could generate chimera when injected into blastocysts. Ground state pluripotent stem cells often refer to mESCs cultured with MEK1 and GSK3β inhibitors (2i) in a chemically defined medium\(^{73-75}\). ESCs in 2i culture can also differentiate very well both in vivo and in vitro, in terms of forming teratomas and living chimeras. However, the epigenetic features are quite different in 2i cultured ESCs compared to naïve ESCs including genome-wide DNA methylation levels\(^{76-79}\). The hall marks for ground state pluripotency include driving Oct4 transcription by its distal enhancer, retaining a pre-inactivation X chromosome state in female lines, global reduction in DNA methylation and reduced deposition of H3K27me3 on developmental regulatory gene promoters. Although a lot about naïve and primed pluripotency have been known, the foundational mechanisms that govern ground state pluripotency remain to be fully established, especially when stem cell research is transitioning to the newly defined ground state pluripotency, due to its chemically defined nature and convenience to derivation of clinic related cell types.

3. ESC as a tool for iPGC in vitro model
Currently Murine ESCs (mESCs) are cultured in two different states, naïve (serum+LIF) and ground state (2i+LIF). mESCs are in vitro derived pluripotent cells from the inner cell mass of E3.5 blastocysts isolated from timed pregnant female mice and are able to differentiate into all four germ layers that compose the entire body: ectoderm, mesoderm, endoderm, and the germline\(^{80-83}\). Previous
studies have shown that ESCs cultured in naïve state could differentiate into
germ cell like cells expressing germline genes by the method of 2-D
differentiation\textsuperscript{29,37} or forming EBs\textsuperscript{27,28,30,32-38}. By comparing these two methods, Wei et al. suggested that Stella-GFP\textsuperscript{+} cells from the EB method showed closer resemblance to the in vivo PGCs with regard to methylation levels of Peg3 and Igf2r DMRs and germline gene expression levels\textsuperscript{37}, indicating that 3-D structure is more in favor of in vitro germ cell like cell differentiation. Moreover, Wei et al. was the first to characterize differentiated in vitro PGCs focusing on genes expressed in specification and pre-gonadal stage. Although this study provided many preliminary data to understand in vitro PGCs in terms of specification, Wei et al. seeded ESCs in low-attachment plates to form variable sizes of EBs, utilized a single transgenic reporter line to isolate the PGC like cells and overlooked detailed analysis of global DNA methylation level and epigenetics relative to endogenous pre-gonadal germline. Therefore, more defined studies need to be performed to really characterize an in vitro PGC population that is correspondent to endogenous pre-gonadal germline. To build upon the present research for deriving pre-gonadal PGCs from ESCs, we devised a 3-D differentiation method based on forming EBs in hanging drops (defined starting cell numbers and differentiation period), that is correspondent to E9.5-E10.5 pre-gonadal PGCs in vivo in Chapter 2. Using our model, I have shown that a potential player in pluripotency, PRMT5, is required for iPGC derivation in vitro.
In concurrent with our studies in Chapter 2, Hayashi et al. invented a method to derive PGC-like cells (PGCLCs) starting from 2i+LIF cultured ground state pluripotent ESCs\textsuperscript{4,84,85}. To generate PGCLCs, this method first involved a 2-D differentiation to Epiblast-like cells (EpiLCs) in the presence of activin A and bFGF, on fibronectin coated 12-well plates starting with 100,000 cells/well. After 2 days of EpiLC induction, EpiLCs were disassociated and plated as floating aggregates in low-cell-binding 96-well plates (2,000 cells/well) in the presence of a cocktail of growth factors and cytokines, BMP4 (500ng/ml), LIF (1000u/ml), Stem Cell Factor (SCF, 100ng/ml), BMP8b (500ng/ml) and Epidermal Growth Factor (EGF, 50ng/ml)\textsuperscript{85}. Initially Hayashi et al. utilized a transgenic reporter ESC line with Blimp1-mVenus and Stella-ECFP (BVSC) and isolated BVSC positive PGCLCs from Day4-Day6 of PGCLC induction\textsuperscript{84}. At the end of this paper, the authors identified surface markers CD61 (also named integrin β3) and SSEA1 to isolate the same PGCLC population. Both BVSC\textsuperscript{+} and CD61\textsuperscript{+}/SSEA1\textsuperscript{+} PGCLCs are able to further differentiate in reconstituted gonads into haploid gametes (spermatozoa and oocytes), which could be fertilized and generate live young\textsuperscript{4,84}. This is the first study showing the creation of functional gametes from a pluripotent cell source able to produce live young. Following this study, I performed PGCLC induction using Prmt5 null EpiLCs and found that PGCLCs were reduced in half in Chapter 3, which is similar to the result I obtained when using the EB method in Chapter 2. Taken together our results indicate that either the EB method or the generation of PGCLCs can be used to study the mechanism of PRMT5 activity in PGC formation \textit{in vitro}.
4. PRMT5 is an important protein arginine methyltransferase (PRMT) involved in critical biological processes

**Protein arginine methyltransferase 5 (PRMT5)** is a type II PRMT that modifies symmetrical di-methylated arginines (SDMA) in glycine and arginine-rich motifs of proteins involved in cancer biology, neurogenesis and reprogramming\(^{86}\).

There are three type II PRMTs that have been described: PRMT5, PRMT7\(^{87,88}\) and PRMT10 (unpublished communication from Dr. Steven Clarke group), out of which PRMT5 is the mostly well characterized. PRMT5 modifies a large number of protein substrates, including both cytoplasmic and nuclear proteins\(^{2,77,78,89-99}\).

The cytoplasmic proteins include Sm-class proteins composing the Sm core in spliceosomes\(^{100,101}\). The nuclear proteins include arginines on tails of multiple histones H2A\(^2\), H3\(^{90,98}\) and H4\(^2,94\) as well as germline RNA binding proteins such as VASA\(^{97}\), PIWI\(^{95}\), MILI and MIWI\(^{102,103}\). Therefore, the molecular function of PRMT5 is implicated in regulating splicing, transcription, RNA biogenesis and transposon repression.

Most interestingly, PRMT5 is required for governing the pluripotency in naïve ESCs cultured in serum+LIF; PRMT5 is required for the *Drosophila* germline and hypothesized to be essential for mammalian germline. Detailed discussion is as follows:

**PRMT5 in *Drosophila* germline**
The *Drosophila* homolog of *Prmt5*, *dart5*, is a grand-childless gene\(^9\). Loss of functional allele of *dart5* results in sex-dependent germline phenotypes in flies. In males, homozygous *dart5-1* (a mutant allele with *piggyBac* transposon inserted in exon 2 of *dart5*) flies are infertile due to the lack of mature spermatocytes although homozygous *dart5-1* females are fertile with slightly reduced fecundity. However, when mated with wild-type male flies, the fertilized embryos are devoid of pole cells, thus are completely agametic, due to failure of nuage formation in the pre-fertilized oocyte and pole plasm assembly in fertilized embryo. Also, DART5 (also known as dPRMT5 and Capsuleen (Csul)) was shown to be responsible for SDMA modification of PIWI proteins required for piRNA (also known as rasiRNAs-repeat associated small interfering RNAs) biogenesis in *Drosophila*\(^9\). Loss of DART5 activity resulted in reduction of piRNA levels and accumulation of retrotransposons in the ovary\(^9\). The mechanism is that DART5 is required for the association between Tud and Aub/AGO3 and loss of DART5 resulted in lower *roo* piRNA (a subtype of piRNA) loading onto Aub\(^10\). In summary, these data suggests that PRMT5 is involved in the initiation of the germline as well as germline development when piRNA biogenesis is required in *Drosophila*.

Although mammalian germline forms by signal induction from BMPs, other than the pre-formation in *Drosophila*, PRMT5 is also indicated to be important in mammalian germline by functioning together with the mammalian germline master regulator BLIMP1\(^10\).
BLIMP1 and PRMT5 in mammalian germline

BLIMP1 is a transcriptional repressor containing a SET domain and Kruppel-type zinc fingers, which enable its DNA-binding activity\(^{25,106}\). BLIMP1 is known as a master regulator of terminal B cell differentiation into plasma cells through repression of the mature B-cell program\(^{107-113}\). Global deletion of Blimp1 in mouse results in embryonic lethality by E10.5\(^{2,114,115}\) and reduction of Alkaline Phosphotase (AP) positive PGCs at E9.5 in a dose-dependent manner\(^{12,84,114,115}\). AP positive Blimp1 null PGC-like cells fail to repress somatic lineage genes such as the homeobox genes Hoxa1 and Hoxb1 by single cell PCR\(^{12,23,115}\). Another study has shown that compared to the somatic neighboring cells, Blimp1 null PGC-like cells fail to repress genes of multiple somatic lineages that are normally repressed in wild-type PGCs, such as genes involved in pattern specification, morphogenesis and DNA methylation, indicating that BLIMP1 functions as a repressor of somatic gene programs to facilitate PGC fate\(^{84,116}\).

At the same time, Ancelin et al. showed that BLIMP1 physically interacts with PRMT5 (Protein arginine methyltransferase 5) by co-immunoprecipitation (co-IP) in 293T cells. PRMT5 was enriched at a BLIMP1 concensus motif within the Dhx38 locus as a result from chromatin immunoprecipitation (ChIP) followed by PCR using E10.5 mouse genital ridges, although the epigenetic marks directed by PRMT5: symmetrical dimethylation of arginine 3 on histone H2A/H4 (H2A/H4R3sme2), were not enriched in the same locus\(^{2,23}\). The authors
concluded that failure to identify the H2A/H4R3sme2 in the Dhx38 BLIMP1-PRMT5 binding sites was due to low number of PGCs (300 PGCs per embryo at E10.5) in the genital ridges that precludes efficient ChIP analysis. Therefore, the authors turned to an in vitro cell line to address this, over-expression of Myc-Blimp1 in P19 cells (an embryonic carcinoma cell line) down regulated Dhx38, and an increased enrichment of H2A/H4R3sme2 on BLIMP1 targeted consensus sequence was seen in Dhx38 locus\(^2,117\). Taken together, these results led to a model that the interaction of BLIMP1 with PRMT5 results in recruitment to key Blimp1-consensus sequences, resulting in the deposition of H2A/H4R3sme2 at these sites, to repress somatic gene expression and promote PGC development. However, lack of cellular material (around 300 cells/E10.5 genital ridge from data of our lab) restricts efforts to clarify PRMT5’s role in germline regulation. Whether BLIMP1 and PRMT5 physically interact in a pure PGC population remains to be proved. Moreover, a complete loss of function of Prmt5 has never been performed in mammalian germline.

**PRMT5 in splicing**

PRMT5 is able to modify SmB/B’, SmD1/D3 proteins in *Drosophila*, mouse cells and human cells\(^45,92,118\). In flies, DART5 (*Drosophila Prmt5*) and DART7 (*Drosophila Prmt7*) were both required for methylating SmB and SmD1/D3 proteins\(^92\). However, snRNP assembly in either dart5 or dart7 mutants was unaffected, due to the fact that methylation of Sm proteins is not required for Sm-core assembly.
Different from *Drosophila*, methylation of the Sm proteins is indeed required for efficient association with the **SMN (survival of the motor neurons)** complex in both human HeLa cells\(^{119}\) and murine **Neural Progenitor Cells (NPCs)**\(^{45}\). In HeLa cells, both PRMT5 and PRMT7 are required for methylation of the Sm proteins and snRNP assembly. However, whether splicing is affected in *Prmt5* or *Prmt7* Knock down (KD) HeLa cells remains to be determined. In Bezzi et al., loss of PRMT5 resulted in increased apoptosis and affected homeostasis of NPCs. *Prmt5* null NPCs showed differentially spliced events mainly in the categories of **retained introns (RI)**. Among the 300 genes with affected splicing events, the authors focused on alternatively spliced *Mdm4* (a P53 inhibitor), because MDM4 was down-regulated after inhibiting splicing machinery\(^{120}\). In *Prmt5* null NPCs, *Mdm4* transcripts comprised a short unstable form relative to the control, leading to the reduction of full length MDM4 protein. As a result, the repression of P53 pathway by MDM4 was released, leading to increased cell death. However, the phenotype by loss of PRMT5 was only partially rescued with a *Trp53^-/-* background, indicating that a P53 independent pathway for regulating cell survival is present.

**PRMT5 in pluripotency**

In Tee et al., PRMT5 was shown to safeguard naïve pluripotency in mouse ESCs by modifying R3 of pre-deposited histone H2A (H2AR3sme2) in the cytoplasm\(^{23}\). Knock down of *Prmt5* by shRNA in mESCs cultured in serum showed precocious
differentiation, i.e., down regulation of Oct4 and Sox2 and up regulation of somatic genes such as FoxA2, Gata4 and HoxD9. Over expression of a mutated H2A that cannot be methylated at R3 lead to partial resemblance to Prmt5 knock down phenotype\textsuperscript{23}. In this paper, symmetrical di-methylation of arginine 3 on histone H4 (H4R3sme2) was not reduced in Prmt5 KD naïve ESCs. However, this is possibly due to the non-specificity of the antibodies recognizing unmodified H4, because the same antibody from the same catalog number (Abcam, #ab5823) still detects a band corresponding to H4 in Prmt5 null MEFs (in collaboration with Dr. Mark Bedford). Using a new antibody from Active Motif, a reduction of H4R3sme2 is observed in Prmt5 null MEFs (Dr. Mark Bedford unpublished) and Prmt5 null ESCs cultured in 2i+LIF (our data in Chapter 3). Therefore, H4R3sme2 and H2AR3sme2 are possibly both required for repression of somatic genes.

In primed pluripotent stem cells such as hESCs cultured with bFGF, PRMT5 is not required for pluripotency, but instead for cell proliferation\textsuperscript{117}. KD of Prmt5 in hESCs resulted in no change of OCT4 and NANOG protein levels. Instead, affected cell proliferation was seen with loss of PRMT5 using competition assays. This was due to induction of P57, resulting in cell cycle arrest in G1/G0 phase. Given that PRMT5 is expressed in all states of pluripotency and the role of PRMT5 in naïve and primed pluripotency is known, it is important to test the function of PRMT5 in ground state pluripotency, therefore the 2i+LIF cultures, to
understand the different mechanisms mediated by a common factor in different states of pluripotency.

5. Generation of inducible knock out of Prmt5 ESCs in 2i culture

Tee et al. showed that Prmt5\(^{-/-}\) blastocysts cannot give rise to ESC outgrowths in serum culture\(^{23}\). To overcome this difficulty, ideally an inducible knock out of Prmt5 ESC line should be derived. Therefore, a CreER system or CrePR system should be utilized in the establishment of models. CreER is a Cre recombinase fused with estrogen receptor (ER) moiety that is respondent to tamoxiften to translocate from cytosol into the nucleus, in order to enable excision of the critical sequence flanked by two loxP sites and delete gene of interest. Similar to CreER, CrePR is Cre recombinase fused with progesterone receptor and induced by mifepristone\(^{121}\). Using CreER or CrePR driven by a universal expressing promoter such as Rosa26 or Actin, the knockout (KO) could be induced in a time-specific manner. On top of the time-specific control, CreER or CrePR could also be driven by a tissue-specific promoter, or used together to allow inducible KO of two different genes.

6. Generation of conditional germline knock out of Prmt5 in vivo

Tee et al. also shows that when mating Prmt5\(^{+/-}\) mice together, no Prmt5\(^{-/-}\) embryos are recovered at E5.5 while normal Mendelian ratio of Prmt5\(^{-/-}\) blastocysts is found with no morphological changes compared to the controls.
Given this embryonic lethality, study of early events of PGC specification (starting at around E6.0) is impossible using the traditional knock out of $Prmt5^{23}$.

**Aims and Significance of the Dissertation**

In summary, the studies discussed above indicate that early events in PGC are largely not understood. In humans this is due to the challenges in acquiring consented human samples at early ages. In mice, this is due to the challenges of studying a small migratory cell population that is allocated at E6.5 to E7.5 soon after implantation. Thus, an *in vitro* model for studying PGCs is of immediate necessity to help broaden existing knowledge of PGCs. However, *in vivo* studies are still required to prove that the phenomenon studied *in vitro* can be recapitulated in the embryo. ESCs serves as a great tool for generating this *in vitro* model because ESCs are pluripotent and share transcriptional and epigenetic similarities as PGCs.

PRMT5 is an essential component for pluripotency and a potential player in mammalian germline formation. Understanding the molecular events centered on PRMT5 is crucial to identify the intrinsic pathways that underlie genomic totipotency. Furthermore, detailed understanding of these mechanisms will be useful in applying these processes to induce plasticity during cellular reprogramming of somatic cells to *induced pluripotent stem cells (iPSCs)*, as well as trans-differentiation from a terminally differentiated cell to PGCs and derivation of PGCs in a dish from iPSCs.
Therefore, the aims of this thesis were to:

1. Devise an ESC-derived model to generate pre-gonadal *in vitro* PGCs (iPGCs) without the use of transgenic reporters to test potential germline modifiers, such as *Prmt5*.
2. Devise an inducible knock out model of *Prmt5* to address its function in ground state pluripotent murine ESCs.
3. Devise a germline conditional knock out model of *Prmt5* to address its role in mammalian germline formation.

Taken together, these studies demonstrated that the invention of a novel ESC-based differentiation strategy is capable of isolating PGCs *in vitro*, with transcriptional and epigenetic features reminiscent of pre-gonadal PGCs to test hypothesized germline modifiers. Development of new genetic tools to evaluate PRMT5’s role in ground state pluripotency and mammalian germline formation were employed, including the generation of inducible and tissue-specific knock out models. Advanced techniques such as paired-end RNA sequencing was also used. Finally, we combined both the *in vitro* and *in vivo* systems to answer the question about PRMT5 in gene regulation and RNA splicing.


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CHAPTER 2

IDENTIFYING THE POTENTIAL ROLE OF PRMT5 IN MAMMALIAN GERMLINE DIFFERENTIATION USING AN IN VITRO TOOL THAT STAGES PRIMORDIAL GERM CELLS DERIVED FROM MOUSE EMBRYONIC STEM CELLS
Data from this chapter was adapted from the published paper:


Introduction

The pre-gonadal molecular events underlying mammalian germline specification and early development are not well characterized because of technical challenges and limited materials. For human germline, early stage embryos are very difficult to obtain because germline has formed early in embryogenesis before pregnancy is realized. Therefore, mouse model is used to perform studies for germ cell development¹.

Although the mouse model has provided much knowledge about the mammalian germline, the challenge to understand the very early stage of germline initiation is still present. Therefore, it is highly favorable to devise a system that provides scalable sources of primordial germ cells (PGCs) that are equivalent to pre-gonadal stage endogenous PGCs, to facilitate large-scale biochemical assays and fast screenings of critical factors involved in early PGC formation.
Previous reports suggested that *in vitro* PGC like cells could be derived in multiple research groups, using either 2-D differentiation method with cells attached to a tissue culture plate, or 3-D differentiation method by forming embryoid bodies (EBs) either in hanging drops or low-attachment plates\(^2\)\(^-\)\(^12\). However, most of the studies based on reporter pluripotent stem cell lines or used a single surface marker for PGC isolation. In addition, absent in most studies is a detailed description for staging the *in vitro* PGCs to the correspondent endogenous developmental stage, which is helpful for studying PGCs in a particular window of reprogramming since PGCs are undergoing dynamic epigenetic and transcriptional changes throughout development, see in Chapter 1. In our study here in Chapter 2, we devised an *in vitro* differentiation system starting from murine embryonic stem cells (mESCs) to generate a tool that allows the staging of *in vitro* PGCs to the relevant endogenous counterparts.

In the mouse model, precursors of the germline are initially separated as 4-6 cells in the embryonic proximal epiblast with the expression of *Blimp1* (or *Prdm1*) at around E5.5-E6.25\(^13\). At around E7.5, there are about 40 cells at the base of the allantois ready to migrate out, expressing germline genes such as *Stella* and *Prdm14* etc.\(^13\)\(^-\)\(^15\). Since then the germ cells are specified and therefore referred as PGCs. From E8.0-E8.5, the PGCs migrate out of the allantois and into the embryonic hindgut endoderm. Coincident with the migration, these PGCs undergo a pause in G2 phase and start to show genome-wide down regulation of DNA methylation (referred to as reprogramming phase I)\(^15\)\(^,\)\(^16\). At E9.5, PGCs
start to proliferate robustly while migrating through the hindgut, and hit the genital ridge at around E10.5 with expression of the gonocyte gene *Mouse vasa homologue (Mvh)*. By E11.5, PGCs colonize the genital ridge or the gonads with approximately 1,000-2,000 cells in number each embryo \(^{16-18}\). For critical developmental steps of mammalian germline, please see Chapter 1, Figure 1-1 through Figure 1-4.

The factors that specify and sustain pre-gonadal PGCs (prior to E10.5) are not well understood due to the material limits. Tremendous efforts have been made to explore the very initiation of the germline using the mouse model. At the same time, the derivation of PGCs from the *in vitro* models is improved in concurrent with our studies in Chapter 2. For example, the advance of inducing PGC like cells (PGCLCs), with a middle step of forming Epiblast like cells (EpiLCs) from ground state pluripotent stem cells \(^{19-21}\), is emerging, using a cocktail of cytokines including BMP4. Recently it is shown that PGCLCs could be derived in EpiLCs overexpressing three germline transcriptional factors, *Blimp1*, *Prdm14* and *Tfap2c* (also known as *AP2γ*), bypassing the addition of cytokines into the culture \(^{22,23}\). Therefore, *Blimp1*, *Prdm14* and *Tfap2c* are considered efficient to establish a regulatory network for germline transcriptome and epigenome.

Recently, Aramaki et al. (2013) showed that *Wnt3*\(^{-/}\) epiblasts fail to induce the germ cell fate by embryo culture experiments, in the presence of the cytokine cocktail including BMP4 (the same as for PGCLC induction) \(^{24}\). In *Wnt3*\(^{-/}\) EpiLC
derived aggregates, the BMP4-SMAD signaling pathway was still intact whereas the WNT-β-CATENIN pathway was impaired, as shown by failure to turn on T (also known as Brachyury). Furthermore, β-catenin−/− embryos failed to initiate PGC fate in the proximal epiblast. Using $T^{137/137}$ (the $T^{137}$ allele is with a transgene insertion into $T$ and disrupts $T$ expression) embryos, the authors showed that the initial expression of Blimp1-Venus (BV) transgenic reporter was not affected from E6.25, but instead, the BLIMP1 protein was absent from BV+ cells at E7.5. Interestingly, T is required for the expression of TFAP2C and PRDM14, which are crucial for PGC development. Therefore, WNT3 in the epiblast is crucial for PGCs to respond to BMP4 signaling and to express critical germline key factors such Blimp1, Prdm14 and Tfap2c.

One of the first and most characterized master regulators of early PGCs is the transcriptional repressor B-lymphocyte induced maturation protein1 (Blimp1) also named as PRD1-BF1 and RIZ (PR) domain 1 (Prdm1). BLIMP1 expression is detected in PGCs as early as E6.25 and persists until E11.5. Loss of BLIMP1 had a dosage effect in terms of PGC numbers: Blimp1+/− embryos exhibited a 50% of PGC reduction at E9.5 and Blimp1−/− embryos showed a complete loss of PGCs. It is hypothesized that the target of BLIMP1 include the Hox genes, in particular, Hoxb1, since Blimp1−/− PGC-like cells displayed up-regulated Hoxb1 expression. However, direct binding of BLIMP1 on the Hoxb1 locus has never been shown.
Initially in Ohinata et al. (2005), the Homeobox genes $HoxB1$ and $HoxA1$ were de-repressed at around E7.25 in TNAP positive PGC like cells, whereas the control cells showed no expression of the $Hox$ genes\textsuperscript{29}. This was the first study indicating that BLIMP1 is a transcriptional repressor in the germline to repress lineage differentiation associated genes to facilitate a germ cell fate. However, prior to that, the mesoderm program (including $HoxB1$ and $T$) is actually present in the early PGCs \textsuperscript{30,31}. In Yabuta et al. (2006), the single-cell gene profiling of PGCs as well as surrounding somatic cells from E6.75-E8.25 was performed. Both $HoxB1$ and $HoxA1$ were found to express in some of the PGCs at E6.75 but not after. Interestingly, the primitive streak marker $T$, is present in PGCs from E6.75-E7.75 and become silent in E8.25\textsuperscript{31}. Another principle mesodermal gene $Fgf8$ is also present in E7.25 PGCs\textsuperscript{30}, indicating that the mesodermal program is present in the earliest PGCs.

$Prdm14$ is another PR domain containing transcriptional factor that is crucial for PGC cell fate initiation and germline development. Induced ectopic expression of $Prdm14$ alone in EpiLCs is efficient enough to form PGCLCs\textsuperscript{22}. $Prdm14$ RNA is found to express in PGCs as early as E6.75\textsuperscript{31,32}, earlier than the expression of $Stella$. $Prdm14^{-/-}$ embryos showed reduced number of PGCs as early as E7.25 and lost Alkaline Phosphatase (AP) positive PGCs by E12.5. $Prdm14$ deficient PGCs remain repression of $HoxB1$ but fail to repress $HoxA1$. $Stella$ and $Sox2$ were found to be down regulated in $Prdm14^{-/-}$ PGCs. In addition, Prdm14 deficient PGCs showed stalled epigenetic reprogramming (failure to down
regulate H3K9me2 and up regulate H3K27me3) and failed to form embryonic germ cell (EGC) colonies in the presence of Leukemia inhibitory factor (LIF). The molecular function of \textit{Prdm14} is to antagonize fibroblast growth factor (FGF) signaling, repress \textit{Dnmt3b} and promote active DNA demethylation through the eleven translocation (TET), in order to maintain a hypomethylation genome of PGCs\textsuperscript{33,34}.

\textit{Tfap2c} (also known as \textit{AP2γ}) is the other transcriptional factor among the top three regulators of PGC formation. In \textit{Tfap2c} mutants, PGCs were lost at around E8.0\textsuperscript{35} and \textit{Tfap2c}\textsuperscript{-/-} EBs showed high expression of somatic genes such as \textit{HoxA1} and \textit{HoxB1}. Recently, Magnusdottir et al. (2013) performed chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) for BLIMP1 and \textit{AP2γ} in P19 embryonic carcinoma (EC) cells overexpressing BLIMP1, PRDM14 and \textit{AP2γ}\textsuperscript{23}. They first showed that by introducing the three factors into EC cells, \textit{T} and \textit{Dnmt3b} were repressed whereas germline genes such as \textit{Nanos3}, \textit{Dnd1}, \textit{Stella} and \textit{Mvh} were activated, resembling the endogenous germ cells. Then the authors utilized this model to show that BLIMP1 bound to gene promoters encoding transcription factors, cell cycle and developmental regulators, with most of its targets repressed. Interestingly, in BLIMP1-binding sites, the binding motif for the AP2 family was enriched. The ChIP-seq for \textit{AP2γ} suggested that \textit{AP2γ} is enriched in several somatic genes such as \textit{Hoxa11}, \textit{HoxB13} and \textit{T}, indicating that \textit{AP2γ} is another somatic gene repressor in germline development.
Going back to *Blimp1*, the mechanism by which BLIMP1 represses somatic gene expression in PGCs is hypothesized to involve recruitment of protein arginine methyltransferase 5 (PRMT5) and localization to the gene loci flanked by consensus targeting sequences recognized by BLIMP1 \(^{25}\). This is based on the fact that BLIMP1 and PRMT5 physically interact in human 293T cells and PRMT5 is bound to a repressed gene *Dhx38* in a region enriching for BLIMP1 targeting sequences using E10.5 genital ridge lysates \(^{25}\). However, a direct interaction between BLIMP1 and PRMT5 in a pure PGC population, or whether a loss of PRMT5 phenocopies *Blimp1* mutants has never been shown.

In this study, we devised an *in vitro* model of deriving PGCs of endogenous relevance from a pluripotent stem cell (PSC) source: murine embryonic stem cells (mESC) and showed that *in vitro* PGCs (iPGCs) are closely resembling E9.5-10.5 endogenous PGCs. Then we utilized an ESC line with loss of BLIMP1 to validate our system. Finally, we performed an shRNA knock down of *Prmt5* in ESCs followed by iPGC induction to show that PRMT5 is a potential factor for mammalian germline formation.

**Results**

*cKit*\(^{bright}\) refines an OCT4\(^+\)/SSEA1\(^+\) iPGC population in embryoid bodies (EBs) derived from murine embryonic stem cells (mESCs)
Given that ESCs and PGCs all express pluripotent genes Oct4, Nanog and Sox2, our initial scheme is to use an Oct4-gfp reporter ESC line $^{36}$ and induce differentiation by the hanging drop method to form EBs with 300 cells/drop ($20 \mu l$) in medium without LIF (Figure 2-1A) and the iPGCs should reside in the OCT4 positive population after most of the cells turn down OCT4 upon differentiation. EBs could be maintained in culture for up to 8 days in hanging drops with increasing loss of viability (Figure 2-S1A, S1B). Using flow cytometry, we show that GFP is not reduced from the majority of cells until the fourth day of differentiation (Figure 2-1B). On day 5 of differentiation, a shoulder of GFP$^{\text{bright}}$ cells appear and a distinct peak of GFP$^+$ cells form by day 6 (Figure 2-1B, arrow).

To generate a transgene-free method of isolating iPGCs which is independent of reporter lines and thus could be used with ESCs with a variety of genetic backgrounds, we correlate expression of OCT4 in day 6 EBs with the cell surface marker SSEA1 that is also expressed in endogenous germline $^{37,38}$. We identify small clusters of cells co-express OCT4 and SSEA1 in EBs (Figure 2-1C). To increase another transgene-free marker for iPGC isolation, we also use the membrane-localized tyrosine kinase receptor cKIT to further define the iPGC population, because cKIT is highly expressed by endogenous PGCs from E7.5-E13.5 $^{28,31,39}$. Flow cytometry combining either two out of the three markers show a side peak population at day 6 of differentiation in EBs, SSEA1$^+$/cKIT$^+$ (Figure 2-1D) and Oct4-GFP$^+$/cKIT$^+$ (Figure 2-S1C) respectively.
To investigate the PGC identity in SSEA1⁺/cKIT⁺ population, we fraction these cells into SSEA1⁺/cKITbright (green), SSEA1⁺/cKITmid (light blue), and SSEA1⁺/cKITdim (dark blue), with SSEA1⁻/cKIT⁻ cells (red) as somatic negative control, to perform quantitative real-time PCR (qRT-PCR) to determine expression of PGC-expressed genes (Figure 2-1E). PGC specific genes including cKit, Blimp1, Stella and Dazl are mostly enriched in SSEA1⁺/cKITbright fraction compared to SSEA1⁺/cKITmid and SSEA1⁺/cKITdim fractions (Figure 2-1F). However, genes that are highly expressed in somatic cells like the Hox genes are highly expressed in SSEA1⁻/cKIT⁻ and SSEA1⁺/cKITdim relative to SSEA1⁺/cKITbright cells. Taken together, we conclude that in EBs at day 6 of differentiation the SSEA1⁺/cKITbright fraction include the putative PGCs that pertain the most PGC identity. We next evaluate how the three fractions enrich for Oct4-GFP and find equal enrichment of Oct4-GFP in SSEA1⁺ cells independent of cKIT intensity (Figure 2-S1D), showing that Oct4-GFP and SSEA1 report the same population when combined with cKIT. In addition, we tested the iPGC production among different genetic backgrounds of mESCs and found that all backgrounds produce iPGCs in a range of a 2-4% on average of live EBs (Figure 2-1G). Therefore, we use SSEA1⁺/cKITbright to isolate iPGCs in the following studies and this method gives rise to similar numbers of iPGCs among different genetic background.

*Day 6 iPGCs display germline signature genes and a pre-gonadal PGC identity*
To compare the expression of PGC genes between Day 6 iPGCs and the endogenous germ cells, we perform Fluorescence Activated Cell Sorting (FACS) to isolate GFP⁺ endogenous PGCs from Oct4-gfp embryos at E9.5 and 10.5, which also show positive for SSEA1 and cKIT (Figure 2-2A-C). We confirm that the sorted GFP⁺ cells have PGC identity by showing expression of cKit, Blimp1, Stella, and Mvh relative to the GFP⁻ somatic cells by qRT-PCR (Figure 2-2D).

Given that germ cell population is quite heterogeneous, next we perform single cell analysis of expression levels of five signature PGC genes, such as Blimp1, Stella, Prdm14, Dnd1 and Dazl, among day 6 iPGCs, E9.5 and E10.5 endogenous PGCs. In total, we analyze 38 single ESCs (as a control for undifferentiated cells, Figure 2-2E), 34 single E9.5 endogenous PGCs (Figure 2-2F), 24 single E10.5 PGCs (Figure 2-2G) and 30 day 6 iPGCs (Figure 2-2H). In undifferentiated ESCs, 17 out of 38 cells (44%) expressed Blimp1. Of the 17 Blimp1⁺ cells, 6 do not express Stella and 12 do not express Dnd1. In contrast to ESCs, 100% of E9.5 and E10.5 PGCs from the embryo and iPGCs express Blimp1 (Figure 2-2F-H). Heat maps of the single cell analysis show that E9.5 PGCs are relatively homogeneous in terms of expression of each gene compared to E10.5 PGCs and iPGCs (Figure 2-2G, H). Critically, only one cell in the iPGC cohort was not a germ cell (Figure 2-2H, asterisk).

We next examine expression levels of each gene for all cells that co-express
Blimp1, Stella, Dnd1, and Prdm14 relative to levels in SSEA1+/cKitbright iPGCs. By comparing ESCs to iPGCs, we find that the 4 Blimp1+ ESCs that co-express Stella, Prdm14 and Dnd1 (10.5%) display significantly lower expression levels than those in iPGCs (Figure 2-2I-L). However, iPGCs are indistinguishable from E10.5 PGCs with regard to Blimp1, Prdm14, and Dnd1 expression levels (Figure 2-2I,K-L). In particular, for cells that also co-express Dazl, ESCs display significantly diminished Dazl levels, while no significant difference is found between iPGCs and E10.5 endogenous PGCs (Figure 2-2M). Stella levels were statistically different between all groups, with SSEA1+/cKitbright iPGCs on average expressing intermediate levels between E9.5 and E10.5 embryonic PGCs (Figure 2-2J). We hypothesize that the intermediate levels of Stella in iPGCs between e9.5 and e10.5 PGCs may suggest that iPGCs are developmentally corresponding to a period of germ cell between E9.5 and E10.5.

To further confirm iPGCs are equivalent to endogenous PGCs between E9.5 and E10.5, we expect the Day 6 EB-derived SSEA1+/cKITbright iPGCs not to express MVH protein although mvh transcripts are seen. To address this, we performed immunofluorescence (IF) for MVH, which is first detectable in gonadal PGCs at E11.5 40-42. IF analysis of E10.5 embryos against MVH and OCT4 confirms that E10.5 OCT4+ PGCs are negative for MVH protein, whereas gonadal stage PGCs are MVH positive (Figure 2-3A). IF on iPGCs reveals that MVH protein is negative, therefore these iPGCs are definitely pre-gonadal (Figure 2-3B). In addition, we evaluate H3K27me3 in SSEA1+/cKITbright iPGCs, a
histone modification that is increasing at PGC specification from E8.25 and depleted from the PGC genome from E11.5-E12.5 \(^{16,43}\) and we observe a high nuclear content of H3K27me3 in iPGCs (Figure 2-3C). Taken together this data suggests that iPGCs are pre-reprogrammed and younger than E11.5.

We also evaluate DNA demethylation at non-imprinted genes-\(Xist\) and \(\text{Intracisternal A Particle 1 (IAP)}\) and differentially methylated region (DMR) of imprinted genes such as \(\text{Snrpn}\), which is erased by E12.5 \(^{44,45}\). (Figure 2-3D). In the putative iPGCs, methylation at the \(\text{Snrpn}\) DMR is modestly reduced to 38.7\% compared to 49.6\% in ESCs, while \(Xist\) and \(IAP\) methylation levels are the same as ESCs. To correlate this partial demethylation of \(\text{Snrpn}\) in endogenous PGCs, we performed bisulfite sequencing of Oct4-GFP\(^+\) endogenous PGCs at E9.5 and E10.5. Methylation at the \(\text{Snrpn}\) DMR in E9.5 and E10.5 PGCs from the embryo were 47.6\% and 54.2\% respectively, consistent with previously reports \(^{45}\). Taken together, using single cell analysis, immunofluorescence and bisulfite sequencing, our data strongly demonstrate that the iPGCs isolated from EBs at day 6 of differentiation correspond to a E9.5-E10.5 stage germ cell in vitro.

\(\text{BLIMP1 is specifically required for iPGC differentiation from EBs}\)

In order to put the iPGC system to practical usage, such as screening for germline essential factors, validation that iPGCs faithfully recapitulate endogenous events when a gene of interest is lost. Therefore, we decide to
derive iPGCs from Blimp1 deficient ESCs to check whether it phenocopies the endogenous phenotypes with loss of BLIMP1 since dosage of BLIMP1 is essential for the specification of PGCs in vivo\textsuperscript{13,27}. We derive Blimp1\textsuperscript{fl/fl} ESCs from E3.5 blastocysts and perform a Y chromosome FISH to identify the sex of the line (Figure 2-4A), and generate three independent Blimp1 null sub-lines (Blimp1\textsuperscript{Δ/Δ}) via introduction of CRE recombinase into cells followed by sub-cloning. Clones are confirmed by Southern blot to verify Blimp1 deletion (Figure 2-4B). Then we perform flow cytometry for SSEA1 (a pluripotent marker) (Figure 2-4C) and Teratoma assays (Figure 2-4D) to examine the overall self-renewal and pluripotency. In both assays, all Blimp1\textsuperscript{Δ/Δ} lines were indistinguishable from parental Blimp1\textsuperscript{fl/fl} cells, indicating that loss of BLIMP1 does not cause gross defects in overall ESC self-renewal or differentiation.

Finally, to evaluate iPGC formation, we performed paired differentiation experiments with Blimp1\textsuperscript{fl/fl} and Blimp1\textsuperscript{Δ/Δ} lines and evaluated iPGC differentiation by flow cytometry (Figure 2-4E). Quantification of SSEA1\textsuperscript{+}/cKIT\textsuperscript{bright} cells reveal that iPGCs constitute approximately 3-4\% of the live cell EB population in the parental Blimp1\textsuperscript{fl/fl} line at day 6 (Figure 2-4F). In contrast, all Blimp1\textsuperscript{Δ/Δ} sub-lines display between a 70-90\% decrease in SSEA1\textsuperscript{+}/cKIT\textsuperscript{bright} iPGCs, with the average percentage constituting less than 1\% of the EB in all three sub-lines examined (Figure 2-4F,G). Functionally, this demonstrates that sorting for SSEA1\textsuperscript{+}/cKIT\textsuperscript{bright} iPGCs captures a Blimp1-dependent PGC population in vitro.
*PRMT5 is identified as a potential critical germline factor using iPGC differentiation*

Above data has suggested that our iPGC model is very robust and could be applied to screen for germline modifiers that capitulate the endogenous phenotypes. In order to test the hypothesis whether PRMT5 is required for mammalian germline formation, before generating a Prmt5 knock out which might be time consuming and cost inefficient, we decide to first identify PRMT5’s role using the iPGC model to pioneer the building of Prmt5 deficient mouse model. To confirm that iPGCs express PRMT5 in the nucleus as endogenous E9.5 and E10.5 PGCs, we performed IF on dropped sorted SSEA1+/cKITbright iPGCs derived from V6.5 ESCs and find PRMT5 is present in the nucleus of the iPGCs (Figure 2-5A, 5B).

To generate a cell line with a knock down of Prmt5, we used lentivirus bearing a shRNA against Prmt5 or scrambled shRNA to transduce V6.5 ESCs, followed by sub-cloning. We obtained 2 sublines of ESC with the most efficient Prmt5 knock down (KD), line 1-4, 1-8. qRT-PCR (Figure 2-6A) and Western blot (Figure 2-6B, 6C) against PRMT5 among control, 1-4 and 1-8 revealed that both Prmt5 RNA and PRMT5 protein is reduced about 50% in 1-4 and 1-8, but not much further. We reason that this is due to the fact that complete loss of PRMT5 results in cell death as Prmt5−/− E3.5 blastocysts lose the ability to derive ESC outgrowth and no Prmt5−/− embryos are recovered at E5.5 ⁴⁶. Next we examined the iPGC
differentiation among control, 1-4 and 1-8 using flow cytometry (Figure 2-6D, 6E). By gating on the SSEA1+/cKIT bright population, we observe that control ESCs show that on average iPGCs constitute about 1.5% of the live EB population, whereas 1-4 iPGCs constitute about 0.5% on average and 1-8 iPGCs constitute less than 0.1%, with no difference of the percentage of the live population of EB among all samples (data now shown).

Taken together, our data suggests that 1. iPGC system functions as a very useful in vitro system to produce scalable source of iPGCs for large-scale experiments; 2. iPGC differentiation serves as a practical tool to screen for germline essential factors that faithfully recapitulate phenotypes seen in vivo; 3. PRMT5 is a potential germline important factor as shown by reduction of iPGC numbers derived from Prmt5 KD ESCs. Since Prmt5 KD and Blimp1 Δ/Δ ESCs show similar phenotypes of reduced iPGCs, further studies of generating a Prmt5 conditional knock out in the germline are highly favorable to tease apart mechanistic role of PRMT5 in mammalian germline formation, in particular, the function dependent or independent of BLIMP1.

Discussion

Emerging cell populations in the early embryo are challenging to investigate due to low numbers and technical challenges. Here we used mESCs from a variety of genetic backgrounds to derive transgene-free, pre-gonadal PGCs with high
expression of PGC signature genes. We show that using the strategy sorting for SSEA1+/cKIT^{bright}, iPGC population with an identity equivalent of endogenous PGCs between E9.5 and E10.5.

In the current study, our model could be used to successfully asset the molecular events in PGC formation before gonadal colonization, gonadal reprograming and sex determination. Our model is successfully validated by using a Blimp1 null ESC line to derive iPGCs compared to the Blimp1^{fl/fl} controls that Blimp1 null ESCs give rise to no iPGCs whereas the control generate 3-4% iPGCs. This is faithfully representing the phenotypes happening in the Blimp1^-/- embryos that E9.5 Blimp1^-/- have no AP positive PGCs. In addition, our model provides a scalable source of iPGCs that could be utilized for assays requiring bigger number of cells. For example, we could generate 150,000-175,000 iPGCs equivalent to E9.5-E10.5 PGCs at day 6 of differentiation of 50 plates of EBs, which takes 1 hour to set up from two wells of undifferentiated ESCs and another 1 hour for FACS at the day of harvest. In contrast, 100 E10.5 embryos would be required and pooled together to obtain approximately 100,000 PGCs via FACS, assuming around 1,000 PGCs could be recovered per embryo at this developmental age.

We also showed that this model could be applied to test unknown potential germline factors, such as PRMT5. Previous reports show that PRMT5 may collaborate with BLIMP1 to repress somatic genes in pre-gonadal PGCs. We
propose that loss of PRMT5 should resemble the phenotypes of loss of BLIMP1. Indeed, we found that by using an shRNA to knock down Prmt5 to about 50% in ESCs, the iPgc population significantly reduced in the KD relative to control, indicating that PRMT5 is a crucial germline factor that may interact with BLIMP1 to ensure germ cell fate. However, more detailed experiments should be performed to tease out the accurate role of PRMT5 in pre-gonadal PGC differentiation.

In conclusion, we propose that the ESC-to-PGC differentiation model is an essential tool for examining molecular events in PGC development, which bypass the challenges of difficulties to perform biochemical or molecular assays requiring big cell numbers. This method allows us to quickly identify potential genes that are required in early PGC formation and could be applied to screen for other crucial germline factors combining siRNA libraries and IF technologies. For example, EBs could be made in 96-well low attachment plates with different siRNAs in each well to differentiate for 6 days. At day 6, the EBs could be trypsinized into single cells and be performed IF of co-staining SSEA1 and cKIT and read by a fluorescence plate reader.

Materials and methods

Ethics Statement

Mouse embryo dissection, breeding colony maintenance, and animal surgery
were all 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.

**Cell Culture and EB Differentiation**

All ESC lines in this study were maintained as described previously with lot-tested FBS (Hyclone Lot #ATJ33070) on inactivated CF-1 mouse embryonic fibroblasts (MEFs)\(^{47}\). Cells were passaged every three days at 5,300 cells/cm\(^2\). For EB formation, ESCs were subjected to MEF depletion by plating a single cell suspension on tissue culture dishes twice for five minutes each. Cells were seeded in drops of 20 microliters each containing 300 cells on the lids of Petri dishes with 5mL PBS in the plate bottom and cultured in the absence of LIF for six days, with addition of 3.5mL PBS on day 3 of differentiation. For ESC derivation, E3.5 blastocysts were isolated from homozygous \textit{Blimp1}\(^{fl/fl}\) (C57BL6/J) crosses and cultured in ESC media containing PD98059 (Cell Signaling) for four days. ESC lines were then passaged and maintained routinely. To generate \textit{Blimp1} null ESCs, \textit{Blimp1}\(^{fl/fl}\) cells were transfected with pCAG-Cre:Gfp43 plasmids and sorted to generate sub-lines.

**Mice**

\textit{Oct4-gfp} embryos were dissected and dissociated with TrypLE (Invitrogen) prior to flow cytometry or FACS. For teratoma analysis, 100,000 ESCs were injected
into the testicles of SCID recipient mice and collected 6 weeks after transplant for histology.

**Flow cytometry and FACS**

Staining for SSEA1 (DSHB, 1:200) and cKit (BD, 1:200) was performed on ice. Indirect labeling was performed with Cy5-conjugated goat anti-mouse IgG and IgM (1:500) and PE-conjugated goat anti-rat IgG (1:1000) (Jackson ImmunoResearch). 7AAD or DAPI were added prior to all acquisitions to examine only live cells for downstream analyses with FlowJo software (TreeStar).

**Immunostaining**

Embryoid bodies were fixed and embedded in paraffin according to standard protocols. For iPGC stains, cells were sorted by FACS and plated onto poly-lysine coated cover slips. The following antibodies were used at the indicated dilutions: SSEA1 (DSHB, 1:100), Oct4 (1:100, Santa Cruz), Mvh (1:100, Abcam), and H3K27me3 (1:500, Millipore). All samples were incubated with primary antibodies overnight at 4°C. Sections were washed, incubated with FITC anti-mouse IgM, TRITC anti-goat IgG, or FITC/TRITC conjugated anti-rabbit IgG antibodies (Jackson Immunoresearch) for 30 minutes at room temperature. Y chromosome FISH was performed on chromosome spreads.

**Real-time PCR**
RNA was extracted from sorted samples using the RNEasy Micro Kit (Qiagen) and reversetranscribed using Superscript RT II (Invitrogen). All gene expression analysis was performed using commercially available TaqMan Gene Expression Assays (Applied Biosystems), with the exception of Id4, which was examined by SYBR Green PCR (Roche). See Table 2-1 for additional primer information. CT values were normalized to Gapdh expression and expressed as fold change relative to a control cell type referenced in each experiment.

Single-cell Real Time RT-PCR

Single cells were sorted by FACS and subjected to reverse transcription and specific target amplification of relevant genes using the Fluidigm BioMark 48.48 dynamic gene expression system according to manufacturer’s instructions, with PCR performed by the UCLA Genotyping and Sequencing core facility. A dilution series of cells were used as detection controls and also to establish primer correlation coefficients and ensure linear amplification of amplicons. Heat map data was generated using Fluidigm Real Time PCR Analysis software.

Bisulfite Sequencing

Genomic DNA was isolated from sorted samples (Zymo Research). Bisulfite conversion was performed using the EZ DNA Methylation Kit according to manufacturer’s instructions (Zymo Research). PCR was performed on bisulfite converted genomic DNA and cloned into pCR2.1- TOPO (Invitrogen). Clones were sequenced and aligned using Lasergene software (DNASTAR). See Table
2-1 for PCR primer information.

**Southern Blot**

Prdm1/Blimp1 deletion was verified with dUTP-digoxigenin-labeled probe generated by PCR upstream of the deleted exons of Blimp1 (fwd:5'-CTCGTGGCTCTTGTGTGTGT -3', rev:5'-AACGCTGTACCCATGACTCC -3'), after digestion with EcoRI. Detection of wild type (15 kb), flox, (13.5 kb), and KO (10 kb) alleles of Blimp1 have been described 48.

ACKNOWLEDGEMENTS

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Figure 2-1. Transgene-free method for isolating iPGCs from embryoid bodies.
A: V6.5 embryoid bodies in hanging drops at days 4, 5, and 6 of differentiation.
Scale bar=500 microns. B: Oct4-Gfp expression (red) relative to V6.5 EBs (black) at days 4, 5 and 6 of differentiation. Arrows indicate shoulder of Oct4-Gfp<sup>bright</sup> cells at day 5 and an Oct4-Gfp<sup>bright</sup> peak at day 6. C: Immunofluorescence of EBs at day 6 for Oct4 (red) and SSEA1 (green). Double positive cells localize in discreet clusters (arrow). Scale bar=20 microns. D: Flow cytometry plot of V6.5 day 6 EBs stained for SSEA1 and cKit. Oval gate defines the SSEA1<sup>+</sup>/cKit<sup>+</sup> side population. E: Flow plot day 6 EBs from V6.5 ESCs fractionated by expression of SSEA1 and cKit into SSEA1<sup>+</sup>/cKit<sup>bright</sup> (green), SSEA1<sup>+</sup>/cKit<sup>mid</sup> (light blue), SSEA1<sup>+</sup>/cKit<sup>dim</sup> (dark blue), and SSEA1<sup>-</sup>/cKit<sup>-</sup> cells (red) populations. Quadrant gates are drawn to demonstrate the criteria for selecting SSEA1<sup>+</sup>/cKit<sup>bright</sup> cells. The remaining cKit<sup>+</sup> population was split into two equal fractions, mid and dim. F:
Semi-quantitative real-time PCR from the populations isolated in E, with levels normalized to Gapdh. SSEA1+/cKit$^{\text{bright}}$ cells are set at 1.0. Data is from two biological replicates each performed in technical duplicate. Error bars represent s.e.m. G: Percentage of live iPGCs acquired from differentiation of ESCs of different genetic backgrounds. Each line was tested at least seven independent times. Error bars represent s.d.
Figure 2-2. Developmental staging of pre-gonadal iPGCs at single cell resolution.

A: Bright field image of representative e9.5 Oct4-gfp embryo. Dotted line indicates where the embryo was bisected at somite 13 for FACS. Scale bar=1
mm. B: Whole mount confocal microscopy of live embryos with migratory Oct4-gfp+ PGCs within the hindgut (arrows). C: Flow cytometry of the bisected lower half of e9.5 Oct4-gfp embryos. Oct4-gfp+ PGCs (circled gate) are also positive for SSEA1 and cKit. D: Real-time RT-PCR of Gfp+ and Gfp- cells. Error bar denotes s.d. E-H: Gene expression analysis at single cell resolution for ESCs (E), e9.5 PGCs (F), e10.5 PGCs (G), and iPGCs (H) represented as a heat map of CT values with expression ranging from not detected (black) to high (yellow). A cell titration was performed as a control to ensure linear amplification of each primer set. Each cell was evaluated for the expression of each gene in technical triplicate. I-M: Semi-quantitative analysis of single cell real time PCR in E-H of cells that co-express Blimp1, Stella, Prdm14, and Dnd1 expressed relative to the average delta CT expression level for each gene in single iPGCs. *p< 1e-03, **p<1 e-04, ***p<1 e-06, NS=not significant.
Figure 2-3. iPGCs have characteristics of pre-gonadal, pre-reprogrammed in vivo PGCs. A: Immunofluorescence of pre-gonadal e10.5 PGCs stained for Oct4 (red) and Mvh (green). e13.5 male gonadal PGCs were stained as a positive control. Dotted circles mark the testis cords. B: Sorted SSEA1+/cKitbright iPGCs stained for Mvh (green, left) or secondary antibody alone (right). C: iPGCs stained for H3K27m3 (red, left), and secondary antibody alone (right). Arrows point to individual iPGCs. D: Bisulfite sequencing of ESCs, iPGCs, and endogenous e9.5 PGCs for Snrpn, the Xist promoter, and IAP. Circles represent individual CG dinucleotides, black = methylated and white = unmethylated cytosines. Arrows indicate individual alleles that display characteristic demethylation. N.D. = not determined.
Figure 2-4. Blimp1 is required for the differentiation of iPSCs from ESCs.

A: DNA-FISH for the Y chromosome in Blimp1^{fl/fl} ESCs. B: Southern blot for detection of wild type (WT), flox, and knock-out (KO) alleles of Blimp1. C: Flow cytometry for SSEA1 on undifferentiated ESCs. D: Representative histological sections from Blimp1^{fl/fl} and Blimp1^{Δ/Δ} teratomas. All lines were capable of differentiation to ectoderm (Ecto), mesoderm (Meso) and endoderm (Endo). Scale bar=100 microns. E: Representative paired EB differentiations of Blimp1^{fl/fl} and Blimp1^{Δ/Δ} ESCs. Quadrant gates indicate criteria for gating SSEA1^{+}/cKit^{bright} iPSCs, which are contained within the rectangular gate (black lines). F: Percentage iPSC yield in the control Blimp1^{fl/fl} line and Blimp1^{Δ/Δ} sublines. Error bars represent s.e.m. G: Quantification of data from F, expressed as
a percent of the *Blimp1*^flu^ iPGC yield from each paired experiment. Error bars represent the standard error of the mean. *p< 0.05, ** p< 1x10^-7.

Figure 2-5. PRMT5 is present in day 6 SSEA1^+/ckit^bright^ iPGCs. A: Schematic plot of obtaining iPGCs and staining for dropped cells on poly-lysine coated slides. B: PRMT5 is present in the nucleus of dropped V6.5 wild type iPGCs.
Figure 2-6. iPGC differentiation is compromised with reduced levels of PRMT5.

A: Gene expression level of PRMT5 in control, 1-4 and 1-8 relative to Gapdh. B: Western blot showing PRMT5 protein level of Ctrl, 1-4, 1-8 quantified in C. D: Representative of flow cytometry plot of iPGCs (SSEA1+/cKit^{bright}) populations among Ctrl, 1-4 and 1-8 and quantified in E. Loss of PRMT5 causes reduction of more than 50% iPGCs relative to control.
Figure 2-S1. Kinetics of EB formation and the transcriptional identity of iPGCs.

A: Oct4-gfp embryoid bodies at days 5-8 of differentiation. Scale bar=500 microns. B: Quantification of EB cell viability recorded as the percent of 7AAD-cells at each time point by flow cytometry. C: Flow cytometry of the live cell EB fraction for Oct4-gfp and cKit at the corresponding time point. Blue oval indicates the Oct4-gfp+/cKit+ side population, which first appears at day 6. Oct4-gfp+/cKitbright cells correspond to iPGCs. D: Oct4-gfp EBs at day 6 were stained with SSEA1 and cKit, and Oct4-gfp expression was examined in SSEA1+/cKitbright, SSEA1+/cKitmid and SSEA1+/cKitdim populations.
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RT=RT-PCR, BS=bisulfite sequencing-PCR, FP=forward primer, RP=reverse primer

Table 2-1. Primers used in this study.
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CHAPTER 3

PRMT5 IS AN ESSENTIAL SURVIVAL FACTOR FOR GROUND STATE PLURIPOTENCY AND PRIMORDIAL GERM CELLS
PRMT5 is an essential survival factor for ground state pluripotency and primordial germ cells

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Summary

PRMT5 is a type II protein arginine methyltransferase (PRMT) with roles in cancer, reprogramming and neurogenesis. In pluripotent stem cells PRMT5 prevents naïve stem cell differentiation however its role in ground state pluripotency is unknown. Here, we show that PRMT5 functions as a critical survival factor in the ground state by regulating splicing of mRNAs associated with RNA processing, the DNA damage response and chromatin. Loss of PRMT5 leads to an increase in gamma H2AX foci and apoptosis without inducing differentiation. In primordial germ cells (PGCs) PRMT5 also regulates survival with a minor role in PGC specification. Taken together, our study demonstrates that PRMT5 is essential for ground state pluripotency and PGC development where it safeguards pre-mRNA splicing upstream of cell survival but does not directly regulate differentiation.

Highlights

1. Pluripotent ground state stem cells and germ cells require PRMT5 for survival.
2. RNA identity in ground state stem cells involves PRMT5-regulated exon skipping.
3. PRMT5 does not have a major role in PGC specification.
**Introduction**

Pluripotent stem cells are critical in vitro cell types that serve as a scalable source of cells for differentiation into clinically relevant cell types, or a novel tool to understand mechanisms associated with embryonic lineage differentiation. Three states of pluripotency have been described, ground state, naïve and primed. The ground state refers to pluripotent stem cells cultured with inhibitors of extracellular signal related kinases and glycogen synthase kinase 3 (2i) together with Leukemia Inhibitory Factor (LIF) (Ying et al., 2008). Ground state cells are hypomethylated similar to cells of the pre-implantation embryo and primordial germ cells (PGCs), and they exhibit uniform expression of pluripotency genes, low expression of differentiation genes and RNA polymerase II paused proximal promoters (Ficz et al., 2013; Leitch et al., 2013) (Bustamante-Marín et al., 2013; Habibi et al., 2013; Hackett et al., 2013b; Marks et al., 2012). The naïve state refers to mouse pluripotent stem cells cultured with LIF in the presence of serum. Naïve cells are hypothesized to resemble early epiblast and exhibit substantial cytosine methylation in a CG sequence context, histone H3 lysine 27 trimethylation (H3K27me3), bivalent chromatin domains, precocious expression of differentiation genes and variable expression of *Oct4, Nanog* and *Sox2* (Ficz et al., 2013; Leitch et al., 2013) (Bustamante-Marín et al., 2013; Habibi et al., 2013; Hackett et al., 2013b; Marks et al., 2012). Ground state and naïve pluripotency are interconvertible in culture, and the epigenome and transcriptome are sufficiently malleable in both states to allow contribution to germline chimeras (Bradley et al., 1984; Marks et al., 2012; Ying et al., 2008).
The primed state refers to pluripotent stem cells derived from post-implantation mouse epiblasts or human blastocysts in media containing Fibroblast Growth Factor 2 and Knockout Serum Replacer (Brons et al., 2007; Tesar et al., 2007; Thomson, 1998). Primed cells exhibit robust CG methylation, however mouse primed stem cells do not contribute to chimeras at high frequency, or at all (Brons et al., 2007; Tesar et al., 2007). Given the epigenetic, transcriptional and functional differences between pluripotent states, it is anticipated that different molecular pathways will be required to sustain their unique identities. Indeed PR domain zinc finger protein 14 (Prdm14) has a different role in the regulation of ground state and naïve pluripotency and is required for PGC differentiation \textit{in vivo} and \textit{in vitro} (Ficz et al., 2013; Grabole et al., 2013; Leitch et al., 2013; Nakaki et al., 2013; Yamaji et al., 2008; 2013).

Protein arginine methyltransferase 5 (PRMT5) is a type II arginine methyltransferase that mediates symmetrical dimethylation of arginine (SDMA) in protein substrates. SDMA facilitates interaction with tudor domain-containing proteins, or alternatively can inhibit protein interactions by removing hydrogen bond donors (Bedford and Clarke, 2009). In pluripotency, PRMT5 is required to establish naïve pluripotent stem cells \textit{in vitro}, and a short interfering RNA (shRNA) knock down of Prmt5 in the naïve state causes down-regulation of pluripotency-associated RNAs and up-regulation of differentiation genes (Tee et al., 2010). In the germline, PRMT5 is also hypothesized to repress differentiation genes (Ancelin et al., 2006), however this remains to be proven. The major
mechanism by which PRMT5 safeguards naïve pluripotency is through SDMA of pre-deposited histone H2A in the cytoplasm, which regulates gene expression in the nucleus. However, it is unclear whether this mechanism operates downstream of PRMT5 in other cell types.

In the current study we evaluated the role of PRMT5 in regulating ground state pluripotency and discovered that PRMT5 is required for survival and not repression of genes involved in differentiation. In the germline we discovered that PRMT5 is required for survival with only modest roles in PGC differentiation. Taken together, mechanisms that function to regulate naïve pluripotency can have different roles in other pluripotency-associated cell types including the ground state and germline.

**Results**

*PRMT5 is essential for survival of ground state pluripotent stem cells*

To address the hypothesis that PRMT5 is required in the pluripotent ground state, we obtained *Prmt5^{3floX-2ht;}* + conditional knockout-first ESCs from the European Conditional Mouse Mutagenesis (EUCOMM) project (Skarnes et al., 2011) and generated germline chimeras in a C57BL/6 background. In order to convert the allele to conditional-ready (Fig 3-1A), we crossed heterozygotes to mice constitutively expressing *Flpase* and screened for mice with the *Prmt5^{2floX;}* + allele (*Prmt5^{fl/+}*). Homozygous *Prmt5^{fl/+}* breeding stocks were established which exhibited normal litter size and fertility. In order to create an inducible knockout,
we crossed Prmt5^{fl/fl} to Rosa26^{CreERT2/CreERT2} (CreER). Siblings were intercrossed to obtain Prmt5^{fl/fl} or Prmt5^{fl/+} mice in a homozygous CreER background. Inducible heterozygous or homozygous ESC lines were made from E3.5 blastocysts with the Prmt5^{fl/fl};CreER ESCs called inducible Prmt5 knockouts (iPKO), and the Prmt5^{fl/+};CreER ESCs were called inducible Prmt5 heterozygotes (iPHet, Fig 3-1B). All experiments were performed after a minimum of 5 passages in 2i + LIF.

To induce a deletion at the Prmt5 locus tamoxifen (4-OHT) was added to the 2i + LIF media on the day of plating and the media was replaced 48 hours later with fresh 2i + LIF media without 4-OHT. Using Western blot analysis we show that this strategy results in near undetectable levels of PRMT5 protein in iPKO treated cells on day 5 relative to iPHet treated controls (Fig 3-1C). Given this result we used the same format to induce a homozygous or heterozygous deletion in Prmt5 iPKO and iPHet ESCs respectively for all future experiments. Next we evaluated the phenotype of ground state iPKO ESCs with a homozygous deletion in Prmt5 and discovered that growth was compromised starting at day 3, which was further exacerbated by day five (Fig 3-1D). Even though growth was affected, the morphology of iPHet and iPKO treated ESCs cells was indistinguishable during this period (data not shown).

To determine whether the reduction in cell growth was due to accumulation of cells in a specific phase of the cell cycle, we performed cell cycle analysis, and
found that treated iPKO cells had equivalent numbers of cells in each phase relative to iPHets, indicating that the growth phenotype was not due to cell cycle arrest (Fig 3-1E). To address whether the growth retarded iPKO cells could be passaged, we harvested with trypsin and seeded in 2i+LIF. Twenty-four hours after plating we found that most iPKO mutant cells did not attach, however the few iPKO surviving ESCS formed alkaline phosphatase (AP) colonies with abnormal cobble-stone morphology (arrows, AP’M-, Fig 3-1F). By the second passage, the iPKO mutant cells were gone.

Based on this result, our initial hypothesis was that a deletion in Pmrt5 caused ground state cells to die by apoptosis. To address this, we performed western blot on day 5 and show that cleaved PARP (cPARP) is elevated in the nucleus and cytoplasm of iPKO treated ESCs (Fig 3-1G). Consistent with this, using flow cytometry for AnnexinV and 7AAD (markers for apoptosis and viability respectively), we show that the percentage of AnnexinV positive cells was also increased at day 5 in iPKO cells (Fig 3-1G).

In addition to evaluating apoptosis, we also explored the possibility of senescence by first staining for Ki67 (Fig 3-1H). We discovered that entire colonies of iPKO treated ESCs were mostly negative for Ki67 (red arrows, Fig 3-1H), whereas iPHet treated controls were positive (white arrows). Loss of Ki67 alone is not sufficient to define senescence. Therefore, we stained for tri-methylation of lysine 9 on histone H3 (H3K9me3), a marker for senescence
associated heterochromatin foci (Sadaie et al., 2013) and show that treated iPKO ESC nuclei have significantly more H3K9me3 foci (red arrows, Fig 3-1I and quantified in Fig 3-1J) relative to iPHet treated controls. Finally, we counted the number of gamma H2AX positive foci per nucleus, which is associated with both senescence and DNA damage (Mah et al., 2010) and found that iPKO treated ESCs have significantly more gammaH2AX foci per nucleus than iPHet treated controls (Fig 3-1K and quantified in 3-1L). Taken together, our data supports a model in which loss of Prmt5 in ground state pluripotency causes an arrest in cell growth, hallmarks of cellular senescence together with increased DNA damage and apoptosis within five days of inducing a homozygous deletion in Prmt5.

A mutation in Prmt5 affects SDMA of histones and expression of DNA damage response genes

To understand the molecular mechanism underlying these phenotypes we first performed western blot to examine SDMA of H2A and H4 (Fig 3-2A). Consistent with previous reports, an induced deletion in Prmt5 caused loss of Histone H2A containing SDMA of arginine 3 (H2AR3sme2) from the nuclear fraction (Tee et al., 2010). We also saw a modest loss of SDMA at arginine 3 in Histone H4 (H4R3sme2). Unlike the naive state where H2AR3sme2 is identified in the cytoplasm, we found that 2i + LIF cultured ESCs have no cytoplasmic H2AR3sme2. Instead we identified a 25KDa band that we speculate is non-specific because a deletion in Prmt5 had no affect on its levels.
The SDMA changes in histone H2A and H4 were puzzling as PRMT5 protein is enriched in the cytoplasm and not in the nucleus (Fig 3-1C, 1I). Therefore, the most likely explanation is that a small amount of PRMT5 is found in the nucleus of ground state ESCs, which we confirmed by over-exposing immunoblots of nuclear fractions (Fig 3-S2A). Therefore the SDMA changes to chromatin in 2i + LIF ESCs are most likely due to a small amount of nuclear PRMT5 rather than modification of pre-deposited cytoplasmic H2A. Furthermore, our Western blots indicate that the levels of H2AR3sme2 are very low in control 2i + LIF ESCs. To quantify this we used HPLC to purify histones followed by Mass Spectrometry and show that the majority of H2AR3 in 2i + LIF cultured ESCs is unmodified (Fig 3-S2B). Taken together, given the cytoplasmic localization of PRMT5 and the very limited inclusion of H2AR3sme2 in the chromatin of control ground state ESCs, we hypothesize that the phenotypes in Prmt5 iPKO ESCs may be due to mechanisms beyond SDMA of histones.

To address this, we turned to RNA-sequencing (RNA-seq). All experiments were performed five days after initiating the 48 hour 4-OHT treatment as in Fig 3-1. Using the criteria FDR<0.05 and fold change >2, we identified 394 genes that were differentially expressed between iPHet and iPKO treated cells (Fig 3-2B, Table 3-S1). Gene ontology (GO) analysis using the criteria of biological process revealed that up-regulated genes in iPKO mutant ESCs (232 genes) included categories such as “calcium ion transport” and “di-, tri-valent inorganic cation transport”. Unexpectedly, we identified categories such as “male gamete
“generation” and “spermatogenesis” as being up-regulated in the iPKO ESCs including Piwil4/Miwi2, Stag3, Taf7l, Dazl and D1Pas1 (Fig 3-2C). Interestingly, the down-regulated genes in iPKO mutant cells (162 genes) belong to GO categories such as “neuron differentiation” and “cell projection morphogenesis” (Fig 3-2C). This is consistent with recent work identifying a role for PRMT5 in neural progenitor cell (NPC) homeostasis (Bezzi et al., 2013). Absent in our differentially expressed gene lists were an increase in differentiation genes including Fgf5, Gata4, Gata6, HoxA3, HoxD9 and FoxA2 which are up-regulated in serum + LIF cultured ESCs with a shRNA knock down of Prmt5 (Tee et al., 2010). Therefore, our data indicates that gene expression changes down-stream of Prmt5 in ground state pluripotency are different to those previously reported for naïve.

Interestingly, we did not find the term “apoptosis” enriched in the GO terms suggesting that PRMT5 is not simply inducing the apoptotic pathway. Instead, we observed alterations in DNA damage response and cell proliferation genes, including down-regulation of Fancl and E2f8 and up-regulation of Dlec1 in iPKO mutant ESCs (Fig 3-2D). Fancl is the L component of the catalytic core of Fanconi Anemia Complementation group (FANC) which regulates DNA interstrand crosslink repair (Garner and Smogorzewska, 2011; Meetei et al., 2003). E2F8 represses E2f1 to enable a cell cycle response to DNA damage. A depletion of E2f8 together with E2f7 leads to massive apoptosis in mouse embryos (Li et al., 2008; Panagiotis Zalmas et al., 2008). Finally, Dlec1 is a
tumor suppressor and negative regulator of cell growth (Daigo et al., 1999; Senchenko et al., 2003). Taken together, our data suggest that loss of Prmt5 leads to an attenuated DNA damage response due to repression of DNA damage response machinery. Quantitative reverse transcriptase PCR (qRT-PCR) was performed to confirm differentially expressed genes identified by RNA-Seq between iPHet and iPKO (Fig 3-S2C).

To determine whether a conserved cohort of differentially expressed genes were found in other Prmt5 mutant cell types, we performed a meta-analysis of Prmt5 mutant and control NPCs and MEFs (Bezzi et al., 2013). Using a cut-off of >2-fold differential expression, we discovered 25 up-regulated genes in common between Prmt5 mutant 2i + LIF ESCs and Prmt5 KO NPCs and 14 common down-regulated genes (Fig 3-2E, Table 3-S1). In NPCs PRMT5 attenuates the P53 pathway, however we did not find any common genes known to be activated by P53 in the Prmt5 mutant 2i+LIF ESCs. Instead, we found that Fancl was down-regulated in both cell types, suggesting that the DNA damage response may be impaired in both NPCs and 2i + LIF ESCs. Interestingly, in Prmt5 mutant MEFs, using the same cut-off we found no overlap in differentially expressed genes with ground state mutant ESCs (data not shown). Taken together, our data demonstrate that loss of PRMT5 causes unique RNA expression changes between ground state ESCs, NPCs and MEFs, and apoptosis in the iPKO ESCs is independent of P53 induced transcription of cell cycle and apoptotic regulators.
Loss of PRMT5 leads to severe abnormalities in splicing

Given that Prmt5 mutant ESCs have hallmarks of DNA damage combined with repression of genes associated with the DNA damage response, we next investigated the potential source of this phenotype. Given that 2i + LIF is a feeder-free chemically defined system (no MEFs), we reasoned that the source of DNA damage must be intrinsic to the ESCs. In mammalian cells PRMT5 is required for spliceosome assembly, a fundamental macromolecular machine responsible for removing introns from all RNA precursors to mRNA. Therefore, we hypothesized that 2i + LIF cultured ESCs are vulnerable to constitutive splicing abnormalities. To address this we used Multivariate Analysis of Transcript Splicing (MATS) with a pair-wise analysis among four pairs of iPHet vs. iPKO replicates. We identified more than 1,000 abnormal splicing events in iPKO mutant ESCs compared to iPHet controls (Fig 3-3A). In particular, we observed a consistent problem with skipping exons (SE) in the Prmt5 mutant ESCs. In contrast, retention of introns was a relatively minor problem suggesting that constitutive splicing was only modestly affected (Fig 3-3A, Table 3-S2). Using the IGV genome browser we confirm that exon 7 in the Prmt5 locus corresponded to a SE in iPKO cells, as expected given the exon no longer exists (Fig 3-3B). The skipped SE in the Prmt5 RNA of iPKO mutants was confirmed by RT-PCR using primers flanking exon 7 (Fig 3-3C).

In order to illustrate the relationships between replicates we plotted the number of abnormal splicing events in common between samples using a Venn Diagram...
We identified 283 overlapping abnormal splicing events between three of the four replicate pairs, with 107 events in common across all four pairs (Fig 3-3D). This high degree of variability between replicates is consistent with the data of (Bezzi et al., 2013) who identified 320 overlapping affected genes in 2 out of 3 replicates of NPCs. However, in contrast to NPCs, our common set of alternatively spliced RNAs reveal defects in genes associated with “mRNA processing”, “RNA splicing”, “Cell cycle” and “Chromatin modification” (Fig 3-3E).

In the category of “mRNA processing” and “RNA splicing”, we identified abnormal splicing of breast carcinoma amplified sequence 2 (Bcas2), a gene that encodes a protein of the PSO4 core complex required for pre-mRNA splicing (Chen et al., 2013; Legerski, 2009) (Fig 3-3E). BCAS2 also functions in the DNA damage response, and is required to facilitate recruitment of DNA repair machinery (Wan and Huang, 2014). In addition, we discovered abnormal splicing of heterogeneous nuclear ribonucleoproteins (hnRNPs) Hnmpa2b1 and Hnmph1, as well as pre-mRNA splicing factors (PRPFs) including Prpf39 and Prpf40b, which together suggest that ground state pluripotency is particularly sensitive to RNA processing events controlled by PRMT5.

Besides “RNA processing”, we identified genes involved in the DNA damage response in the group “Cell cycle”, for example, Rif1 (Rap1 interacting factor 1 homolog), Pms2 (postmeiotic segregation increased 2) and Mdm4 (transformed mouse 3T3 cell double minute 4) (Fig 3-3E). Rif1 is required for resection of
double strand breaks, and a homozygous mutation in Rif1 causes embryo lethality (Buonomo et al., 2009; Chapman et al., 2013). PMS2 is a critical component of the DNA mismatch repair pathway (Iyer et al., 2006). Abnormal splicing of Mdm4 is a known splicing target of PRMT5 in NPCs (Bezzi et al., 2013).

Another category of interest was abnormal splicing of genes involved in chromatin including euchromatic histone lysine N-methyltransferase 2 (Ehmt2) and suppressor of variegation 4-20 homolog 2 (Suv420h2). Ehmt2 is responsible for di-methylation of histone H3 lysine 9 (H3K9me2), a prominent mark in transcriptional repression (Tachibana et al., 2002; 2005) (Fig 3-3E). This gene has fourteen alternatively spliced transcripts, of which six are protein-coding (www.ensemble.org). Analysis of the Ehmt2 locus in iPKO cells identified both SE and RI events at exon 23 and intron 22 (Fig 3-3F and validated in 3-3G). These abnormal splicing events are within the region coding for the methyltransferase domain responsible for H3K9me2, with the SE creating RNAs that encode a premature stop codon. Therefore, we performed a western blot to evaluate the levels of H3K9me2 in iPKO and iPHet treated cells, and consistent with abnormal splicing of Ehmt2, we show that the global levels of H3K9me2 are reduced in iPKO cells, whereas global levels of tri-methylation of K27 on H3 (H3K27me3) are modestly increased (Fig 3-3H). Taken together, loss of PRMT5 causes abnormal splicing of genes involved in RNA processing, the DNA
damage response and chromatin organization for which the phenotypic outcome is poor proliferation, an increase in DNA damage and cell death.

**PRMT5 is dynamically expressed in the mammalian germline during development**

Given the unexpected role for Prmt5 in survival of ground state pluripotent stem cells together with a significant increase in germ cell-expressed genes in the iPKO mutants, we next evaluated the role of Prmt5 in germline differentiation. In the mouse, PRMT5 is hypothesized to interact with the major PGC determinant BLIMP1 to regulate PGC fate and repress differentiation genes similar to its role in naïve pluripotency.

Using immunofluorescence (IF) we show that PRMT5 is initially localized to both the nucleus and cytoplasm of OCT4+ PGCs at E9.5 with enriched expression in PGCs relative to somatic cells starting at E10.5 when PGCs begin to express the gonadal stage germline gene Mouse Vasa Homologue (MVH) (arrows, Fig 3-4A). At E11.5 PRMT5 is highly enriched in the cytoplasm of both sexes (arrows, Fig 3-4A). However, between E11.5-E13.5 and coinciding with sex determination, PRMT5 localizes in a sexually dimorphic pattern. In males (Fig 3-4B), PRMT5 transitions back into the nucleus, and from E16.5 to birth is also enriched in small discreet cytoplasmic foci mostly independent from MVH+ foci (Fig 3-4B). In females (Fig 3-4C), PRMT5 remains in the cytoplasm for the remainder of
gestation and does not enrich in obvious cytoplasmic foci. A summary of PRMT5 localization is shown in Fig 3-4D.

**PGCs survival is compromised between E10.5 to E13.5**

To evaluate the role of PRMT5 in PGC development we crossed *Prmt5*^{fl/fl} to *Blimp1-Cre;+* (BC) transgenic mice. *Blimp1* is expressed in PGC-precursors in the epiblast at E6.0 and the BC tool is reported to exhibit 55%-75% recombination efficiency in PGCs by E7.5 (Ohinata et al., 2005). We evaluated recombination rate at E9.0 and discovered that recombination has occurred in 85% of E9.0 PGCs and by E13.5 recombination efficiency is 100% (Fig 3-S5A). Crossing *Prmt5*^{fl/fl} females to *Prmt5*^{fl/+}; BC males yielded male and female *Prmt5*^{fl/-}; BC (PCKO) pups at the expected Mendelian ratio. Analysis of gonads from females and males at P1-2 showed a complete lack of germ cells both by histology (Male, Fig 3-5A; Female, Fig 3-5B; arrows pointing to germ cells) as well as by IF (Male, Fig 3-5C; Female, Fig 3-5D; arrows pointing to germ cells).

To identify the embryonic stage when PGCs are lost we first evaluated E13.5 embryos and found both male and female PCKO embryos completely lacked MVH positive PGCs in the gonads (Fig 3-5E and 5F respectively). However gonad formation was otherwise normal, including formation of seminiferous cords containing SOX9 positive sertoli cells in males (Fig 3-5E and 5G). At E11.5 PGC number in PCKO genital ridges was significantly reduced (Fig 3-5H, quantified in 5J). At E9.5 PGC number and location in the embryo is normal (Fig 3-5I, Fig 3-
Morphologically we found PCKO embryos at E9.5 were smaller than littermate controls (Fig 3-S5C), however somite number was within the normal range for E9.5, and embryo turning had occurred indicating that the embryos were not delayed, but rather mildly growth retarded. This growth retardation phenotype was transient and most PCKO embryos were indistinguishable from controls at E11.5 (Fig 3-S5D). Taken together our data suggests that PRMT5 regulates PGC survival between E10.5-E13.5.

*PCKO PGCs exit cell cycle and have increased apoptosis, increased H3K9me3 foci and fail to implement the gonadal PGC program*

Loss of PGCs in the PCKO embryos could be due to either failed migration and/or PGC apoptosis. Analysis of E10.5 embryos revealed a PGC migration pattern that was indistinguishable from controls (Fig 3-6A), suggesting that migration to the genital ridges was unaffected. However, analysis of cPARP reveals a statistically significant increase in the number of apoptotic OCT4⁺ PGCs in PCKO embryos (Fig 3-6A and B).

Next we evaluated Ki67 which was negative in a majority of 2i + LIF cultured ESCs with a null mutation in *Prmt5*. We found that similar to ground state ESCs, the majority of OCT4⁺ PGCs in PCKO embryos at E9.5 were also negative for Ki67 (Fig 3-6C and quantified in D). Loss of Ki67 at E9.5 is critical because 24 hours prior, wild type PGCs enter a long G2 pause (Seki et al., 2007). Therefore, we hypothesized that PCKO PGCs do not successfully exit from G2. To test this
we evaluated PCKO and control embryos for CYCLIN B1 (CCNB1), which is cytoplasmic in G2 and translocates to the nucleus in early M (Fig 3-6E). Our data demonstrate that the fraction of PGCs in G2 or G2/M is similar between control and PCKO mutant embryos (Fig 3-6F). However, given that 40% of PGCs in PCKO embryos are negative for CCNB1 and 40% of PGCs have lost Ki67 staining, we propose that the CCNB1 negative PGCs in PCKO embryos at E10.5 have exited the cell cycle.

Given that H3K9me3 foci are enriched in Prmt5 mutant ground state ESCs, we also evaluated H3K9me3 in surviving PGCs at E10.5 (Fig 3-6G). Similar to ESCs we show that H3K9me3 foci are more distinct (red arrows, Fig 3-6G) in OCT4⁺ PCKO PGCs at E10.5 compared to controls (white arrows). Finally we examined expression of MVH at E11.5. MVH protein expression is initiated at E10.5 in control PGCs, then becomes highly enriched in the PGC cytoplasm from E11.5-E13.5 (Toyooka et al., 2000; Vincent et al., 2011). Our data reveals that MVH is not enriched in PCKO PGCs at E11.5 (Fig 3-6H), indicating that the gonadal PGC program is not correctly executed in the Prmt5 mutant germline. Taken together, our data demonstrate that a conditional mutation in Prmt5 causes PGCs to exit the cell cycle at or before E9.5, accumulate H3K9me3 and progressively undergo apoptosis without fully executing the gonadal stage germline program as demonstrated by failed induction of MVH.

*PRMT5 does not have a major effect on PGC specification prior to E9.5*
PRMT5 is hypothesized to interact with BLIMP1 in the germline to repress somatic cell differentiation and enable germ cell fate (Ancelin et al., 2006). Therefore, we were surprised that PRMT5 mutants had normal numbers of PGCs at E9.5. One explanation is that PRMT5 protein may still be present in some PCKO PGCs prior to E13.5. Using IF at E10.5 we found PRMT5 protein in OCT4⁺ PCKO PGCs, similar in intensity to the surrounding somatic cells (Fig 3-7A). Therefore, we speculate that the lack of phenotype in PCKO embryos before E9.5 may be due to the presence of some residual PRMT5 protein.

To address this, we turned to the PGC-like cell (PGCLC) differentiation model from Epiblast-like cells (EpiLC) (Hayashi et al., 2011). In order to examine PGCLC induction in the absence of PRMT5, we first added 4-OHT to undifferentiated iPKO and iPHet ESCs prior to EpiLC differentiation. Addition 4-OHT for 48 hours in the ESC state resulted in loss of PRMT5 protein as expected (Fig 3-7B). However, differentiation of EpiLCs and induction of PGCLCs from the iPKO mutant ESCs yielded small balls of dead cells which was not useful to address our hypothesis (data not shown). Therefore, we initiated 4-OHT in the EpiLC state, and assayed PRMT5 expression on day 2 (Fig 3-7C). Our data reveals that PRMT5 protein is expressed at background levels in both iPHet and iPKO EpiLCs consistent with the down regulation of PRMT5 in mouse epiblast (Tee et al., 2010), and therefore indicates that PGCLC induction in this model is initiated from cells depleted of PRMT5.
To assay PGCLC induction we created floating aggregates from equivalent numbers of iPHet and iPKO treated EpiLCs (Fig 3-7D). This yielded aggregates of similar size and morphology at day 4. In order to evaluate PGCLC induction we performed flow cytometry using the PGCLC surface markers SSEA1 and CD61 at day 4 (Fig 3-7E). These markers are known to mark PGCLCs equivalent to E9.5-E11.5 (Hayashi et al., 2011). We show that deleting Prmt5 in EpiLCs results in a 50% reduction in the percentage of PGCLCs in iPKO floating aggregates relative to iPHet controls (Fig 3-7E). Therefore, PRMT5 modulates PGCLC formation/survival in vitro but is not essential for PGCLC specification per se, given that we were still able to generate PGCLCs in this model. Similar to the in vitro experiments, inducing a deletion in the Prmt5fl/fl or Prmt5fl/+;CreER embryos at E6.5 still resulted in the formation of PGCs in the Prmt5fl/fl genotype indicating that PGC formation can occur in the absence of Prmt5.

Discussion

PRMT5 is dynamic protein required for cellular state and fate. Our work revealed that PRMT5 plays a novel role in survival of ground state pluripotent cells and immature germline progenitors in the embryo. This is in contrast to naïve pluripotent stem cells, where PRMT5 safeguards pluripotency (Tee et al., 2010) and primed human pluripotent stem cells where PRMT5 regulates proliferation (Gkountela et al., 2014). A summary of the major mechanisms by which PRMT5 safeguards survival of ground state pluripotent stem cells as revealed by this work in Fig 3-7G.
Ground state pluripotent stem cells and PGCs are distinct cell types, yet they share tremendous overlap with regard to molecular regulation and identity. In particular, both cell types rely on PRDM14 to repress \textit{de novo} DNA methyltransferases and attenuate fibroblast growth factor signaling (Ficz et al., 2013; Grabole et al., 2013; Leitch et al., 2013; Nakaki et al., 2013; Yamaji et al., 2008; 2013). Furthermore, the genomes of both PGCs and ground state pluripotent stem cells are considerably hypomethylated, and TET methylcytosine dioxygenases are necessary to facilitate locus-specific demethylation during transition to their unique epigenetic ground states (Ficz et al., 2013; Leitch et al., 2013) (Habibi et al., 2013; Hackett et al., 2013a; 2013b; Kobayashi et al., 2013; Marks et al., 2012; Seisenberger et al., 2012; Vincent et al., 2013; Yamaguchi et al., 2013). Our data suggests that PGCs and ground state pluripotent stem cells are exquisitely sensitive to loss of PRMT5 leading to immediate cell death such that \textit{Prmt5} mutant cells cannot contribute to embryogenesis (Tee et al., 2010) and will not participate in the transmission of DNA from one generation to the next through the germline. Thus PRMT5 is a true survival factor for the species.

It was previously hypothesized that PRMT5 functions together with BLIMP1 to regulate germline induction by repressing somatic cell gene expression (Ancelin et al., 2006). Our data unequivocally demonstrate that PRMT5 is required for PGC formation. However, contrary to its purported role, we show that PRMT5 does not have a major role in PGC specification. Instead we show that PRMT5
regulates PGC survival at the time when PGCs exit G2 and initiate rapid proliferation to complete epigenetic reprogramming. In *Drosophila*, *Prmt5 (dart5)* is required for PGC specification by ensuring Tudor localizes to the posterior pole (Anne et al., 2007; Gonsalvez et al., 2006). In mammals, tudor proteins are required in the male germline after E13.5 to ensure transposon repression by piRNAs (Chuma and Nakano, 2012; Reuter et al., 2009; Tanaka et al., 2011; Vagin et al., 2009; Yabuta et al., 2011). The sex-specific localization of PRMT5 to the nucleus and processing bodies of male germ cells after E13.5 suggests that PRMT5’s conserved role with *Drosophila* may involve modulating the intracellular localization and function of mammalian tudor family proteins which repress transposons (Kirino et al., 2009; 2010; Nishida et al., 2009; Vagin et al., 2009). Future studies using *Prmt5*/*m* mice can be used to definitively address this.

In ground state pluripotency we discovered that PRMT5 sits at the top of a hierarchy that closely regulates RNA sequence identity by restraining abnormal splicing. In NPCs, PRMT5 also regulates splicing (Bezzi et al., 2013), however the downstream RNAs affected in NPCs are more closely aligned with promoting a P53 apoptotic response than augmenting deregulation of the splicing machinery and attenuating the DNA damage response. Similar to NPCs, we found that *Mdm4* RNA is abnormally spliced in ground state pluripotent stem cells (Fig 3-7F). However, given the lack of P53 target gene induction, our data indicates that ground state pluripotent stem cells are less sensitive to the disabling effects of *Mdm4*, and that alternate mechanisms are utilized to clear.
abnormally spliced cells from the population. In ground state pluripotency, we speculate that self-renewing stem cells have increased sensitivity to DNA damage, which is further exacerbated by their hypomethylated genome. We propose that ground state pluripotent stem cells use *Prmt5*-driven splicing of DNA damage response genes as an essential mechanism to guard against the propagation of DNA damage in the population in the absence of a robust cell cycle checkpoint system.

In summary, by combining RNA processing with regulation of the DNA damage response, ground state pluripotent stem cells have evolved an elegant feedback mechanism that does not rely on P53 or cell cycle checkpoints to ensure that damaged cells will not accumulate in the population, and that only cells of the correct transcriptional make-up will survive.

**Experimental Procedures**

*Mouse strains and genotyping*

The *Prmt5* knockout first ESCs were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM; [http://www.knockoutmouse.org](http://www.knockoutmouse.org)) and were injected into B6 (Cg)-*Tyr<sup>2J</sup>*/J blastocysts (Stock No.000058, The Jackson Laboratory). Male chimeras were backcrossed to C57BL/6J females for germline transmission. To generate the *Prmt5 Flox* allele, the βgal-neomycin cassette was removed by crossing *Prmt5* knockout first mice with β-actin-Flpe transgenic mice [B6.Cg-Tg(ACTFLP3)9205Dym/J; Stock No.005703, The Jackson Laboratory] to
generate \textit{Prmt5}^{fl/fl} mice. 4-hydroxytamoxifen (4-OHT) inducible knockouts were created by crossing \textit{Prmt5}^{fl/fl} with \textit{Rosa26}^{CreERT2/CreERT2} mice [B6.129-Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj} J; Stock No. 008463, The Jackson Laboratory]. At the same time, \textit{Prmt5}^{fl/fl} mice were crossed to \textit{Blimp1-Cre} [B6.Cg-Tg(Prdm1-Cre)1Masu/J; Stock No. 008827, The Jackson Laboratory] to generate male mice with the genotype of \textit{Prmt5}^{fl/+}; \textit{BC} (Blimp1-Cre). Germline conditional knockout mice are generated by crossing \textit{Prmt5}^{fl/+}; \textit{BC} males with \textit{Prmt5}^{fl/fl} females.

Primers used to identify \textit{Prmt5}^{fl/fl} were Prmt5-For: 5'-TTCTTTCTAAGGTGCAGCAGAGGC-3', Prmt5-Rev: 5'-TTGCCTCTTTCCTCTGGTGCTGGG-3'. The mutant band is 371bp and the wild type band is 422bp. All other genotyping methods and primers can be found at \url{http://jaxmice.jax.org/index.html}.

For additional experimental details please refer to Extended Experimental Procedures

\textbf{Author Contributions}

Ziwei Li: performed and designed the experiments, wrote the manuscript.

Juehua Yu: Computational analysis of RNA-Seq data.

Ashley Cass: Computational analysis of RNA Seq data.

Linzi Hosohama: Performed experiments.

You Feng: Performed and designed experiments.

Sanjeet Patel: Performed and designed experiments.
Sonal Chaudhari: Performed experiments.
Kevin Nee: Performed experiments.
Steven G. Clarke: Designed experiments.
Xinshu Xiao: Designed computational analysis of RNA Seq data.
Amander T. Clark: Designed experiments, wrote the manuscript, maintained all required institutional compliances for biological safety and animal work.

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Figure 3-1. PRMT5 is essential for survival of ground state pluripotent stem cells. (A) Schematic design of the targeting vector. (B) Schematic model of ESC derivation. (C) Western blot of iPHet and iPKO ESCs five days after treatment with 4-OHT. (D) Growth curve of iPHet and iPKO ESCs, n = 2 replicates. (E) Representative cell cycle analysis using flow cytometry. (F) AP staining and quantification of iPHet and iPKO ESCs one passage after treatment with 4-OHT. M− = abnormal morphology. Significance calculated using t-test with mean+SEM. Scale bar = 100µm. (G) Western blot and AnnexinV/7AAD flow cytometry. (H) IF for Ki67. White arrows = Ki67+, red arrows = Ki67−. (I) IF for H3K9me3 and PRMT5. Red arrows = H3K9me3 foci. (J) Quantification of number of H3K9me3 foci. (K) IF for gamma H2AX. (L) Quantification of gamma H2AX positive foci. Statistics = Mann Whitney test with data expressed as mean+standard error of
mean (SEM) unless otherwise stated. Significance = p<0.05. Scale bar = 20µm unless otherwise stated.

Figure 3-2. Loss of PRMT5 affects SDMA of histones and gene expressions. (A) Western blot of H2AR3sme2 and H4R3sme2. (B) Scatter plot of iPHet vs. iPKO RNA-seq data. (C) GO analysis from DAVID for biological process showing the top five categories of up (red) or down (green) regulated genes in iPKO cells. (D) Heat map of up- or down-regulated genes in iPKO cells with 2-fold cutoff, FDR<0.05. (E) Venn diagram of overlapping genes between NPC and ESC in knockout vs. controls. RNA-Seq on ESC was performed five days after initiating 4-OHT treatment.
Figure 3-3. Loss of PRMT5 leads to abnormalities in RNA splicing. (A) Pair-wise analysis of differential splicing events in each iPKO replicate relative to the paired iPHet control. A3SS, alternative 3' splice site; A5SS, alternative 5' splice site; MXE, mutual exclusive exons; RI, retained introns; SE, skipped exons. (B) Sashimi plot of the Prmt5 locus. Exon 7 (boxed) is a SE in iPKO cells. (C) Prmt5 SE is confirmed with RT-PCR. (D) Venn Diagram of pair-wise analysis of MATS, showing overlapping differential splicing events among replicates. (E) Representative GO terms for common differentially spliced genes identified in three out of four replicate pairs. (F) Sashimi plot of Ehmt2 with boxed region shows intron 22 and exon 23. (G) RT-PCR validation of RI and SE events in Ehmt2 locus. (H) Western blot showing H3K9me2 and H3K27me3. All analyses on ESCs were performed five days after initiating 4-OHT treatment. Significance calculated using t-test with mean+SEM.
Figure 3-4. PRMT5 is dynamically expressed in the mammalian germline during embryogenesis. (A) IF for PRMT5 (green) and OCT4⁺ PGCs (arrows) at E9.5 and E10.5, and MVH⁺ PGCs (arrows) at E11.5 (red). (B) PRMT5 (green) in male gonads at E13.5, E16.5 and P1-2. MVH (red) marks germ cells, with PRMT5 foci (white arrow heads) or MVH foci (red arrow heads). (C) PRMT5 (green) expression in female gonads at E13.5, E16.5 and P1-2. MVH (red) marks germ cells. (D) Summary of expression of PRMT5 in the mammalian germline. Scale bar = 20μm.
**Figure 3-5.** PGCs survival is compromised between E10.5 to E13.5. (A) P1-2 male gonad and (B) P1-2 female gonad in control (Ctrl) and PCKO embryos. Arrows indicate germ cells. Scale bar = 100µm. (C-D) IF for PRMT5 (green) and MVH (red) in (C) P1-2 male, and (D) P1-2 female gonads. L=Leydig cell. Arrows indicate germ cells. (E-F) IF for MVH (red) (E) male, and (F) female gonads at E13.5. In males (E) sertoli cells are marked with SOX9 (green). Dashed circle indicates a testis cord. Scale bar = 50µm. (G) Higher power image at E13.5. Dashed circle indicates a cross-section through a testis cord. Arrows point to MVH+ germ cells. (H) IF of the indifferent gonads at E11.5 for the germ cell markers STELLA (green) and MVH (red). Arrows indicate a small germ cell cluster in the PCKO mutants. Scale bar = 100µm. (I) IF of germ cells at E9.5 for STELLA (green) and OCT4 (red). (J) Quantification of germ cell number between control and PCKO at different gestational stages. Statistics = t-test with data
shown as mean±SEM. Significance = p<0.05. Scale bar = 20µm unless otherwise stated.

**Figure 3-6.** PCKO PGCs exit the cell cycle and fail to progress into MVH positive germ cells. (A) IF of E10.5 embryos showing OCT4+ (red) PGCs with cPARP (green). Arrows indicate apoptotic PGCs. (B) Quantification of apoptotic PGCs in control and PCKO embryos at E10.5. (C) IF of E9.5 embryos for Ki67 showing OCT4+ PGCs (arrows). Scale bar = 10µm. (D) Quantification of Ki67 negative OCT4+ PGCs at E9.5. (E) IF at E10.5 for OCT4+ PGCs and CCNB1. White arrow = PGC in G2, red arrow = PGC in G2/M, yellow arrow = CCNB1 negative PGC. Scale bar = 10µm. (F) Quantification of CCNB1 expression. (G) IF at E10.5 for OCT4+ PGCs and H3K9me3. White arrows = control PGCs. Red arrows = PCKO PGCs with increased H3K9me3 foci. (H) IF at E11.5 for PGCs (arrows)
with MVH (red) and STELLA (green). Statistics = t-test with data shown as mean±SEM. Significance = p<0.05. Scale bars = 20µm unless otherwise stated.

Figure 3-7. PRMT5 does not have a major affect PGC specification prior to E9.5.
(A) IF at E10.5 for OCT4⁺ PGCs (red) and PRMT5 (green). Scale bar=20µm.
Arrows show PGCs. (B) IF for PRMT5 (green) in iPHet and iPKO ESCs five days after treatment with 4-OHT. (C) IF for PRMT5 (green) in EpiLCs treated for 24hrs with 4-OHT. (D) Aggregates derived from treated EpiLCs at day 4. Scale bar=100µm. (E) Flow cytometry to detect PGCLCs (square gates) in aggregates
derived from iPHet and iPKO EpiLCs. n=2 replicates. (F) Quantification of PGC numbers in E9.5 inducible PCKO embryos after administering 4-OHT of E6.5 pregnant female. (G) Summary of PRMT5’s role in the ground state pluripotency and in the mammalian germline. Statistics = t-test with data shown as mean+SEM. Significance = p<0.05. Scale bar is 10µm unless otherwise stated.

Supplemental Figure Legends

Figure 3-S2, related to Figure 2. (A) Over-exposed Western blot of iPHet and iPKO nuclear fractions 5 days after initiating 4-OHT. (B) Mass Spec plot from Orbitrap. Arrows indicate to unmodified H2A.1. n=2 biological replicates. (C) qRT-PCR validation of gene expression alternations from RNA-seq in biological replicates, n=2 biological replicates in technical duplicate.

Figure 3-S5, related to Figure 5. (A) Recombination rate of Blimp1-Cre (BC). BC was crossed to YFP lox-stop-lox mice and recombination rate was calculated based on the fraction of GFP+ (GFP antibody also recognizes YFP, cat#GFP-1020, Aves Labs) out of STELLA+ (E9.0) or MVH+ (E13.5) germ cells. (B) Wholemount IF for E9.5 PGCs identified by STELLA and OCT4. (C) E9.5 embryos from control and PCKO. (D) E11.5 embryos of PCKO and Control. The majority of PCKO embryos are equivalent size to the control at E11.5.
Supplemental Figures
Figure S5
Extended Experimental Procedures

Embryonic stem cell (ESC) derivation

Mice were time-mated and plugs were checked the next morning before 10 AM. The embryo was timed E0.5 the day when the plug was seen. At E3.5, the pregnant female was sacrificed. The uterus along with the ovary was fully recovered from the female mouse and placed in pre-warmed M2 medium (M7167, Sigma). Blastocysts were flushed from the uterine horns with M2 medium by an 18-21 gauge needle. Successfully flushed blastocysts were transferred to inactivated mouse embryonic fibroblast (MEF) feeder layer plated <24 hours earlier in a 4-well plate, with pre-warmed embryonic stem cell (ESC) medium containing 15% FBS (Gibco lot tested), 1xpenicillin-streptomycin/glutamine (Invitrogen), 0.1mM MEM NEAA (Invitrogen), 0.055mM 2-mercaptoethanol, 1000U/ml LIF (Millipore) in Knockout DMEM (Invitrogen) with addition of 0.5µM PD0325901 inhibitor. The initial ESC outgrowth was observed within 2-5 days of plating. Then the ESC outgrowth was passaged 1:1 to another 4-well pre-plated with inactivated MEFs before freezing to create stocks and transitioning to 2i+ LIF. The male ESC lines PC1 (iPHet) and PC4 (iPKO) were used for the majority of experiments.

ESC culture, PGC like cell (PGCLC) differentiation

ESCs were transitioned into the 2i + LIF by using the following media: 1:1 mixture of DMEM-F12/N2 (DMEM-F12 supplemented with N-2) and Neurobasal/B27
(Neurobasal supplemented with B27) with 1xpenicillin-streptomycin, 0.1mM MEM 
NEAA, 0.1mM 2-mercaptoethanol, 1000IU/ml LIF, 1µM PD0325901 and 3µM 
CHIR99021. ESCs were passaged using 0.05% Trypsin and neutralized with 2 
volumes of 1mg/ml Trypsin Inhibitor and seeded at fixed numbers each passage 
(either at 50K or 200K, 200K for PGCLC experiments) on human plasma 
fibronectin (1mg/ml) coated plates. PGCLC derivation from Epiblast like cells 
(EpiLCs), was performed as described previously (Hayashi and Saitou, 2013; 
Hayashi et al., 2011).

Western blot
Protein fractions were isolated using the QProteome Cell Compartment Kit 
(Qiagen) according to manufacture's instructions. Protein was quantified using 
the BCA Kit (Thermo), analyzed by electro-phoreses on 12% NuPAGE Novex 
Bis-Tris gels (Invitrogen) and transferred to Hybond ECL Nitrocellulose 
Membrane (GE Healthcare) according to standard procedures. Primary 
antibodies (1:1000): PRMT5 (07405, Millipore), ACTIN (sc47778, Santa Cruz), 
cPARP (5625, Cell Signaling), H2A (ab18255, Abcam), H2AR3sme2 (A gift from 
Dr. Mark Bedford from MD Anderson Cancer Center, University of Texas), 
H4R3sme2 (61188, Active Motif), H3K9me2 (ab1220, Abcam), H3K27me3 
(07449, Millipore) and H3 (06755, Millipore). Secondary HRP-conjugate 
antibodies were from Molecular Probes and Santa Cruz, all used at 1:5000. Blots 
were developed using ECL Western Blotting Detection Kit (GE Healthcare).
Immunofluorescence (IF)

Immunofluorescence was performed either in fresh cell cultures on chamber slides, or on paraffin embedded sections or on wholemount embryos. Cells were cultured normally on fibronectin coated chamber slides with seeding cell numbers calculated according to the surface area of wells. For fresh cell and paraffin sections, tissues were fixed in 4% PFA for 10 min and permeabilized in 0.5% Triton for 1hr followed by standard IF protocol as described (Vincent et al., 2011). For wholemount IF, embryos were fixed in 4% PFA for 1hr, permeabilized in 1% Triton for 1hr, and blocked in 10% FBS in staining buffer (PBS with 0.2% Triton) for 3hrs before addition of the primary antibodies overnight. Antibody incubations and washes were performed in 1% FBS in staining buffer and the incubation of the secondary antibodies was also overnight. After the final washes, the embryos were cleared in 50% glycerol overnight before mounted in glycerol mounting media on glass slides. All images were taken and processed using a Zeiss LSM780 confocal microscope. The primary antibodies used for IF (dilution 1:100) are as follows: Ki67 (556003, BD Pharmingen), PRMT5 (07405, Millipore; ab12191, Abcam), H3K9me3 (ab8898, Abcam), gammaH2AX (07164, Millipore), OCT4 (sc8628, Santa Cruz), MVH (ab13840, Abcam; AF2030, R&D systems), SOX9 (ab5535, Millipore), STELLA (sc67249, Santa Cruz), CYCLINB1 (4138, Cell Signaling), GFP (GFP-1020, Aves Labs) and cPARP (5625, Cell Signaling). The secondary antibodies used (1:400) include Donkey anti Rabbit Alexa488, Alexa594; Donkey anti Mouse Alexa488; Donkey anti Goat Alexa594 (Jackson Immunoresearch).
Flow Cytometry

Cells were dissociated with 0.05% Trypsin (Invitrogen) at 37°C for 5 min followed by neutralization with 1mg/ml Trypsin Inhibitor and collected by centrifugation at 1,000 rpm for 5 min. For apoptosis analysis, cells were stained with AnnexinV-FITC (640905, Biolegend) or AnnexinV-APC (640919, Biolegend) together with 7AAD (BD Pharmingen) in 1x binding buffer (422201, Biolegend) according to the manufacture’s protocols. For PGCLC flow, disassociated cells were stained in PBS plus 1% BSA with conjugated primary antibodies: CD61-PE (104307, Biolegend) at 1:20 and SSEA1-eFluor660 (50-8813-41, eBioscience) at 1:20 for 15 min on ice, followed by 1 wash with PBS plus 1% BSA. 7-AAD (BD Pharmingen) was used as a viability dye at 1:50 dilution. Analysis was performed using LSR II or LSR Fortessa (Becton Dickinson) and FlowJo software (Tree Star Inc).

EdU Analysis

EdU was added at a 30mM concentration and cells were incubated for 1 hour before EdU analysis was performed according to manufacture’s instructions using the Click-it EdU Flow cytometry Assay Kit (C10425, Invitrogen). EdU labeled cells were then incubated with DAPI at a 1mg/ml concentration on ice for 30 min. EdU/DAPI labeled cells were analyzed by flow cytometry on either an LSR II or LSR Fortessa (BD).
**RNA-seq library preparation and bioinformatic analysis**

Total RNA extractions from iPHet and iPKO ESCs were performed with Qiagen RNeasy Mini kit using 1-2 million cells. The RNA-seq libraries were prepared by UCLA sequencing core using the Illumina TruSeq RNA Sample Preparation kit. At least 35 million, 100-bp paired-end reads were mapped to the GRCm38/mm10 version of the mouse genome per replicate per sample. Genes were labeled as significantly differentially expressed if the FDR value <0.05 as called by cuffdiff (Trapnell et al., 2013). To identify differential splicing events, MATS 3.0.6 beta or Python MATS 3.0.8 (Shen et al., 2012) was used for counting junction reads. Only significant events occurring in at least three replicates were taken into consideration. Splicing events were labeled significant using the criteria FDR<0.1. All sequencing data have been submitted to the Gene Expression Omnibus repository and are available under BioProject ID PRJNA245758. GO term analysis was performed with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al., 2008) using the biological process (BP) pathway.

**Realtime reverse transcriptase (RT)-PCR and MATS validation**

Total RNA from iPHet and iPKO ESCs was extracted using RNeasy Mini kit (Qiagen). 1µg RNA was treated by DNase I (Invitrogen) for 15 min at room temperature followed by addition of 2mM EDTA and heat inactivation at 65°C for 10 min. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacture’s instruction. 1µl cDNA
product was used for qRT-PCR in each reaction. For validation of gene expression, *Prmt5* and *Gapdh* are purchased from Taqman expression assays (Applied Biosystems), other primers were designed against each transcript using MacVector software and the primer lists are listed in Supplemental Table S3. For MATS validation, primers were designed using MacVector to distinguish differential splicing events and semi-quantitative PCRs were performed (*Prmt5*: 35 cycles and all others: 40 cycles) and analyzed by gel electrophoresis. Gel intensity from each experiment was analyzed by ImageJ software.

**HPLC separation and Mass Spectrometry of Histone H2A.1**

Histones extracted from iPHet and iPKO ESCs were separated by RP-HPLC as described previously (Lin and Garcia, 2012) using a 4.6-mm Vydac C18 column (4.6 mm internal diameter × 250 mm). Peak fraction of H2A.1 was speed dried and dissolved in 50µl H2O. Samples were then loaded again to OMIX C18 ziptip (Agilent Technologies, A5700310), washed with 0.1% formic acid and eluted with formic acid/acetonitrile/H2O (0.1/50/50). The eluent was introduced directly onto an LTQ-Orbitrap mass spectrometer by electrospray ionization. Unit resolution was achieved by operating the instrument at 30,000 resolution. Different charge states of the target histone with varied modifications were ion isolated for better comparison.
**4-OHT preparations for cell culture and injections**

4-OHT (T176, Sigma) was firstly dissolved in 100% ethanol with a concentration of 100mg/ml and frozen down in aliquots at -20°C. Before use, aliquots of 4-OHT were warmed up at 65°C for 10 min to fully dissolve. For medium use, ideal amount of 4-OHT was added into 50ml medium and kept at 4°C for 2 weeks as the longest if not used. For mouse injections, 4-OHT was resuspended in corn oil (C8267, Sigma) at the concentration of 10mg/ml with vortexing. 250 µl 4-OHT in corn oil was given to E6.5 pregnant female at a single dose intraperitoneally in the bio-containment area of the vivarium.

**Supplemental References**


CHAPTER 4

CONCLUSION
Conclusion

The primary goal of the study presented in this dissertation is to identify and characterize the mechanisms that drive ground state pluripotency and the earliest stages of germline establishment using both in vitro and in vivo models. In Chapter 2, we developed the means to differentiate and isolate in vitro PGCs (iPGCs) equivalent to E9.5-E10.5 PGCs and validated that this system could recapitulate phenotypes in vivo when using mutant ESC lines\(^1\). In particular, PRMT5 was suggested as a potential critical germline factor for pre-gonadal PGC formation, because iPGC derivation was severely affected when deriving from a Prmt5 knock down (KD) ESC line. The best KD achieved was around 50% in our experiments and the Prmt5\(^{-/-}\) blastocysts failed to derive ESC outgrowth in previous reports\(^2\), indicating that PRMT5 is essential for ESC survival. Following these studies, in Chapter 3 we devised conditional knock out (KO) tools to deplete PRMT5 both in ground state stem cells with inducible CreER, and in endogenous germline with tissue-specific Cre (Blimp1-Cre). We found that PRMT5 is an essential survival factor for ground state pluripotency and for mammalian germline, leading to interesting discussions about PRMT5’s roles among different states of pluripotency, different types of tissues, and different stages of germline formation.

**Role of PRMT5 in different states of pluripotency**

During the time for this thesis work, PRMT5’s role in naïve (serum+LIF)\(^2\) and primed (hESC with FGF2)\(^3\) states of pluripotency was recovered: PRMT5 is
required to safeguard the undifferentiated state of ESCs in naïve culture, and PRMT5 regulates proliferation instead of pluripotency in primed hESCs. In addition to the investigation of PRMT5 in primed state as a side project, I carry out studies in this thesis, which explored PRMT5 in ground state pluripotency as an essential survival factor. Given that loss of a common factor expressing in all states of pluripotency presents different outcomes, why and how PRMT5 plays a different role among different pluripotent states needs to be addressed.

One explanation might be the epigenetics intrinsic to each pluripotent state. Compared to the naïve state, ESCs in 2i displayed lower levels of lineage specific gene expression, reduced bivalent domains and reduced pol II pausing on developmental genes, etc\(^4\sin{6}^6\). In our study, we did not observe pre-deposited H2A with SDMA modification on arginine 3 (H2AR3sme2) in 2i, which was seen in ESC with serum+LIF in Chapter 3. Instead, in ground state ESCs, H2AR3sme2 was present only in the nuclear fraction and composed a small proportion of total H2A.1 population (Chapter 3). H2AR3sme2 levels were sensitive to loss of PRMT5 but no de-repression of somatic genes was seen. It is possible that H2AR3sme2 is a minor repressive mark in ground state pluripotency and not a direct repressor for somatic gene expression.

Secondly, in comparison to ESC in serum+LIF, 2i+LIF ESCs highly express genes involved in glutathione metabolism\(^1\sin{6}^6\), a reducing pathway to functionally reduce reactive oxygen species (ROS) in the cell. Also an up-regulation of P16,
P19 and P21 protein levels was seen with 2i+LIF in contrast to serum+LIF. These proteins are indicators of DNA damage response (DDR), suggesting active DDR is present in 2i cultures relative to serum+LIF. In our studies in Chapter 3, Annexin V positive cells were high in population even in the control, suggesting that there is inherent cell death in the 2i system. Therefore, it is possible that PRMT5 is of ultra importance for ground state stem cells, where DDR is already present. Because loss of PRMT5 impaired DDR and increased damaged DNA suggested by increased gamma H2AX foci.

Thirdly, Primed hESCs with Prmt5 KD behave differently from naïve ESCs\textsuperscript{2,3}. PRMT5 regulates P57 to maintain a short G1 phase in hESCs cultured in FGF but not greatly changes transcriptional profile\textsuperscript{2,3}. This is possibly due to a species difference or a difference among pluripotent states, as the epigenome and transcriptome are quite different in each state. To tease this apart, PRMT5’s role in the murine EpiSC needs to be evaluated. In addition, it is also interesting to investigate the diverse roles of PRMT5 in splicing between naïve and primed states of pluripotency, as well as unique genes affected by splicing in each pluripotent state.

**Role of PRMT5 between pluripotent/multipotent progenitors and differentiated cells**

In this study, we performed a meta-analysis of our RNA-seq data in iPKO ESCs with 2 published data sets\textsuperscript{3,7}, to address effects following the absence of PRMT5.
Firstly, we analyzed differentially expressed genes in *Prmt5* KO among ground state ESCs, neural progenitor cells (NPCs) and murine embryonic fibroblast (MEF). There were about 400 genes altered in 2i ESCs, 900 genes altered in NPCs and only 20 genes changed in MEFs with a cutoff of 2 fold change, FDR<0.05. It appears that PRMT5 has a greater role in transcriptional regulation in NPCs relative to 2i+LIF ESCs or MEFs, given that a larger number of RNAs were affected.

In MATS analysis, with a cutoff of 20% difference (calculated by the software to distinguish different splicing events), there are about 600 genes alternatively spliced in 2i ESCs, 140 genes in NPCs and only 40 genes in MEFs. Again, MEFs are the minimal affected cell type by loss of PRMT5. 2i ESCs and NPCs share around 80 genes commonly disrupted and many of them belong to the biological processes of RNA processing and splicing, suggesting that affected genes in RNA splicing will amplify the differential splicing events and result in high variability among biological replicates\textsuperscript{4-7}. In particular, we found *Mdm4*, an upstream inhibitor of P53, is alternatively spliced in 2i ESCs similar as in NPCs described in Bezzi et al.\textsuperscript{7}. With a skipped exon event in *Mdm4*, the original long form of *Mdm4* transcript becomes a short unstable form that is submissive to degradation, resulting in reduced amount of functional protein. However, in contrast to the NPCs, we did not observe any up regulations of genes directly activated by P53, indicating that apoptosis and senescence mediated by P53 pathway is not the major cause of the phenotypes. To completely exclude P53
pathway and have a P53 free background to study P53 independent events, 2i+LIF Prmt5 KO ESCs with a background of Trp53−/− should be generated to address P53-independent cell death and proliferation arrest in Prmt5 mutant ESCs.

The difference of genes affected by splicing between ESCs and NPCs could be due to two reasons: 1- the alternative splicing of splicing regulators, 2- transcriptional profiles distinctive in each cell type, because splicing will mostly affect actively transcribed genes. In addition, PRMT5 is hypothesized to modify symmetrical di-methylation of arginines (SDMAs) on other splicing components with SDMA modification, such as LSM4, U1-70K, ASF/SF2 and KSRP/ZBP28. Notably, PRMT5 is not the only type II protein arginine methyltransferase (PRMT). PRMT7 is another purported type II PRMT involved in regulating splicing through modifying SDMAs in HeLa cells9 and PRMT10 is predicted to be a novel type II PRMT (unpublished from our collaborator Dr. Steven Clarke). How other type II PRMTs contribute to the constitutive and alternative splicing in different cell types remains to be addressed.

One potential way to address this is to use SYM10 and SYM11 antibodies, which are recognizing SDMA to precipitate modified proteins from control and Prmt5−/− ESC lysates. Mass Spectrometry analysis of immunoprecipitates should be performed to identify PRMT5 specific SDMA modifications and the corresponding protein populations. Another way to address this is to generate ESC lines with
loss of PRMT7 or loss of both PRMT5/7 at the same time, to investigate independent roles of PRMT5 or 7, or synergistic roles of PRMT5 and 7, in terms of methylating critical histone candidates and components of splicing machinery.

**Role of PRMT5 in the germline**

The fundamental advance we describe here is for the first time showing that PRMT5 is indeed required for mammalian germline formation. Although PRMT5 has been hypothesized to co-repress somatic gene expression together with BLIMP1 in early PGCs\(^{10}\), our data suggested that loss of PRMT5 has direct effects on cell survival and proliferation, which is the essential cause for reduced PGC number. Absent in our study is whether PRMT5 is involved in repressing somatic genes in the germline through the covalent histone modification H2A/H4R3sme2. Further studies are needed using the *in vitro* PGCLC derivation as well as sorted endogenous PGCs to evaluate whether PRMT5 is required to repress somatic program. CD61\(^+\)/SSEA1\(^+\) PGCLCs or early PCKO PGCs (if possible) should be purified and isolated, to perform paired-end RNA sequencing on ultra small cell sample, to verify alternatively spliced genes and changes of gene expressions that may affect cell survival, or unique genes specially affected in PGCs.

Based on our expression map of PRMT5 in different embryonic stages and sexes, we predicted that PRMT5 has diverse roles in different stages of germline development, or even in different sexes. Prior to E9.5, we used two different *in
vivo approaches \((Prmt5^{fl/fl}\) with either CreER or Blimp1-Cre) and one in vitro approach (PGCLC derivation from iPKO EpiLCs) to show that in the knock out PGCs are still present at a developmental stage corresponding to E9.5. This suggests that PRMT5 does not have a major role in PGC specification using our assays. Instead, our research indicated that PRMT5 is indeed required for PGC survival from E10.5 to E13.5. How PRMT5 affects later stage PGCs, especially when sex determination starts and DNA methylation is re-established, needs to be further determined by inducing conditional knock out of \(Prmt5\) at E11.5 and E13.5 or even later in the germline. Because PRMT5 has been reported to modify SDMAs on gonadal germline RNA-binding proteins, such as MVH and the MIWI\(^{11-13}\).

**PRMT5’s role in human germline**

Although we have clarified PRMT5’s role in early murine PGC formation and 2i+LIF mESC survival in Chapter 2 and 3, the role of PRMT5 in human endogenous PGCs (hPGCs) has never been determined. In parallel with building the mouse iPGC model, our lab has derived a strategy to generate in vitro PGCs (iPGCs) from FGF cultured hESCs, which express \(Oct4\), \(Blimp1\) and \(Nanos3\) and correspond to a stage prior to gonadal colonization and loss of 5mC\(^{14}\). We staged iPGCs isolated from hESC differentiation strategy to be correspondent to a stage younger than E8.5 of murine embryogenesis, when genome-wide 5mC is still present\(^{15-19}\), which suggests that iPGC number may still remain the same because in Chapter 3 PGCs are not changed prior to E9.5 with \(Prmt5\) KO.
Therefore, it is still unknown whether PRMT5 plays an essential role in hPGC formation at late stage of migration and the beginning of colonization correspondent to E10.5 in the mouse.

One way to address this is to improve current strategy of generating human iPGCs, by either starting from a ground state human pluripotent stem cell (PSC) source (which needs more investigation and research to achieve), or by overexpressing transcriptional factors to promote germline development, such as Blimp1, Prdm14 and Nanos3 in the starting PSC population. After we improve the human iPGC model to derive iPGCs equivalent to a pre-gonadal or gonadal stage, we could knock down Prmt5 in the starting cells or during the iPGC induction. Another way to address this is to generate Prmt5 KO primate models using the CRISPR/Cas9 technologies and evaluate germline formation in different embryonic gestational stages with timed pregnancy experiments.

**Summary**

In conclusion (Figure 4-1), what we discovered here is crucial to generate a cost efficient system, for identifying crucial germline genes and understanding how PRMT5 (one of the crucial genes) is essential for ground state pluripotency and the mammalian germline, as well as derivation of functional PGCLCs from ground state PSC source. This dissertation pointed out that a key component may function differently in different states of pluripotency and broadened the knowledge of current stem cell biology and germ cell biology (Figure 4-1). This
study uncovered links among arginine methylation, splicing and cell survival, providing important input and reference for basic understanding and utilization of pluripotency. Finally, this study paved the way for, and shed light on further sophisticated studies about type II PRMTs in stem cells and the mammalian germline.

Figure 4-1. Divergent roles of PRMT5 in different states of pluripotency and in the mammalian early germline. The function of PRMT5 in lineage committed cells still remains to be explored.


