Review Article

Emerging clues for regenerative medicine in infertility: an overview for cell sources and results with a sensitizing modifier PRMT5

Jiayi Duan[1], Qi Li[2], Ziwei Li[1]

[1]Department of Surgery, The 1st Hospital Affiliated to Kunming Medical School, Kunming 650032, China; [2]Department of Dentistry, Renji Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200001, China

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Correspondence to: Ziwei Li. Department of Surgery, The 1st Hospital Affiliated to Kunming Medical School, Kunming 650032, China.
Email: cocolzw@yeah.net.

Abstract: Germ cells are the most important cell type for generation continuation in all sexually reproductive species. Pluripotent stem cells (PSCs) are important cell resources for disease modeling and tissue-specific differentiation in regenerative medicine. Ground-state naïve PSCs share similar transcriptional and epigenetic features with primordial germ cells (PGCs) and PGC like cells can be derived from PSCs in mice. As a critical regulator in PSCs and PGCs, PRMT5 has a versatile role in regulating cell function both in pluripotency and early germline. This review discusses current progress from mammalian PSCs to germ cell differentiation and PRMT5's important role in PSCs and PGCs, which will help future research and clinical investigation.

Keywords: Protein arginine methyltransferase 5 (PRMT5); pluripotent stem cells (PSCs); germ cells

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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 it is genetically malleable through transgenics or embryonic stem cells (ESCs) and these genetic changes can be passed through the germline (6-10). Very recently, early germline development has been scrutinized using Cynomolgus monkeys (Macaca fascicularis), which shows primate PGCs originate from the nascent amnion (11).

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 (12-20) and approximately 40 germ cells are first specified at E7.5 (13,21-23). Germ cell number does not significantly increase until after the germ cells have entered the gonad at around E11.5 (24-27). In Sasaki *et al.*, Cynomolgus germ cells are characterized from E36–E55 [Carnegie stage (CS) 17–23 and early fetal corresponding to E9.5–E12.5 in mice) (11). As early as E11 in monkeys, fewer than 10 $SOX17^+/TFAP2C^+$ cells were

found in 3/5 embryos. Given this small number, large-scale biochemical studies that require a significant number of cells are impossible. The murine 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 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: ESCs and induced pluripotent stem cells (iPSCs) (28-38):

The efforts for generating functional gametes from ESCs started as early as 2003 by independent investigators from Dr. Hans Scholer's group (30) and Dr. George Daley's group (39). Hubner *et al.* from Scholer group identified a 2-D differentiation method using attached cell cultures with a *gcOct4-GFP* reporter ESC line, resulting in oocyte-like structure as early as Day (D) 26 and blastocyst-like structure at D 43 (30). 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 embryoid bodies (EBs) using the hanging drop method (39). 30 µL droplets containing 200 cells were plated on the inverted petri-dish lid as hanging drops and collected into non-attachment plates at D 3. After 3–4 more days of differentiation, SSEA1$^+$ 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.

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 (28,29,31-38). 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$^+$ cells derived from EBs (39). However in Hubner *et al.*, a pregonadal germline marker *ckit* and a gonadal marker *Vasa* were both used for sorted gcOct4-GFP$^+$/cKit$^+$ populations, and oocyte markers *ZP 1-3* were used for the oocyte-like structures from 2-D differentiation (30). 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$^+$ cells from both adherent differentiation and EB differentiation (38). 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.

Secondly, most of these studies require ESC lines with a transgenic reporter that is often hard to obtain and create, such as *gcOct4-GFP* (30), *Oct4ΔPE-GFP* (29,32), *Dazl-GFP*(36), *Mvh-RFP* (31) and *Stella-GFP* (35,38), or rely on a single surface marker such as SSEA1 (39), which is not exclusively expressed in the germline (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 under different genetic backgrounds, which usually takes five 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-More than one reporter is often required to stage PGC like cells to endogenous equivalents. Therefore, a differentiation method without the use of transgenic mice, but with more than one marker to pinpoint the correct stage of *in vitro* PGCs is highly favorable. Significant progress has been made recently by inducing murine (m) ESCs/iPSCs into PGC like cells (mPGCLCs) passing through an intermediate epiblast-like cell (EpiLC) stage. These PGCLCs could contribute to spermatogenesis and oogenesis with an *in vivo* maturation step and are able to fertilize into live young (4,41). Moreover, human (h) ESCs/iPSCs have been induced into hPGCLCs using similar methods and media (42,43), predicting that human gametes would one day be produced in a dish to cure infertility. However, the first step is to bypass the ethical concerns.

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 transgenic mice. 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 guidance 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 induced PGC (iPGC) differentiation in a dish.

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. Although non-human primates could be utilized as a new model, studies are extremely rare and difficult to perform due to difficulty in sample preparation and collection besides ethical and spending issues. Therefore, mouse models have served 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 (ExE) and visceral endoderm (VE), such as Bone Morphogenic Protein (BMP) 2, BMP4 and BMP8b (12,15-20,44,45). By E7.25, PGCs start to express *Stella/Dppa3*. Together with *Blimp1*, these are the earliest known markers of founder PGCs (13,46). 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 (23,47). 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.

Germ cell specification

The inductive signals for germ cell specification have been identified using knock out studies (48) 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), 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 (18,49,50). BMP8b is expressed in ExE from E5.5 and the phenotype with loss of BMP8b resembled loss of BMP4 (20,51). BMP2 is expressed in 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 (52,53). Since the downstream part of BMP signaling is mediated and amplified through the mothers against decapentaplegic (SMAD) proteins, similar phenotypes were observed in certain SMAD mutants such as *Smad1* (12,17,54), *Smad4* (55,56) and *Smad5* (15,16,57). *Smad1* and *Smad5* are ubiquitously expressed in the epiblast and *Smad4* is ubiquitously expressed during gastrulation. *Smad1*$^{-/-}$ and *Smad5*$^{-/-}$ embryos completely lacked PGCs. *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 (3), it is shown that 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 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*.

To understand PGC specification at a single cell level, Saitou *et al.* [2002] (46) 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 (13). *Blimp1*$^{-/-}$ 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 leads to reduced PGC number as early as E7.25 and *Prdm14*$^{-/-}$ male/female animals were both sterile (58). 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 (59). 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 a recent study that overexpressing the three factors in a cell type derived from PSCs results in generation of functional germline.

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) (25,27). These pre-gonadal epigenetic changes in concomitant with PGC migration are termed Reprogramming Phase I (25,60-64). 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 (27). 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.

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 (60,61,63,65-68). Recent studies suggest that the ten-eleven translocation (TET) family proteins play critical roles in mediating active demethylation of the imprinted genes (68,69). Yamaguchi *et al.* [2012] (68) used paternal *Tet1* knockout mice (paternal^{KO}; progenies from *Tet1*$^{-/-}$ male X wild-type female) to analyze the effect of the paternal TET1 loss on the offspring (68), because *Tet1*$^{-/-}$ females had meiotic defects. In E9.5 paternal^{KO} embryos, 11–46 out of 81 expressed imprinted genes were dysregulated and the *Peg10* differentially methylated region (DMR) remained fully methylated compared to the controls. By performing reduced representative bisulfite 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, Dawlaty *et al.* [2013] (69) found that *Tet1/Tet2* deficiency partially compromised imprinting (69) 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.

Different states of ESCs in culture

In recent years three PSCs states have been described: primed, naïve and ground state naïve. Primed PSCs are traditionally cultured with 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) (70-73) cultured in bFGF. Naïve PSCs 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 naïve PSCs often refer to mESCs cultured with MEK1 and GSK3 inhibitors (2i) in a chemically defined medium (74-76). 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 (77-80). 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.

Efforts have been spent on generating ground state naïve human ESCs by adding a few compounds into the media including a combination of 2i, FGF and hLIF (81,82). In these studies, naïve-like hESCs could resemble transcriptional profile as human preimplantation embryo (82) and from interspecies chimeras after injection into mouse morula (81). There are other studies reporting that supplementing 2i and FGF could contribute to naïve-like hESC in the presence (83) or absence of hLIF (84). Theunissen *et al.* compared the previous four reports and identified 2i and FGF are necessary in all media and optimized the media with three more inhibitors (BRAF, ROCK and SRC inhibitors) to facilitate cell expansion plus activin-A (85). Following the naïve-like hESCs, Cynomolgus monkey naïve-like ESCs/iPSCs have been converted to facilitate neural differentiation (86).

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 naive (2i+LIF). mESCs are *in vitro* derived pluripotent cells from the ICM 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 (87,88). 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 (30,38) or forming EBs (28,29,31,33-39). By comparing these two methods, Wei *et al.* suggested that Stella-GFP$^+$ 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 (38), 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, Vincent *et al.* 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* (63,89).

Hayashi *et al.* invented a method to derive PGC-like cells (PGCLCs) starting from 2i + LIF cultured ground state pluripotent ESCs (4,41,90). 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 (500 ng/mL), LIF (1,000 /mL), Stem Cell Factor (SCF, 100 ng/mL), BMP8b (500 ng/mL) and Epidermal Growth Factor (EGF, 50 ng/mL) (90). Initially Hayashi *et al.* utilized a transgenic reporter ESC line with *Blimp1-mVenus* and *Stella-ECFP* (BVSC) and isolated BVSC positive PGCLCs from D 4–6 of PGCLC induction (41). At the end of this paper, the authors identified surface markers CD61 (also named integrin 3) and SSEA1 to isolate the same PGCLC population. Both BVSC$^+$ and CD61$^+$/SSEA1$^+$ PGCLCs are able to further differentiate in reconstituted gonads into haploid gametes (spermatozoa and oocytes), which could be fertilized and generate live young (4,41). This is the first study showing the creation of functional gametes from a pluripotent cell source able to produce live young.

Based on the mPGCLC induction, there are two groups reporting the induction of hPGCLCs from primed hPSCs (believed to mimic an epiblast-like status) on feeders (43) or

feeder-free using MEF-conditioned media (91). To explore hPGCLC induction in a chemically defined environment, Sasaki *et al.* demonstrated robust hPGCLC induction with the cells expressing gonadal transcriptome similar to Cynomolgus monkeys and human gonadal PGCs (42).

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 (92). There are three type II PRMTs that have been described: PRMT5, PRMT7 (93,94) and PRMT10, out of which PRMT5 is the mostly well characterized. PRMT5 modifies a large number of protein substrates, including both cytoplasmic and nuclear proteins (2,78,80,95-105). The cytoplasmic proteins include Sm-class proteins composing the Sm core in spliceosomes (106,107). The nuclear proteins include arginines on tails of multiple histones H2A (2), H3 (96,104) and H4 (2,99) as well as germline RNA binding proteins such as VASA (103), PIWI (101), MILI and MIWI (102,108). 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 is essential for mammalian germline. Detailed discussion is as follows:

PRMT5 in Drosophila germline

The *Drosophila* homolog of *Prmt5*, *dart5*, is a grand-childless gene (95). 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* (101). Loss of DART5 activity resulted in reduction of piRNA levels and accumulation of retrotransposons in the ovary (101). 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 (109). 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 (2).

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 (27,110). BLIMP1 is known as a master regulator of terminal B cell differentiation into plasma cells through repression of the mature B-cell program (89,111-116). Global deletion of *Blimp1* in mouse results in embryonic lethality by E10.5 (2,117,118) and reduction of Alkaline Phosphatase (AP) positive PGCs at E9.5 in a dose-dependent manner (13,41,117,118). 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 (13,26,118). 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 (41,119).

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 consensus 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 (2,26). 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 (2,120). 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 remain to be proved. Li *et al.* has utilized a conditional Cre, *Blimp1-Cre* to knock out *Prmt5* specifically in the germline as early as E6.25, resulting in reduction of germ cell numbers as early as E10.5 and a complete "wipe-out" of germ cells at E13.5 in both male and female mouse embryos (121). The defected germ cells exit cell cycle, undergo apoptosis and arrest in G2/M.

PRMT5 in splicing

PRMT5 is able to modify SmB/B', SmD1/D3 proteins in *Drosophila*, mouse cells and human cells (72,97,122). In flies, DART5 (*Drosophila Prmt5*) and DART7 (*Drosophila Prmt7*) were both required for methylating SmB and SmD1/D3 proteins (97). 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 (122) and murine Neural Progenitor Cells (NPCs) (72). 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.* (72) 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 (72,123). 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 (26). 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 (26). 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. Therefore, H4R3sme2 and H2AR3sme2 are possibly both required for repression of somatic genes.

In primed PSCs such as hESCs cultured with bFGF, PRMT5 is not required for pluripotency, but instead for cell proliferation (120). 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. Tee *et al.*

showed that *Prmt5*$^{-/-}$ blastocysts cannot give rise to ESC outgrowths in serum culture (26).

In Li *et al.*, ground state naïve mESCs lacking PRMT5 also exhibited many defects in pools of RNA transcripts including go terms "RNA processing", "Cell cycle", "DNA damage response" and "Epigenetic modification" etc., with compromised *Mdm4* splicing acting as a known downstream indicator of aberrant splicing. Moreover, the SDMAs on SmB, SmD1/D3 were missing without PRMT5 (121).

Summary

This review summarizes current trends in progress about making early mammalian germ cells *in vitro* starting from a PSC type either in mouse or human. The studies within the field have greatly inspired the investigation for gametogenesis in a dish to cure infertility, which is an exacerbating health problem worldwide. PRMT5 is a critical modifier important for and exhibits functional difference in multi-species pluripotent states. And PRMT5 has different functions in fly and mouse germline probably due to evolution. Therefore, it is interesting to investigate PRMT5's role systematically in mouse and human PSCs under primed, naïve and ground state naive pluripotent states. It is also interesting to investigate PRMT5's role in the germline from early non-human primate embryos. Given that PSCs and PGCs share intrinsic genetic and epigenetic characteristics, exploration about PRMT5 in core regulation network will not only provide clues for regenerative and reproductive medicine, but also hint the research for cancer stem cell maintenance and clinical treatment for potential type of cancers.

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Footnote

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/amj.2017.05.16). The authors have no conflicts of interest to declare.

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