Single Cell Analysis Facilitates Staging of Blimp1-Dependent Primordial Germ Cells Derived from Mouse Embryonic Stem Cells

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Abstract
The cell intrinsic programming that regulates mammalian primordial germ cell (PGC) development in the pre-gonadal stage is challenging to investigate. To overcome this we created a transgene-free method for generating PGCs in vitro (iPGCs) from mouse embryonic stem cells (ESCs). Using labeling for SSEA1 and cKit, two cell surface molecules used previously to isolate presumptive PGCs, we show that not all SSEA1+/cKit- double positive cells exhibit a PGC identity. Instead, we determined that selecting for cKitbright cells within the SSEA1+ fraction significantly enriches for the putative iPGC population. Single cell analysis comparing SSEA1+/cKitbright iPGCs to ESCs and embryonic PGCs demonstrates that 97% of single iPGCs co-express PGC signature genes Blimp1, Stella, Dnd1, Prdm14 and Dazl at similar levels to e9.5–10.5 PGCs, whereas 90% of single mouse ESC do not co-express PGC signature genes. For the 10% of ESCs that co-express PGC signature genes, the levels are significantly lower than iPGCs. Microarray analysis shows that iPSCs are transcriptionally distinct from ESCs and repress gene ontology groups associated with mesoderm and heart development. At the level of chromatin, iPSCs contain 5-methyl cytosine bases in their DNA that are imprinted and non-imprinted loci, and are enriched in histone H3 lysine 27 trimethylation, yet do not have detectable levels of Mvh protein, consistent with a Blimp1-positive pre-gonadal PGC identity. In order to determine whether iPSC formation is dependent upon Blimp1, we generated Blimp1 null ESCs and found that loss of Blimp1 significantly depletes SSEA1/cKitbright iPSCs. Taken together, the generation of Blimp1-positive iPSCs from ESCs constitutes a robust model for examining cell-intrinsic regulation of PGCs during the Blimp1-positive stage of development.

Introduction
The molecular events that regulate cell fate decisions in post-implantation mammalian embryonic development are largely uncharacterized due to the challenge in identifying and isolating small populations of specific precursor cells that are developmentally transient in the early embryo. In particular, precursors of the germ cell lineage are initially set aside as four to six cells in the murine embryonic epiblast, which proliferate and migrate through the primitive streak to generate the initial founder primordial germ cell (PGC) pool of approximately forty cells at the base of the allantois at embryonic (e) day e7.5 [1,2]. The PGCs migrate out of the allantois and into the embryonic hindgut endoderm at e8.0–8.5 where they continue to proliferate and begin to accumulate nuclear histone H3 lysine 27 trimethylation (H3K27me3) [3]. By e10.5–e11.0 a single embryo has approximately 1,000–2,000 PGCs, which exit the hindgut and begin colonization of the indifferent gonad and express Mvh protein [3,4,5].

The transcription factors that specify and sustain PGC identity prior to gonadal colonization are not well understood. One of the most characterized regulators of PGC fate is the transcriptional repressor B-lysophocyte-induced maturation protein 1 (Blimp1), the transcriptional product of the PRD1-BF1 and RIZ (PR) domain 1 (Prdm1) gene. Blimp1 expression is detected in epiblast-derived PGCs and persists until e11.5, when PGCs have colonized the gonad [2,6]. Loss of one Prdm1 allele significantly reduces PGC numbers in the allantois, with the loss of both causing almost a complete loss of PGCs [2]. The major direct target of Blimp1 in PGCs is hypothesized to be Hobl [7]. However, direct binding of Blimp1 at the Hobl locus in PGCs has not been demonstrated.

The mechanism by which Blimp1 mediates gene repression is hypothesized to involve recruitment of the chromatin-remodeling enzyme Protein arginine methyltransferase 5 (Prmt5) to chromatin...
However, genome-wide analysis of PGC chromatin is currently not feasible due to the challenge in performing chromatin immunoprecipitation on small cell numbers, necessitating the development of a scalable model to accurately capture the Blimp1-positive phase of PGC development.

The differentiation of pluripotent stem cells, including embryonic stem cells (ESCs), has emerged as a novel technology for generating sufficient numbers of embryonic progenitors at-scale to evaluate embryonic lineage development. A number of methods for identifying in vitro PGCs (iPGCs) have been described that mostly involve use of integrated fluorescent reporters, including Oct4-delta-PE-GFP [8,9,10,11,12], Stella-GFP [13,14,15], Dazl-Gfp [16] and Mvh-LacZ/Gfp transgenes [10,17,18]. A small number of studies have used Stage Specific Embryonic Antigen 1 (SSEA1) to enrich for germ cells [19,20], but the identity of PGCs from ESCs within the SSEA1+ fraction has not been interrogated at a single cell level. Furthermore, the majority of PGC differentiation studies have been designed to characterize the post-colonized Blimp1-negative PGC. Therefore, the goal of the current study is to generate a robust ESC differentiation system to acquire PGCs in the Blimp1-positive stage of development for future in-depth analysis of the pre-gonadal stage.

Results

Oct4+ refines an Oct4+/SSEA1+ iPGC population in embryoid bodies

To identify pre-gonadal iPGCs with differentiation, we first used Oct4-Gfp ESCs [21] to generate hanging-drop embryoid bodies (EBs) containing 300 cells per drop (Figure 1A). EBs could be maintained for up to 8 days in this system (Figure S1A), but cell viability decreased rapidly after day 6 from 69% to 19% by day 8 (Figure S1B). Using flow cytometry we show that Gfp is retained in the majority of cells in the first four days of differentiation (Figure 1B), reminiscent of sustained Oct4 expression in vivo in both PGCs and embryonic somatic cells up to e8.5 [22,23]. On day 5 of differentiation, we observed the emergence of a shoulder of Gfpbright cells and the formation of a distinct Gfp+ peak by day 6 (Figure 1B, arrow).

To generate a transgene-free method of iPGC differentiation, we correlated expression of Oct4 protein in day 6 EBs derived from V6.5 ESCs with the cell surface marker SSEA1. In the embryo, SSEA1 is highly expressed on Blimp1-positive stage PGCs and PGC precursors derived from epiblast stem cells [24,25]. We found that Oct4 is co-expressed with SSEA1 in small cell clusters at day 6 of differentiation by immunofluorescence (Figure 1C). Given that Oct4 and SSEA1 are also expressed by undifferentiated ESCs, we used the membrane-localized tyrosine kinase receptor cKit to assist in further defining the iPGC population within either the SSEA1+ or Oct4+ fractions. cKit is highly expressed by endogenous PGCs from e7.25 to e13.5 [7,22,26] and is not expressed by epiblast cells [22]. Indeed, flow cytometry analysis of V6.5 ESC-derived EBs at day 6 of differentiation revealed a discreet a side population of SSEA1+/cKit+ cells (Figure 1D). A side population of Oct4-Gfp+/cKit+ cells was also identified beginning at day 6 of differentiation in EBs derived from Oct4-gfp ESCs, and this was sustained to day 8 (Figure S1C).

To interrogate PGC identity in specific fractions of SSEA1+/cKit+ cells when the population is first identified at day 6, we used real time PCR to determine relative levels of PGC-expressed genes in discreet cKit+ fractions. These fractions include SSEA1+/cKitbright (green), SSEA1+/cKitdim (light blue), and SSEA1+/cKit- cells (red) as a negative control (Figure 1E). cKitbright cells were selected based on increased signal intensity above the main population. PGC genes including cKit, Blimp1, Stella and Dazl were all enriched in the SSEA1+/cKitbright fraction, with lower expression in the cKit+/cKit- and cKitdim fractions of SSEA1+ cells (Figure 1F). Mvh was also expressed in the SSEA1+/cKitbright fraction, but was not specifically enriched in SSEA1+/cKitbright cells relative to cKit+/cKit- and cKitdim. Furthermore, analysis of Mvh levels in SSEA1+/cKitbright cells at day 8 of differentiation also did not show an increase relative to day 6 (data not shown). In contrast, transcription factors expressed in somatic cells such as Hoxal and Hoxbl were highly expressed in SSEA1-/cKit- and SSEA1+/cKitdim relative to SSEA1+/cKitbright cells. Together, we conclude that not every SSEA1+/cKit+ cell in EBs at day 6 of differentiation is a putative PGC, and that selecting for cKitbright cells enriches for the iPGCs beginning at day 6 of differentiation and persisting until day 8. We next determined if Oct4-gfp could further sub-fractionate the putative SSEA1+/cKitbright putative iPGC population, and found equal enrichment of Oct4-gfp in all SSEA1+ cells regardless of cKit intensity (Figure S1D). Therefore the use of the Oct4-Gfp reporter together with SSEA1 and cKit does not further refine the isolation of putative iPSCs, but instead shows that Oct4-Gfp and SSEA1 report the same population when used with cKit.

We next evaluated the yield of SSEA1+/cKitbright or Oct4-gfp/cKitbright cells (called iPGCs) isolated at day 6 of differentiation (Figure 1G). We determined that 1–4% of total live EB cells exhibited an iPGC surface signature, and there was no statistical difference between genetic backgrounds (Figure 1G). Furthermore, gene expression profiling of Oct4-gfp/cKitbright cells from the Oct4-gfp line and SSEA1+/cKitbright cells from J1 EBs at day 6 revealed enriched expression of cKit, Stella, Blimp1, Dazl and Mvh relative to the SSEA1-/cKit- somatic cell controls (Figure S1E). Conversely, somatic gene expression as documented by Hoxal and Hoxbl were not enriched in the putative iPSCs relative to somatic cells (Figure S1F).

To test identity of SSEA1+/cKitbright cells derived from V6.5 ESCs we sorted putative iPSCs and cultured them on mouse embryonic fibroblasts (MEFs) supplemented with basic Fibroblast Growth Factor 2 (FGF2), Stem Cell Factor (SCF), Leukemia Inhibitory Factor (LIF) and retinoic acid (RA), a driver of PGC proliferation (Figure 1H). This assay has been used previously to confirm PGC identity relative to undifferentiated ESCs, which respond to RA by undergoing differentiation and becoming alkaline phosphatase (AP) negative [13,19,20]. AP+ colony forming ability is almost exclusively associated with SSEA1+/cKitbright population when compared to sorted undifferentiated ESCs, SSEA1+/cKitmid or SSEA1+/cKit- cells plated at equivalent numbers (Figure 1I). The three non-iPGC populations generate mostly mixed colonies or AP negative colonies. Withdrawal of RA, FGF2 and SCF while retaining LIF in the media of RA-treated SSEA1+/cKitbright sorted cells results in the formation of self-renewing pluripotent embryonic germ cells (EGCs), which could be maintained for at least 10 passages (Figure 1J).

Day 6 iPGCs have a pre-gonadal, pre-reprogrammed PGC identity

Day 6 EB-derived SSEA1+/cKitbright or Oct4-Gfp/Ckitbright iPSCs consistently express Dazl and Mvh RNA in addition to Blimp1 (Figure 1F & Figure S1E). Therefore we could hypothesize that putative iPSCs correspond to newly colonized PGCs that have expressed Mvh protein and have potentially undergone whole genome reprogramming. To address this, we performed
immunofluorescence for Mvh, which is first detectable in gonadal PGCs at e11.5 [27,28,29]. We also evaluated DNA demethylation at imprinted and non-imprinted genes, which is erased by e12.5 [30,31]. Immunofluorescence analysis of e10.5 embryos with antibodies against Mvh and Oct4 confirms that e10.5 Oct4- positive PGCs are negative for Mvh protein, whereas gonadal-stage PGCs are Mvh positive (Figure 2A). Analysis of SSEA1+ / cKitbright sorted iPGCs derived from V6.5 ESCs reveals that Mvh protein is not detectable above background (Figure 2B). We also tested J1 ESC-derived iPGCs and were unable to detect Mvh protein similar to V6.5 iPGCs (data not shown). Furthermore we evaluated H3K27m3 in SSEA1+/ cKitbright iPGCs, a histone modification that is depleted from the PGC genome from e11.5– e12.5 [4]. We found a high nuclear content of H3K27me3 in iPGCs (Figure 2C). Together this data suggests that the SSEA1+ / cKitbright iPGCs are pre-programmed and younger than e11.5.

Figure 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-Gfpbright cells at day 5 and an Oct4-Gfpbright 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+/cKit+ side population. E: Flow plot day 6 EBs from V6.5 ESCs fractionated by expression of SSEA1 and cKit into SSEA1+ / cKitbright (green), SSEA1+/cKitmid (light blue), SSEA1+/cKitdim (dark blue), and SSEA1-/cKit- cells (red) populations. Quadrant gates are drawn to demonstrate the criteria for selecting SSEA1+/cKit+ bright cells. The remaining cKit+ 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+/cKitbright 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. H: Diagrammatic representation of iPGC replating assay. I: Quantification of alkaline phosphatase (AP) staining of colonies derived from indicated cell populations after 5 days of culture. Right, representative images of colony types. Scale bar = 500 microns. J: Self-renewing EGCs at passage 10 derived from RA/FGF2/LIF/SCF cultured iPGCs, followed by routine passaging in the presence of LIF only. Error bars represent s.d. doi:10.1371/journal.pone.0028960.g001
To further confirm a pre-reprogrammed identity, we next evaluated the methylation status of an imprinted gene (Snrpn) and two non-imprinted loci, Xist and Intracisternal A Particle 1 (IAP), by bisulfite sequencing (Figure 2D). Analysis of undifferentiated ESCs shows that the differentially methylated region (DMR) of Snrpn is 49.6% methylated, the Xist promoter is 66.3% methylated, and IAP is 83.5% methylated. In the putative iPGCs, methylation at the Snrpn DMR is modestly reduced to 38.7%, while Xist and IAP methylation levels are the same as ESCs. To determine if the DMR of Snrpn also exhibits partial demethylation in endogenous PGCs, we performed bisulfite sequencing of sorted PGCs from e9.5 and e10.5 Oct4-gfp embryos. Methylation at the Snrpn DMR in e9.5 and e10.5 PGCs from the embryo were still present (47.6% and 54.2% respectively) consistent with previously published findings [31]. Furthermore, we observed evidence of demethylation at the 5′ and 3′ ends of these clones in endogenous PGCs at e9.5 and two sequences at e10.5 similar to what was observed in iPGCs (Figure 2D, arrows). Taken together, using real time PCR, immunofluorescence and bisulfite sequencing, our data strongly argue that the Blimp1-positive PGCs isolated from EBs at day 6 of differentiation correspond to a pre-e11.5 stage germ cell in vitro.

Germ line signature genes including Blimp1 are co-expressed in single SSEA1+/cKitbright cells

To determine whether the relative levels of PGC signature genes in SSEA1+/cKitbright cells are comparable to the levels found in PGCs sorted from the embryo prior to e11.5, we sorted Gfp+ cells from Oct4-gfp embryos at 9.5 and e10.5 (Figure 3A–C, shown is e9.5). A distinct Gfp+ population was detected from e9.5 to at least e13.5 (Figure 3C and data not shown). Sorted Oct4-gfp+ PGCs from the embryo are SSEA1+ and exhibit bright cKit+ staining (Figure 3C). We confirmed that the Gfp+ cells are PGCs due to enriched expression of cKit, Blimp1, Stella, and Mvh relative to the Gfp- somatic cells at a population level by real time PCR (Figure 3D). Detection of cKit and Blimp1 RNA in the Gfp-popolation was not unexpected as these genes are also expressed in endothelial and hematopoietic cells during early embryogenesis [32,33].

We next evaluated the transcriptional identity of undifferentiated ESCs, iPGCs and embryonic PGCs at e9.5 and e10.5 at a single cell level by examining expression of five signature PGC markers (Blimp1, Stella, Prdm14, Dnd1 and Dazl) using the BioMark Fluidigm Real Time PCR platform (Figure 3E–H). We evaluated 38 single undifferentiated ESCs (Figure 3E), 34 single embryonic Oct4-Gfp+ PGCs at e9.5 (Figure 3F), 24 single Oct4-Gfp+ PGCs at e10.5 (Figure 3G), and 30 iPGCs from day 6 EBs (Figure 3H). In undifferentiated ESCs, 17 of the 38 cells (44%) expressed Blimp1. Of the 17 Blimp1+ cells, 6 did not express Stella and 12 did not express Dazl. In contrast to ESCs where less than 50% of cells expressed Blimp1, 100% of e9.5 and e10.5 PGCs from the embryo and iPGCs expressed Blimp1 (Figure 3F–H). Heat maps of the single cell analysis indicate that e9.5 PGCs are relatively homogeneous when comparing individual cells to each other for each gene, whereas at e10.5 and in iPGCs expression levels between individual cells is more heterogeneous (Figure 3G,H). Critically, only one cell in the iPGC cohort was not a germ cell (Figure 3H, asterisk).

Figure 2. 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 AP. Circles represent individual CG dinucleotides, black = methylated and white = unmethylated cytosines. Arrows indicate individual alleles that display characteristic demethylation. N.D. = not determined. doi:10.1371/journal.pone.0028960.g002
Figure 3. 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-
We next examined expression levels of each gene for all cells that co-expressed Blimp1, Stella, Prdm14, and Dnd1 relative to levels in SSEA1+/cKitbright iPGCs. We first compared ESCs to iPGCs and found that of the 4 Blimp1+ ESCs that co-expressed Stella, Prdm14 and Dnd1 (10.5%), the transcript levels were significantly lower than those in iPGCs (Figure 3I–L). However, comparison of iPGCs to embryonic e10.5 PGCs revealed no significant difference with regard to Blimp1, Prdm14, and Dnd1 expression levels (Figure 3I,K–L). In single cells that also co-expressed Dazl, ESCs displayed significantly diminished Dazl levels, but no significant difference was found between iPGCs and c10.5 endogenous PGCs (Figure 3M). Stella levels were statistically different between all groups, with SSEA1+/cKitbright iPGCs on average expressing intermediate levels between c9.5 and c10.5 embryonic PGCs (Figure 3J). We propose that the intermediate levels of Stella in iPGCs between c9.5 and c10.5 may indicate that iPGCs are developmentally equivalent to a period of germ cell differentiation between c9.5 and c10.5.

Gene expression profiling by microarray reveals iPGCs repress a mesoderm transcriptional program and identifies a novel marker of in vitro PGC formation from ESCs

Although analysis of five critical PGC-expressed genes at a single cell level was informative for ensuring that >96% of SSEA1+/cKitbright iPGCs have a Blimp1+ PGC identity, our next goal was to obtain a more comprehensive transcriptional portrait of iPGCs derived from day 6 EBs by performing microarray analysis using Affymetrix Mouse Genome chips followed by D-Chip analysis and examine expression of ESC-expressed genes and somatic genes (Figure 4A,B). We profiled the SSEA1+/cKitbright...
fraction from V6.5 EBs at day 6 of differentiation (Samples A and B). Oct4-Gfp+/cKit<sup>bright</sup> cells from day 6 Oct4<sup>-</sup> EBs (Samples C and D), undifferentiated V6.5 SSEA1+/cKit<sup>+</sup> ESCs (Samples E and F), and SSEA1<sup>-</sup>/cKit<sup>-</sup> and Oct4<sup>-</sup>/cKit<sup>-</sup> day 6 EB cells (Samples G–J).

Cluster analysis of genes that are differentially expressed at greater than three-fold between undifferentiated ESCs and iPGCs (548 genes, p<0.01) generated four major transcriptional clusters (Figure 4A & Table S1). Gene ontology (GO) analysis of Cluster I (enriched in ESCs but not iPGCs or somatic cells) identified genes associated with transcription factor activity and DNA binding. Cluster II (enriched in iPGCs but not ESCs or somatic cells) revealed enrichment in genes associated with hydrolyase activity, cytoplasmic proteins and MAPK signaling pathways. Genes in Cluster III (enriched in ESCs and somatic cells but not iPGCs) were associated with GO terms for stress fibers and actin filament bundle genes. Finally, GO analysis of Cluster IV (repressed in iPGCs and ESCs but not somatic cells) revealed genes associated with mesoderm formation including heart and blood development, and morphogenesis. Together, these data suggest that iPGCs repress genes associated with mesoderm differentiation, similar to what has been proposed for endogenous PGC formation through the microarray was validated by real time RT-PCR from Oct4-Gfp+/cKit<sup>bright</sup> iPGCs (Figure 4B). Similarly, expression of additional somatic genes (Hoxa2 and Hoxc5) which were not evaluated earlier (Figure S1F) revealed undetectable expression in iPGCs, whereas somatic cells were positive.

Next, we compared our microarray data between iPGCs and undifferentiated ESCs to identify a marker that could distinguish between these two cell types. We identified <i>Inhibitor of DNA binding 4</i> (Id4) as being significantly higher in iPGCs relative to ESCs. We confirmed the microarray data showing significant enrichment of Id4 RNA in independently collected iPGC samples relative to undifferentiated ESCs (Figure 4C). To determine if Id4 protein is expressed in iPGCs, we performed immunohistochemistry of day 6 EBs with SSEA1 and Id4, and identified Id4 positive cells within the clusters of SSEA1+ cells (Figure 4D). Likewise, immunohistochemistry of e10.5 embryos shows that Id4 protein is expressed in SSEA1+ PGCs (Figure 4E, arrow). However, Id4 was also expressed in the surrounding embryonic somatic cells. Taken together, Id4 is a new marker for distinguishing iPGCs from undifferentiated ESCs, but does not distinguish PGCs from somatic cells of the embryo.

**Blimp1 is specifically required for iPGC differentiation from EBs**

Dosage of <i>Blimp1</i> is essential for the specification of PGCs in vivo [2,32]. To determine if the emergence of SSEA1+/cKit<sup>bright</sup> PGCs in vivo is similarly dependent upon <i>Blimp1</i> expression, we derived <i>Blimp1<sup>fl/fl</sup></i> ESCs from e3.5 blastocysts. We performed Y chromosome FISH to identify a male line (Figure 5A), and generated three independent <i>Blimp1<sup>fl/fl</sup></i> knockout sub-lines (<i>Blimp1<sup>fl/+/fl</sup></i>) via transfection of Cre recombinase fused to Gfp followed by re-plating of Gfp+ cells at limiting dilutions. Clones were screened by Southern blot to verify <i>Blimp1</i> deletion (Figure 5B). To compare overall self-renewal and pluripotency in <i>Blimp1<sup>fl/+/fl</sup></i> cells relative to the parental line, we performed flow cytometry for SSEA1 under self-renewing conditions in the presence of LIF (Figure 5C), and teratoma analysis by injection of undifferentiated ESCs into the testicles of SCID mice (Figure 5D). In both assays, all <i>Blimp1<sup>fl/+/fl</sup></i> lines were indistinguishable from parental <i>Blimp1<sup>+/+</sup></i> cells, indicating that loss of <i>Blimp1</i> does not cause gross defects in overall ESC self-renewal or differentiation.

Finally, to evaluate in vitro PGC formation, we performed paired differentiation experiments with <i>Blimp1<sup>fl/+/fl</sup></i> and <i>Blimp1<sup>fl/+/+</sup></i> lines and evaluated iPGC differentiation by flow cytometry (Figure 5E). Quantification of SSEA1+/cKit<sup>+</sup> cells revealed that iPGCs constitute approximately 3–4% of the live cell population in the parental <i>Blimp1<sup>fl/+</sup></i> line at day 6 (Figure 5E). In contrast, all <i>Blimp1<sup>fl/+/+</sup></i> sub-lines displayed between a 70–90% decrease in SSEA1+/cKit<sup>+</sup>iPGCs, with the average percentage constituting less than 1% of the EB in all three sub-lines examined (Figure 5F,G). Functionally, this demonstrates that sorting for SSEA1+/cKit<sup>+</sup>iPGCs captures a <i>Blimp1</i>-dependent PGC population in vivo, whereas iPGCs do not exhibit the same reliance on <i>Blimp1</i> as in vivo PGCs.

**Discussion**

Emerging cell populations in the early embryo are challenging to investigate. Therefore, we used mouse ESCs from multiple genetic backgrounds to differentiate transgene-free, pre-gonadal stage PGCs where 100% of the single iPGCs express <i>Blimp1</i> in vitro. Here we show that sorting for the cKit<sup>bright</sup> fraction of SSEA1+ cells at day 6 of differentiation when the population is first discernable yields an iPGC population with an identity suggestive of PGCs younger than e11.5.

One of the major challenges in the ESC and PGC fields has been to distinguish early progenitor PGCs from undifferentiated ESCs due to their similar expression patterns. Indeed, e11.5 PGCs isolated from the genital ridge prior to sex determination cluster very closely to undifferentiated ESCs in 2-dimensional principle component analysis after microarray [11]. Therefore, it has been proposed that ESCs originate from a progenitor germ cell consistent with detectable expression of PGC-signature genes, including <i>Dazl</i> and <i>Tissue non specific alkaline phosphatase</i> in the undifferentiated state [34,35]. Although our studies do not address the origin of ESCs, our data does indicate that a small nascent PGC-like population corresponding to about 10% of cells can be identified in an ESC culture in the self-renewing state, agreeing strongly with previous work which demonstrated that <i>Dazl</i> null ESCs exhibit reduced expression of PGC-signature genes [8]. However, our data also show that despite co-expression of germ cell genes in these 10% of cells, the transcript levels are significantly lower than the levels found in <i>bona fide</i> PGCs isolated from the embryo between e9.5–e10.5 as well as the iPGCs. Taken together, our data argues that the majority of undifferentiated ESCs are not PGCs, and that a single cell analysis is critical to uncouple differences between ESCs and progenitor PGCs.

In the current study, we identified Id4 as a new marker enriched in iPGCs relative to undifferentiated ESCs. Id4 was recently found to be a germ line marker expressed in gonocytes and spermatogenesis of postnatal and adult murine gonads [36]. We extend these findings to show that Id4 is expressed during the earliest stage of germ line development, prior to gonadal colonization (Figure 4E). Interestingly, Id4 similar to Stella constitutes a marker for defining PGC identity yet has no functional role in specifying PGC fate [13,36,37]. However, by combining Id4, SSEA1, and Oct4 expression in day 6 EBs, we propose a model for germ line formation in vitro that involves the generation of multiple SSEA1+/Oct4+ niches during EB formation, with Id4+ iPGCs emerging from within these niches (Figure 6). We propose that similar to PGC development in the allantois of the embryo, the tight clustering of SSEA1+/Oct4+ cells creates a microenvironment in the EB to protect the iPGCs against somatic cell differentiation signals [38]. Given that Id4+ cells constitute only a subpopulation of cells within SSEA1+ clusters, we hypothesize that the clusters
are composed of a heterogeneous mixture of immature cells, including epiblast-like cells (Stella negative, Blimp1 negative), PGC precursors (Stella negative, Blimp1 positive) and definitive Id4-positive cKit bright iPGCs (Figure 6). Whether the SSEA1+/cKit bright PGCs emerge from a subpopulation of the SSEA1+/cKit mid fraction of cells remains to be determined. However, our data strongly argue that iPGCs do not differentiate from SSEA1+/cKit dim cells, which have no colony forming potential, and express high levels of Hoxa1 and Hoxb1, indicating commitment to a somatic fate.

Although our data suggest that the iPGCs are younger than e11.5 of development due to lack of Mvh protein expression, it is conceivable that iPGCs at day 6 are more similar to e11.5 in some aspects, but have not received the appropriate cues to express Mvh protein. The signals that promote Mvh protein expression in PGCs at e11.5 are not well understood, but one study has indicated that gonadal somatic cells are involved in this process [29]. Lack of Mvh protein expression in our model suggests that the hanging drop EB system by day 6 of differentiation does not provide the necessary signals to promote developmental progression to Mvh protein-positive iPGCs. This result implies that progression of iPGCs in vitro may require a gonadal niche to promote differentiation to the Mvh protein-positive stage. Indeed, while this manuscript was under review, Hayashi and colleagues demonstrated that a neonatal seminiferous tubule niche was necessary to promote differentiation of ESC-derived PGCs, which this group called PGC like cells (PGCLCs), into functional post-meiotic male germ cells [39]. In these studies, PGCLCs were isolated using SSEA1 and Integrin Beta 3 and were hypothesized to be equivalent to e9.5 of development. Similar to this group, iPGCs isolated at day 6 also express significantly high levels of Integrin Beta 3 RNA (Figure S2).

In the current study, we successfully acquired PGCs in the Blimp1-positive stage of development. Blimp1 is not expressed in

**Figure 5. Blimp1 is required for the differentiation of iPGCs from ESCs.** A: DNA-FISH for the Y chromosome in Blimp1fl/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 Blimp1fl/fl and Blimp1Δ/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 Blimp1fl/fl and Blimp1Δ/Δ ESCs. Quadrant gates indicate criteria for gating SSEA1+/cKit bright iPGCs, which are contained within the rectangular gate (black lines). F: Percentage iPGC yield in the control Blimp1fl/fl line and Blimp1Δ/Δ sub-lines. Error bars represent s.e.m. G: Quantification of data from F, expressed as a percent of the Blimp1fl/fl iPGC yield from each paired experiment. Error bars represent the standard error of the mean. *p<0.05, **p<1x10^-5.

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meiotic or post-meiotic cells and therefore our model is not useful for evaluating meiotic progression; however, we propose that this model can be used to successfully evaluate molecular events in PGC formation prior to gonadal colonization, gonadal reprogramming and sex determination. As an example of the utility of our model, one hundred male e10.5 embryos would be required to obtain approximately 100,000 PGCs via FACS, if we estimate that there are 1,000 PGCs per embryo at this developmental age [5]. In contrast, generating iPGCs equivalent to e9.5–10.5 of development using ESC differentiation required 50 plates of hanging drop EBs, which takes 1 hour to set up from only two wells of undifferentiated ESCs. This yields on average 150,000–175,000 Blimp1-positive iPGCs at day 6 of differentiation, resulting in more than 100-fold enrichment in cell numbers over embryonic dissections [40].

In conclusion, we propose that the ESC-to-PGC differentiation model is an essential tool for examining molecular events in PGC development. In this study we developed a model that specifically captures the Blimp1-positive stage of male PGC formation prior to the expression of Mvh protein. This period of germ cell development (prior to e11.5) is uniquely regulated in mammals and is not conserved with lower model organisms such as Drosophila, C. elegans, frog, and chick (for example, the role of Blimp1). Therefore, creating models that study the initial formation of mammalian PGCs such as the one described here, as well as extending this model to female ESC lines, will be critical to our understanding of the mechanisms that govern fundamental principles of inheritance via the germ line.

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) [41]. Cells were passaged every three days at 5,300 cells/cm². 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 5 mL PBS in the plate bottom and cultured in the absence of LIF for six days, with addition of 3.5 mL PBS on day 3 of differentiation. For ESC derivation, e3.5 blastocysts were isolated from homozygous Blimp1flox/flox (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 Blimp1 null ESCs, Blimp1flox/flox cells were transfected with pCAG-Cre:Gfp [42] and sorted to generate sublines.

Mice

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 [40].

iPGC Colony Assay

iPGCs were sorted from EBs by FACS and re-plated on inactivated CF-1 MEFs. iPGCs were cultured in ESC media
supplemented with 15 ng/ml bFGF2 (R&D), 30 ng/ml SCF (Peprotech), and 2 micromolar retinoic acid (Sigma), for five days as described previously [15]. Cells were cultured for five days with daily media changes followed by assaying for AP activity. EGCs were derived from iPGCs by culture of iPGCs for five days with LIF/SCF/bFGF2/RA, followed by passaging in LIF-only containing media for subsequent passages.

Flow cytometry and FACS
Staining for SSEA1 (DSHB, 1:200) and cKit (BD, 1:200) was performed on ice. Indirect labeling was performed with Cy3-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. SSEA1 and Id4 immunohistochemical detection was performed using anti-Id4 (1:100, Novus Biologicals) and anti-SSEA1 (DSHB) with standard protocols (Vector Labs).

Real-time PCR
RNA was extracted from sorted samples using the RNEasy Micro Kit (Qiagen) and reverse-transcribed 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 S2 for additional primer information. CT values were normalized to Gapdh expression and also to establish primer correlation coefficients and ensure transcriptional cluster in Figure 4A differentially expressed more genes corresponding to Affymetrix probe sets identified from each microarray presented in Figure 4A.

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.

Microarray and Data Analysis
RNA extraction, labeling amplification and hybridization to Affymetrix Mouse Genome 430 2.0 arrays were performed as previously described [41]. Analysis was performed using model-based expression and invariant set probe normalization using D-Chip software [43]. Gene ontology (GO) terms were identified using DAVID [44,45]. Microarray data is deposited under GEO accession number GSE33121.

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 S2 for PCR primer information.

Southern Blot
Pdn1/Blimp1 deletion was verified with dUTP-digoxigenin-labeled probe generated by PCR upstream of the deleted exons of Blimp1 (fwd:5’-CTCTTGAGGCCTTGTGTGTG-3’, rev:5’-AACGCTGTACCCATGACTCC -3’), after digestion with EcoRI. Detection of wild type (15 kb), 13.5 kb), and KO (10 kb) alleles of Blimp1 have been described [46].

Supporting Information
Figure 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+/cKit 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+/cKit cells. E: SSEA1 gene expression data for Oct4-gfp and J1-derived iPGCs and somatic cells. F: Somatic gene expression data for Oct4-gfp and J1-derived iPGCs and somatic cells. (TIF)

Figure S2 Integrin Beta 3 is enriched in iPGCs. Normalized signal intensity from probe sets for Integrin beta 3 (Itgb3) for ESCs and iPGCs were determined from the microarray presented in Figure 4A. (TIF)

Table S1 DAVID Gene Ontology of transcriptional clusters identified between ESCs and iPGCs. Gene ontology analysis of genes corresponding to Affymetrix probe sets identified from each transcriptional cluster in Figure 4A differentially expressed more than 3-fold with p<0.01. (DOCX)

Table S2 Primers used in this study. (DOCX)

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Author Contributions
Conceived and designed the experiments: JJV ATC. Performed the experiments: JJV ZL SAL XL MOE SVDP SKT SG AGL. Analyzed the data: JJV ATC. Contributed reagents/materials/analysis tools: JJV. Wrote the paper: JJV ATC.
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