Developmental Cell

Comparative Principles of DNA Methylation Reprogramming during Human and Mouse In Vitro Primordial Germ Cell Specification

Graphical Abstract

Duration of epigenetic germline reprogramming
- ~ 35 days
- > 7 days

Highlights
- Defined specification of human in vitro PGCLCs from naive ESCs
- Human and mouse epigenetic germline reprogramming tempo differs significantly
- Demethylation-resistant regions are enriched in TEs and repressive chromatin marks
- Mouse in vitro PGCLCs show expression of transposon-derived piRNAs

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In Brief
von Meyenn et al. establish a system for human primordial germ cell specification in vitro to characterize early events of epigenetic reprogramming during formation of the human germline relative to the mouse. Reprogramming in human versus mouse shows significantly different developmental timing that may be explained by differential DNMT3 and UHRF1 regulation.
Comparative Principles of DNA Methylation Reprogramming during Human and Mouse In Vitro Primordial Germ Cell Specification

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SUMMARY

Primordial germ cell (PGC) development is characterized by global epigenetic remodeling, which resets genomic potential and establishes an epigenetic ground state. Here we recapitulate PGC specification in vitro from naive embryonic stem cells and characterize the early events of epigenetic reprogramming during the formation of the human and mouse germ line. Following rapid de novo DNA methylation during priming to epiblast-like cells, methylation is globally erased in PGC-like cells. Repressive chromatin marks (H3K9me2/3) and transposable elements are enriched at demethylation-resistant regions, while active chromatin marks (H3K4me3 or H3K27ac) are more prominent at regions that demethylate faster. The dynamics of specification and epigenetic reprogramming show species-specific differences, in particular markedly slower reprogramming kinetics in the human germline. Differences in developmental kinetics may be explained by differential regulation of epigenetic modifiers. Our work establishes a robust and faithful experimental system of the early events of epigenetic reprogramming and regulation in the germline.

INTRODUCTION

Primordial germ cells (PGCs) are the precursors of the fully differentiated gametes, oocytes, and sperm, establishing during their development the prerequisites of the totipotent state. Upon their specification PGCs undergo global epigenetic reprogramming, erasing epigenetic memory and re-establishing an epigenetic ground state (Clark, 2015; von Meyenn and Reik, 2015; Reik and Surani, 2015). Our basic understanding of mammalian PGC specification and epigenetic reprogramming stems largely from work in the mouse. However, recent work is beginning to shed light on human germ line development and epigenetic reprogramming (Saitou and Miyauichi, 2016; Surani, 2015). In the mouse, after exit from naive pluripotency in the inner cell mass (ICM) and priming for differentiation, a small cluster of ~40 PGCs is detectable in the epiblast around embryonic day 7.25 (E7.25). Subsequently PGCs migrate through the hindgut to the developing genital ridges (E8–E10.5) where they proliferate extensively before sexual differentiation commences. Human PGCs (hPGCs) are specified around E12–E16 (developmental week 2), and, while the early migratory phase (weeks 3–5) of in vivo hPGC development is currently not accessible to experimental analysis, gonadal hPGCs have recently been isolated and characterized molecularly (Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015). This in vivo work has shown that hPGCs are characterized by the expression of known PGC marker genes such as BLIMP1, PRDM14, or DPPA3 but also express human specific genes such as SOX17. Similar to epigenetic reprogramming in mouse PGCs (mPGCs), in vivo hPGCs have erased DNA methylation globally by week 5.5, presumably starting during the migratory phase, resulting in a hypomethylated epigenetic ground state.

Given the relative inaccessibility and difficulties in manipulating PGCs in vivo, the development of an in vitro differentiation system is highly desirable. Spontaneously differentiating human and mouse cells expressing germ cell markers isolated from embryoid bodies (EBs) were initially used as a proxy for in vitro generation of gametes or PGCs (Daley, 2007; Saitou and Yamaji, 2010), and some erasure of DNA methylation was documented in the mouse system (Vincent et al., 2013). However, only more recent studies have demonstrated the potential to faithfully reconstitute early mouse and human PGC specification in vitro (Hayashi et al., 2011; Irie et al., 2015; Sasaki et al., 2015; Sugawa et al., 2015). Some limited results suggested that DNA methylation reprogramming takes place, but no systematic genome-scale analysis has been carried out (Hayashi et al., 2011; Tang et al., 2015).

The specification of mouse PGC-like cells (mPGCLCs) closely recapitulates in vivo PGC specification (Hayashi et al., 2011). Naïve ICM-like embryonic stem cells (ESCs) (Nichols and Smith, 2012) are differentiated to epiblast-like cells (EpiLCs), which closely mimic the in vivo epiblast state around E6.25, when mPGCs are first specified. “Germline-competent” EpiLCs then
progress toward mPGCLCs, which have the potential to generate oocytes (Hayashi et al., 2012) and spermatid-like cells (Zhou et al., 2016). Human PGCLC (hPGCLC) specification protocols thus far started from already “germline-competent pluripotent stem cells” (Irie et al., 2015), thereby not fully recapitulating the in vivo hPGC specification and raising the possibility that key epigenetic steps during the priming phase were missed (Saitou and Miyauchi, 2016). The recent establishment of naive hESC culture conditions (Guo et al., 2016; Takashima et al., 2014; Theunissen et al., 2014, 2016), which more closely resemble the in vivo state of naive human ICM cells, provides a promising opportunity to reconstitute more faithfully hPGC specification in vitro.

Here we report the establishment of a protocol for in vitro hPGCLC specification from naive hESCs and investigate the early events of DNA methylation remodeling prior to and during PGCLC specification. We have also undertaken a comparative analysis of epigenetic reprogramming at single base resolution during human and mouse in vitro germline development and have identified conserved as well as divergent mechanisms regulating the observed DNA methylation dynamics. This work establishes a tractable model system for the precise study of epigenetic reprogramming in the germline, and describes the principles and dynamics of DNA methylation remodeling during early PGC specification.

RESULTS

Generation of Human and Mouse PGCLCs from Naive ESCs

Using a similar strategy to that described for mPGCLC specification (Hayashi et al., 2011) we differentiated naive hESCs toward hEpiLCs in serum-free N2B27 medium containing transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), and knockout serum replacement (KSR) (Figure 1A). Naive hESC colonies’ typical domed shape was lost and hEpiLCs adopted a flat, primed cell morphology by day 4 (Figure 1B), a characteristic also observed during mPGCLC differentiation (Hayashi et al., 2011). Next we aggregated hEpiLCs to EBs (day 0) and induced hPGCLC specification by adding bone morphogenetic protein 4 (BMP4), stem cell factor (SCF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF). Using a GFP reporter expressed under the control of the OCT4-dPE promoter (Theunissen et al., 2014) we were able to monitor the activity of the naive and germ cell-specific OCT4-dPE promoter (Theunissen et al., 2014; Yeom et al., 1996) in the EBs, suggesting the formation of hPGCLCs (Figure 1B). Since previous in vivo studies have shown that hPGC development is not completed by week 5.5 (Tang et al., 2015), we aimed to progress hPGCLC differentiation as far as possible and naive ESCs, primed EpiLCs, and sorted cKIT- human or SSEA1+/CD61+ mPGCLCs, found that PGCLCs cluster separately from naive and primed cells (Figures S1F and S1G). While mPGCLCs showed a preference to separate by time points, suggesting a temporal progression, the different hPGCLC time points were intermingled, indicating smaller temporal changes. Principal component analysis (PCA) confirmed these observations (Figures 1G and 1H) and showed that during specification of PGCLCs from naive ESCs, some of the transcriptional variance between naive and primed cells (PC2) was reversed during primed to PGCLC differentiation, suggesting re-establishment of a more “naive” transcriptional signature in PGCLCs.

Global Epigenetic Changes during Human and Mouse PGCLC Specification

To gain insights into epigenetic reprogramming during in vitro PGCLC specification, we performed whole-genome bisulfite sequencing of human and mouse PGCLCs and primed EpiLCs (Table S1), and in our analysis included published in vivo and in vitro datasets (Ficz et al., 2013; Guo et al., 2014; Okae et al., 2014; Seisenberger et al., 2012; Takashima et al., 2014; Tang et al., 2015; Wang et al., 2014) (Figures 2 and S2). After fertilization, the highly methylated epigenome of sperm and to a lesser extent of oocytes is globally erased during progression to ICM and naive ESCs (~29% in hESCs and ~31% in mESCs). Subsequently, there was a strong de novo methylation activity during priming of human and mouse EpiLCs, which increased the average CpG methylation levels to ~65% (Figures 2A and 2B), equivalent to the methylation levels found in mouse epiblast at E6.5 (Seisenberger et al., 2012). We confirmed this remethylation using liquid chromatography followed by mass spectrometry, showing an increase from approximately 1.5%–4% of all cytosines being methylated from the naive ESC state to day-4 hEpiLCs and day-2 mEpiLCs, respectively (Figures S2A and S2B). We also observed an approximately 2-fold increase in 5-hydroxymethylcytosine (5hmC) levels in mouse and human EpiLCs compared with naive ESCs. Interestingly, the remethylation phase during priming took twice as long in humans than in mice, suggesting a different regulation of the de novo methylation machinery. In line with increased methylation activity, the expression levels of the de novo DNMTs 3A and 3B were upregulated in both mouse and human primed cells, albeit the increase in mouse was much more pronounced (Figures 2C and S2C).

Next we analyzed epigenetic changes during early PGC specification. mPGCLCs rapidly lost global methylation, reaching
levels of around 40% CpG methylation after 4 days and 24% CpG methylation at day 6, similar to in vivo PGCs at E10.5 (~28%) or E11.5 (~20%) (Seisenberger et al., 2012). In contrast, hPGCLCs demethylated much more slowly, gradually decreasing global levels of CpG methylation from approximately 68% at day 4 to 55% at day 12 (Figures 2A and 2B). In line with this, previous reports using immunofluorescence to assess 5hmC (Irie et al., 2015), while in vivo hPGCs demethylate to 68% at day 4 to 55% at day 12 (Figures 2A and 2B). In line with decreasing global levels of CpG methylation from approximately 100% at day 6, similar to in vivo PGCs at E10.5 (CpG methylation at day 6, similar to in vivo PGCs at E10.5), hPGCLCs demethylated much more slowly, gradually decreasing global levels of CpG methylation from approximately 68% at day 4 to 55% at day 12 (Figures 2A and 2B). In line with decreasing global levels of CpG methylation from approximately 100% at day 6, similar to in vivo PGCs at E10.5 (CpG methylation at day 6, similar to in vivo PGCs at E10.5), hPGCLCs demethylated much more slowly, gradually decreasing global levels of CpG methylation from approximately 68% at day 4 to 55% at day 12 (Figures 2A and 2B). In line with decreasing global levels of CpG methylation from approximately 100% at day 6, similar to in vivo PGCs at E10.5 (CpG methylation at day 6, similar to in vivo PGCs at E10.5), hPGCLCs demethylated much more slowly, gradually decreasing global levels of CpG methylation from approximately 68% at day 4 to 55% at day 12 (Figures 2A and 2B).
CGI-containing promoters remained at low levels of methylation throughout all time points with a small increase during EpiLC priming. Next we analyzed the methylation at known differentially methylated regions (DMRs) of imprinted genes (Figures 2F and S2G). Methylation of paternal or maternal DMRs was exclusively found in either sperm or oocytes, respectively; after fertilization the combined levels were maintained at around 50% into ICM cells, indicating faithful maintenance of imprinting. Naive mESCs and mEpiLCs maintained a similar methylation pattern of imprinted DMRs, which notably were subsequently erased during mPGCLC formation, with substantial erasure in day-6 mPGCLCs. In vivo mPGCs also demethylate the imprinted DMRs, starting around E10.5/E11.5 with complete erasure by E13.5. In contrast, naive hESCs had erased almost all imprinted DMRs, as previously reported (Pastor et al., 2016) and, as a consequence, imprinted DMRs were not re-established during hEpiLC priming and remained demethylated during hPGCLC specification at levels comparable with in vivo hPGCs.

To identify specific unique regions showing different methylation dynamics compared with the whole genome during the early phase of human epigenetic resetting, we performed k-means clustering of 2-kb probes of the human genome, excluding probes overlapping with repetitive elements. Seven clusters were generated and the enrichment of specific genomic features compared with the whole genomes was assessed. Published datasets from human sperm, oocytes (Okae et al., 2014), ICM (Guo et al., 2014), naive ESCs (Takashima et al., 2014), in vivo PGCs (Tang et al., 2015), and mouse sperm, oocytes, ICM (Wang et al., 2014), naive ESCs (Ficz et al., 2013), epiblast, and in vivo PGCs (Seisenberger et al., 2012) were included in the analysis. Biological replicates were pooled and average levels were used for the analysis. See also Figure S2.
Regulation of Local Methylation Dynamics

Having found that the feature composition and proximity to repetitive elements correlates with the methylation dynamics, we first compared the local methylation levels of primed day-4 hEpiLCs with day-12 hPGCLCs. The overall methylation distribution showed that the genome was not demethylated uniformly; we thus constructed a background model of demethylation from primed day-4 hEpiLCs to day-12 hPGCLCs and tested for probes with significantly (p < 0.05) higher (red) or lower (blue) levels of DNA methylation (Figure 3A).

A subset of probes with significantly lower levels of DNA methylation in day-12 hPGCLCs overlapped with CGIs, which remained largely methylated throughout the whole time course (compare clusters 2 and 7 in Figure 2G). We then looked for genomic features overlapping with the more highly methylated regions (Figure 3A) and found that probes overlapping with gene bodies or the repetitive elements SVA and L1Pa, which had previously been found to resist demethylation in in vivo hPGCs (Tang et al., 2015), retained higher levels of DNA methylation in day-12 hPGCLCs. This can also be seen in the illustrated example (Figure 3B), where SVA overlapping probes (green shading) or gene bodies of ASXL2 or RAB10 (red shading) retained higher levels of methylation, even in in vivo week-5.5 hPGCs. Increased DNMT3B binding and de novo methylation at transcribed genes has been reported previously (Baubec et al., 2015) but we did not find a correlation between persistence of gene body methylation and transcription in day-12 hPGCLCs (Figure S3A).

We next compared the methylation levels of the probes identified with significantly higher (red) or lower (blue) levels of DNA methylation (Figure 3A) across the whole time course of in vitro hPGCLC specification, including earlier and later in vivo datasets (Figure 3C). Regions that partially resisted demethylation during hPGCLC specification (red) showed higher methylation in naive hEpiLCs and hICM but acquired methylation levels comparable with that of the whole genome upon priming and remethylation (day-4 hEpiLCs). During subsequent hPGCLC differentiation, these regions retained higher levels of methylation until in vivo week-5.5 hPGCs and only became almost completely demethylated in week-7 hPGCs, indicating that DNA demethylation kinetics differ significantly across the genome.

To better understand the regulation of local methylation dynamics, we performed a similar analysis on the mouse datasets (Figure 3D) and compared the methylation levels of day-2 mEpiLCs with globally demethylated day-6 mPGCLCs. A defined set of regions retained high levels of methylation in mPGCLCs and these were enriched in intracisternal A particle (IAP) transposable elements (TEs), but there was no enrichment in gene body methylation as observed in human. As illustrated in Figure S3B, not all regions that retain methylation in mPGCLCs (and in vivo mPGCs) are IAP associated. We therefore overlaid the methylation comparison with available chromatin immunoprecipitation sequencing (ChIP-seq) data from day-6 mPGCLCs (Kurimoto et al., 2015) or in vivo mPGCs (Liu et al., 2014). Regions retaining high levels of DNA methylation were enriched in the repressive histone marks histone 3 lysine 9 trimethylation (H3K9me3) or H3K9me2 (Figures 3D and S3C), which have been shown to play a pivotal role in recruitment of the DNA methylation machinery (Citterio et al., 2004; Karagianni et al., 2008; Rothbart et al., 2012). Conversely, regions with low levels of DNA methylation in day-6 mPGCLCs were enriched in the activating histone marks H3K4me3 or H3K27ac (Figures 3D and S3C).

The transcript levels of the H3K9 methyllases EHMT1 and EHMT2 were reduced in PGCLCs and in vivo PGCs of both species, while the expression of the H3K9 demethylases KDM3A and KDM3B was increased in only mPGCLCs and in vivo PGCs of both species (Figures 3E and S3D), but not in hPGCLCs.

Regulation of Transposable Elements in PGCLCs

About half of the mammalian genome is composed of interspersed repetitive elements resulting from replicative insertion events of TEs (Burns and Boeke, 2012; Lander et al., 2001; Mouse Genome Sequencing Consortium et al., 2002). DNA methylation is important for TE repression in somatic cells, and other mechanisms including histone modifications or PIWI-interacting RNAs (piRNAs) control TEs upon global demethylation (Friedli and Trono, 2015; Iwasaki et al., 2015). We analyzed the average methylation levels of major classes of human and mouse TEs, including long and short interspersed elements (LINEs and SINEs), long terminal repeats, human SVA retrotransposons, and mouse IAPs (the most active murine TE class).

All TEs gained methylation during priming from naive hESCs to hEpiLCs and only became demethylated slowly during hPGCLC specification (Figure 4A), with SVA elements retaining most methylation, while in vivo hPGCs showed demethylation with only SVA and human endogenous retrovirus K (hERVK) TEs retaining some methylation at week 7 (Tang et al., 2015). Naive hESCs showed high expression of SVA TEs but transcription of TEs was generally low in hPGCLCs and hPGCs, with the exception of hERVK elements, which showed some expression in all datasets (Figure S4A).

In contrast, there was extensive demethylation of TEs during mPGCLC development (Figure 4B), again resembling levels of in vivo mPGCs around E10.5/E11.5. IAPs retained higher levels of methylation in vitro, as they do in vivo. Analysis of poly(A)-enriched RNA-seq datasets (Figure 4C) showed increased expression of IAPs and ERVK in day-6 mPGCLCs. Transcription of other TEs remained low in all mPGCLC datasets, suggesting that additional repressive mechanisms are controlling TE expression. Previous studies using total RNA-seq have also shown increased TE expression in hypomethylated in vivo mPGCs and an involvement of piRNAs in controlling TE expression (Molaro et al., 2014). piRNAs are germline-specific 24- to 31-nt-long small RNAs (smRNAs) which have been shown to regulate the activity of TEs in the germline (Aravin et al., 2007). Due to the lack of suitable mammalian experimental models, the mechanisms controlling the generation of mature piRNAs and their molecular TE-silencing activity are still enigmatic (Iwasaki et al., 2015). Notably, mPGCLCs do express the relevant enzymes required for piRNA biogenesis and activity, namely Miwi2 and Mili (Figure 4D) while expression of MILI and MIWI2 in hPGCLC is lower than in in vivo hPGCs (Figure S4B), perhaps as a consequence of the fact that TEs are not yet demethylated and hence not prone to transcriptional activation.

We generated small RNA-seq libraries from mPGCLCs and in vivo E15.5 male prospermatogonia (Table S1) to assess the expression of piRNAs. mPGCLCs and prospermatogonia
Figure 3. Regulation of Local Methylation Dynamics during Human and Mouse PGCLC Specification

(A) Scatter plots of CpG methylation percentages over probes spanning 50 CpGs comparing primed day-4 hEpiLCs and day-12 hPGCLCs. The scatter plots were overlaid with red or blue to highlight probes with higher or lower levels of CpG methylation than the background model (p < 0.05) or labeled to highlight CpG density, overlap with gene bodies, SVA, or L1Pa elements.

(B) Representative bisulfite-sequencing data showing a part of chromosome 2 from hICM, hESC, primed hEpiLC, hPGCLC, and in vivo hPGC datasets. Regions retaining higher levels of DNA methylation and overlapping with SVA elements or gene bodies are shaded in green or red, respectively. The position of genes, CGIs, and SVA or L1PA elements is shown in the top panel.

(C) Box plots of the CpG methylation levels of probes defined in (A) as methylated higher or lower than the background model. Shown are samples across the whole hPGC/hPGCLC specification period. The middle line indicates the median of the data, the upper and lower extremities of the box show the 25th and 75th percentiles, and the upper and lower black whiskers show the median ± the interquartile range (25%–75%) multiplied by 2. Any individual points that fall outside this range are shown as filled circles. Each circle represents a single probe.

(legend continued on next page)
showed strong enrichment in 24- to 31-nt-long smRNAs with high numbers mapping to gene-derived piRNAs (Li et al., 2013) and >50% of all smRNAs mapping to TEs (Figures 4E, 4F, and S4C). In contrast, mESC-derived smRNAs were mostly 22–23-nt-long microRNAs (miRNAs). Furthermore, we found characteristics of piRNAs (Iwasaki et al., 2015) in the smRNAs from mPGCLC and prospermatogonia samples that mapped to repetitive elements (defined by repeatmasker); smRNAs mapping to TEs had a tendency for U at the 5’ end (Figures 4G and S4D) and also a high frequency of exactly 10-nt spaced 5’ to 5’ overlaps (“ping-pong signature”) (Figure 4H). Similarly, we found high numbers of smRNAs mapping both sense and antisense to repetitive elements in mPGCLCs (Figure 4I). Notably, therefore, in vitro mPGCLCs express transposon-derived piRNAs at levels comparable with those of in vivo prospermatogonia.

**DISCUSSION**

Global DNA demethylation is a key characteristic of mammalian PGC (and early embryo) development and allows the germ cell lineage to create a blank slate (“tabula rasa”) (Clark, 2015) with an underlying pluripotent characteristic, possibly a prerequisite for the subsequent generation of the totipotent zygote (Reik and Surani, 2015). Here we have developed a protocol for hPGCLC formation from naive ESCs, hence recapitulating in vivo priming and specification, and studied the early events of DNA methylation reprogramming in human and mouse PGCLCs. This approach has also allowed us to characterize the DNA methylation changes during the initial priming phase toward EpIiLCs, which formed the basis for the subsequent demethylation during PGOLC specification, and to obtain insights into the regulation of epigenetic resetting in human and mouse. Notably, there are some key differences in the regulation of epigenetic modifiers, which may underlie the very different reprogramming kinetics in human and mouse.

We discovered that human and mouse PGCLCs can be induced from naive pluripotent stem cells using similar methodologies, despite the fact that the transcriptional networks regulating human and mouse PGC specification differ in several aspects (Saitou and Miyachi, 2016). Notably, in vitro hPGCLC development was significantly delayed compared with mPGCLC specification, which is in agreement with the different developmental timing in vivo (Irie et al., 2014). Interestingly, the rate of de novo methylation during priming to EpiLCs was approxi-

mately slower epigenetic reprogramming kinetics in human versus mouse PGCLCs.
Demethylation was, however, not uniform across the genome. Regions overlapping with young and active TEs (Friedli and Trono, 2015) partially resist DNA demethylation in PGCLCs, as they do in in vivo PGCs (Gkountela et al., 2015; Guo et al., 2015; Kobayashi et al., 2013; Seisenberger et al., 2012; Tang et al., 2015). IAPs, which are the youngest and most active TEs in the mouse germline, are most resistant to demethylation, while none of the human TE families were as resistant, consistent with human TEs being more endogenized (Friedli and Trono, 2015). In addition to TEs, we found a strong correlation of H3K9me2/3 enrichment at regions with retained DNA methylation during mPGCLC specification and, conversely, an enrichment of H3K4me3 and H3K27ac at regions with faster than average demethylation. This suggests that the underlying chromatin signature influences both the global and the local demethylation rate in mouse germline development.

Loss of DNA methylation has generally been linked to activation of retrotransposons (Bourc’his and Bestor, 2004; Walsh et al., 1998), and in vivo piRNAs have been found to control TE expression (Iwasaki et al., 2015). Indeed, TE expression was low in substantially hypomethylated day-6 mPGCLCs and we found expression of piRNAs in in vitro mPGCLCs, suggesting that TE expression is also restrained by smRNA-dependent mechanisms in vitro. Since loss of piRNA activity causes male sterility (Carmell et al., 2007; Cheng et al., 2014; Kuramochi-Miyagawa et al., 2004), mPGCLCs would seem to represent a good experimental system for the investigation of piRNA biology in the future. In hPGCLCs we found some extent of hERVK reactivation followed by progressive repression in hPGCs, also suggesting the activity of a DNA methylation-independent repressive mechanism in the human germline. Notably, we also observed a specific increase in the expression of...
of SVA elements in naive hESCs but not in similarly hypomethylated week-5.5 hPGCs, suggesting that the expression of SVA might be a specific marker of naive hESCs (Theunissen et al., 2016).

We found a loss of primary methylation imprints in naive hESCs (confirming a recent study [Pastor et al., 2016]), which were not re-established during priming to HêpiLcs. Abnormal imprinting is linked to a range of human developmental disorders and malignancies (Butler, 2009). While it is hoped that future developments of naive hESC derivation and culture protocols will resolve this issue, especially for the application of hESCs, whether there are any adverse implications of loss of imprinting for germline development is unclear at present. Finally, the current PGCLC system enables the characterization of early events of epigenetic reprogramming and its regulation in the mammalian germline, but further developments are required to also capture the later events of human PGC development. These will reveal the regulation and importance of piRNAs in the human germline and also shed light on the subsequent events of epigenetic reprogramming not assessed thus far.

EXPERIMENTAL PROCEDURES

Human hESC Culture and hPGCLC Differentiation

Naive H9 and naive WIBR3 OCT4-dPE-GFP hESCs were propagated in serum-free N2B27 medium (N2 & B27; Life Technologies) supplemented with 20 ng/mL LIF (Cambridge Stem Cell Institute [SCI]), 1 μM MEK inhibitor PD0325901 (SOCI), 3 μM GSK inhibitor CHIR99021 (SOCI), and 2 μM protein kinase C inhibitor Gö6983 (Sigma-Aldrich), as described previously (Guo et al., 2016; Takashima et al., 2014). The medium was refreshed every day and cells were passaged every 4–5 days. hEpiLcs were induced by plating 2 × 10^4 naive hESCs on a well of a 6-well plate coated with growth factor reduced Matrigel (Corning) in N2B27 medium supplemented with 1 ng/mL TGF-β1 (PeproTech), 12 ng/mL bFGF (SCI), and 1% KSR (Gibco). The medium was changed every day. hPGCLCs were induced by plating 3–4 × 10^3 day-2 day-3 hEpiLcs in a well of an Ultra-Low attachment U-bottom 96-well plate (Corning) in GK15 medium (Glasgow’s minimal essential medium [Life Technologies] with 15% KSR [Life Technologies], 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM β-mercaptoethanol) supplemented with 500 ng/mL BMP4 (R&D Systems), 20 ng/mL HGF (SCI), 100 ng/mL NFG (R&D Systems), and 50 ng/mL mEGF (R&D Systems). Cells were cultured in 5% O2 and 5% CO2 in a humidified incubator at 37°C.

Mouse mESC Culture and mPGCLC Differentiation

Naive E14 or BvSc mESCs were cultured feeder-free in N2B27 supplemented with 10 ng/mL LIF (SCI), 1 μM MEK inhibitor PD0325901 (SOCI), and 3 μM GSK3 inhibitor CHIR99021 (SOCI), together known as 2i (Ying et al., 2008). The medium was refreshed every day and cells were passaged every 2–3 days. mEpiLcs were induced by plating 1 × 10^5 naive mESCs on a well of a 12-well plate coated with human plasma fibronectin (Millipore, FC010) in N2B27 medium supplemented with 20 ng/mL activin A (SCI), 12 ng/mL bFGF (SCI), and 1% KSR (Gibco) (Hayashi et al., 2011). The medium was changed every day. mPGCLCs were induced by plating 2 × 10^3 day-2 day-3 mEpiLcs in a well of an Ultra-Low attachment U-bottom 96-well plate (Corning) in GK15 medium supplemented with 500 ng/mL BMP4 (R&D Systems), 10 ng/mL HGF (SCI), 100 ng/mL mNFG (R&D Systems), and 50 ng/mL mEGF (R&D Systems). Cells were cultured in 5% O2 and 5% CO2 in a humidified incubator at 37°C.

In Vivo Prospermatogonia Collection

Embryonic samples were collected from timed matings of C57BI/6J female mice expressing an Oct-4-GFP transgene in the developing gonad (Yoshimizu et al., 1999). Prospermatogonia were isolated as described previously (Seisenberger et al., 2012). All animal work carried out as part of this study is covered by a project license (to W.R.) under the 1986 Animal (Scientific Procedures) Act, and is further regulated by the Babraham Institute Animal Welfare, Experimentation, and Ethics Committee.

RNA-Seq, Mapping, and Analysis

Extracted total RNA was DNase treated and poly(A) enriched. RNA-seq libraries were prepared using the TruSeq RNA Library Prep Kit V2 (Illumina) or a modified SMART-Seq2 protocol (Picelli et al., 2014). Sequencing was performed on Illumina HiSeq 2000 instruments and RNA-seq sequences were trimmed using Trim Galore (v0.4.1). High-throughput sequencing of all libraries was carried out with single-end protocols on a HiSeq 2000 instrument (Illumina).

Bisulfite Sequencing, Mapping, and Analysis

Whole-genome bisulfite libraries were generated using the Illumina TruSeq Small RNA Library Preparation Kit (RS-200-0012) with the following modifications. A total of 100 to 1 μg RNA input material was used. cDNA samples were run on 10% Novex PAGE gels for purification and the gel piece between the 145- and 160-bp marker excised, and cDNA was eluted from the gel in freshly prepared 0.3 M NaCl by rotation overnight at 4°C. The C DNA was precipitated in ETOH overnight; from the supernatant the cDNA was resuspended in 10 μL of EB buffer and the library was quantified using the high-sensitivity DNA chips on the Agilent Bioanalyzer. High-throughput sequencing of all libraries was carried out with single-end protocols on a HiSeq 2000 instrument (Illumina).

Small RNA-Seq, Mapping, and Analysis

smRNA-seq libraries were generated using the Illumina TruSeq Small RNA Library Preparation Kit (RS-200-0012) with the following modifications. A total of 10 to 1 μg RNA input material was used. cDNA samples were run on 10% Novex PAGE gels for purification and the gel piece between the 145- and 160-bp marker excised, and cDNA was eluted from the gel in freshly prepared 0.3 M NaCl by rotation overnight at 4°C. The C DNA was precipitated in ETOH overnight; from the supernatant the cDNA was resuspended in 10 μL of EB buffer and the library was quantified using the high-sensitivity DNA chips on the Agilent Bioanalyzer. High-throughput sequencing of all libraries was carried out with single-end protocols on a HiSeq 2000 instrument (Illumina).
or single-end mode for read 2; defaults. Reads were then deduplicated and CpG methylation calls were extracted from the deduplicated mapping output ignoring the first 6 bp of each read to reduce the methylation bias typically observed in PBAT libraries using the Bismark methylation extractor (v0.14.4.4) with the following parameters: (a) paired-end mode: --ignore 6 --ignore_r 2 6; (b) single-end mode: --ignore 6.

Raw sequence reads from WBGS libraries were trimmed to remove poor-quality reads and adapter contamination using Trim Galore (v0.4.1). The remaining sequences were mapped using Bismark (v0.14.4.4) (Krueger and Andrews, 2011) with default parameters to the mouse reference genome GRCm38 or the human reference genome GRCh37 in paired-end mode. Reads were then deduplicated and CpG methylation calls were extracted from the deduplicated mapping output using the Bismark methylation extractor (v0.14.4.4) in paired-end mode.

CpG methylation calls were analyzed using R and SeqMonk software. Global CpG methylation levels of pooled replicates were illustrated using bean plots. The genome was divided into consecutive 20-kb probes covered by at least 10 CpGs, and percentage methylation was calculated using the bisulfite feature methylation pipeline in SeqMonk.

Probe trend plots were generated by calculating average CpG methylation levels of 1-kbp 500-bp overlapping probes from 5 kbp upstream of the transcriptional start site through gene bodies (which were scaled for visualization) to 5 kbp downstream of the transcriptional end site.

For analysis of specific genome features, these were defined as follows using the Ensembl gene set annotations for mouse and human: exons (probes overlapping exons), introns (probes overlapping introns), promoters (probes overlapping 1,000 bp upstream of genes), CGI promoters (promoters containing or within 250 bp of a CGI), non-CGI promoters (all other promoters), intergenic (probes not overlapping with gene bodies). Annotations for mouse and human germline imprint control regions were obtained from Tomizawa et al. (2011) and Court et al. (2014). Pseudocolor heatmaps representing average methylation levels were generated using the R “heatmap.2” function without further clustering, scaling, or normalization.

For k-means clustering, average CpG methylation across 2-kb probes of the human genome were calculated using the bisulfite feature methylation pipeline in SeqMonk, excluding probes overlapping with repetitive elements. Seven clusters were generated, and enrichment of specific genomic features was assessed by counting the percentage overlap of probes in each cluster with the specific genomic features and comparing these with the whole genome.

Scatter plots visualizing the changes in global methylation were generated by plotting the percentage methylation over probes defined to contain 50 CpGs each. Scatter plots were colored according to the probe density or the density of the indicated overlapping genomic feature. Published raw CHIP-seq data were trimmed to remove poor-quality reads, adapters, and barcode sequences using Trim Galore (v0.4.1). Trimmed data were mapped using Bowtie2 against the mouse reference genome GRCm38 and filtered to remove non-primary alignments or alignments with MAPQ <20. Read-count enrichments were overlaid on the methylation scatter plots. Pseudocolor scatter plots were generated using R.

Correlation between gene body methylation and gene expression was computed from average CpG methylation across gene bodies using the bisulfite feature methylation pipeline in SeqMonk and correlating these values with the respective gene expression values for each gene.

Repeat Analysis
Repeat locations for a pre-defined set of repeat classes of interest were extracted from the pre-masked retrotransposon libraries (mouse, retrotransposon v4.0.3, library version 20130422; human, retrotransposon v4.0.5, library version 2014031). Repeat instances within 2 kb of an annotated gene in the Ensembl gene set were removed to avoid mixing signals from genomic expression with specific expression of repetitive sequences.

RNA-seq sequences were processed and mapped as described above (RNA-Seq, Mapping, and Analysis). Non-directional overlaps were quantitated between the mapped RNA-seq reads and the repeat instances. Summed counts for all instances of each class of repeat were calculated, and these were corrected for both the total length of all repeats and the size of the individual libraries to generate RPKM (reads per kilobase of transcript per million mapped reads) expression values. The matrix of expression values and samples was plotted using the R heatmap library.

Bisulfite sequencing libraries were processed and mapped as described above (Bisulfite Sequencing, Mapping, and Analysis). Methylation levels at the repeat instances were quantitated by summing up all methylation calls and non-methylation calls for all instances of each class of repeat and calculating the percentage of methylated calls over all calls. The matrix of expression values and samples was plotted using the R heatmap library.

See also Supplemental Experimental Procedures.

ACCESS NUMBERS
The accession number for the next-generation sequencing data reported in this study is GEO: GSE86586.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.09.015.

AUTHOR CONTRIBUTIONS
F.v.M. and W.R. conceived and designed the study; F.v.M. performed experiments and analyzed data; R.V.B. prepared and analyzed small RNA-seq libraries; S.A. analyzed high-throughput sequencing data; F.S. performed bioinformatics processing; R.O. generated naive WIBR3 OCT4-dPE-GFP cells; W.D. performed in vivo collection of mPGCs; P.J.R.-G helped design the study; F.v.M. and W.R. wrote the manuscript with input from all authors; W.R. supervised the study.

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Note Added in Proof

During the revision of this manuscript another study reported global loss of DNA methylation and imprint erasure in vitro mPGCLCs (Miyoshi et al. 2016), supporting the usefulness of the PGCLC system and confirming our observations. REF: Miyoshi, N., Stel, J. M., Shioda, K., Qu, N., Odahima, J., Mitsunaga, S., et al. (2016). Erasure of DNA methylation, genomic imprints, and epimutations in a primordial germ-cell model derived from mouse pluripotent stem cells. Proceedings of the National Academy of Sciences of the United States of America. http://doi.org/10.1073/pnas.1610259113.