Epigenetic Regulation during Primordial Germ Cell Development and Differentiation

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Keywords
Epigenetics · Epigenome · Epitranscriptome · Germline · Non-coding RNA · Primordial germ cells · Small RNA

Abstract
Germline development varies significantly across metazoans. However, mammalian primordial germ cell (PGC) development has key conserved landmarks, including a critical period of epigenetic reprogramming that precedes sex-specific differentiation and gametogenesis. Epigenetic alterations in the germline are of unique importance due to their potential to impact the next generation. Therefore, regulation of, and by, the non-coding genome is of utmost importance during these epigenomic events. Here, we detail the key chromatin changes that occur during mammalian PGC development and how these interact with the expression of non-coding RNAs alongside broader epitranscriptomic changes. We identify gaps in our current knowledge, in particular regarding epigenetic regulation in the human germline, and we highlight important areas of future research.

Introduction

Across metazoans there are a diverse array of mechanisms of primordial germ cell (PGC) specification which have important implications for the evolution of the vertebrate body plan [Extavour and Akam, 2003; Evans et al., 2014]. In mammals, despite notable embryological differences, many features of PGC development are conserved [Kobayashi and Surani, 2018; Dion and Leitch, 2020; Hancock et al., 2021]. PGCs are induced by the action of BMP signals on either the pluripotent epiblast [Ohinata et al., 2009; Kobayashi et al., 2017] or, perhaps, its recent descendants, such as the amnion in cynomolgus macaques [Sasaki et al., 2016] (Fig. 1a). This leads to the up-regulation of PGC specification genes (including BLIMP1 and TFAP2C), although species-specific differences have been noted [Irie et al., 2015; Tang et al., 2015; Kojima et al., 2017; Kobayashi et al., 2017; Sybirna et al., 2020]. This is accompanied by maintenance of OCT4 and re-expression of many pluripotency-associated transcription factors [Leitch and Smith, 2013]. Thereafter, PGCs migrate to the genital ridge, where upon colonisation a process of genome-wide epigenetic reprogramming is completed prior to sex-specific differentiation [Hajkova, 2011; Leitch et al., 2013a]. To our knowledge, no mammalian
species studied so far deviates from this basic developmental blueprint. Like many specification factors, induction of a small number of key transcription factors appears to drive segregation of the germline [Nakaki et al., 2013; Magnusdottir and Surani, 2014]. Perhaps unsurprisingly, this is accompanied by epigenomic changes such that upon arrival at the genital ridges, PGCs are already epigenetically distinguishable from their new somatic cell neighbours, which have a distinctive developmental origin [Seki et al., 2007; Hajkova, 2011]. Here, we will briefly summarise these early epigenetic changes that provide the template for genome-wide epigenetic reprogramming, which is subsequently discussed in detail. Next, we consider how non-coding RNAs map onto these key early germline events and summarise their known germline functions, before finally suggesting important unanswered questions that might constitute future areas of research into the function of the non-coding genome in early mammalian germ cells. Filling in these gaps in our knowledge is a key priority because, in contrast to any other cell lineage, epigenetic alterations in the germline have the potential to impact the development and health of the next generation.

**Fig. 1.** Mammalian models of primordial germ cell specification. a Simplified depictions of cylindrical mouse (left) and bilaminar human (right) embryos with mouse primordial germ cells (mPGCs) specified at embryonic day (E) 6.25 at the posterior epiblast, and human PGCs (hPGCs) thought to be specified around week 2–3 putatively at the early amnion and/or posterior epiblast. b In vitro models of PGC-like cell (PGCLC) differentiation for mouse (top) and human (bottom). Mouse PGCLCs (mPGCLCs) are derived from 2-inhibitor (2i: PD0325901, CHIR99021)/Leukemia Inhibitory Factor (LIF), embryonic stem cells (mESCs), then induced to epiblast-like cells (mEpiLCs) upon 40–48 h culture with Activin A and fibroblast growth factor 2 (FGF2). mPGCLCs are then isolated from embryoid body (EB) formation upon 4–6 days of treatment with cytokines bone morphogenetic factor 4 (BMP4) (and optionally with BMP8A/B), stem cell factor (SCF), LIF, and epidermal growth factor (EGF). Human PGCLCs (hPGCLCs) are derived from conventional human ESCs (hESCs) or induced pluripotent stem cells (iPSCs) in Essential-8 (E8) or conventional hESC media grown in 1 of 3 protocols. (1) 4-inhibitor hESC (4i: CHIR99021, PD0325901, SB205820, SP600125), FGF2, LIF, and transforming growth factor beta 2 (TGFβ2) grown on feeder layers; (2) pre-mesoderm (pre-ME) cells grown in Activin A, glycogen synthase kinase 3 beta inhibitor (GSK3βi: CHIR99021), and Rho-associated protein kinase inhibitor (ROCKi) for 12 h; or (3) incipient mesoderm-like cells (iMeLC) grown in Activin A, GSK3βi, ROCKi for 2 days. hPGCLCs are isolated from day 4 EBs upon treatment of either of these cell states with ROCKi and cytokines BMP2 or 4, SCF, LIF, and EGF.
**Setting the Scene – Epigenetic Changes in Pre-gonadal PGCs**

Initial epigenetic changes triggered by mouse PGC specification (starting around E6.25) have largely been quantified by immunofluorescence staining (Fig. 2). This revealed that the repressive histone modification H3K9me2 becomes globally depleted from around E8.0 [Seki et al., 2005; Hajkova et al., 2008]. Conversely, H3K9me3 levels remain high at pericentric heterochromatin, with punctae observed in immunostainings, whereas repressive H3K27me3 levels become progressively enriched.
Repressive modifications of histones H2A and H4 (H2A/H4R3me2) also increase, due to the nuclear translocation of Prmt5 and its association with Blimp1 [Ancelin et al., 2006]. Notably, there is a decline in DNA methylation starting from around E8.5 [Seki et al., 2005; Guibert et al., 2012; Seisenberger et al., 2012] which is associated with downregulation of de novo methyltransferases Dnmt3a and 3b, as well as the Dnmt1 co-factor Uhrf1 [Seki et al., 2005; Kurimoto et al., 2008; Ohno et al., 2013]. However, clearly some DNA methyltransferase activity is maintained as many loci retain high levels of DNA methylation during this period, and conditional deletion of Dnmt1 in pre-gonadal PGCs results in precocious depletion of DNA methylation, in particular at imprinting control regions, meiotic genes, and endogenous retrovirus-intracisternal A particle (ERV-IAP) retrotransposons [Hargan-Calvopina et al., 2016]. How DNA methylation levels are regulated at different genomic regions remains poorly characterised (discussed below).

The small number of newly specified PGCs has been a limiting factor for more detailed epigenomic analyses. Instead, the mPGC-like cell (mPGCLC) system (Fig. 1b) has been used to generate sufficient cell numbers to perform high resolution chromatin-immunoprecipitation followed by next-generation sequencing (ChIP-seq). As mPGCLCs are induced from epiblast-like cells (mEpiLCs), they show a widespread reorganisation of the repressive marks H3K27me3 and H3K9me2 [Kurimoto et al., 2015], in keeping with the in vivo data. These germline-competent mEpiLCs were noted to harbour relatively low levels of H3K27me3 across developmental regulators compared with mESCs which cannot directly give rise to PGCs. This perhaps allows mEpiLCs to make rapid transcriptional responses upon receipt of appropriate signalling cues. It was additionally noted that in early mPGCLCs, H3K27me3 marks are recruited across promoters of developmental regulators of somatic lineages – perhaps acting to reinforce their silencing in the germline. Curiously, later (Day 6) mPGCLCs display concomitant upregulation of the active H3K4me3 mark across a number of these genes, marking these promoters as bivalent yet still repressed regions – the significance of which remains unknown. Meanwhile, unlike mEpiLCs, epiblast-derived stem cells (mEpiSCs) show reduced competence for germline fate [Hayashi and Surani, 2009; Hayashi et al., 2011]. These mEpiSCs lose H3K27me3 but gain more stable repressive DNA methylation marks especially over germline-related promoters when compared to germline-competent in vivo E6.25 epiblast cells [Hayashi and Surani, 2009; Zylicz et al., 2015], which may contribute to the reduced germline competence of mEpiSCs. Intriguingly, a recent report has described the derivation of self-renewing formative stem cells that exhibit similar properties to mEpiLCs, including the ability to directly give rise to mPGCLCs [Kinoshita et al., 2021]. Expansion culture of in vivo-sourced mPGCs may provide an alternative in vitro system in the future but remains challenging despite some recent advances [Leitch et al., 2013b; Borkowska and Leitch, 2021].

Chromatin dynamics have not been studied in newly-specified, pre-migratory human PGCs in vivo due to the inherent challenges in accessing early post-implantation human embryos for research (Fig. 3). Human (h)PGCLCs are thus the only available tool that might provide an approximation of nascent hPGCs (Fig. 1b). However, with no in vivo comparisons of newly-specified PGCs, it remains unclear exactly which stage of development hPGCLCs represent [Dion and Leitch, 2020]. Of note, recent analysis of an extremely rare week 3 (CS 7) gastrulating human embryo identified the putative presence of hPGCs [Tyser et al., 2020], and there has been (infrequent) detection of hPGC markers in late week 2 ex vivo cultured human embryos [Chen et al., 2019]. If further rare embryos do become available, or if ex vivo embryo culture beyond day 14 [Clark et al., 2021] reveals nascent hPGCs, this might provide further relevant comparisons to more accurately stage hPGCLCs. Nevertheless, hPGCLCs will likely continue to be the mainstay of investigation into the early human germ line. hPGCLCs have not been reported to show large-scale global changes in DNA methylation following induction [Irie et al., 2015; Murase et al., 2020], and there are differing reports as to whether DNA demethylation proceeds following further in vitro culture [Gell et al., 2020; Murase et al., 2020]. This may be in part due to differential regulation of the DNA methyltransferase enzymes and UHRF1 in the different induction and culture systems. Immunostainings have shown decreased H3K9me2 and slightly higher H3K27me3 staining [Sasaki et al., 2015], in keeping with data from the mouse and also with immunostainings from the earliest hPGCs obtained thus far [Gkountela et al., 2013; Tang et al., 2015]. Additionally, ATAC-seq of hPGCLCs identified regions of accessible chromatin that significantly overlap with naive-pluripotency related TFAP2C-bound enhancers. Indeed, the deletion of the TFAP2C-bound Pou5F1/OCT4 distal enhancer perturbed hPGCLC induction [Chen et al., 2018].

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However, no rigorous genome-wide analysis of histone modifications in hPGCLCs has yet been reported but would be of interest to the field, especially if compared with fetal hPGC samples.

Thus, following specification, and during migration to the genital ridges, PGCs establish a distinctive epigenome characterized by low levels of DNA methylation and H3K-9me2, as well as elevated H3K27me3 and H2A/H4R3me2s. However, this appears to act as a prelude to a more complex and complete epigenetic reprogramming event upon arrival at the genital ridge, associated with, and/or leading to, further profound changes in epigenetic regulation.

**Fig. 3.** Epigenome and non-coding RNA dynamics in human PGC development. Human germline development proceeds asynchronously with founder gonadal PGCs (orange cells), then mitotic-arrest (MA, purple cells) fetal germ cells (hFGCs) from week 10 with both subpopulations persistent up till week 26. In females, in addition to founder hPGCs, retinoic acid-responsive (RA, pink cells), meiotic (Mei, salmon cells), and oogenesis (Oo, red cells) hFGCs emerge from weeks 11, 14, and 18, respectively, with all 4 subpopulations persistent up till week 26. Broken vertical lines denote postulated dynamics. Dots represent H3K9me3 punctae over chromocentres. Question marks denote uncertain or absence of data.

**Back to the Future – Epigenetic Reprogramming in Gonadal PGCs**

**DNA Methylation**

**Mouse**

Since the first reports of DNA methylation changes in mouse PGCs, it has been clear that DNA methylation drops across almost all DNA elements during migration and following colonisation of the genital ridge [Monk et al., 1987; Kafri et al., 1992; Hajkova et al., 2002]. These findings have been replicated in more recent studies utilising genome-wide approaches, demonstrating that
During epigenetic reprogramming in the gonad, DNA methylation reaches its nadir, achieving levels lower than at any other stage of development [Seisenberger et al., 2012; Hill et al., 2018]. The exact timing of DNA demethylation differs according to genetic background (which does make direct comparison between different studies challenging), however, in most cases this commences around E11.0 and is complete by E13.5. Critically, it is during this process that genomic imprints are erased [Hajkova et al., 2002; Seisenberger et al., 2012]. The removal of DNA methylation from imprinted differentially methylated regions (DMRs) is a unique event during mammalian development and allows subsequent re-establishment of sex-specific genomic imprints. In males, imprint re-establishment appears coupled to global increases in DNA methylation, a process that begins soon after epigenetic reprogramming [Lees-Murdock and Walsh, 2008], with substantial restoration of global DNA methylation clearly evident by E16.5 [Li et al., 2004; Seisenberger et al., 2012]. The histone modification H3K-36me2, deposited by NSD-1 over broad euchromatic regions occupying 59% of the genome, has recently been shown to play a critical role in this de novo DNA methylation process in mouse spermatogonia by E16.5, particularly over non-transposable element (TE) regions [Shirane et al., 2020]. In contrast, low levels of DNA methylation are maintained in mouse oogonia until oocyte growth commences postnatally [Smallwood et al., 2011]. Intriguingly, a recent in vitro study inducing oocyte-like cells from pluripotent stem cells by transcription factor overexpression has demonstrated that at least some of the de novo DNA methylation that occurs during oocyte growth can occur independently of the preceding process of epigenetic reprogramming [Hamazaki et al., 2021].

Despite the loss of DNA methylation from promoter and regulatory elements, there is not a generalised transcriptional activation. However, loss of DNA methylation has been associated with activation of selected germline-specific genes [Maatouk et al., 2006; Hackett et al., 2012b; Velasco et al., 2010], and more recently, a larger group of germline reprogramming responsive (GRR) genes have been described. These GRR genes become upregulated as a result of promoter DNA demethylation, and robust expression also requires simultaneous depletion of polyclamp marks [Hill et al., 2018]. How these findings relate to the global hypertranscription reported in mPGCs [Percharde et al., 2017] has not been specifically addressed and is an important topic for future study (see later). In particular, it will be interesting to determine when hypertranscription commences (it was noted as early as E9.5) and how this relates to the epigenomic landscape at this timepoint.

Most TEs are also subject to DNA demethylation [Seisenberger et al., 2012; Ohno et al., 2013], however, this does not result in widespread upregulation of expression, with the exception of specific evolutionary young elements such as certain loci of ERV-IAP retrotransposons (IAPA_MM, IAPEZI), long interspersed nuclear element-1 (L1) (L1mdTF_Ii, L1MdA_Ii/III), long terminal repeat (LTR) elements (MMERVK10C), and early transposon (ETn) elements [Ohno et al., 2013; Hill et al., 2018]. This may suggest both an alternative mechanism for maintaining transcriptional silencing of those elements that are DNA demethylated but not expressed, as well as a method of post-transcriptional silencing of those that are upregulated. In contrast, the majority of L1A and ERV-IAP retrotransposons retain substantial levels of DNA methylation [Hajkova et al., 2002; Seisenberger et al., 2012], which may be required for their transcriptional repression. Which locus-specific mechanisms maintain the relatively higher levels of DNA methylation on these loci during reprogramming remains largely unknown, although there is some evidence that repressive histone modifications play a role [Liu et al., 2014]. Notably, when mESCs are transitioned from serum to 2-inhibitor(2i) + leukemia inhibitory factor (LIF) + vitamin C conditions there is DNA demethylation and loss of H3K9me2 at certain TE loci. This is accompanied by an initial burst of TE de-repression which is subsequently quelled by gain of H3K27me3 [Walter et al., 2016]. Intriguingly, this switch in repressive histone modifications is reminiscent of the global changes that occur in pregonadal PGCs [Seki et al., 2005].

However, this classic view of relatively limited TE expression in PGCs is challenged by the above-mentioned observation of hypertranscription in mPGCs between E9.5 and E15.5, including higher expression of TEs in mPGCs than in somatic cells [Percharde et al., 2017]. This elevated expression of TEs is evident at E9.5 when DNA methylation remains high and is observed in TEs that both retain and lose DNA methylation at later timepoints. Therefore, as for single copy genes, future work that combines cell number-normalised TE expression analysis with epigenomic analysis would be of particular interest. Other important factors that impact whether (and which) TEs show elevated expression upon demethylation include the additional activatory mechanisms such as transcription factors that are recruited to their loci [Kunarso et al., 2010; Pontis et al., 2019], and their evolutionary age,
with older TEs carrying a high mutational burden that may impact their competence for transcription [Rollins et al., 2006; Castro-Diaz et al., 2014, 2015]. Additionally, subsequent higher TE expression may not be detected in steady-state RNA-seq datasets due to the activity of post-transcriptional mechanisms in controlling transcript levels. Nevertheless, the postulated presence of TE transcripts, even at these early PGC stages, may potentially suggest the involvement of post-transcriptional mechanisms in preventing deleterious retrotransposition events (see later discussion).

The mechanism of DNA demethylation has sparked intense debate. The rapid reduction in DNA methylation following colonisation of the gonad that occurs without a change in doubling time [Tam and Snow, 1981], and possibly even within a single cell cycle [Hajkova et al., 2010], has suggested an active process. However, a mechanism of active DNA demethylation that could explain this global loss of DNA methylation has not yet been described in mammals. With the discovery of Tet enzymes the possibility of replication-coupled, Tet-assisted passive demethylation by hydroxylation of 5mC seemed both an attractive proposition and a highly plausible mechanism [Dawlaty et al., 2011; Hackett et al., 2013; Yamaguchi et al., 2013]. However, direct measurements of 5hmC levels in mPGCs reveal levels that are an order of magnitude lower than would be required for this to be the major mechanism [Hill et al., 2018]. Furthermore, deletion of Tet1 in mPGCs drops 5hmC by a further order of magnitude, while DNA demethylation proceeds unencumbered. In fact, the role of Tet1-mediated hydroxylation in mPGCs appears to be to protect newly demethylated DNA from inappropriate re-acquisition of DNA methylation, a mechanism which mirrors the role of Tet3 in the zygote [Amouroux et al., 2016; Hill et al., 2018]. This mechanistic distinction is consistent with the observation that imprint abnormalities occur only sporadically following colonisation of the gonad [Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015; Li et al., 2017a, 2020]. In these studies, human germ cells were isolated via cKIT or AP/cKIT surface marker cell sorting from embryos between 5 and 24 weeks, a period that spans epigenetic reprogramming and subsequent differentiation of hPGCs. Additionally, single-cell analysis of more mature fetal germ cells (hFGCs) has revealed sex-specific sub-populations emerging from week 9.

In keeping with the mouse, hPGCs arriving at the gonad already have lower methylation levels than the surrounding tissue or that of the inner cell mass (ICM). Once in the gonads, hPGCs continue DNA demethylation, with levels decreasing to a trough of approximately 5% by week 7, as measured by bulk whole genome bisulfite sequencing [Guo et al., 2015; Tang et al., 2015]. Using single cell bisulfite sequencing for hPGCs, Li et al. [2020] both corroborated earlier findings and also revealed the DNA methylation states of later hFGCs. Most intriguingly, all subpopulations of male and female hFGCs analysed showed persistent low levels of DNA methylation (4–10%). Overall, this indicates that the very low DNA methylation established in gonadal hPGCs are maintained in hFGCs for months. While this mirrors the low levels of DNA methylation maintained in mouse oogonia, it contrasts markedly with the rapid restoration of DNA methylation observed in male gonocytes (also known as spermatagonia [McCarrey, 2013]) in mice. When exactly DNA remethylation occurs during sex-specific differentiation of hFGCs therefore remains an open and interesting area for future study.
Gene bodies, enhancers, promoters, and intergenic regions all show very low methylation levels, as do CpG islands [Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015; Li et al., 2020]. Promoter methylation falls to 10% in germ cells compared to 80% in the soma, while only 12% of demethylated promoter-controlled genes show upregulation in hPGCs. These upregulated genes are mainly associated with germline processes, such as DAZL and piRNA pathway associated genes DDX4 and PI-WIL1/2. Meiotic gene expression is found upregulated only in female hFGC subpopulations from week 11 onwards [Li et al., 2017a; Chitishvili et al., 2020], but these meiotic genes are not transcribed in male hFGCs at matched time points, despite similarly low levels of DNA methylation, suggesting roles for activatory processes perhaps linked to dynamic signalling as germ cells mature within the gonadal niche.

DNA methylation at most imprinted DMRs in hPGCs has already fallen to below somatic and earlier ICM levels during migration, but like the mouse, imprint erasure appears to be completed only after gonadal colonisation [Guo et al., 2015; Tang et al., 2015; Li et al., 2020]. The maternally methylated DMRs of PEG10 and IGF2R appear to show slower dynamics [Tang et al., 2015; Li et al., 2020] but are completely erased in mature sperm.

Notably, several genomic regions were found to retain methylation at a higher level than the genome average and are termed “escapees” [Tang et al., 2015; Dietmann et al., 2020]. Interestingly, these correspond to gene ontology terms relating to brain-expressed loci, with potential links to neurological and metabolic disorders in humans, albeit with the caveat that neuronal genes are statistically much larger in terms of genome size [Sahakyan and Balasubramanian, 2016; Lopes et al., 2021], and possibly more likely to be enriched in genomic analyses. A similar trend was seen in recently profiled pig PGCs [Zhu et al., 2021]. Thus, it has been proposed that DNA methylation states at these regions might be heritable, with implications for transgenerational inheritance of epialleles [Tang et al., 2015; Dietmann et al., 2020] (discussed below).

Although TEs are highly variable between species, the overall rules of DNA methylation at TEs appear to follow the pattern observed in mouse in both human [Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015; Li et al., 2020] and pig PGCs [Zhu et al., 2021]. While DNA demethylation follows the genome-wide pattern at most TEs, DNA methylation is retained at specific TE families – most notably evolutionary young TEs, for instance L1HS and AluY in humans, PRE-1 in pigs, and IAPEz in mouse [Zhu et al., 2021]. Single-cell ATAC-seq probing chromatin accessibility meanwhile showed the primate-exclusive SINE-VNTR-Alus (SVAs), followed by the HERVKs, HERV1s, Alu elements and SINEs had enrichment for accessibility in hPGCs and hFGCs compared to the soma [Li et al., 2020]. However, despite DNA demethylation and increased accessibility, TE expression remains low compared to their juxtaposed neighbouring somatic cells, as measured by standard scRNA or bulk RNA-seq techniques. The only exception noted so far is that levels of SVA transcripts were noted to creep upwards from week 5.5 to week 9, while HERVK and HERVH transcripts are also detectable [Tang et al., 2015; Li et al., 2020; Pontis et al., 2021].

Less information is available regarding the mechanism of DNA demethylation in humans. UHRF1, and to a lesser extent DNMT3B, are repressed in migratory cells, however DNMT1 still remains expressed in all germ cells stages at a similar or higher levels than the soma [Guo et al., 2015; Tang et al., 2015; Li et al., 2017a]. The down-regulation of the former 2 factors in migratory PGCs is supported by cross-species studies in pig: DNMT3B and UHRF1 were both shown to be expressed at lower levels than the epiblast and soma [Zhu et al., 2021]. Of the TET enzymes, TET1 shows the highest expression compared to the soma in gonadal germ cells [Guo et al., 2015; Tang et al., 2015], with stainings suggesting 5hmC is present in migratory PGCs, before falling rapidly at the gonad at week 7 [Tang et al., 2015]. Intriguingly, the BER pathway was found to be upregulated in both human and pig PGCs [Guo et al., 2015; Zhu et al., 2021].

**Histone Modifications**

In addition to DNA demethylation, the chromatin landscape also changes during reprogramming of mPGCs, suggesting the presence of global epigenome reprogramming that includes global alterations in histone modifications, histone exchange, and changes to nuclear architecture.

**Mouse**

Alteration to histone modifications in mPGCs have generally been considered to occur in two main phases, with distinct changes noted in pre-gonadal and gonadal PGCs (Fig. 2). In early mPGCs, H3K27me3, H3K4me2, and H3K9ac levels rise from E7.5 to E11.5. H3K9me2 declines during this window, although H3K9me3 stays relatively constant over constitutive heterochromatin [Seki et al., 2005, 2007; Hajkova et al., 2008]. At E11.5, repressive marks (H3K9me3 and H4/H2AR3me2) and permissive
mark (H3K9ac) diminish [Hajkova et al., 2008], while different studies observe either loss [Hajkova et al., 2008; Mansour et al., 2012] or maintenance of H3K27me3 [Kagiwada et al., 2013] during this time window. It has also been observed that the histone variant H2A.Z is removed from demethylating mPGCs, with H1 chaperone NAP1 entering the nucleus at the point where 5mC drops in the gonad, while H3.3 chaperone HIRA is also expressed [Hajkova et al., 2008]. Replacement of canonical histones with their histone variant counterparts has been suggested as one mechanism which could underly the rapid disappearance of these marks [Hajkova et al., 2008], although dynamic changes in linker H1 and the histone chaperone NAP1 were not observed in a subsequent study [Kagiwada et al., 2013]. More recently, dynamic regulation of histone H1 subtypes has been observed in vivo using knock-in mouse lines in which endogenous H1 subtypes are tagged [Izzo et al., 2017]. Notably, H1.4 and H1.10 appear to aggregate at the nuclear membrane around E11.25 before becoming undetectable at E11.5, before re-appearing again at the nuclear envelope at E11.75 and spreading throughout the nucleus, emphasising a degree of dynamic control that could easily be missed by using alternative methods [Izzo et al., 2017].

Following reprogramming, chromatin re-compacts with nuclei shrinking [Hajkova et al., 2008; Kagiwada et al., 2013], chromocenters returning, and H3K9me3 and H3K27me3 being re-established [Hajkova et al., 2008]. Meanwhile, H3K9me2 slightly increases from E13.5 [Abe et al., 2011] but remains low compared to the soma [Deguchi et al., 2013], while H3K9ac and H4/H2AR3me2 do not appear to return after reprogramming [Hajkova et al., 2008]. For histone H1, differences between the sexes begin to show post-reprogramming, with female mPGCs showing higher H1.1 levels than the soma and male mPGCs showing lower H1.4 than the surrounding soma [Izzo et al., 2017]. The function of a small number of histone modifying enzymes/complexes has been directly assessed in PGCs. Deletion of polycomb repressive complex 2 (PRC2) component embryonic ectoderm development (Eed) and the accompanying H3K27me3 loss in mPGCs lead to defects in both male and female mPGCs, with the female germ-line being more sensitive to premature upregulation of sex-specific differentiation genes at E11.5 and E13.5 [Lowe et al., 2019], emphasising sexually-divergent dependencies on H3K27me3 and perhaps on additional histone modifications yet to be studied. The related histone mark H2AK119ub, which is deposited by polycomb repressive complex 1 (PRC1), is also present at E12.5 [Yo-
kobayashi et al., 2013]. Co-regulation by PRC1 and PRC2 complexes is well described [Laugesen et al., 2019]; however, knockout of PRC1 component Rnf2 in mPGCs does not affect H3K27me3 deposition. Instead, it leads to a downregulation of Oct4 in female mPGCs at E12.5, while double knockout of Rnf2 and Ring1, another PRC1 component, leads to a reduction of mPGCs in both sexes. Loss of H2AK119ub affects female mPGCs more than male, with the major defects occurring in the regulation of meiosis. Female cells begin the process of meiosis at E13.5 in wildtype PGCs, but upregulation of Stra8, Rec8, and Syp3 occurs at E11.5 in Rnf knock-out cells when retinoic acid (RA) is produced in the gonad [Yokobayashi et al., 2013]. Male germ cells enter mitotic arrest at E13.5 [McLaren, 1984; Vergouwen et al., 1991] with meiotic entry prevented due to the degradation of RA by Cyp26b1 [Bowles et al., 2006]. However, knockout of PRC1 components is insufficient to trigger precocious meiotic entry. Notably, when Rnf2 knock-out PGCs of either sex are treated with RA in vitro, an early and increased expression of Stra8 is observed, suggesting that PRC1 plays a role in repressing the meiotic programme in both sexes [Yokobayashi et al., 2013]. Deletion of the X-linked H3K27me3 demethylase Utx appears to reduce the germline transmission of male mESCs in chimaeric animals, and when chimaeric embryos are assessed, Utx null mPGCs exhibited reprogramming abnormalities in the gonad with higher levels of H3K27me3 than controls. However, such abnormalities are compatible with fertility in males, while the knockout is embryonic lethal in females [Mansour et al., 2012; Welstead et al., 2012]. Meanwhile, the aforementioned arginine methyltransferase Prmt5 forms a complex with Blimp1 in the germline at E8.0, allowing translocation into the nucleus and leading to methylation of H2A/H4R3 in germ cells [Ancelin et al., 2006]. Deletion of Prmt5 in mPGCs leads to a loss of H2A/H4R3me2 and apoptosis, with complete loss of germ cells by E15.5–E16.5 [Kim et al., 2014; Li et al., 2015]. Knock-out mPGCs show activation of the DNA damage response [Kim et al., 2014] and splicing defects [Li et al., 2015], but also higher expression of TE elements, including IAP-LTRs, IAP-GAG, and L1 elements, indicating a role for Prmt5 in TE silencing in early mPGCs [Kim et al., 2014]. Later, in male gonadal mPGCs, Prmt5 translocates back to the cytoplasm where it methylates PIWI family proteins [Vagin et al., 2009; Kim et al., 2014]. This post-translational modification enables interaction with Tudor-domain proteins, essential for subsequent PIWI-interacting RNA (piRNA) biogenesis and TE silencing by DNA methylation [Aravin et al., 2008] (discussed below). Thus, Prmt5
is implicated in a range of epigenetic mechanisms to regulate TE expression, including histone modifications, non-coding RNAs, and DNA methylation.

Human
In humans, only immunofluorescence data are currently available [Gkountela et al., 2013, 2015; Guo et al., 2015; Tang et al., 2015; Eguizabal et al., 2016]. These show that H3K9me3 and H3K9me2 are depleted in hPGCs in comparison to neighbouring somatic cells, although distinct and bright punctae of H3K9me3 remain within the nucleus. H3K27me3, although initially enriched at week 4, is thereafter downregulated in hPGCs in comparison to somatic neighbours. Active marks such as H3K4me1/3 increase during this time period to levels comparable to the soma, while H3K9ac shows a marked increase even above the levels observed in neighbouring cells [Eguizabal et al., 2016]. The higher numbers of hPGCs make epigenomic studies using ChIP-seq or CUT&RUN [Brind’Amour et al., 2015; Skene and Henikoff, 2017] feasible despite the challenges in obtaining samples, and locus specific information awaits such studies. However, the asynchrony of hPGC development will be a challenge in interpreting such data, unless the distinct germ subpopulations are cleanly isolated using unique cell-surface markers [Li et al., 2020; Mishra et al., 2021]. Indeed, detailed immunostaining of histone modifications in different hFGC subpopulations (stained with the appropriate stage-specific hFGC markers), has not yet been undertaken.

More globally, in both mouse and human PGCs, nuclear size appears to increase during this window, suggesting a widespread dismantling of chromatin structure while demethylation occurs [Hajkova et al., 2008; Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015]. This may be necessary to allow the final stages of DNA demethylation or alternative functions such as recruiting the DNA repair pathway for rapid active DNA demethylation.

Lost in Translation? Non-Coding RNAs and the Epitranscriptome in Early Germline Development

In addition to covalent modifications of chromatin, roles for small (<200 nt) and long (>200 nt) non-coding RNAs as well as RNA modifications have also been explored in the context of mammalian germline development (Fig. 2, 3).

Germline small RNAs include ~22 nt microRNAs (miRNAs), 25–32 nt PIWI-interacting RNAs (piRNAs), as well as ~22 nt endogenous-silencing RNAs (endo-siRNAs). Each have distinct biogenesis pathways, with the small RNAs acting as guide RNAs for their associated Argonaute proteins, leading primarily to post-transcriptional gene silencing via Watson-Crick base pairing.

miRNAs
miRNAs may play a role in triggering mPGC specification, with the inhibition of let-7 miRNA biogenesis by Lin28a appearing to be required for PGC development [West et al., 2009]. In an in vivo chimera assay, the depletion of Lin28a led to a reduction of the Stella-positive mPGCs in the gonad, which was rescued upon the introduction of Blimp1 with an ectopic 3′-UTR carrying a mutated let-7 miRNA target site [West et al., 2009]. This suggests a model in which downregulation of let-7 via its repressor Lin28a is required to allow appropriate expression of key PGC specification gene Blimp1. However, let-7 miRNAs are also known suppressors of the pluripotent state, whilst encouraging the somatic programme, whereas Lin28 antagonises this, maintaining pluripotency. So, whether this regulatory circuit is required for specification versus maintenance of the germline fate is less clear. Meanwhile, the role of LIN28A in hPGCLC specification remains as of yet unstudied.

Thereafter, in mouse gonadal germ cells, the miR-17/92 family, miR-181 family, and miR-290 cluster, among a handful of others, were observed to be highly expressed, with putative roles in proliferation of bipotential early mPGCs [Hayashi et al., 2008; Bhin et al., 2015; Fernandez-Perez et al., 2018], while depletion of somatic let-7 miRNAs continued to be observed. This putative function was ascertained via the use of a conditional germ-line-specific knockout (AP-Cre) of the miRNA biogenesis factor Dicer1 in PGCs [Hayashi et al., 2008; Maatouk et al., 2008], which identified defective proliferation of mPGCs as well as arrested spermatogenesis. In addition, while Dicer is important for the biogenesis of miRNA, it is also required for the biogenesis of other small RNA types such as endo-siRNA, with additional roles in post-transcriptional gene silencing [Czech et al., 2008; Kim et al., 2009], thus confounding the interpretation of the specific roles of miRNAs in mPGCs.

A chief observation by the above studies was the high expression of all members of the miR-290 cluster in mPGCs. This cluster of 7 miRNA hairpins, largely sharing the same seed sequence, is associated with developmentally regulated expression in pluripotent cells, with its expression robustly seen in the ICM of mouse blastocysts, as well as in naive mESCs [Parchem et al., 2014]. Intrigu-
ingly, miR-290 expression drops upon the specification of the epiblast as well as in mEpiSCs, both sharing primed-pluripotent states. Meanwhile, miR-290 cluster expression picked up again exclusively in the germline in the embryo, with additional trophoblast expression also continuously seen [Parchem et al., 2014; Paikari et al., 2017]. This pattern of behavior contrasted with that of a related family of miRNAs, the miR-302 cluster, which is upregulated in E5.5–6.5 epiblasts, and remains present in late epiblasts, but is subsequently absent in mPGCs upon their specification. Taken together, this has led to the model of alternating expression levels of these clusters in the progression from ICM/naive-mESC, to epiblast, and then germline cell fate.

Crucially, the generation of a miR-290 knock-out mouse demonstrated the key role of this cluster, with pervasive (yet incompletely penetrant) embryonic lethality. In embryos that survived, a vast reduction in mPGC numbers was seen at both E11.5 and E13.5. Females were rendered infertile, while males remained fertile due to the continued viability of the very few mPGCs that survived [Medeiros et al., 2011]. A further study also revealed catastrophic placenta defects that could also explain part of the embryonic lethality seen, as the miR-290 cluster is indeed observed to be expressed in the trophoblast [Paikari et al., 2017]. This experiment together with Dicer knockouts described above therefore bolsters the observation that miRNAs are important for the regulation of gene networks in mPGCs.

In the human, no studies have yet comprehensively profiled the miRNAs of isolated hPGCs nor of their in vitro counterpart, the BMP-induced hPGCLCs. However, germ cell cancers, and the seminoma cell line TCAM-2, have been extensively profiled for miRNAs, with the equivalent of the miR-290 cluster in the human, the miR-371 cluster (composed of 3 hairpins, producing 6 mature miRNAs), spotted to be highly abundant [Murray et al., 2011; Novotny et al., 2012]. In addition, naive-hESCs have also been profiled for miRNAs, with a similar observation noted [Faridani et al., 2016; Dodsworth et al., 2020]. Therefore, it may be reasonable to expect that the miR-371 cluster is also expressed in the human germline, considering the similar behavior of this cluster in both mouse and human naive ESCs, as well as its presence in seminoma whose transcriptome overlaps somewhat with that of hPGCs. A concerted approach towards profiling the miRNAs in human germline development and identifying their mRNA targets will thus certainly be useful to the field.

Upon sex specification later in gonadal germ cell development, miRNA profiles in mouse fetal germ cells (mFGCs) diverge. In particular, miR-17/92 and the let-7 families decreased in female mFGCs as they entered meiosis, while their levels increased in male mFGCs as they entered mitotic arrest at E13.5 [Hayashi et al., 2008; Bhin et al., 2015]. miR-29b was meanwhile found to be exclusively expressed in female mFGCs from E13.5 with putative targets Dnmt3a and Dnmt3b [Takada et al., 2009], potentially contributing to low DNA methylation levels in contrast to their remethylated male counterparts at the same time points. Full profiles of miRNAs in both male and female mFGCs beyond E15.5, as well as hFGCs, however, remain unknown.

**piRNAs**

piRNAs are restricted to metazoa, with signatures of 2′-O-methyl-modified 3′-ends and a 5′-U bias, while deploying the PIWI-clade of Argonautes (PIWI proteins), distinguishing them from miRNAs [Weick and Miska, 2014; Czech et al., 2018; Özata et al., 2020]. In mammals, their expression is restricted to the germline, with a range of lengths seen depending on the PIWI protein they are found associated with.

Unlike miRNAs, piRNAs do not play a role in PGC specification but instead appear later during sex-specific differentiation. In the mouse, 3 PIWI proteins are expressed in the male only (Piwil1/Miwi, Piwil2/Mili, and Piwil4/Miwi2), with their individual knockouts leading to spermatogenic defects and complete male infertility [Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007]. Piwil2/Mili expression begins in both sexes at E12.5, followed by male-exclusive Piwil4/Miwi2 at a window of E13.5 to postnatal day (P)3, then male-exclusive Piwil1/Miwi from P14 at the beginning of the pachytene-stage of meiosis [Aravin et al., 2008]. The coincidence of Piwil2/Mili and Piwil4/Miwi2 in male mFGCs yields 26–29 nt long piRNAs (pre-pachytene piRNAs). Longer 30 nt pachytene piRNAs (not discussed here) are produced in the male adult upon the co-expression of Piwil2/Mili and Piwil1/Miwi from P14. In particular, Piwil2/Mili and other PIWI pathway members have been shown to be strongly activated upon DNA demethylation [Hackett et al., 2012b], suggesting a direct role of DNA methylation regulation on their expression.

The knockout of either Piwil2/Mili and Piwil4/Miwi2 leads to the arrest of spermatogenesis at early prophase of meiosis I (pre-pachytene). Molecularly, these knockouts correspond to the upregulation of TEs, in particular, evolutionary young L1s (as the case for Piwil4/Miwi2 and
In the developing human germline, piRNAs have been detected mainly by oxidising whole fetal gonadal samples to enrich for 2′-O-methyl-modified 3′-end small RNAs. In summary, piRNAs could not be detected when first trimester samples were studied. Among second trimester samples, Gainetdinov et al. [2017] were able to investigate the very low levels of oxidized male week 20 piRNA dataset from Williams et al. [2015] to conclude that these pre-pachytene piRNA target the evolutionary youngest TEs, as do later pachytene piRNAs, but that later pachytene piRNAs also map to older TEs. Meanwhile, Reznik et al. [2019] claimed to identify piRNAs with a ping-pong signature, although they could only study the 0.53% of Repbase-mapped small RNA reads in their non-oxidized heterogenous testis small RNA samples which are otherwise populated with somatic small RNA types. For female samples, 28/27 nt-peak piRNAs were detected in Williams et al. [2015] and Roovers et al. [2015] oxidized late second trimester samples, with a large divergence in TE-mapping rates noted (2% versus 30% respectively) and with the detection of ping-pong signatures only in Williams et al. [2015]. In all papers, no attempt was made to isolate pure germ cell populations. A more concerted approach to rigorously profile the emergence of piRNAs and their sequences in the context of the human germline, and with attention to the specific germ-subpopulations, is therefore warranted.

Additionally, microscopy-based descriptions of PIWI paralogue distribution and expression dynamics in the developing human germline have been noted. Cytoplasmic PIWIL1 was detected only in female mitotic-hPGCs, with single granules seen later in hFGCs in females, but not males [Fernandes et al., 2018]. Cytoplasmic PIWIL2 was detected in both male and female hPGCs and hFGCs [Fernandes et al., 2018], while PIWIL4 localised to the perinuclear/inter-mitochondrial cement in both sexes [Fernandes et al., 2018; Reznik et al., 2019], with instances of nuclear PIWIL4 localisation [Reznik et al., 2019; Guo et al., 2021] in male hFGCs also reported. PIWIL3 was not detected in the developing fetus [Fernandes et al., 2018], which has only been observed in adult oocytes instead [Roovers et al., 2015; Fernandes et al., 2018; Yang et al., 2019].

**endo-siRNAs**

Unlike piRNAs, endo-siRNAs are more ubiquitously expressed [Kim et al., 2009]. They derive from double-stranded RNA duplexes which can occur as a consequence of convergent sense- and antisense-transcription across the genome, which can typically occur across TE sequences when they run antisense to gene bodies or on self-complementary single-stranded RNA sequences.
Duplexes are recognised as a substrate for DICER1 cleavage, thus merging with the miRNA biogenesis pathway. These endo-siRNA are then loaded into AGO1-4, silencing targets based on complementary seed-based pairing [Xia et al., 2013; Piatek and Werner, 2014; Svoboda, 2014; Taborska et al., 2019].

While a clear role for the endo-siRNA pathway is seen in adult murine oocytes [Murchison et al., 2007; Nagaraja et al., 2008], with the involvement of an oocyte-specific Dicer isoform [Flemr et al., 2013], much lower levels of endo-siRNAs were noticed to be upregulated in early mPGC development coinciding with the onset of DNA demethylation [Berrens et al., 2017]. The same observation was noted in parallel, with a Dnmt1 conditional knockout in mESCs artificially causing passive demethylation, whereupon endo-siRNAs were upregulated to silence TEs before the establishment of repressive histone marks. However, any functional roles for these endo-siRNAs in mPGC development were not assessed. A hint of the role of endo-siRNA in PGC development can however be delineated with the observation that a more severe impact on spermatogenesis resulted as a consequence of conditional Dicer1 knockout, compared to Dgcr8 knockout (Vasa-Cre), with Dicer1 implicated in both miRNA and endo-siRNA biogenesis, whereas Dgcr8 is only implicated in miRNA biogenesis [Zimmermann et al., 2014].

**lncRNAs**

Long non-coding RNAs (lncRNAs) are distinguished from small non-coding RNAs by having sizes greater than 200 nt, while similarly being non-protein coding [Hombach and Kretz, 2016]. A small proportion have been reported to play roles in development by different mechanisms of action, such as in transcriptional, post-transcriptional, and epigenetic regulation of chromatin in either cis or trans [Liu and Lim, 2018].

**Xist/Xact**

In female mPGCs, the inactive X chromosome is reactivated alongside DNA demethylation with a decrease in Xist clouds between E9.5 and E11.5, followed by a slight window of X dosage excess until E14.5 [Sugimoto and Abe, 2007; Chuva de Sousa Lopes et al., 2008; Sangrithi et al., 2017], an event recapitulated in the demethylated ICM [Mak et al., 2004], thus ensuring that each oocyte contains an active X chromosome for early embryonic development after fertilisation. This dosage excess in mPGCs normalises back down to an X:autosome (X:A) ratio of 1 in developing oogonia from E15.5 [Li et al., 2017b; Sangrithi et al., 2017].

In the human, earlier work suggested that the X chromosome was already activated in hPGCs due to the noted lack of H3K27me3 foci over the X chromosome in week 7 hPGCs compared to neighbouring somatic cells [Tang et al., 2015]. A recent study by Chitishvili et al. [2020] proposed a more subtle and unique human germline behaviour in reactivation. The X was claimed to be reactivated but slightly dampened, such that X:A ratios were <1. The bi-allelic presence of the primate-exclusive X chromosome active lncRNA XACT was also noted, doubling as a unique marker of hPGCs. X reactivation was also noted in later hFGC subpopulations, followed by an XIST-independent inactive state in oogeneric hFGCs. However, a different study relying on earlier bulk RNA datasets [Sangrithi et al., 2017] observed the opposite behaviour with X chromosome dosage excess observed during initial reactivation, followed by a return to an X:A ratio of 1 in hPGCs. Further, more detailed studies will therefore be needed to resolve these contrasting observations.

**RNA Modifications**

**m6A**

Post-transcriptional modifications of transcribed RNA, or the epitranscriptome, can also contribute to the regulatory landscape of developing cells. The N6-methyl-
Polyuridylation

Terminal uridylyl transferases (TUTases) are known to polymerise untemplated U nucleotides at the 3′-end of RNA, or occasionally a single U addition, known to impact the regulation of the transcript, as well as the let-7 family of miRNAs as mentioned earlier [Thornton et al., 2012; Lim et al., 2014]. In relation to TEs, knockouts of Tut4/7 were recently shown to cause the surge of L1 retrotransposition events in mammalian cells [Strzyz, 2018; Warkocki et al., 2018]. Indeed L1s were found to be 3′-uridylated in human cells and mouse testes [Warkocki et al., 2018]. Their roles in mammalian germline development (in particular PGCs) are hitherto unknown, although a role was identified for the TUTases in the clearance of transcripts in later development in both mouse spermatogenesis and oogenesis [Morgan et al., 2017, 2019].

In both these cases, the roles of post-transcriptional covalent modification or tailing of RNA in shaping the regulatory landscapes in developing hPGCs remain uncharacterised and present an opportunity for further work.

Prospects

Model Organisms for Mechanistic Studies

For mechanistic studies, the mouse will remain an essential tool due to the ease of embryo and gene manipulation. Indeed, the lack of available tools and/or limited access to embryos makes it difficult to conceive that new molecular mechanisms will be uncovered in other mammalian model organisms or in human fetal samples. While the PGCLC system has revolutionised the field, any mechanistic insights will inevitably require in vivo confirmation. Recent work on PGCs in pigs [Kobayashi et al., 2017; Zhu et al., 2021] and rats [Leitch et al., 2010; Northrup et al., 2011; Encinas et al., 2012; Kobayashi et al., 2020], as well as the observation of female PGC piRNAs in a non-murine rodent, the golden hamster [Hauswa et al., 2021; Ishino et al., 2021; Loubalova et al., 2021; Zhang et al., 2021], do show promise and will hopefully make an increasing contribution to complement mouse studies in the years to come. While recent studies in non-human primates have been illuminating, their use does carry an increased ethical burden, particularly for studies into fundamental mechanisms that could be first undertaken in other species. Without doubt, mechanistic studies in model organisms will continue to be crucial and allow confirmatory studies in human using available fetal tissue and in vitro systems.

Developmental Timing and the Problem of Asynchrony

It has become clear, especially from single cell studies, that while mPGCs differentiate into either mitotically (male) or meiotically (female) arrested gonocytes from E13.5, around a week after they were first specified, hPGCs persist much longer, with asynchronous differentiation into later hFGCs observed [Li et al., 2017a; Chitiashvili et al., 2020; Hwang et al., 2020; Guo et al., 2021]. In particular, founder hPGC subpopulations persist up till the latest examined timepoint of week 26, while male mitotic-arrest hFGCs start to emerge from week 10, and 3 sequential subpopulations of female hFGCs (RA-responsive, meiotic, and oogenic) emerge from week 11, 14, and 18, respectively, whilst also all persisting up until week 26 [Li et al., 2017a] (Fig. 3). This vastly prolonged differentiation process and coexisting germ cell subpopulations in different stages of maturity differs from that of the mouse and will have implications on both their differing biology as well as the bulk study of human germ cells beyond week 10, with single-cell studies or unique cell-surface markers required for the isolation of pure subpopulations [Li et al., 2020; Mishra et al., 2021].

ncRNAs in the Germline – A Long Way to Go

Genetic perturbation of mPGCs as well as small RNA profiling of isolated, fluorescently-marked mPGCs have allowed the characterisation of some of the roles of miRNAs, piRNAs, and endo-siRNAs in germline development. However, far less is known about the role of IncRNAs. Indeed, we hope this review serves to highlights that our overall understanding of the role of the non-coding genome during early germline development remains limited even in the mouse. In particular, the interactions between ncRNAs and underlying chromatin requires fur-
Epigenetic Regulation in PGC Development

Regulating TEs in the DNA Hypomethylated State

In male mPGCs the window of the demethylated state is short (E11.5–E15.5), with piRNAs emergent from E13.5, whereas the demethylated state persists postnatally in female germ cells. In male and female hPGCs, this demethylated state extends for a much longer period (week 5 to week 26, the latest characterised time point). A locus-specific characterisation of repressive histone marks during PGC development using low-input techniques such as ULI-ChIP or CUT&RUN/CUT&Tag may help shed light on any additional layers of transcriptional repression of TEs. Specific care could also be made to explore TE transcript levels at the individual locus level, rather than the bulk family analytic methods predominantly used [Lanciano and Cristo-fari, 2020; Stow et al., 2021]. Which additional epigenetic mechanisms help to suppress the activity of TEs during this demethylated time window remain unknown. Given the recent report of hypertranscription in mPGCs, this seems particularly pertinent [Percharde et al., 2017]. It will be interesting to assess if hypertranscription is observed in PGCs of other species, including humans. Intriguingly, in the human testis, high transcript abundance has been observed to evade reprogramming or are in fact targeted for active maintenance remains an open question. Such loci might have implications for transgenerational epigenetic inheritance (TEI). However, although TEI is a well described mechanism in other model organisms [Castel and Martienssen, 2013; Heard and Martienssen, 2014; Miska and Ferguson-Smith, 2016; Perez and Lehner, 2019; Miska and Rechavi, 2021] it remains a largely unproven notion in mammals [Heard and Martienssen, 2014; Otterdijk and Michels, 2016; Miska and Ferguson-Smith, 2016; Blake and Watson, 2016]. Such loci would have to further evade zygotic epigenetic reprogramming and the passive DNA demethylation that occurs during pre-implantation development to have an effect on the next generation and/or continue their passage through the germline. Indeed, recent studies have demonstrated that newly introduced epialleles are completely erased during passage through the naive cell state [Carlini et al., 2021] and that metastable IAP epialleles are highly resistant to environmental perturbations in the first place [Bertozzi et al., 2021]. The studies that have rigorously addressed this question have not shown DNA methylation marks that can be maintained following even a single passage through the entire germline cycle [Radford et al., 2014; Kazach-enka et al., 2018].
Despite significant advances in our understanding of epigenetic reprogramming and the role of the non-coding genome in mammalian germline development, this review has highlighted that many basic processes and mechanisms remain poorly understood. New technologies are emerging as a key enabling factor, overcoming the challenges of small cell numbers, limited access to samples, and cellular heterogeneity that have long challenged germline studies. Improved single-cell transcriptomic protocols and analysis pipelines, single-cellomics, low-input methods for assessing histone modifications and small non-coding RNAs, and epitranscriptome co-sequencing of long reads may help to provide mechanistic insight in the future. However, these technologies must be applied with rigour, to the right experimental system, and interpreted with a caution that reflects the complexity of epigenetic regulation in the germline. Indeed, that so many questions remain unanswered likely reflects the intricate interactions of overlapping regulatory systems, or alternatively, that we are still missing key parts of the puzzle. As such, while there is a recent ferment in favour of studying human biology directly in human cells, organoids, or embryo-models [Posfai et al., 2021], it seems just as likely that major advances in our understanding of human biology will continue to flow from research on model organisms. This may also be aided by CRISPR/Cas9 technology, which makes multiple genetic manipulations in mammals feasible, allowing more complex genetic experiments in the future. Regardless of model organism or approach, there remain a great many mysteries to uncover in germline epigenetics and the non-coding genome, which will keep the field busy in the decades to come.

Acknowledgements

We thank Petra Hajkova and Michelle Percharde for their comments on the manuscript.

Funding Sources

H.G.L. is supported by MRC Core Funding (MC- A652-5QA10) and BBSRC Responsive Mode Grant (BB/R002703/1). H.G.L. also acknowledges support from the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC). E.A.M. is supported by Cancer Research UK (C13474/A18583, C6946/A14492) and the Wellcome Trust (219475/Z/19/Z, 092096/Z/10/Z).

Conflict of Interest Statement

The authors have no conflicts of interests to declare.

Author Contributions

All authors contributed to writing this review.

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