Epigenetic events in mammalian germ-cell development: reprogramming and beyond

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Abstract | The epigenetic profile of germ cells, which is defined by modifications of DNA and chromatin, changes dynamically during their development. Many of the changes are associated with the acquisition of the capacity to support post-fertilization development. Our knowledge of this aspect has greatly increased— for example, insights into how the re-establishment of parental imprints is regulated. In addition, an emerging theme from recent studies is that epigenetic modifiers have key roles in germ-cell development itself — for example, epigenetics contributes to the gene-expression programme that is required for germ-cell development, regulation of meiosis and genomic integrity. Understanding epigenetic regulation in germ cells has implications for reproductive engineering technologies and human health.

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Epigenetics refers to a collection of mechanisms and phenomena that define the phenotype of a cell without affecting the genotype[1]. In molecular terms, it represents a range of chromatin modifications including DNA methylation, histone modifications, remodelling of nucleosomes and higher order chromatin reorganization. These epigenetic modifications constitute a unique profile in each cell and define cellular identity by regulating gene expression. Epigenetic profiles are modifiable during cellular differentiation, but heritability is an important aspect of epigenetics: it ensures that daughter cells have the same phenotype as the parental cell.

The process of germ-cell development is regulated by both genetic and epigenetic mechanisms[2–5]. Among the various cell types that constitute an animal body, germ cells are unique in that they can give rise to a new organism. On fertilization, the products of germ-cell development, the oocyte and sperm cell, fuse to form a zygote, which is totipotent — it can develop a whole new organism[2]. For the zygote to acquire this totipotency, germ cells and the zygote undergo extensive epigenetic reprogramming[2,3]. In mammalian germ cells, reprogramming also strips existing parental imprints — epigenetic marks that ensure parental-origin-specific monoallelic expression of about a hundred mammalian imprinted genes in the next generation — and establishes new ones that are different in male and female gametes.

The role of epigenetics in germ cells can be viewed differently from that in somatic cells. During somatic cell differentiation, cells start in a pluripotent state and make a series of decisions about their fates, thereby giving rise to a range of cell types[6]. Their gene-expression programmes become more restricted and potentially locked in by changes in epigenetic modifications. However, germ cells are different in that, once their fate has been determined during early development, there is no need for developmental decisions to be made. Instead, germ cells have a specific fate and go through a series of epigenetic events that are unique to this cell type. The aspects of germ-cell development that are relevant to these epigenetic events are the need for a unique gene-expression programme that is different from somatic cells, the fact that germ cells undergo meiosis and the particular importance of maintaining genomic integrity in these cells.

In this Review, we discuss dynamic epigenetic changes that occur during mammalian germ-cell development. Recent studies have identified a number of epigenetic modifiers, including DNA methyltransferases, histone-modification enzymes and their regulatory factors, that have crucial influences on germ-cell development. There is also an increasing understanding of the mechanisms of the epigenetic reprogramming that takes place during germ-cell development — for example, how imprints are re-established in the male and female germ cells. Our discussion follows the temporal progression of events during germ-cell development,

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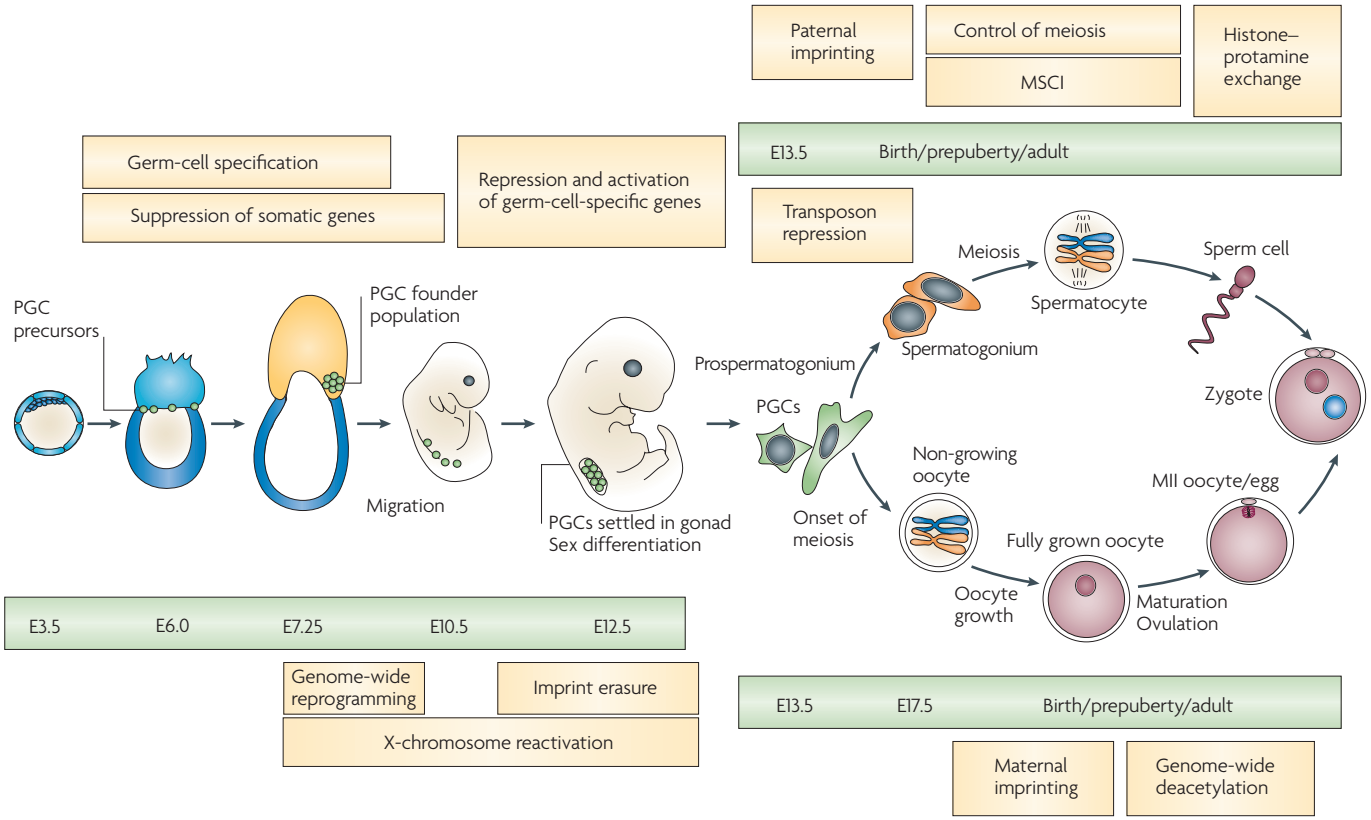

Figure 1 | **Germ cell development and associated epigenetic events in mice.** Chronology of mouse germ cell development and the main epigenetic events that occur. PGCs (primordial germ cells) first emerge at embryonic day 7.25 (E7.25) as a cluster of about 20 cells. Subsequently, they rapidly proliferate with an average doubling time of approximately 16 hours. Before they stop dividing at E13.5, their number reaches up to about 26,000. MSCI, meiotic sex-chromosome inactivation.

DNA methylation
A covalent modification that occurs predominantly at CpG dinucelotides in the vertebrate genome. It is catalysed by DNA methyltransferases and converts cytosines to 5-methylcytosines. It represses transcription directly by inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl-CpG-binding proteins and their associated repressive chromatin-remodelling activities.

Histone modifications
Histones undergo post-translational modifications that alter their interactions with DNA and nuclear proteins. In particular, the tails of histones H3 and H4 can be covalently modified at several residues. Modifications of the tail include methylation, acetylation, phosphorylation and ubiquitylation, and influence several biological processes, including gene expression, DNA repair and chromosome condensation.

and we describe the epigenetic changes and their contributions to germ-cell-specific functions at each stage. Understanding the epigenetic changes that take place during germ-cell development has important implications for animal cloning, assisted reproductive technologies and human health.

Germ-cell specification and differentiation

Determination and maintenance of the germ-cell fate. In post-implantation mammalian embryos, a population of pluripotent cells in the epiblast gives rise to primordial germ cells (PGCs), the fate of which is specified by tissue interaction during gastrulation. In mice, PGCs first emerge inside the extra-embryonic mesoderm at the posterior end of the primitive streak as a cluster of cells at embryonic day 7.25 (E7.25) (REFS 7–9) (FIG. 1). Before the final specification of PGCs, their precursors are induced within the proximal epiblast cell population by signals from the adjacent extra-embryonic ectoderm[10–15]. A transcriptional regulator, B-lymphocyte maturation-induced protein 1 (BLIMP1, also known as PR-domain-containing 1), is expressed specifically in the precursor cells as early as E6.25 (REF. 16), and this molecule is essential for PGC specification.

The PGC precursors need to suppress the somatic gene-expression programme, and epigenetic modifications might be important for this suppression. PGC-like cells in *Blimp1*-null embryos have aberrant expression of the Hox genes[16], which are normally repressed in PGCs. This suggests that BLIMP1 is crucial for suppression of the somatic programme, which might ensure that the PGC precursors and nascent PGCs are restricted to the germ-cell fate. In organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, this repression involves global inhibition of RNA polymerase II (RNAPII)-dependent transcription[17–19]. In *D. melanogaster*, the pole cells that develop into PGCs also have reduced levels of histone H3 lysine 4 methylation (H3K4me), a mark that is associated with the permissive (active) state, and are enriched in H3K9me, a mark that is associated with repression, suggesting a role for epigenetic modifications in suppressing the somatic programme[20]. Here, maternally inherited molecules such as the products of *gcl* (germ-cell-less)[21,22], *pgc* (polar granule component)[23,24] and *nanos*[20,25] are involved in the transcriptional quiescence. Therefore, suppression of somatic differentiation through transcriptional regulation might be an evolutionarily conserved theme for germ-cell specification. However, as RNAPII is clearly active in nascent PGCs in mice, the molecules and mechanisms that regulate the process might differ between species.

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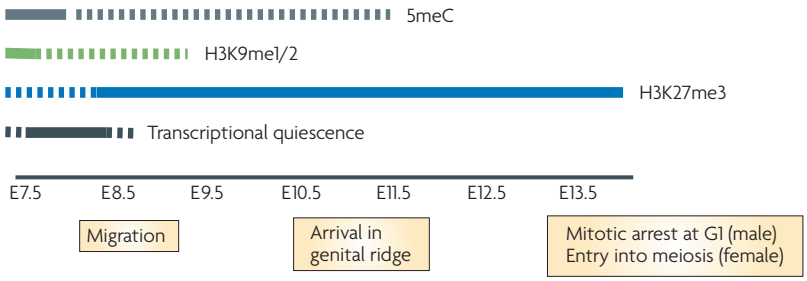

Figure 2 | **Epigenetic reprogramming in primordial germ cells (PGCs).** Changes in epigenetic modifications that occur during the genome-wide reprogramming that takes place during mouse PGC development. Dashed lines indicate that the level of the epigenetic modification is lower during these periods than that during the periods shown by solid lines. 5meC, 5-methylcytosine (the product of DNA methylation).

How BLIMP1 regulates germ-cell specification and suppresses the somatic genes is currently obscure. Although BLIMP1 has a histone-methyltransferase motif, such an activity has not been detected for this protein. As discussed below, BLIMP1 binds to a histone-arginine methyltransferase, PRMT5 (protein arginine *N*-methyltransferase 5), to repress premature expression of some germ-cell-specific genes in more advanced PGCs[26], and it is possible that epigenetic modification might also contribute to the somatic repression role of BLIMP1.

Genome-wide epigenetic changes during early PGC differentiation. An important recent insight into germ-cell development comes from findings that unique epigenetic and transcriptional changes are seen in further differentiating, migrating PGCs[27,28]. When the germ-cell fate is established at E7.25, levels of genome-wide DNA methylation, H3K9 dimethylation (H3K9me2) and H3K27 trimethylation (H3K27me3) — all marks that are associated with transcriptional repression — are similar to those in surrounding somatic cells. Subsequently, H3K9me2 starts to be erased at E7.5 and DNA methylation decreases after about E8.0, with the level of the former being clearly lower than in the neighbouring somatic cells by E8.75 (REFS 27,28) (FIG. 2). Following this initial decrease in these two repressive marks, the level of H3K27me3, another repressive mark, starts to be upregulated after E8.25 and most PGCs show significantly higher levels of this mark at E9.5 (REF. 28) (FIG. 2). It is likely that this upregulation of H3K27me3 complements the erasure of H3K9me2 to maintain a proper repressive chromatin state of the PGC genome. The observation indicates that the PGC genome is relatively free of repressive chromatin between E7.5 and E8.25, which could result in deregulated transcription. However, global RNAPII-dependent transcription is transiently repressed during this period, as demonstrated by the absence of both 5-bromouridine 5′ triphosphate (BrUTP) incorporation and RNAPII C-terminal-domain phosphorylation[28]. As RNAPII is active in nascent PGCs, as mentioned above, this transcriptional quiescence seems indifferent to the suppression of the somatic programme.

The global changes in repressive marks in migrating PGCs might reflect the reprogramming of the PGC genome, which is eventually necessary for the zygote to acquire totipotency. Recent studies of chromatin modifications in embryonic stem cells (ES cells) showed that their pluripotency might be ensured by bivalent chromatin — that is, coincidence of H3K27me and H3K4me — at genes that encode key developmental transcription factors[29,30]. Such modifications might temporarily keep the developmental genes poised for activation in undifferentiated ES cells. The increased level of H3K27me3 and loss of other repressive marks in PGCs seem to make the PCG genome partly resemble such a chromatin state. Understanding the epigenetic profiles of ES cells and germ cells should facilitate research on the exciting possibility of deriving functional gametes from pluripotent cells in culture (BOX 1).

Regulation of post-migratory PGC-specific genes by epigenetic mechanisms. Recent studies have shown that changes in epigenetic modifications also have important roles in the regulation of post-migratory PGC-specific genes. Genes such as *Ddx4* (DEAD box polypeptide 4, also known as *Mvh*), *Sycp3* (synaptonemal complex protein 3) and *Dazl* (deleted in azoospermia-like) are induced after migrating PGCs have entered the genital ridge between E10.5 and E11.5 (FIG. 1). DNA-methylation analysis revealed that, despite the genome-wide decrease in DNA methylation after E8.0, the flanking regions of these genes remain methylated at E10.5, but become hypomethylated by E13.5 when they are expressed[31]. Furthermore, these genes are derepressed in E9.5 embryos that lack the maintenance DNA methyltransferase, DNMT1 (REF. 31). The results suggest that DNA methylation regulates the timing of activation of these genes. This demethylation might be part of the second wave of demethylation that occurs around E11.5 (REF. 32) (see below).

A recent study has shown that histone methylation that is mediated by BLIMP1 and its associated histone-arginine methyltransferase PRMT5 also regulates PGC-specific genes in post-migratory PGCs[26]. In migrating PGCs, a nuclear-localized BLIMP1–PRMT5 complex mediates dimethylation of histone H2AR3 and H4R3. After PGCs have settled in the genital ridge, the BLIMP1–PRMT5 complex translocates to the cytoplasm and the levels of H2AR3me2 and H4R3me2 are diminished. Subsequently, *Dhx38* (DEAH box polypeptide 38), a putative target of BLIMP1–PRMT5, is upregulated, suggesting that arginine methylation prevents premature expression of this gene[26]. *Dhx38* encodes a protein that contains a DEAD box, which is an RNA-helicase motif, and its homologue in *C. elegans* is involved in germ-cell development[33]. It is interesting that H4R3me is associated with activation of other genes[34,35] and H3R8me, which is also mediated by PRMT5, can repress transcription[36]. Together, these findings suggest that H2AR3me2 and H4R3me2 that are mediated by BLIMP1–PRMT5 might have a novel repressive role in PGCs.

Nucleosome
The basic structural subunit of chromatin, responsible in part for the compactness of a chromosome. Each nucleosome consists of a sequence of DNA wrapped around a histone core, which is a histone octamer containing two copies of each of the core histones: H2A, H2B, H3 and H4.

Pluripotent
Able to give rise to a wide range of, but not all, cell lineages (usually all fetal lineages and a subset of extraembryonic lineages).

Epiblast
An embryonic lineage that is derived from the inner cell mass of the blastocyst, which gives rise to the body of the fetus.

Gastrulation
A process of cell and tissue movements whereby the cells of the blastula are rearranged to form a three-layered body plan, which consists of the outer ectoderm, inner ectoderm and interstitial mesoderm.

Primitive streak
A transitory embryonic structure, which is present as a strip of cells, that pre-figures the anterior–posterior axis of the embryo. During gastrulation embryonic cells progress through the streak.

Embryonic stem cell
A type of pluripotent stem cell that is derived from the inner cell mass of the early embryo. Pluripotent cells are capable of generating virtually all cell types of the organism.

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Blastocyst
An early stage of mammalian embryonic development at which the first cell lineages become established.

Embryoid body
Spherical structure formed by differentiating ES cells in culture, which resembles the early embryo.

Erasure and establishment of parental imprints

Imprint erasure in PGCs. When they arrive at the genital ridge, which occurs by E11.5, PGCs undergo extensive epigenetic reprogramming, including the erasure of parental imprints (FIG. 1). The erasure of imprints is reflected by demethylation of the imprinted loci, which occurs concomitantly with demethylation of other regions[32]. The erasure occurs at different imprinted loci at different times between E10.5 and E12.5, as shown in a study of cloned embryos that were produced from PGC nuclei[37]. Since the imprint erasure at each locus is a rapid process that is completed within one day of development, this might be an active demethylation process[32].

In somatic cells of female mammals, one of the two X chromosomes is inactivated so that the dosage of the genes on this chromosome is equalized between males and females. The inactive X chromosome is reactivated during female germ-cell development. It had been thought that this reactivation occurs around the time of imprint erasure[38,39]. However, more recent studies showed that it is initiated in the migratory stage[40] or at an even earlier stage[41]. So, X-chromosome reactivation occurs progressively over a prolonged period and is completed in post-migratory PGCs. The initiation of reactivation in migrating PGCs is reminiscent of the X reactivation in inner-cell-mass cells of female blastocysts, but the mechanisms of these processes are yet to be clarified.

Not all sequences show DNA demethylation in post-migratory PGCs. For example, DNA methylation of the intra-cisternal A particle (IAP) retrotransposon family is only partially reprogrammed[42]. Incomplete removal of epigenetic marks in the germ line can lead to epigenetic inheritance from one generation to the next, evidence of which is now accumulating in both mice and humans[43–45]. Together with the fact that epigenetic marks can be influenced by environmental factors, it has been suggested that this phenomenon could provide a basis for adaptive evolution and/or heritable disease susceptibility without changes in DNA sequence[43–45] (BOX 2).

Imprinting in the male and female germline. Once the parental imprints have been erased, new imprints must be re-established according to the gender of the animal. This re-establishment occurs only after sex determination has been initiated, and male and female germ-cell development diverges to give rise to sperm or oocytes, respectively. In mice, the gonads of males and females become morphologically distinguishable by E12.5. In female embryos, germ cells arrest in meiotic prophase at around E13.0, whereas male germ cells enter into G1-phase mitotic arrest at a similar time (FIGS 1,2). A number of environmental cues, including retinoic acid signals from the mesonephroi[46], determine the timing of entry into meiosis by germ cells.

G1-arrested male germ cells are called prospermatogonia or gonocytes (FIG. 1). Paternal methylation imprints, which have been identified at just three loci, are progressively established in these cells between E14.5 and the newborn stage[47–51]. A germline-specific gene-knockout study indicated that the *de novo* DNA methyltransferase, DNMT3A, has a central role in the *de novo* methylation process of all known paternally methylated loci, and another *de novo* methyltransferase, DNMT3B, is involved only at the *Rasgrf1* (RAS protein-specific guanine nucleotide-releasing factor 1) locus[50,52]. The reason why *Rasgrf1* requires an additional enzyme is unknown, but this could be related to the presence of several retrotransposon sequences at this locus (see below). The establishment of paternal methylation imprints at all loci requires another member of the Dnmt3 family, DNMT3L, which is highly expressed in prospermatogonia[50,52–54]. This protein has no DNA-methyltransferase activity but forms a complex with DNMT3A and/or DNMT3B and stimulates their activities.

The established methylation imprints are then maintained throughout the rest of male germ-cell development. Notably, germline stem (GS) cells — which are established from neonatal testes, can be maintained stably in culture and can give rise to sperm when transplanted into testes — possess paternal methylation imprints, whereas their multipotent derivatives, mGS cells, show partial demethylation at these sites, similar to ES cells[55]. GS cells and mGS cells provide invaluable tools for germ-cell study and reproductive engineering.

In the female germline, the initiation of DNA-methylation imprinting occurs after birth, during the oocyte growth[56,57]. The growing oocytes are at the diplotene stage of meiotic prophase I, and the *de novo* methylation process is complete by the fully-grown oocyte stage (FIG. 1). Both DNMT3A and DNMT3L also have essential roles in this process[52,58,59], but DNMT3B seems dispensable[52].

Recent studies have started to provide some clues on the mechanism by which the DNMT3A–DNMT3L complex recognizes the imprinted loci (and some retrotransposons, see below). A crystallographic analysis of the complexed C-terminal domains of DNMT3A and DNMT3L revealed a tetrameric structure with two active sites[60]. This structure suggests that DNA regions

Box 1 | Derivation of germ cells from embryonic stem cells

Various types of somatic cell, including blood cells and neural cells, have been obtained from embryonic stem (ES) cells in culture dishes. Recent studies have revealed that it is also possible to generate gametes from ES cells[108–111]. Gametes or gamete-like cells were derived when mouse ES cells were cultured under various differentiation conditions including simple monolayer culture (oocyte)[108], embryoid-body formation (sperm)[109], embryoid-body formation followed by treatment with retinoic acid (sperm)[110] and retinoic acid induction alone (sperm)[111]. In the most successful case, ES-derived sperm cells were able to fertilize oocytes after intracytoplasmic injection and support embryonic development to term[111]. The resultant pups, however, had abnormalities in DNA methylation at imprinted loci and survived only up to five months, indicating that reprogramming of the germ-cell genome was not properly accomplished. When we fully understand the mechanisms of germ-cell reprogramming, we might be able to derive appropriately reprogrammed, functional gametes from cultured cells, which will allow new approaches to reproductive engineering, although ethical and safety issues must be carefully considered.

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Box 2 | **Transgenerational influence of epigenetic alterations in germ cells**

Recent studies have suggested that exposure to chemicals and malnutrition conditions can affect not only the children of the affected individuals, but also their grandchildren. This might be attributable to epigenetic alterations that occur in fetal germ cells. When gestating female rats were exposed to some endocrine disruptors, the number of spermatogenic cells decreased in the F1 generation. This effect was transmittable through the male germ line to subsequent generations, and this was correlated with altered DNA-methylation patterns[112]. In another example, exposure to methyl-donor supplementation during midgestation affected the epigenetic status of fetal germ cells[113]. The mouse A^{vy} gene, which influences coat colour, is regulated by the DNA methylation status of an intracisternal A particle (IAP) retrotransposon inserted at pseudoexon 1A of the gene. The methyl-donor supplementation shifted the coat colour of the F2 generation to a darker one. This suggests that the methyl donor directly or indirectly affected the epigenetic status of A^{vy} in fetal germ cells. Finally, epidemiological studies have indicated that grandchildren of malnourished women show low birth weight[114], that grandchildren of men who were well-fed before adolescence have a greater risk of mortality from diabetes, and that descendants of men who suffered famine have low cardiovascular mortality[115]. In these cases, it is possible that the nutrition status caused epigenetic alterations in germ cells, but further studies are needed to confirm this possibility.

with a 10-nucleotide CpG interval are a preferred substrate, and these are found in many imprinted loci[60]. However, there are many other regions in the genome with the same CpG spacing. Another study showed that DNMT3L interacts with unmodified H3K4 (REF. 61), which might restrict targets to regions without H3K4me. Together, both nucleosome modification and CpG spacing might provide the basis for the recognition of the imprinted loci by DNMT3A–DNMT3L (REF. 60) (FIG. 3). The differential methylation of the imprinted loci in the male and female germlines might require additional factors. In the case of paternally imprinted *H19*, protection of this locus from *de novo* DNA methylation in oocytes requires CCCTC-binding factor (CTCF), which is known to bind to an unmethylated *H19* (H19 fetal liver mRNA) regulatory region[62].

X-chromosome inactivation in female mice is imprinted in pre-implantation embryos and the extra-embryonic tissues of post-implantation embryos, and in both cases the paternal X chromosome is preferentially inactivated. This imprinted X inactivation depends on both an activating imprint on the maternal X chromosome and an inactivating imprint on the paternal X chromosome. As mentioned above, the inactive X chromosome is reactivated in female PGCs, but maintenance of the active state of the maternal X chromosome beyond fertilization requires an imprint. Nuclear transplantation experiments showed that this maternal imprint is set on the X chromosomes during the growth of the oocyte[63], as with the imprints at autosomal loci. As the maternal X chromosome from *Dnmt3a/Dnmt3b* double-mutant oocytes seems to have normal imprints[64], the mechanism of this imprinting might be different from that of autosomal imprinting.

Parthenogenesis, which is a successful development of unfertilized eggs, is observed in many vertebrate species and, if it were possible in mammals, would provide a way to produce clones of livestock animals. However, imprinting is a major barrier to parthenogenetic development in mammals because expression levels of the imprinted genes, which include many important developmental genes, are unbalanced in such embryos. When the imprinted genes are appropriately modified by genetic engineering and developmental manipulation, however, it was possible to derive adult female mice with two maternal genomes and no paternal complement[65,66] (BOX 3). As the method involves genetically engineered animals and highly complex nuclear-transfer technologies, its direct application to livestock seems difficult.

Epigenetic silencing of retrotransposons

Only germ cells can transmit genetic information to the next generation. Therefore, transposons, which mobilize in the genome and might cause insertional mutations, have to be strictly controlled in these cells. Approximately 40–50% of the mammalian genome is occupied by retrotransposons, which mobilize through an RNA intermediate, although many of them are truncated or have accumulated mutations. Mammalian retrotransposons include short interspersed nuclear elements (SINEs), long interspersed nuclear elements (LINEs) and endogenous retroviruses (long terminal repeat (LTR)-type retrotransposons).

One way to control transposable elements is through epigenetic mechanisms[67]. In the male germline, all retrotransposon sequences undergo *de novo* DNA methylation during the fetal prospermatogonium stage[50], concomitant with the *de novo* methylation of the imprinted loci (FIG. 1). Gene-knockout studies in mice showed both common and differential target specificities of DNMT3A and DNMT3B with respect to these sequences: SINEB1 is mainly methylated by DNMT3A, whereas LINE1 and IAP are methylated by both DNMT3A and DNMT3B (REF. 50). By contrast, DNMT3L is required for methylation of all these sequences[50], indicating the crucial function and broad specificity of this factor in *de novo* DNA methylation (FIG. 3).

The functional importance of DNA methylation in retrotransposon silencing and germ-cell development was first seen in *Dnmt3L* knockout mice. The LINE and IAP retrotransposons, of which *de novo* methylation was prevented by *Dnmt3L* mutations, were highly transcribed in spermatogonia and spermatocytes[53,54,68]. The mutations also caused meiotic failure with widespread non-homologous chromosome synapsis and progressive loss of germ cells by the mid-pachytene stage (TABLE 1). This resulted in complete azoospermia in older animals. The non-homologous synapsis could arise from illegitimate interactions between dispersed retrotransposon sequences that were unmasked by demethylation or from single- or double-strand breaks that were produced during replicative retrotransposition[53].

Recently, a link between a small-RNA pathway and DNA methylation of retrotransposons was discovered[69] (FIG. 3). MILI, a member of the Piwi subfamily of Argonaute proteins, is expressed in the male and female gonads as early as E12.5 (REF. 70) and interacts with a class of small RNAs called piwi-interacting RNAs (piRNAs)[69,71]. In *Mili*-mutant testis, LINE1 and IAP retrotransposons were

X-chromosome inactivation
The process that occurs in female mammals by which gene expression from one of the pair of X chromosomes is downregulated to match the levels of gene expression from the single X chromosome that is present in males. The inactivation process involves a range of epigenetic mechanisms on the inactivated chromosome, including changes in DNA methylation and histone modifications.

Chromosome synapsis
The association or pairing of the two pairs of sister chromatids (representing homologous chromosomes) that occurs at the start of meiosis.

Argonaute proteins
Argonaute proteins are the central components of RNA-silencing mechanisms. They provide the platform for target-mRNA recognition by short guide RNA strands and the catalytic activity for mRNA cleavage.

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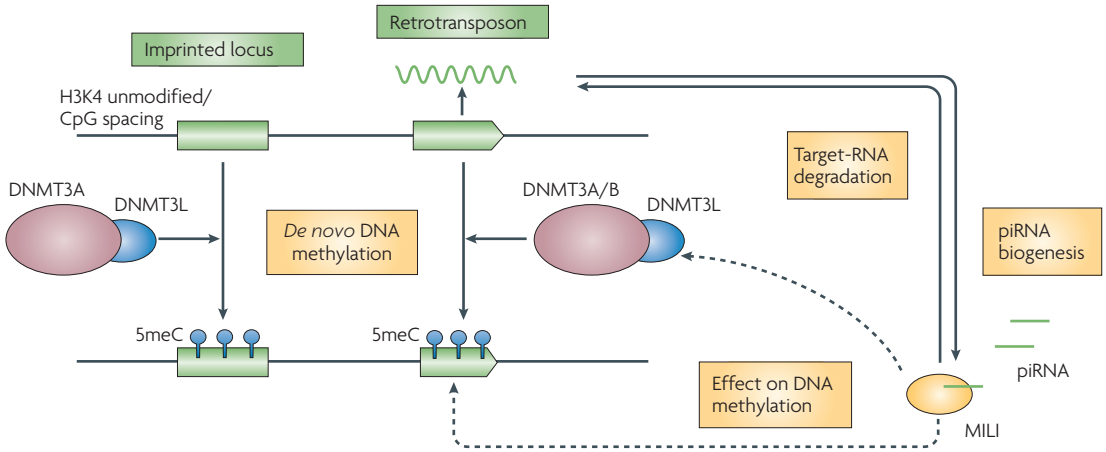

Figure 3 | **Cross-talk between DNA methylation, histone marks and the piRNA pathway in male germ cells.** In prospermatogonia of the fetal testis, *de novo* DNA methylation of paternally imprinted loci and retrotransposons occurs. The DNMT3A (DNA methyltransferase 3A)–DNMT3L complex recognizes its targets, such as imprinted loci, by sensing unmodified histone 3 lysine 4 (H3K4) and CpG spacing at 10-nucleotide intervals. Both DNMT3A–DNMT3L and DNMT3B–DNMT3L complexes methylate retrotransposons but how these sequences are recognized is unknown. MILI, a member of the Argonaute family of proteins, is involved in the piwi-interacting RNA (piRNA) pathway, but recent studies have shown that it also has a role in DNA methylation of retrotransposons. Whether MILI functions in *de novo* methylation or maintenance methylation is currently unknown. 5meC, 5-methylcytosine (the product of DNA methylation).

derepressed[69], which is consistent with the fact that a large proportion of piRNAs from pre-pachytene spermatocytes[69] and prospermatogonia (Kuramochi-Miyagawa, S., Watanabe, T., H.S. and Nakano, T., unpublished data) were retrotransposon-derived. As RIWI, a member of the rat Piwi family, co-purifies with an RNA-cleavage activity[72], it is likely that the suppression involves RNA-guided cleavage of target RNAs. Interestingly, however, the *Mili* mutants also showed decreased DNA methylation at LINE1 retrotransposons[69] and meiotic defects[73], similar to the phenotype of *Dnmt3L* mutants. At present, how the MILI–piRNA complex leads to DNA methylation is an open question. Recent studies in *Drosophila* suggest a link between H3K9me3 and H3K9me2, heterochromatin protein 1 (HP1) and Piwi proteins[74,75], and it is interesting to ask whether such a link exists in the mammalian germline.

Dnmt3L-mutant females can produce fully mature oocytes (although embryos derived from them die *in utero* because of the imprinting defects)[58,59]. However, a recent study showed that loss of LSH (lymphoid-specific helicase), a member of the SNF2-helicase family of chromatin remodelling proteins, leads to DNA demethylation and derepression of IAP retrotransposons in pachytene oocytes[76]. This suggests a role for DNA methylation in retrotransposon suppression in oocytes as well. Furthermore, the mutant oocytes showed demethylation of repeats at pericentric heterochromatin as well as incomplete chromosome synapsis, leading to a severe loss of oocytes at an early postnatal stage. In addition to DNA methylation, oocytes also use small-RNA pathways to silence retrotransposons (REF. 77 and Watanabe, T. and H.S., unpublished data). So, both male and female germ cells use multiple mechanisms for defence against retrotransposons.

Pericentric heterochromatin
This is the highly compacted chromatin region that is juxtaposed to centromeres and contains large blocks of tandem repeats. It is irreversibly silenced and remains so throughout the cell cycle.

Histone variants
Structurally distinct, non-typical versions of the histone proteins. They are encoded by independent genes and often subject to regulation that is distinct from that of the canonical histones.

Epigenetic regulation of meiosis

Germ cells undergo several types of change in their epigenetic profile during the various stages of meiosis. For example, in premeiotic PGCs and spermatogonia, unique patterns of histone modifications such as low H3K9me2 levels are observed[27,78], but these patterns are dynamically changed upon the initiation of meiosis, especially in male germ cells[79] (FIG. 4).

The role of histone variants. Male germ cells also express an unusually high number of histone variants, including TH2A, TH2B, TH3, H3.3A, H3.3B and HT1, which are incorporated in the nucleosomes of spermatogonia and/or spermatocytes[5]. In oocytes, somatic histone H1 is replaced by an oocyte-specific variant H1FOO during meiotic prophase I (REF. 5). The changes in histone composition and modification might contribute to a chromatin state that is required for meiosis to take place correctly, and for the further maturation of the gametes (see REF. 5 for a detailed review of the role of histone variants during meiosis).

Crucial functions of histone methyltransferases in meiotic prophase. The functional significance of histone modifications during the process of meiosis is particularly clear from gene-knockout studies (TABLE 1). Double mutations of the H3K9 trimethyltransferase genes *Suv39h1* and *Suv39h2* cause abnormal meiotic prophase in the testis[79]. The mutant spermatocytes lack pericentric H3K9me3 just before and during early meiotic prophase, and meiotic chromosomes in these mutant cells undergo non-homologous interactions, predominantly at centromeres, and delayed synapsis. Meiosis is arrested at the pachytene stage in these cells, which triggers pronounced apoptosis of spermatocytes. The results suggest that specific

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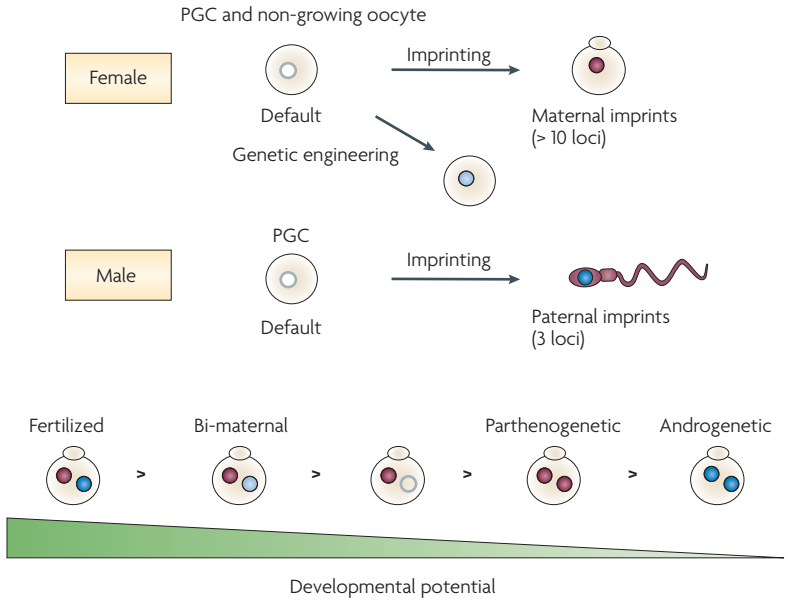
Box 3 | **Production of bi-maternal mice**

Kono *et al.* produced mice with two oocyte-derived genomes and no sperm-derived genome using tricks (see below) to adjust the expression levels of imprinted genes[65,66]. The mice were initially referred to as parthenogenetic but are now called bi-maternal mice. The success relied on the fact that the number of loci that are imprinted in the male germ cells (the paternally imprinted loci) is smaller (only three are known) than that of the maternally imprinted loci (more than ten). In their previous experiments, eggs were reconstituted so that they have one genome from fully grown (imprinted) oocytes and the other from newborn or non-growing (non-imprinted or default) oocytes, using a serial nuclear-transfer technology that they devised. These eggs (bottom, middle) developed better than the control parthenogenetic embryos (second from the right) for three embryonic days[116]. The extended development was probably due to the fact that, in the reconstituted eggs, all loci except the three paternally imprinted loci from the newborn oocytes had appropriate imprints. To adjust the expression levels of the three loci, non-growing oocytes were obtained from mice in which either one (*Igf2* (insulin-like growth factor 2)) or two (*Igf2* and *Dlk/Gtl2*) of the paternally imprinted loci were genetically engineered so that the genes in these loci are appropriately expressed. These engineered non-growing oocytes were then used to reconstitute eggs. This resulted in a low (0.5%, one locus)[65] or surprisingly high (30%, after correction for the expected frequency of the desired genotype, both loci)[66] production rate of adult bi-maternal mice (second from the left). The results clearly indicate that imprinting is the main barrier to parthenogenesis in mammals and that sperm-derived RNAs or proteins are unnecessary for full development.



PGC, primordial germ cell.

changes in modification of pericentric chromatin by SUV39H might be necessary for the proper progression of meiotic prophase. Abnormalities of female germ cells have also been observed in these double mutants[79], but the molecular details of these defects are unknown.

A recent study showed that H3K9 mono- and dimethylation by EHMT2 (euchromatic histone-lysine *N*-methyltransferase 2, also known as G9a) is also essential for early meiotic progression[80]. A germ-cell-specific homozygous mutation of *Ehmt2* caused arrest of meiosis at the early pachytene stage, and synapsis between homologous chromosomes was not properly formed in either testis or ovary. In the mutant spermatocytes, most H3K9me and H3K9me2 signals were lost, but H3K9me3 was unaffected. Expression of some genes was upregulated in EHMT2-deficient germ cells, suggesting that silencing of these genes by EHMT2-mediated H3K9 mono- and dimethylation might be essential for proper synapsis.

A functional link between H3K4 methylation and meiosis-specific gene expression has also been demonstrated[81]. PRDM9 (PR domain-containing 9, also known as Meisetz) is an H3K4 trimethyltransferase that causes transcriptional activation. It is specifically expressed in early meiotic germ cells both in the testis and ovary, and analysis of *Prdm9*-null mice revealed that it is necessary for synapsis and recombination of homologous chromosomes during meiotic prophase. In PRDM9-deficient spermatocytes, expression of a number of autosomal genes, including those that are specifically expressed in meiotic germ cells, was repressed. The results suggest that the genes that are activated by the function of PRDM9 might include those that are involved in the synapsis of homologous chromosomes and/or recombination. However, it is also possible that H3K4me3 that is introduced by PRDM9, as well as H3K9me and H3K9me2 that are mediated by EHMT2, is important for specific chromosomal structures that are required for events such as the search for homologous chromosomes and synapsis. Further studies are needed to test this possibility.

Mechanisms of meiotic sex-chromosome inactivation. In male germ cells, X and Y chromosomes undergo synapsis only within the pseudo-autosomal region during prophase I. Their chromatin is subsequently condensed to form a macrochromatin body that is called the XY or sex body, and the chromosomes become transcriptionally silent[82]. This form of X-chromosome inactivation is called meiotic sex-chromosome inactivation (MSCI) (FIGS 1,4), and might be necessary for synapsis of the pseudo-autosomal regions[83]. It is also suggested that the XY body might mask asynapsed axes of non-pseudo-autosomal regions to prevent sensing by the pachytene checkpoint machinery and subsequent meiotic arrest[83].

MSCI is mediated by some key molecules including the histone H2A variant, H2AX, and its regulatory proteins[83,84]. During the leptotene phase of meiosis, H2AX that is phosphorylated at Ser139 (γH2AX), which is known to recruit the DNA repair machinery to damaged chromatin, is localized at sites of DNA double-strand breaks. γH2AX subsequently disappears from autosomes by pachytene when synapsis is completed. γH2AX accumulates in the XY body at the zygotene–pachytene transition, and analysis of H2AX-deficient mice demonstrated that this is essential for XY-body formation, MSCI and synapsis between the sex chromosomes[83]. The functional importance and mechanisms of H2AX phosphorylation are suggested by studies of additional molecules such as ATR (ataxia telangiectasia and RAD3-related) and the tumour suppressor BRCA1 (breast cancer 1). ATR, a member of the PI3-like kinase family, co-localizes with γH2AX only on X and Y chromosomes at the onset of their inactivation[84]. Studies of BRCA1-deficient spermatocytes indicated that localization of ATR on XY chromatin and proper formation of the XY body

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Table 1 | **Impaired meiosis caused by defects in epigenetic modifiers**

Gene	Function of protein	Mutant phenotype	Refs
Suv39h1 and *Suv39h2*	H3K9 trimethyltransferase	(Male) Arrest at mid to late pachytene and apoptosis in double mutants; impaired chromosome synapsis; impaired modification of pericentric heterochromatin	79
Ehmt2	H3K9 mono- and dimethyltransferase	(Male and female) Arrest at early pachytene and apoptosis; impaired chromosome synapsis; deregulation of target genes	80
Prdm9	H3K4 trimethyltransferase	(Male and female) Arrest at early pachytene and apoptosis; impaired chromosome synapsis and recombination; impaired activation of meiosis-specific genes; impaired XY body formation	81
Dnmt3a	*De novo* DNA methyltransferase	(Male) Arrest at pachytene and apoptosis; imprinting failure	52
Dnmt3L	Regulator of DNA methylation	(Male) Arrest at pachytene and apoptosis; impaired chromosome synapsis; imprinting failure; derepression of retrotransposons	53, 54, 68
Lsh	Chromatin remodelling protein of SNF2-helicase family	(Female) Arrest and apoptosis at diplotene; impaired chromosome synapsis and recombination; demethylation of retrotransposons and tandem repeats	76
Scmh1	Polycomb group protein	(Male) Apoptosis at late pachytene; abnormal chromatin modifications at XY body	86
Mili	Piwi family protein; small RNA regulation	(Male) Arrest at early pachytene and apoptosis; derepression of retrotransposons	70, 71

Dnmt3a, DNA methyltransferase 3a; *Ehmt2*, euchromatic histone-lysine *N*-methyltransferase 2, also known as *G9a*; *Lsh*, lymphoid-specific helicase; *Prdm9*, PR domain-containing 9, also known as *Meisetz*; *Scmh1*, sex comb on midleg homologue 1.

depends on the function of BRCA1, and that γH2AX localizes on sex chromosomes in an ATR-dependent manner[84].

The XY body shows characteristic changes in histone modifications including deacetylation of histones H3 and H4 and dimethylation of H3K9 during pachytene, consistent with the inactive state[85]. Also, H3K4me2 and H3K4me3 are over- and under-represented, respectively. Interestingly, loss of function of PRDM9 causes a failure of XY-body formation[81]. This suggests that PRDM9 might methylate autosomal loci that are crucial for XY-body formation to keep them transcriptionally active.

SCMH1 (sex comb on midleg homologue 1), which is a component of Polycomb repressive complex 1 (PRC1) is involved in histone modifications of the XY body and progression of meiotic prophase[86]. In cells that lack functional SCMH1, meiosis arrests at late pachytene[86]. In normal spermatocytes, PRC1 components as well as H3K27me3 are excluded from the XY body at late pachytene, whereas they are abnormally retained on the XY body in *Scmh1*-mutant spermatocytes. A failure of H3K9me and H3K9me2 accumulation in the XY body and a failure of phosphorylated RNAPII exclusion were also observed in the mutants. Therefore, SCMH1 is required for specific changes in histone modification in the XY body. However, in spite of the aberrant histone modifications, the inactivation of XY chromosomes is not affected by the *Scmh1* mutation, and it is currently unclear why meiosis is arrested[86].

It has been suggested that MSCI might have a role in imprinted X-chromosome inactivation. A model has been proposed in which imprinted X inactivation results from inheritance of an X chromosome that has been pre-inactivated by MSCI from the paternal germline[87]. This model is supported by the observation that the X chromosome is persistently repressed in postmeiotic haploid cells and retains repressive modifications such as H3K9me2 (REFS 88,89). However, other studies showed that autosomal transgenes carrying an X-inactivation centre do not undergo MSCI but can induce imprinted inactivation of the inserted region[90]. Furthermore, it was shown that the paternal X chromosome is transcribed at zygotic gene activation in female embryos, arguing against the pre-inactivation model[90]. Therefore, the relationship between MSCI and imprinted X inactivation is yet to be clarified.

Histone deacetylation in maturating oocytes and segregation of meiotic chromosomes. In mouse oocytes, histones H3 and H4 are generally acetylated at prophase I of meiosis, but they rapidly become deacetylated at metaphase I (MI) by histone deacetylases (HDACs)[91] (FIG. 4). *In vitro* culture of germinal-vesicle-stage oocytes that have been arrested at prophase I in the presence of trichostatin A, an inhibitor of HDACs, showed that neither meiotic maturation, fertilization nor preimplantation development is affected[92]. However, a closer examination revealed that chromosomes are not properly aligned at the metaphase plate in MII oocytes, and that aneuploidy is frequent in single-cell zygotes. Consequently, about half of embryos derived from the trichostatin-A-treated oocytes died *in utero*. Importantly, histones remained acetylated in the oocytes of older (10-month old) female mice, suggesting that the high incidence of aneuploidy (such as trisomy 21 in humans) in older pregnancies might be due to inadequate histone deacetylation. Histone deacetylation might be involved in specific chromosomal structure that is important for chromosome segregation.

Epigenetic changes during gamete maturation

After meiosis, both male and female germ cells undergo final developmental changes, at the level of both their morphology and the epigenome, to allow them to carry out their roles in fertilization and the initial stages of zygotic development. In haploid round spermatids, global nuclear remodelling occurs, although some histone

Aneuploidy
Presence of an abnormal number of chromosomes. For example, in the case of trisomies, an extra copy of a chromosome is present.

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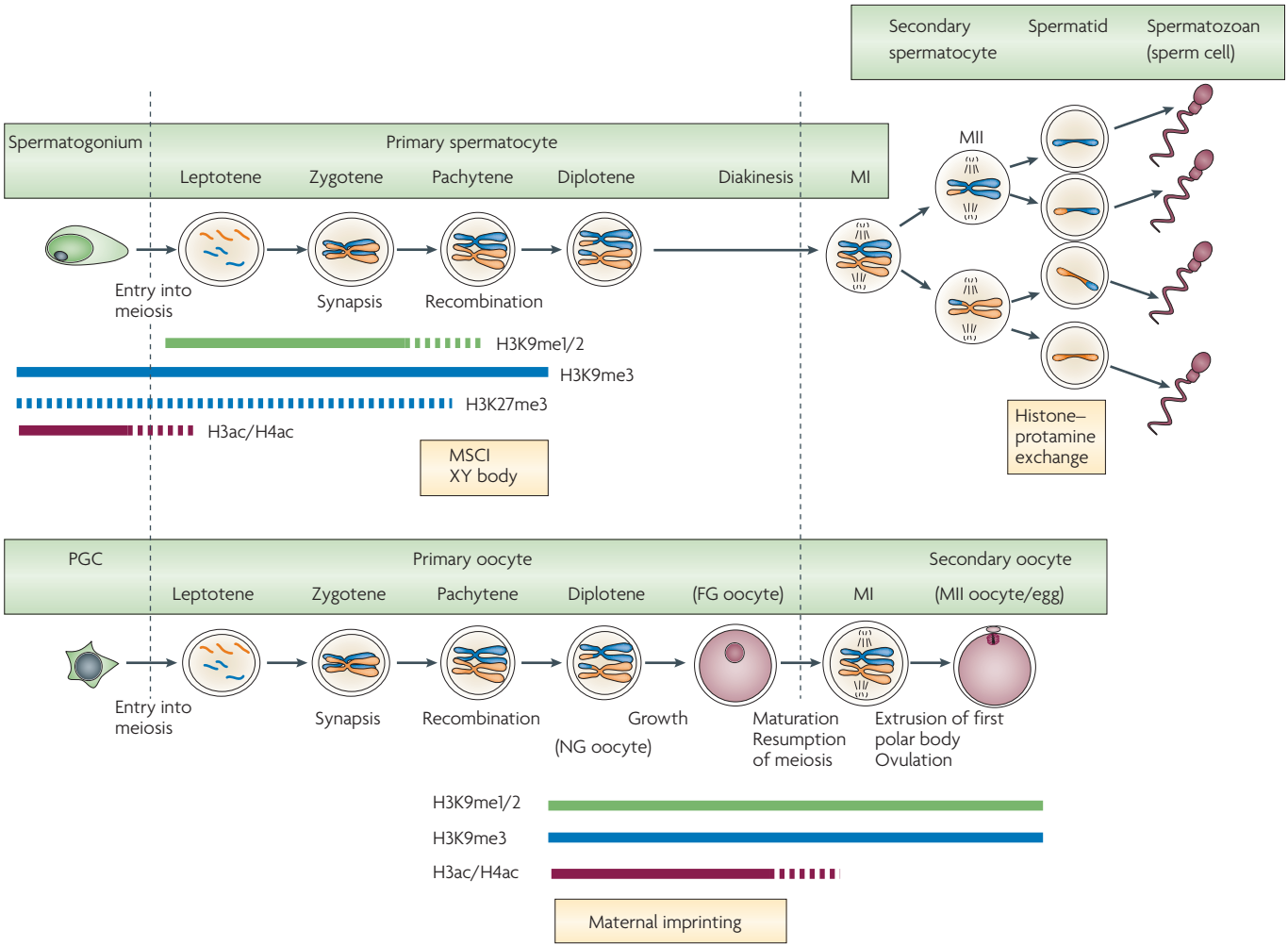

Figure 4 | **Epigenetic changes that occur during meiosis in male and female gametogenesis.** Changes in epigenetic modifications that occur at various stages of meiosis in male and female germ-cell development are shown. Dashed lines indicate that the level of epigenetic modification is lower during these periods than that during the periods shown by the solid lines. FG, fully grown; MSCI, meiotic sex-chromosome inactivation; MI, metaphase I; MII, metaphase II; NG, non-growing; PGC, primordial germ cell.

marks such as H3K9me2 on the inactive X chromosome are retained[88,89]. A testis-specific linker histone variant H1T2 appears at this stage and has an important role in chromatin condensation during spermiogenesis[93]. Later, another linker histone variant HILS1 (histone-1-like protein in spermatids 1) appears in elongated spermatids. In the process of histone–protamine exchange, histones are first replaced by TNP1 (transition protein 1) and TNP2 and then by protamines[5,94], and phosphorylation and dephosphorylation of these proteins regulate the process[5]. Very recently, the JmjC-domain-containing histone demethylase 2A (JHDM2A, also known as JMJD1A), which is an H3K9me1/2-specific demethylase, was shown to be necessary for the specific activation of *Tnp1* and *Prm1* (protamine 1)[95]. The incorporation of protamines into sperm chromatin induces DNA compaction, which is necessary for the formation of spermatozoa and for providing a safe environment for the genome, resistant to physical damage and chemical agents. However, an interesting twist is that mammalian sperm chromatin retains up to 15% of the spermatid histones, and some regions — such as the human protamine gene cluster and imprinted *IGF2* (insulin-like growth factor 2) — have been reported to be histone-rich[96]. The presence of somatic-like chromatin in the sperm nucleus could provide a means to transmit epigenetic information to the offspring.

The genome-wide DNA methylation pattern changes little during spermiogenesis, as its acquisition has been completed by the end of the pachytene spermatocyte stage[97]. However, there is evidence that specific loci such as *Pgk2* (phosphoglycerate kinase 2) become *de novo* methylated as late as the sperm-maturation period in the epididymis[98], the mechanism of which is unknown. As *Pgk2* is only expressed in spermatocytes and spermatids, this methylation might preclude unnecessary activation of this gene during post-fertilization development.

A recent report showed that there are numerous intra- and inter-individual differences in DNA methylation in human sperm samples[99], which could contribute

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to phenotypic differences in the next generation (see also BOX 3). Furthermore, it has been reported that sperm samples from oligospermic patients often contain DNA-methylation defects at the imprinted loci[100,101]. The significance of these findings and also when and how these changes arise are important questions for future studies.

In oocytes, except for the rapid deacetylation of histones H3 and H4 at the germinal-vesicle breakdown mentioned above, histone modifications seem to remain unchanged throughout meiosis (FIG. 4). After the first meiotic division, ovulation occurs by releasing the secondary oocyte that is arrested at MII from the ovarian follicle. The ovulated MII oocyte is now ready to fuse with a sperm cell and the second meiotic division is completed after fertilization. Subsequently, the paternal and maternal genomes of the zygote undergo further reprogramming to acquire ultimate totipotency. The oocyte contributes not only the maternal genome and its associated epigenetic information, but also some factors that are required for post-fertilization reprogramming, which are present in either the cytoplasm or the nucleus[102]. These factors are important for the development of both fertilized embryos and nuclear-transferred embryos. Identification of the reprogramming factors, which might include transcription factors and epigenetic modifiers, is an important subject for future study.

Conclusion

It has become clear that many epigenetic modifiers, including DNA methyltransferases, histone-modification enzymes and their regulatory proteins, have essential roles in germ-cell development. Mutations that affect some of these factors cause early germ-cell loss, whereas mutations that affect the others cause arrest at specific meiotic stages and subsequent apoptosis. The functions of these modifiers are either reprogramming of the germ-cell genome, repression or activation of the downstream target genes, or establishment of a chromatin state that is appropriate for germ-cell-specific events such as chromosome pairing and genetic crossing over. Furthermore, the functional links between DNA methylation, histone modifications and even small-RNA metabolism in germ cells are beginning to be understood, as shown by the recent studies on DNMT3L and MILI (REFS 61,69).

Some epigenetic modifiers are specifically expressed in germ cells whereas others are more widely expressed; this has implications for future studies. The crucial roles of germ-cell-specific genes such as *Dnmt3L* and *Prdm9* were revealed by conventional knockout studies[58,59,81]; the importance of the widely expressed genes such as *Dnmt3a* and *Ehmt2* became explicit only when a germline-specific conditional knockout strategy was adopted[52,80], because null mutants were lethal. Therefore, the current collection of known epigenetic modifiers that are essential for germ cells is probably just the tip of an iceberg, which suggests that we will need to study many more genes. Although conditional knockout continues to be a key technology, recently established GS cells, which can be stably maintained in culture, are genetically modifiable and can differentiate to give rise to functional sperm when transplanted into infertile testes, might offer an alternative experimental system[103].

Recent advances in epigenomic analysis technologies that allow high-resolution mapping of chromatin modifications across the genome (for example, REF. 29) also show promise for future progress in this field. These technologies will allow us to know the normal epigenomic landscape of germ cells and epigenomic changes that occur in mutants and disease conditions, and to identify the downstream targets of epigenetic modifiers. However, challenges exist in this respect, such as the amount of germ-cell samples that are available to researchers. Further improvement of small-scale chromatin immunoprecipitation[104] and unbiased DNA-amplification methods[105] will make the technologies much more useful. Nuclear-transfer technologies, which allow the expansion of cells possessing a specific epigenetic profile in cloned embryos[37], and single-cell analysis technologies including live cell imaging are also important. To know the whole picture of the epigenetic regulation of germ-cell development, further studies as well as improvements of these key technologies are needed.

Finally, some of the findings described here will have an immediate impact on studies of human fertility: mice harbouring mutations in epigenetic modifier genes can be models for human infertility conditions. However, increasing knowledge of epigenetic regulation of germ-cell development will affect a wider biomedical area. For example, one goal of current germ-cell research is the efficient and stable derivation of functional gametes from pluripotent stem cells in culture (BOX 1), success of which depends crucially on knowledge of genetic and epigenetic mechanisms of germ cells. Together with the use of recently described induced pluripotent stem cells[106,107], such a technology will allow a new approach for saving endangered species and overcoming infertility. In addition, epigenetic changes that are induced by environmental stress and trans-generational epigenetic inheritance, which could have relevance to many human diseases, will become an exciting area, for which research on germ cells is particularly important. The far-reaching implications of these studies are improvements in animal cloning, livestock husbandry, assisted reproductive technologies and human health.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene

ATR | BLIMP1 | BRCA1 | CTCF | *Dazl* | *Ddx4* | *Dhx38* | DNMT1 | DNMT3A | DNMT3B | DNMT3L | EHMT2 | *gcl* | *H19* | H1FOO | H1T2 | H2AX | H3.3A | H3.3B | HILS1 | HP1 | *IGF2* | JHDM2A | LSH | MILI | *nanos* | *pgc* | *Pgk2* | PRDM9 | *Prm1* | PRMT5 | *Rasgrf1* | SCMH1 | *Suv39h1* | *Suv39h2* | *Sycp3* | TH2A | TH2B | TNP1 | TNP2

FURTHER INFORMATION

Hiroyuki Sasaki's homepage:
http://www.nig.ac.jp/section/sasaki/sasaki-e.html
Cell Resource Center for Biomedical Research:
http://www.idac.tohoku.ac.jp/en/organization/cancer_cell_repository/index.html

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