REPRODUCTION
REVIEW

Fine-tuning evolution: germ-line epigenetics and inheritance

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Abstract

In mice, epiblast cells found both the germ-line and somatic lineages in the developing embryo. These epiblast cells carry epigenetic information from both parents that is required for development and cell function in the fetus and during post-natal life. However, germ cells must establish an epigenetic program that supports totipotency and the configuration of parent-specific epigenetic states in the gametes. To achieve this, the epigenetic information inherited by the primordial germ cells at specification is erased and new epigenetic states are established during development of the male and female germ-lines. Errors in this process can lead to transmission of epimutations through the germ-line, which have the potential to affect development and disease in the parent's progeny. This review discusses epigenetic reprogramming in the germ-line and the transmission of epigenetic information to the following generation.
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Introduction

Epigenetic mechanisms involve mitotically heritable chromatin modifications that regulate global gene expression patterns and underpin cellular identity and function throughout life. This is achieved through mechanisms involving DNA methylation, histone modifications and non-coding RNAs. Not surprisingly, altered epigenetic states (epimutations) in somatic cells have been associated with a growing list of illnesses including cancer, behavioural disorders and metabolic diseases. In most cases, these altered epigenetic states have only been studied in somatic cells. However, some epigenetic information is transmitted via the gametes to a parent's progeny, in whom it can affect development and fitness.

Gametes originate from the developing germ cells, which establish the essential genetic and epigenetic information required for the zygote to obtain complete developmental potential – a quality known as totipotency. Therefore, germ-line epimutations may have heritable effects on the development of the resulting embryo and may be the cause of a variety of inherited disorders and diseases. Determining how epigenetic information is established in the developing germ-line and the early embryo is crucial for understanding germ-line transmission of the epigenome and how this will affect the next generation.

The genome undergoes two rounds of extensive epigenetic reprogramming during embryonic development. In pre-implantation embryos, specific epigenetic modifications are erased and reset in order to establish essential developmental gene expression patterns (Howlett & Reik 1991, Santos *et al*. 2002, Borgel *et al*. 2010, Smallwood *et al*. 2011, Gillich *et al*. 2012, Smallwood & Kelsey 2012, Smith *et al*. 2012). However, some epigenetic modifications, such as those that regulate imprinted genes, escape this remodelling and are maintained in somatic lineages into adulthood (Reik *et al*. 2001, Lane *et al*. 2003, Weaver *et al*. 2009, Borgel *et al*. 2010, Smith *et al*. 2012; Fig. 1). Genomic imprinting is an inherited epigenetic mechanism that regulates specific genes such that only one of the two inherited alleles is expressed, dependent on its parental origin. Thus, epigenetic modifications regulating these imprinted genes are retained to maintain a parent-of-origin genomic identity. A second round of epigenetic reprogramming takes place in the primordial germ cells (PGCs), which found the male and female germ-lines (Seki *et al*. 2005, 2007, Seisenberger *et al*. 2012; Fig. 1). This reprogramming is more extensive and includes epigenetic modifications that were maintained during pre-implantation reprogramming (Fig. 1). The erasure of the existing epigenome in PGCs facilitates establishment of sex-specific epigenetic profiles crucial for normal germ-line development and inherited epigenetic mechanisms such as genomic imprinting (Surani 2001, 2007, Surani *et al*. 2007, Saitou & Yamaji 2010, Hackett *et al*. 2013). This review discusses the establishment of the germ-line epigenome, its maintenance in the early embryo and evidence for multi-generational epigenetic inheritance.

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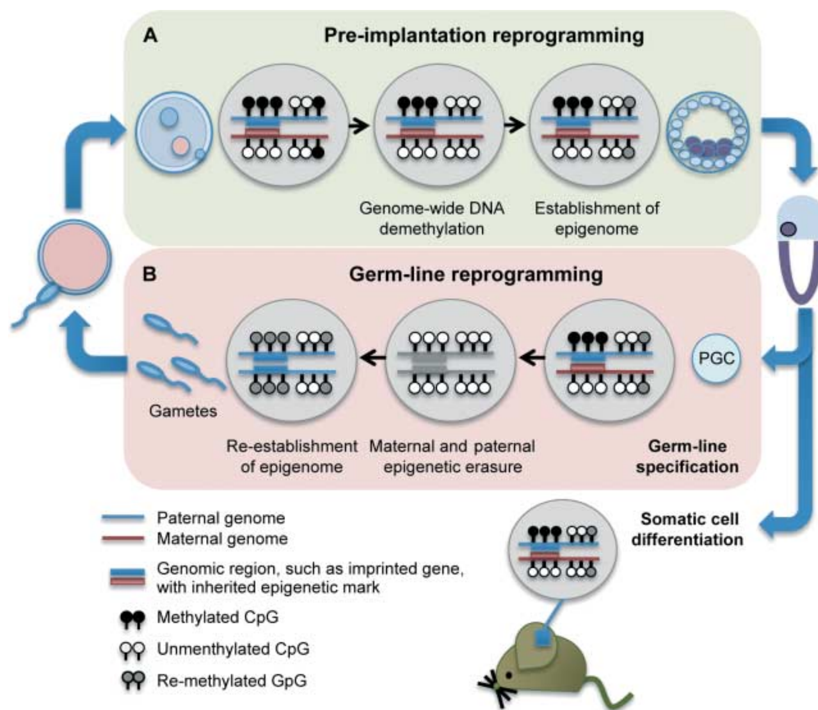

Figure 1 Epigenetic reprogramming during pre-implantation and germ-line development in the mouse. (A) In pre-implantation embryos, epigenetic information is erased allowing establishment of developmental specific epigenetic programmes. However, imprinted genes and possibly other inherited epigenetic modifications are protected from this reprogramming and maintained into adulthood. (B) A second, more extensive round of epigenetic reprogramming occurs in the germ-line resulting in epigenetic erasure at non-imprinted genes and imprinted genes. Epigenetic modifications are then re-established in a sex-specific manner.

Germ-line specification and the germ cell epigenome

In common with other species, including invertebrates such as *Caenorhabditis elegans*, mammalian germ cell development involves molecular segregation from somatic differentiation and the retention of developmental potential. This is achieved partly through epigenetic mechanisms that repress genes involved in somatic cell differentiation and/or activate genes that promote germ cell identity (Strome & Lehmann 2007, Saitou & Yamaji 2010). In mice, proximal epiblast cells give rise to both PGCs and somatic lineages within the embryo. These epiblast cells carry essential epigenetic information, including parent-specific epigenetic marks, which are required for normal somatic development in the embryo (Barton *et al*. 1984, McGrath & Solter 1984, Surani *et al*. 1984, Surani 1991, 1994). Thus, the epigenetic information present in the epiblast must be removed from PGCs following their specification to establish a new sex-specific epigenetic profile appropriate for germ cell function and development (Fig. 1).

In mice, PGC commitment occurs during embryonic (E) days 6–7.25 and is induced by signals from the neighbouring extra-embryonic tissue. These signals induce the production of B-lymphocyte-induced maturation protein 1 (*Prdm1* (Blimp1)), which in turn represses a number of somatic genes, such as homeobox genes *Hoxa1* and *Hoxb1* (Ohinata *et al*. 2005, Kurimoto *et al*. 2008*a*). PRDM1 functions in a complex containing the arginine methyltransferase PRMT5 and establishes methylation of histones, such as histone 2A (H2A) and H4 (Ancelin *et al*. 2006). Moreover, PRDM1 and the transcription factor *Prdm14* are considered essential for re-expression of genes promoting pluripotency (e.g. *Sox2*) in early PGCs (Kurimoto *et al*. 2008*b*, Yamaji *et al*. 2008, Magnusdottir *et al*. 2012). PGC commitment also coincides with repression of the '*de novo*' DNA methyltransferases 3a (*Dnmt3a*) and 3b (*Dnmt3b*) (Seki *et al*. 2005, Yabuta *et al*. 2006, Kurimoto *et al*. 2008*b*). Conversely, the maintenance *Dnmt1*, which adds methyl groups to hemimethylated DNA, is expressed at relatively constant levels in E6.75–E7.75 PGCs and the neighbouring somatic cells (Seki *et al*. 2005, Yabuta *et al*. 2006). Based on immunofluorescence, early PGCs also exhibit similar DNA methylation patterns to somatic cells (Seki *et al*. 2005). Likewise, at E6.5–E7.0, whole-mount immunofluorescence revealed similar levels of histone modifications, such as methylation of histone 3 at lysines 4, 9 and 27 (H3K4me2/3, H3K9me1/2/3 and H3K27me2/3) and acetylation of lysine 9 (H3K9Ac), in PGCs and the adjacent somatic cells (Seki *et al*. 2007). Thus, at specification, PGCs are epigenetically similar to the neighbouring somatic cells but rapidly diverge through the expression of genes, including *Prdm1*, *Prmt5* and *Prdm14*, that drive PGC specification and repress somatic fate.

Remodelling the early PGC epigenome

After germ-line commitment, PGCs proliferate and migrate from the base of the allantois, through the hindgut and enter the developing gonad at E10.5 (Fig. 2).

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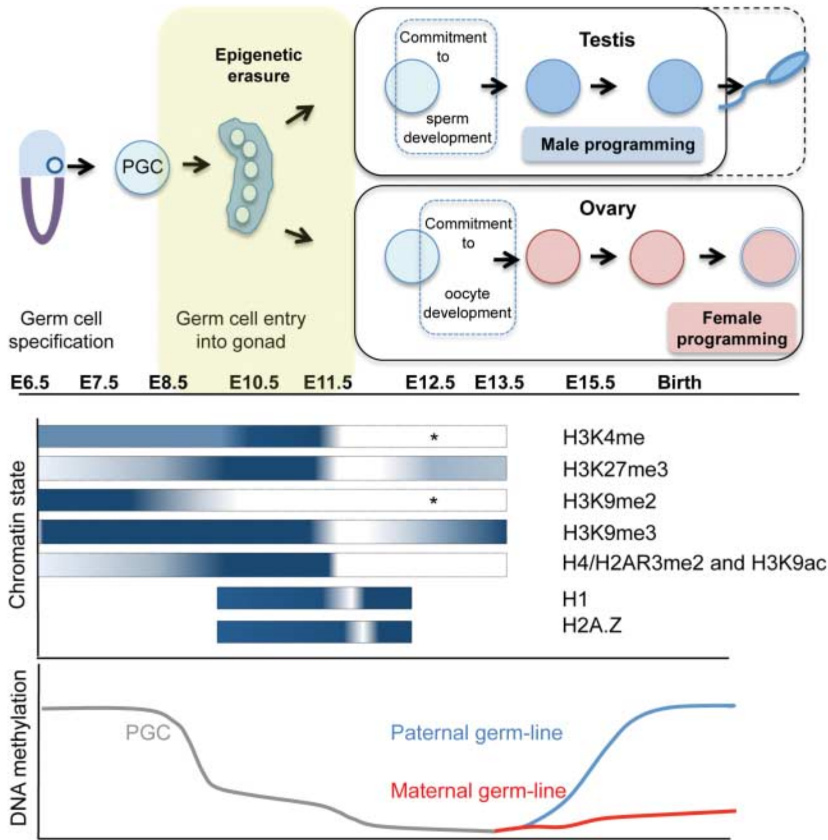

Figure 2 Epigenetic reprogramming relative to developmental events in mouse germ cells. PGCs are derived from the pluripotent epiblast at E6–E7.25 and migrate to the developing gonads by E10.5. During migration, PGCs initiate genome-wide epigenetic erasure, including the removal of H3K9me2, significant DNA demethylation and enrichment of H3K27me3. The germ cells enter the gonad at E10.5 and undergo further epigenetic erasure, including the removal of DNA methylation on imprinted genes and TEs. This phase is thought to involve the removal of H3K27me3, H3K9me3 and H2A/H4 R3 methylation, removal of histone variants H1 and H2A.Z and reorganisation of chromatin state. Events primarily based on the reports from Seki *et al.* (2005), Hajkova *et al.* (2008) and Kagiwada *et al.* (2013), although these reports differ in the extent of the chromatin remodelling that takes place. After germ-line sex determination, sex-specific epigenetic modifications are established in the male and female germ-lines. While some re-establishment of H3K27me3 and H3K9me3 occurs from E12.5, the extent and gene specificity remain unknown. The establishment of other histone modifications (e.g. H3K9me2 and H3K4me) also remains poorly described (*). In male germ cells DNA re-methylation is initiated from E15.5 but is not completed until late spermatogenesis. DNA methylation in female germ cells is less extensive and occurs later than in male germ cells.

Between E7.75 and E8.75, PGCs transiently enter G2 cell cycle arrest and undergo genome-wide epigenetic remodelling (Seki *et al.* 2005). This includes gradual but complete demethylation of H3K9me2, which coincides with reduced levels of GLP (EHMT1), a histone methyltransferase that catalyses H3K9 methylation (Seki *et al.* 2005, Tachibana *et al.* 2005, Yabuta *et al.* 2006, Seki *et al.* 2007, Hajkova *et al.* 2008, Kurimoto *et al.* 2008*b*; Fig. 2). Loss of H3K9me2 is followed by enrichment of H3K27me3 before the release of PGCs from G2 arrest at E9.0 (Seki *et al.* 2005, 2007, Hajkova *et al.* 2008; Fig. 2). Increased levels of methylated arginine 3 in H2A and/or H4 (H2A/H4 R3) have also been reported in PGCs at E8.5 (Ancelin *et al.* 2006). G2 arrest in PGCs is accompanied by reduced polymerase II-dependent transcription, which may prevent aberrant gene expression during the transition between the repressive modifications, H3K9me2 and H3K27me3 (Seki *et al.* 2007). Relatively high levels of the permissive histone marks, H3K9Ac and H3K4me2, were detected in E8.5 and E10.5 PGCs (Hajkova *et al.* 2008). However, an alternative study detected only a transient up-regulation of H3K9Ac and H3K4me in E10.5 PGCs (Seki *et al.* 2005). Nevertheless, these data combined indicate that PGC specification initiates a significant period of epigenetic remodelling in these cells.

Upon entry into the gonads, PGCs undergo further chromatin remodelling, including loss of H2A/H4 R3 methylation (Ancelin *et al.* 2006). Similarly, H3K9me3 and H3K27me3 are removed from mouse PGCs at E11.5 and re-established soon after (Hajkova *et al.* 2008), indicating that these histone modifications undergo reprogramming. A similar loss in H3K27me3 has also been observed in human PGCs, although levels then remained low (Gkountela *et al.* 2013). Conversely, alternative reports indicate that H3K27me3 is maintained at relatively stable levels in mouse PGCs until E12.5 (Seki *et al.* 2005, Kagiwada *et al.* 2013). These data suggest that the reduction of H3K27me3 in PGC at E11.5 may be coupled to fluctuating levels during the cell cycle, rather than epigenetic reprogramming (Kagiwada *et al.* 2013).

Other aspects of chromatin remodelling have also been observed in conjunction with reprogramming of histone modifications in E11.5 PGCs (Hajkova *et al.* 2008). These include accumulation of the histone chaperones NAP1 and HIRA in PGC nuclei, rapid depletion of linker histone H1 and H2A.Z and morphological changes such as expansion of nuclei and temporary loss of visible chromophores (Hajkova *et al.* 2008). Based on the rapid nature of this process, the authors suggested that histone modifications

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are reprogrammed through a histone replacement mechanism (Hajkova *et al*. 2008). In accord with this, histones, including H1 and H2A.Z, were detected in the cytoplasm of a few PGCs, indicating that they might be transported for degradation (Hajkova *et al*. 2008). However, PGCs lacking a H3K27me2/3 demethylase, UTX (Kdm6a), have aberrant chromatin dynamics and fail to form pluripotent embryonic germ cells (Mansour *et al*. 2012), indicating that UTX, and similar enzymes, may also be involved in the removal of histone modifications from PGCs. Another recent study did not detect the chromatin changes reported by Hajkova *et al*. (2008) and suggests that a histone replacement mechanism is unlikely to regulate reprogramming at this time (Kagiwada *et al*. 2013). The reason for the differences between these studies remains unclear, but it is apparent that the extent of chromatin reprogramming and mechanisms involved in resetting histone modifications remain to be clarified. Nevertheless, such mechanisms could play an important role in providing an epigenetic ground state for the establishment of sex-specific histone modifications in PGCs that influence the development in the next generation.

In addition to histone remodelling, PGCs undergo extensive genome-wide DNA demethylation, which is initiated in migrating PGCs and completed by E13.5, after population of the gonads (Popp *et al*. 2010, Guibert *et al*. 2012, Seisenberger *et al*. 2012, Hackett *et al*. 2013, Kagiwada *et al*. 2013). Recent genome-wide profiling of DNA methylation using bisulphite sequencing demonstrated that global DNA methylation is decreased from 71 to 30% in PGCs between E6.5 and E9.5 and further decreased to 14 and 7% after entry of PGCs into the developing testis and ovary respectively (Seisenberger *et al*. 2012). Importantly, this wave of DNA demethylation includes genomic regions that initially preserved their methylated state during the zygotic reprogramming (e.g. imprinted genes). Moreover, the majority of imprinted gene differentially methylated regions (DMRs) methylation is maintained during migration, and demethylation of these regions is only completed once PGCs enter the genital ridges from E10.5 (Seisenberger *et al*. 2012). This is consistent with the notion that PGCs need to enter the gonad before erasure of imprints is initiated, perhaps by signalling from the somatic cells (Surani 2007). However, analyses based on the dynamics of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) suggest that erasure of some imprints commences before the entry into the genital ridges, although gene-by-gene differences in demethylation rates were noted (Hackett *et al*. 2013, Kagiwada *et al*. 2013). This indicates that the process is temporally defined and its initiation may be independent of gonadal signals. DNA demethylation during this period also includes transposable elements (TEs) such as LINE and SINE's, although intracisternal-A-particles (IAPs) remain methylated. Interestingly, demethylation of LINE1 elements in germ cells did not result in aberrant expression (Seisenberger *et al*. 2012). This suggests that other mechanisms are in place to repress transcription during this phase of reprogramming (Seisenberger *et al*. 2012). These could include RNA binding proteins, which are highly represented in PGCs and a number of other mechanisms. In PGCs, there are a subset of genes that suppresses TE activity, including Tex19.1, Mili, Mov10l1 and Asz1. A recent report demonstrates that these genes are primarily regulated by DNA methylation, rather than histone modification (Hackett *et al*. 2012). Moreover, as transcriptional activity was detected for these genes during reprogramming, it was suggested that they may provide a mechanism to silence TEs in the absence of methylation, thereby protecting genome integrity (Hackett *et al*. 2012).

While the genome is hypomethylated in both sexes by E13.5, methylation is retained at some loci (Seisenberger *et al*. 2012, Hackett *et al*. 2013). Most recently inserted retrotransposon sequences, particularly IAPs, are substantially resistant to demethylation in PGCs, ensuring their repression and maintaining genome integrity (Lane *et al*. 2003, Popp *et al*. 2010, Guibert *et al*. 2012, Seisenberger *et al*. 2012, Hackett *et al*. 2013). However, some single-copy CpG islands (CGIs) in the genome were also found to be resistant to demethylation (Seisenberger *et al*. 2012, Hackett *et al*. 2013). Although the functional significance of this remains unknown, these CGIs may provide candidates for inter-generational epigenetic inheritance (Seisenberger *et al*. 2012, Hackett *et al*. 2013), which is discussed in more detail later in this review.

The mechanisms involved in removal of methyl groups from DNA in mammalian PGCs remain a matter of debate. Recent studies have provided substantial insights into this process but disagree on whether passive and/or active mechanisms are involved. Of the known enzymes required for DNMT activity (including *Dnmt1*, *Dnmt3a/b/l* and *Uhrf1*), all but *Dnmt1* are down-regulated in PGCs after E7.25–E7.5 (Hajkova *et al*. 2002, Seki *et al*. 2005, Yabuta *et al*. 2006, Kurimoto *et al*. 2008*a*, Kagiwada *et al*. 2013). As UHRF1 is required for recruitment of DNMT1 and maintenance methylation (Sharif *et al*. 2007), it is highly unlikely that PGCs undergoing demethylation contain DNMT activity. Therefore, when PGCs are highly proliferative, it is likely that passive, replication-dependent reduction contributes significantly to DNA demethylation. However, active mechanisms are also thought to contribute to PGC demethylation. Activation-induced cytidine deaminase (AID)-dependent DNA demethylation has been proposed to mediate DNA demethylation in PGCs that have entered the developing gonads. However, only a moderately higher level of DNA methylation is maintained in germ cells deficient for AID, suggesting other mechanisms are involved (Bhutani *et al*. 2010, Popp *et al*. 2010). The base excision repair (BER) and the ten to

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eleven translocation (TET) pathways have also been implicated in DNA demethylation in PGCs and during reprogramming in the zygote, ES cells and pre-implantation embryos (Hajkova *et al.* 2010, Ito *et al.* 2010, Hackett *et al.* 2012). However, of the genes involved in the three known active demethylation pathways (TET, AID and BER pathways), substantial levels of transcription were observed only for *Tet1*, suggesting that only the TET1–TDG pathway can operate in PGCs (Kagiwada *et al.* 2013). Moreover, *Tet1*-deficient PGCs have been shown to undergo genome-wide DNA demethylation (Yamaguchi *et al.* 2012), suggesting that *Tet1* is not required for demethylation in PGCs. In contrast, another study detected both TET1 and TET2 protein in PGCs undergoing reprogramming between E9.5 and E11.5 (Hackett *et al.* 2013). This expression coincided with a reduction of 5mC and an elevation of 5hmC (Hackett *et al.* 2013). Additionally, microRNA knockdown of *Tet1* and *Tet2* in PGCs blocked DNA demethylation, while constitutive overexpression promoted 5mC erasure (Hackett *et al.* 2013). This provides substantial evidence of the involvement of TET-mediated 5mC-to-5hmC conversion in demethylation. After conversion, levels of 5hmC declined between E10.5 and E13.5, which correlated with the rate of DNA replication suggesting that clearance of 5hmC is a passive process (Hackett *et al.* 2013). Furthermore, analysis using hairpin bisulphite sequencing allowed the identification of hemimethylated sequences in PGCs at E9.5 and E10.5, which were subsequently completely unmethylated by E13.5 (Seisenberger *et al.* 2012). Thus, it appears that DNA demethylation in PGCs is likely to employ both active and passive mechanisms.

Epigenetic reprogramming of PGCs is rapidly followed by germ-line sex determination, which sets the bipotential PGCs on a path to produce either male or female gametes. Sex-specific germ-line development is marked by the entry of XY (male) germ cells into mitotic arrest and XX (female) germ cells into meiosis, processes that involve signalling by *Fgf9*, *Nodal*, *Tgf/Activin* and retinoic acid (Baltus *et al.* 2006, Koubova *et al.* 2006, DeFalco & Capel 2009, Western 2009, Bowles *et al.* 2010, Miles & Western 2012, Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013). Although poorly understood, these signalling mechanisms are associated with important changes in gene expression profiles, including the repression of genes that regulate pluripotency (e.g. *Pou5f1* (*Oct4*), *Sox2* and *Nanog*) and activation of genes that drive sex-specific germ-line development. These programs also drive establishment of sex-specific epigenetic profiles important for gametogenesis and development in the next generation.

Re-establishment of the germ-line epigenome

Epigenetic erasure in the germ-line creates a chromatin landscape permissive for re-establishment of the male and female epigenomes. Although there is global re-methylation of H3K27me2/3 and H3K9me3 in both XX and XY germ cells from E12.5 (Hajkova *et al.* 2008; Fig. 2), the extent and higher resolution patterning of these and most other epigenetic modifications remain unknown. However, the re-establishment of DNA methylation has been more extensively studied, particularly at imprinted loci and TEs. Re-establishment of DNA methylation is mediated by the *de novo* DNMTs (DNMT3A and DNMT3L), which are strongly up-regulated in quiescent male germ cells. Conditional knockout studies have demonstrated that both *Dnmt3a* and *Dnmt3l* are essential for re-methylation of imprinted genes and for methylation of other sequences including TEs (Bourc'his *et al.* 2001, Chedin *et al.* 2002, Hata *et al.* 2002, Bourc'his & Bestor 2004, Kaneda *et al.* 2004, Sakai *et al.* 2004, Webster *et al.* 2005, Kato *et al.* 2007, Lucifero *et al.* 2007, Shovlin *et al.* 2007).

Genomic imprinting is an inherited epigenetic state that results in a parent-of-origin-specific expression of ~140 genes, transposons and microRNAs in the therian (eutherian and marsupial) mammals (http://igc.otago.ac.nz/home.html and http://www.mousebook.org/catalog.php?catalog=imprinting). The molecular mechanisms regulating genomic imprinting are well characterised at only a few loci (Constancia *et al.* 1998, Reik & Walter 1998, Edwards & Ferguson-Smith 2007, Ideraabdullah *et al.* 2008, Sha 2008, Kim *et al.* 2009). However, the majority of imprinted genes are marked by DNA methylation (or histone modifications) that differs between the maternal and paternal alleles and is thought to regulate parent-specific expression of the gene(s) at that locus (Fig. 3). Establishment of parent-specific DNA methylation (differential methylation) at imprinted loci occurs within the male and female germ-lines and is maintained through zygotic reprogramming, possibly by mechanisms involving DNMT1, DPPA3/Stella and TRIM28 (Nakamura *et al.* 2007, Cirio *et al.* 2008, Messerschmidt *et al.* 2012), and in many somatic tissues throughout life (Reik & Walter 2001, Bartolomei & Ferguson-Smith 2011). In mice, male germ cells acquire DNA methylation from around E15.5, coinciding with a male germ cell-specific period of mitotic arrest. Imprinted genes such as *Rasgrf1*, *Meg3* (*Gtl2*) and *H19* start to acquire methylation in male germ cells between E12.5 and E17.5 but are not completely methylated until late spermatogenesis (Li *et al.* 2004). In contrast, female germ cells acquire methylation after birth, although the levels of DNA methylation may be significantly lower than those in the sperm (Davis *et al.* 2000, Li *et al.* 2004, Lucifero *et al.* 2004, Smith *et al.* 2012).

Imprinted genes are usually located in clusters and are co-regulated by imprinting control regions (ICRs) that contain DMRs. In developing germ cells, DNA methylation inherited from the previous generation is erased and is re-established in a sex-specific manner at DMRs, single-copy genes and TEs. For example, paternally

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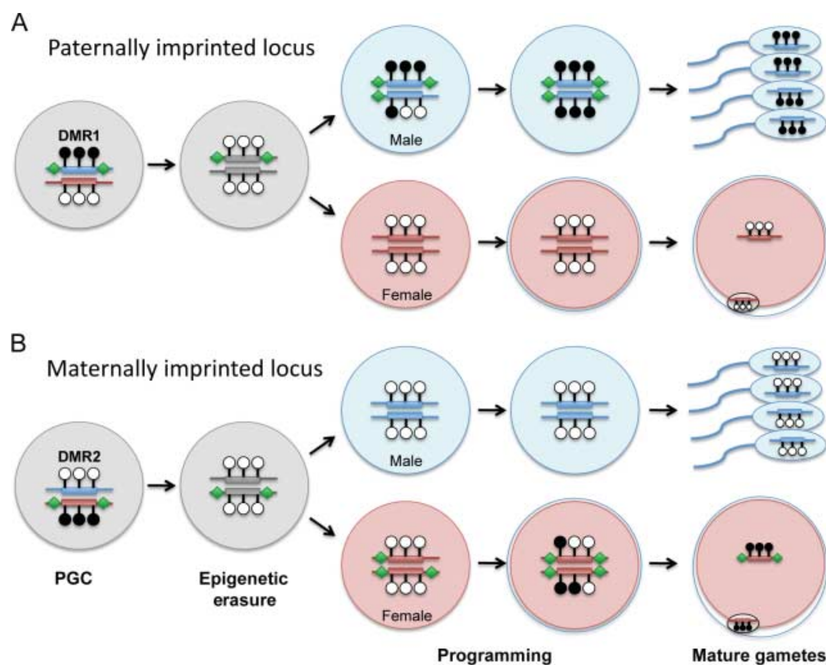

Figure 3 Epigenetic erasure and establishment of a paternally and maternally imprinted locus in male and female germ cells. In the PGCs, the blue allele was inherited from the father and the pink allele was inherited from the mother. New parent-specific epigenetic marks are acquired after germ cell sex determination. Black circles represent methylated CpG sites and open (white) circles indicate unmethylated sites. (A) A paternally imprinted gene acquires methylation on both alleles in the male germ-line while the female germ-line remains unmethylated. (B) A maternally imprinted gene acquires methylation on both alleles in the female germ-line while the male germ-line remains unmethylated. Other epigenetic marks (green diamonds) may be maintained during germ-line epigenetic erasure and direct the establishment of the new parent-specific marks such as DNA methylation. DMR, differentially methylated region.

methylated DMRs become methylated on both alleles in male germ cells but remain unmethylated in female germ cells (Fig. 3). Interestingly, remethylation of germ-line DMRs may occur asynchronously. For example, at the mouse H19 ICR, the previously methylated paternal allele becomes re-methylated before the previously unmethylated maternal allele (Davis *et al*. 1999, 2000, Li *et al*. 2004). As both alleles exist in an unmethylated state after erasure, other marks must be retained to direct re-methylation to the appropriate sites in an ordered manner (Fig. 3). In somatic cells, the methylated allele within ICRs is associated with H4K20me3 and H3K9me3, while the unmethylated allele is enriched for H3K4me2 and acetylated H3 (H3ac; Delaval *et al*. 2007, Henckel *et al*. 2009, Arnaud 2010). Similarly, H3K4me2 and H3ac are enriched at unmethylated ICRs before the global exchange of histones for protamines in spermatogonia (Delaval *et al*. 2007, Henckel *et al*. 2009, Arnaud 2010). Maternal DPPA3/Stella, TRIM28 and H3K9me2 may be required for maintaining imprints in the zygote (Nakamura *et al*. 2007, Messerschmidt *et al*. 2012, Szabo & Pfeifer 2012). Interestingly, G9a, a euchromatin-localised histone methyltransferase, catalyses H3K9 dimethylation and is required for the establishment of methylation at specific loci (Dong *et al*. 2008, Tachibana *et al*. 2008, Ikegami *et al*. 2011, Leung *et al*. 2011). Combined, these studies led to speculation that histone modifications marking allele-specific methylation may contribute to the maintenance of imprinting during zygotic reprogramming (Delaval *et al*. 2007, Abramowitz & Bartolomei 2012).

Repetitive DNA elements, including minor satellite sequences and some TEs, such as IAPs maintain a relatively high level of methylation during epigenetic erasure and are completely remethylated by E17.5 (Hajkova *et al*. 2002, Lane *et al*. 2003, Lees-Murdock *et al*. 2003, Seisenberger *et al*. 2012). DNA methylation of these elements prevents their transposition during germ-line development, thereby maintaining genome integrity. Methylation of TEs involves small RNAs, particularly P-element-induced wimpy testes (PIWI)-interacting small RNAs (piRNAs). piRNAs interact with PIWI-like proteins and functionally related TUDOR proteins, with piRNAs guiding RNA-induced transcriptional silencing, *de novo* DNA methylation and potentially the establishment of histone methylation (e.g. H3K9me3 and H3K9me2) on target sequences (Aravin & Bourc'his 2008, Aravin *et al*. 2008, Kuramochi-Miyagawa *et al*. 2008, Siomi *et al*. 2011, Bamezai *et al*. 2012). The extent to which the PIWI–piRNA axis is required for other, non-constitutive epigenetic regulation in the developing germ-line remains unclear. However, PIWIs may interact with piRNAs to direct DNA methylation and histone modifications that mediate both silencing and activation of target genes (Bamezai *et al*. 2012).

Ultimately, these and other mechanisms result in establishment of the epigenetic modifications within the gametes that are transmitted to the next generation. As most nucleosomes are replaced by protamines during spermatogenesis (Balhorn *et al*. 1977, Gatewood *et al*. 1987), it has long been thought that histones play an insignificant role in transmission of this information through the male germ-line. However, some nucleosomes are retained and are preferentially located at the promoters of developmental regulators, such as

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Hox genes, *Sox2*, *Brachyury(T)*, *Cdx2* and *Gata6* (Tanphaichitr *et al.* 1978, Gatewood *et al.* 1987, Hammoud *et al.* 2009, Brykczynska *et al.* 2010). Therefore, the maintenance of histone modifications in sperm may provide a mechanism to control expression of specific gene sets in the next generation and for the embryo to recognise the paternally inherited genome (Tanphaichitr *et al.* 1978, Gatewood *et al.* 1987, Hammoud *et al.* 2009). It remains unclear when this information is established during sperm development and whether these histone modifications regulate paternal gene expression in the father's progeny. However, recent studies on *C. elegans* have functionally linked piRNAs and histone modifications to multi-generational epigenetic inheritance (Ashe *et al.* 2012, Buckley *et al.* 2012, Gu *et al.* 2012). These studies provide a link between epigenetic patterning in the developing germ-line and a memory mechanism that facilitates transmission of this information to the next generation.

Multi-generational epigenetic inheritance

It has been widely speculated that the germ-line epigenome is subject to alteration by environmental influences, thereby allowing evolutionary adaptation through mechanisms such as epigenetic modifications. An increasing number of studies suggest that exposure of a pregnant mother to particular environmental influences could alter the epigenome in the fetal somatic and/or germ cells (Fig. 4a; Anway *et al.* 2005, Skinner & Guerrero-Bosagna 2009, Skinner 2010). For example, potential epigenetic effects were reported in individuals who experienced the Dutch Hunger Winter (1944–1945). Nineteen-year-old men who were pre-natally exposed to this famine had different phenotypes depending on the timing of exposure. Malnutrition during the first trimester of pregnancy resulted in higher rates of obesity than exposure in the last trimester (Ravelli *et al.* 1976). Similarly, the imprinted *IGF2* gene was less methylated in individuals exposed to famine in the peri-conception period compared with their unexposed, same-sex siblings (Heijmans *et al.* 2008). Although these observations are of significant interest, it is very difficult to determine whether these environmental factors cause changes in the F1 germ-line and are transmitted to the following generations in human subjects (Fig. 4a).

To circumvent these limitations, other models have been investigated. Analyses of rodent models have revealed altered glucose metabolism (e.g. reduced insulin secretion and insulin resistance) in pups born to malnourished mothers. Interestingly, similar phenotypes were observed in the second and third generations (Dahri *et al.* 1991, Martin *et al.* 2000, Benyshek *et al.* 2004, 2006). However, the second-generation animals were bred from F1 dams with reduced insulin secretion and the third generation was obtained from insulin-resistant F2 dams. Therefore, it remains possible that the second- and third-generation phenotypes may be the result of the maternal environment, which confounds identification of trans-generational effects in these studies. The confounding effects of maternal environment may be avoided if similar phenotypes could be

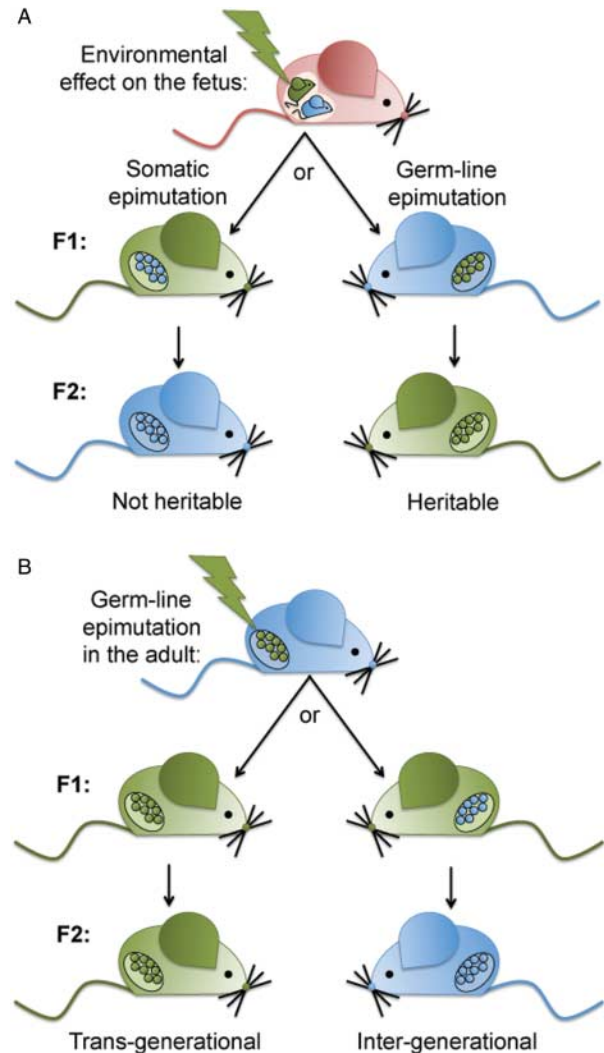

Figure 4 Models of epigenetic inheritance. (A) Chemicals or environmental agents could alter the epigenome of the fetus (F1), resulting in either somatic (blue germ cells, left) or germ-line epimutations (green germ cells, right). Somatic epimutations may affect the F1 fetus but are not heritable between generations (left). Germ-line epimutations will be inherited in the F2 animals and may affect development, assuming that the epimutation is not reprogrammed in the pre-implantation embryo. (B) If a germ-line epimutation is inherited and maintained over several generations, it may mediate trans-generational affects. However, if such an epimutation is reprogrammed in the germ-line of the F1 individual and is not inherited by the following generations, it may have only an inter-generational affect. Blue and pink mice are epigenetically normal male and female mice respectively. Green mice carry a somatic epimutation. Blue germ cells are epigenetically normal and green germ cells carry an epimutation.

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demonstrated by transmission through the paternal germ-line. A similar study demonstrated that a maternal high-fat diet results in increased body length and insulin insensitivity in the mother's pups. While this phenotype was maternally and paternally transmitted over the first two generations, in the third generation, increased body length was only detected in the female offspring and was only paternally transmitted (Dunn & Bale 2011). Although imprinted genes have been implicated, the molecular mechanisms underlying inheritance of these phenotypes remain unknown.

Other studies have more directly demonstrated that mutations in epigenetic modifiers can result in paternal inheritance of epigenetic effects (Chong *et al*. 2007). Haploinsufficiency of *Dnmt1* and *Smarca5* in male mice caused changes in expression of the maternally inherited A^{vy} gene in genetically wild-type offspring (Chong *et al*. 2007). This demonstrates a *trans*-effect on maternal gene expression that is apparently mediated through a paternally inherited epigenetic mechanism. Although the identity and function of the pathways involved are yet to be understood, the authors speculated that non-coding RNAs might provide a molecular mechanism for inheritance of the epigenetic effects in these models. In support of this, studies on *C. elegans* demonstrate that molecular mechanisms underlying paternal epigenetic inheritance involve small non-coding RNAs, particularly piRNAs, and histone-modifying proteins (Ashe *et al*. 2012, Buckley *et al*. 2012, Gu *et al*. 2012). Indeed, recent studies have used RNA sequencing to identify sperm RNAs that could be passed to the next generation (Krawetz *et al*. 2011, Peng *et al*. 2012, Sendler *et al*. 2013), where they might contribute to patterning the epigenetic state.

Induction of epimutations in the germ-line could involve mutations in genes regulating epigenetic state (Chong *et al*. 2007) or be mediated through environmental influences, such as chemicals and toxins (Anway *et al*. 2005). Hence, even if the embryo is genetically normal, environmentally induced germ-line epimutations may be transmitted through the gametes and underlie phenotypic variation, including diseases and disorders in the parent's offspring (Fig. 4). As variation in epigenetic patterning is likely to affect many target genes, germ-line epimutations could underlie a range of developmental disorders in the next generation.

Trans-generational vs inter-generational effects

Trans-generational effects are considered to result from epimutations established in the germ-line that are stable through multiple generations (Fig. 4b). To be transmitted across multiple generations, epimutations must survive germ-line reprogramming. This could occur through two different mechanisms. First, an epimutation in the germ-line may be resistant to, or lack factors that mediate, epigenetic erasure during germ cell development. Alternatively, there could be a persistent change in the memory mechanism governing where specific epigenetic modification(s) are re-established in the chromatin of developing germ cells (Fig. 3). Either way, trans-generational epimutations could contribute significantly to evolutionary mechanisms that respond to environmental change at a more rapid rate than selection for genetic mutations. However, if such mechanisms are to be responsive to environmental cues and avoid the relative persistence of a genetic mutation, germ-line epimutations underlying trans-generational changes should be reversible or changeable.

Epimutations induced in the F1 germ-line, either through impacts on the fetus during pregnancy or on the germ-line in post-natal life of individuals of the F1 generation, may also affect the F2 generation but not subsequent generations (Fig. 4). Such effects can be considered inter-generational, as the effect is not maintained past the F1 progeny. Although potentially less important in an evolutionary sense, relatively short-lived inter-generational epigenetic changes could have significant impacts on human health, especially if the germ-lines of consecutive or non-consecutive generations are exposed to the same or similar environmental influences such as chemicals, drugs or toxins. Therefore, it may be less important to understand whether an epimutation persists in a trans-generational or inter-generational sense and initially more important to understand how epigenetic information is transmitted through the germ-line.

Early embryonic reprogramming

To mediate the effects in the fetus or during post-natal life, epimutations must be sustained through pre-implantation development. However, fertilisation induces genome-wide epigenetic reprogramming that is thought to allow reacquisition of totipotency in the zygote (Howlett & Reik 1991, Santos *et al*. 2002, Borgel *et al*. 2010, Smallwood *et al*. 2011, Gillich *et al*. 2012, Smallwood & Kelsey 2012, Smith *et al*. 2012). There have been several other studies that have reached somewhat varied conclusions regarding the extent of epigenetic reprogramming during pre-implantation development. Studies in a variety of mammalian models using immunofluorescence suggest that up to 90% of DNA methylation is removed after the first cell cycle (Mayer *et al*. 2000, Dean *et al*. 2001, Reik *et al*. 2001, Santos *et al*. 2002). Bisulphite analysis at specific loci also revealed DNA demethylation in the zygote, although to a lesser extent than that indicated by immunofluorescence (Oswald *et al*. 2000, Lane *et al*. 2003). Moreover, in a recent study using global DNA bisulphite sequencing, it was demonstrated that oocytes are initially hypomethylated compared with sperm (Smith *et al*. 2012). However, during pre-implantation reprogramming, the paternal genome is demethylated

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such that the DNA methylation levels in the zygote are similar to those in the oocyte (Smith *et al*. 2012). Extensive analysis of CGIs using reduced-representation bisulphite sequencing demonstrated that significant loss of 5mC occurred during pre-implantation development, although the levels of 5mC in the blastocyst were higher than those expected (Smallwood *et al*. 2011). This is consistent with the studies that have shown resistance of some non-imprinted genes to demethylation during pre-implantation development (Borgel *et al*. 2010, Smith *et al*. 2012) and that the paternal pronucleus maintains a relatively high level of DNA methylation well into embryonic development (Li & O'Neill 2012). Data obtained from human zygotes are also inconsistent; in some cases, ∼30% of the DNA methylation is lost from the paternal pronucleus, while other studies show no loss of methylation (Fulka *et al*. 2004). Similarly, no loss of DNA methylation was observed in either pronucleus of bovine or rabbit zygotes sourced *in vivo* and only partial demethylation was observed in bovine zygotes (Beaujean *et al*. 2004). Therefore, while reprogramming appears to be widespread during pre-implantation development, the data are variable and it appears likely that in addition to imprinted genes, DNA methylation and possibly other epigenetic information may be maintained at some non-imprinted genes.

The clearest examples of inherited epigenetic effects in offspring involve imprinted genes, for which some parent-specific epigenetic marks are maintained throughout life. How this epigenetic information is maintained through pre-implantation reprogramming is poorly understood. However, recent experiments have demonstrated that maternal loss of proteins encoded by *Stella*, *Zfp57* and *Trim28* is associated with loss of genomic imprinting in the zygote and have provided some insights into this process (Nakamura *et al*. 2007, Li *et al*. 2008, Messerschmidt *et al*. 2012, Szabo & Pfeifer 2012). Although it is not yet clear how these proteins protect imprints, loss of these and other maternal proteins may also affect other epigenetic modifications that would normally be retained through pre-implantation embryonic development. Further insights into these, and other similar mechanisms, will be central to understanding how epigenetic information is processed in early development and how epigenetic information provided by the gametes is transmitted through this stage to affect later development.

Conclusions

Both female and male germ cells are derived from PGCs, which undergo genome-wide reprogramming during their migration to the developing gonads. Reprogramming in the germ-line is essential to reset parent-specific epigenetic information, such as genomic imprints, and is potentially required for establishment of sex-specific germ-line development and identity. It is clear that epigenetic information is established during germ-line development and is passed from parent to progeny through the gametes. Thus, epimutations acquired in the parental generation have the potential to be transmitted and affect the development in the next generation(s). This has significant implications for our understanding of inherited disorders and the long-term impact of chemicals and drugs in our environment. Moreover, trans-generational inheritance of environmentally induced epigenetic change may contribute to an organism's ability to adapt to environmental influences. Further studies examining the molecular mechanisms for inter-generational and trans-generational epigenetic effects are essential for understanding the potential for the environment to directly affect a parent's offspring and induce multi-generationally inherited traits and diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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