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DNA Methylation and Demethylation: A Pathway to Gametogenesis and Development

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SUMMARY

The generation of gametes falls between two reprogramming phases. These phases are characterised by profound periods of transcriptional activity, which define and reinforce lineage decisions. The control of these transcriptional programs and the interpretation of the underlying genetic instruction is the task of the epigenome. As such, dynamic processes during reprogramming are critical for the development of the germ line and its resetting, which propels that developmental process forward and provides the transfer of genetic and epigenetic information between generations. Central in this reprogramming is the addition and subtraction of DNA methylation and its oxidative products, coupled to the mechanisms at play to achieve this goal. The activities competent to add DNA methylation, and identification of those enzymes able to modify it, have heralded a new chapter in our understanding of the complexities that dictate and direct cellular fates. How the early embryos makes use of these marks and how they are modulated will give us insight into cellular differentiation and reprogramming critical for health and into the process of aging. This review details some of these processes and the activities essential to achieve the immortality of the mammalian germ line.

"[DNA methylation dynamics] influence lineage and cell-fate decisions during early development and serve to broker branches points that define cell fate, plasticity and pluripotency."

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INTRODUCTION

A seamless continuum of cycles of programming and reprogramming transfers genetic material between generations. These cycles are characterised by the erasure and re-establishment of the interpretative epigenome, which defines and then reinforces cellular fate and supplies the forward only direction we often refer to as development (Hemberger et al., 2009). The newly formed zygote, with a singular genetic plan, gives rise to an ever more complex organism, culminating in a balanced collection of differentiated tissues that remain in their fixed roles throughout the lifetime of the organism. These very processes, which both read and write information and instruction to the underlying genetic code, have fascinated biologists for decades.

Waddington defined the term epigenesis to explain the levels of cellular differentiation culminating in the fully formed organism. Here, cell types arising from common progenitors are canalised to separate fates and defined by a series of descending cellular potentials (Waddington, 1940). This very principle was challenged first by somatic nuclear cloning, effectively reanimating the embryonic stage of life of the organism (Gurdon, 1962; McGrath

Abbreviations: 5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5meC, 5-methylcytosine; CpA/G, cytosine-adenosine/guanosine dinucleotide; CXXC, zinc finger protein binding domain to non-methylated CpG; E, embryonic day; **Proteins**: AID, activation-induced deaminase; APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; DNMT, DNA methyltransferase; DNMT1o/s, DNA (cytosine-5)-methyltransferase1, oocyte/somatic form; DNMT3a/b/L, DNA (cytosine-5)-methyltransferase 3a/b/-like; MBD4, methyl-CpG binding domain 4; NP95, nuclear protein 95; TDG, thymine DNA glycosylase; TET, ten–eleven translocation dioxygenase.

and Solter, 1983; Campbell et al., 1996), and later by reprogramming of a differentiated cell to generate induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Collectively, these two strategies successfully challenged the forward-only direction of development—a breakthrough that led to a Nobel Prize in 2012 for Sir John Gurdon and Shinya Yamanaka. Normally, this retrograde process is not possible or at least not apparent in vivo. Thus, the role of the epigenome is both to restrict and to permit the transfer of characteristics of a species between generations via the germ line.

Our fascination with the four component deoxyribonucleic acid bases that are the building blocks of life has rarely diminished. Early on, it became apparent from hydrolysates of genomic DNA that some bases had other modified forms, such as alkylation groups added to them. Among the most prevalent was a methyl group attached to cytosine that was later verified to be a bona fide biomolecule. These modified bases helped establish the conceptual understanding of a DNA epigenome and a significant part of the wider field we now call epigenetics. In the post-genomics era and some 70 years on from the first identification of 5-methyl cytosine (5meC) (Hotchkiss, 1948), the divisions of labour between the genetic and the epigenetic components of nuclear DNA are clearer. The identification of new cytosine modifications—for example hydroxymethylcytosine (5hmC), whereas others were rediscovered (Penn et al., 1972)—continue to pique our interests in the interplay between these two features of the DNA portion of the epigenome.

DNA methyltransferases (DNMTs) are known to add a methyl group to the carbon-five position of the heterocyclic ring of cytosine. Activities able to further modify this methyl group have recently been discovered. The ten–eleven-translocation (TET) family of three genes all encode the 5mC dioxygenase superfamily activity that is able to modify 5meC to 5hmC and, under appropriate conditions, may catalyse two further oxidative steps to generate 5-formyl (5fC) and 5-carboxylcytosine (5caC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Wu and Zhang, 2011). When paired with genetic approaches, Next-Generation Sequencing (NGS) has paved the way to uncovering these marks, thereby revealing the complexity of these processes in cellular and developmental biology and healthy aging in humans (Ficz et al., 2011).

In this review, I will outline the enzymatic activities involved in mammalian developmental programming and reprogramming cycles, and point out some of their complex partnerships that are essential for full-term development. I will try to establish roles for these reprogramming activities where they are known, and outline significant and slightly obscure mechanistic pathways that modulate DNA methylation in the context of the germ line and early development in the mouse—particularly since genetic manipulations via constitutive and conditional deletion of key players in the DNA methylation pathway have revealed many basic principles for their epigenetic roles. An important caveat to our understanding, however, is that these marks are not fixed throughout the organism's life, often demonstrating exceptional roles in totipotent and pluripotent tissues.

GLOBAL EPIGENETIC REMODELLING IN MAMMALS

DNA methylation is the most comprehensively studied epigenetic modification in mammals (Robertson and Wolffe, 2000). This is undoubtedly a function of our understanding of how methylation marks are inherited during each replication cycle, wherein the cytosine–guanosine dinucleotide (CpG) context is faithfully perpetuated (Razin and Riggs, 1980; Stein et al., 1982; Bird, 2002). It is this very heritability that informs our understanding of this mark as an essential player that reinforces cellular fate following lineage specification in development, where transcriptional states and epigenetic features together define differentiating cell types (Santos et al., 2002). DNA methylation is usually regarded as a repressive transcriptional mark, largely based on studies that identified DNA methylation at promoters of silent genes. This dogma in the canon of DNA methylation has been repeated faithfully, although high-resolution next-generation sequencing of DNA methylation together with genome-wide transcriptomics are revealing a different picture (Suzuki and Bird, 2008). Genome-defence, a special case of transcriptional regulation (repression) required to abate the myriad of retrotransposable elements that litter the mammalian genome and pose a constant threat, remains an essential role for DNA methylation (Bestor, 1998). DNA methylation is also thought to play an independent role in genome stability, especially in and around centromeric satellite sequences and telomeres (Lehnertz et al., 2003).

DNA methylation is globally reprogrammed in two defined periods during mammalian development. First, in the perpetual cycles that ensure the immortality of the germ line, re-establishment and maintenance of DNA methylation is essential (Monk et al., 1987; Reik et al., 2001). Next-generation sequencing of bisulphite-treated DNA from primordial germ cells, gametes, and early embryos is beginning to create significant and accurate base-pair resolution of DNA methylation profiles and supplies quantitative data indicating that genomic methylation is a dynamic process (Popp et al., 2010; Seisenberger et al., 2012; Smith et al., 2012). Second, following fertilisation, the highly specialised gametes must undergo remodelling to establish the ephemeral zygote stage (Santos and Dean, 2004). In the mouse, this period is highly protracted and as such may represent the most extreme case of asymmetric DNA methylation between the two parental haploid genomes among mammals (Dean et al., 2001).

During the post-fertilisation reprogramming window, remodelling of the highly methylated sperm occurs rapidly and is in stark contrast to the female pronucleus, which has just completed meiosis (Mayer et al., 2000; Santos et al., 2002). The nucleoprotamine-based chromatin in sperm is configured in a nearly dehydrated state while that of the oocyte contains a very somatic-cell-like chromatin of

nucleohistone (McLay and Clarke, 2003). In the zygote, reconciling these very different chromatin states is imperative for development to proceed. Running in parallel to this remodelling of both male and female chromatin is the first active loss of DNA methylation, where paternal DNA methylation is rapidly lost prior to replication (Mayer et al., 2000; Oswald et al., 2000). Maternal DNA methylation is retained and, if anything, undergoes cycles of replacement by de novo methylation in the hours following fertilisation and in advance of the first S phase, with little or no net change in total maternal methylation during this first cell cycle.

The first S phase in the newly formed zygote is initiated some 8–10 hr after fertilisation. This cues the first opportunity for passive demethylation to act as a demethylation mechanism (Rougier et al., 1998). The conditions for such a loss of DNA methylation are perfect: The abundance of DNA methyltransferase 1 (DNMT1), the maintenance methylase, is largely neutralised by the active exclusion of DNMT1 protein to the subcortical region (Carlson et al., 1992; Howell et al., 2001), where it resides throughout most of the period of preimplantation, thus supporting a stepwise loss of DNA methylation ostensibly from the maternal genome (Ratnam et al., 2002). Moreover, nuclear protein 95 (NP95) (or UHFR1), an obligate chaperone of DNMT1, is like-wise excluded in the same subcortical space during this period, yet is apparently not functionally associated with DNMT1 (unpublished; Oda et al., 2013).

In contrast to primordial-germ-cell erasure, germ-line imprinted DNA methylation is retained and inherited during this early zygotic reprogramming period. This occurs as a consequence of residual DNMT1s, the somatic form of DNMT1, which operates in the milieu of the highly abundant oocyte form, DNMT1o (Cirio et al., 2008; Dean, 2008). Only on conditional deletion of DNMT1 do we see the loss of imprinted methylation marks from maternal-knockout oocytes fertilised by wild-type males (Hirasawa et al., 2008). This confirms that below the level of resolution of antibody detection, the imprinted loci are undergoing maintenance methylation while the majority of other CpG and non-CpG features lose their methylation in the zygote. Interestingly imprinted genes are not the only group that resists this global loss of DNA methylation. The highly aggressive intracisternal A particle (IAP) retrotransposon family, unique to the mouse, retains DNA methylation in a similar manner to that of imprinted genes, and begs the question as to which class of molecules first employed this mechanism of methylation maintenance during reprogramming (Lane et al., 2003). Importantly, DNA methylation is also retained in centromeric regions in the male pronucleus, where major and minor satellite sequences rely on it to maintain genome integrity and to ensure chromosome segregation at mitosis (Santos et al., 2005; Probst et al., 2007). Here, global loss of paternal DNA methylation coincides with asymmetric expression of satellite RNA transcripts derived predominantly from the paternal haploid genome. These transcripts may be essential to direct ongoing DNA methylation to non-CpG sites in the pericentric satellite region, utilizing a mechanism reminiscent of transcription-dependent regulation of DNA methylation during oogenesis (Chotalia et al., 2009). This essential prelude to chromocentre formation highlights transcriptional roles in the first cell cycle that are essential for development after zygotic genome activation occurs at the two-cell stage (Probst et al., 2010).

DNA METHYLATION REPROGRAMMING

The asymmetric loss of DNA methylation from the paternal pronucleus in a shared cytoplasm is a remarkable feature, particularly since extensive remodelling of chromatin immediately following fertilisation must provide unrestricted opportunity and access to common cytoplasmic factors for both male and female genomes. It is reasonable to assume that this asymmetry is achieved by the nature and epigenome of the contributing, mature gametes. Some explanation may be found in considering the activities and processes within the individual gametes just prior to fertilisation (Buehr and McLaren, 1993; Dean, 2013).

At fertilisation, the mammalian egg is poised to complete meiosis, a process initiated in the embryo. At embryonic day 13.5 (E13.5), the erasure process in primordial germ cells is completed and female gonocytes are arrested in meiotic prophase—the state of the genome that constitutes the beginning of oogenesis (Dokshin et al., 2013). This period from E13.5 to puberty represents a hiatus from the replicative repair and genome reprogramming that occur in dividing cells; in this respect, the oocyte is both unique in its abilities and restrictions. It is in the pre-natal ovary where the genome is restored to a virtually DNA-methylation-free landscape. At E13.5, approximately 5% of the entire genome contains DNA methylation a significant change from the somatic precursor population of an E6.5 day embryo where ~70% CG methylation is found (Seisenberger et al., 2012). Oocyte growth and maturation are initiated postnatally in females, coincident with de novo DNA methylation establishment, which is a product of the DNA methyltransferase DNMT3A together with its non-enzymatic co-factor DNMT3L (Lucifero et al., 2007; Smallwood et al., 2011). Thus DNA methylation returns to the genome via the combined and coordinated activities of DNMT3A/DNMT3L, chromatin components associated with histone modifications, and targeting molecules that escort the DNA methylation enzymes to specific genomic addresses (Quenneville et al., 2011). Methylation is thereby added back in oocytes in a prescriptive manner, by unknown mechanisms, leading to step-wise reacquisition of DNA methylation. This process is accompanied by active transcription that richly supplies the oocyte with RNA and protein.

High-resolution, genome-wide DNA methylation profiles have recently produced interesting and unexpected results that reveal a late-maturation phase, with dynamic methylation added immediately prior to ovulation of the metaphase II (MII) oocyte. Using high-throughput, next-generation sequencing of reduced-representational bisulphite libraries (Meissner et al., 2005), Smallwood and colleagues identified approximately 90 CpG islands (promoter regions with high CG content) that became methylated between the

germinal-vesicle stage and that of the mature MII oocyte, resulting in an extraordinarily high number (>1,000 CpG islands) of methylated sites in addition to imprinted regions (Smallwood et al., 2011). Among those genes with significant CpG-island methylation was the de novo DNA methyltransferase family member *Dnmt3b*, which, like *Dnmt1s*, is transcriptionally silent at this time. This observation typifies an ever-growing concept where epigenetic modifiers are themselves regulated by their cognate marks (Hemberger et al., 2009).

Recent molecular studies have supplied values for DNA methylation on CpG dinucleotides in mature gametes. These values set DNA methylation at ~40% (Kobayashi et al., 2012; Shirane et al., 2013) for oocytes, and approximately double that value for sperm at 80% (Popp et al., 2010; Kobayashi et al., 2012). The mechanism underlying specific loss of such high levels of DNA methylation from the paternal pronucleus has long been the source of much interest, speculation, and debate (Ooi and Bestor, 2008; Gehring et al., 2009; Wu and Zhang, 2010).

THE PATHWAY TO ACTIVE DEMETHYLATION: MYSTERY AND MECHANISM

Active demethylation was first suggested in fertilised oocytes using molecular analysis and indirect immunofluorescence. Using informative alleles and direct visualization, it became apparent that the two parental alleles were treated asymmetrically in the shared cytoplasm of the fertilised egg (Mayer et al., 2000; Oswald et al., 2000). Loss of DNA methylation was thought to rapidly alter the epigenetic landscape, and this reprogramming would thereby harness those enzymes able to remove DNA methylation and hence reactivate gene expression. Moreover, this was not solely restricted to the mouse as most other mammalian embryos are able to alter DNA methylation of a paternal pronucleus, albeit to vastly different degrees (Dean et al., 2001; Beaujean et al., 2004; Fulka et al., 2004). The hunt for a mammalian demethylase orthologue of ROSI and DEMETER, both plant enzymes able to directly break the carbon–carbon bond in 5meC, has failed (Agius et al., 2006; Morales-Ruiz et al., 2006). Instead, there has been a redirect of attention to enzymes involved in DNA repair pathways as they can achieve the same de facto removal of the methyl group. But what was acting upstream to initiate a repair event? Candidate enzymes known to have deaminase activity and shown to be able to deaminate cytosine as well as 5meC have been evaluated (Morgan et al., 2005; Teperek-Tkacz et al., 2011). Members of the activation-induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (AID/APOBEC) family of deaminases are prime candidates, particularly the activation-induced cytidine deaminase (AICDA) responsible for class-switch recombination and somatic hypermutation in activated B cells. These enzymes catalyse the loss of the amine group from methyl cytidine, converting it into thymidine. This base conversion necessitates that the base excision/nucleotide excision/mismatch repair pathway restore the original cytidine, thereby resulting in the net loss of the 5'-methyl group from the cytidine base (Franchini et al., 2012).

A number of groups have pursued this alternative avenue of investigation, focusing on base-excision repair and nucleotide-excision repair activities and their inhibition or depletion, to infer participation in the active demethylation of the paternal pronucleus (Hajkova et al., 2010; Wossidlo et al., 2010). In support of this mechanism, inhibitors of poly-ADP-ribose polymerase 1 (PARP1), a member of the base-excision repair pathway, and of apurinic/apyrimidinic endonuclease 1 (APE1), an essential downstream enzyme needed to generate an abasic site as part of the restoration of the C-G base pair, were found to retain paternal-specific DNA methylation (Hajkova et al., 2010). It is worth stating that *Parp1*-null mice are viable and fertile, and despite showing impaired response to repair, they are not otherwise significantly incapacitated (Pacchierotti et al., 2011). Further, no definitive activity upstream of the demethylation that might be responsible for the methylation loss was identified. Indeed, these authors also excluded a potential role for de novo DNMT3a/3b acting as deaminases in primordial germ cells, contrary to the report in human cells (Kangaspeska et al., 2008; Metivier et al., 2008), even though DNMT3a is abundant in mature eggs and the fertilised oocyte (Lucifero et al., 2007; Hirasawa et al., 2008).

Active demethylation, in the most rigorous sense, involves direct loss of the methyl group from the 5'-position of methylcytosine and is thought to occur outside of S phase. Several reports have proposed mechanisms that meet this criterion: Arising from a candidate-based screen, components of the transcription elongation complex, elongator complex protein 3 (ELP3), have been implicated in the active loss of DNA methylation. When ELP3 and related activities (ELP1 and ELP4) were depleted via interference (RNAi), paternal methylation was maintained, as measured by direct live-cell imaging using a CXXC–EGFP fusion protein approach, which served as a reporter for paternal demethylation as it binds to unmethylated CpG-rich regions in the absence of DNA methylation (Okada et al., 2010). Why such an activity would be involved in this process was not immediately apparent: ELP factors contain a radical S-adenosyl methionine (SAM) domain that can demethylate via a direct interaction with the methyl group of 5meC, leading to the formation of formaldehyde as a neutral leaving group (Wu and Zhang, 2010); this is not the first time such a mechanism of free-radical elimination has been proposed. Among the first candidate demethylases was methyl-binding domain protein 2 (MBD2), first described in cancer cells (Bhattacharya et al., 1999). Its demethylating activity has not, however, been independently verified in noncancerous cells, and *Mbd2*-null oocytes still undergo loss of DNA methylation in the same timeframe as the matched controls (Santos et al., 2002).

Very recently, the gonad-specific expression (*Gse*) gene transcript was knocked down by RNAi and evaluated using immunofluorescence and bisulphite sequencing of Line 1 elements as well as several other pluripotency genes

known to have promoter methylation. While imprinted genes (e.g. *H19*) were unaffected, Line 1 elements and other features showed a convincing increase in DNA methylation upon *Gse* knockdown, consistent with a role for this protein in the loss of DNA methylation (Hatanaka et al., 2013). How this might take place is completely unknown, although clues might be found in considering roles in conjunction with the TET family of activities.

All TET family members encode 5-methylcytosine oxidase activity that is able to modify 5meC to 5hmC. TET1 was first identified in Purkinje cells of the brain, and later in mouse embryonic stem cells (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009); since then, two other family members, TET2 and TET3, both possessing the dioxygenase motif, have been identified (Ito et al., 2010; Ko et al., 2010). These activities commonly require Fe(II) and alpha-ketoglutarate binding to complete the oxidation of 5me to 5hmC. More importantly, there is strict spatiotemporal regulation of the TET dioxygenases, with TET3 found to accumulate in eggs and fertilised oocytes (Iqbal et al., 2011; Wossidlo et al., 2011). Interestingly the TET3 transcript is quickly degraded after fertilisation, leaving little or none by the two-cell stage (Iqbal et al., 2011). This activity and abundance profile was the first indication that loss of 5meC could occur without the removal of the base, but by merely modifying the methyl group in situ. Antibody tools were quickly made available to detect 5hmC, thereby allowing the evaluation of TET-dependent reprogramming of the fertilised oocyte (Ficz et al., 2011; Inoue and Zhang, 2011; Iqbal et al., 2011). Double labelling of mature, pronuclear-staged fertilised oocytes clearly demonstrated that the paternal loss of DNA methylation coincided with a gain of 5hmC (Inoue and Zhang, 2011; Iqbal et al., 2011). Staining preimplantation stages with an antibody to 5hmC indicated that a passive, replication-based loss of signal occurred. 5hmC marks cannot be maintained, as neither the de novo DNA methylase, DNMT3a, nor the maintenance methylase, DNMT1, are able to restore the 5mC (Inoue and Zhang, 2011; Inoue et al., 2011).

The 30-fold enrichment of transcript compared to TET1 and TET2 indicated that TET3 was the enzyme likely responsible for the 5hmC accumulation in the zygote (Iqbal et al., 2011). The abundance of TET3 transcripts in the egg and fertilised oocyte has been confirmed by a number of studies using a variety of inbred mouse strains (Wossidlo et al., 2011). To investigate a mechanistic connection between TET3 and hydroxymethylation, Wossidlo et al. (2011) used a small interfering RNA (siRNA) approach to deplete the considerable store of TET3 transcript. Reduction of TET3 abrogated 5hmC, but more importantly this resulted in a commensurate increase in 5mC—thus cementing the model whereby 5mC was directly modified to form 5hmC in situ. This reciprocal asymmetry was not restricted to the mouse, but was widely conserved in both rabbit and bovine zygotes (Wossidlo et al., 2011). Gu et al. (2011) used a genetic approach to conditionally delete *Tet3* from the oocyte or germ cell; subsequent immunofluorescence staining for 5hmC in the zygote confirming that TET3 was responsible for the 5hmC in the paternal pronucleus. In contrast to the findings of Wossidlo et al. (2011), maternal deletion of TET3 activity that resulted in the loss of 5hmC was not accompanied by an increase in 5mC. Furthermore, normal programming of the proximal enhancer of *Oct4* was affected by maternal deletion of *Tet3* and a reduced ability to reprogram somatic nuclei following somatic nuclear transfer was also reported (Gu et al., 2011).

These early results formally gave credence to the model of 5hmC as an oxidative intermediate in the active loss of DNA methylation. As such, an expectation that the oxidative end products formyl (5fC) and carboxyl cytosine (5caC) existed seemed inevitable. Ultra-sensitive isotopic methods had sought to establish these intermediates as downstream products in tissues undergoing DNA demethylation. Despite confirming the widespread presence of 5hmC, no further derivatives were identified (Globisch et al., 2010). Casting a wider net to cell types where demethylation might be taking place, several labs have identified 5fc and/or 5caC in embryonic stem cells (He et al., 2011; Ito et al., 2011; Pfaffeneder et al., 2011). Furthermore, biochemical results to identify 5caC recognition sites led to the suggestion that thymidine DNA glycosylase (TDG) might be involved in a DNA-repair-based mechanism. Thus, loss of the carboxyl group to restore a cytosine does not require a decarboxylase per se but rather the presence of TDG to resolve the cytidine-to-guanidine pairing (He et al., 2011; Maiti and Drohat, 2011).

Formyl and carboxyl cytosine are also found in the fertilised zygote and the ensuing preimplantation stages of development. These oxidative products can be detected, using antibodies, in the paternal pronucleus during the first cell cycle and thereafter in each preimplantation stage. Exquisite quality chromosome spreads of staged embryos throughout preimplantation showed that, rather than being eliminated by an enzymatic process, these marks were observed to decline, in keeping with replication-based half-fold dilution, suggesting that they were stable and implying a function separate from the demethylation pathway (Inoue and Zhang, 2011).

TETs AND DNMTs: MARKS AND MECHANISM

Conversion of 5meC to 5hmC in the paternal pronucleus of the zygote is dependent on TET3 enzymatic activity. Thereafter, iterative oxidation to 5fC and 5caC is likewise a result of the same enzymatic activity (Tan and Shi, 2012). The dynamics of this process are not continuous, however, and despite the presence of substrate, the female pronucleus remains methylated, only losing DNA methylation as a consequence of passive replication loss owing to the exclusion of DNMT1 (Cardoso and Leonhardt, 1999). Despite the extraordinary sensitivity of anti-5hmC antibodies, 5hmC is not detectable before pronuclear stage 3a (prior to replication), when DNA replication is first initiated. Gan et al. (2013) confirmed that sperm is relatively highly hydroxymethylated, at levels slightly lower than embryonic stem cells. 5fC is present and detectable in both compartments of the zygote from the earliest stages after

fertilisation, suggesting that it may also be inherited by both gametes. Significant levels of 5caC are only detected by 8-hr post-fertilisation in the male pronucleus, at a time when 5fC is also abundant. Thus, the simultaneous appearance of the three TET3-dependent modifications suggests that, while there is evidence for a step-wise progression, it is by no means quantitative. As such, all four marks are both present and subsequently decline during cleavage-stage cell divisions.

Expression of the family of TET dioxygenases and their partnership with DNMTs is particularly relevant when considering the dynamic acquisition and loss of the epigenetic quartet of modified cytosine. Interestingly, the trio of TET dioxygenases is rarely expressed in any single cell type. During oogenesis, TET3 transcript is first apparent early on post-natally in the primary follicle and accumulates throughout this period (Gu et al., 2011). At fertilisation, reverse transcriptase PCR indicates there is a further increase compared to the ovulated MII oocyte (Gu et al., 2011). Nonetheless, TET3 is the only cytosine hydroxylase present in detectable amounts at the time of fertilisation (Gu et al., 2011).

Despite the early presence of the *Tet3* transcript, it is not immediately clear whether the 5hmC inherited by the oocyte derives from TET3 or another TET member. TET1 activity is essential for germ-cell development and deletion of TET1 results in the specific loss of female meiosis-specific gene expression, suggesting it has a demethylation role at a small number of key functional loci rather than in genome wide-loss of methylation (Yamaguchi et al., 2012). Female primordial germ cells are virtually without DNA methylation at the time of meiotic arrest (Guibert et al., 2012; Seisenberger et al., 2012). Hence it is reasonable to assume that the 5hmC accumulation in the germinal vesicle-staged oocyte must occur in parallel with re-establishment of DNA methylation (by DNMT3a/3L) or shortly thereafter, but in the absence of DNA replication. This is notable because the fertilised oocyte is replete with TET protein, yet little conversion of 5meC to 5hmC takes place prior to replication (Gu et al., 2011; Wossidlo et al., 2011). Conditional deletion of *Tet3* will be required to fully evaluate this prospect.

Commensurate with the rich provision of *Tet3* transcripts, TET3 protein can be detected in the germinal vesicle-stage oocyte– yet the female pronucleus remains unmodified and possesses minimal 5hmC. Gu et al. (2011) detected TET3 protein exclusively in the male pronucleus. Such definitive asymmetry of the protein is consistent with the asymmetry of the 5hmC, 5fC, and 5caC populations in the paternal genome during the first cell cycle. It is important to remember that these marks are not exclusive to the paternal compartment; rather, they are merely more abundant in the male pronucleus than the female. While there are clearly significant quantitative variations reported between mouse strains, the impression sometimes conveyed is not supported by the data (Inoue et al., 2011; Iqbal et al., 2011; Salvaing et al., 2012).

The parental asymmetry of 5hmC in zygotes is maintained thereafter during preimplantation, suggesting that these oxidative marks may serve another purpose, such as offering an enduring stamp of parental origin much like that reinforced at imprinted genes throughout the lifetime of an organism. This asymmetry also sets the essential placement for further oxidative modifications in the genome. In replicating cells, DNMT1 restores methylation to the new strand in conjunction with NP95 to maintain DNA methylation at CpG sites (Sharif et al., 2007). Further modifications of 5meC, however, require the targeting of TET dioxygenases to some of these same genomic loci. This targeting may be achieved, at least in the case of TET1 and TET3, via its CXXC domain, which binds to unmethylated CpG sites densely clustered in CpG islands. This binding motif thus places TET dioxygenases at promoter regions, providing a mechanistic link with the DNA methylation machinery that likewise possesses this CpG zinc-finger domain. Together with a significant cohort of other CXXC-containing proteins, TET dioxygenases and DNMTs combine to create a complex with bivalent functions at the level of cytosine modification(s) that can specify both transcriptional activation, by direct binding of TET1 at pluripotency genes, and repressive chromatin configurations, via the polycomb repressive complex PRC2 (Spruijt et al., 2013).

MBD4 AND TDG: REMOVING THE OXIDATIVE PRODUCTS

Active demethylation in the fertilised oocyte has been suggested to take place via base-excision repair. The precise pathway and the normal resolution of the mismatch event, however, are far from the canonical pathway described for embryonic stem cells or differentiated somatic cells (Sjolund et al., 2013). The mismatch repair pathway is activated as a result of an altered or damaged nucleotide base pair. A frequent transition mutation, needing repair, is that of a 5meC to thymine that passively arises by spontaneous hydrolytic deamination but may also result as a consequence of enzymatic activity of one of the AID/APOBEC family enzymes. There are two activities capable of removing the mismatched thymine–guanosine base pairing, that of methyl binding domain 4 (MBD4) and thymine DNA glycosylase (TDG) (Cortazar et al., 2007). MBD4 is highly specialised in this role, and is able to remove both thymine and the oxidised-and-deaminated 5′-hydroxymethyluracil (5hmU), a product of 5hmC, as its preferred substrate, thereby restoring the cytosine–guanosine pairing (Morera et al., 2012). TDG has long been implicated in DNA repair, but of particular significance is its ability to function as an epigenetic regulator in the removal of the serial oxidation products of 5hmC, 5fC, and 5caC (Cortazar et al., 2007; Hashimoto et al., 2012; Morera et al., 2012). This broad repertoire of activity can take place in single-stranded or duplex DNA with high efficiency. Interestingly, TDG has reduced efficiency to excise 5hmU in single-stranded DNA, but along with MBD4 and single-strand selective monofunctional uracil DNA glycosylase 1 (SMUG1), TDG has good efficiency with this mismatch in the duplex. MBD4, SMUG1, and other glycosylase family

members alone have little or no capacity to remove the 5fC and 5caC derivatives (Morera et al., 2012). What emerges is a complex network of repair activities that, when coupled to their individual enzyme specificities, complement the downstream serial-oxidative products arising from 5meC, thus creating an additional level of regulation (Fig. 1). This coupled interaction has recently been shown to have significant impact on transcriptional processes in embryonic stem cells (Shen et al., 2013; Song et al., 2013).

This specific division of labour helps to explain the somewhat unexpected pattern and timing of dynamic fluctuation of the downstream oxidative products of 5meC. The mature oocyte has little or no TDG or MBD4 (Hajkova et al., 2010; BioGPS). Consequently, any and all of the 5fC and 5caC generated from 5hmC present in both the MII oocyte and sperm (Gan et al., 2013) is destined to undergo passive reduction during preimplantation development owing to the absence of any enzymes able to remove it. By the blastocyst stage, however, TDG is expressed and able to resolve this mismatch, abrogating the need for a decarboxylase to restore the cytosine–guanosine base pair. As such, there is the real possibility that 5fC and 5caC are much more than simply intermediates of 5meC metabolism; they may instead influence transcriptional activation of the zygotic genome at the two-cell stage and beyond. Extending these principles from embryonic stem cells would imply specific, functional roles for each of the iterative marks of 5hmC early in development (Shen et al., 2013; Song et al., 2013).

HISTONES: ASYMMETRY AMPLIFIERS

In the simplest scenario, metabolism of DNA methylation would progress if 5meC was converted to 5hmC and, in turn, restored as the demethylated product cytosine. In this mechanism, the most reduced form of the base is replaced quantitatively by the next iterative oxidised state. That all four states can exist simultaneously implies that a more sophisticated form of regulation must exist. Indeed this is the case for the loss of 5meC from the male, but not the female, pronucleus after fertilisation. This lends credence to the idea that a read-write-and-protect mechanism for 5meC must be able to operate in the first cell cycle. This is accomplished, in part, in the maternal pronucleus owing to a key feature of chromatin that is absent from the male pronucleus.

It has been known for some time that maternal and paternal chromatin have asymmetric modifications of core histones that are temporally put in place during the immediate post-fertilisation period (Arney et al., 2002;

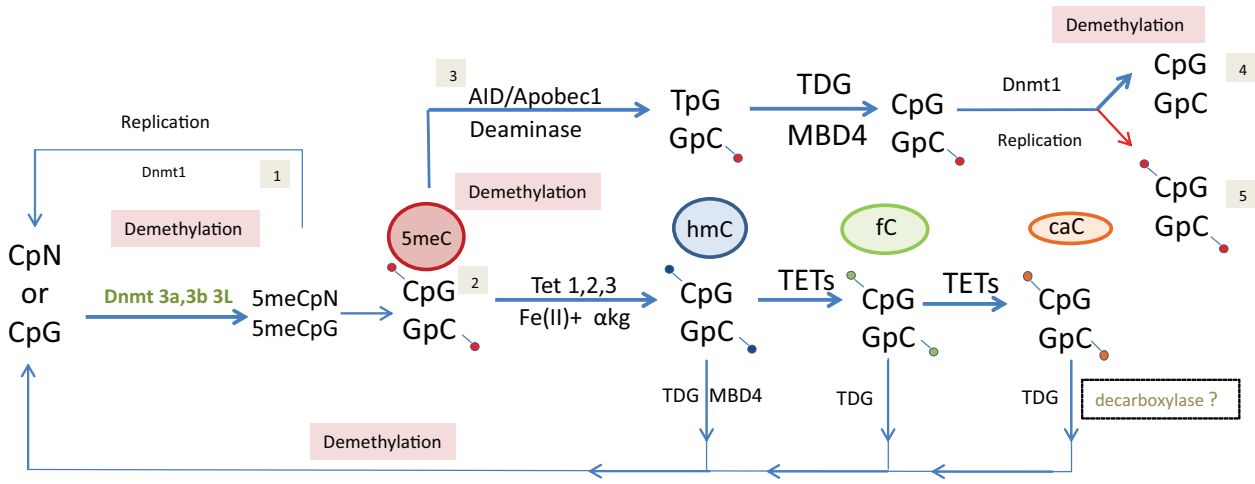

Figure 1. Pathways to demethylation. The acquisition of DNA methylation is the product of de novo methylation by DNMT3a, 3b, and 3L at cytosine either in the canonical dinucleotide CpG or in one of the non-CpG contexts. (1) In the non-CpG context, during replication, DNA methylation cannot be read by the maintenance methyltransferase DNMT1, and hence results in loss of DNA methylation. In the CpG context, this methylation can be faithfully replicated. (2) In the presence of the TET dioxygenases and their appropriate cofactors Fe (II) and the metabolic intermediate α-ketoglutarate, the 5meC group is oxidised serially: 5-hydroxylmethyl cytosine (hmC) becomes formyl cytosine (fC) and then carboxy cytosine (caC), culminating in a return to the unmodified base cytosine. Iterative oxidation is only possible in the absence of TDG and MBD4, however, as is the case for the fertilised mouse oocyte and the early cleavage-stage embryos. This mechanism may offer some explanation for the observed demethylation in these stages. TDG can act on and remove any of the iterative oxidative products of 5meC. MDB4 shares only partial function with TDG, as it is only able to repair the mismatched 5hmC. (3) Methyl cytosine may also be modified by another pathway. The presence of AID or APOBEC1 directs the methyl group along a different path. A mismatched thymine–guanosine arising from deamination of 5meC is repaired by TDG/MBD4, leaving a hemi-methylated CpG, which in replicating cells or tissues, leaves one fully methylated strand and one fully demethylated strand. (4) Loss of DNA methylation at unmethylated CpG sites supplies the substrate for the binding of CXXC-domain proteins such as de novo methylases. This type of passive demethylation has been described during the reprogramming of primordial germ cells. (5) The fully methylated strand is now a substrate for methyl binding domain proteins (MBDs), and thus defines a branch point from a common progenitor, the hemi-methylated dinucleotide.

Santos et al., 2005). As a consequence, key histone modifications that are ordinarily associated and mechanistically linked with DNA methylation must be uncoupled for active demethylation of the paternal pronucleus to proceed (Liu et al., 2013). As such, at the time of fertilisation, the female pronucleus has already organised its chromatin to include dimethylated histone H3 lysine 9 (H3K9me2), a post-translational modification of the histone tail that interacts intimately with DNA methylation to reinforce a silent transcriptional configuration (Santos et al., 2005). The male pronucleus will also incorporate H3K9me2 into its chromatin, but in a replication-dependent manner and only after initiation of the early loss of DNA methylation, at a time when 5hmC is becoming readily detectable. Recent studies reveal that this protection of the female pronucleus is accomplished through direct binding of the H3K9me2 by the maternal factor Stella (or DPPA3), a pluripotency factor normally associated with embryonic stem cells and primordial germ cells (Nakamura et al., 2012). Although Stella is found in both compartments of the fertilised oocyte, extraction of fertilised oocytes prior to fixation and staining displays a clear retention of Stella in chromatin in the female pronucleus whereas it is readily removed from the male pronucleus (Nakamura et al., 2012). This binding, in turn, affords additional protection from TET3 and subsequent oxidation to 5hmC, but exactly how this takes place is at present unknown. This association operates both genome-wide and in the context of specific imprinted genes, where histone-based chromatin in the sperm bound to Stella is similarly protected and DNA methylation is retained (Nakamura et al., 2012).

ASYMMETRIC DNA METHYLATION

DNA methylation is added de novo during germ-cell development. The context for this methylation may depart significantly from the CpG dinucleotide, however, since it is thereafter maintained by DNMT1. Cytosine methylation can be found in other symmetric (CHG) and asymmetric (CHH) configurations (where H is adenosine, thymine or cytosine) in addition to hemi-methylated CpG. This pattern is found commonly in the epigenome of plants, where specific chromo-methylases are able to maintain it by acting in repression of retrotransposons and repetitive elements (Law and Jacobsen, 2010). In mammals, especially in embryonic stem cells, recent reports have reignited an interest in non-CpG methylation (Lister et al., 2009; Stadler et al., 2011). Early on, Ramsahoye et al. (2000) reported significant levels of non-CpG methylation comprising 15–20% of the total DNA methylation in embryonic stem cells. This non-CpG methylation is not entirely random, preferring cytosine-adenosine dinucleotides (CpA) to other contexts, which may hint at functional significance. Considerable scepticism concerning methodology and variability of reporting, however, demanded more intensive investigation to resolve the issue. Recently, analysis of human and mouse embryonic stem cell and induced pluripotent stem cells, at base-pair resolution, indicates a significant proportion of non-CpG methylation, rising as high as 25% overall, with specific enrichment at gene bodies, lending support for their role in the epigenome (Lister et al., 2009).

Non-CpG methylation is not exclusive to pluripotent cells in culture. Oocytes and sperm both accumulate significant levels of non-CpG methylation, with differential temporal dynamics for acquisition and loss. At CG-rich genomic regions, mature oocytes contained approximately 40% of their total cytosine methylation within asymmetric sites, with nearly 27% falling within the CpHpHp category (Tomizawa et al., 2011). Furthermore, in-depth whole-genome bisulphite analysis indicated that this non-CpG methylation was dynamic and that non-growing oocytes had little or none, although germinal vesicle-stage oocytes had nearly 66%. Mechanistically, conditional maternal deletion of *Dnmt3a*/*3L* eliminated non-CpG methylation in germinal vesicle-stage oocytes (Shirane et al., 2013). In contrast to the oocyte, mature sperm contains less than 0.5% non-CpG methylation. Detailing the dynamic process in sperm maturation identified that during the mitotically arrested pro-spermatogonial stage, non-CpG methylation accumulates in a DNMT3a-dependent fashion. Upon mitotic resumption, this asymmetric methylation, found predominantly in the CpA context, is lost (Ichiyanagi et al., 2013). In the male germ line, restoration of DNA methylation additionally requires the PIWI pathway of small RNAs, which also serves an essential role in the silencing of retrotransposons. Paternally imprinted genes have essentially expanded this mechanism such that mature sperm is highly methylated (Kobayashi et al., 2012).

Despite the abundance of non-CpG methylation in the oocyte, this signal is quickly eroded owing to the failure of DNMT1 to maintain it during post-fertilisation replication. As such, these genomic signatures, which have been inherited transgenerationally, are largely lost by the blastocyst stage. This creates the interesting prospect that the paternal compartment is largely influenced by the early, active loss of CpG methylation whereas the female is passively demethylated, losing mostly non-CpG methylation (Fig. 2). Whether or not asymmetric loss of methylation from the male and female pronuclei is functionally significance for the zygote and subsequent embryo is still not known. What remains possible is that with an increase in 5meC, the threat of mutation by deamination of cytosine, the most common such event in mammals, creates an additional need for enhanced DNA-repair capacity in the growing oocyte beyond the requirements arising from active transcription and meiotic recombination (Baarends et al., 2001).

CONCLUSION

Genome-wide studies with single-base resolution are changing the face of the epigenetic roles for DNA methylation. Technology development to accommodate the small cell numbers of embryos undergoing reprogramming have provided a detailed and deeper understanding of the state

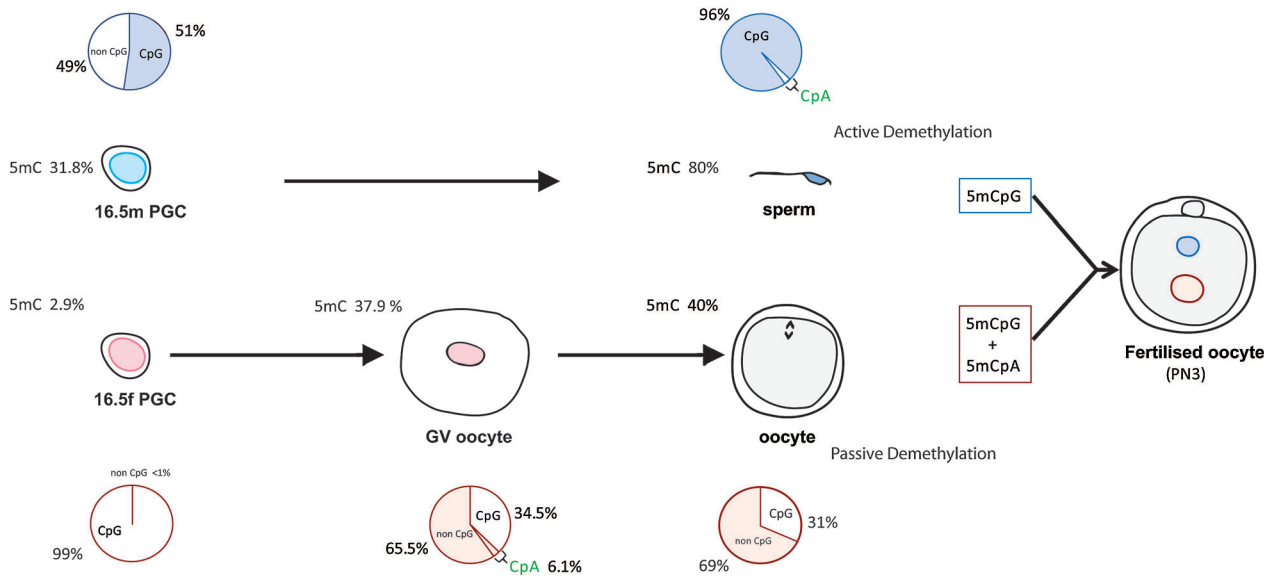

Figure 2. Dynamic reprogramming of DNA methylation during gamete maturation and on fertilisation. DNA methylation asymmetry starts in the gonocytes. In the developing germ line, erasure of DNA methylation is completed by E13.5 in females and E14.5 in males. At this time, the female gonocytes arrest at meiotic prophase and cease to divide. As such, oocyte growth and maturation is completed in the absence of DNA replication. De novo DNA methylation in females coincides with oocyte growth and is initiated post-natally. The trajectory in males is different. Male gonocytes are temporarily arrested in mitosis at E14.5, but resume mitotic activity shortly thereafter. At this time, de novo DNA methylation is resumed such that there is a significant increase in the average DNA methylation to 31.8%. Interestingly, this adds DNA methylation equally in both the canonical CpG context as well as the noncanonical or asymmetric CpHpGp and CpHpHp sites. Post-natal resumption of mitosis consequently results in a loss of virtually all noncanonical DNA methylation, with mature sperm acquiring an average DNA methylation of 80%. This is in stark contrast to the situation in the female germ line. At E16.5, female gonocytes still have little or no DNA methylation (average DNA methylation 2.9%); only methylated CpG sites are detected. The post-natal period for the female germ line is typified by significant addition of DNA methylation genome-wide. The fully grown oocyte, known as the germinal vesicle (GV)-stage oocyte, now has a complex mixture of DNA methylation (average level at 37.9%) with a majority of methyl CpG in the noncanonical form (65.5% non-CpG). In each reproductive cycle, GV oocytes undergo a transition from meiosis I to meiosis II, producing the ovulated MII oocyte. In this last 48-hr period, an additional 10% of DNA methylation (average 40% at ovulation) is added, leaving the oocyte with a predominantly asymmetrical methylated pattern of cytosine. Thus at fertilisation, the remodelling of maternal and paternal chromatin takes place on vastly different epigenomes. The loss of DNA methylation from paternal alleles allows for a rapid depletion of genomic methylation, suggesting an active process during early post-fertilisation stages. In the female, where DNMT1-dependant maintenance of imprinted methylation marks is required, the majority of DNA methylation will not be maintained as it is in the non-CpG context, and therefore is not read or restored by DNMT1. Thus, passive demethylation in the female occurs even in the presence of DNMT1. As such, prior to zygotic genome activation at the two-cell stage, DNA methylation has already been significantly reprogrammed. These dynamic changes in the epigenetic landscape may well have a significant influence on the maturation of gametes and implications for fertility in animals and humans.

and genomic positioning of DNA methylation, the activities which add and erase the marks, and the mechanism by which this is possible. The long-standing view about the stability of DNA methylation versus the dynamic and cyclic nature of this mark, as modified in the consecutive oxidative metabolism supplied by the TET dioxygenases, continues to open new avenues for investigation. What appears to be emerging is a highly dynamic and tightly integrated system that encompasses chromatin-based regulation in conjunction with DNA methylation machinery to specify and reinforce transcriptional instructions. These influence lineage and cell-fate decisions during early development and serve to broker branches points that define cell fate, plasticity, and pluripotency.

Defining the basics is just the very beginning of our understanding of how the overlying epigenome orchestrates the program of development and ensures the ultimate immortality of the germ line. The exquisite beauty of the details is being revealed daily in an unrelenting pace. New technologies, especially the advent of single-cell epigenomics combined with high resolution imaging, will drive the field forward to supply a clearer understanding of the mechanistic roles for the marks supplied by DNA methylation and their significance in gametogenesis and development.

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