REPRODUCTION

REVIEW

Epigenetic dynamics during preimplantation development

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Abstract

Successful mammalian development requires descendants of single-cell zygotes to differentiate into diverse cell types even though they contain the same genetic material. Preimplantation dynamics are first driven by the necessity of reprogramming haploid parental epigenomes to reach a totipotent state. This process requires extensive erasure of epigenetic marks shortly after fertilization. During the few short days after formation of the zygote, epigenetic programs are established and are essential for the first lineage decisions and differentiation. Here we review the current understanding of DNA methylation and histone modification dynamics responsible for these early changes during mammalian preimplantation development. In particular, we highlight insights that have been gained through next-generation sequencing technologies comparing human embryos to other models as well as the recent discoveries of active DNA demethylation mechanisms at play during preimplantation.

Reproduction (2015) **150** R109–R120

From zygote to blastocyst

Mammalian preimplantation development is a time of dynamic change in which the fertilized egg undergoes cleavage divisions developing into a morula and then a blastocyst with the first two distinct cell lineages (inner cell mass (ICM) and trophectoderm (TE)). This developmental period is characterized by three major transitions, each of which entails pronounced changes in the pattern of gene expression. The first transition is the maternal-to-zygotic transition (MZT), which serves three functions: i) to destroy oocyte-specific transcripts (e.g., H1oo (Tanaka *et al*. 2001)), ii) to replace maternal transcripts that are common to the oocyte and early embryo with zygotic transcripts and iii) to facilitate the reprogramming of the early embryo by generating novel transcripts that are not expressed in the oocyte (Latham *et al*. 1991). In mice, zygotic gene activation initiates during the 1-cell stage, and is clearly evident by the 2-cell stage (Latham *et al*. 1991, Schultz *et al*. 1993). Coincident with genome activation is the implementation of a chromatin-based transcriptionally-repressive state (Nothias *et al*. 1995, Schultz 2002) and more efficient use of TATA-less promoters (Majumder & DePamphilis 1994), which are likely to play a major role in establishing the appropriate pattern of gene expression required for successful development.

The second developmental transition is compaction, which occurs during the 8-cell stage, when the first morphological differentiation occurs due to adhesive interactions between the blastomeres generating a tightly organized and less distinct mass of cells (Fleming *et al*. 2001). Accompanying compaction are pronounced biochemical changes through which blastomeres acquire characteristics resembling somatic cells, reflected in such features as ion transport, metabolism, cellular architecture and gene expression pattern (Fleming *et al*. 2001, Kidder & Winterhager 2001). Following compaction, cleavage divisions allocate cells to the inside of the developing morula. These inner cells are set aside between the 8-cell and 16-cell stage, and then again between the 16-cell stage and the 32-cell stage (Pedersen *et al*. 1986). The inner cells of the morula give rise to the ICM cells from which the embryo proper is derived, whereas the outer cells differentiate exclusively into the TE, which gives rise to extraembryonic tissues (Yamanaka *et al*. 2006). The TE is a fluid-transporting epithelium that is responsible for forming the blastocoel cavity and is essential for continued development and differentiation of the ICM (Biggers *et al*. 1988, Watson *et al*. 1990). Distinct differentiation first occurs in the blastocyst and is characterized by differences in gene expression between the ICM and

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-15-0180
Online version via www.reproduction-online.org

TE cells (Nichols & Gardner 1984, Pesce & Scholer 2001). Additionally, by the time of implantation the primitive endoderm has differentiated from the ICM/epiblast and resides as a single-cell layer on the blastocoel cavity side of the ICM/EPI (reviewed in Schrode *et al*. (2013)).

These dynamic morphological, cellular and molecular events are driven by gene expression changes facilitated by epigenetic phenomenon, including DNA methylation and histone modifications at sites throughout the genome. Below we review the current understanding of the mechanisms responsible for regulation of epigenetic programming and re-programming that occur during mammalian preimplantation.

DNA methylation dynamics in the preimplantation mouse embryo

In mammalian cells, the predominant form of DNA methylation occurs at CpG dinucleotides. Throughout the genome, non-promoter associated CpGs are generally found methylated. However, the majority of protein coding genes have regions of high-density CpG dinucleotides termed CpG islands. In most cell types the methylation status at these promoter associated CpG islands correlate with the transcriptional activity of the locus – actively transcribed genes generally are not methylated while silenced genes are often found to be heavily methylated in the promoter island. Additionally, there is growing evidence that CpG islands found outside of transcription start sites play functional roles (Saxonov *et al*. 2006, Illingworth *et al*. 2010, Maunakea *et al*. 2010). While DNA methylation at gene promoters is traditionally thought to act as a binary switch (methylated, silent; unmethylated, active), it appears that CpG density, not just presence of methylation alone, also contributes to regulation of expression. For example, methylation at low CpG dense promoters still allows for transcriptional activity (Fouse *et al*. 2008). Furthermore, there are numerous examples, particularly of non-coding RNAs, that are transcribed, although the allele is heavily methylated (Bartolomei *et al*. 1993, Takada *et al*. 2002, Sleutels *et al*. 2003). These examples highlight that while there are general correlations of methylation status and gene activity, individual loci vary greatly.

In mammals, the molecular machinery responsible for adding a methyl group to cytosine residues (resulting 5-methylcytosine (5mC)) has been identified as a family of DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b,and Dnmt3l). Dnmt3a and Dnmt3b are responsible for *de novo* methylation and play partially redundant but independently essential roles during early development. This includes methylation of repeat regions, imprinted loci, as well as genes involved in lineage decisions (Okano *et al*. 1999, Bourc'his *et al*. 2001, Kaneda *et al*. 2004). More specifically, Dnmt3a and Dnmt3b help to establish *de novo* methylation in the blastocyst, allowing global 5mC levels to increase to that of somatic cells following implantation (Smith *et al*. 2012). Dnmt3a is maternally loaded in the oocyte and is the predominate methyltransferase in the oocyte and zygote (Kaneda *et al*. 2004, Kato *et al*. 2007), whereas Dnmt3b is transcribed upon zygotic genome activation (Watanabe *et al*. 2002) and is the primary mediator of *de novo* methylation during implantation (Borgel *et al*. 2010). Knockout studies in mice show that each of the Dnmts is required for viability (Li *et al*. 1992, Okano *et al*. 1999), highlighting the essential nature of *de novo* and maintenance methylation during development.

Dnmt1 has two functional transcripts that are expressed during development – Dnmt1s is expressed in somatic cells while Dnmt1o is specifically expressed as an oocyte specific form (Rouleau *et al*. 1992, Gaudet *et al*. 1998, Mertineit *et al*. 1998). Unlike Dnmt3a and Dnmt3b, Dnmt1 maintains CpG methylation by recognizing hemimethylated DNA and methlyating the unmethlyated strand, ensuring 5mC is maintained through DNA synthesis (Leonhardt *et al*. 1992, Arand *et al*. 2012). Targeting of Dnmt1 to replication foci occurs in most proliferating cells (Kishikawa *et al*. 2003, Bostick *et al*. 2007), however, Dnmt1o/s is largely excluded from the nucleus during early preimplantation stages (Howell *et al*. 2001), likely to allow for the large-scale demethylation that occurs to both haploid genomes (Figs 1 and 2).

Both sperm and oocytes contain parent- of- origin specific 5mC patterns. Therefore, at the time of fertilization the two haploid genomes arrive with diverse epigenomic signatures. Both parental pronuclei undergo dramatic global demethylation, presumably to ensure similar epigenetic information at the two parental alleles of the majority of genes (imprinted loci being one exception) as well as to program the newly formed zygote to a totipotent state. The male haploid genome is heavily methylated in sperm, where between 80 and 90% of all CpG dinucleotides are methylated (Mayer *et al*. 2000, Oswald *et al*. 2000, Santos *et al*. 2002). Global DNA methylation levels in the maternal haploid genome are approximately half that of the sperm (Howlett & Reik 1991, Smallwood *et al*. 2011, Peat *et al*. 2014). Shortly after fertilization, the two parental genomes undergo distinct but equally dramatic waves of DNA demethylation. The paternal genome undergoes active, replication-independent demethylation within the first several hours post-fertilization. In contrast, the maternal genome largely undergoes passive, cell division-dependent diffusion of methylation, resulting in demethylation over the course of preimplantation development.

Active DNA demethylation during preimplantation development by Tet3 oxidation of 5mC

Demethylation begins immediately in the newly formed embryo, prior to the first cell division. By the time the

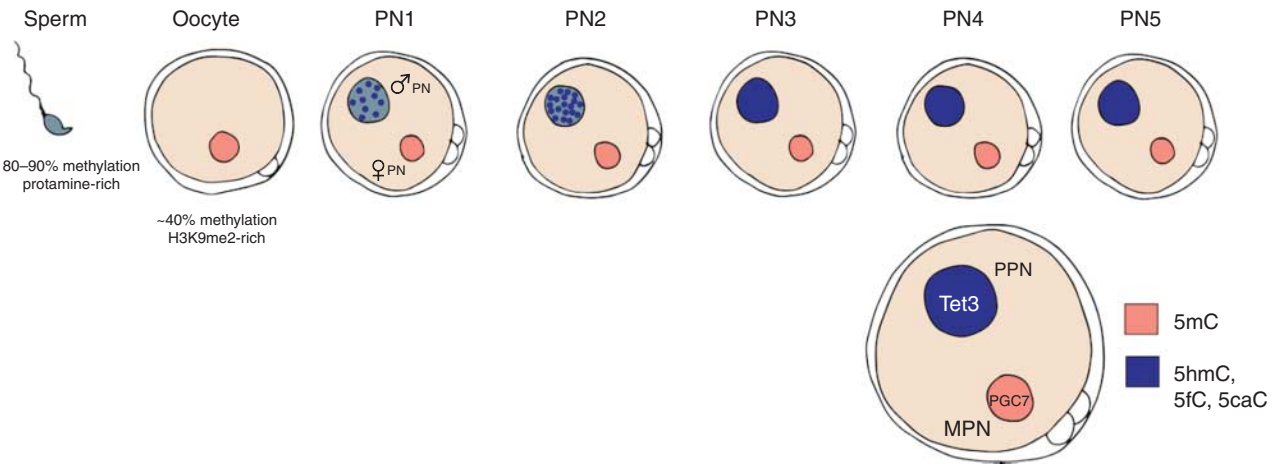

Figure 1 DNA demethylation in the zygote. Distinct demethylation dynamics occur in the maternal (pink) and paternal (blue) pronuclei prior to fusion. At fertilization, the maternal haploid genome has ~40% methylation compared to nearly 90% methylation in the paternal haploid genome. Upon fertilization and continuing through pronuclear stage PN2, the paternal genome undergoes Tet3-dependent demethylation. PGC7, which preferentially binds to H3K9me2-rich chromatin, protects the maternal genome from Tet3 activity during PN stages. By PN5 stage, the bulk of paternal 5mC is gone and little change has occurred to maternal methylation. PPN, paternal pronucleus; MPN, maternal pronucleus.

embryo reaches the morula stage, the genome is almost completely devoid of DNA methylation (Santos *et al.* 2002). Despite wide-spread global demethylation, a few regions of the genome are protected, including imprinted loci and active retrotransposons like intracisternal A particle (IAP) elements (Lane *et al.* 2003). The large-scale demethylation begins with the rapid, active demethylation of the paternal haploid genome.

The differences between demethylation dynamics within the maternal and paternal pronuclei are thought to arise from their distinct architecture. The paternal genome is packed mostly around protamines, which are disassembled after fertilization and re-organized with histone-containing nucleosomes (Braun 2001, Balhorn 2007). The maternal genome is largely assembled around H3K9me2-rich histones. These structural distinctions between the two haploid genomes at pronuclear stage 0 (PN0) is thought to greatly influence the timing of bulk genome-wide demethylation (Santos *et al.* 2005), the kinetics of which are different between the maternal and paternal pronuclei (Fig. 1). Examination of global DNA methylation by immunofluorescence showed that the paternal pronucleus undergoes division-independent demethylation (Santos *et al.* 2002). When the zygote reaches the PN3 stage (~4 h after fertilization), there is already a dramatic loss of 5mC observed in the paternal pronucleus but little change in the maternal pronucleus (Mayer *et al.* 2000, Oswald *et al.* 2000). By the time of the first cell division (24 h after fertilization), there is no 5mC signal detected in the paternal PN, indicating near-complete loss of 5mC methylation. Even though the two parental genomes occupy the same nucleus after PN fusion, the differences in 5mC levels are apparent beyond the 4-cell stage (Santos *et al.* 2002).

Early studies of demethylation dynamics in mice based on immunofluorescence conflicted somewhat with bisulfite DNA sequencing data sets, which did not show as dramatic a loss of 5mC (Oswald *et al.* 2000). It was not until the realization that the 5mC oxidation product 5-hydroxymethylcytosine (5hmC) is present *in vivo* that this discrepancy was resolved. Traditional bisulfite treatment does not distinguish between 5mC and 5hmC (Huang *et al.* 2010), while the antibodies used for immunofluorescence specifically (and only) detect 5mC. It has been subsequently shown that TET enzymes mediate the oxidation of 5mC to 5hmC (as well as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)) *in vivo* and that TET proteins are expressed and differentially localized during preimplantation development (Gu *et al.* 2011, He *et al.* 2011, Iqbal *et al.* 2011, Wossidlo *et al.* 2011). Specifically, TET3 primarily localizes to the paternal pronucleus (Fig. 1) and is thought to be responsible for the observed rapid demethylation. Importantly, Gu *et al.* (2011) showed that the loss of 5mC corresponds with a concomitant gain in 5hmC. In both pronuclei, 5mC is present until PN3, and by late PN3, there is a detectable decrease in 5mC and an increase in 5hmC (Fig. 1; Gu *et al.* 2011, Iqbal *et al.* 2011, Wossidlo *et al.* 2011). It was also shown that Tet proteins convert 5mC to 5fC and 5caC as well, suggesting that Tet-mediated oxidation results in three oxidative forms for cytosine *in vivo* (Inoue 2011), ultimately resulting in replacement of the oxidized base with unmethylated cytosine by base excision repair or replication-dependent diffusion. Additionally, oxidation of 5mC has been shown in other mammalian zygotes, indicating a conserved mechanism of demethylation (Wossidlo *et al.* 2011).

Surprisingly, deletion of Tet3 activity results in retention of 5mC in the paternal pronucleus and inappropriate gene activation at many loci, but only mild global phenotype (reduced viability (Gu *et al.* 2011)). Tet3 mediated

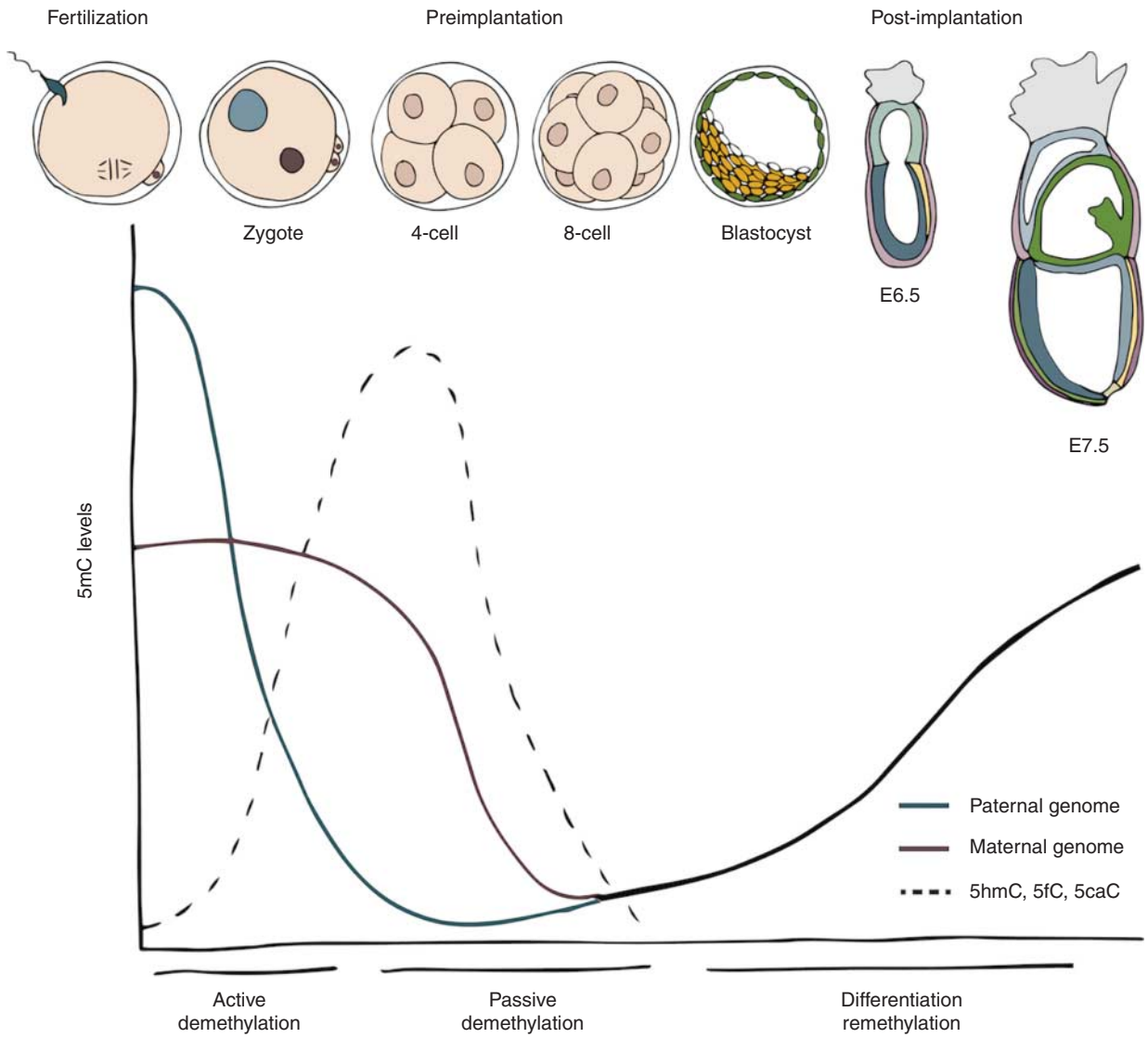

Figure 2 DNA methylation dynamics from fertilization through gastrulation. The three main phases of methylation change are illustrated. During zygotic stages, the paternal genome undergoes active demethylation (blue line). This active demethylation is evident by sharply increased levels of oxidative products (5mC to 5hmC, 5fC and 5caC) in the paternal pronucleus. Passive replication-dependent demethylation (predominantly in the maternal pronucleus) occurs though exclusion of DNMTs from the nucleus during early preimplantation (red line). Lowest levels of methylation are reached between the morula to blastocyst stage, when methylation levels begin to rise. During blastocyst formation and gastrulation, the genome becomes re-methylated to levels consistent with somatic cells (black line).

hydrolysis of 5mC also occurs during reprogramming after SCNT cloning – Tet3 localizes to the pseudo-pronucleus in recombined zygotes. In SCNT embryos made with Tet3-null host oocytes, there is no 5hmC present in the pseudo-pronucleus, further indicating the role of Tet3 in active demethylation (Wossidlo *et al.* 2011, Gu *et al.* 2011).

These recent studies offered the prevailing idea that demethylation during preimplantation development occurred via i) active DNA demethylation of the paternal pronuclei mediated by Tet3 and thymine DNA glycosylase (TDG)-mediated base excision repair (reviewed in Kohli & Zhang (2013)) and ii) passive, replication-dependent dilution loss of methylation of the maternal genome due to lack of Dnmt1 in the nucleus (Howell *et al.* 2001).

However, recent work is shifting the hypotheses about the mechanisms responsible *in vivo*. Using whole genome approaches to assess cytosine methylation patterns, it has been shown that Tet3-mediated demethylation is only partially responsible for paternal demethylation and that active demethylation also occurs in the maternal PN (Guo *et al.* 2014*a*, Shen *et al.* 2014). Furthermore, although Tet3-mediated oxidation is required for active demethylation, TDG-mediated base excision repair is not (Guo *et al.* 2014*a*). Additionally, it appears that there is conflicting evidence regarding the

role that replication-dependent demethylation plays in removing methylation from the paternal genome. By blocking replication of the paternal pronucleus, Shen *et al*. (2014) showed diminished demethylation in the paternal pronucleus even though Tet3 activity is present, indicating that replication is also involved in the demethylation of the paternal PN. Adding additional ambiguity is the fact that that Tet3-mediated demethylation is largely dispensable for successful development (Peat *et al*. 2014, Shen *et al*. 2014, Inoue *et al*. 2015).

Taken together the mechanisms that reprogram sperm and oocyte specific DNA methylation are not mutually exclusive as once predicted, and these recent stories indicate that there are likely unknown mechanisms also contributing to DNA methylation dynamics during preimplantation. With these advances, there are three main modes of DNA demethylation: i) active Tet3-mediated oxidation (predominantly in the paternal pronucleus); ii) replication-dependent dilution of Tet3-oxidative products, which plays a major role in demethylation of the paternal pronucleus and iii) replication-dependent (Tet3-independent) dilution of 5mC (predominantly in the maternal pronucleus). As our technical abilities evolve, it will be interesting to determine the interplay of these mechanisms within the same cells *in vivo*, define the specific loci at which each occurs and identify whether there are differing roles influencing cell fate decisions.

If Tet3 is present in the oocyte but only acts primarily on the paternal genome, there must be a protective mechanism to prevent conversion of maternal 5mC. One candidate for this maternal genome protection is PGC7/Stella, a DNA-binding protein expressed during germ cell specification and in gonads and oocytes (Saitou *et al*. 2002, Sato *et al*. 2002). PGC7 null embryos fail to complete preimplantation and there is a loss of 5mC in both pronuclei, indicating a protective role in the maternal pronucleus. Additionally, PGC7/Stella is targeted to differentially methylated regions (DMRs) of imprinted genes in the early embryo (Nakamura *et al*. 2007), supporting a functional role in blocking Tet3-mediated demethylation.

PGC7/Stella is able to protect the maternal pronucleus by binding to H3K9me2, which is a distinguishing feature of the maternal pronucleus. Loss of H3K9me2 by ectopic Jndm2a, a H3K9 methylation/dimethylation-specific demethylase, leads to loss of 5mC in both the maternal and parental pronuclei. PGC7/Stella also binds to H3K9me2 regions of the paternal pronucleus, including DMRs, which are not subject to protamine replacement. Tet3 is inhibited by PGC7/Stella, thus offering protection from active demethylation (Nakamura *et al*. 2012).

Imprinted loci are protected from demethylation

While most of the genome undergoes global DNA demethylation, imprinted loci are protected and retain parent of origin DMRs (Branco 2008, Cirio 2008, Hirasawa 2008). It is clear that Dnmt1 is required for the maintenance of these imprinted sites (Bourc'his *et al*. 2001), even though it is largely excluded from the nucleus (Hirasawa 2008). Stella is also known to protect these loci, including some imprinted sites of the paternal genome. Additionally, Zfp57, a KRAB zinc finger protein, and Trim28 have also been shown to be required for integrity of ICRs in the early embryo. Trim28 interacts with Zfp57 to target it to specific imprinted sites, resulting in recruitment of repressive complexes including NuRD, SETDB1 and DMNTs (Iyengar *et al*. 2011, Quenneville *et al*. 2011, Zuo *et al*. 2012). While loss of maternal Zfp57 can be rescued by paternal expression, loss of maternal Trim28 is lethal (Li *et al*. 2008, Messerschmidt *et al*. 2012), due in part to the variation in loss of imprinted expression (Messerschmidt *et al*. 2012).

Early DNA demethylation dynamics in other mammalian species

Preimplantation DNA demethylation dynamics are largely the same in mouse and human embryos. However, this is not the case in all mammals, indicating distinct epigenetic reprogramming in different species. During the PN stages and in the first cell divisions, human, mouse and rat zygotes lose the majority of their paternal 5mC (Dean *et al*. 2001, Zaitseva *et al*. 2007). In contrast, both bovine and goat embryos retain an intermediate level of 5mC in the paternal pronuclei (Park *et al*. 2010, Wossidlo *et al*. 2010). Strikingly, sheep, pig and rabbit embryos retain 5mC during the PN stages and throughout preimplantation development (Beaujean *et al*. 2004, Jeong *et al*. 2007, Reis e Silva *et al*. 2012). In sheep, levels of 5mC drop during the 2-cell stage, but then increase at the 16-cell stage, and the ICM maintains levels of DNA methylation but the TE levels decrease dramatically (Young & Beaujean 2004).

These comparative studies illustrate the differences in timing and degree of 5mC loss during preimplantation among different mammalian species. These differences may be due in part to variation in zygotic genome activation, but they may also hint at differences in methylation reprogramming requirements needed to reach a totipotent state. These data also support the idea that active, Tet3-mediated demethylation in mice is not required for normal preimplantation development (Peat *et al*. 2014, Shen *et al*. 2014).

Next-generation sequencing to assess global DNA methylation dynamics during preimplantation development

Next-generation sequencing, including reduced representation bisulfite sequencing (RRBS) and whole-genome bisulfite sequencing (WGBS) now allow

assessment of global DNA methylation reprogramming with high resolution even from limited numbers of cells. Confirming earlier work, methylation across the genome is observed at relatively low levels in oocytes and early preimplantation stages, while sperm and post-implantation embryos have methylation similar to that of somatic cells (Smith *et al*. 2012, Guo *et al*. 2014*a*,*b*, Peat *et al*. 2014). These newer technologies have allowed for refined assessment of methylation changes across the genome during precise developmental stages, examination of specific classes of DNA sequence elements and comparison of mouse and human preimplantation embryos.

DNA methylation dynamics in human preimplantation embryos

Two groups recently examined genome-wide DNA methylation changes in human oocyte, sperm, zygote, pre- and post-implantation stages, using RRBS and WGBS (Smith *et al*. 2012, Guo *et al*. 2014*a*). Similar to mouse, both groups found that human sperm is highly methylated (although less than mouse sperm) and human oocytes have intermediate levels of methylation. The post-fertilization demethylation kinetics are also similar in the human zygote (Smith *et al*. 2012). Guo *et al*. (2014*a*) note that the greatest loss of DNA methylation occurred between the 1- to 2-cell stage in human embryos, rather than during PN stages (as is the case in the mouse). This could indicate that differences in the rate of active demethylation also correlates with the timing of zygotic genome activation, which occurs later in humans (Beaujean *et al*. 2004). Because bisulfite sequencing used in these studies did not distinguish between 5mC and the oxidative products of Tet-mediated demethylation, the distinct timing in mouse and human embryos may reflect a difference in the oxidation rates of 5mC or Tet activity between species. Paternal genome demethylation in humans is similar to observations in mouse zygotes, in that the majority of methylation is rapidly lost and only low levels of methylation remain during preimplantation. Levels of methylation in the maternal pronucleus are similar to mice, but the genomic regions that are demethylated are divergent. Additionally, unlike in mice, the majority of sperm and oocyte-specific DMRs regain their full methylation following implantation (Smith *et al*. 2012, Guo *et al*. 2014*a*).

Comparison of genome-wide methylation with single-cell RNA sequencing data (Yan *et al*. 2013) confirmed the previously observed negative correlation between promoter methylation and gene expression and highlighted that this inverse relationship strengthens after the MZT in human embryos (Smith *et al*. 2012, Guo *et al*. 2014*b*). Genes that had increased promoter methylation after the blastocyst stage showed a predicted decrease in expression in post-implantation-stage embryos (Guo *et al*. 2014*b*). Also as expected, changes in DNA methylation during preimplantation influence the repression of transposable elements. SINE/variable number of tandem repeat/Alu elements (SVAs) expression increases after the 2-cell stage, when rapid demethylation occurs. This expression is maintained until the morula stage, when expression decreases, presumably as the genome is re-methylated – a trend which continues post-implantation (Smith *et al*. 2012, Guo *et al*. 2014*b*).

While many repeat elements undergo loss of DNA methylation and increased expression, the evolutionary age of the transposable element appears to influence the retention of methylation during preimplantation development. Evolutionarily younger elements, which are still capable of transposition are relatively resistant to demethylation while their evolutionarily older counterparts that have lost the ability to jump are readily demethylated along with coding genes. This might hint at the evolutionary origins of methylation/demethylation dynamics in mammalian preimplantation development (Wang *et al*. 2014).

Histone modifications during preimplantation

In addition to DNA methylation changes, chromatin organization and histone modifications play a critical role in establishing a totipotent embryo, as well as directing the first lineage decisions. Chromatin is a highly organized and dynamic nuclear structure containing DNA, histones and many other proteins. Nucleosomes, the basic building block of chromatin are comprised of two each of histone H2A, H2B, H3 and H4. It is well established that the N-terminal tails of these core histones are subject to post-translational modifications (PTMs), which play a fundamental role in influencing gene expression patterns among disparate cell types (Fischle *et al*. 2003). Histone PTMs include acetylation, methylation, phosphorylation, ubiquitination and others, which occur at specific amino acid residues catalyzed by specific enzymes (Strahl & Allis 2000, Tan *et al*. 2011). Additional complexity arises in that methylation at lysines or arginines may exist in distinct forms: mono-, di-, or trimethyl for lysines and mono- or dimethyl on arginine residues (Kouzarides 2007). A general theme has emerged in which PTMs are catalyzed by opposite functional pairs of enzymes.

Many studies have revealed functional themes where histone PTMs correlate with gene expression patterns. For example, lysine acetylation is commonly considered to be an active mark that correlates with chromatin accessibility and active transcription, whereas histone lysine methylation can be either active or repressive depending on the particular lysine residue that is modified (Tsukada *et al*. 2006, Bernstein *et al*. 2007). Recent large-scale efforts supported by the Roadmap Epigenomics Project are defining 'chromatin states' in many diverse tissues – that is, combinations of histone modifications, DNA methylation and transcription factor binding that correlate with

functional property of a particular locus (http://www.roadmapepigenomics.org/publications/).

Histone modification during preimplantation embryo development

Studies of early embryonic development have shown that shortly after fertilization, many histone modifications are observed asymmetrically in the parental haploid genomes prior to PN fusion (summarized in Fig. 3). For example, in mice, H3K27ac, H4K5ac and H4K16ac are only detectable in the paternal PN of early zygotes (Adenot *et al*. 1997, Stein *et al*. 1997, Hayashi-Takanaka *et al*. 2011). Conversely, all forms of H3K4 methylation (me1, me2 and me3) are observed in maternal PN (Lepikhov & Walter 2004, Santenard *et al*. 2010), and H3K9me2 and me3 are also significantly higher in the maternal PN (Lepikhov & Walter 2004, Wongtawan *et al*. 2011, Beaujean 2014). H3K27me1 is present in both PNs, but H3K27me2 and -me3 occur extensively in the maternal PN (Erhardt *et al*. 2003, Santos *et al*. 2005, Santenard *et al*. 2010). Additionally, H3K9me3S10P, H3K36me3 and H4K20me3 are also found exclusively in the maternal PN at early post-fertilization stages (Boskovic *et al*. 2012, Ribeiro-Mason *et al*. 2012, Beaujean 2014). Although the functional significance of these asymmetric PTMs remains largely unknown, it highlights the distinct reprogramming that is required for the paternal and maternal PN for proper embryonic genome activation and embryo development (Ribeiro-Mason *et al*. 2012, Beaujean 2014).

Histone PTMs also play key roles in remodeling of chromatin configuration and DNA methylation. In mice, the increase of H3K79me by forced expression of DOT1L causes premature chromocenter formation and developmental arrest of 2-cell embryos (Ooga *et al*. 2013). Additionally, deletion of the methyltransferase Setdb1 in mouse embryonic stem cells leads to the reduction of H3K9me3 and an overall decrease of DNA methylation levels at specific loci (Leung *et al*. 2014). In porcine embryos, disturbed H3K4me3-H3K27me3 balance after knockdown of demethylase Kdm5b can cause increased expressions of Tet family members (Huang *et al*. 2015), which are found to be crucial for the interactions between histone modification and DNA methylation in mouse embryonic stem cells (Sui *et al*. 2012).

Functional studies of the roles of specific modifications are just beginning, using genetic strategies to add or remove specific enzymatic activities to embryos. For example in mice, hyperacetylation of histone H4 mediated by knockdown of HDAC1 causes developmental delay (Ma & Schultz 2008). Knockdown of either *Ing2* (H3K4me3 methyltransferase activity) or RNF20 (histone H2B monoubiquitination) results in arrest at the morula stage (Ooga *et al*. 2015, Zhou *et al*. 2015). Depletion of H4K20me1 by knockout of the *PR-Set7* gene induces early embryonic lethality prior to the 8-cell stage (Oda *et al*. 2009), and our lab has shown critical roles of H3K36me3 during preimplantation development by knockdown of CTR9/PAF1 (Zhang *et al*. 2013*a*). Other recent examples include studies in mice showing that maternal-specific H3K9me3 is enriched at the *Xist* promoter region and prevents maternal *Xist* activation (Fukuda *et al*. 2014); increased H3K4me2 results in abnormal expression of *eIF-4C*/*Oct4* and arrest at the 2-cell stage (Shao *et al*. 2008); and that PRC1 binding to H3K27me3 plays an indispensable role in embryonic genome activation and developmental progression (Posfai *et al*. 2012). Although precise in the removal of specific gene function, these studies highlight the difficulty in assigning specific function to a particular modification or enzymatic activity since the phenotype is often developmental failure and misregulation of many genes. It is only very recently that next-generation technologies allow for very low input such that

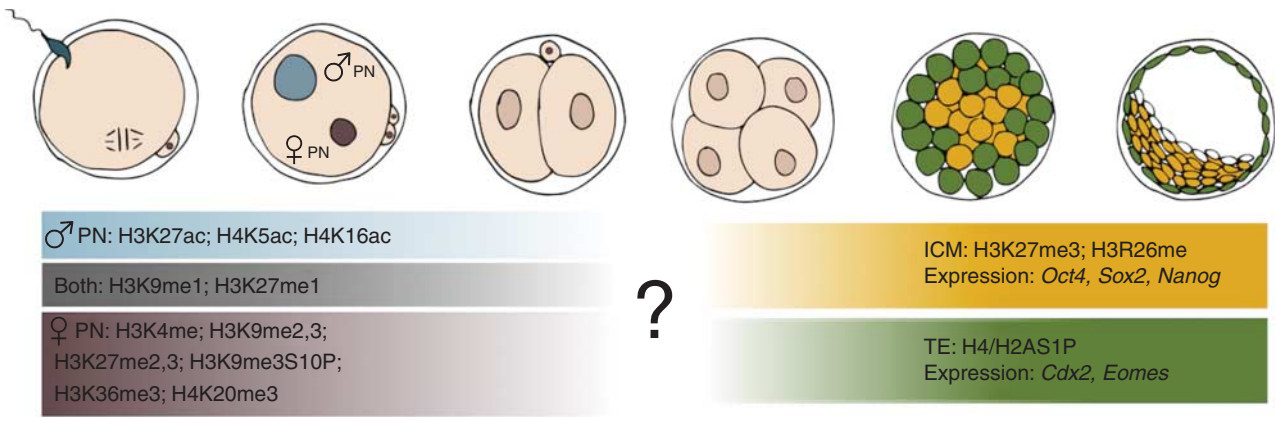

Figure 3 Differential histone modification during preimplantation development. After fertilization, the parental pronuclei are differentially enriched with many distinct histone modifications (left side: list of PTMs). Little data is available regarding the timing and mechanisms resulting after pronuclear fusion that result in largely homogenous PTMs during cleavage stages (indicated by the question mark). By the time ICM and TE begin to differentiate, these cell lineages have acquired distinct epigenetic signatures and gene expression patterns (right side).

investigators can determine which loci across the genome are altered in these knockout/knockdown embryos (Brind'Amour *et al*. 2015).

Relevant to artificial reproductive technologies, histone modifications are sensitive to manipulations during preimplantation, potentially altering epigenomic patterns (Feil & Fraga 2012, Dupont *et al*. 2012). For example, in the mouse, H3K4me3 is significantly lower in the *in vitro* fertilized embryos compared with *in vivo* fertilized embryos (Wu *et al*. 2012). Similarly lower levels of H3K27me3 are found in the ICM of heated-sperm-derived blastocysts when compared to untreated-sperm-derived blastocysts (Chao *et al*. 2012), and cryopreservation can alter H4K12ac patterns in both oocytes and zygotes (Suo *et al*. 2010). Despite these observations, it remains unclear if altered PTM levels persist in offspring or if surviving individuals contain appropriate epigenomic information – possibly correcting the epigenome during cell lineage differentiation at post-implantation stages.

Histone modifications in ICM and TE lineage specification

In mouse embryos, transcription factors such as Oct4, Sox2 and Nanog are enriched in cells of the ICM and function to both promote pluripotency and resist differentiation. Conversely, in TE, transcription factors such as Cdx2 and Eomes become upregulated, promoting differentiation. In contrast to the mouse, Oct4 and Cdx2 are co-expressed in the ICM and TE of bovine and porcine embryos, and the mechanisms of molecular differentiation remain largely unknown (Kirchhof *et al*. 2000).

This first lineage specification is critical for implantation and successful development. DNA methylation has been shown to be dispensable for growth and differentiation of the extraembryonic lineages (Sakaue *et al*. 2010), suggesting that appropriate histone modifications may provide key epigenetic information directing gene expression and lineage specification. Once the TE and ICM become distinct, they exhibit asymmetries in specific histone PTMs. For example, in the mouse, H4- and H2AS1P are increased in the TE cells (Sarmento *et al*. 2004), while H3K27me3 is enriched in the ICM (Erhardt *et al*. 2003). At the 4-cell stage, blastomeres have different levels of methylated H3R26me and those cells with higher H3R26me are more likely to result in ICM cell fate. Overexpression of the H3R26 methyltransferase CARM1 results in increased expression Nanog and Sox2, suggesting that pluripotency factor expression is influenced by locus-specific H3R26me (Torres-Padilla *et al*. 2007). Other examples include studies showing that repressive H3K9me3 at the *Cdx2* promoter is important for maintaining pluripotency and loss of associated methyltransferase ESET in early embryos results in ICM failure (Yeap *et al*. 2009). However, in TE lineage, Suv39h methyltransferase mediates repressive H3K9me3 at ICM-specific gene promoters in the TE lineage (Alder *et al*. 2010, Rugg-Gunn *et al*. 2010). These studies highlight that even the same histone modification can be finely tuned by distinct enzymes to influence lineage specification in different cell populations.

There are ever growing observations of locus specific enrichment of histone modifications correlating with lineage decisions during preimplantation development. For example, H3K4me3 and H3K27me3 are enriched at promoters of genes exclusively expressed in ICM or TE in both murine and bovine embryos (Dahl *et al*. 2010, Herrmann *et al*. 2013). It was also recently shown that loss of repressive H3K27me3 participation at TE-specific genes is essential for TE lineage development and embryo implantation (Saha *et al*. 2013, Paul & Knott 2014). In addition to methylation of histone H3 residues, acetylation of histone H4 (H4K8ac and H4K12ac) has also been implicated in early lineage specification (VerMilyea *et al*. 2009, Zhang *et al*. 2013*b*).

A handful of histone-modifying proteins thought to be central to epigenetic programing during development have knock-out phenotypes only apparent after preimplantation. These include members of Polycomb Repressive Complex 2 (Eed, Ezh2 and Suz12) as well as the H3K9 methyltransferases G9a and Eset. It remains unclear if the timing of null phenotypes is due to functional redundancy with other genes or maternal loading of RNA/protein, or if the modifications they perform are in fact not required until gastrulation (or later). There are a few histone-modifying enzyme knock-out phenotypes in mice that do result in lethality during preimplantation, some of which show lineage-specific defects. Loss of the histone H3K9 demethylase Jmjd2C results in morula arrest and null embryos show reduced levels of ICM-specific gene transcription, suggesting a failure to maintain pluripotency (Wang *et al*. 2010). Similarly, null embryos of several members of the NuRD complex (Sin3A, Suds3, Arid4b (McDonel *et al*. 2009)) and PAF1 complex (Ctr9 and Rtf1 (Ding *et al*. 2009, Zhang *et al*. 2013*a*)) do form blastocysts but show defects in ICM proliferation as a major cause of developmental lethality and failure. It is perhaps not so surprising that knockout of genes with distinct functions (such as Sin3A and Ctr9) result in similar defects in maintenance of ICM potency, which is of the utmost importance for continued development and requires myriad proteins to accomplish.

Moving forward

As described above, a wide array of covalent histone modifications are now recognized to occur *in vivo* and correlate with distinct transcriptional states and/or chromatin conformation. However, knowledge about the role of histone modifications during development is mostly limited to reports of changes in global patterns – apparent by immunofluorescence with antibodies

directed against specific modifications (reviewed in Beaujean (2014)). While these descriptive studies are an essential beginning, little is known about the functional importance of these modifications. *In vivo* analysis of the role of histone modifications at specific loci during early development is only just beginning, and the relative lack of functional data is due to several factors including: i) limitations in our ability to efficiently generate maternal and zygote null embryos at the same time, ii) limitations in our ability to assess histone modifications at specific loci from very small numbers of cells and iii) an inability to alter specific modifications at specific loci. Due to the combinatorial nature of the histone code and the difficulty in functionally preventing one particular modification at one locus *in vivo*, it is currently not feasible to simply ask, 'What is the role of a specific histone modification at a specific genomic locus during development'. Fortunately, this type of epigenetic engineering has come to the fore and many groups are currently working to develop *in vivo* epigenetic targeting tools.

With greatly enhanced access to next-generation sequencing technologies, there is ever-growing opportunity to probe genome-wide methylation patterns at single-base/nucleosome resolution in diverse cell populations, and improved techniques are pushing WGBS towards single-cell sequencing. Additionally, multiple methods are now readily available for the discrimination of 5mC and 5hmC at a single base resolution. Combining DNA methylation analysis with ChIP-seq and RNA-seq during preimplantation development will allow for a comprehensive cataloguing of early epigenetic reprogramming dynamics. Cross-species comparison of these dynamics at specific loci and the capability to functionally test the importance of specific modifications will allow for deeper understanding of how epigenetic dynamics influence preimplantation development, the transition from gametes to totipotency and the requirements of lineage differentiation.

Declaration of interest

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

Funding

This was supported in part by March of Dime Research Grant #6-FY11-367 and NIH 1R21HD078942-01 to J Mager.

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Received 17 April 2015
First decision 11 May 2015
Revised manuscript received 26 May 2015
Accepted 1 June 2015