Review

Fei Gao and Sanjoy K. Das*

Epigenetic regulations through DNA methylation and hydroxymethylation: clues for early pregnancy in decidualization

Abstract: DNA methylation at cytosines is an important epigenetic modification that participates in gene expression regulation without changing the original DNA sequence. With the rapid progress of high-throughput sequencing techniques, whole-genome distribution of methylated cytosines and their regulatory mechanism have been revealed gradually. This has allowed the uncovering of the critical roles played by DNA methylation in the maintenance of cell pluripotency, determination of cell fate during development, and in diverse diseases. Recently, rediscovery of 5-hydroxymethylcytosine, and other types of modification on DNA, have uncovered more dynamic aspects of cell methylome regulation. The interaction of DNA methylation and other epigenetic changes remodel the chromatin structure and determine the state of gene transcription, not only permanently, but also transiently under certain stimuli. The uterus is a reproductive organ that experiences dramatic hormone stimulated changes during the estrous cycle and pregnancy, and thus provides us with a unique model for studying the dynamic regulation of epigenetic modifications. In this article, we review the current findings on the roles of genomic DNA methylation and hydroxymethylation in the regulation of gene expression, and discuss the progress of studies for these epigenetic changes in the uterus during implantation and decidualization.

Keywords: decidualization; DNA hydroxymethylation; DNA methylation; epigenetics; uterus.

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List of abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; 5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5hmU, 5-hydroxymethyluracil; 5mC, 5- methylcytosine; ADD, ATRX–DNMT3–DNMT3-like domain; AID, activation-induced deaminase; APOBEC, apolipoprotein B mRNA-editing enzyme complex; BER, base excision repair; BRG1, brm/SWI-2 related gene 1; CBX4, chromobox 4; CGI, CpG island; CTCF, CCCTC-binding factor; DNMT, DNA methyltransferase; DPPA3, developmental pluripotency-associated protein 3; ESC, embryonic stem cell; Esrrb, estrogen related receptor beta; H3K4me1, monomethylated histone H3 lysine 4; H3K4me3, trimethylated histone H3 lysine 4; H3K9me2, dimethylated histone H3 lysine 9; H3K9me3, trimethylated histone H3 lysine 9; H3K27Ac, acetylated histone H3 lysine 27; H3K27me3, trimethylated histone H3 lysine 27; HDAC, histone deacetylase; IAP, intra-cisternal-A-particle; IDAX, CXXC finger protein 4; Kdm1b, lysine (K)-specific demethylase 1B; LSH, lymphoid specific helicase; MBD3, methyl-CpG binding domain protein 3; MeCP2, methyl CpG binding protein 2; MSRF, methylation-sensitive restriction fingerprinting; Nanog, Nanog homeobox; OCT4, octamer-binding transcription factor 4; PCNA, proliferating cell nuclear antigen; PGCs, primordial germ cells; ICRs, imprinting control regions; PHD, plant homeo domain; PRC, polycomb repressive complex; REST, repressor element 1 silencing transcription factor; RING, really interesting new gene domain; SET, suvar3-9, enhancer-of-zeste, trithorax domain; SIN3A, SIN3 transcription; regulator family member A; SOX2, (sex determining region Y)-box 2; SUMO, small ubiquitin-like modifier T, thymidine; TDG, thymine-DNA glycosylase; TET, ten-eleven translocation protein; TSS, transcriptional starting site; UHRF1, ubiquitin-like with PHD and ring finger domains 1.

Introduction

Epigenetic regulation in its strictly modern concept is defined as stably heritable changes in a chromosome without alterations to the DNA sequence (1). Epigenetic processes mainly include DNA methylation, histone modification, nucleosome positioning, and non-coding RNA. Heredity is transmission between dividing cells or between generations of an organism. However, dynamic changes of classical epigenetic markers for controlling differential gene expression is still loosely described as 'epigenetic' regulation, although they may not always fulfill the strict 'heredity' definition. DNA methylation is one of the best-studied epigenetic phenomena in plants, fungi, and animals, and our understanding of it has been accelerated recently by the rapid development of 'next generation sequencing' techniques. In the past several years, the rediscovery of hydroxymethylation and its catalytic enzymes has started to bridge our understanding of methylation and demethylation, and revealed more dynamic changes than previously known, which, in turn, has tremendously expanded our knowledge of DNA methylome. In this review, we will discuss recent progress on studying DNA methylation and hydroxymethylation in mammalian epigenetic regulation and its relevance in the reproductive system, particularly in pregnant uteri that experience highly tempo-spatial activation and silencing of genes at a high rate.

Aspects of methylated DNA

DNA methyltransferase

DNA methylation is catalyzed by the DNA methyltransferase (DNMT) family to generate 5-methylcytosine (5mC) by adding a methyl group to the 5-position of cytosine (2). In mammals, the DNMT family has three members: DNMT1, DNMT3A, and DNMT3B.

DNMT1 is responsible for the maintenance of DNA methylation inherited from parental cells (2). DNMT1 prefers to bind on hemi-methylated DNA over unmethylated DNA structurally during S phase (3). Proliferating cell nuclear antigen (PCNA) forms a complex with DNMT1 at the foci of newly replicated DNA to copy the methyl pattern from template strands (2). In order to correctly load DNMT1 onto newly replicated DNA, ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is needed for the recognition of hemi-methylated sites through its SET and RING finger-associated (SRA) domain to direct DNMT1 to the foci, which ensures accurate preservation of DNA methylation in cell cycles (4). Additionally, some locally specific mechanisms may also adapt to this catalytic machinery, for example, UHRF1 binding to bi- or trimethylated histone H3 lysine 9 (H3K9me2/3) is needed for DNMT1 activity at peri-centric heterochromatin (5).

DNMT3A and DNMT3B are responsible for *de novo* DNA methylation in cells (2). They are highly homologous but take distinct duties in different cell contexts and developmental stages. DNMT3B prevails more in early embryos for attainment of methylation to suppress germ line expressed genes during the transition from blastocyst to the postimplantation epiblast (6). DNMT3A mainly functions later on in germ line cells to establish parental imprints and in differentiate somatic cells to set lineage specific DNA methylation patterns (7). Interestingly, a recent analysis using computed hidden Markov models in embryonic stem cells (ESCs) with individual or combined mutation of the *Dnmt* family genes argues against a strict enzyme specific functional categorization in certain contexts. For example, DNMT1 has considerable *de novo* methylation activity at certain repetitive elements and single copy sequences, whereas DNMT3A and DNMT3B are also essential to maintain symmetrical CpG methylation at distinct hemimethylated sites in ESCs (8).

DNMT3L shares an ADD domain with DNMT3A/B for binding unmethylated histone H3 lysine 4, but is scarce of the catalytic domain (9). Nonetheless, it is essential for the establishment of imprints in the germ line, as a cofactor coupled with DNMT3A/B (10).

Distribution and function of methylated DNA

In general, 5mC is associated with silencing of genomic DNA regions. The methyl group of 5mC protrudes into the major groove of duplex DNA, therefore, inhibiting transcription potentially in two ways: it prevents transcriptional factors binding, and it interacts with methyl-binding proteins to further recruit factors with transcription-suppression capabilities (11).

Four to six percent of cytosines in the genome are methylated in human cells. Globally higher levels of methylation are detected in ESCs than in somatic cells (12). In most mammal cells, DNA methylation occurs predominantly at CpG dinucleotides. Arbitrary and empirical criteria based on computational methods define (G+C) and CpG enriched region as CpG islands (CGIs) (13). As shown in Figure 1A, CGIs are distributed across different regions of the genome, at 48%, 27%, and 25% levels in

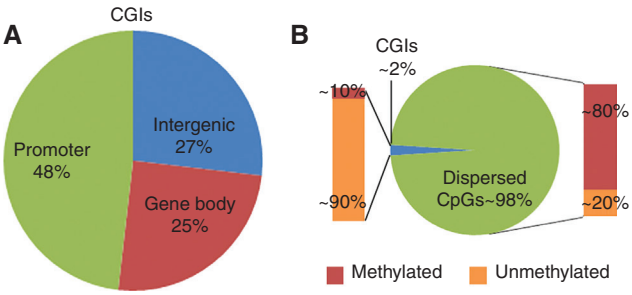

Figure 1 Genomic distribution of CpG islands (CGIs) and methylation status of CpGs.
(A) Percent distribution of CGIs in the promoter, intergenic, and gene body regions of the whole genome. (B) Percent distribution of DNA methylation status of the dispersed CpG and CGI regions on the whole genome.

the promoter, intergenic, and gene body regions, respectively. While, in general, CpG methylation occurs in an inverse correlation between cytosine methylation and CpG density, dispersed CpG, representing most of the genome (~98%), has high levels of cytosine methylation (~80%); however, CGIs (~2%) remain largely unmethylated (~90%) (Figure 1B) (12–14).

About half of human gene promoters are associated with CGIs, most of which are primarily unmethylated, as shown by an illustration of a gene model (Figure 2). Unmethylated promoters related transcriptional starting sites (TSS) are usually depleted of nucleosomes, marked with trimethylation of histone H3 lysine 4 (H3K4me3) and bordered by nucleosomes containing histone variant H2A.Z, accommodating local chromatin a transcriptionally active structure (11). CGI methylation mainly happens in allele-specific gene silencing on the inactive X chromosome in females or in parental genomic imprinting and rarely in most promoter regions. In certain somatic cells, promoter CGI methylation for tissue-specific gene silencing helps to specify cell fate (15). Other CGIs, that are not associated with annotated promoters, are more frequently methylated during development, showing novel regulatory mechanism from distal regions or unknown promoters (13). CpG methylation in non-CGI promoters also can directly regulate gene expression (11). In contrast to the promoter region, gene bodies are mostly poor in CpGs and widely methylated, as indicated on a representative gene model (Figure 2) (12, 13). Moreover, methylation of CpGs in gene bodies has positive correlation with gene transcription (12). Extensive existence of methylated repetitive repeat elements within gene bodies may protect gene from transcription initiation from wrong sites, while at the same time permitting elongation propagating through that area. This intragenic methylation may also regulate transcription initiating from cell or tissue specific alternative promoters in gene bodies (16). Another function of methylation within gene bodies is implicated for transcript splicing, by the fact that exons attain more methylation than introns, and sharp transitions of methylation are featured at exon–intron boundaries (17).

DNA sequence itself can determine local methylation status, revealing the links between genetic information and epigenetic regulations. The conserved sequence of immediate adjacent bases surrounding CpGs has been shown to correlate highly with allele-specific DNA methylation (18). Diverse motifs are also found to be preferred for DNA methylation. Small methylation-determining regions within different DNA elements are identified by truncation analysis, which can mediate both hypomethylation and *de novo* methylation in *cis* and can be modulated at different developmental stages (19). Similarly, unmethylated CGIs are also protected from methylation by DNMT3A/B using a common sequence-based structure (20). DNA binding factors, such as CTCF and REST, can also regulate local DNA methylation at binding regions (21).

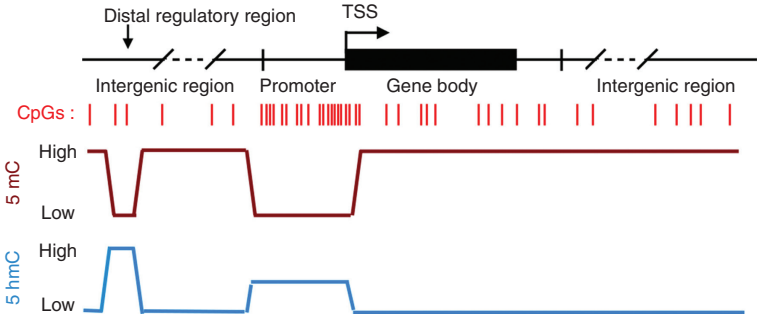

Figure 2 A diagrammatic representation of CpG sites and the status of methylated CpG (5mC) and hydroxymethylated CpG (5hmC) on different locations (promoter, intergenic, and gene body) of a gene.
Note that 5mC and 5hmC are distributed in an opposite fashion and that CpGs are enriched primarily in the promoter region.

Recently, DNA methylomes at single-base resolution revealed more non-CpG methylation in diverse cell types. In ESCs, about one-fourth of 5mC is in non-CpG sites, such as CHG or CHH (H=A, C, or T), whereas in differentiated fibroblasts, almost all 5mC are in CpG sites (12). Non-CpG methylation disappears upon differentiation of ESCs and can be restored in an induced pluripotent state (12). This was shown in mouse germinal vesicle oocytes experiencing active growth, cycle arrested fetal spermatogonia, and in adult brains (22, 23). This non-CpG methylation may depend on DNMT3A/B-DNMT3L complex in different cell contexts (8, 22). Methylated non-CpGs closely resemble the distribution pattern of methylated CpG on the whole genome (12). Non-CpG methylation is strongly related to neighboring sequence (12) and CpG methylation (8). There are distinct features of methylation between CpG and non-CpG. In ESCs, non-CpG sites show a relatively lower level of methylation (10%–40%) compared to CpG (80%–100%) (12). While mCpG sites are symmetrically methylated on both strands, surprisingly mCHG sites are highly asymmetrical and distributed only on one strand (12). Further exploration is needed to determine how the mechanisms work that faithfully maintains methylation without two hemi-methylated CHG sites. These findings have unraveled previously underappreciated similarities between DNA methylation in animals and plants. However, more studies are still needed to understand non-CpG methylation and its function in mammals.

Aspects of hydroxymethylated DNA

Ten-eleven translocation (TET) proteins

The TET proteins have three members (TET1, TET2, and TET3) and are comprised of 2-oxoglutarate and Fe(II)-dependent dioxygenases family, which are responsible for creating 5-hydroxymethylcytosine (5hmC) (24, 25). The TET1 protein was first found capable of catalyzing the conversion of 5mC into 5hmC (25). Similar to DNMTs, TET1 and TET3 have the DNA-binding domain CXXC to recognize CpG sites. TET2 lacks this domain; however, as an ancestral TET2 protein, the CXXC domain, IDAX helps to recruit TET2 to target genes and regulate its stability (25). Pre-existing 5mC is necessary for 5hmC production, as 5hmC is eradicated in *Dnmt1/3a/3b* triple mutant ESCs, which is absent of 5mC (26). Whether 5hmC is faithfully maintained during DNA replication by TET proteins as DNMT1 does for 5mC still awaits further exploration.

Expression levels of TET1 and TET2 are high in ESCs, but decrease dramatically once differentiation occurs. Meanwhile, TET3 shows an inverse pattern that suggests the distinct functions of TET1, TET2, and TET3 (25). TET1 and TET2 involve regulating pluripotency in stem cells through a possibly reciprocal feedback process. TET1 and TET2 control promoter hypomethylation of pluripotency-maintaining genes (*Nanog*, *Esrrb*, etc.) to maintain their expression in ESCs (24). Conversely, OCT4/SOX2 complex directly regulates TET1 and TET2 transcription (25). Knockdown of TET1 in ESCs causes skewed differentiation into the endoderm-mesoderm lineage and a bias towards trophectoderm differentiation (25, 27). In addition to hydroxylase activity, TET1 also controls DNA methylation by binding to CpG-rich regions to prevent unwanted DNA methyltransferase activity, which imparts differential maintenance of unmethylated state at TET1 targets and potentially works as a guardian for epigenetic fidelity (28). An unexpected role in transcriptional repression of TET1 independent of its catalytic activity is uncovered while being associated with the SIN3A co-repressor complex or being co-recruited with the PRC2 complex at overlapping target genes (24, 26). The aforementioned dual roles of TET1 are supported by TET1 being enriched at genes with either H3K4me3 monovalent or H3K4me3/ (trimethylated histone 3 lysine 27) H3K27me3 bivalent modifications (26, 28). However, the importance of TET1 on pluripotency in ESCs is still controversial, as *Tet1* mutant mouse can survive for postnatal development (27). Another *in vivo* role of TET1 is its specific regulation on demethylation and the activation of meiotic genes in the germ line, indicated by observations of reduced oocytes production and subfertility in *Tet1* mutant female mice (24). Not surprisingly, the three members of TET proteins may have some redundant functions. *Tet1* and *Tet2* double mutants have more pronounced defects in the female germ line than *Tet1* mutants, and upregulated *Tet3* may compensate for their roles in the maintenance of ESC pluripotency (27). Beyond potential roles in ESCs, TET2 controls 5hmC accumulation at regulatory regions of genes in the fetal brain, which will be demethylated and activated later on for memory formation towards adulthood (23). TET2 is also critical for hematopoiesis and mutation of TET2 relates to decreased 5hmC during myeloid tumorigenesis (25). TET3 is highly expressed in oocytes and zygotes to control the rapid conversion of 5mC to 5hmC, which may be responsible for immediate demethylation of paternal pronucleus upon fertilization (25).

Distribution of 5hmC

Techniques based on methylation-sensitive restriction enzymes and sodium bisulfite treatment of DNA are incapable of distinguishing 5mC and 5hmC. Therefore, several chemical/enzymatic modification based methods were developed to selectively convert 5hmC. Conjugation of those techniques with bisulfite sequencing makes the base-resolution mapping of 5hmC possible, which has revealed more accurate global distribution of 5hmC than affinity based enrichment and sequencing methods (24, 29). Compared to the relatively constant level of 5mC, 5hmC shows more variability between different cell and tissue types. The brain has a much higher portion of 5hmC than other organs (23). ESCs has relatively lower ratio of 5hmC. 5hmC (~0.03% of all nucleosides) has far lower abundance compared to 5mC (~0.8%) in the genome of mouse ESC (30). In contrast with high abundance of 5mC in non-CpG context, 5hmC mostly shows in CpG sites in ESCs (29) and central neuron system (23). The high level of asymmetric distribution of hydroxymethylation is another incomprehensible feature of 5hmC, however, this observation needs confirmation by enhanced depth of sequencing due to its low abundance (29).

5hmC is mostly associated with euchromatin containing actively transcribed genes. The balance between 5hmC and 5mC is different between genomic regions (24). As indicated in Figure 2, 5hmC is highly enriched in distal-regulatory elements, which includes enhancers, insulators, p300-binding sites and DNase I hypersensitive sites, in human and mouse ESCs (21, 29). The vast majority of repetitive elements are highly enriched with 5mC but not 5hmC (29). In those regions, distribution of 5hmC shows reverse correlation with 5mC. 5hmC is also distributed in the exons of gene bodies as 5mC but is mostly depleted in CGIs (25, 26). In promoters, 5hmC is mostly enriched in those with low to moderate CpG contents (29), while most affinity based methods found that 5hmC is enriched in CGIs close to TSS and highly related with active transcription (26, 28), which, indicating technical bias, may affect the interpretation of data. 5hmC is especially enriched at the start sites of genes whose promoters bear H3K4me3/H3K27me3 bivalent marks, suggesting a model in which 5hmC contributes to the 'poised' chromatin signature found at developmentally-regulated genes in ESCs (25, 29). Enrichment of 5hmC is also correlated with the monomethylated histone H3 lysine 4 (H3K4me1) markers at the poised enhancers, but not with the acetylated histone H3 lysine 27 (H3K27Ac) markers at active enhancers, indicating a poised epigenetic state or demethylation intermediate (24).

Function of hydroxymethylated DNA and DNA demethylation

The mechanism of DNA hydroxymethylation remodeling epigenetic signature is still under vigorous investigation and many novel regulatory machineries have been unraveled. 5hmC may change the chromatin structure by precluding the methyl-binding proteins that only have affinity for 5mC, therefore, disrupting binding of the transcription regulatory machinery after conversion (31). Conversely, 5hmC sequence prefers to be bound by specific coregulatory proteins, such as chromatin modifiers MBD3 and BRG1, to uniquely control gene de-repression event (32).

5hmC may also serve as an intermediate product for DNA demethylation. As no direct demethylase has been found so far, indirect demethylation through passive or active pathways occurs. As shown in Figure 3, in the model of passive DNA demethylation, after 5mC conversion to 5hmC in template strands, large amounts of newly synthesized chains become unmethylated during rapid division of cells, for example, the maternal genome loses methylation gradually for the purpose of reprogramming in early embryos from the zygote to blastocyst stages (25). Meanwhile, in the model of active DNA demethylation, several pathways possibly mediate this process. First, the activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex (APOBEC) family of cytidine deaminases converts 5mC and 5hmC into thymidine (T) and 5-hydroxymethyluracil (5hmU), respectively, followed by thymine-DNA glycosylase (TDG)-mediated T or 5hmU base excision, and finally ended with unmethylated cytosines by downstream repair (33). TDG is necessary for active demethylation of tissue-specific promoters and enhancers that are developmentally and hormonally regulated (33). AID/APOBEC assisted demethylation has been uncovered in primordial germ cells (PGCs) and slowly dividing brain cells (34). Second, TET proteins can generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) from 5mC or successively from 5hmC (35). 5fC and 5caC are excised and repaired to regenerate unmodified cytosines by mammalian TDG and the base excision repair (BER) pathway (35, 36). Finally, another possible player of demethylation could be DNMT3A/B, the same enzymes for *de novo* DNA methylation, which are characterized with 5hmC dehydroxymethylases activity *in vitro* (37). Related to this, an earlier finding about hormonal controlled cyclic methylation implies that DNMT3A/B may have deaminase activity for rapid demethylation (38).

Global demethylation and remethylation, processes known as epigenetic reprogramming, only occur twice

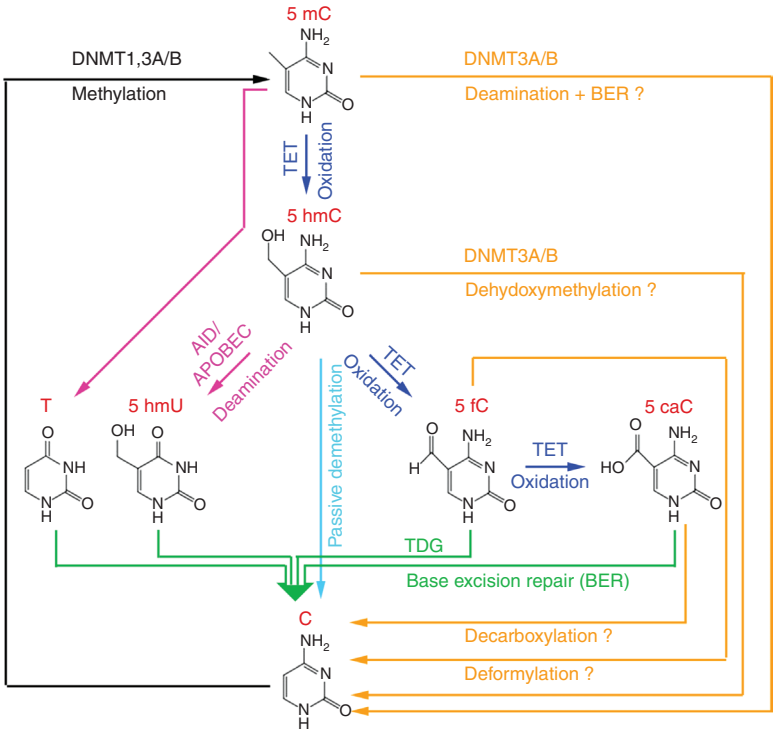

Figure 3 Scheme depicting enzymatic steps involved in the regulation of DNA methylation and demethylation through oxidation, deamination, passive demethylation, and other putative pathways.

in the life-cycle of mammals and are under tight regulation by TET family members via mediating conversion to 5hmC. The first occurrence of reprogramming arises soon after fertilization, but it is asymmetrical between the parental genomes found in preimplantation embryos. Paternal DNA loses methylation rapidly after fertilization and before the first division of the zygote, whereas maternal DNA is gradually demethylated during embryonic cleavage (24, 39). Nonetheless, most of the imprinting control regions (ICRs), and a few other regions, which are differently methylated between male and female gametes, escape from this global erasure to assure proper transmission of allele specific imprints between generations (10). During this process, developmental pluripotency-associated protein 3 (DPPA3) protects the maternal chromatin and certain paternal imprinted loci from TET3-mediated conversion of 5mC to 5hmC via local binding to harbored H3K9me2 (40). The second lifetime occurrence of reprogramming happens during PGCs expansion, migration, and entry into the gonads, which involves TET1 and TET2 driven transformation to 5hmC (34). Similar to the first wave, there is extensive loci correspondent to the intra-cisternal-A-particles (IAPs), but not imprinted loci, showing a resistance to global demethylation theorized to be for protecting genomic integrity in PGCs (41).

DNA methylation and histone modification

DNA methylation and hydroxymethylation determine the methylome throughout the whole genome. It is worth noting that DNA methylation at promoter sites and other regulatory regions acts as a mechanism to maintain gene silencing; however, the depletion of DNA methylation does not necessarily cause gene activation per se, but rather renders the gene permissive state for activation. Therefore, it is important to combine the methylation status of DNA location and other *cis*- or *trans*- modification to determine the state of gene expression.

As nucleosome components, histones have diverse posttranslational modifications, representing another layer of important epigenetic regulation. Modifications on DNA and histones define a state of the chromatin, directing a particular transcriptional program, and thus, a distinctive cellular fate. Compared to strict dependence between DNA methylation and histone modification established in fungi and plants, the relationship is more intricate in mammals (42). Whole-genome profiling of both modifications finds that DNA methylation patterns are better correlated with histone methylation patterns than

with the underlying genome sequence context (14). The active transcription starting sites bound by methylated histone H3K4 are usually not preferred by the DNMT3A/B-DNMT3L complex as a catalytic target, thus this modification correlates with unmethylated CGIs (9, 14). Knockout of *Kdm1b* [lysine (K)-specific demethylase 1B] in oocytes leads to increased histone H3K4 methylation and a failure to acquire maternal DNA methylation for many imprinted genes (43). Histone H3 lysine 36 methylation, residing in actively transcribed gene body with methylated cytosine but depleted from CGI promoter, can be bound by Dnmt3A and colocalized with methylated cytosine (44).

In contrast to the above histone markers correlated with active transcription, suppressive histone modifications have complicated and uncertain correlation with DNA methylation in mammalian genomes. Although H3K9me3 presents together with 5mC on chromatins (45), mutation of histone H3 lysine 9 trimethyltransferases only has limited effects on DNA methylation at constitutive heterochromatin or endogenous retroelements (46).

Another marker of H3K27me3 occurs mostly independent of DNA methylation; in certain contexts, H3K27me3 and DNA methylation even antagonize each other (45). However, in cancer cells *de novo* DNA methylation targets a large number of CGIs marked by H3K27me3, which indicates a PRC mediated distinct mechanism of initiating DNA methylation during carcinogenesis (42, 45). Coordination of different histone markers increases the complexity of the effort to correlate those modifications with DNA methylation. In pluripotent cells, bivalent modifications by colocalized H3K4me3 and H3K27me3 occur in a large number of gene promoters, which entails a plasticity for transcriptional regulation, allowing a rapid switch between repression and activation during differentiation (47). Such bivalent genes are associated with developmental functions and repressed in ESCs, but poised for activation upon differentiation. This makes the H3K27me3 a more flexible marker than a repressive one, which could be a reason for the complicated correlation with DNA methylation. Enrichment of 5hmC, highly related with active transcription and shown to overlapping with those bivalent regions, supports the poised status of genes in ESCs (25, 26).

Histone deacetylation has been suggested as another type of modification related to DNA methylation and gene silencing, as it has been shown that methylated DNA, methyl-CpG-binding protein MeCP2, and several DNA methylation regulators, such as UHRF1 and LSH, recruit histone deacetylases (HDACs) to repress transcription (48).

It is worth noting that, in certain conditions, histone modification can modulate transcriptional activity without cooperation of DNA methylation in controlling tissue specific gene expression (49), which can make them act either in parallel or reciprocally to coordinate the gene silencing.

DNA methylation and uterine physiology

The common dogma that DNA methylation arises during differentiation and remains stable for life has been challenged by more and more findings that transient and inducible alteration of DNA methylation takes place for the regulation of gene transcription responding to physiological or environmental changes. For example, a rapid and dynamic process of methylation/demethylation in the brain occurs during memory formation and in response to stimuli, highly associated with expression changes of particular genes (50).

It is well known that hormones can control gene expression through epigenetic regulation. Hormones can induce frequent methylation/demethylation on promoter CpG sites to regulate cyclic transcriptional activation of target genes (38). In diverse reproductive events, hormone-driven temporal changes of DNA methylation have been revealed. For example, to control seasonal reproductive neuroendocrine function in the hamster, thyroid hormone reversibly reduces promoter DNA methylation and upregulates expression of photoperiodic sensor gene *dio3* (51). During transition from follicle to corpus luteum after ovulation, LH induced DNA methylation and histone modifications are associated with repression of the *inhibin* α expression in the ovary (52). In the post-partum mouse, uterine oxytocin receptor exhibits a negative correlation between mRNA transcription and the methylation of its estrogen responsive element (ERE)-containing promoter, revealing a rapid physiologically inducible change of methylation within 24 h (53).

DNA methylation in cyclic uterus

The uterus is a hormone controlled organ that experiences cyclic physiological changes. Coordinated interaction of estrogen and progesterone directs proliferation and differentiation of compartmental uterine cells during the estrous/menstrual cycles. Long-term and transgenerational adverse effects can be acquired by the chronic exposure to endocrine disrupting compounds, such as phytoestrogens, toxins, and other environmental estrogens, which cause aberrant DNA methylation and an

increased risk of developmental defects, cancer, infertility, and other reproductive disorders as reviewed elsewhere (54). Pathological conditions in the uterus, including endometriosis, endometrial carcinoma, adenomyosis, leiomyoma, and other diseases cause irregularity of methylation globally or at specific loci (55). Beyond these accumulated epigenetic changes, more evidence has revealed that uterine DNA methylation also responds to acute stimulus provoked by hormones. One example is how phytoestrogen genistein promotes DNA demethylation of the steroidogenic factor 1 (SF-1) promoter in endometrial stromal cells, which can then be inherited to daughter cells (56).

In several species, the pattern of DNA methylation and DNMTs expression changing with reproductive cycles has been investigated. Simple measures of global DNA methylation do not find much change across the estrous cycle in bovine uteri (57). In the human endometrium, the expression of DNMTs are shown to vary with changes from one phase to another during the menstrual cycle at either mRNA or protein levels, and are potentially related to hormone fluctuation (58–61). However, it is difficult to provide a convincing profile of cyclic regulation on DNMTs with these discordant results, due to common variance in clinical studies and experimental designs, such as dissimilar sources, limited number of participants, and individual difference of tissue samples (62).

DNA methylation and uterine receptivity

Pregnancy causes dramatic change in endocrine signals, which are transmitted to temporally adapt the uterus for hosting the fetus. Implantation, the initial interaction between embryo and maternal uterus, needs synchronized activation of the blastocyst and receptive uterus, which is primed to the receptive stage by hormones and downstream molecular regulatory network (63). The difference with bovine uterine receptivity is indicated to be correlated with differential DNA methylation at global levels, concomitant gene transcription, and expression of DNMTs (64). Inhibiting DNA methylation by 5-aza-2′-decytocine (5-aza-dC) or suppression of DNMTs shows a correlation with the upregulation of E-Cadherin for advocating adhesive ability of endometrial epithelium cell line (65). However, increased expression of DNMT1 shows a positive correlation with enhanced endometrial receptivity in a clinical human IVF study (66). In our pilot study using a mouse model, application of 5-aza-dC while establishing receptivity (days 3–4) seems to have minimal impact on embryo attachment and the number of implantation sites, however, the proliferation of local stromal cells is comprised profoundly, indicating the predominant adverse effects in the postimplantation uterus (67). Another study administrating 5-aza-dC immediately after fertilization (day 1) through day 4 shows opposing effects on receptivity and moderate reduction in the expression of DNMTs (68), possibly due to increased chronic cytotoxicity by extended drug treatment. It is also possible that untested and potentially harmful effects on hormone production are at fault, as DNA methylation of crucial genes involves corpus lutea formation (52).

DNA methylation and uterine decidualization

In mammals with hemochorial placenta, including humans and rodents, endometrial stroma cells extensively proliferate and differentiate into decidual cells under the direction of signals released by invading embryos after implantation (63). Differentiated decidual cells show huge changes in the transcriptome compared with non-differentiated counterparts (69). Once pregnancy is terminated, the uterus recovers to an unpregnant state and reenters menstrual cycles after shedding and degeneration of decidual residues and regeneration of the epithelium. It is plausible to hypothesize that DNA methylation plays a role in regulating gene transcription during such a highly dynamic process. Although the transcriptional changes have been studied vigorously (69), the understanding of epigenetic regulation on this just starts. In an *in vitro* model of decidualization using estrogen, progesterone, cAMP or a different combination of treated primary or cloned human endometrial stroma cells, *DNMT*s exhibit a varied expression pattern with mostly transient or sustained decrease (59, 60, 70, 71), except *DNMT1* shows temporal upregulation in the early stage (61, 70). The discrepancy between studies could derive from different cell sources, treatment recipes, and other experimental variance (62, 70). DNA methylation inhibitor 5-aza-dC induces certain cell morphological and molecular changes similar with decidualized cells treated by hormonal recipe; however, these effects are not exactly the same as induced by hormones, which indicates some other effects independent of its demethylation activity (72). In a similar *in vitro* model, no obvious change of global DNA methylation after induced decidualization is detected by another group (71). Consequently, without a gene specific survey,

those observations cannot reveal the real change of DNA methylation during decidualization.

In order to screen differentially methylated genes during the progression of decidualization, we profiled the change of DNA methylation for the first time through a non-biased approach, using a methylation-sensitive restriction fingerprinting (MSRF) technique, in an experimentally induced decidualization model in mice (67). Multiple genes are identified showing hyper- or hypomethylation in regulatory elements and correlate well with their down- or upregulated expression in the decidual horn compared with an undifferentiated counterpart, clearly showing changes of DNA methylation during this biological event. Enhanced expression of DNMT1 and DNMT3A in decidua, together with the fact that 5-aza-dC significantly prohibits the maintenance of decidualization and aberrantly upregulates two hyper-methylated genes (*Bcl3* and *Slc16a3*), strongly supports an essential role of this epigenetic regulation for successful postimplantation decidual development (Figure 4). The negative effects of 5-aza-dC on decidualization is also supported by another study using a mouse model (68). Recently, appreciable differential methylation on a single chromatin is uncovered comparing fertile and infertile decidua from genetically different mice (73). There is considerable discrepancy between the aforementioned studies in humans and mice on regulation of DNMTs expression and the effects of DNA methylation inhibitor. Several factors are probably involved in the divergence, such as incomplete reflection of *in vitro* conditions for *in vivo* physiology, difference of species [decidualization is only induced during pregnancy in mice, but occurs cyclically in human during the secretory phase without the presence of embryos, implicating distinctly a potential role in acquiring receptivity (63, 74)] and differential regulation of enzymes [DNMT3B is barely detectable in the mouse uterus (67), but important in human decidualized stroma cells (70)]. In spite of the discovery of a set of genes with differential methylation in our study, the number of those genes is relatively low, which may be caused by low resolution and other limitations of MSRF strategy.

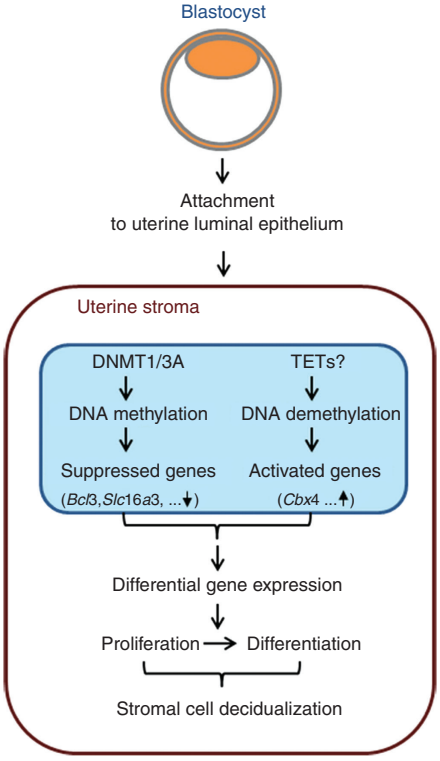

Figure 4 A proposed model for stromal cell decidualization at the site of implantation via DNA methylation/demethylation for differential gene expression.

Interestingly, chromobox 4 (*CBX4)* is one of the confirmed differentially methylated genes and is highly inducible in decidua (67). CBX4 is known as a component of polycomb repressive complex 1 (PRC1), which is known as epigenetic regulatory machinery for gene silencing. Through PRC1-dependent and -independent mechanisms, CBX4 participates in the regulation of cell proliferation, differentiation, and senescence (75), which are also actively involved during decidualization (63). PRC1 has diverse compositional variations and ability to perform transcriptional suppression dependent or independent of PRC2 catalyzed H3K27me3 (76). In respect to histone modification, decline of H3K27me3 and increase of acetyl-H3 and H4 at promoters of decidual marker genes *PRL* and *IGFBP1* have been shown to involve chromatin remodeling in the *in vitro* human endometrial decidualization model (77). Strong evidence *in vivo* has also found that H3K27me3 mediates silencing of inflammatory chemokine genes in decidua to adapt the immune tolerance at the feto-maternal interface (78). CBX4 has also been found to modulate stability and activity of DNMT3A as a SUMO-E3 ligase (79), which may link it back to the regulation of DNA methylation locally, as both of them are inducible in the decidual bed. It will be very interesting to explore the function of CBX4 and PRCs and to find the relationship between DNA methylation and histone modification in decidual transformation.

Concluding remarks

DNA methylation, as a well-established epigenetic marker, has attracted the efforts of biomedical researchers from

diverse fields for several decades. Combining our new knowledge of DNA methylome with histone modification and non-coding RNAs will provide important insights into the mechanisms of chromatin remodeling and regulation of gene transcription, which is instrumental to comprehensive understanding of developmental reprogramming and disease formation.

The pregnant uterus provides a good model for exploring the relationship between transient epigenetic regulation and transcription, due to its highly dynamic but reversible molecular and cellular changes during pregnancy or the menstrual cycle. Although emerging evidence suggests that epigenetic regulation is critical to finely tune multiple biological events in the uterus during pregnancy, limited progress has been made towards fully understanding these mechanisms, especially DNA methylation (62, 73). Heterogeneous composition of cell types, differential regulation of compartments, and difficulty of recapitulation via *in vitro* models makes the study of pregnant uteri biologically and technically challenging. However, rapidly developing next-generation sequencing techniques and DNA chemical modifying approaches allow for single-base resolution mapping of methylation and other newly found modifications on DNA. Applying those powerful technical tools and the appropriate experimental designs will help us to acquire a high-resolution view of epigenetic landscapes for the dynamically changing uterus. Integration of newly identified epigenetic information with data of transcriptome and proteome will advance our knowledge of the molecular mechanism behind uterine implantation and decidualization during early pregnancy.

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