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Regulation of CpG methylation by Dnmt and Tet in pluripotent stem cells

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Abstract. Vertebrate genomes are highly methylated at cytosine residues in CpG sequences. CpG methylation plays an important role in epigenetic gene silencing and genome stability. Compared with other epigenetic modifications, CpG methylation is thought to be relatively stable; however, it is sometimes affected by environmental changes, leading to epigenetic instability and disease. CpG methylation is reversible and regulated by DNA methyltransferases and demethylases including ten-eleven translocation. Here, we discuss CpG methylation instability and the regulation of CpG methylation by DNA methyltransferases and ten-eleven translocation in pluripotent stem cells.

Key words: CpG methylation, CRISPR/Cas, DNA methyltransferase, Embryonic stem cells, Ten-eleven translocation

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In vertebrate genomic DNA, the cytosine residues at the fifth position in CpG sequences are often methylated [1]. Most CpG sequences are located in nongenic repeated sequences, whereas some CpGs are located in the promoter regions of housekeeping genes and tissue-specific genes, where they regulate gene expression [2–4]. Although there are some methods for controlling gene expression, CpG methylation is one of the epigenetic hallmarks determining chromatin structure and controlling gene expression. The mechanism by which CpG methylation represses gene expression is not completely understood; however, several molecular mechanisms have been suggested, including blockage of transcription factor binding, recruitment of histone deacetylases (HDACs) via methyl-CpG-binding domain proteins (e.g., MeCP2, MBD1, and MBD2), recruitment of heterochromatin proteins (e.g., HP1), and transcription of non-coding RNA (e.g., Xist) [5–7].

In the past two decades, researchers have clearly demonstrated that CpG methylation plays a very important role in the normal development of mammalian embryos by regulating gene expression through genomic imprinting, X chromosome inactivation, and genomic stabilization [8–12]. Because CpG methylation is more stably maintained than chromatin modifications, CpG methylation is observed in imprinted genes, transposons, and the inactive X chromosome, which require long-term silencing throughout development and life. However, CpG methylation is reversible, sometimes changing during embryonic development and differentiation, and is even affected by environmental changes. Supplementation of the maternal diet with genistein (the major phytoestrogen in soy) in mice during gestation shifts the coat color of heterozygous viable yellow agouti (A^{vy}/a) offspring toward pseudoagouti [13]. This marked phenotypic change is associated with increased methylation by *in utero* dietary genistein at six CpG sites in a retrotransposon upstream of the transcription start site of the *Agouti* gene. In addition, numerous studies have evaluated altered CpG methylation, which causes several diseases. For instance, the most representative disease, cancer, often shows undesired gene silencing in tumor suppressor genes because of CpG hypermethylation.

Therefore, it is important to understand the role of molecules that write, maintain, and erase CpG methylation. It has been reported that DNA methyltranseferases (Dnmts) function as methylation writers and maintainers, and ten-eleven translocation (Tet) acts as a methylation eraser (Fig. 1). Regulation of DNA methylation has been widely examined in pluripotent stem cells, including embryonic stem cells (ESCs). In this review, we discuss the regulation of CpG methylation by the Dnmt and Tet proteins in pluripotent stem cells.

DNA Methyltransferase (Dnmt)

Maintenance- and *de novo*-type DNA methyltransferase activity have been reported in vertebrates. Maintenance-type Dnmt1, which shows a preference for hemi-methylated DNA, faithfully maintains the methylation state during cell division [14–17]. In contrast, two other DNA methyltransferases, Dnmt3a and Dnmt3b, are responsible for the creation of new methylation patterns during the early stages of embryogenesis and possess *de novo*-type DNA methylation activity [18, 19]. Both Dnmt3a and Dnmt3b contain an N-terminal regulatory domain that includes the PWWP and plant homeodomain (PHD) and a C-terminal catalytic domain with highly conserved motifs (I, IV, VI, VIII, IX, and X) (Fig. 1A). Although Dnmt3L shares homology with Dnmt3a and Dnmt3b, it lacks intrinsic DNA methyltransferase activity [20]. Dnmt3L physically associates with Dnmt3a and possibly with Dnmt3b to increase methylation efficiency and plays a critical role in germ cells [21–24]. Dnmt2 contains the complete sequence motifs as DNA cytosine-methyltransferases, but do not have global *de novo* or maintenance methylation activity for DNA [25].

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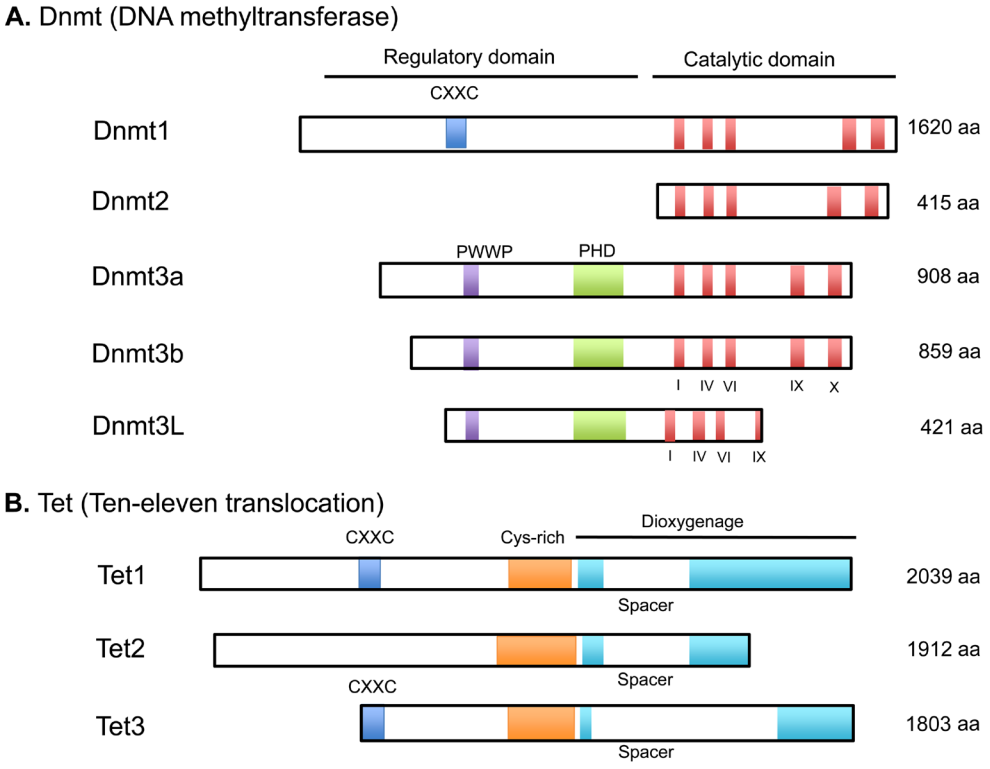

Fig. 1. Schematic diagrams of Dnmt and Tet proteins in mice. A. Dnmts contain an N-terminal regulatory domain and C-terminal catalytic domain. The catalytic domain of each contains conserved methyltransferase motifs (Roman numerals). The regulatory motifs differ between maintenance- and *de novo*-type methyltransferases. These regions control protein interactions as well as targeting. Cys-rich refers to the cysteine rich motif in Dnmt1. B. Tet proteins contain two conserved domains, the Cys-rich domain and the dioxygenase domain. Tet1 and Tet3 contain the CXXC domain in their N-termini, but the Tet2 CXXC domain has been lost during evolution. The numbers represent the amino acid numbers.

According to gene targeting experiments in mice, Dnmt1 and Dnmt3b knockout (KO) mice show embryonic lethality and Dnmt3a KO mice show postnatal lethality at 4–8 weeks [16, 19]. In contrast, Dnmt3L is not essential for embryonic development but is necessary for the establishment of methylation imprints in gametes [21]. The phenotype of Dnmt3a conditional KO mice in germ cells is indistinguishable from that of Dnmt3L KO mice, indicating that both Dnmt3a and Dnmt3L are essential for the methylation of imprinted loci in germ cells [26].

Ten-eleven Translocation (Tet)

Numerous studies have evaluated the methylating machinery, including Dnmts, but few studies have examined the mechanisms of demethylation and the major players. Although several CpG demethylation factors have been reported previously, most studies were not conclusive [27, 28]. Currently, major active demethylation is thought to be mediated by the ten-eleven translocation (Tet) family of proteins, which was recently identified as a new family of enzymes [29]. All three Tet proteins, Tet1, Tet2, and Tet3, contain a C-terminal 2-oxoglutarate- and Fe(II)-dependent dioxygenase domain (Fig. 1B). This dioxygenase domain catalyzes the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and the subsequent generation of 5-formylcytosine (5fC) and

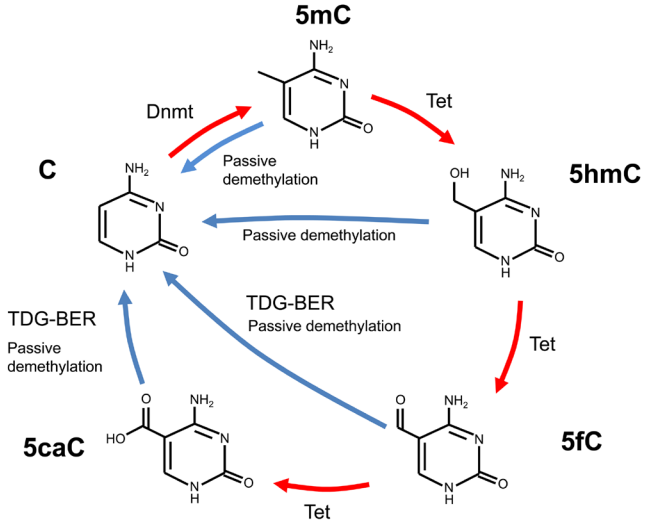

Fig. 2. Regulation of DNA methylation and demethylation by Dnmts and Tets. C, cytosine; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine.

5-carboxylcytosine (5caC), both of which are removed by thymine DNA glycosylase and base excision repair [30–32] (Fig. 2). As a result, the Tet-mediated oxidation pathway followed by thymine DNA glycosylase (TDG)-base excision repair (BER) leads to active demethylation of 5mC. In contrast, 5hmC cannot be recognized by Dnmt1 during DNA replication [33]; therefore, the conversion of 5mC to 5hmC inhibits the maintenance of existing DNA methylation patterns, leading to passive demethylation in proliferating cells. Tet1 and Tet3 contain another conserved domain, an N-terminal CXXC zinc finger domain with high affinity for clustered unmethylated CpG. While Tet2 lost this motif during evolution, the CXXC domain that was previously a part of Tet2 now exists as a different gene called IDAX/CXXC4 [34].

Experiments using KO mice showed that a homozygous mutation in Tet3 leads to neonatal lethality mainly because paternal genome conversion of 5mC into 5hmC fails to occur in early embryos [35]. In contrast, Tet1 and Tet2 KO mice are viable [36]; however, Tet1 KO significantly reduces female germ cell numbers and fertility [37]. Moreover, Tet1 and Tet2 are necessary to erase methylation imprints in primordial germ cells [38–41].

Instability of DNA Methylation in Embryonic Stem Cells

We previously reported the instability of CpG methylation in mouse preimplantation embryos. For example, blastocyst-stage embryos derived from *in vitro* fertilized zygotes or parthenogenetically activated oocytes showed a significant loss of CpG methylation in major and minor satellite repeats or in differentially methylated regions of several imprinted genes [42, 43]. This hypomethylation may be caused by artificial treatment, such as superovulation [44] and *in vitro* culture [45]. ESCs are established from the inner cell mass of blastocyst-stage embryos [46, 47]; therefore, altered DNA methylation in blastocysts may affect the CpG methylation status of their derivative ESCs. In fact, CpG methylation differences between *in vitro*- and *in vivo*-derived blastocysts are inherited by ESCs at early passages [48]. In contrast, epigenetic differences are lost during prolonged culture of these ESCs, and an epigenetic drift in each ESC line was preferentially observed [48, 49]. We found that ESCs derived from somatic cell nuclear transfer embryos also exhibited more dramatic epigenetic changes during long-term culture *in vitro* [49]. The epigenetic drift in these ESCs may be partially caused by small molecules contained in the ESC culture medium. For example, vitamin C, which is abundant in KnockOut™ Serum Replacement (Thermo Fisher Scientific, Waltham, MA, USA), a popular ESC culture medium, induces Tet activity in ESCs to promote DNA demethylation [50]. In addition, global demethylation occurred more frequently in female ESCs compared to in male ESCs because of lower expression of Dnmt3a and Dnmt3b [51].

Once ESC lines are generated, ESCs lose pluripotency during prolonged *in vitro* culture [52]. Several studies have indicated that accumulation of epigenetic alterations over time is associated with the loss of pluripotency in ESCs. Indeed, epigenetic alterations that occur in ESCs persist until later developmental stages and are correlated with abnormal phenotypes in completely ESC-derived mice [53]. Humpherys *et al*. observed variations in imprinted gene expression in most of the mice cloned from the same donor ESC line [54], indicating epigenetic drift in the same ESC line. Thus, epigenetic alterations appear and accumulate during culture and may have a detrimental effect on survival.

This CpG methylation instability may be induced by an unbalanced Dnmt or Tet expression pattern. Among the Dnmts, several alternative splicing variants lack regulatory and/or catalytic regions. In particular, Dnmt3b has nearly 40 different isoforms generated by alternative splicing and/or alternative promoter usage, and the ratio of its isoforms is different among cell types [55–57]. We recently found that mouse Dnmt3b lacking exon 6 (Dnmt3bΔ6) is highly expressed in *in vitro* manipulated embryos and their derivative ESCs, which exhibit a loss of CpG methylation [43]. *In vitro* methylation activity assays revealed that Dnmt3bΔ6 had lower methylation activity than the normal Dnmt3b. Gopalakrishnan *et al*. also report that this isoform is highly expressed in tumors and induced pluripotent stem cells and that ectopic overexpression results in repetitive element hypomethylation [58]. To understand the role of each enzyme, disruption of each or all Dnmt and Tet genes in ESC lines is necessary.

Gene Targeting of Dnmts and Tets in ESCs

The role of Dnmts in mouse ESCs has been widely studied using conventional gene targeting methods. For example, disruption of Dnmt1 in mouse ESCs leads to genome-wide loss of CpG methylation [16]. In addition, ESCs lacking both *de novo* methyltransferases, Dnmt3a and Dnmt3b, are unable to methylate proviral genomes and repetitive elements [19]. Moreover, Tsumura *et al.* established ESCs deficient for all three DNA methyltransferases, including Dnmt1, Dnmt3a, and Dnmt3b (Dnmt triple KO, Dnmt TKO) [59], and showed that ESCs without CpG methylation maintain stem cell properties, proliferation ability, heterochromatic domains marked with H3K9 trimethylation, and euploidy. Although reduced DNA methylation in mammalian somatic cells typically results in growth defects, cell death, activation of retrotransposons, and genome instability [60–65], mouse ESCs can tolerate the CpG hypomethylation status caused by inactivation of all Dnmts. This indicates that gene silencing and chromatin structures are regulated differently in undifferentiated ESCs. In fact, the proliferation of TKO cells is comparable to that of undifferentiated wild-type cells, but their growth is delayed upon differentiation by the formation of embryoid bodies [59].

Recently, clustered regularly at interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nucleases were developed as an epoch-making genome engineering technology known as CRISPR/Cas [66]. This system only requires the Cas9 nuclease and single-guide RNA (sgRNA) complementary to a target sequence. The CRISPR/Cas system makes the generation of knockout cells and animals more convenient than the conventional targeting method. Dnmt KO ESC lines have already been generated in mice using conventional methods [16, 19, 59], but they have not been generated in human cells because of the low targeting efficiency in human pluripotent stem cells. Because DNMT3B mutations cause immunodeficiency, chromosomal instability, and facial anomalies (ICF syndrome) in humans [19, 61, 67], we generated DNMT3B KO lines from human induced pluripotent stem cells using the CRISPR/Cas system [68]. We found that CpGs in pericentromeric regions (satellite 2) lose CpG methylation, as previously observed in ICF

syndrome; therefore, this DNMT3B KO cell line may be a good model for studying the syndrome. While Dnmt TKOs are viable in mouse ESCs despite the global loss of DNA methylation, Liao *et al.* reported that DNMT TKOs are lethal in human ESCs [69]. Disruption of DNMT3A and/or DNMT3B results in viable human ESCs with distinct effects on their DNA methylation landscapes, whereas human ESCs showed rapid cell death after DNMT1 deletion, indicating that DNMT1 is essential for human ESCs but not for mouse ESCs.

Similarly to studies of Dnmts, Tet KO ESCs were generated using both conventional gene targeting methods [36, 70] and the CRISPR/Cas system [71–73]. These reports showed that ESCs lacking all three Tet proteins (Tet TKO) were largely normal but undifferentiated. However, Tet1/2 double KO and Tet TKO ESCs showed depleted 5hmC and impaired ESC differentiation, resulting in poor differentiation of embryoid bodies and teratoma formation. Global gene expression and methylome analyses of Tet TKO ESCs revealed promoter [70] or enhancer [73] hypermethylation and deregulation of genes implicated in embryonic development and differentiation. In contrast, functional analyses of Tet TKO ESCs revealed other roles for Tet proteins. For example, Lu *et al.* indicated a role for Tet proteins in regulating the two-cell embryo-like state under ESC culture conditions [73]. In addition, they showed that Tet TKO ESCs exhibit increased telomere–sister chromatid exchange and elongated telomeres. More recently, our collaborators showed that 5hmC generated by Tet proteins localized at sites of DNA damage and repair, playing an essential role in ensuring genome integrity [74]. The CRISPR/Cas system enables the direct production of genetic KO mice via the introduction of the Cas9/sgRNA complex into zygotes, which is easier than conventional targeting methods using ESCs and chimera mice. We and other groups reported the direct production of Tet KO mice using the CRISPR/Cas system [71, 75] and found that these mice could assist with functional analyses of Tet proteins *in vivo*. Interestingly, one-step generation of Tet 1/2 multiple KO mice has also been reported [71].

Perspective

The role of each of the Dnmt and Tet genes has been analyzed in detail. However, Dnmts and/or Tets function cooperatively (e.g., Dnmt3a and Dnmt3L), suggesting the necessity of functional analysis using multiple KO cells. Fortunately, CRISPR/Cas enables the generation of multiple KO ESC lines easily, with various combinations of KO cell lines deficient in Dnmt, Tet, and other candidate genes. Particularly, Tet1 overlaps functionally with Tet2 in ESCs and primordial germ cells, but it is not well-understood how they share these roles. Thus, further studies are needed to understand epigenetic regulation in ESCs.

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