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DNA methylation and hydroxymethylation in stem cells

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Abstract

In mammals, DNA methylation and hydroxymethylation are specific epigenetic mechanisms that can contribute to the regulation of gene expression and cellular functions. DNA methylation is important for the function of embryonic stem cells and adult stem cells (such as haematopoietic stem cells, neural stem cells and germline stem cells), and changes in DNA methylation patterns are essential for successful nuclear reprogramming. In the past several years, the rediscovery of hydroxymethylation and the TET enzymes expanded our insights tremendously and uncovered more dynamic aspects of cytosine methylation regulation. Here, we review the current knowledge and highlight the most recent advances in DNA methylation and hydroxymethylation in embryonic stem cells, induced pluripotent stem cells and several well-studied adult stems cells. Our current understanding of stem cell epigenetics and new advances in the field will undoubtedly stimulate further clinical applications of regenerative medicine in the future.

Keywords

epigenetics; hydroxymethylation; methylation; stem cells; adult stem cells

INTRODUCTION

Epigenetics refer to the temporal and spatial control of gene expression during the development of organisms, contributing to cell identities. Epigenetic regulation is mainly via chromatin modification, with the packaging of DNA with histone and histone-binding proteins. Chromatin modifications are divided into several categories, including DNA modifications for cytosine methylation and the recently rediscovered hydroxymethylation, histone post-translational modifications (such as methylation, ubiquitylation, acetylation and phosphorylation) and adenosine triphosphate-dependent chromatin remodelling and non-coding RNA-mediated pathways. In recent years, there has been substantial progress

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

towards understanding the roles of histone modifications and DNA methylation, particularly in stem cell function and differentiation.

In mammals, stem cells are undifferentiated cells that can differentiate into specialized cells and self-renew through mitosis. In general, there are two types of stem cells: embryonic stem cells (ESCs), which are derived from the inner cell mass of blastocysts, and adult stem cells, which reside in various tissues that replenish or repair adult tissues. Two properties generally define a stem cell: the capacity for long-term self-renewal and the ability to differentiate into one or more specialized cell types. These stem cell identities and functions are highly involved in epigenetic regulation, sensing external environmental cues. For instance, DNA methylation, histone modifications and nucleosome remodelling are widely studied epigenetic modifications that are critical to ensure proper lineage commitment and cell fate determination. Like histone modification, methylation of DNA is an essential epigenetic control mechanism in embryonic development, sex chromosome silencing, repression of retrotransposons and imprinting. 5-Methylcytosine (5mC) can be oxidized to 5-hydroxymethylcytosine (5hmC) by the TET enzymes TET1, TET2 and TET3.[1,2] 5hmC was first reported in bacteriophages in 1952[3] and was rediscovered recently in human and mouse brains, as well as ESCs.[1,2] Similar to 5mC, 5hmC has been implicated in embryonic development and stem cell regulation and function, among other processes. Here, we focus on the current knowledge of as well as progress on DNA methylation and hydroxymethylation in ESCs and several well-studied adult stem cells.

EMBRYONIC AND INDUCED PLURIPOTENT STEM CELLS

Embryonic stem cells are isolated from the inner cell mass of the developing blastocyst. They have huge potential in regenerative medicine and hold out great therapeutic promise for many diseases. ESCs can be cultured indefinitely without compromising their pluripotency, and they can be differentiated into different cell types *in vitro*, which gives them their tremendous therapeutic promise. Because human ESCs (hESCs) are derived from fertilized eggs, using them for clinical application can pose ethical issues. As an alternative, induced pluripotent stem cells (iPSCs) bypass these ethical issues. iPSCs are induced from somatic cells by using cocktails of transcription factors, such as Oct4, Sox2, Klf4 and c-Myc.[4,5] In 2014, the world's first clinical trial of an iPSC-based therapy was launched in Japan, in which retinal tissue from stem cells created from skin cells were used for human patients in clinics, marking a momentous step towards regenerative medicine using iPSCs.

Induced pluripotent stem cells provide an inexhaustible source of cells for drug screening, for disease modelling from patient-specific cell lines, and for regeneration therapies once differentiated into certain cell types, as well as *in vitro* modelling of embryonic development processes. In the past several years, numerous studies have contributed to our understanding of how pluripotency is established and how to guide those iPSCs to desired cell types. Because iPSC reprogramming is a long, inefficient and complex process, understanding the mechanism will reveal more efficient reprogramming methods and make safer stem cells that are suitable for clinical application. In this section, we review DNA (hydroxy)methylation in pluripotent stem cells.

Methylation in embryonic stem cell and induced pluripotent stem cell reprogramming

DNA methylation is a DNA modification that usually occurs at CpG dinucleotides. CpG methylation in mammals is a specific epigenetic mechanism that can contribute to the regulation of gene expression.[6] In addition to CpG methylation, a methyl group can be added to a cytosine that is not upstream of a guanine; this form of DNA methylation is called non-CpG methylation and is abundant in plants.[7] In mammals, there are also reports of non-CpG methylation, such as in ESCs.[8–10] More recent publications have described significant levels of non-CpG methylation in some other somatic cell types.[11–15] In cells, DNA methylation is maintained by DNA methyltransferase 1 (DNMT1) and initiated by *de novo* DNA methyltransferase DNMT3a/b and cofactor DNMT3L. *Dnmt1* is essential for mouse embryonic development, and *Dnmt1* null mouse ESCs (mESCs) have normal self-renewal but are impaired for differentiation.[16,17] *Dnmt3a* and *Dnmt3b* are essential for mouse early development. Inactivation of both genes by gene targeting blocks *de novo* methylation in ESCs and early embryos, but in general, it has no effect on the maintenance of imprinted methylation patterns.[18] However, for repetitive sequences including LINE-1 promoters in mESCs, Dnmt3a and Dnmt3b were found to compensate for inefficient maintenance methylation by Dnmt1.[19] Although DNA methylation by DNMT1 or DNMT3a/b plays a crucial role in development, mESCs are fully functional for self-renewal in the complete absence of DNA methylation in triple-knockout *Dnmt1*$^{-/-}$*Dnmt3a*$^{-/-}$*Dnmt3b*$^{-/-}$ ESCs (Table 1).[20]

DNA methylation is important in gene regulation, genomic imprinting and X-chromosome inactivation.[21] DNA methylation changes are essential for successful iPSC reprogramming, evidenced by the necessity for loss of promoter methylation in many pluripotent genes.[5] DNA methylation changes are observed to follow histone modification changes and occur exclusively in the late stage of iPSC reprogramming.[22] If the loss of DNA methylation at pluripotent genes is not achieved, cells will be only partially reprogrammed.[23] Low-passage mouse and human iPSCs harbour residual DNA methylation `epigenetic memory' characteristic of their somatic tissue of origin, which favours lineages related to the donor cell when differentiating, while restricting alternative cell fates.[24,25] The DNA methyltransferase inhibitor 5-azacytidine applied during reprogramming improves reprogramming efficiency by around tenfold.[26] These findings suggest that demethylation in general plays important roles in reprogramming, and DNA demethylation is an inefficient step in establishing pluripotency (Table 1). In contrast to DNA demethylation, DNA *de novo* methylation does not contribute significantly to iPSC reprogramming.[27] Two *de novo* DNA methyltransferase-encoding genes, *Dnmt3a* and *Dnmt3b*, are highly expressed in ESCs and strongly induced after establishment of pluripotency. However, *de novo* DNA methylation is not critical and is dispensable for nuclear reprogramming of somatic cells to a pluripotent state (Table 1).[28] This suggests that the silencing of somatic genes may be initiated mainly via different mechanisms, such as H3K27 methylation or H3K9 methylation, as evidenced by the essential role of Polycomb repressive complex 2 function and H3K9 methyltransferases in reprogramming.[29–31]

Hydroxymethylation in embryonic and induced pluripotent stem cells—5-Hydroxymethylcytosine levels are high in mESCs and hESCs. For example, in mESCs,

5hmC consists of 0.04% of all nucleotides, or 5–10% of total methylcytosine (mC).[2] The modification from mC to hydroxymethylcytosine (hmC) suggests that a hydroxylated methyl group could be an intermediate for oxidative demethylation or a stable modification, leading to mC binding protein affinity changes at 5hmC loci or the recruitment of 5hmC selective binding proteins. All three TETs can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), with an abundance in the order of 5mC>5hmC>5fC>5caC in tissues.[2,32] Both formylcytosine and carboxylcytosine can be excised by thymine DNA glycosylase (TDG), which triggers subsequent base excision repair, suggesting a potential role for active demethylation (Figure 1).[33,34] These mechanisms implicate 5hmC function in pluripotency establishment and differentiation.

Based on reports, 5hmC is involved in the differentiation process.[35,36] Tet1 and Tet2 are abundantly expressed in mESCs.[37] Biochemically, Tet1 and Tet2 seem to have different characteristics in mESCs. Tet1 depletion diminishes 5hmC levels at gene transcription start sites, whereas Tet2 depletion is predominantly associated with decreased 5hmC in gene bodies.[38] Depletion of 5hmC by the double knockout (DKO) of *Tet1* and *Tet2* leads to cells that remain pluripotent but causes developmental defects in chimeric embryos (Table 1).[39] The $Tet1^{-/-}Tet2^{-/-}$ DKO mouse is compatible with development but promotes hypermethylation and compromises imprinting. The DKO of *Tet1* and *Tet2* results in partially penetrant embryonic and neonatal abnormalities associated with perinatal lethality in about half the mutants. Moreover, combined loss of all three TET enzymes restricts the normal differentiation of ESCs, and *Tet* null ESCs contribute poorly to developing embryos and cannot support development (Table 1).[40]

5-Hydroxymethylcytosine can be further oxidized to 5fC and 5caC, both of which could be repaired by TDG to produce unmodified cytosine.[33,34,41] Given this biochemical property, a compelling model is that hmC, formylcytosine and carboxylcytosine serve as intermediates of an active DNA demethylation process. Many regulators have been reported in the regulation of this stepwise process, linking this mechanism to various cellular functions. For example, in mESCs, TET proteins are direct substrates of calpain, which is a family of calcium-dependent proteases. Calpain1 mediates TET1 and TET2 stability post-transcriptionally, and calpain2 regulates TET3 levels during differentiation.[42]

In addition to cytosine modification, cells also have 5-hydroxymethyluracil (5hmU), which is the product of reactive oxygen species. A recent report shows that Tet-induced oxidation is not limited to mC but that thymine is also a substrate, which can be catalysed to hydroxymethyluracil (Figure 1). Thus, 5hmU may have an additional function in cells, besides triggering the DNA repair pathway.[43]

TET proteins and 5-hydroxymethylcytosine in pluripotent reprogramming— The iPSC reprogramming process is accompanied by increasing 5hmC, and most of the parental somatic cells lack 5hmC. Given the model that DNA demethylation may be modified by a TET base excision repair process, many groups have studied whether TET-mediated 5hmC is involved in reprogramming, and how it contributes to this stepwise process.[44–47] TET1, in synergy with NANOG, enhances the efficiency of reprogramming. Co-expression of NANOG and TET1 increases 5hmC levels at the top-ranked common

target loci, *Esrrb* and *Oct4*, resulting in the priming of their expression before reprogramming to naive pluripotency.[44] In another study, Tet1 overexpression promotes the formation of reprogrammed colonies from fibroblasts, and the authors suggest that Tet1 functions via accelerated *Oct4* transcriptional activation by demethylation of its promoter.[46] This model is strongly supported by an experiment wherein Tet1 was able to replace Oct4 to generate fully pluripotent iPSCs (Table 1). Reprogramming of mouse embryonic fibroblasts deficient in TDG was also impaired. The impediments of reprogramming are caused at least in part by defective activation of some microRNAs (miRNAs), which depend on demethylation promoted by TET and TDG.[48] Similar to many other systems, the TET-regulated process during reprogramming has complex layers of regulation. miR-22 is both a repressor of TET proteins and a powerful oncogene in the mammary epithelium and haematopoietic system.[49,50] miR-29a/b are also involved in TET regulation.[50] As miR-22 and 29a/b are differentially expressed between fibroblasts and iPSCs,[51] it is likely that those miRNAs post-transcriptionally modulate TET, thus inhibiting somatic cell reprogramming.

Interestingly, or perhaps unexpectedly, ascorbic acid (vitamin C) is able to modulate Tet activity. Several reports demonstrate that vitamin C increases 5hmC levels by threefold to sevenfold in cellular DNA,[52–55] pointing to it as a direct regulator of Tet activity and a factor facilitating DNA demethylation. Mechanistically, vitamin C may promote replication-dependent and passive DNA demethylation by enhancing 5hmC formation and accelerate active DNA demethylation by enhancing formation of 5fC and 5caC. In iPSC reprogramming, vitamin C is thought to overcome the senescence block and facilitate the pre-iPSC transition,[56] enhance the activity of H3K36 demethylases[57] and prevent imprinted Dlk1-Dio3 erasure,[58] all of which are important for increasing reprogramming efficiency. These findings imply that Tet1 has a positive impact on reprogramming. However, one study showed that when switched from standard iPSC culture medium to modified medium enriched with vitamin C, *Tet1*$^{-/-}$ mouse embryonic fibroblast cells have improved efficiency. This contradictory finding reveals the relationship between vitamin C and Tet1 in reprogramming. Based on this report, some believe that depending on the presence or absence of vitamin C, Tet1 either positively or negatively regulates somatic cell reprogramming.[55]

Methylome in embryonic stem cells—With the reduced cost of next-generation sequencing and technological advances, many genome-wide analyses of DNA methylation and hydroxymethylation are being applied to ESCs. These studies yield more perspectives on the function of (hydroxy)methylation on a large scale. A bisulfate sequencing study at single-base resolution in hESCs and fibroblasts revealed two important features of DNA methylation.[11] First, DNA methylation is generally depleted at DNA–protein interaction sites regardless of pluripotency, and, second, nearly 25% of methylation loci in ESCs are in a non-CG context; non-CG methylation showed enrichment in gene bodies and depletion in protein binding sites and enhancers. Non-CG methylation reduced significantly upon induced differentiation of the ESCs and was restored in iPSCs.[11] Nevertheless, its functions are still unclear. Analysis of the correlation of DNA methylation and histone modifications of chromatin states and DNA methylation status at promoters and most CpG islands revealed that the methylation state of H3K4 is a good indicator of promoter DNA

methylation levels in mammalian cells.[59–61] Base-resolution maps of DNA methylation for hESCs and hESC-derived cell types [mesendoderm, trophoblast cells, neural progenitor cells and mesenchymal stem cells (MSCs)] reveal that promoters for genes expressed preferentially at later stages are often CG poor and have DNA methylation when silenced. Interestingly, the early developmental regulatory genes are often located in large genomic domains called DNA methylation valleys that are generally devoid of DNA methylation in most lineages.[62]

Studies of 5hmC profiling using various strategies such as affinity purification-based sequencing[63,64] and TET-assisted bisulfite sequencing methodology (TAB-seq),[65] in stem cells reveal similar patterns as 5mC. 5hmC is enriched at promoter and enhancer regions, particularly at promoters bearing dual H3K27me3 and H3K4me3 marks.[64,66] Restriction endonuclease AbaSI coupled with sequencing Aba/TAB-seq common sites yielded no significant differences in general; nevertheless, 5hmC on non-CpG sites was non-detectable by TAB-Seq, whereas Aba-Seq suggests there is indeed DNA hydroxymethylation on non-CpG sites in stem cells.[67]

Studies have highlighted large-scale `hot spots' of aberrant epigenomic reprogramming that frequently exhibit aberrant DNA methylation patterns, as well as hydroxymethylation patterns in iPSCs, relative to ESCs.[47,68,69] These studies suggest iPSCs may not be identical to ES cells. Interestingly, essentially all of these hmC hot spots overlap with mC hot spots, and those mC hotspots overlap with regions with H3K9me3 domains in differentiated cells, pointing to a potential model wherein H3K9me3 may act as an epigenetic barrier for reprogramming, thereby impeding DNMT3/TET recruitment at these loci.[70]

ADULT STEM CELLS

To meet the demands of subsequent development, the embryo forms germline stem cells (GSCs) and somatic stem cells for reproduction and organogenesis, respectively.[71,72] As with ESCs, these adult stem cells are undifferentiated and reside in a special microenvironment, termed the `niche', which is located variously depending on the tissue type.[72] Adult stem cells are an essential component of tissue homeostasis, as they have been demonstrated to play indispensable roles in physiological tissue renewal and tissue repair following injury.[73,74] Recently, consensus became that adult stem cells might be able to differentiate into any cell type present in their parent germ layers.[75,76] For example, haematopoietic stem cells (HSCs) and MSCs in bone marrow are derived from the mesoderm, and neural stem cells (NSCs) in the central nervous system (CNS) are derived from the ectoderm.[74,77,78] The use of human adult stem cells in research and therapy attracts intense interest, as adult stem cells can be harvested from patients and are not considered to be as controversial as ESCs. Given the fact that adult stem cells must be functional in the whole life span of the organism, a delicate balance between self-renewal and differentiation has to be maintained.[73] The underlying mechanisms, including epigenetic regulation, that control this delicate balance are important to our understanding of adult stem cell regulation and the therapeutic use of adult stem cells in human disease. In the succeeding sections, we summarize and discuss the roles of DNA methylation and demethylation in some well-studied adult stem cells.

Adult haematopoietic stem cells

Haematopoietic stem cells are the multipotent blood cells, and all haematopoietic lineages, including the myeloid and lymphoid lineages, derive from a pool of HSCs.[79,80] HSCs start to appear after primitive erythrocytes are generated in the yolk sac and placenta, then in the aorta–gonad–mesonephros region of the embryo.[79–81] HSCs then migrate to the foetal liver and thereafter emerge in the bone marrow, the final and predominant site of haematopoiesis throughout adulthood.[80] Two groups simultaneously identified the osteoblastic cells, primarily those lining the trabecular bone surface, as the pivotal component of the HSCs niche.[82,83] As HSCs represent one of the first recognized adult stem cell types, many *in vitro* and *in vivo* analyses have revealed that many genes, such as DNA (de)methylating enzymes, histone modifiers and factors involved in miRNA synthesis, are indispensable for HSC development and function.[84,85] In mouse aged HSCs, 1600 genes have been roughly profiled to be repressed with age, including a number involved in chromatin remodelling, histone deacetylases and *de novo* DNA methylation.[86] Notably, *Dnmts* and *Tet2* are found to be differentially expressed among young and old HSCs.[87,88]

Here, we summarize the roles of DNA methylation and TET protein in adult HSCs.

DNA methylation in adult haematopoietic stem cells—Three major DNA methyltransferases are found to be vital throughout mammalian development: the absence of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in mouse germline leads to the lethality of mice at gastrulation, E9.5 and postnatal 3 weeks, respectively.[16,18] Studies using conditional Dnmts-knockout mice have clarified the essential roles of DNA methylation in adult HSCs.[89–92] Defects of self-renewal and niche retention were observed in *Dnmt1*$^{-/-}$ HSCs.[90] Reduced activity of *Dnmt1* in HSCs resulted in an increased expression of myeloerythroid progenitor regulators, including *Gata1*, *Id2* and *Cepba*, and caused uncontrolled differentiation from HSCs into myeloerythroid progeny, rather than lymphoid progeny (Figure 2a).[89] It seems that the promoters of myeloerythroid progenitor regulators are preferentially methylated in HSCs, with a critical threshold to balance the myeloid output and regulate differentiation of lymphoid tissue appropriately. On the other hand, *de novo* DNA methyltransferases DNMT3a and DNMT3b also play critical roles in HSC self-renewal and differentiation. Conditional knockout of DNMT3a in the haematopoietic compartment leads to the expansion of HSC numbers, while in the meantime driving a gradual impairment of HSC differentiation over serial transplantation (Figure 2b).[92] Both increased and decreased methylation have been observed in *Dnmt3a*$^{-/-}$ HSCs at distinct loci, including an extensively hypermethylated CpG island. HSC multipotency genes are upregulated in *Dnmt3a*$^{-/-}$ HSCs, whereas HSC differentiation factors are downregulated, and the progeny of HSCs exhibit global hypomethylation and incomplete repression of HSC-specific genes, such as *Runx1* and *Vasn*.[92] A recent paper reported that loss of both *Dnmt3a* and *Dnmt3b* in HSCs leads to an even more severe arrest of HSC differentiation (Figure 2b).[91] However, a specific role for *Dnmt3b* in permitting the differentiation of HSCs is also reported.[91] The mild *in vivo* phenotype of *Dnmt3b*$^{-/-}$ HSCs suggests that DNMT3a can compensate for the vast majority of DNMT3b loss, but DNMT3b is not completely competent in the reverse situation. The authors hypothesized that the target specificity of the remaining catalytically functional DNMT3b was different, which might result in aberrant DNA methylation

patterns, such as CpG island hypermethylation. Together, these data suggest that DNA methylation plays a critical role in the HSC regulatory genes, thereby enabling efficient self-renewal and differentiation.

TET proteins in adult haematopoietic stem cells—TET proteins that regulate active DNA demethylation have important roles in stem cell self-renewal and differentiation. Unlike the universal roles of all three TETs in ESCs and iPSCs, so far, only TET2 has been closely linked to HSC function.[93–95] TET2 acts as a key tumour suppressor and is mutated/deleted with high frequencies (up to 20–40% of the total cases) in multiple forms of myeloid malignancies, indicating the dynamic regulation of DNA methylation and demethylation may be critical for the fate of adult HSCs.[96] In a mouse model, conditional deletion of *Tet2* in the haematopoietic compartment, not only $Tet2^{-/-}$ but also $Tet2^{+/-}$, leads to increased HSC self-renewal and extramedullary haematopoiesis *in vivo*, suggesting that TET2 regulates adult HSC maintenance.[94] Inactivation of *Tet2* in mouse significantly affects haematopoiesis, including myeloid and lymphoid differentiation, gives the cells competitive abilities and finally leads to malignancy development.[97] The myeloid progenitor cells generated from $Tet2^{-/-}$ mice have been found with increased expression of the self-renewal regulators *Meis1* and *Evi1*, and at the same time with reduced expression of multiple myeloidspecific factors (including *Cebpa*, *Cebpδ*, *Mpo* and *Csf1*), suggesting that they have gained self-renewal ability in the absence of Tet2.[94] In a recent study of zebrafish TET proteins, based on morpholino-mediated knockdown technology, loss of TET2 specifically caused the suppressed expression of lineage-specific genes (*Scl*, *Gata-1* and *Cmyb*), inhibited erythropoiesis and resulted in dysregulation of the formation and differentiation of erythroid progenitors.[98] It has been found that the status of 5hmC and demethylation in the intermediate CpG promoters (ICPs) of those lineage-specific genes, particularly at specific regions or CpG sites of these ICPs, significantly affects erythropoiesis.[98] In contrast, a subsequent similar study, based on zinc finger nuclease technology, revealed neither change in *Cmyb* and *CD41* expression nor defect in embryonic erythropoiesis.[99] The homozygous $Tet2^{m/m}$ zebrafish lines showed decreased levels of 5hmC specifically in haematopoietic cells of the kidney marrow rather than other cell types, which indicates the unique function of TET2 in haematopoietic cells that cannot be compensated for by other TETs.[99] As indirect regulators of normal haematopoiesis, a number of miRNAs are found to inhibit TET2 expression and cellular 5hmC.[100] *In vivo* expression of TET2-targeting miRNAs, including those preferentially overexpressed in TET2 wild-type acute myeloid leukaemia, affects normal haematopoiesis, which results in haematopoietic expansion and/or myeloid differentiation bias; these phenotypes can be rescued by co-expression of TET2.[100]

In contrast to the relevant function of TET2 in HSCs, TET1 shows limited impact on haematopoiesis.[94] However, as a fusion partner of the mixed-lineage leukaemia (MLL) gene, TET1 exhibits an indispensable oncogenic role in the development of MLL-rearranged leukaemia, in which TET1 is significantly upregulated, with an accompanying global increase of the 5-hmC level.[101,102] This feature enables TET1 to be served as a potential target for future therapeutic intervention of this presently therapy-resistant cancer.[102]

Adult neural stem cells

Adult neural stem cells are self-renewing and multipotent cells that generate the main compartments of the CNS.[103,104] The discovery of adult NSCs is a landmark in our understanding of adult brain plasticity, as it overturns the dogma that the adult mammalian CNS does not generate new neurons.[105,106] In the neural system, the stem cell niche is found in endothelial cells located at the base of the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus.[107–109] Adult NSCs can give rise primarily to neurons and glial cells, including astrocytes and oligodendrocytes,[110] and the process to generate various functional neural cell types from adult NSCs is defined as adult neurogenesis.[111–113] Adult neurogenesis can be recognized as a classic case of stem cell differentiation processes, in which epigenetic regulators delicately manipulate the spatial and temporal expression of key genes in NSCs and determine the proliferation, fate specification and differentiation of NSCs.[113,114]

DNA methylation in adult neural stem cells—DNA methylation plays a critical role in synaptic plasticity related to learning and memory via the regulation of specific gene expression.[115–117] To explore the role of DNA methylation in the CNS, a number of conditional knockout mouse models have been generated. DNMT1 is ubiquitously expressed in mouse brains, and the ablation of *Dnmt1* in neuronal progenitor cells results in DNA hypomethylation, activates the JAK–STAT astrogliogenic pathway and accelerates the glial differentiation process.[118,119] *Dnmt3a* is activated around E10.5 in mouse neuronal precursor cells and remains active in postmitotic neurons in the adult.[117] Mice lacking functional DNMT3a in the entire CNS are born apparently healthy but die prematurely with the acquisition of developmental defects.[120] With the deletion of *Dnmt3a,* impaired postnatal neurogenesis is seen in both the subventricular zone and SGZ, i.e. tenfold fewer neurons are differentiated from *Dnmt3a*-null NSCs.[121] Self-renewing postnatal NSCs are repressed though *Dnmt3a*-mediated methylation on proximal promoters. In addition, *Dnmt3a* also competes with Polycomb, via nonproximal promoter methylation, and promotes the transcription of targets, including neurogenic genes.[121]

Methyl-CpG-binding protein 1 (MBD1), a protein that binds to hypermethylated gene promoters, has been reported as an indispensable and specific regulator for adult NSCs in a DNA methylation-dependent manner. *Mbd1*$^{-/-}$ mice are born without obvious developmental defects throughout their life span, but their adult neurogenesis is severely impaired (e.g. they have spatial learning defects).[122] Fibroblast growth factor 2 (*Fgf2*) is a mitogen for adult neural progenitors, and its hypermethylated promoter can be bound by MBD1; therefore, loss of MBD1 induces hypomethylation in the *Fgf2* promoter and increases its expression in adult NSCs, which results in the inhibition of NSC differentiation.[123] Active DNA methylation/demethylation is reported to play critical roles in neurogenesis, in which growth arrest and DNA damage-inducible protein 45b (Gadd45b) is considered a key regulator in promoting adult neurogenesis, while MeCP2, another DNA methyl-CpG-binding protein, in mature neurons can interpret reversible DNA methylation under the regulation of Dnmts and Gadd45b proteins.[117,124] Moreover, some miRNAs, such as miR-184 and miR-137, respectively, function as the direct target of MBD1 and MeCP2 and inhibit neuronal differentiation from adult NSCs.[125–127] For instance, MeCP2-mediated

epigenetic regulation of miR-137 involves coregulation by Sox2, a core transcription factor in stem cells, and regulates the fate of adult NSCs.[127]

DNA hydroxymethylation and TET in adult neural stem cells—TET-mediated hydroxymethylation in CNS is also important because 5hmC is highly enriched in the brain, approximately tenfold higher than in ESCs.[128] The specific distribution of 5hmC in mammalian brain and its roles in gene regulation suggest that 5hmC is important in neuronal development and may play a role in neurological diseases.[129] The marked gain of 5hmC in mouse neuronal cells from postnatal neurodevelopment through adulthood has been reported by our group, and the additional genome-wide analyses of 5hmC distribution in human cerebellum further revealed conserved characteristics of 5hmC in mammals.[130] Given the fact that 5hmC is found at such high levels in brain and that dynamic 5hmC readers have been identified, 5hmC is thought to be not just an intermediate in DNA demethylation. The distribution and localization of 5mC and 5hmC within chromatin, as well as interactions with 5mC-binding and 5hmC-binding proteins, are altered throughout development, and these changes parallel neuronal differentiation and development.[131] In addition, consistent with the idea that 5hmC contributes to neuronal differentiation, a subset of 5hmC-binding proteins are found specifically expressed in NCSs and the brain.[132]

TET1-mediated 5mC/5hmC conversion indicates that 5hmC is more likely to undergo deamination than 5mC by the activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex family of cytidine deaminases to complete the demethylation cycle.[133] Overexpression of TET1 or AID in the dentate gyrus results in a dramatic decrease of CpG methylation levels on two neuronal activity-related genes, *Bdnf* and *Fgf1b*, but not on the promoter of non-neuronal genes.[133] In conditional knockout mice, loss of *Tet1* results in a 45% decrease of NCSs in the SGZ, and the neurosphere derived from *Tet1*$^{-/-}$ mice shows impaired growth function.[134] A number of genes involved in NSC proliferation are found to be both hypermethylated and downregulated in *Tet1*$^{-/-}$mice.[134] Overexpression of *Tet2* and *Tet3*, combined with the loss of *Ezh2*, increases 5hmC formation and leads to defects in neuronal differentiation.[135] Moreover, depletion of *Tet3* in *Xenopus* significantly inhibits master neuronal development genes, including *Pax6*, *Rx* and *Six6* in the eye.[136]

Germline stem cells

During post-implantation mammalian embryo development, on E6.25, a small cluster of posterior proximal epiblasts separates from the somatic cells and assumes the germline cell fate. Soon after that, these cells are induced by signals from extra-embryonic ectoderm to become primordial germ cells (PGCs) on E7.25, resulting in PGC specification.[137,138] From PGC migration to early residence in gonad, genome-wide epigenetic changes take place, known as the second wave of reprogramming (E7.75–E12.5),[139,140] through which PGCs escape the somatic cell fate and gain the unipotency of developing into GSCs.[141] Given the nature of producing gametes for transmitting genetic information from generation to generation, GSCs are also considered as true `immortal stem cells'.[142] In model invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, long-term self-renewing GSCs exist in both male and female invertebrates.[72] In mammals, on the other hand, male mammals maintain GSCs in the testes for spermato-genesis,[143] whereas whether

postnatal female mammals also possess GSCs remains controversial.[144–146] Mouse GSCs have been successfully cultured *in vitro* and can be used to repopulate germ cell-depleted testes and restore fertility.[147,148] Interestingly, cultured mouse GSCs can produce ESC-like cells, which are capable of differentiating into different cell types.[147,149]

DNA methylation and demethylation in adult germline stem cells—Stage-specific high-resolution profiles of DNA methylation, 5hmC, histone modifications/variants and RNA-seq in adult GSCs (AGSCs) and during spermatogenesis have been investigated recently.[150] For the first time, the DNA methylation profiles of AGSCs and ESCs were found to differ at loci for germline development.[150] Comparison of AGSCs to ESCs yields about 330 differentially methylated promoters of genes that are silenced in ESCs while active in germline development. Between E16.5 PGCs and AGSCs, a number of key genes associated with meiosis lose DNA methylation, and many of the key genes responsible for cell migration become silenced. Remarkably, gametogenesis occurs without significant changes in DNA methylation and instead involves transcription of DNA-methylated promoters bearing high RNA pol2, H3K9ac and H3K4me3 and low CG content and 5hmC. Moreover, enhancers of pluripotency genes, like *Nanog*, *Sox2* and *Prdm14*, are often hypomethylated and bivalent in mature sperm, suggesting the potential for active DNA methylation removal in the zygote.[151] These results together suggest that DNA methylation and demethylation patterns are changing between cell stages, regulating the transcriptome to ensure successful acquisition of pluripotency and germline development committed to gametogenesis.

TET and DNA hydroxymethylation in primordial germ cells—The identity of GSCs is directly and firmly linked with the PGC reprogramming process. In principle, PGC reprogramming must both repress the ongoing somatic programme and activate the germ cell transcriptional network to ensure the successful gain of unipotency for GSCs, leading to gametogenesis.[139,152] In wild-type PGCs, distinct distributions of 5mC and 5hmC have been observed during different stages of germ cell reprogramming, highlighting the importance of the dynamic alteration of DNA methylation in proper germ cell maintenance.[153–155] The processes of global demethylation of PGCs and establishment of imprinting are accompanied by conversion from 5mC to 5hmC, driven by TET1 and TET2.[155] In support of this, increased imprinting defects are seen in *Tet1*$^{-/-}$*Tet2*$^{-/-}$DKO mice.[39] In addition, *Tet3* is expressed later in gametogenesis (E16.5), as well as during spermatogenesis and in the oocytes, but not in early-stage germ cells.[39,154,156–158] The ablation of *Tet3* in mouse oocytes results in epigenetic abnormalities in the paternal genome.[157] Therefore, epigenetic abnormalities seen in *Tet1*$^{-/-}$*Tet2*$^{-/-}$DKO mice may be due to a combination of epigenetically aberrant gametes and to only partial restoration of 5hmC content by *Tet3* expression.[39] Alternatively, relative hypermethylation in AID-deficient PGCs has been confirmed by analysis of individual loci in the genome, in which AID deficiency strongly influences genome-wide DNA demethylation.[159]

Taken together, DNA methylation and hydroxymethylation play multiple roles in germline stem cell-associated unipotency acquisition and gametogenesis commitment. Consequently,

a better understanding of GSCs could be key to providing alternative therapeutic strategies for infertility and degenerative diseases in the future.

Other adult stem cells

Adult stem cells also refer to many other types, such as mammary stem cells, MSCs, intestinal stem cells (ISCs), endothelial stem cells and olfactory adult stem cells, in which DNA methylation and demethylation play important roles in the regulation of their self-renewal and differentiation. For example, DNA methylation at enhancers is changing dynamically during ISC development. The loss of DNA methylation at these enhancers leads to abnormal ISC differentiation.[160] Differentially methylated regions (DMRs) at enhancers are positively linked to gene expression in differentiated villus cells.[161] Moreover, promoter demethylation of achaete scute-like 2 (*Ascl2*), an essential transcription factor in maintaining ISC identity, results in the aberrant upregulation of ASCL2 in gastric cancer.[162] In breast cancer stem cells (bCSCs), a whole-genome promoter microarray analysis shows that 68 DMRs are more hypomethylated in bCSCs than in non-bCSCs.[163] These DMRs are significantly enriched in genes coding for transcription growth factor (TGF)-β signalling-related proteins, and interestingly, the hypomethylation of DMRs correlates to an overexpression of TGF-β signalling genes in a series of 109 breast tumours, which implies that DNA methylation patterns affect tumour malignancy by regulating the transcription of genes relevant to bCSC multipotency and differentiation.[163] In a recent study, MSCs were reprogrammed to iPSCs and then redifferentiated into MSCs again.[164] Although redifferentiated MSCs (from MSC-derived iPSCs) share the same morphology and gene expression profiles with primary MSCs, the DNA methylation patterns vary, with tissue-specific, senescence-associated and age-related DNA methylation patterns erased during reprogramming. An impaired ability to suppress T-cell growth is also seen in the redifferentiated MSCs, indicating an appropriate DNA methylation pattern is important for maintaining the normal immunomodulatory function of MSCs.[164] 5hmC patterning is reported to have an impact on transcriptional regulation in mouse main olfactory epithelium development, and the 5hmC level in the gene body is positively correlated with transcription levels across different cell types, including olfactory stem cells.[165] TET3 overexpression interferes with the correct targeting of axons to the olfactory bulb in mature olfactory neurons.[165]

Together, DNA methylation and demethylation play critical roles in adult stem cell self-renewal and differentiation. The dynamic change of DNA methylation is required to maintain the delicate regulation of adult stem cell fates. Although it is clear from the evidence that DNA methylation and demethylation are necessary for controlling adult stem cell proliferation and differentiation, their precise contributions in each lineage programme remain unclear. Notably, future studies will need to take into account the extensive cross-talk between DNA modification and other epigenetic mechanisms, such as histone modification and non-coding RNA, for a better understanding of the regulation of adult stem cells.

CONCLUSIONS AND OUTLOOK

In summary, the goal of regenerative medicine is to replace lost or damaged cell tissues through stem cell therapy. This can be accomplished potentially using pluripotent or adult stem cells directly from bodies or from engineered somatic cells via reprogramming or transdifferentiation. A profound knowledge of the molecular mechanisms, such as DNA methylation or hydroxymethylation, underlying embryonic and adult stem cell function and a detailed characterization of the driving forces related to these cells, such as iPSC reprogramming, may enable us to design better strategies. These strategies will aim towards the generation of higher-quality stem cells that will be suitable for clinical application. In the past several years, the rediscovery of hydroxymethylation tremendously expanded our insight and uncovered more dynamic aspects of cellular methylation regulation on stem cell identity and function. Our current understanding of epigenetics and possible advances of the future will pave the way towards exploitation of novel strategies to prevent or cure diseases.

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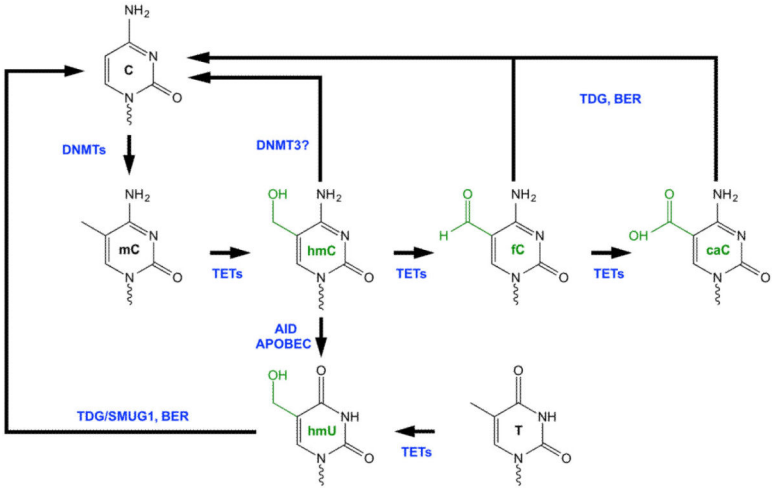

Figure 1.

Hydroxymethylcytosine (hmC)-dependent DNA demethylation pathway. Cytosines (C) that are methylated to methylcytosine (mC) by DNA methyltransferases (DNMTs) can be converted to hmC by TET enzymes (TETs). Subsequently, hmC can be oxidized to formylcytosine (fC) and carboxylcytosine (caC) by TETs or deaminated to hydroxymethyluracil (hmU) by activation-induced deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC). These products can then be excised by thymine DNA glycosylase (TDG) with or without SMUG1, followed by base excision repair (BER). DNMT3 may contribute to DNA demethylation by dehydroxymethylation, but further experiments are needed to confirm this pathway. In addition, thymine (T) is also severed as a substrate of TETs and can be catalysed to hmU.

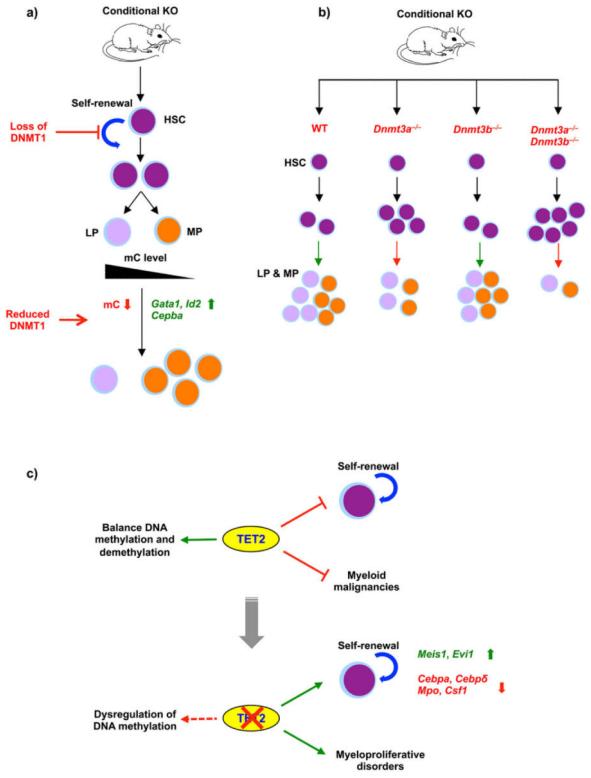

Figure 2.

Roles of DNMTs and TET2 in regulating haematopoietic stem cell (HSC) self-renewal and differentiation. (a) HSCs are derived from conditional knockout (KO) mice. Loss of DNMT1 results in the inhibition of HSC self-renewal. Reduced DNMT1 activity leads to DNA hypomethylation in the promoters of the myeloerythroid progenitor regulators and causes preferential differentiation from HSCs into myeloerythroid progeny (MP), rather than lymphoid progeny (LP). (b) Conditional KO of *Dnmt3a* in the haematopoietic compartment increases HSC numbers and impairs HSC differentiation, while double KO of *Dnmt3a* and *Dnmt3b* in HSCs leads to a more severe arrest of HSC differentiation. Alternatively, *Dnmt3b*$^{-/-}$ HSCs show a mild *in vivo* phenotype, indicating that DNMT3a can compensate for the vast majority of DNMT3b loss. (c) Under normal conditions, TET2 suppresses HSC self-renewal and myeloid malignancies; it is highly involved in DNA demethylation and contributes to balancing the critical threshold of DNA methylation status. Loss of TET2 function, usually caused by mutation or deletion, results in the dysregulation of DNA methylation, increased self-renewal and aberrant differentiation of HSCs and initiates myeloproliferative disorders.

Table 1

Function of Dnmts and Tets in induced pluripotent stem cell (iPSC) reprogramming and embryonic stem cell (ESC) function

Dnmts/Tets	Function
iPSC reprogramming	
Dnmt1	Downregulation of Dnmt1 in reprogramming facilitates transition towards bona fide iPSCs.[23]
Dnmt3a/b	No significant effect in mouse embryonic fibroblasts (MEF) reprogramming; DNMT3a knockdown from a screening study finds it moderately promotes iPSC formation in human cells .[27,28]
Tet1	Depletion reduces iPSC reprogramming efficiency.[44,46,57]
Tet2	Depletion reduces iPSC reprogramming efficiency.[44,45]
Tet3	TET3 alone has little effect on reprogramming. MEFs from triple-knockout Tet1/2/3 fail to generate iPSCs.[48]
ESC function	
Dnmt1 $^{-/-}$	Severely decreased 5-methylcytosine levels block differentiation in chimera assays.[17]
Dnmt3a$^{-/-}$ or *3b*$^{-/-}$	High-passage *Dnmt3a*-deficient or *Dnmt3b*-deficient ES cells were unable to form teratomas.[166]
Dnmt1$^{-/-}$ *3a*$^{-/-}$ *3b*$^{-/-}$	The self-renewal of TKO cells was similar to that of wild-type cells, but their growth was delayed during embryoid bodies differentiation.[20]
Tet1 $^{-/-}$	Does not affect pluripotency but skews differentiation towards extra-embryonic lineages in the teratoma.[101]
Tet2 $^{-/-}$	Does not affect pluripotency.[39]
Tet1$^{-/-}$ *Tet2*$^{-/-}$	Remains pluripotent but exhibits skewed differentiation defects towards extra-embryonic lineages.[39]
Tet1$^{-/-}$ *Tet2*$^{-/-}$ *Tet3*$^{-/-}$	ESCs contribute poorly to chimeric embryos and cannot support development.[40]