Crosstalk Between DNA and Histones: Tet’s New Role in Embryonic Stem Cells

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Abstract: Embryonic stem (ES) cells are characterized by the expression of an extensive and interconnected network of pluripotency factors which are downregulated in specialized cells. Epigenetic mechanisms, including DNA methylation and histone modifications, are also important in maintaining this pluripotency program in ES cells and in guiding correct differentiation of the developing embryo. Methylation of the cytosine base of DNA blocks gene expression in all cell types and further modifications of methylated cytosine have recently been discovered. These new modifications, putative intermediates in a pathway to erase DNA methylation marks, are catalyzed by the ten-eleven translocation (Tet) proteins, specifically by Tet1 and Tet2 in ES cells. Surprisingly, Tet1 shows repressive along with active effects on gene expression depending on its distribution throughout the genome and co-localization with Polycomb Repressive Complex 2 (PRC2). PRC2 di- and tri-methylates lysine 27 of histone 3 (H3K27me2/3 activity), marking genes for repression. In ES cells, almost all gene loci containing the repressive H3K27me3 modification also bear the active H3K4me3 modification, creating “bivalent domains” which mark important developmental regulators for timely activation. Incorporation of Tet1 into the bivalent domain paradigm is a new and exciting development in the epigenetics field, and the ramifications of this novel crosstalk between DNA and histone modifications need to be further investigated. This knowledge would aid reprogramming of specialized cells back into pluripotent stem cells and advance understanding of epigenetic perturbations in cancer.

INTRODUCTION

Embryonic stem (ES) cells are isolated from the inner cell mass of the early-stage blastocyst and can be characterized by their pluripotency and capacity for self-renewal. The pluripotent stem cell program is driven by the “core” transcription factors Oct4, Sox2 and Nanog which form a stable autoregulatory loop that can also poise the cell for differentiation [1]. The pluripotent state also displays characteristic epigenetic marks, including bivalent histone domains on developmental regulators [2] and prominent non-CpG methylation throughout the genome [3].

Histone modifications can be either activating or repressive depending on the location and the chemical mark. Two canonical modifications are trimethylation at lysine 27 of histone H3 (H3K27me3), which is a repressive mark catalyzed by Polycomb Repressive Complex 2 (PRC2), and trimethylation at lysine 4 of histone H3 (H3K4me3), which is an activating mark catalyzed by the Trithorax family of proteins [4]. H3K27me3 and H3K4me3 co-localize on a significant number of developmental regulators in ES cells, forming the distinctive “bivalent domain” which poises these gene loci for activation upon differentiation [5].

Keywords: Tet, ES cells, Polycomb repressive complex, DNA modification, Histone modification, Epigenetics, 5mC, 5hmC.

DNA methylation was thought to be responsible for long-term and stable repression of gene expression and has been implicated in X-inactivation, genomic imprinting and retrotransposon silencing [6]. In adult somatic cells, DNA methylation and the resultant 5-methylcytosine (5mC) mark predominantly occur at the cytosine of CpG dinucleotides, but in ES cells near a quarter of all DNA methylation happens in a non-CpG context [3]. Recently, global demethylation events have been characterized in the zygote and developing primordial germ cells [7-10], and extensive turnover of loci-specific methylation has been observed during cellular differentiation [3, 11], changing our conception of DNA methylation into that of a more dynamic mark.

The discovery of the ten-eleven translocation (Tet) family of DNA hydroxylases revolutionized the epigenetics field, sparking an incredibly rapid investigation of possible DNA demethylation pathways mediated by Tet and of the protein family’s functional significance in multiple important gene regulation contexts, including cancer and ES cells. There are three members within the Tet family of proteins, namely Tet1, Tet2 and Tet3, and this review will primarily focus on the first two members, which are upregulated in the pluripotent state and downregulated during differentiation [12]. While many details about the regulation of DNA methylation are still unknown, an exciting discovery involves the novel dual functions of Tet1 occupancy on specific gene loci in the ES cell [13-15]. These recent studies link DNA modification status with that of the surrounding histone marks,
and this crosstalk is important to keep in mind for future manipulations of gene regulation, whether in the context of induced pluripotent stem (iPS) cells or cancer treatment.

**ROLE OF TET IN DNA DEMETHYLATION**

Tet1 is a Fe(II)- and alpha-ketoglutarate (α-KG)-dependent DNA hydroxylase that was first discovered as a fusion partner of the mixed lineage leukemia (MLL) gene in a rare form of acute myeloid leukemia (AML) containing the t(10; 11)(q22;q23) translocation [16, 17]. Tet1 and Tet2, as well as Tet3, became subjects of intense research once they were found to have catalytic activity on 5-methylcytosine (5mC) [18], converting the initial cytosine modification to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) through successive oxidation steps [10, 19-21]. 5hmC accumulates in cells at appreciable levels of around a hundred to several thousand modified bases per million unmodified C, but 5fC and 5caC levels are barely detectable, even in cell types enriched with the Tet family of proteins [21]. However, accumulation of 5caC levels can be observed when thymine-DNA glycosylase, responsible for excision of 5caC, is depleted in mouse embryonic stem cells [21].

These new cytosine modifications are widely believed to be the first steps of active DNA demethylation, but there has only been one study showing a robust mechanism for replication-independent demethylation [22]. There are currently three theories about how active demethylation can be completed from Tet-mediated cytosine modifications [23] see Fig. (1). First, 5fC or 5caC could be excised by thymine DNA glycosylase (TDG) [20] and subsequently replaced with unmodified C through the Base Excision Repair (BER) pathway. Second, 5hmC could be deaminated by the activation induced deaminase/apolipoprotein B editing complex (AID/APOBEC) family of cytidine deaminases to form 5-hydroxymethyluracil (5hmU), which would then also be excised by a DNA glycosylase and subject to BER [24]. Third, 5caC could be directly decarboxylated to generate unmodified cytosine, though there is yet no direct evidence of decarboxylase. The second mechanism involving AID/ APOBEC and mediated by Tet1 has been observed in vivo in the adult mouse brain, though the research group did not rule out the presence of other contributing mechanisms [22]. AID deficiency also weakens global demethylation in mouse primordial germ cells [25]. However, the two BER pathways are considered unlikely to be solely responsible for the global demethylation events seen in embryonic development, as large-scale BER would put excessive strain on genome stability [26]. Furthermore, a recent study has revealed no detectable deamination of 5hmC by AID/APOBEC in vitro, which the authors attribute to 5hmC’s increased steric bulk compared to cytosine and 5mC [27]. This study challenges the viability of the AID/APOBEC demethylation pathway. The search for a 5caC decarboxylase – or another yet-undiscovered mechanism for genome-wide demethylation - will undoubtedly continue in the future.

A pathway for passive demethylation using 5hmC has also been elucidated. On the basis of findings from others that Tet3-mediated oxidation of 5mC to 5hmC is important for global demethylation of the paternal genome in zygotes and preimplantation embryos [28-30], Inoue et al. suggested that loss of 5hmC during preimplantation is likely a DNA replication-dependent passive process by use of an immunostaining approach [31, 32]. Nonetheless, caution should be exercised, because immunostaining is not quantitative and therefore it is possible that only a portion of 5mC is converted to 5hmC whereas the remaining portion of 5hmC is removed by an alternative pathway [33].

**Fig. (1).** Dual roles of Tet1 in embryonic stem cells. (A) The conventionally recognized role of Tet1 as a transcriptional activator. Even though the pathways for active demethylation have not been fully elucidated, 5hmC, 5fC and 5caC are still generally accepted as intermediates in the demethylation pathway. (B) The novel role of Tet1 as a transcriptional repressor. Tet1 indirectly mediates binding of EZH2 to gene promoters to create the repressive H3K27me3 mark, leading to heterochromatin and repression of gene expression.
ROLE OF TET IN EMBRYONIC STEM CELLS

The role of 5hmC and the Tet family of proteins in ES cell pluripotency, self-renewal and lineage specification has been discussed ever since the function of Tet1 was first discovered [18]. Both Tet1 and Tet2 are upregulated in ES cells – though Tet1 shows up to 5-fold higher expression than Tet2 – and their expression levels drop after induced differentiation; similarly, 5hmC is also enriched in ES cells and downregulated during development [18, 34, 35]. Tet1 and Tet2 have been shown to be binding targets of Oct4 [34], incorporating the proteins into well-characterized pluripotency machinery, but the necessity of Tet1/2 to pluripotency maintenance is still unclear. Several groups have shown significant loss of ES cell morphology after Tet1 knockdown, coinciding with a decrease in 5hmC levels and downregulation of Nanog through hypermethylaiton of its promoter [12, 18]. Other groups have observed bias in lineage specification after knockdown of Tet1 or Tet2, skewing ES cells towards trophoectoderm or mesoderm commitment by upregulating specific differentiation genes such as Cdx2, Gata6, Eomes and Elf5 [12, 34]. Interestingly, Koh et al. found that Tet1 and Tet2 showed antagonistic effects against each other at several developmental regulators, suggesting that, despite having similar function, the proteins may be responsible for two different lineage specifications [34]. Koh et al. [34] also saw no effect on Nanog expression or the stem cell phenotype after Tet1 or Tet2 knockdown, an observation that was verified by other groups [15]. Similarly, Dawlaty et al. [36] were able to generate Tet1 knockout ES cells that retained pluripotency and in fact developed into knockout pups. They also generated viable Tet1 knockout mice through crosses of heterozygous Tet1 +/- mice, though the mutant progeny were also generated viable Tet1 knockout mice through crosses of heterozygous Tet1 +/- mice, though the mutant progeny were slightly smaller in body size than wild-type mice [36]. It is possible that the effects of Tet1 knockout were compensated for by Tet2 activity, and it will be important to generate double knockout ES cells and mice in order to fully test for the role of Tet proteins in pluripotency.

The molecular mechanisms behind Tet1 and Tet2’s functions in ES cells are not clearly proven, though, surprisingly, demethylation of bound loci is not the only effect of Tet1 occupancy. Many genome-wide studies of Tet, 5mC and 5hmC occupancy have been conducted in order to better understand the functions and interactions of these epigenetic marks [13, 15, 23, 37-42], and new techniques have been created to detect 5hmC on genome-wide scale [43, 44] and more recently also at single-base resolution [45-47].

A majority of the genome-wide studies discovered new complexities in Tet1 and 5hmC signaling in ES cells. First, Tet1 and 5hmC did not colocalize as extensively as would be expected from their association, though both were clustered in gene-rich areas of the genome [15, 26, 37]. Tet1 binds DNA through its N terminus CXXC domain, which has been shown to bind preferentially to unmodified, CpG rich DNA [15]. In light of this binding preference, it is not surprising that Tet1 is heavily enriched at high CpG promoters and exons [15, 26, 37], which have been previously associated with low DNA methylation [48]. However, 5hmC was shown to be excluded from high CpG density promoters, even though the mark is enriched according to CpG density within the gene body [15]. One group found only a 30% overlap of Tet1 and 5hmC peaks within the ES genome, distributed roughly equally between promoter, intron, exon and intergenic regions [15]. At these overlapping regions, Tet1 knockdown downregulates 5hmC levels and upregulates 5mC levels, but Tet1 knockdown does not affect 5hmC levels at other gene loci [15]. The disparity between Tet1 and 5hmC loci does not, by itself, prove a new function for either Tet1 or 5hmC. Tet1 could be acting on 5mC without fully binding to the DNA, and 5hmC could be too transient an intermediate between 5mC and further oxidation derivatives to be detected at Tet1-bound loci. However, when looking for differential gene expression between control and Tet1-knockdown cells, researchers observed both Tet1-activated and Tet1-repressed targets, direct evidence for a novel repressive function of Tet1 [13-15, 26]. In fact, both groups observed a greater number of Tet1-repressed targets, i.e. genes that are upregulated upon Tet1 knockdown, than Tet1-activated targets, or genes that are downregulated upon Tet1 knockdown [13-15, 26]. This finding, combined with other evidence, suggested a new mechanism of gene expression by Tet1 outside of hydroxymethylation.

REGULATION OF iPSC CELLS BY TET2

An intriguing area of interest is the study of induced pluripotent stem cells (iPS) and the ability of these cells to be used in science and medicine. These cells are generated by reprogramming somatic cells by using pluripotency factors Oct4, Sox2, Klf4 and c-MYC (referred to as OSKM) [49]. Very recently, Doege et al. found reprogramming by OSKM led to Parp1 (ADP-ribose polymerase-1) and Tet2 recruitment to the loci of Nanog and Esrerb, previously known pluripotency loci [50]. Although somatic epigenetic signatures are lost in iPSC reprogramming [51] and both genes were previously indicated in epigenetic remodeling, this was the first evidence for their role in the reprogramming of pluripotency loci. They found that Tet2, but not Tet1 or Tet3, was significantly induced in somatic cells transformed with OSKM both at early reprogramming stages and at the iPSC stage. Moreover, loss of Parp1 or Tet2 led to decreased chromatin-active histone modifications at pluripotency loci. Reduction of Tet2 blocked induction of hydroxymethylation at pluripotency loci while methylation changes varied. This, combined with the early induction of hydroxymethylation at these loci, suggests that 5hmC potentially serves as a distinct epigenetic mark from 5mC. Together these results reveal a new function of Tet2 in the induction of pluripotency as well as suggesting that hydroxymethylation potentially acts outside the demethylation pathway and might promote chromatin remodeling [50].

THE POLYCOMB REPRESSIVE COMPLEX IN EMBRYONIC STEM CELLS

Another key component to the control of ES cell differentiation is the regulation of the Polycomb Repressive Complexes 1 and 2 (i.e., PRC1 and PRC2), with a majority of the existing scientific research focused on PRC2. The PRC1/2 complexes create repressive histone marks on their target genes, thus blocking expression of these genes. The repressive marks known to be catalyzed by PRC1 and PRC2, to date, are monoubiquitylation of histone H2AK119 (H2AK 119u1) and dimethylation and trimethylation of histone
H3K27 [52-58]. While the Polycomb complexes have been known for several years to be important regulators of cell fate decision [59, 60], it was not until recently that this complex was clearly tied to the maintenance and differentiation of ES cells [61]. In 2006, several groups showed simultaneously that Polycomb regulated targets were transcriptionally repressed in both human and mouse ES cells and reactivated during ES cell differentiation [62-64]. Since then, more evidence has accumulated that highlights the importance of these complexes in the regulation of embryonic stem cells. For example, knockout of SUZ12, EZH2, PCL2, EED or JARID2, all components of PRC2, in embryonic stem cells results in the inability of these stem cells to undergo proper differentiation and the expression of higher levels of pluripotency markers (reviewed in [65]). Those studies have demonstrated that PRC1 and PRC2 are essential components of ES cell maintenance and differentiation.

**TET1 INTERACTION WITH THE POLYCOMB REPRESSIVE COMPLEX IN EMBRYONIC STEM CELLS**

While it was known that both Tet1 expression and PRC1 and PRC2 regulation were required to maintain embryonic stem cells, there was little to no functional connection between Tet1 and PRC1/2 until very recently. In 2011, Wu et al. [13, 14, 23, 26] connected Tet1-repressed targets to the recruitment of a repressive histone mark, H3K27me3, to the gene promoter. They also discovered that Tet1 knockdown substantially compromises the ability of Ezh2 to bind to PRC2/Tet1 co-bound gene loci in ES cells [13, 14, 23, 26] see Fig. (1). Importantly, Nanog overexpression in Tet1-knock down cells cannot fully rescue Ezh2 binding to its target loci, showing that Tet1’s effects on Ezh2 do not result from disruption of core pluripotency machinery [13, 14, 23, 26]. No stable interactions have yet been observed between Tet1 and Ezh2, and Tet1 knockdown does not reduce PRC2 expression or stability [13, 14, 23, 26], producing an intriguing question – how does Tet1 affect PRC2 binding without direct interaction with its subunits?

**FUTURE WORK**

There is still much we do not know about DNA modifications and about epigenetic regulation in general. New techniques have been developed to distinguish 5hmC from 5mC, even at single-base resolution [45-47], but these methods need to be further refined to be able to determine all layers of DNA modifications (5mC, 5hmC, 5caC and 5fC) at a single cell level. Once detection methods for all epigenetic marks and protein-DNA binding have reached this degree of resolution, we will be able to better understand how crosstalk interactions occur between epigenetic regulatory complexes and their resultant marks.

More work is also required to better understand the connection between reading, writing and interpreting the histone code, both on its own and as it relates to other epigenetic factors including methylation/hydroxymethylation. Specifically in ES cells, a majority of the research so far has focused on the function and localization of Tet1, and Tet2 must be incorporated into this regulatory picture. Other epigenetic mechanisms should also be integrated, including the further oxidation derivatives of 5hmC, other histone modifications and ES cell-specific non-coding RNAs. This future work will provide a better understanding of stem cell pluripotency and of crosstalk between the different mechanisms of gene regulation.

**CONFLICT OF INTEREST**

The author(s) confirm that this article content has no conflicts of interest.

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**ABBREVIATIONS**

| Abbreviation | Definition |
|--------------|------------|
| ES | Embryonic Stem |
| Tet | Ten-Eleven Translocation |
| PRC | Polycomb Repressive Complex |
| H3K27me3 | Trimethylation At Lysine 27 of Histone H3 |
| H3K4me3 | Trimethylation At Lysine 4 of Histone H3 |
| 5mC | 5-Methylcytosine |
| 5hmC | 5-Hydroxymethylcytosine |
| 5fC | 5-Formylcytosine |
| 5caC | 5-Carboxylcytosine |
| iPS | Pluripotent Stem |
| MLL | Mixed Lineage Leukemia |
| AML | Acute Myeloid Leukemia |
| TDG | Thymine DNA Glycosylase |
| BER | Base Excision Repair |
| AID | Activation Induced Deaminase |
| APOBEC | Apolipoprotein B Editing Complex |

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