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Review

DNA demethylation, Tet proteins and 5-hydroxymethylcytosine in epigenetic reprogramming: An emerging complex story

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ABSTRACT

Epigenetic reprogramming involves processes that lead to the erasure of epigenetic information, reverting the chromatin template to a less differentiated state. Extensive epigenetic reprogramming occurs both naturally during mammalian development in the early embryo and the developing germ line, and artificially in various *in vitro* reprogramming systems. Global DNA demethylation appears to be a shared attribute of reprogramming events, and understanding DNA methylation dynamics is thus of considerable interest. Recently, the Tet enzymes, which catalyse the iterative oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine, have emerged as potential drivers of epigenetic reprogramming. Although some of the recent studies point towards the direct role of Tet proteins in the removal of DNA methylation, the accumulating evidence suggests that the processes underlying DNA methylation dynamics might be more complex. Here, we review the current evidence, highlighting the agreements and the discrepancies between the suggested models and the experimental evidence.

Contents

1. Introduction

Development of an organism starts with a totipotent zygote. Through series of cell divisions and the differentiation processes, this cell will eventually give rise to the whole organism containing hundreds of specialised cell types carrying out diverse physiological functions. While the cells at the onset of development have the capacity to generate all cell types (*i.e.* are toti- or pluripotent), this developmental capacity is progressively lost as cells undertake cell fate decisions [1]. At the molecular level, the memory of these differentiation events is laid down in a complex layer of epigenetic modifications at both the DNA and the chromatin level. In accordance with the unidirectional character of the developmental progress, the key acquired epigenetic modifications are stable and inherited through subsequent cell divisions. This paradigm is, however, challenged during cellular reprogramming that requires de-differentiation (reprogramming of somatic nucleus through nuclear transfer to the oocyte — SCNT,

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generation of induced pluripotent stem cells — iPS) or a change in cell fate (transdifferentiation).

The desire to reverse cell fate and thus to challenge the directionality of development has inspired generations of cell biologists; however despite intense efforts of numerous research teams, the molecular processes underlying cellular reprogramming remain mostly unknown. At the molecular level, cellular reprogramming must involve erasure of epigenetic information, resulting in reversion of the chromatin template to a less differentiated state characterised by low DNA methylation levels [2] and high chromatin plasticity [3]. Interestingly, both repressive histone methylation [4] and DNA methylation [5] have been identified as molecular barriers to successful reprogramming process. While histone modifications have been known to be highly dynamic for quite some time, and the mechanism of removal of histone methylation through jumanji domain containing histone demethylases has been described on the molecular level [6], molecular mechanisms underlying removal of DNA methylation have only now begun to be unravelled [7]. Recent discovery of the Tet family of oxygenases, which catalyse the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine and higher oxidative derivatives [8–10] (Fig. 1), has opened up a long sought after mechanistic route for DNA demethylation. Since the discovery of their enzymatic activity in 2009 [10], Tet enzymes have been implicated in reprogramming processes *in vivo* and *in vitro*, and linked with both active and passive mechanisms of DNA demethylation. The accumulating evidence, however, suggests that the exact molecular role of Tet proteins might be more complex than originally anticipated, and that additional mechanisms of DNA demethylation are potentially at play, at least during the major changes in DNA methylation associated with *in vivo* epigenetic reprogramming. This review summarises our current understanding of DNA (de)methylation dynamics during the processes of experimental epigenetic reprogramming *in vitro*, as well as during the developmental epigenetic reprogramming *in vivo*, with particular focus on the role of Tet driven 5mC oxidation.

2. DNA demethylation, Tet proteins and 5mC oxidative derivatives

Conceptually, the mechanisms of DNA demethylation can be distinguished by their dependence on DNA replication (Fig. 2): DNA methylation patterns are typically maintained in a faithful manner due to the activity of Dnmt1 DNA methyltransferase, which associates with the replication fork through its binding to PCNA and Uhrf1 and provides specific activity on the hemi-methylated newly replicated DNA [11–13]. Loss of this maintenance methylation activity results in **passive DNA demethylation** (Figs. 2A, B), a gradual loss of DNA methylation demonstrated in systems lacking Dnmt1 or Uhrf1 tethering Dnmt1 to the replication fork and the hemi-methylated DNA [12,13].

As an alternative mechanism, **active DNA demethylation** would lead to the removal of 5mC in a replication independent manner (Figs. 2C, D). Several molecular mechanisms of active DNA demethylation have been proposed; these include “reverse” enzymatic reaction driven by DNA methyltransferases in the absence of S-adenosylmethionine (SAM, a donor of methyl group) [14–16], or an involvement of MBD binding proteins [17,18]. It should be, however, noted that these mechanisms still await *in vivo* validation.

In a manner similar to flowering plants that utilise 5mC specific glycosylases Dme and Ros1 [19], DNA repair has also been implicated in the active DNA demethylation processes in higher vertebrates, including in *Xenopus*[20], and in both mouse zygotes and developing germ cells (see below) [21]. As the protein family of 5mC specific DNA glycosylases seems to have evolved independently in flowering plants with no direct sequence homology in higher vertebrates, the described role of DNA repair in DNA demethylation in these organisms might require the existence of an additional 5mC modification that would trigger the observed DNA repair response. In alignment with this idea, 5mC has been suggested to be modified to thymine by the enzymatic activity of DNA deaminases [22] and consequently activation induced DNA deaminase (Aid, also known as Aicda) has been implicated in DNA demethylation processes in both zebrafish and mouse development [23,24], and in *in vitro* reprogramming systems [25]. It should, however, be noted that DNA deaminases (including Aid) generally prefer unmodified cytosines in a single stranded context as their substrate, and hence their potential activity on 5mC is very limited, at least *in vitro*[26]. Further experimental evidence is thus required to clarify the extent of the contribution of this molecular pathway to observed instances of DNA demethylation.

The discovery of the Tet family of enzymes converting 5mC to 5-hydroxymethyl cytosine (5hmC), and its higher oxidative products 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), and the evidence for the presence of 5hmC in mammalian DNA, opened up a new possible mechanistic route for 5mC removal [8–10,27]. Tet (1–3) enzymes can oxidise 5mC to generate 5hmC that can be diluted through subsequent rounds of replication due to the low enzymatic activity of Dnmt1 on the hemi-hydroxymethylated DNA (Fig. 2B) [28]. In an alternative scenario, 5hmC can be further oxidised to 5fC and 5caC; both of these modified bases have been shown to be targeted by TDG DNA glycosylase and the lesion subsequently processed through the BER DNA repair pathway [8] (Fig. 2C). Tet driven 5mC oxidation thus provides a direct mechanistic route for both passive and active DNA demethylation. However, in view of current findings discussed below, it is likely that additional, as yet unidentified, molecular pathway(s) of DNA demethylation may exist. Global changes in DNA methylation observed in various reprogramming systems are thus likely to require a concerted action of several DNA demethylation mechanisms.

3. Epigenetic reprogramming and waves of global DNA demethylation *in vivo*

3.1. Zygotic DNA demethylation

Extensive epigenetic reprogramming occurs at two stages during normal development: in the early zygote, immediately following fertilisation; and in the primordial germ cells of the developing embryo (Fig. 3). In mammals, following fertilisation, the highly condensed and methylated paternal genome is decondensed through protamine removal and replacement with the histone variant H3.3 [29]. This is immediately followed by extensive and rapid DNA demethylation [30,31], while the maternal methylome remains mostly unchanged [32,33]. From the two cell stage onwards, both the paternal and maternal genomes undergo progressive loss of methylation until the blastocyst

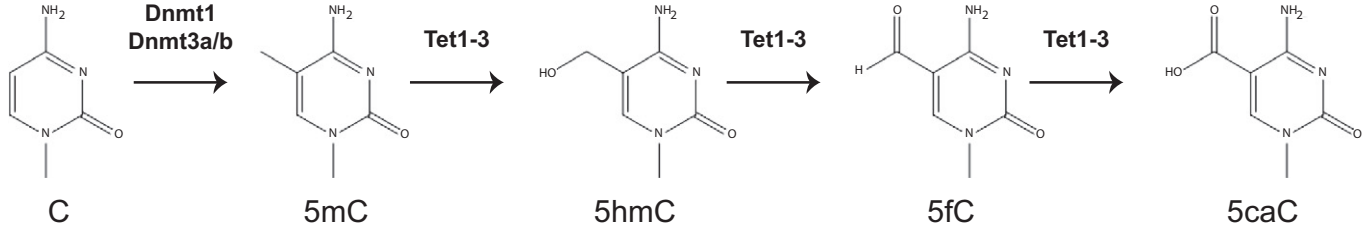

Fig. 1. Overview of enzymatic cytosine modifications observed in mammalian DNA.