DNA (de)methylation: the passive route to naivety?

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Abstract:

Mouse pluripotent embryonic stem cells can exist in distinct, yet interchangeable, epigenetic states dictated by their culture environment. Previous reports have shown that naïve pluripotent cells grown in the presence of 2i are characterised by global DNA hypomethylation and changes in abundance and distribution of histone modifications. New research provides insights regarding how this might be achieved.

Global changes in the abundance and localisation of various epigenetic marks regulate gene expression. The underlying mechanisms are thus of wide interest as they potentially provide means to enhance our ability to manipulate cell fates.

Culture conditions have a profound impact on the molecular properties of pluripotent stem cell lines [1]. Traditionally grown in serum containing medium on a fibroblast feeder layer, transfer of mouse embryonic stem (ES) cells to a defined medium containing dual inhibition of MEK and GSK3B (2i medium) leads to profound gene expression and epigenetic changes [2-5]. Notably, cells cultured in 2i display a dramatic global decrease in DNA methylation (5mC) levels to a degree of hypomethylation comparable to the *in vivo* epiblast from blastocysts, from which ES cells are derived [3-5]. However, despite substantial gene expression and epigenetic differences, ES cells grown in either condition retain key characteristics of pluripotency and contribute to chimaeras following injection back into the preimplantation embryo.

Although the global loss of 5mC in cells cultured in 2i has been well documented [3-5], the mechanism of this loss is less clear . The loss of DNA methylation has been associated with downregulation of the *de novo* methyltransferase machinery – Dnmt3a, Dnmt3b and Dnmt3L [3,5]. In this context, *Prdm14*, a transcriptional and epigenetic regulator that is upregulated under 2i conditions may play a key role [3,5-7]. In addition, a role for the Tet enzymes that convert 5mC to 5hmC has also been proposed as an alternative or complimentary mechanism [5,8]. Culture in 2i also leads to a redistribution of, amongst others, the repressive histone modification H3K27me3 [2] and a decrease in H3K9me2 [3,9]. Last but not least, changes in the expression of many epigenetic modifiers accompany alterations in epigenetic modifications, which suggests that ES cells cultured in 2i represent a distinct yet interchangeable epigenetic state [3].

A new study by Reik, Stunnenberg and colleagues [10], provide further insights on the mechanism driving hypomethylation in naïve mouse pluripotent cells. The authors measured the level and distribution of DNA modifications during the transition from serum to 2i; the resulting datasets were used to instruct a mathematical model of possible molecular mechanism(s) behind global DNA demethylation [10] . The model attributes loss of DNA methylation to one of three possible pathways – loss of

maintenance methylation, loss of *de novo* DNA methylation activity or active DNA demethylation (represented by 5hmC formation in this model). The model was instructed by an impressive array of datasets (including liquid chromatography followed by mass spectrometry, reduced representation bisulphite sequencing, whole genome bisulphite sequencing and Tet-assisted bisulphite sequencing) derived from ES cells collected at multiple intervals during the transition. When fed into the model, the results suggested that the major contributor to the resulting global hypomethylation is the decreased activity of maintenance DNA methylation.

A range of deletion mutant ES cells were subjected to the same experimental procedure to test the predictions made from the model. Strikingly, in keeping with the models predictions, *Tet1/Tet2/Tet3* triple knockout ES cells undergo rapid and complete DNA methylation with dynamics comparable to wild type cells. Thus, although a small number of loci were found to be dependent on activity of Tet enzymes, there does not seem to be a substantive role for them in this global demethylation process. Since the *de novo* methylation machinery is rapidly downregulated on transition to 2i/LIF, so predictably deletion of *Dnmt3a* and *Dnmt3b* does not have a major impact on the process. Loss of these enzymes in cells cultured in serum also has only a minor impact on DNA methylation over the short timescale of the study. The authors therefore also dismiss the *de novo* methyltransferases as a major contributing factor. Only a loss of *Dnmt1* leads to a more rapid and substantial loss of DNA methylation than that observed upon transition of cells to 2i. This suggests that there might be ongoing Dnmt1 activity throughout the demethylation process, in agreement with the observed stable Dnmt1 RNA and protein levels.

As the expression of Dnmt1 does not change between culture conditions, and the authors confirm that the enzyme remains active, how else could maintenance methylation be impacted? The authors demonstrate that the Dnmt1 co-factor Uhrf1 is present at a lower level in 2i, although the protein remains readily detectable in 2i conditions. Also, in comparison to data generated from *Uhrf1* null ES cells, the methylation profiles of cells in 2i clearly indicate an ongoing role for Uhrf1 in maintaining DNA methylation at least at certain loci, including genomic imprints. Therefore an additional mechanism regulating locus specific Uhrf1 recruitment must play an important role during the demethylation upon transition to 2i.

Uhrf1 is recruited by binding to hemimethylated DNA through its SRA domain, but Uhrf1 also has a Tudor domain capable of binding H3K9me2/3 [11]. As H3K9me2 is globally reduced in 2i conditions [3,9,10], it is possible that this may affect Uhrf1 recruitment. In keeping with this, the authors show a small yet significant increase in DNA methylation at regions where H3K9me2 is present in 2i. Therefore the authors suggest that reduced Uhrf1 protein levels as well as decreased H3K9me2 levels upon transfer to 2i may lead to sufficient impairment of maintenance DNA methylation to explain the demethylation process. More provocatively, they suggest that this mechanism applies not just to the artificial culture environment, but is sufficient to explain *in vivo* reprogramming events such as the loss of DNA methylation in the preimplantation embryo and during primordial germ cell development.

Can the proposed model explain the whole complexity of DNA demethylation upon transition to naïve pluripotency? While the finding that DNA demethylation proceeds largely unaltered by deletion of the *Tet* enzymes provides strong evidence that their role is non-essential in this context, the picture is less clear for the *de novo* methyltransferases. Although DNA methylation is maintained following acute deletion of *Dnmt3a/Dnmt3b* in serum, prolonged culture has been shown to lead to significant DNA hypomethylation [3,12]. It is possible that changes to the chromatin template in 2i conditions could lead to a more rapid decline than observed in serum; perhaps this is relevant even over the timescales studied. The elegant final model put forward by the authors suggests an interplay between changes in histone modification levels and the activity of the maintenance DNA methylation machinery. An interaction between altered histone code and Dnmt targeting could equally apply to the *de novo* methyltransferases. As Dnmt3a/Dnmt3b are rapidly downregulated in 2i conditions the relevance of deleting them in this context is unclear. Perhaps of more interest would be to assess whether their forced expression renders cells resistant to the demethylating effects of 2i.

Notably, a role for Prdm14 is worthy of consideration since *Prdm14* null ES cells maintain high levels of DNA methylation despite culture in 2i/LIF [3,6,7]. *Dnmt3a* and *Dnmt3b* are direct targets of Prdm14 [5,13] and their levels increase in *Prdm14* null cells while *Dnmt1* levels remain unchanged [3,7]. Interestingly, *Uhrf1* levels also increase in *Prdm14* null PGCs [6], although both Prdm14 and Blimp1 are required for efficient downregulation of *Uhrf1* observed in early mouse PGCs. This suggests

possible differences between events in pluripotent cells *in vitro* and reprogramming events *in vivo* [14]. Another direct target of Prdm14 is *Glp*, an important co-factor for the histone methyltransferase G9a [13,15], which is known to deposit H3K9me2 modification. Previously reported upregulation of Prdm14 in 2i conditions [3,4] may thus lead to observed lower Glp [3] and hence lower H3K9me2 [3,9,10] potentially providing a direct mechanistic link between Prdm14 and the discussed impaired maintenance of DNA methylation in naive pluripotency (Figure 1). In this context, further investigation of the methylation pattern in Prdm14 null cells as well as an assessment of DNA methylation of *G9a* null ES cells on transition to 2i would be of significant interest.

The recently published work represents a significant resource and advances a model of how DNA demethylation proceeds. Time will tell whether this model will need to be refined as we learn more about the epigenetic landscape of naïve pluripotent cells, the mechanisms by which methylation marks are removed from DNA and the possible interaction between these factors. On-going studies in cultured cells might be informative in furthering our mechanistic understanding of *in vivo* epigenetic reprogramming events, or may well in fact benefit from lessons learned from the embryo.

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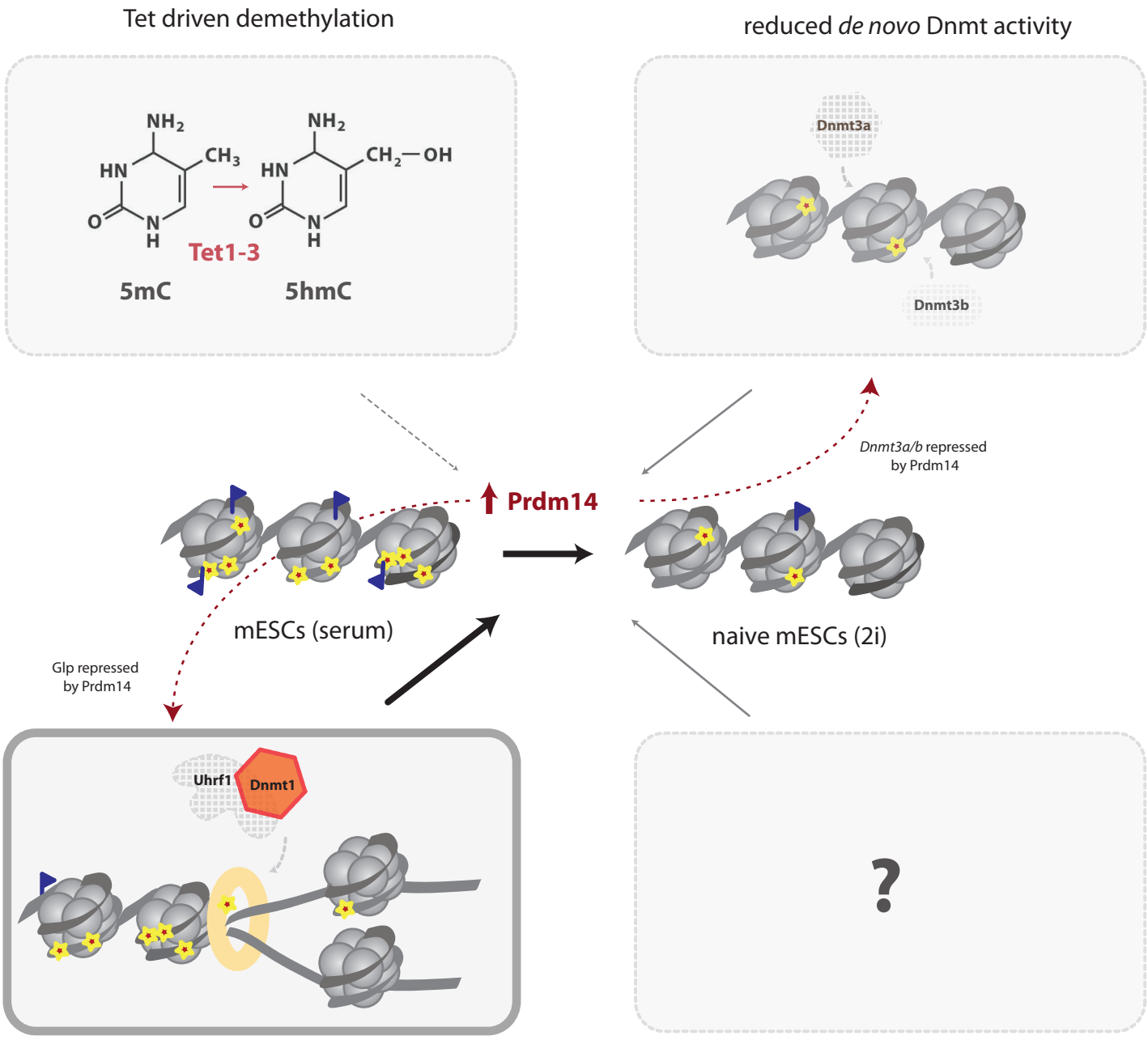
Figure legends:

Figure 1: Overview of the molecular pathways thought to contribute to the observed global DNA hypomethylation in naïve mESCs cultured in 2i conditions, including the proposed role of Prdm14 in the observed global DNA hypomethylation.


Tet driven demethylation
reduced *de novo* Dnmt activity
Tet1-3
5mC
5hmC
Dnmt3a
Dnmt3b
Prdm14
Dnmt3a/b repressed by Prdm14
mESCs (serum)
naive mESCs (2i)
Glp repressed by Prdm14
Uhrf1
Dnmt1
?
passive DNA demethylation (reduced Uhrf1 and inefficient targetting of Uhrf1/Dnmt1 complex to replication foci due to reduced H3K9me2/3)
other mechanism(s)
H3K9me2/3
5mC
Uhrf1/Np95
PCNA