DNA methylation and the preservation of cell identity

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Abstract

DNA methylation is a major epigenetic modification of vertebrate genomes that is mostly associated with transcriptional repression. During embryogenesis, DNA methylation together with other epigenetic factors plays an essential role in selecting and maintaining cell identity. Recent technological advances are now allowing for the exploration of this mark at unprecedented resolution. This has resulted in a wealth of studies describing the developmental roles of DNA methylation in various vertebrate model systems. It is now evident that in certain contexts DNA methylation can act as a key regulator of cell identity establishment, whereas in many other cases the quantity of DNA methylation will merely reflect other upstream regulatory changes. For example, a number of studies have indicated that DNA methylation might be dispensable for pluripotency stages of embryonic development. Nevertheless, targeted deposition and removal of DNA methylation by DNMTs and TET proteins, respectively, appears to be required for vertebrate gastrulation. Here we review the roles of DNA methylation in the establishment and maintenance of cell identity during development, with a special emphasis on insights obtained from *in vivo* studies.

Introduction

Vertebrate development is characterized by major epigenome remodeling processes that drive and sustain cell identity in the developing embryo [1]. Perhaps one of the best-studied epigenetic mechanisms that is known to be highly dynamic during embryogenesis is DNA methylation (mC). mC is a covalent modification of cytosine within genomic DNA that in vertebrates predominantly occurs on the CpG dinucleotide and is frequently associated with long-term transcriptional repression [2]. Examples of such cellular processes include X chromosome inactivation, genomic imprinting and silencing of repetitive DNA elements [3,4]. mC is established and maintained by the action of the maintenance and *de novo* DNA methyltransferase enzymes DNMT1 and DNMT3A/B/L, respectively [5]. Conversely, mC can also be removed by dilution through genome replication without maintenance or inactivation of DNMTs, or by active demethylation mediated by the Ten-eleven translocation (TET) proteins and the TDG pathway [6]. Cellular interpretation of the mC signal is complex and is thought to involve at least two different pathways. The first and likely predominant pathway involves the recruitment of methyl CpG-binding proteins that target protein complexes with histone deacetylase activity to nearby genes to silence transcription [7-10]. Another possible mode of action of mC is the direct interference with transcription factor binding sites [11-13]. Notably, a number of recent studies suggest that the functions of mC and its oxidized derivatives such as hydroxymethylation (hmC) could be far more complex than previously thought, and that depending on the cell type, mC could recruit a wide variety of transcriptional modulators and even be compatible with transcriptional activation in certain biological contexts [14-16]. The fact that mC can be stably propagated through cell divisions underlies its potential to serve as a form of cellular epigenetic memory that is implicated in embryogenesis, cellular differentiation and reprogramming. The last decade has seen a technological revolution that has enabled the interrogation of mC state on a genome-wide scale and at single base resolution. Studies utilizing these whole-genome methodologies have provided many important insights regarding the genomic location and spatio-temporal dynamics of mC in various experimental models [17-23]. Through the combination of high throughput mC profiling techniques and functional studies in diverse model systems, our understanding of mC function in genome regulation is rapidly advancing. Here we

review the recent advances regarding the roles of mC in cell identity establishment and preservation during vertebrate embryogenesis.

Global methylome reprogramming and pluripotency establishment

While predominantly stable in somatic cells, mC displays remarkable dynamics during mammalian preimplantation development and germline formation [24]. Upon fertilization, the paternal pronucleus becomes almost completely demethylated followed by a decrease in mC levels in the maternal pronucleus and nearly complete embryonic hypomethylation in the blastocyst. The exact mechanisms by which these processes take place remains a topic of dispute, however it is likely that both active and passive mechanisms play a role [24,25]. Such global mC reprograming can also be induced *in vitro* when embryonic stem cells (ESCs) are transferred from serum-containing media to a media containing two small molecular inhibitors (2i) that target the kinases MEK and GSK3 [26-28]. 2i conditions closely resemble the environment of the blastocyst inner cell mass (ICM), and trigger global mC erasure and a switch to the naïve pluripotent cell state.

Those observations notwithstanding, the exact requirements for mC erasure during the establishment of pluripotency remain unclear. For example, *in vitro* reprogramming to the pluripotent state by ectopic expression of Oct4, Sox2, c-Myc, and Klf4 transcription factors in somatic cells results in fully competent induced pluripotent stem cells (iPSCs) [29] even though those cells do not pass through a complete global demethylation event [30]. This could be the reason why cells generated by *in vitro* reprograming are known to bear epigenetic abnormalities and to retain somatic memory [31-33]. This effect is less pronounced, however, if the cells are subjected to somatic nuclear transfer (SCNT) and reprogrammed by factors present in the ooplasm, as recently demonstrated in a study that compared SCNT ESC and *in vitro* iPSC methylomes generated from same somatic cells [34]. Concordantly, it was recently shown that only naïve human ESCs and preimplantation embryos express the HERVK endogenous retrovirus LTR5HS when compared to primed human ESCs and iPSCs, and that this expression was caused by the hypomethylated state of those cells [35]. While the function of this peculiar embryonic event is not known, it has been postulated that it might play a role in early embryonic immune defence.

Anamniotes such as fish and frogs lack global mC erasure [20,21] and yet achieve the competence to form similar tissues as mammals. Interestingly, in early *Xenopus* embryos mC is uncoupled from transcriptional repression [14] and individual cells within the embryo have been shown to express markers that are not characteristic of their position [36]. These and other studies [reviewed in 37] suggest that both mammals and anamniotes pass through an early phase of open and permissive chromatin that facilitates pluripotency establishment and likely promotes hypomethylation in mammals. A recent report utilizing single cell transcriptomics demonstrated that rare cells in heterogeneous ESC population cycle through a transient state that is characterized by MERVL/Zscan4 expression, global hypomethylation and significant chromatin decompaction [38]. The MERVL transcriptional network was previously shown to be upregulated in two-cell mouse embryos [39]. Using inducible *Dnmt1* knockout cells, Eckersley-Maslin et al. demonstrated that the observed global hypomethylation is not the cause but rather a consequence of this transient state, as hypomethylation was not sufficient to induce MERVL expression. Thus, it appears that the increase in chromatin accessibility is an upstream event that facilitates MERVL expression. This in turn promotes the expression of translation inhibitors that block DNMT function and cause global hypomethylation. Altogether, these data demonstrate that hypomethylated ESCs display transcriptional features of preimplantation embryos. Nevertheless, mC erasure is not an evolutionarily conserved feature of vertebrate pluripotency, and is likely simply a consequence of an early open chromatin configuration. Whether this major mC remodelling event evolved in mammals to prevent the transmission of potential epimutations from the previous generation remains to be determined.

TET proteins control vertebrate gastrulation

Until recently, mC was considered a stable epigenetic mark associated with establishment of permanent cell identity choices. The fact that somatic cells can be reprogrammed to a pluripotent state challenged that notion and demonstrated that such epigenetic barriers can be overridden. This was followed by the discovery that mC can be oxidized to hmC, and subsequently to 5-formylcytosine (fC) and 5-carboxylcytosine (caC) through the action of TET enzymes [6]. The TET protein family in mammals consists of three members: TET1, TET2, and TET3. However, pinpointing the exact developmental roles of TET proteins has proven to be challenging. This is

mostly due to the fact that TET proteins display considerable functional redundancy during embryogenesis [40]. Recent loss of function studies describing the simultaneous targeting of all three TET family members have started to provide important insights into the developmental functions of TET proteins. Our recent work revealed that the transcriptional regulation of key genes and pathways involved in body plan formation in zebrafish, *Xenopus tropicalis* and mouse is driven by active DNA demethylation of a functionally conserved set of enhancer elements [41]. These enhancers are characterized by TET-dependent mC to hmC conversion during the phylotypic period of vertebrate development and subsequent mC/hmC loss. Importantly, these regions are located in the vicinity of genes belonging to Wnt, Notch/Delta and TGF-β pathways, thus linking active DNA demethylation to fundamental developmental processes such as cell fate specification, cell proliferation, embryo polarity establishment and body axis patterning (Figure 1a). These observations are in line with the severe phenotypes caused by the loss of Tet1/2/3 proteins in zebrafish [41]. The majority of triple Tet morphants did not survive gastrulation whereas the remainder displayed shortened embryonic axes, malformed eyes and head structures as well as reduced pigmentation. A severe gastrulation phenotype was also recently observed in a triple TET knockout (TKO) mouse model generated through germline deletion of *Tet1/2/3* [42]. TKO embryos developed successfully until embryonic day E6.5 and, despite the dysregulation of several hundred genes, they appeared virtually indistinguishable from their wild type counterparts up until that stage. At E7.5, the affected embryos were much smaller in size than the controls and displayed defects in primitive streak patterning as well as problems with mesoderm specification. Interestingly, it appears that such a phenotype was at least partly caused by hyperactive Nodal signalling, as Nodal inhibition partially restored embryonic patterning. The defects in Nodal signalling were due to reduced expression of *Lefty1* and *Lefty2* genes, well known inhibitors of Nodal and members of the TGF-β signalling pathway [42]. Importantly, more recent work on mouse triple TET knockouts [43] linked the Wnt pathway to the loss of TET function, thus further supporting the initial observations that TET proteins might regulate key developmental pathways [41]. These triple knockout embryos at E8 – E8.5 stages displayed hyperactive Wnt signalling and impaired differentiation of neuro-mesodermal progenitors [43]. Together, these data demonstrate an evolutionarily conserved role for TET proteins in cell fate determination associated with gastrulation and body plan formation in early vertebrate embryos.

mC as an epigenetic barrier during PGC differentiation

The mC reprograming events that occur in mammalian primordial germ cells (PGCs) are even more drastic than the mC erasure observed in preimplantation embryos [24]. This mC remodelling reduces the overall mC levels to its lowest point during the mammalian life cycle [44,45]. One of the major functions of this process is likely the erasure of parental imprints that subsequently allows for their proper gender-specific resetting. PGC demethylation occurs in two steps; the first phase involves passive demethylation caused by the exclusion of UHRF1 from the nucleus and repression of DNMT3 proteins, whereas the second phase is carried out through active TET-mediated demethylation [44]. This second wave of active demethylation targets imprinting control regions (ICRs) as well as promoters of germline-specific genes. Until recently, the exact reason for why this process is divided in two stages was unknown. A recent report utilizing a PGC-specific *Dnmt1* knockout mouse, demonstrated that DNMT1 guards PGCs from premature differentiation during the first stage of global demethylation [46] (Figure 1b). DNMT1 expression in PGCs is required to repress a set of meiotic genes during stage I demethylation, including the meiotic licensing factor DAZL that is crucial for the differentiation of both male and female PGCs. PGC-specific depletion of DNMT1 resulted in precocious germline differentiation and led to hypogonadism and infertility in both males and females [46]. This work provides an example of how locus-specific mC can serve as an epigenetic barrier that prevents premature transitions in cell identity. Altogether, it appears that mC, at least in the mammalian germline, evolved as in important regulator of the timing of germline induction.

Conclusions and future perspectives

The roles of mC during vertebrate embryogenesis have been under intense investigation for more than three decades. Very few studies, however, managed to demonstrate a regulatory role for this epigenetic mark during this dynamic period of the vertebrate life cycle. This has resulted in the roles of mC during vertebrate embryogenesis being challenged [47]. Indeed, in mammalian cells mC appears to be locally influenced by the presence of cell type-specific transcription factors [48,49], and it is likely that DNA methylation is generally not required for pluripotency [50]. Nevertheless, recent studies carried out in various vertebrate models suggest that mC might play an important role in orchestrating early embryonic development through the regulation of

essential developmental pathways [41-43]. Additionally, maternal deposition of H3K4me3 and H3K27me3 histone marks in the early embryo appears to be regulated through a logic of hypomethylated islands, and uncoupled from zygotic genome activation [51].

In zebrafish and mouse embryos, the loss of TET proteins results in severe gastrulation phenotypes caused by aberrant cell fate specification choices. These problems are the result of improper use of early signalling pathways such as Wnt and TGF-β. Importantly, in both zebrafish and mouse the loss of TET proteins resulted in reduced chromatin accessibility at enhancer regions suggestive of an upstream regulatory role TET proteins might play in enhancer activation [41,43]. Thus, it appears that mC could act as a highly evolutionarily conserved safe-lock that has to be removed by the activity of TET proteins in order for gastrulation to complete. A similar safe-lock function for mC was also recently observed during mammalian PGC specification [46].

While the precise roles of global demethylation during mammalian preimplantation development await further clarification, recent data suggest that such a hypomethylated state might be required for the proper expression of retroviral elements during this developmental period [35]. Future studies will greatly benefit from single cell DNA sequencing technologies that allow for the exploration of mC patterns at an unprecedented resolution, and the generation of cell-type specific methylome maps of early embryos. Finally, the recent advances in (epi)genome editing technologies will enable the functional testing of the role of mC itself in the regulation of developmental enhancers and their target genes. Altogether mC provides a versatile input to early embryonic development by regulating and maintaining cell identity choices through a variety of molecular mechanisms.

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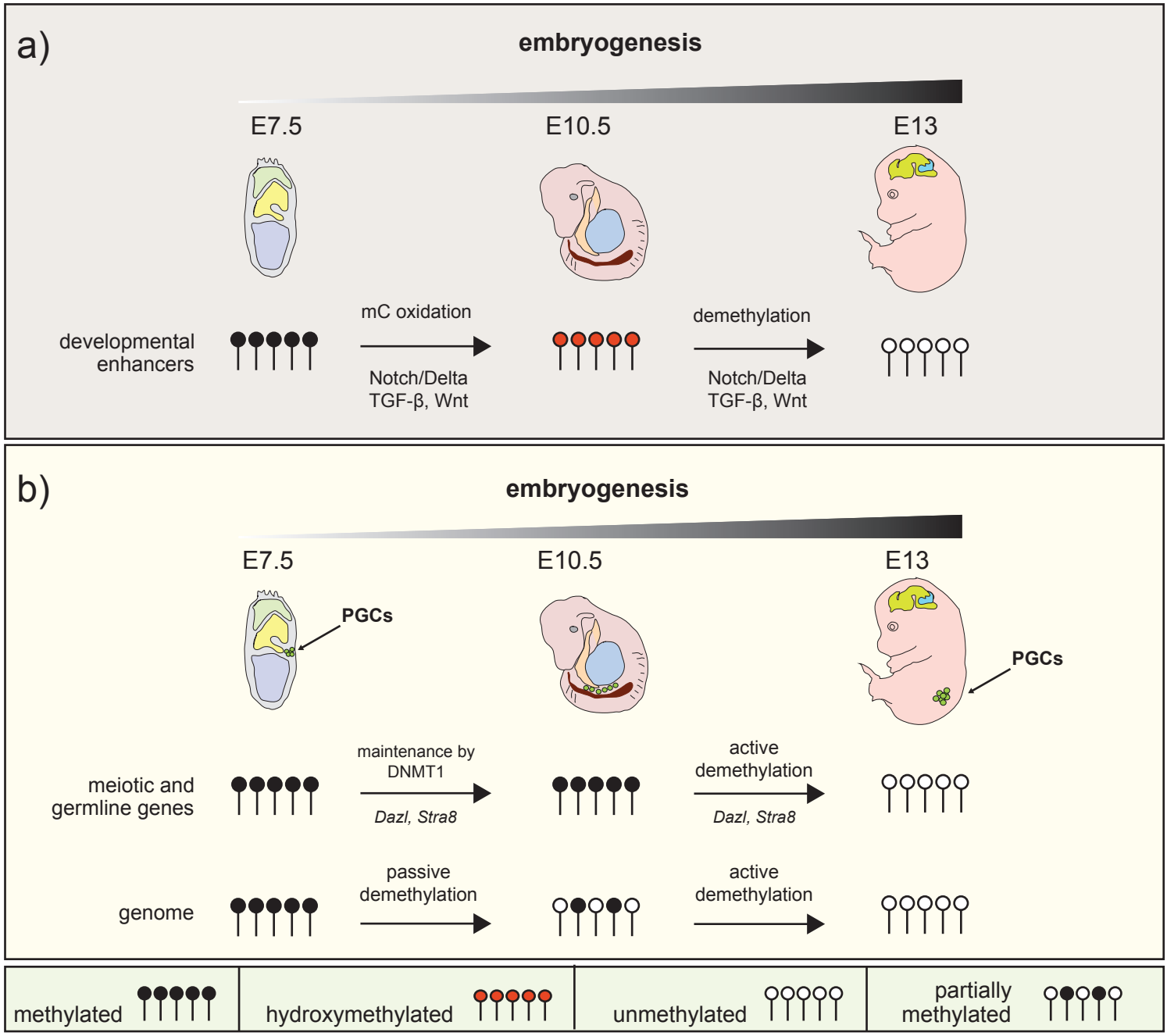
Figure legends

Figure 1. mC and the regulation of cell identity during embryogenesis a) In vertebrate embryos, TET-dependent oxidation takes place on a subset of developmental enhancers linked to TGF-β, Notch/Delta and Wnt pathways during body plan formation. This demethylation event involves the 5-hydroxymethylcytosine (hmC) intermediate. **b)** During the first wave of global demethylation in primordial germ cells (PGCs), DNMT1 maintains a number of germline and meiotic gene promoters methylated thereby preventing precocious germline differentiation. These promoters lose mC only during the second wave of demethylation when their expression is needed.

Acknowledgments

R.L. was supported by a Sylvia and Charles Viertel Senior Medical Research Fellowship. O.B. is supported by an Australian Research Council Discovery Early Career Researcher Award (DECRA; DE140101962)

Figure 1


a)
embryogenesis
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developmental enhancers
mC oxidation
Notch/Delta
TGF-β, Wnt
demethylation
Notch/Delta
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b)
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PGCs
PGCs
meiotic and germline genes
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Dazl, Stra8
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methylated
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