DNA methylation and chromatin dynamics in embryonic stem cell regulation

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Abstract

Introduction

Self-renewal and pluripotency are two most prominent properties of stem cells that are regulated by intrinsic factors that maintain intricate balances among important molecular factors. Many of transcription factors that are involved in the regulation of stemness genes or differentiation-related genes ensure its timely expression for the maintenance or differentiation of stem cells during development. In addition to the genetic factors, the epigenetic regulation has been well recognized as a critical process in the control and maintenance of stem cell properties. This article will highlight recent advances on the epigenetic regulation in stem cells, especially in the context of DNA methylation, chromatin dynamics, and nuclear remodeling histone deacetylase (NasRD) complex in the regulation of stem cell self-renewal and maintenance. Furthermore, we briefly discuss impact of adoption of stem cell technologies in regenerative medicine.

Conclusion

Studies on epigenetic regulation in stem cells will advance our understanding towards how pluripotent stem cells acquire differentiation competency that leads to the initiation of somatic cell growth control, an important topic that is currently largely unclear. Detailed understanding of molecular regulations during embryonic stem cell differentiation and development will contribute to our understanding on developmental process with utility for regenerative therapeutics.

Introduction

Divergence of stem cell fate—self-renew or differentiation—can be measured as shifts in patterns of gene expressions and echoes dynamics and reversibility of epigenetic modifications. Epigenetics is the study of heritable changes in gene activity that are independent of nucleotide sequences. In this process, regulation of genetic activity is achieved through modification of chromatin structure, post-translational histone tail modification, and DNA methylation by enzymes and protein complexes. Chromatin structure is the component that is important in its relation to the activity level of genes in the region. In general, loosely packaged euchromatin is shown to associate more with RNA polymerases, while tightly packaged heterochromatin regions often contain inactive genes (Figure 1a).

DNA methylation is one of critical epigenetic modifications of the genome. In addition, post-transcriptional modifications such as acetylation and methylation of histone tail residues significantly alter chromatin structure and dynamics of gene expression (Figure 1a). Pluripotent stem cells have been resourceful to understand molecular mechanisms of epigenetics. Extensive studies in recent years have identified epigenetic mechanisms that directly affect the "stemness" properties of stem cells. While many are complex and their mechanistic frameworks need to be further outlined, interplay between DNA elements and epigenetic processes makes up a hallmark of pluripotency and lineage specific differentiation. As genome-wide epigenetic reprogramming coincides with acquisition of pluripotency in the early embryo, it is reasonable to describe epigenetic and chromatin dynamics for mechanistic explanation of the stemness. In this review, we discuss epigenetic regulation of stem cell self-renewal and maintenance through the process involving chromatin structure modification, DNA methylation, and NuRD complex. Recent groundbreaking discovery of induced pluripotent stem cells (iPSCs) sparked its potential in regenerative medicine and advanced the field of stem cell research. Roles of epigenetic modification in iPSCs and for regenerative medicine will also be discussed.

DNA methylation and chromatin structure in stem cell regulation

DNA methylation is a heritable epigenetic modification that plays a critical role in transcriptional repression, genomic imprinting, suppression of retrotransposable element, X-chromosome inactivation, and configuration of local chromatin structure. While it is the only epigenetic modification on DNA, roles of DNA methylation in epigenetics largely coincide with the chromatin structure and genomic location it resides and regulates. Catalytic transfer of a methyl group to position C5 of a cytosine base (5mC) and DNA methylation patterns in the genome are established and maintained by two general classes of DNA methyltransferases (DNMTs): de novo methyltransferases (DNMT3a and DNMT3b) and maintenance methyltransferase (DNMT1) (Figure 1b). In ESCs, defects in DNA methylation are embryonic lethal, and that mouse DNMT knockout studies demonstrated that Dmnt1 and Dmnt3b
are essential for early embryonic development and that absence of Dnmt3a causes mice to die within a few weeks after birth\(^5,6\).

In mammals, a vast majority of DNA methylation occurs in the symmetric CpG context in the genome\(^7\), and a small amount of non-CpG methylation is observed in ESCs\(^8\). Satellite-rich constitutive heterochromatin regions, both centromeric and pericentromeric, are CpG rich and highly methylated and contribute to higher order chromatin structure. Common gene regulatory elements such as enhancers and transcription factor binding sites contain differentially methylated regions (DMRs), which well represent methylation dynamics and display lineage- or cell-type-specific pattern of methylation (Figure 2a)\(^9\).

Moreover, these regulatory elements can control genomic imprinting via imprinting control regions (ICRs), and differential methylation in ICRs is established during spermatogenesis or oogenesis and maintained throughout development\(^10\). CpG methylation in promoter regions has been shown to have inverse correlation with gene expression. CpG islands (CGIs) found within or proximal promoters are generally unmethylated and associate with active transcription marker histone H3 lysine 4 (H3K4) methylation (Figure 2a)\(^11\). In turn, this correlation becomes weaker in differentiated cells along with loss of H3K4 methylation\(^11\), indicating an important role of chromatin structure in ESCs development.

Furthermore, promoters with low CpG content are more likely methylated in ESCs but demethylated and actively expressed during differentiation in cell-type-specific manner (Figure 2b)\(^12,13,14,15\). While DNMTs establish and maintain DNA methylation patterns, methyl-CpG binding proteins (MBDs) recognize methylated DNA\(^7\). Along with direct binding of DNMTs to histone deacetylases (HDACs), this further facilitates concomitant deacetylation from histones exerting methylation-dependent transcriptional repression\(^16,17\). Taken together, these findings demonstrate significant roles of DNA methylation in transcriptional activity and acquisition of distinct chromatin structure in ES cells.

**DNA methylation in stem cell maintenance and development**

DNA methylation pattern is maintained through cell division cycles via distinct phases of remodelling and reprogramming processes. In somatic cells, the level of CpG methylation reaches around 70 to 80%\(^9\) and maintained as epigenetic memory. Contrastingly, these heritable epigenetic marks including modified histones can be removed during epigenetic reprogramming, and DNA methylation levels are reduced between 5 to 30% in primordial germ cells (PGCs), early embryos, and naïve ESCs\(^18\). Here, DNA demethylation facilitates stem cell homeostasis by providing a mechanism of cellular memory for self-renewal and proliferation and by preventing premature activation of differentiation. During early embryogenesis, two major phases of global DNA

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**Figure 1:** Schematic representation of changes in chromatin structure. a. Chromatin structure is a building block of a chromosome that DNA strands wrapped around histone octamers forming nucleosomes and adopts changes in its structure through epigenetic modifications such as site-specific histone tail modifications—acetylation, methylation, and phosphorylation—at multiple locations. Reversible effects of post-transcriptional modification that influences chromatin structure and hence gene expression are depicted here. Interplay between DNA elements and epigenetic processes make up a hallmark of pluripotency and lineage specific differentiation. Top: loosely packed active/open chromatin. Bottom: condensed inactive/silent chromatin preventing access of transcription factors, RNA Polymerase II, etc. White circles: unmethylated cytosines. Red circles: methylated cytosines (5-methylcytosine (5-mC)). b. DNA methylation takes place at position C5 of a cytosine base catalyzed by two general classes of DNA methyltransferases (DNMTs): de novo methyltransferases (DNMT3a and DNMT3b) and maintenance methyltransferase (DNMT1). Together with modifications described above, epigenetic modifications regulates chromatin organization and gene expression.
DNA methylation can be observed in mammalian germline: where the paternal and maternal genomes undergo passive DNA demethylation and where PGCs derive from the epiblast (Figure 3a)\textsuperscript{10}. During the first phase, the regaining of pluripotency and formation of inner cell mass/ESCs are achieved with erasure of the epigenetic barriers by active conversion of 5mC to 5-hydroxymethylcytosine (5hmC) by hydroxylases TET1 and TET2 (paternal)\textsuperscript{19} or by passive dilution of 5mC during replication (both paternal and maternal) until the blastocyst stage (E3.5). This reduction in genomic 5mC levels establishes embryonic epigenetic state and contributes to subsequent cell-fate commitment with concomitant de novo DNA methylation (Figure 3a). Following de novo methylation, a second reprogramming occurs to erase global 5mC in PGCs. Yet some repetitive sequences such as the intracisternal A particle (IAP) retrotransposon family remained methylated, this step is the most comprehensive DNA demethylation step found in mammalian life cycle which erases remaining methylated sequences such as DMRs, genomic imprints, CGIs and providing genomic plasticity to form a distinct epigenome\textsuperscript{19}.

Prior to differentiation, safeguarding pluripotency and self-renewability is fundamental in stem cell maintenance. Recent studies have been focusing heavily on epigenetic mechanisms in naïve ESCs. Growing under 2i conditions (MEK and glycogen synthase kinase-3 inhibitors), naïve ESCs show characteristics of decreased global 5mC content and loss of de novo DNMT 3a/3b and 3l (Figure 3a)\textsuperscript{20}. Here, PR-domain-containing transcriptional regulator (Prdm14) regulates expression of de novo DNMTs and fibroblast grown factor (FGF) signalling, a key trigger for differentiation, by recruiting polycomb repressive complex 2 (PRC2) and stabilizes naïve pluripotency in ESCs\textsuperscript{21,22}. These findings demonstrate that DNA hypomethylation is crucial for antagonizing the self-activating differentiation signal, resetting the epigenome and re-establishing the pluripotency network, and maintaining germ line fate in vivo\textsuperscript{21,22}. Additionally, downregulation of DNMTs is correlated with the actual elevation of symmetric divisions\textsuperscript{23}, another evidence suggesting the role of demethylation in promoting self-renewal to maintain cells at the most naïve state.

While stemness of ESCs in the culture is characterized by global DNA hypomethylation, subsequent differentiation is accompanied by the accumulation of DNA methylation\textsuperscript{20}. During differentiation, dynamic changes and genome wide redistribution of DNA methylation resolve toward lineage specification as de novo methylation silences pluripotency genes and establishes lineage-specific methylation pattern\textsuperscript{24,25,26}. Moreover, DNA methylation maintains the proper spatial and temporal developmental gene regulation and stabilizes differentiated cellular traits\textsuperscript{27}. Thus, DNA methylation functions as a memory of a cell’s developmental history along with its feature of clonal inheritance. Granting DNA methylation plays a critical role in stem cell maintenance and development, it only partially describes the whole epigenetic regulation mechanism. It has been reported that de novo DNMTs are necessary for retaining a proper cell type identity and efficiently silencing pluripotency genes during differentiation. Notably, DNA methylation and histone modifications act coordinately, and the epigenetic regulations of differentiation-specific genes in stem cells are repressed by alternative chromatin remodelling.

\begin{figure}[ht]
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\caption{DNA methylation changes in stem cells and development. a. DNA methylation patterns in common gene regulatory elements. Gene regulatory elements such as enhancers and transcription factor binding sites contain differentially methylated regions (DMRs) and CpG islands (CGIs) well represent methylation dynamics. Example of DMRs in regulatory elements of imprinted genes differentially expressed from paternal or maternal alleles is shown. CpG islands (CGIs) found within or proximal promoters are generally unmethylated and associate with active transcription marker histone H3 lysine 4 (H3K4) methylation, whereas they can become hypermethylated in differentiated cells along with loss of H3K4 methylation. CpGs located in gene bodies are often found methylated and lead to gene repression. White hexagon: unmethylated DMRs. Red hexagon: methylated DMRs. White circles: unmethylated cytosines. Red circles: methylated cytosines (5-mC). Orange boxes: maternally-expressed genes. Green box: paternally-expressed gene. Blue box: actively-transcribed genes. Gray box: repressed genes. b. Lineage- or cell-type-specific pattern of methylation. Promoters with low CpG content are more likely methylated in ESCs but demethylated and actively expressed during differentiation in cell-type-specific manner.}
\end{figure}
factors\textsuperscript{24}. Along with histone modification, recent studies have been making a progress on studying roles of the Nucleosome Remodelling and Deacetylase (NuRD) complex in stem cell regulation. The details of NuRD complex and its roles will further be discussed in the following.

**Role of NuRD complex in stem cell regulation**

The NuRD complex is a multifunctional epigenetic regulator having critical roles in transcription repression, chromatin assembly, cell cycle progression, genomic stability and has been linked to cancer progression\textsuperscript{26}. Although the NuRD complex is incapable of actively demethylating DNA, it has been shown to be necessary for establishing an unmethylated state at particular promoters\textsuperscript{29}.

The NuRD complex is also essential for ESC lineage commitment by recruiting PRC2 and modulating transcriptional heterogeneity of a wide range of pluripotent genes\textsuperscript{30,31}. NuRD-mediated deacetylation and subsequent trimethylation of histone H3 lysine 27 (H3K27) at NuRD target promoters impose controlled gene expression by downregulating genes that are lineage inappropriate and by activating lineage-specific genes\textsuperscript{31}. Being a multi subunit complex, distinct combinations of non-enzymatic subunits are proposed to be the mechanism behind which the NuRD complex derives its functional specificity and links transcriptional regulatory processes, including histone deacetylation and demethylation, nucleosome mobilization and recruitment of other regulatory proteins\textsuperscript{32}.

The NuRD complex consists of six core subunits, which include histone deacetylase core (HDAC) proteins, histone-binding proteins, metastasis-associated proteins, the methyl-CpG-binding domain (MBD) protein and the chromodomain-helicase-DNA-binding protein\textsuperscript{33}. While histone deacetylation is catalysed by HDAC1 and -2, MBD2 and -3 subunits mediate binding to DNA and transcription factors\textsuperscript{28,34}. MBD2, a subunit of the Mi2-NuRD complex, selectively recognizes and binds CpG island promoters that are highly methylated and is postulated to regulate gene expression through recruitment of co-repressor complex (Figure 3b)\textsuperscript{35,36}. Conversely, MBD3 is incapable of binding methyl-CpG due to amino acid substitution in the binding domain. Yet, a strong negative correlation between
MBD binding and promoter activity as well as enhancer accessibility was identified regardless of their methylation status, suggesting that the MBD subunits are partially responsible for NuRD complex recruitment (Figure 3b)37. However, the molecular mechanisms of transcriptional regulation remain elusive. The NuRD complex has been shown to silence transcription of pluripotency genes to below a threshold allowing the cell to exit self-renewal in response to a loss of self-renewal factors30. MBD2 and MBD3 are assembled into two distinct Mi-2/NuRD-like complexes39, and the MBD3/NuRD complex has been shown to regulate pluripotency (Figure 3b). Mbd3-deficient ESCs failed to silence early embryonic genes, which further resulted in failure to commit to developmental lineages31. MBD3-deficient blastocysts fail to develop into mature epiblasts and expand their Oct4-positive, pluripotent cell population39.

Moreover, the study using primed pluripotent epiblast stem cells (EpiSCs) expressing Nanog-GFP knock-in reporter and reprogrammed into a naïve pluripotent state showed that MBD3 to be a major roadblock during somatic cell reprogramming under 2i condition as its knockdown by RNAi facilitated reprogramming nearly 100 % in short period of time60. This is in line with the suppressing role of MBD3/NuRD on pluripotency gene expression. On the contrary, one study identified MBD3 to be essential in initiating reprogramming of neural stem cells to induced pluripotent stem cells41. In this study, the NuRD complex was shown to directly regulate expression of pluripotency genes in neural stem cells, and combined overexpression with NANOG facilitated reprogramming in both kinetics and efficiency in MEF-derived reprogramming intermediates41. Nonetheless, the NuRD complex was shown to be acutely responsible for the expression of pluripotent genes in MBD3-/- ESCs expressing an inducible MBD3 protein40. Knockdown studies of NuRD-mediated genes, such as Klf4 and Klf5, indicators of stem-like capacity, in the MBD3/- ESCs model demonstrated NuRD’s role in facilitating lineage commitment in ESCs30. In addition, the MBD3/NuRD complex was shown to interact with cyclin-dependent kinase 2-associated protein 1 (CDK2AP1) to regulate pluripotency of ESCs, particularly through the silencing of Oct4 (Figure 3b)42. This interplay between the repressive effects of the NuRD complex and transcription activation signals is critical in facilitating lineage commitment. NuRD-deficient ESCs fail to silence expression of determinant genes and instead express trophectoderm-specific genes in response to extracellular signals30.

Studies investigating the effects of MBD3-deficient ESCs demonstrate the pivotal role NuRD complex plays in epigenetic silencing and the regulation of differentiation responsiveness of pluripotent cells. Further studies behind the regulatory mechanisms underlying its processes need to be conducted to fully realize NuRD’s role in maintaining the stemness of ESCs and facilitation of differentiation.

In addition, identifying additional components of the NuRD complex, such as MBD3 and CDK2AP1, will be critical in providing us with a better insight into how it modulates the transcriptional heterogeneity of ESCs. By better understanding the mechanisms of the NuRD complex, we may be one step closer towards harnessing the potential of iPSCs in regenerative medicine.

**Discussion**

More than three decades of history being identified as a key player in epigenetics, DNA methylation plays a central role in transcriptional repression by providing a bridge between genetic code and epigenetic modifiers. Since DNA methylation machinery regulates gene expression through local chromatin structure, effects and location of DNA methylation are in concordance with state of chromatin configuration. The NuRD complex recruits regulatory proteins to gene regulatory elements to exert transcriptional activation or repression. Perturbation of aforementioned epigenetic modifications has huge impact on the stemness and its sustainability19. Stem cell technology to reprogram somatic cells into iPSCs with a defined set of transcription factors Oct4, Sox2, Klf4, and c-Myc13,44 advanced our knowledge in developmental cell biology. Its mechanistic framework of epigenetic reprogramming to pluripotency is ongoing process and hampered by inefficient and time-consuming process and often being scrutinized. The iPSC induction is achieved through manipulation of cell fate including reprogramming of somatic histone and DNA CpG methylation marks by the coordinated activity of transcription factors and epigenetic modifying enzymes45 and cytoskeletal rearrangement46. Recent two reports on new barriers in iPSC reprogramming in human and mouse reprogramming utilized genome-wide RNAi screens and demonstrated significant amount of genes that hinder somatic cell reprogramming process47,48. These studies achieved a wealth of knowledge on gene function during iPSC reprogramming. Although Yang et al. and Qin et al. employed the unbiased approach of RNAi screening, neither group identified roles of Mbd3 in epigenetic reprogramming while previous reports found Mbd3 as essential41 or as a barrier40 for iPSC reprogramming. A key to this difference could reside in the model system employed. Among the groups, Rais et al. carried out the RNAi knockdown analysis with the secondary cell line that uniformly expresses a reporter gene, hence, minimizing heterogeneity in the cell population being tested.

The growing number of human diseases has been benefitted from stem cell technology and epigenetic reprogramming to dissect disease mechanisms and for enormous therapeutic potential. The aim is to understand mechanisms underlying the pathogenesis and treat diseases to re-establish and maintain proper
epigenetic controls while pharmacological intervention could sustain its compensatory side effects and hold lack of specificity. During reprogramming, the epigenetic landscape shifts from a somatic cell of origin to an ESC through erasure of repressive marks on pluripotency genes and establishment of bivalent domains on lineage specific genes. In this regard, iPSCs are very similar to ES cells; however, iPSCs continue to retain a transcriptional memory of the original cell in early passages that can be attributed to incomplete promoter DNA methylation during reprogramming. Moreover, some evidence indicates uncontrolled differentiation of transplanted ES cells resulting in tumour formation.

Therefore, it is critical to ensure and restrict the use of iPSCs with well-defined biological relevance. With this reason, use of stem cells in regenerative medicine is currently still in a rudimentary stage at practical standpoint and under intensive investigation.

Conclusion
Taken together, further development of stem cell technology is expected to preclude chemical or genomic manipulations and aimed as replacement for damaged tissues and organs.

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