

Review



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Epigenetic regulation in pluripotent stem cells: a key to breaking the epigenetic barrier

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The differentiation and reprogramming of cells are accompanied by drastic changes in the epigenetic profiles of cells. Waddington's classical model clearly describes how differentiating cells acquire their cell identity as the developmental potential of an individual cell population declines towards the terminally differentiated state. The recent discovery of induced pluripotent stem cells as well as of somatic cell nuclear transfer provided evidence that the process of differentiation can be reversed. The identity of somatic cells is strictly protected by an epigenetic barrier, and these cells acquire pluripotency by breaking the epigenetic barrier by reprogramming factors such as Oct3/4, Sox2, Klf4, Myc and LIN28. This review covers the current understanding of the spatio-temporal regulation of epigenetics in pluripotent and differentiated cells, and discusses how cells determine their identity and overcome the epigenetic barrier during the reprogramming process.

1. Introduction

Induced pluripotent stem (iPS) cells are generated by the enforced expression of embryonic transcription factors, most commonly Oct3/4, Sox2, c-Myc and Klf4. In addition to pluripotency, they have infinite capacity for self-renewal [1,2]. iPS cells have been generated from multiple cell types, including keratinocytes [3], mesenchymal cells in fat [4], the oral mucosa [5], dental pulp cells [6], peripheral blood [7] and cord blood [8], as well as skin fibroblasts [2]. The characteristics of fully reprogrammed cells are functionally and molecularly very similar to those of embryonic stem (ES) cells in terms of their morphology, gene expression profile and capacity to differentiate into any of the three germ layers: endoderm, mesoderm and ectoderm. iPS cells could be a useful source for cell transplantation therapy, drug screening and disease modelling [9].

iPS cells are also highlighted as a cell model for epigenetic research. Pluripotent stem cells differentiate into any of the 200–300 specialized cell types with distinct properties. Waddington clearly described how differentiating cells acquire their cell identity, by illustrating differentiating cells as marbles rolling down valleys, with the developmental potential of individual cell populations declining towards the terminally differentiated state at the lowest elevation [10]. Recent genome-wide analyses using high-performance sequencers have uncovered key differences in the epigenetic landscape of pluripotent stem cells compared with that of lineage-committed cells. Waddington's classical model is now widely accepted; it appears that 'Waddington's marbles' are present in different valleys and that the different elevation levels have distinct epigenetic profiles, which are likely to play a role in the irreversibility of the properties of lineage-committed cells and the maintenance of their identity [11].

Cellular reprogramming induces differentiated cells to revert back to undifferentiated cells including pluripotent stem cells. On the basis of Waddington's model, somatic cells in differentiated states maintain their own cell fate and do not normally change from one differentiation pathway to another, although cell fate can be altered by nuclear reprogramming [11–15]. This reversal process can

be achieved by breaking the barrier of the differentiated state, and it provides one of the strategies for investigating the molecular basis of cell identity governed by epigenetic regulation. Because of its observed lower efficiency, the reprogramming process has been depicted as climbing a mountain, because it is much harder to achieve than differentiation, which is a spontaneous process, as sliding down a hill [11]. Therefore, a molecular understanding of the reprogramming process may address the question of how differentiated cells maintain their identity. Induced pluripotency is a process associated with gradual epigenetic changes [16], and thus can be exploited to obtain a molecular understanding of the determination of cell fate, which is mediated by epigenetic changes such as the silencing of retroviral transgenes upon the establishment of pluripotency [17,18], the reactivation of endogenous pluripotency genes [1], the establishment of bivalent chromatin domains in the promoters of developmentally regulated genes [12,19], global DNA hypomethylation, DNA hypermethylation of imprinted gene loci [17], reactivation of the inactive X chromosome in female iPS cells and reorganization of chromatin fibres [20,21].

This review summarizes studies performed to understand the epigenetic signatures associated with pluripotent and differentiated states, and addresses how their unique signatures contribute to the maintenance of pluripotency and how they are established during the reprogramming process.

2. Distinct histone modification profile in pluripotent cells

Recent technical advances have allowed us to map chromatin modifications throughout the genome by combining chromatin immunoprecipitation with DNA microarray analysis (ChIP-chip) or high-performance sequencing (ChIP-seq). Pluripotent stem cells have a unique expression pattern for histone modifiers and distinct distributions of modified histones.

The Polycomb group (PcG) complexes with the activity of H3K27 methylation to repress the expression of developmentally regulated genes in pluripotent stem cells [22,23], whereas the Trithorax group (TrxG) complexes with the activity of H3K4 methylation to activate the expression of genes associated with self-renewal [24]. An active mark, H3K4me₃, is frequently observed in promoter regions of pluripotent stem cells, and is linked to transcriptional activation in general [25–27]. The methylation of H3K4 is mediated by TrxG members such as Set/mixed lineage leukaemia (MLL) methyltransferases. Wdr5, a key component of TrxG, interacts with H3K4me₂, and mediates the transition of H3K4me₂ to H3K4me₃ [28]. The expression of Wdr5 is the highest in undifferentiated ES and iPS cells, and the level decreases during the differentiation process. The expression of Wdr5 along with the reprogramming factors enhances the efficiency of iPS cell generation [24]. Wdr5 physically interacts with Oct3/4, and co-occupies the DNA-binding sites of Oct3/4. Silencing of Wdr5 expression results in decreased expression of Oct3/4 target genes and the loss of self-renewal capacity of ES cells. H3K4 demethylase LSD1 stabilizes global DNA methylation [29] and also maintains an appropriate balance between H3K4 and H3K27 methylation in the regulatory regions of several developmental genes in pluripotent stem cells [30]. The recently reported interaction between LSD1 and Dnmt1

indicated that LSD1 mediates the linkage between DNA methylation and H3K4 demethylation [31].

The methylation of H3K27 is mediated by Polycomb repressive complex 2 (PRC2), which is composed of PcG proteins such as enhancer of zeste 2 (Ezh2), embryonic ectoderm development (Eed) and suppressor of zeste 12 homolog (Suz12) [32,33]. ES cells lacking a single component of the PRC2 complex, such as *Ezh2*, *Eed* or *Suz12*, show partial disruption of self-renewal accompanied by complete depletion of H3K27me₃ [23,34], indicating that each component of the PRC2 complex collaboratively executes H3K27 trimethylation and regulates pluripotency and differentiation [35–38]. The histone methyltransferase activity of Ezh2 is responsible for maintaining H3K27 trimethylation in pluripotent stem cells [36,38,39]. Suz12 interacts with Ezh2, and inhibits protein degradation of Ezh2 [37]. A genome-wide analysis showed that Suz12 is co-localized with H3K27 trimethylation at key development regulators, as well as with highly conserved non-coding elements in ES cells [22]. A subset of Suz12-bound and H3K27me₃-enriched genes are co-occupied by Oct3/4, Sox2 and Nanog. They are preferentially activated during ES cell differentiation, indicating that PRC2 poises differentiation-related genes for rapid gene activation during differentiation in pluripotent stem cells [22,40]. The PRC1 complexes composed of RING1A, RING1B, BMI1 and other proteins exhibit diverse functions in a PRC2-independent manner, such as ubiquitination of lysine 119 of H2A [41,42], and are also involved in the repression of transcription [43,44]. Previous studies in *Drosophila melanogaster* and *Caenorhabditis elegans* demonstrated that PcG proteins bind *cis*-acting DNA sequences and repress transcription, facilitating heterochromatin formation by binding to RNA [45–50]. For example, the incorporation of non-coding RNAs into PRC2 complexes has been observed. The PRC2 complexes interact with *Xist* RNA in mouse ES cells [51], whereas interaction between *HOTAIR* and *SUZ12* has been observed in human fibroblasts. Such a gene repression mechanism may also be employed by mammalian pluripotent stem cells.

Transcriptionally inactive heterochromatin is usually accompanied by H3K9 di- and tri-methylation (H3K9me_{2/3}). Oct3/4 upregulates demethylases for H3K9me_{2/3}, such as *Jmjd1a* and *Jmjd2c*, by interacting with their promoters. Demethylation of H3K9me_{2/3} by these demethylases contributes to the self-renewal of ES cells [52,53]. In fact, depletion of *Jmjd1a* and *Jmjd2c* leads to decreased expression of pluripotency genes and differentiation of ES cells. In contrast, H3K9 methyltransferases have been reported to play an important role in early embryogenesis. G9a is an H3K9 methyltransferase that is essential for embryonic development [54], and has been shown to prevent reprogramming by recruiting Dnmt3a and Dnmt3b to the promoters of Oct3/4 and HP1β [55]. Treatment of cells with a chemical inhibitor specific for G9a increases the efficiency of iPS cell generation [56]. Although the molecular significance of silencing is unknown, ES cells are considered to be a good model for studying the relationship between DNA methylation and histone modifications, because of their high level of *de novo* DNA methyltransferase activity [57]. Endogenous retroviruses (ERVs) are transcriptionally silenced in ES cells. However, the silencing of ERVs is initiated by the H3K9 methyltransferase ESET/SETDB1, with KRAB-associated protein 1 (KAP1, also known as

TRIM28) in a DNA methylation-independent manner [58,59]. This suggests that not only the global level of H3K9me2/3, but also the context-dependent regulation of H3K9 (de)methylation is involved in the maintenance of pluripotency and differentiation. It is unclear whether the level of H3K9me2/3 is lower in pluripotent stem cells [60,61].

The acetylation of histones is also a significant modification observed in pluripotent stem cells. The level of acetylation is generally correlated with transcriptional activation, and is strictly regulated by the balanced actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [62]. RNA interference screening of ES cells for chromatin components showed that a large set of HAT complexes to which Tip60 (TAT-interacting protein 60)/p400 contributes are ES cell development regulators, such as Gata4 and Gata6, and significantly overlap with target genes of Nanog [63,64]. On the other hand, HDAC inhibitors, such as valproic acid and trichostatin A, improve the efficiency of nuclear reprogramming by both nuclear transfer [65,66] and the transduction of pluripotency genes [67], suggesting that histone acetylation is involved in the maintenance and acquisition of pluripotency.

One of the most distinctive features of histone modifications in pluripotent stem cells is hypothesized to be 'bivalent domains', where both the active mark H3K4me3 and the repressive mark H3K27me3 are observed [25,68,69]. These conflicting marks are preferentially observed at promoters of lineage-specific genes in pluripotent stem cells but very rarely in differentiated cells [19,25,27,68,70,71]. This finding indicates that target genes in bivalent domains are 'poised' for expression, which is kept silent by H3K27 trimethylation in pluripotent stem cells and is presumably dependent on the trimethylation of H3K4. For example, while the expression of genes in bivalent domains is low in pluripotent stem cells, it switches to conventional patterns in the presence of active or repressive marks by erasing opposite marks during differentiation [69,72]. Consequently, differentiation-related genes with bivalent domains are expressed only in cells of their specific lineage. The repressive function of H3K27 methylation at lineage-specific loci is also demonstrated by the derepressed expression of these target genes in ES cells lacking key subunits of the H3K27 methyltransferase complex PRC2 [22,23,68]. Thus, the formation of poised chromatin architecture is proposed to be a key mechanism involved in both the maintenance of pluripotency and the developmental potential of pluripotent stem cells.

Incomplete formation of bivalent domains is occasionally observed in partially reprogrammed cells [19]. Furthermore, the *Ink4/Arf* locus is silenced during the early stage of reprogramming with the formation of bivalent chromatin domains, whereas forced silencing of *Ink4/Arf* by shRNA increases the efficiency of iPS cell generation, indicating that the *Ink4/Arf* locus could functionally behave as a barrier to reprogramming [73]. In this way, genes responsible for differentiation are susceptible to the formation of bivalent domains, and keep target genes poised for transcriptional activation in pluripotent stem cells [12].

In the last decade, it has come to be widely accepted that the bivalent domain is one of the most distinctive features of pluripotent stem cells. However, a recent study revised the role of the bivalent domain in pluripotent stem cells. Mouse ES cells cultured in leukaemia inhibitory factor (LIF)-containing and

feeder-free medium with two small-molecule kinase inhibitors (2i) exhibited ground-state pluripotency. These naive ES cells exhibited a decreased amount of H3K27me3 on the bivalent domain compared with that observed in mouse ES cells under conventional culture conditions. The distribution of H3K4me3 in naive ES cells is similar to that in ES cells under 2i-free culture conditions, demonstrating that the bivalent domain is transiently formed during differentiation [74].

The replacement of canonical histones with specific variant forms has emerged as a key mechanism of modulation of nucleosome dynamics and chromatin structure. Incorporation of histone variants alters the interaction surfaces and overall stability of nucleosomes, including localized changes in chromatin structure and the formation of specialized chromosomal domains [75–77]. Some of the histone variants are considered to play an important role in differentiation or reprogramming. In ES cells, H2AZ, a highly conserved variant of H2A, is preferentially incorporated into the bivalent domains of developmentally important genes [78,79]. The depletion of H2AZ by RNA interference in ES cells results in the expulsion of PcG proteins from the bivalent domains, leading to derepression of genes that are silenced by the PcG complexes, although H2AZ is not required for the maintenance of stemness in ES cells [78].

In addition to H2AZ, macroH2A, one of the histone variants incorporated mainly in heterochromatin [80], was recently identified as a regulator of reprogramming [81]. In addition to variants of core histones, differential composition of a linker histone H1 has been observed [82]. The possible roles of other histone variants in differentiation or reprogramming also need to be evaluated.

3. DNA methylation and demethylation: modulating the barrier for reprogramming

DNA methylation maintains long-lasting cell memories, and is therefore considered to be a pivotal epigenetic barrier to cellular reprogramming [83]. During reprogramming, the activation of endogenous pluripotency genes including *Oct3/4* and *Nanog* is accompanied by erasing the methylation of cytosines at their promoter regions. Insufficient DNA demethylation at the promoter regions, which is occasionally observed in partially reprogrammed iPS cells, fails to produce the robust reactivation of pluripotency genes [1,84–86]. In addition, the differential patterns of DNA methylation that are associated with genomic imprinting, retrotransposon silencing and X chromosome inactivation are observed between differentiated and pluripotent stem cells and among a series of pluripotent stem-cell lines [27,86–89], indicating that DNA methylation may be a suitable epigenetic marker for characterizing pluripotent stem-cell lines. Although it is unclear how such differential levels of DNA methylation arise, functional linkage between DNA methylation and reprogramming has been demonstrated. The inhibition of DNA methylation by chemical compounds or RNA interference targeting DNA methyltransferase can increase the efficiency of iPS cell generation [19].

Recent analyses using a high-performance sequencer have enabled mapping of DNA methylation with high resolution and have revealed an intriguing distribution of methylated cytosine in pluripotent stem cells. Since DNA methylation

is frequently observed at CpG islands, which contain a high frequency of CpG sites, it is considered that the frequency of CpG sequences was positively correlated with the susceptibility to DNA methylation. However, the most recent studies of genome-wide DNA methylation status in pluripotent stem cells have produced observations that differ from the widely accepted model. The methylation levels of CpGs in pluripotent stem cells were negatively correlated with the local CpG density.

In ES and iPS cells, regions with high CpG density exhibited low DNA methylation, whereas those with low CpG density exhibited high DNA methylation [27,87,90,91]. Regions with low CpG density are frequently observed in the promoters of tissue-specific genes [91], implying that the mechanism responsible for DNA methylation in the regulation of tissue-specific genes is different from that for DNA methylation in the regulation of other genes. Intriguingly, DNA hypermethylation at the promoters of these tissue-specific genes with low CpG density is accompanied by bivalent chromatin in ES and iPS cells [91,92]. The relevance of this uniquely low CpG methylation level in pluripotent stem cells with bivalent domains is yet to be investigated at the molecular level; such information would provide important clues regarding the mechanisms of epigenetic regulation during differentiation.

A single-base-resolution methylome analysis by whole-genome bisulphite sequencing (WGBS) also highlighted the significance of non-CG methylation in pluripotent stem cells [86,89]. Surprisingly, approximately one-quarter of all methylated cytosines in ES and iPS cells occurred in a non-CpG context, whereas most of the methylated cytosines in somatic cells were observed in CpG sequences. These pluripotent stem cell-specific non-CpG methylation sites tend to be located in the exonic regions of actively transcribed genes [86]. The existence of DNA methylation in cytosine of non-CpG may be linked to the fidelity of DNA methylation, which was proposed in a previous study of DNA demethylase Tet1 [93].

Studies using mice harbouring mutant DNA methyltransferases showed the importance of strict regulation of DNA methylation during the normal developmental process. Dnmt1 and Dnmt3a/Dnmt3b are enzymes essential for the maintenance and establishment of DNA methylation, respectively [87,94,95]. The loss of *Dnmt1* causes the loss of two-thirds of total DNA methylation, thus leading to embryonic lethality [96]. Embryos with mutant *Dnmt3b* appear to be normal in early developmental stages but show multiple developmental defects in the later stages [97]. The conditional deletion of *Dnmt3b* in mouse embryonic fibroblasts leads to a partial loss of DNA methylation [98]. However, although the Dnmt family plays an essential role in both the developmental process and the reprogramming of germ cells, de novo methylation by Dnmt3a and Dnmt3b is dispensable for the induction of iPS cells [99].

The mechanism by which methylated cytosine is converted into unmodified cytosine during the reprogramming process is elusive. However, with regard to DNA demethylation at the global level, two possible mechanisms have been proposed: a replication-independent 'active' DNA demethylation pathway and a replication-dependent 'passive' DNA demethylation pathway. The existence of active DNA demethylation is demonstrated by the base-excision

repair (BER) machinery in plants and in fertilized eggs of animals. Previous studies have suggested TDG [100,101], MBD4 [102], AID/APOBEC [103], GADD45A [104] and MBD2B [105–107] as candidate DNA demethylases in mammalian cells [108–111], and the coordinated action of these factors is required for active DNA demethylation through the BER machinery [112]. However, the roles of these molecules in active DNA demethylation in mammals are controversial.

The recent findings of Tet family proteins as candidate DNA demethylases have advanced our understanding of DNA demethylation in pluripotent stem cells and other tissues [113–119]. Tet family proteins catalyze the conversion of methylcytosine to 5-hydroxymethylcytosine (5hmC) in an Fe(II)- and α -ketoglutarate-dependent manner. Tet proteins have been implicated in ES cell maintenance and lineage specification *in vitro*. Tet1 and Tet2 are highly expressed in mouse ES cells, and are downregulated upon cell differentiation [116,119]. The silencing of *Tet1* expression by RNA interference downregulates the expression of pluripotency genes such as *Nanog*, *Esrrb*, *Klf4*, *Prdm14*, *Lefty1* and *Lefty2*, and increases the trans-differentiation potential of ES cells to extra-embryonic lineages [93,116,119–121]. Genome-wide analysis using a high-performance sequencer revealed the presence of an intricate relationship between Tet1 and the expression of its target genes [93,120,122,123].

Tet1 preferentially binds to the gene body and GC-rich sequences in promoter regions of both transcriptionally active and repressive genes [122]. The Tet1-binding sites overlap with PcG-target sites [123]. Consistently, the proteomic analyses identified Sin3a, a component of the PcG protein complex, as a binding partner of Tet1 [93]. Knockdown of Tet1 decreases the expression of PcG-target genes and pluripotency-related genes [120], indicating that gene regulation by Tet1 cannot be completely explained by the collaborative functioning with PcG. The involvement of chromatin remodellers in Tet1-mediated gene regulation has also been reported. The Mbd3/NURD complexes were identified as a 'reader' of 5hmC. The Mbd3/NURD complexes directly recognize 5hmC and control the expression of Tet1-target genes [124]. Functioning of Mbd3/NURD complexes as a reader of 5hmC may affect the regulation of Tet1-target genes through modification with 5hmC. Knockdown of Tet1 in ES cells produces a phenotype similar to that of Mbd3-knockdown ES cells with increased expression of trophoblast markers, implying a functional link between Tet1 and Mbd3. In addition to 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) were detected as intermediates of the oxidation reaction mediated by Tet1 [125], and 5caC is subject to base excision by thymine–DNA glycosylase (TDG) in ES cells [126]. This suggests that the oxidation of Tet1 followed by base excision of 5caC by TDG is a possible pathway for active DNA demethylation in ES cells. The functional relevance of Tet1 in active DNA demethylation and the maintenance of pluripotency has been demonstrated *in vitro*; however, Tet1 mutant mice are viable and fertile. Moreover, ES cells from mutant mice did not show any aberrations in the maintenance of pluripotency [127]. Although the involvement of Tet1-mediated active DNA demethylation in the maintenance of pluripotency is of great interest, further studies are necessary to elucidate the role(s) of Tet1 in pluripotent stem cells.

4. Implication of other epigenetic regulations: chromatin remodelling, high-order structure and non-coding RNA

Adenosine triphosphate (ATP)-dependent chromatin remodelling factors, which are capable of mobilizing or displacing nucleosomes at both the global and the locus-specific level [128–130], regulate gene expression programmes in early development and cell fate decisions [129,131,132].

SWI/SNF (switch/sucrose non-fermentable) complexes trigger the ejection of nucleosomes. The SWI/SNF factor is composed of two complexes Brg/Brahma-associated factors (BAF) and polybromo BAF (PBAF), and contributes to the self-renewal, proliferation or differentiation of ES cells. BRG1, a catalytic subunit of BAF, regulates the self-renewal and pluripotency of ES cells [133]. Knockdown of BRG1 by RNA interference results in morphological changes and a decreased proliferation of ES cells. BRG1 binds to the promoter regions of pluripotency genes such as *Oct3/4*, *Sox2* and *Nanog*. Decreased expression of BRG1 downregulates the expression of pluripotency genes including *Oct3/4*, *Sox2* and *Sall4*, accompanied by upregulation of differentiation-related genes such as *Gata4* and *Gata6*. The involvement of esBAF, an ES cell-specific BAF complex, in self-renewal and pluripotency has also been reported [134–137]. esBAF enhances the binding of *Oct3/4* to the target promoters, and facilitates the reprogramming of fibroblasts [138]. Although the molecular mechanism of esBAF-mediated facilitation of reprogramming has been explained, Brg1, the subunit of esBAF, also facilitates PcG function and represses the expression of classical PcG targets such as Hox genes, the expression of which is essential for the maintenance of pluripotency [139]. The CHD (chromodomain helicase DNA-binding) family complexes, as well as SWI/SNF complexes, trigger the ejection of nucleosomes, and are involved in the cell identity and function of ES cells [140–146]. CHD complexes are composed of the CHD enzymes, methyl-CpG-binding domain 3 (MBD3) and HDACs. The NuRD complex includes CHD3, CHD4 and MBD3, and is responsible for the deacetylation and trimethylation of H3K27. In addition, the NuRD complex is essential for maintaining both pluripotency and developmental transitions in early embryogenesis [140,147,148]. In addition to the function of the complexes, the roles of each component of the complexes in pluripotency have been reported. For example, CHD1 targets *Oct3/4*-binding sites, and is required for efficient reprogramming of fibroblasts to the pluripotent stem-cell state. The silencing of CHD1 expression by RNA interference blocks normal differentiation and the accumulation of heterochromatin [144]. The deletion of HDAC1 results in aberrant differentiation of ES cells, the effects of which include preferential differentiation toward the mesodermal and ectodermal lineages at the expense of endoderm [149], and leads to embryonic lethality [150–153]. ES cells lacking MBD3 express trophoblastic markers and show aberrant differentiation with sustained high expression of *Oct3/4* [140,146]. The restriction of interaction of MBD3 with the SWI/SNF component BRG1 to pluripotent stem cells [137] implies that crosstalk among chromatin remodelling complexes regulates the pluripotency of the cells.

The TIP60/p400 complexes belonging to the INO80 family regulate gene transcription by depositing histone variants H2A.Z into chromatin [130]. Knockdown of

TIP60/p400 expression in ES cells resulted in aberrant morphology and a loss of pluripotency. The expression profile of TIP60/p400-silenced cells was similar to that of *Nanog*-silenced cells [63], suggesting that *Nanog* and TIP60/p400 cooperatively maintain the pluripotency of ES cells. Bp1, a member of the ISWI family proteins, is also involved in early embryonic growth and represses the expression of differentiation markers in ES cells [154].

The organization of high-order chromatin structures has emerged as a key machinery of genome regulation [155–157]. ES cells possess loosely compacted euchromatin. They have an increased level of highly condensed heterochromatin that forms transcriptionally inactive regions during the differentiation process [158,159]. Another study using fully reprogrammed iPS cells with high *Nanog* expression and partially reprogrammed iPS cells that were morphologically similar to ES cells but lacked *Nanog* expression revealed that fully reprogrammed cells with high pluripotency lose the ability to form heterochromatin [21]. Not only loci-specific heterochromatin formation, but also other nuclear features, such as the nuclear lamina nucleolus and nuclear speckles, may affect the chromatin architecture. However, the role of the lamina in pluripotency remains controversial. One report showed morphological differences between pluripotent and differentiated cells [20], whereas another report indicated that B-type lamins are not required for ES cells [160].

RNA occasionally acts as a chromatin regulator. MicroRNAs (miRNAs) regulate the post-transcriptional control of gene expression [161]. The involvement of miRNAs in the maintenance of pluripotency was suggested by the finding that the expression of miRNAs is regulated by the core transcriptional regulatory circuit in ES cells [162]. Studies using mice lacking either *Dicer* or *Dgcr8*, which are required for the maturation of all miRNAs, reveal that they play essential roles in the proliferation and differentiation of ES cells [163–165]. However, although the loss of *Dgcr8* results in a cell cycle defect and aberrant differentiation as a result of inability to silence the self-renewal programme of ES cells, *Dgcr8*-deficient mice still maintain self-renewal in ES cells [163]. This complicated regulation of pluripotency by miRNA is now partially explained by antagonism between miRNA-294, a regulator of the cell cycle in ES cells, and the *let-7* family, which is abundantly expressed in somatic cells, in the stabilization of the self-renewing and differentiation status [166]. miRNA-294 downregulates the *let-7* family through stabilization of the *let-7*-negative regulator LIN28 in the self-renewal state. LIN28 is highly expressed in pluripotent stem cells [167,168] and facilitates the reprogramming of somatic cells in collaboration with other pluripotency genes, such as *Oct3/4*, *Sox2* and *Nanog* [169]. *let-7* downregulates MYC in cancer cells through *let-7*-binding sites on MYC 3'UTR, whereas the overexpression of MYC decreases *let-7* expression [170,171]. These observations reveal a direct double-negative feedback loop, and imply similar capacities of MYC and LIN28 to promote induction of pluripotency. However, further study is required, particularly to characterize the autoregulatory loop between MYC and LIN28 in pluripotent stem cells.

Although previous studies have demonstrated similar expression profiles of mRNA and miRNA in different clones of both ES cells and iPS cells, other studies have shown differential expression of a few transcripts from imprinted regions or pluripotency-related genes. Some iPS

cell clones display aberrant silencing at the *Dlk-Dio3* gene cluster on mouse chromosome 12F1, which is associated with a poor cellular contribution to chimeric mice [172]. However, another report demonstrated contradictory evidence in which the stoichiometry of reprogramming factors, but not the imprinting status at the *Dlk1-Dio3* region, strongly interfered with the quality of iPS cells [160]. Hence, further studies using a number of iPS cells with well-characterized methodology are required to elucidate the molecular features that can predict the quality of pluripotent stem cells.

X chromosome inactivation (XCI) is a regulatory mechanism by which one of the two X chromosomes in female cells is silenced [173,174]. A mouse ES cell or fully reprogrammed iPS cell carries two active chromosomes (XaXa) and the differentiation of these cells initiates XCI, leading to the inactivation of either X chromosome to equalize the expression of X-linked genes between the male (XY) and the female (XX). The reprogramming of female mouse fibroblasts faithfully reactivated the silenced X chromosome [175]. However, a previous study showed that some human ES cells and iPS cells from female cells retained an inactive X chromosome [176]. Human pluripotent stem cells share characteristics with mouse epiblast stem cells (EpiSCs), suggesting that human pluripotent stem cells are in a 'primed' pluripotent state, whereas mouse ES cells and iPS cells are in a 'naïve' pluripotent state [177]. Since diverse mechanisms for the initiation of XCI during development have been found in mammals, such differences may be associated with the observed inconsistencies in X chromosome reactivation between mouse and human pluripotent stem cells [178]. There are recent reports of human iPS cells with ground-state pluripotency, demonstrated by X chromosome reactivation, under certain culture conditions [179,180]. Further studies are required for understanding of the molecular mechanism responsible for the inactivation and reactivation of the X chromosome during the reprogramming process [181].

5. Conclusions and perspectives

Pluripotent stem cells have been used as a cell model for understanding the molecular mechanism of cellular differentiation and a source of cells for regenerative medicine. Studies of stem-cell identity and the fate of pluripotent stem cells upon differentiation have advanced remarkably over the last few decades. Many studies, including a recent genome-wide analysis of epigenetic modifications, support the classical 'landscape model' of Waddington, which describes irreversible cell differentiation. Our growing understanding of epigenetic regulation in pluripotent stem cells and their dynamic changes during differentiation can be used to update this model, which represents not only cell fate but also the coupling of developmental potential with the epigenetic status of the cells during differentiation.

The recent discovery of iPS cells has enabled us to dissect epigenetic regulation during reprogramming and differentiation [1,182]. Reprogramming, the reverse of differentiation, is achieved by breaking the barrier of the differentiated state. Dissection of epigenetic regulation during the reprogramming process may provide a description of how cells sustain their fate and may provide candidates for molecules that act as guardians of differentiation. The identification of guardian molecules that are

responsible for the differentiated state of cells will be of use in the efficient generation of iPS cells. Reprogramming factors, including Oct3/4 and Sox2, are thought to be inducers of pluripotency and may also act as 'destroyers' of the differentiated state. It will be of interest to know how reprogramming factors contribute to the destruction of the epigenetic barrier in the early stage of the reprogramming process, and whether such a mechanism directly regulates the master regulators that maintain the differentiated state, or break the differentiated state through genome-wide alteration of epigenetic status.

Pluripotent stem cells are now suggested as an artificial source of tissues, and consequently it is necessary to be able to guarantee their safety in the human body after transplantation. However, both ES cells and iPS cells are produced after long-term culture, and thus harbour clone-to-clone variations in their epigenetic profiles as well as DNA sequences and copy numbers. Difference in iPS cells among clones have been reported [85], and it is therefore important to validate the quality of pluripotent stem cells including ES and iPS cells by genomic and epigenomic analyses.

Methylome analysis may be a good candidate for evaluating the quality of pluripotent stem cells, since DNA methylation is stable and acts as a source of long-term memory. Some residual DNA methylation signatures observed in iPS cells show characteristics of their somatic tissues of origin, implying the presence of epigenetic memory [85,183]. Considering the recent reports of variation among clones in terms of the characteristics of pluripotent stem cells, it is crucial to establish methods for the reliable evaluation of the quality of iPS cells, which should eventually be useful for generating clinical-grade iPS cells for use in regenerative medicine.

Analysis using deep sequencers has also revealed non-negligible differences among individuals in the genome and epigenome. One of the advantages of using iPS cells as pluripotent stem cells is the ability to analyse and compare established iPS cells with the original somatic cells. In other words, with these cells, it is possible to distinguish whether the observed alterations in the genome/epigenome represent aberrations acquired during long-term cell culture or the individual variation that is normally observed among the individuals.

There is increasing evidence of the importance of epigenetic regulation in maintaining pluripotency and the reprogramming process. Current high-performance sequencers make it possible to screen for genomic alterations at the whole-genome level, and can be used to guarantee that the cells are of clinical grade, on the basis of their genomic sequence. It is also important to examine the epigenetic profile of pluripotent stem cells, because the epigenetic landscape represents both the past and the current developmental state, and may be a useful indicator to predict their future potential. Further advances in the understanding of epigenetic regulation hold promise for the molecular understanding of cell fate and the realization of regenerative medicine using pluripotent stem cells.

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