Epigenetic mechanisms of induced pluripotency

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Reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) requires profound alterations in the epigenetic landscape. During reprogramming, a change in chromatin structure resets the gene expression and stabilises self-renewal. Reprogramming is a highly inefficient process, in part due to multiple epigenetic barriers. Although many epigenetic factors have already been shown to affect self-renewal and pluripotency in embryonic stem cells (ESCs), only a few of them have been examined in the context of dedifferentiation. In order to improve current protocols of iPSCs generation, it is essential to identify epigenetic drivers and blockages of somatic cell reprogramming.

Key words: induced pluripotent stem cells, epigenetics, chromatin modifications, DNA methylation.

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Introduction

Reprogramming somatic cells to induced pluripotent stem cells (iPSCs) that can differentiate into any cell type, similarly to embryonic stem cells (ESCs), paved new avenues for regenerative medicine, disease modelling, and drug screening [1]. The process might be induced with nuclear transfer [2, 3], cell fusion [4], or forced expression of transcription factors (i.e. OSKM: Oct4, Sox2, Klf4, c-Myc [5], or other combinations [6]). All of these methods initiate a cascade of transcriptional and epigenetic changes that direct cells toward pluripotency [7, 8].

Epigenetic machinery combines multiple networks of proteins that are interconnected through functional and physical interactions to write, read, and edit chromatin information, thus allowing dynamic regulation of chromatin status and gene expression. Adult somatic cells are characterised by a stable chromatin environment, responsible for silencing genes that are not specific for a particular cell type. Compared to lineage-committed cells, pluripotent stem cells possess a unique epigenetic profile enriched for open, active chromatin modifications, including H3K4me3, H3K36me3, histone acetylation, and hypomethylated DNA (Fig. 1). These marks are frequently found within the regions of pluripotency genes. In contrast, tightly composed heterochromatin is marked with H3K27me3, H3K9me3, and hypermethylated DNA and localises to multiple tissue-specific genes and repetitive elements. Besides, pluripotent stem cells contain increased levels of bivalent domains marked both with H3K4me3 and H3K27me3 at differentiation-related genes. The genes marked with bivalent domains are in a poised state, which means that their expression can be quickly turned on or stably silenced via erasure of H3K27me3 or H3K4me3, respectively. Disturbance of such a delicate balance can result in reduced self-renewal, enhanced differentiation of pluripotent cells, and/or impeded reprogramming of somatic cells to iPSCs [8–11] (Table 1). Hereby, we aim to review current knowledge and understanding of the epigenetic profile, mechanisms, and modifiers that drive or block somatic cell reprogramming.

DNA methylation

DNA methylation profile in induced pluripotent cells

The content of DNA methylation changes dramatically during gametogenesis and cellular differentiation, with near complete erasure in progenitor germ cells and waves of re-methylation during germ cell maturation and fertilisation [12]. Early observations, most based on 5-methylcytosine by immunostaining, indicated that the sperm's genome undergoes dynamic demethylation in the zygote (which occurs without new DNA synthesis) [13], while the embryo's genome loses DNA methylation passively along cellu-

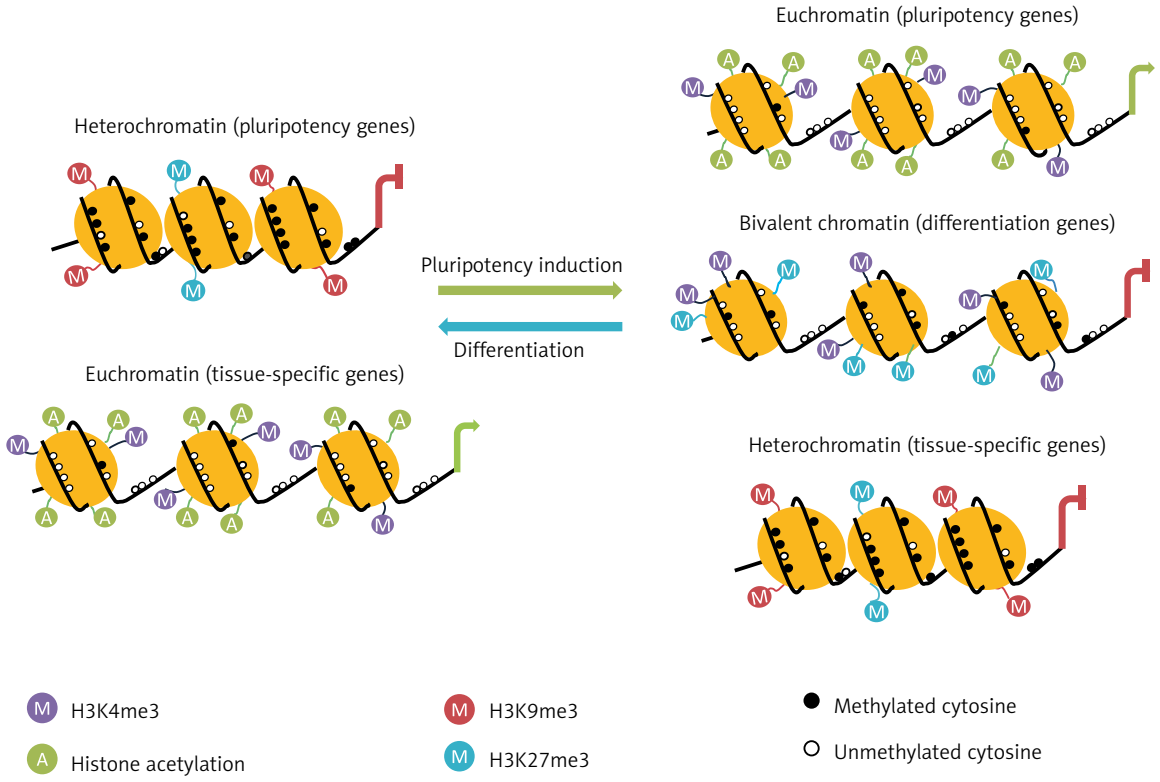

Fig. 1. Schematic representation of the chromatin rearrangements occurring during somatic cell reprogramming and differentiation of pluripotent stem cells

lar divisions [14]. Refinements of these observations have been provided by single-base resolution assays to measure 5-methylcytosine [15, 16], but the core information that DNA methylation is dynamically changed during fertilisation and development remains true. The importance of DNA methylation during development was also reinforced by several studies showing that mice lacking any of the DNA methyltransferases (Dnmt1, Dnmt3a, and Dnmt3b) are not viable or die within a few weeks [17–20]. These observations are evidence that epigenetic events are key to cellular differentiation, and it is logical to extrapolate that these same events have to be reversed during induced reprogramming. Indeed, resetting of the DNA methylation profile appears to be an important part of dedifferentiation. Treatment of somatic cells with agents that block the activity of DNA methyltransferases (e.g. 5-aza-cytidine) contributes to the increased efficiency of iPSC generation [21, 22]. What is more, it facilitates the transition of partially reprogrammed cells into a full state of pluripotency [22].

In contrast, somatic cells show stable DNA methylation. Once the tissue-specific patterns of DNA methylation are established, they will remain virtually unmodified throughout life, with the exception of cellular transformation by carcinogenesis. The paradigm that differentiation is a one-way process was challenged with the description of reprogramming by somatic cell nuclear transfer, initially described using frog cells [2] and later in mammals [3]. A revolution in the field of cellular reprogramming occurred with the description of induced pluripotent (iPS) stem cells [5]. The initial report of iPS cells identified four genes that, when re-expressed in differentiated mouse cells, were sufficient to induce dedifferentiation: Oct4, Sox2, Klf4, and cMyc. The process was shown to be reproducible in human cells using the same four factors [23], and combinations of alternative genes were also described (for example, the use of Nanog and Lin28 in place of Klf4 and Myc) [6].

Oct4 is highly expressed in embryonic stem cells, and it shows decreased expression with differentiation and concomitant increase in DNA methylation of its promoter region. A fundamental characteristic of iPS cells is the re-activation of the endogenous Oct4 gene and other pluripotency factors (like Nanog). This is accompanied by demethylation of their gene promoters, as shown in the original iPSCs [5]. Further research extended the DNA methylation profiling of iPSCs genome-wide, and iPSCs globally present diverse similarities to ESCs: the genome of iPSCs is methylated to a higher extent than the genome of somatic cells; non-CG methylation (which is basically inexistent in somatic cells but abundant in ESCs) is found in iPSCs, and somatic unmethylated genes that are methylated in ESCs revert to a methylated state (and also the reverse situation, i.e. somatic hypermethylated genes can be reprogrammed to an unmethylated state) [24, 25].

Among the differences, several groups have described DMRs (differentially methylated regions) that are unique to iPSCs compared to ESCs. Using MethylC-Seq to evaluate DNA methylation at near whole genome scale, Lister *et al*. [24] identified over a thousand DMRs in the CG context (CG-DMRs), comprising in total 1.68 Mb and ranging in length from 1 kb to 11 kb. They also evaluated DMRs in the non-CG context and found that these are less numerous (29) but much greater in length (typically spanning

Table 1. Epigenetic modifiers and their role in somatic cell reprogramming and biology of pluripotent stem cells

Epigenetic modifier	Reprogramming to iPSCs	ESC biology
esBaf (Swi/Snf complex)	EsBaf overexpression increases reprogramming [70]	EsBaf maintains pluripotency [90, 91]
Chd1	Chd1 depletion hinders reprogramming [73]	Chd1 depletion augments heterochromatisation, reduces self-renewal properties and leads to improper differentiation [73]
Mbd3 (NuRD complex)	Conflicting data: Mbd3 depletion improves reprogramming [82], even to 100% efficiency [81], but is indispensable for iPS generation [83]	Mbd3 depletion up- [92] and downregulates certain pluripotency genes and impedes differentiation capacity [93]
Mbd2 (NuRD complex)	Mbd2 silencing increases reprogramming due to derepression of Nanog [79], overexpression of isoform Mbd2c increases reprogramming [80]	Overexpression of isoform Mbd2a leads to differentiation [80]
Hdac (NuRD complex)	Hdac inhibitors increase reprogramming [21, 75-78]	Hdac1 deletion induces meso- and ectodermal differentiation [94]
Ino family	Ino80 depletion decreases reprogramming efficiency [72]	Tip60-p400 silencing impairs self-renewal and differentiation [95]
Wdr5 (H3K4 methyltransferase Set/Mll complex)	Wdr5 knockdown decreases reprogramming [55]	High Wdr5 expression correlates with pluripotency; loss of Wdr5 induces differentiation [55]
Kdm5b (H3K4me demethylase)	Kdm5b knockdown enhances reprogramming [56]	Highly expressed in ES [96], Kdm5b silencing evokes differentiation [97]
Jhdm1b (H3K36me demethylase)	Jhdm1b overexpression enhances reprogramming [57, 58]	Highly expressed in ES [57]
Ezh2, Eed, Suz12 (H3K27 methyltransferase complex PRC2)	Ezh2 overexpression enhances [60, 62], while Ezh2, Eed, Suz12 silencing reduces reprogramming efficiency [59]	Eed depletion results in loss of pluripotency [98], ESCs lacking Eed, Suz12 are unable to reprogram B cell to pluripotency [61]
Utx (H3K27me2/3 demethylase)	Utx knockdown impairs iPS formation [64]	Utx knockout does not influence pluripotency [64]
Ehmt2/G9a (H3K9 methyltransferase)	Ehmt2/G9a repression increases [21, 68] or moderately decreases [59] reprogramming	Loss of Ehmt2/G9a impedes differentiation [99, 100]
Setdb1 (H3K9 methyltransferase)	Setdb1 inhibition increases [65] or moderately decreases [59] reprogramming	SetDB1 directs retroviral silencing in ESCs [101, 102], its repression induces differentiation, especially into the trophoectoderm lineage [103-106]
Suv39H1/2 (H3K9 methyltransferase)	Suv39H1/2 downregulation increases reprogramming [59, 67]	Suv39H represses LINE and ERV retroelements [107]
H3K9 demethylases	Loss of Kdm3a, Kdm3b, Kdm4b, Kdm4c decreases reprogramming [65, 69]; Kdm4b overexpression promotes conversion from pre-iPSCs to iPSCs [65]	Loss of Kdm3a, Kdm4b, Kdm4c evokes loss of self-renewal and differentiation [69, 108]
Dot1l (H3K79me demethylase)	Dot1l silencing facilitates reprogramming [59]	Dot1l deficiency impairs differentiation due to cell proliferation defects [109, 110]

megabases). The majority of both types of DMRs were typically hypomethylated in iPS compared to ES cells. Another significant difference is that key lineage-specific methylated genes appear to be resistant to resetting, at least in low-passage iPS cells [26]. Differences in DNA methylation appear to be attenuated by continued passaging of iPS cells [27].

Genomic imprinting

Genomic imprinting is a phenomenon resulting from epigenetic marking of an allele depending on its parental origin, which consequently leads to gene expression from either maternal or paternal locus. Many imprinted genes function as growth regulators, so adequate imprinting is crucial for development, especially at its early stages. Imprinting marks are erased in primordial germ cells, re-established in the maturing gametes, and maintained during global DNA demethylation post-fertilization and thereafter [28]. During reprogramming, the cells also undergo DNA demethylation, however, iPSCs generally retain proper imprinting [29]. Nevertheless, a few reports demonstrated that a small percentage of iPS colonies may show variable loss of imprinting, leading either to hyper- or hypomethylation of imprinting control regions and subsequent change in gene expression [30–32]. The most apparent exception to this schema is represented by the Dlk1-Dio3 region, which is frequently hypermethylated and therefore repressed in iPSCs [32–34]. Remarkably, the iPSCs with downregulated Dlk1-Dio3 cluster have reduced ability to fully contribute to chimeras [33–35]. Such a phenomenon might be prevented with ascorbic acid, which was recently shown to inhibit the silencing of genes within the Dlk1-

Dio3 region during reprogramming by interfering with *de novo* DNA methyltransferase, Dnmt3a [36].

X-inactivation

An important feature in determining the state of pluripotency is the reactivation of the inactive X chromosome in female cells. This phenomenon occurs relatively late during reprogramming, reflecting the activation of endogenous transcription factors (i.e. Nanog and Oct4) [37]. Xist (X-inactive specific transcript) large, non-coding RNA molecule is a regulating agent of cis-repression responsible for the X-inactivation. This process involves accumulation of inactive chromatin marker, H3K27me3. In mouse iPSCs reactivation of X chromosome is complete and the expression of Xist is not observed. It was proposed that transcription factors Oct4, Sox2, and Nanog could bind to a coding region of Xist and downregulate its expression [38]. However, the process in human cells is more complicated. Female hESCs are characterised by a large variety of epigenetic status of X chromosome. The same phenomenon was observed in the case of female hiPSCs. There are conflicting results showing that in some cases X-chromosome inactivation is still present in iPSCs [39, 40], while other researchers have demonstrated full X-reactivation [41, 42]. The reason for this event seems to be the length of iPS colony culture. At first the X chromosome remains inactive, but it becomes activated upon the subsequent passages [43, 44].

DNA demethylation mechanisms

Global pattern of DNA methylation is erased during early development [12]. Passive demethylation occurs during DNA replication, whereas active demethylation involves oxidative activity of Tet enzymes: Tet1/2/3, which are responsible for hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). The hydroxylated form of 5mC might be further converted to unmethylated cytosine through subsequent cell divisions or base-excision repair mechanisms [45]. This leads to the generation of an open chromatin environment that allows an access of transcription factors (such as Nanog and Esrrb) to the promoters of genes involved in pluripotency [46, 47]. Catalytic activity of Tet1/2 in cooperation with Nanog enhances reprogramming efficiency [48]. It was proposed that Tet1 is recruited by Nanog to pluripotency-associated genes and facilitates their expression by increasing 5hmC levels [48]. In concordance with this notion, the level of 5hmC and Tet1 activity was shown to be upregulated during the course of dedifferentiation [47, 49]. Moreover, recent reports indicate that loss of the proteins involved in oxidative demethylation abrogates mesenchymal-to-epithelial transition, an event occurring at the initial stages of reprogramming [50]. It seems that erasing DNA methylation promotes expression of pluripotency-related genes that were silenced through hypermethylation in somatic cells.

Chromatin modifications

Euchromatization occurring during generation of iPSCs involves multiple factors that affect higher chromatin structure, nucleosome composition, and location, as well as post-translational histone modifications (Fig. 2) [10]. Acetylation of histones decreases their interaction with DNA, whereas histone methylation creates patterns that might affect the binding or activity of chromatin rearranging complexes. Proteins that are involved in histone modifications interact with each other and with the DNA methylation machinery creating a complex regulatory network [51].

Active histone modifications

H3K4 methylation

The initiation phase of reprogramming evokes changes in the expression profile of multiple genes. Interestingly, both up- and downregulation of expression affects the genes, whose promoters in differentiated cells are associated with a permissive chromatin environment marked mainly by H3K4me3 [52]. This indicates that initiation of reprogramming is tightly linked with an open chromatin state. Another marker of active chromatin, H3K4me2, surrounds both gene promoters and enhancers. Its enrichment does not correlate with immediate upregulation of gene expression during the first phase of reprogramming. Instead, H3K4me2 marks developmental and pluripotency-related genes, which become transcriptionally active at the latter stage of reprogramming [52]. Trimethylation of H3K4, which is generally correlated with gene activation, is catalysed by Trithorax group (TrxG) protein complex [53]. In mammalian cells histone methyltransferase complex Set/Mll, a homologue of TrxG, is responsible for methylation of H3K4. To be catalytically active, Set/Mll requires core subunits such as Wdr5 [54]. It has been shown that Wdr5 becomes overexpressed during iPSCs generation [55]. IPSCs generation is impaired after Wdr5 knockdown, and this effect is most apparent during the initiation phase of reprogramming [55]. This is consistent with the findings that expression of Kdm5b is a barrier in the iPS colony formation: Kdm5b belongs to jumonji C-containing protein complexes, a family of demethylases that triggers demethylation of active histone mark H3K4me1/2/3. Erasing H3K4 methylation impairs reprogramming, whereas knockdown of Kdm5b enhances efficiency of iPSC generation [56].

H3K36 and H3K79 methylation

H3K36me2/3 modification is present across gene bodies that are actively transcribed. Interestingly, global reduction of this mark enhances reprogramming [57, 58]. Wang *et al*. demonstrated that overexpression of a H3K36me2 demethylase, Jhdm1b (Kdm2b), is sufficient to promote iPS colony formation by Oct4 alone [58]. What is more, it was shown that Kdm2b binds promoters of genes that are activated early during reprogramming, including epithelial and pluripotency genes [57]. Another mark of open chromatin structure, methylation of histone H3 at lysine 79 (H3K79me), positively associates with transcriptionally active genes and negatively associates with genes marked by H3K27me3. As described by Onder *et al*., inhibition of Dot1l – H3K79 methyltransferase facilitates more efficient loss of H3K79me2 from somatic genes and increases reprogramming potential [59].

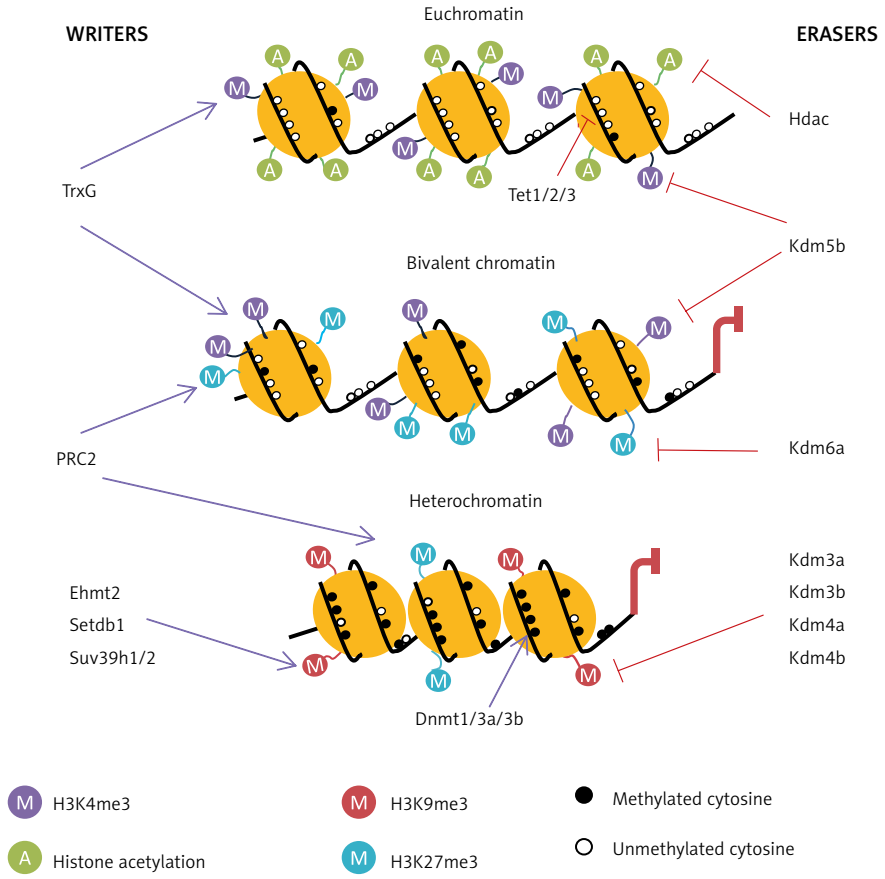

Fig. 2. Epigenetic modifiers responsible for chromatin reorganisation that play a role in reprogramming as drivers or blockages

Repressive histone modifications

H3K27 methylation

Downregulation of somatic genes at the early stage of reprogramming is mediated by PRC2 (Polycomb Repressive Complex 2) that deposits a trimethylation mark at K27 of H3 (H3K27me3). H3K27me3 is associated either with gene silencing or, when co-localised with H3K4me3, with bivalent domains. Recent studies revealed that PRC2 components (Ezh2, Eed, and Suz12) are critical to iPSCs generation [59]. It has been shown that loss of these factors reduces reprogramming efficiency [59, 60]. Moreover, ESCs lacking either PRC1 or PRC2 components lose the ability to reprogram B cells to iPSCs upon cell fusion [61]. In another study, Ding *et al*. observed that Ezh2 expression is induced during the course of reprogramming and is retained at high levels in iPSCs. Consistently with this notion, exogenous overexpression of Ezh2 enhanced reprogramming, while its suppression impaired transition to iPSCs [62]. In contrast, Fragola *et al*. showed that loss of Ezh2 had little effect on iPSCs generation, despite global decrease in H3K27me3 level, due to selective, partial retention of this mark on target genes [63]. Nevertheless, erasing H3K27me3 seems to be an important part of epigenetic reprogramming. For example, the H3K27me2/3 demethylase Utx (Kdm6a) was shown to be essential for iPSCs generation, although its influence on ES cell maintenance of pluripotency or lineage commitment was marginal [64].

H3K9 methylation

H3K9 trimethylation canonical function is the promotion of long-range heterochromatisation, and thus, transcriptional silencing. It has been shown in multiple reports that H3K9 methylation machinery acts as a key epigenetic blockade to reprogramming [65–68]. Repression of H3K9 methyltransferases (Ehmt2, Setdb1, Suv39h1/2) results in increased reprogramming efficiency, which is accompanied by decondensation of chromatin [67, 68]. It has been shown, for example, that transient silencing of Suv39h1/2 promotes reprogramming at its early stages due to decreased level of H3K9me3 within the binding sites of OSKM factors. Thus, loss of H3K9me3 permits an access of OSKM factors to their target regions, including those responsible for pluripotency-related signalling [67]. Consistently with these observations, knockdown of a number of H3K9 demethylases (Kdm3a, Kdm3b, Kdm4a, Kdm4b) blocks reprogramming [65, 69], while exogenous overexpression of Kdm4b induces conversion of pre-iPSCs to iPSCs [65].

ATP-dependent chromatin remodelling complexes

ATP-dependent chromatin-modifying complexes (such as: Swi/Snf, Chd, Ino80, Iswi) induce structural transitions between various chromatin conformations by evicting, re-positioning, or altering the composition of nucleosomes

[51]. Overexpression of the ESC-specific component of Swi/Snf complex, esBaf, assists reprogramming by facilitating Oct4 binding to its target sites [70]. Also, high levels of Baf subunits (Baf155, Brg1) correlate with higher reprogramming efficiency in liver progenitor cells compared to differentiated hepatocytes, while their knockout impedes reprogramming of liver progenitors [71]. Silencing of another chromatin remodeller, Ino80, decreases dedifferentiation due to reduced recruitment of Oct4 and Wdr5 to promoters of pluripotency genes [72].

More attention has been given to the Chd family ATPases, especially to the components of NuRD complex. Chd1 ATPase is necessary to maintain open chromatin structure in stem cells. Its depletion leads to increased heterochromatisation, decreased self-renewal properties, and reduced iPSCs formation [73]. NuRD functions in cooperation with other factors (PRC2, Lsd1, Oct4, Stat3, p300, and esBaf) to balance pluripotency gene expression and to maintain developmental genes in poised or silent state. NuRD complex contains Mi-2α/β ATPases, Hdac1/2 (histone deacetylases) and Mbd2/3 (methyl-CpG binding domain) proteins that drive chromatin condensation [74]. Chemical inhibition of Hdac boosts iPS colony formation through euchromatization due to global histone acetylation [21, 75–78]. It remains to be elucidated, however, whether other proteins that remain acetylated upon HDACi treatment also support somatic cell reprogramming. Recruitment of NuRD complex to methylated loci is mediated by Mbd2. Mbd2 silencing by overexpressed miR302 cluster enhances iPS generation through reactivation of Nanog [79]. Interestingly, Mbd2 effect on dedifferentiation seems to depend on its splicing isoform because Mbd2c (which does not interact with NuRD) increases reprogramming efficiency [80].

Interesting but conflicting data have come from studies on Mbd3 factor. Rais *et al*. demonstrated that Mbd3/NuRD depletion renders reprogramming deterministic, because 100% of the cells become pluripotent [81]. In another study Mbd3 silencing promoted chromatin decondensation at pluripotency genes, thus increasing iPSCs formation efficiency, but not to a deterministic level [82]. In contrast to these reports, it has recently been shown that NuRD complex is indispensable for efficient reprogramming to naïve pluripotent stem cells [83]. These data suggest that the effect of Mbd3 on reprogramming is dependent on cellular context and timing of Mbd3 knockdown.

Histone variants

The composition of nucleosomes affects their stability, susceptibility to histone modifications, and thus chromatin structure [11]. Histone variants are expressed in a cell type-specific manner and their arrangement influences gene expression. For example, somatic reprogramming is enhanced by two histone variants: TH2A and TH2B, which are characteristic for oocytes, testis, and zygotes [84]. In contrast, macroH2A2 and macroH2A1 block reprogramming due to the co-occupancy with H3K27me3 at the pluripotency and developmental genes [85–88]. These observations remain in agreement with previous studies demonstrating that upon somatic cell nuclear transfer to oocytes, macroH2A is rapidly removed from the nucleus [89].

Conclusions

The role of the proteins involved in the regulation of chromatin status in reprogramming is currently being intensively investigated. It needs to be emphasised, however, that the majority of data reported so far have utilised mouse models. Similar mechanisms should also be tested and validated in human cells. What is more, several studies on epigenetic modifiers provide conflicting data, and these discrepancies need to be addressed. Untangling the interconnectivity of the chromatin remodellers during reprogramming will greatly enhance our understanding of the mechanisms involved in this complex process. It will also allow conscious, targeted usage of specific chemical compounds to boost reprogramming or even substitute OSKM cocktail. Many processes accompanying somatic cell reprogramming to iPSCs occur also during carcinogenic reprogramming. Thus, the knowledge stemming from research on the epigenetic mechanisms in reprogramming might also improve our understanding of cancer biology.

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