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Epigenetics: the guardian of pluripotency and differentiation

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Abstract: Multicellular organisms comprise a variety of cell types that have the same genotype but distinct phenotypes. This diversity is possible because of the establishment of a specific epigenetic landscape that stabilizes gene expression profiles that are exclusive for a particular cell type. Accumulating data indicate that the maintenance, loss, and reacquisition of pluripotency are dynamically regulated by epigenetic alterations evoked by a subset of cellular factors. A better understanding of the epigenetic mechanisms involved in stem cell biology and differentiation will improve our ability to use these cells in the clinical context. Here we review current insights into the epigenetic mechanisms implicated in embryonic development and the induction of pluripotency.

Key words: Epigenetics, chromatin modifications, DNA methylation, noncoding RNAs, pluripotent stem cells, differentiation, reprogramming

1. Introduction

Organisms at the multicellular level are composed of various cell types. Although they harbor similar genomes, the cells differ dramatically in terms of their morphology, physiology, and functions. During development, initially totipotent cells proliferate intensively, acquire more specialized phenotypes, and progressively lose their differentiation capacity. These changes are possible due to profound alterations in their gene expression profiles that are evoked and stabilized mainly through epigenetic means. Multifaceted epigenetic regulation comprises complex, interconnected protein networks that affect DNA methylation, posttranslational histone modifications, histone variant composition within nucleosomes, higher-order chromatin arrangement, and noncoding RNA expression (Li et al., 2012; Apostolou and Hochedlinger, 2013). Deregulation of the epigenetic machinery may lead to the loss of self-renewal capacity and abnormal differentiation, which ultimately may induce developmental aberrancies.

Pluripotency is a transient state in vivo. Thus, many of the reported studies in this field utilized cultured in vitro embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts. Cell lines that are obtained in this manner maintain their potential to differentiate into the three germ layers and proliferate virtually indefinitely. Recently, converting somatic cells into so-called induced pluripotent stem cells (iPSCs) was shown to be possible through the forced expression of pluripotency genes (Takahashi and Yamanaka, 2006) or chemical induction (Hou et al., 2013). This conversion is driven by a profound epigenetic reprogramming that enables the reacquisition of the chromatin state characteristic of ESCs (Maherali et al., 2007; Mikkelsen et al., 2008; Mattout et al., 2011).

Due to their multilineage differentiation potential, pluripotent stem cells constitute an attractive source of biological material with wide applicability in regenerative medicine, disease modeling, and drug cytotoxicity testing. Thus, a better understanding of the epigenetic regulation of pluripotency is essential to improve our capacity to control and manipulate these cells and their reprogramming procedures in a clinical setting. Many epigenetic modifiers and mechanisms have been shown to play critical roles in the maintenance and reacquisition of pluripotency. Here we focus on the current state of knowledge related to the epigenetic processes implicated in stem cell biology.

2. An overview on the epigenetic mechanisms in the regulation of pluripotency

Pluripotent stem cells possess a distinctive permissive chromatin structure that facilitates the dynamic transition into more differentiated cell types. In the pluripotent

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state, the chromatin maintains a homogeneously dispersed structure with a high contribution of loose euchromatin and low dosage of heterochromatin. Upon lineage commitment, this ratio changes as the chromatin acquires inactivating markers and becomes heterogeneous with distinctive condensed loci (Ahmed et al., 2010). Open chromatin architecture facilitates in pluripotent stem cells transcriptional hyperactivity, allowing for the high abundance of the genes involved in transcription and epigenetic remodeling, and also in the activation of noncoding transcripts and retroelements (Efroni et al., 2008). This feature contributes to the plasticity of the cells to undergo a differentiation program towards any of the three germ layers.

An open chromatin structure is maintained by chromatin remodelers that actively relax compacted regions and/or affect nucleosome composition, structure, and posttranslational modification of histones. Modifications of the histone residues may alter their physical contact with DNA or with epigenetic modifiers that remodel the chromatin within a given region. In pluripotent cells, histones are frequently acetylated, which removes their negative charge and limits interaction with DNA. In addition, methylation at lysines 4, 36, and 79 of histone 3 facilitate loose chromatin conformation and allow gene transcription: H3K4me3 is enriched at the active gene promoters, while H3K36me3 and HK79me3 are enriched at the gene bodies (Gaspar-Maia et al., 2011; Liang and Zhang, 2013). In contrast, repressed genes are marked with H3K27me3. In pluripotent stem cells, certain differentiation-related genes are localized into bivalent domains containing both active (H3K4me3) and repressive (H3K27me3) markers. A high level of bivalent regions, which are transcriptionally inactive, is characteristic to pluripotency. This so-called poised regions may be switched either to an active or a repressed state through the removal of one of the markers, permitting a fast response to developmental cues (Bernstein et al., 2006; Mikkelsen et al., 2007; Adamo et al., 2011). Another repressive marker, H3K9me3, is enriched in stem cells at the infrequent heterochromatin regions within repetitive and imprinted loci. Furthermore, promoters and enhancers associated with pluripotency have a low DNA methylation level that permits transcriptional activation, while repressive DNA hypermethylation is found alongside H3K9me3 within repetitive and imprinted regions (Mikkelsen et al., 2007; Mikkelsen et al., 2008; Polo et al., 2012).

Changes in the cellular potency are associated with the massive turnover of chromatin modifiers followed by alterations in the epigenetic signature of the cell. Differentiation increases H3K27me3 and H3K9me3 deposition to suppress the pluripotency genes and the genes specific to other tissue types (Mikkelsen et al., 2007). Moreover, differentiation is accompanied by change in DNA methylation profile, whereby pluripotency-associated promoters and enhancers become hypermethylated to ensure stable silencing of their expression (Mohn et al., 2008; Polo et al., 2012). The process involves multiple epigenetic modifiers that mediate DNA methylation changes, histone deacetylation, and trimethylation of H3K27 and H3K9. Lack of those modifiers may lead to sustained pluripotency and impaired differentiation. In contrast, somatic cell reprogramming to iPSCs requires reacquisition of the open chromatin conformation and reestablishment of the pluripotency-specific DNA methylation pattern. Thus, the epigenetic modifiers that drive euchromatization facilitate dedifferentiation, while the heterochromatin factors block the process (Liang and Zhang, 2013). Although the general mechanisms maintaining pluripotency and driving reprogramming and differentiation are relatively well understood, the exact kinetics of the changes and the set of the epigenetic players responsible for these phenomena remain largely unexplored. Improving the understanding of the epigenetic enzymes and substrates, as well as the timing of required alterations, will greatly contribute to our knowledge about these processes. This knowledge is urgently needed to improve culture, reprogramming, and differentiation protocols that will allow precise control and manipulation of various cell types required for regenerative medicine and establishment of in vitro disease models. In this review, we provide a summary of the current understanding of the epigenetic factors involved in reprogramming, stem cell maintenance, and differentiation.

3. Higher chromatin architecture

3.1. Three-dimensional chromatin arrangement

The global chromatin arrangement plays an important role in transcriptional regulation by providing 3D interactions between gene promoters and other regulatory sites (e.g., enhancers) (Kagey et al., 2010). Pluripotent stem cells have a unique three-dimensional chromatin structure. Electron microscopy imaging has shown that their chromatin possesses a homogeneously dispersed architecture, with long ranges of euchromatin and a low amount of condensed foci at the nuclear envelope regions. This spatial arrangement is established by the eight-cell stage of embryonic development, whereas highly condensed chromatin domains reappear upon lineage commitment (Ahmed et al., 2010). Consistently, active chromatin modifications are frequent, while repressive marks are less common in ESCs compared with the more specialized cell types (Hawkins et al., 2010; Mattout et al., 2011).

It was shown that pluripotency-specific genes are involved in the maintenance of diffused chromatin structure. For example, deletion of Oct4 resulted in

chromatin compaction in ESCs (Ahmed et al., 2010). Additional studies indicated that the core pluripotency factors (Oct4, Nanog, and Sox2) create an interacting network between their binding sites, thereby affecting chromatin folding in ESCs (de Wit et al., 2013). The expression of pluripotency factors also relies on adequate chromatin organization. Klf4 was shown to recruit looping factors, cohesins, to the Oct4 distal enhancer, thereby promoting the expression of endogenous Oct4 during reprogramming to iPSCs (Wei et al., 2013). Similarly, DNA fragments interacting with the Nanog promoter in ESCs and iPSCs are frequently bound by DNA looping factors and genes from the pluripotency network (Esrrb, Klf4, and Sox2), thereby enhancing Nanog expression (Apostolou et al., 2013).

3.2. Nucleosome composition

The local chromatin structure is largely dependent on the nucleosome composition, density, and positioning. Histone variants assembled within nucleosomes differ in their susceptibility to posttranslational modifications and in the turnover rate that influences nucleosome stability (Skene and Henikoff, 2013). The dynamic exchange of histones is important for the maintenance of stem cell identity. Knockout of the nucleosome assembly factor HirA results in accelerated differentiation, probably due to the accumulation of unbound histones H3 and H3.3. In contrast, increased binding of mutated H1 to DNA arrests the differentiation potential of ESCs (Meshorer et al., 2006).

The expression of histone variants is specific to a particular cell type. Recent reports provided evidence that different histone isoforms play a major role in the acquisition and maintenance of pluripotency. Variants expressed at high levels in oocytes, testes, and zygotes (TH2A and TH2B) augment reprogramming of somatic cells into iPSCs (Shinagawa et al., 2014). Conversely, the isoform macro-H2A, elevated upon differentiation, constitutes a barrier to reprogramming and needs to be removed from the nucleus of oocytes upon somatic cell nuclear transfer (Pasque et al., 2012). In differentiating cells, this isoform was shown to contribute to the H3K27me3-mediated silencing of the genes implicated in pluripotency and development (Barrero et al., 2013). Moreover, knockout of macro-H2A enhances OSKM-driven reprogramming to iPSCs. Another isoform (H2A.Z) implicated in nucleosome plasticity becomes elevated in pluripotent stem cells (Gaspar-Maia et al., 2013). In ESCs, H2A.Z localizes with the H3K27me3 modification not only to the promoters and distal elements, as observed in somatic cells, but also to the intergenic regions. This broad deposition suggests that H2A.Z may promote nucleosome exchange in pluripotent cell, thereby rendering the chromatin refractory to H3K27me3-mediated condensation (Zhu et al., 2013). Additionally, recent studies have shown that the isoform H3.3, which is incorporated into both active and repressed chromatin in ESCs, localizes to class I and II endogenous retroviruses together with H3K9me3 and TRIM28 to participate in the silencing of these parasitic sequences (Elsasser et al., 2015). This observation suggests that in addition to their nucleosome remodeling function, histone variants may have more subtle and targeted roles in pluripotent stem cells.

3.3. ATP-dependent chromatin modifiers

In addition to histone variants, ATP-dependent chromatin modifiers are involved in the local chromatin arrangement. These large remodeling protein complexes chaperone histone variant exchange and catalyze the removal or repositioning of nucleosomes (Swygert and Peterson, 2014). In pluripotent stem cells, the SWI/SNF complex (comprising Brg1, Baf155, and Brm proteins) is responsible for chromatin decondensation through interaction with Oct4 (Singhal et al., 2010). For this reason, high expression of the Baf subunits (esBaf, Baf155, and Brg1) improves the efficiency of iPSC generation (Singhal et al., 2010; Kleger et al., 2012). Another chromatin modifying factor (Ino80) colocalizes with the core pluripotency genes (Oct4, Nanog, and Sox2) and maintains an open chromatin structure to facilitate the upregulation of the pluripotency network. Loss of Ino80 induces differentiation of ESCs, hinders reprogramming, and impairs blastocyst development (Wang et al., 2014). Additionally, Tip60-p400 from the Ino80 family, which is responsible for histone acetylation, was demonstrated to guard the self-renewal properties of ESCs (Fazzio et al., 2008).

The Chd factors are other chromatin remodeling proteins involved in stem cell functions. Chd1 is essential for the maintenance of an open chromatin structure in ESCs. Its depletion results in heterochromatization and differentiation towards neural lineages. Moreover, Chd1 knockdown impairs the efficiency of iPSC colony formation (Gaspar-Maia et al., 2009). Two other members of the Chd family (Chd3/Mi2α and Chd4/Mi2β) are components of the NuRD complex. This complex contains histone deacetylases: HDAC1 and 2 and the methyl-CpG binding domain proteins (Mbd2/3), which together drive the repression of a target locus. Through interaction with other chromatin remodeling proteins (e.g., Lsd1, esBaf, Prc2, Oct4, and Tet1) (Yildirim et al., 2011; Latos et al., 2012; Reynolds et al., 2012; Whyte et al., 2012), NuRD seems to play a fine-tuning role between pluripotency and differentiation. It has been proposed that the repressive NuRD complex associates with the activating esBaf at the promoters of a number of pluripotency genes to antagonistically regulate their expression (Yildirim et al., 2011), thus mediating lineage commitment (Reynolds et al., 2012). This effect is additionally strengthened by

the NuRD-interacting protein Lsd1, which facilitates the demethylation of H3K4me3 at pluripotency-specific enhancers (Whyte et al., 2012). This result is in accordance with the observations published by Kaji et al. (2006), who demonstrated that ESCs devoid of Mbd3/NuRD exhibited impaired differentiation potential and sustained self-renewal properties independent of LIF signaling. In contrast, the inhibition of the NuRD proteins (Hdac and Mbd2/3) via small molecules or RNAi facilitates the reacquisition of an open chromatin architecture during reprogramming, thereby improving the efficiency of iPSC generation (Huangfu et al., 2008; Hou et al., 2013; Lee et al., 2013; Luo et al., 2013) even to 100%, as was shown in Mbd3 depletion studies (Rais et al., 2013). However, loss of the Mbd3/NuRD complex was also shown to impair the generation of naïve pluripotent stem cells (dos Santos et al., 2014), which indicates that Mbd3 function may vary depending on the cellular context and developmental stage.

3.4. Histone modification

Posttranslational histone modifications affect physical interactions between histones and DNA or other chromatin factors (Figure 1). The transition between a permissive and a repressive chromatin environment is stringently regulated by the histone acetylation process (Eberharter and Becker, 2002). The positive charge of lysine residues on histones is neutralized by the addition of an acetyl group; hence, interaction with negatively charged DNA becomes weaker, which leads to local euchromatization (Kingston and Narlikar, 1999). Usually, histone acetylation is associated with activation of gene transcription, whereas deacetylation is associated with repression (Shang et al., 2000). Apart from the global effect of acetylation on chromatin structure, posttranslational histone modifications (Figure 2), including both acetylation and methylation, play a role as docking sites for other proteins. These chemical modifications may facilitate or impede histone interaction with other factors (so-called readers)

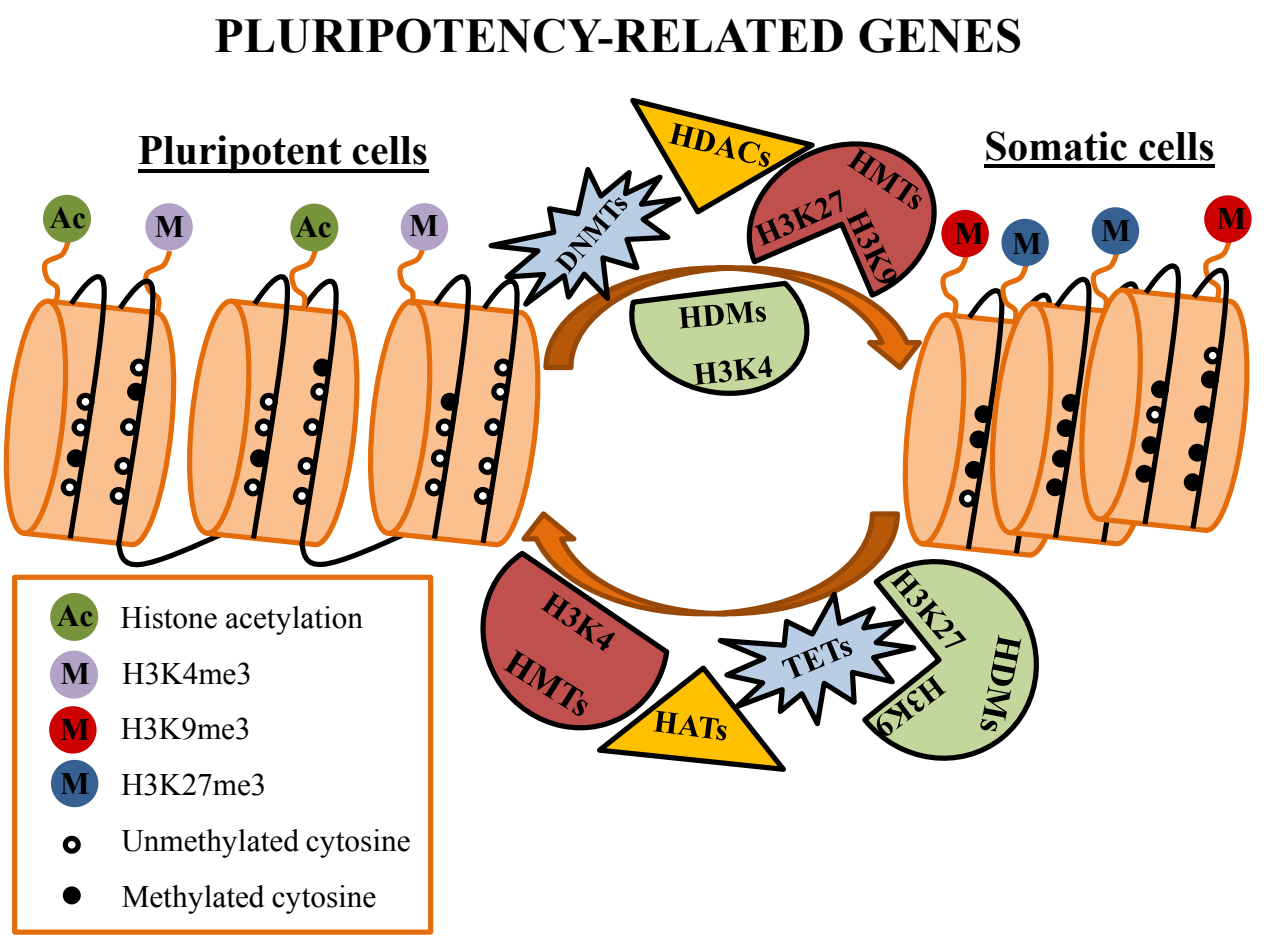

Figure 1. Chromatin reorganization within pluripotency-related genes. DNMTs: DNA methyltransferases; HATs: histone acetylases; HDACs: histone deacetylases; HDMs: histone demethylases; HMTs: histone methyltransferases.

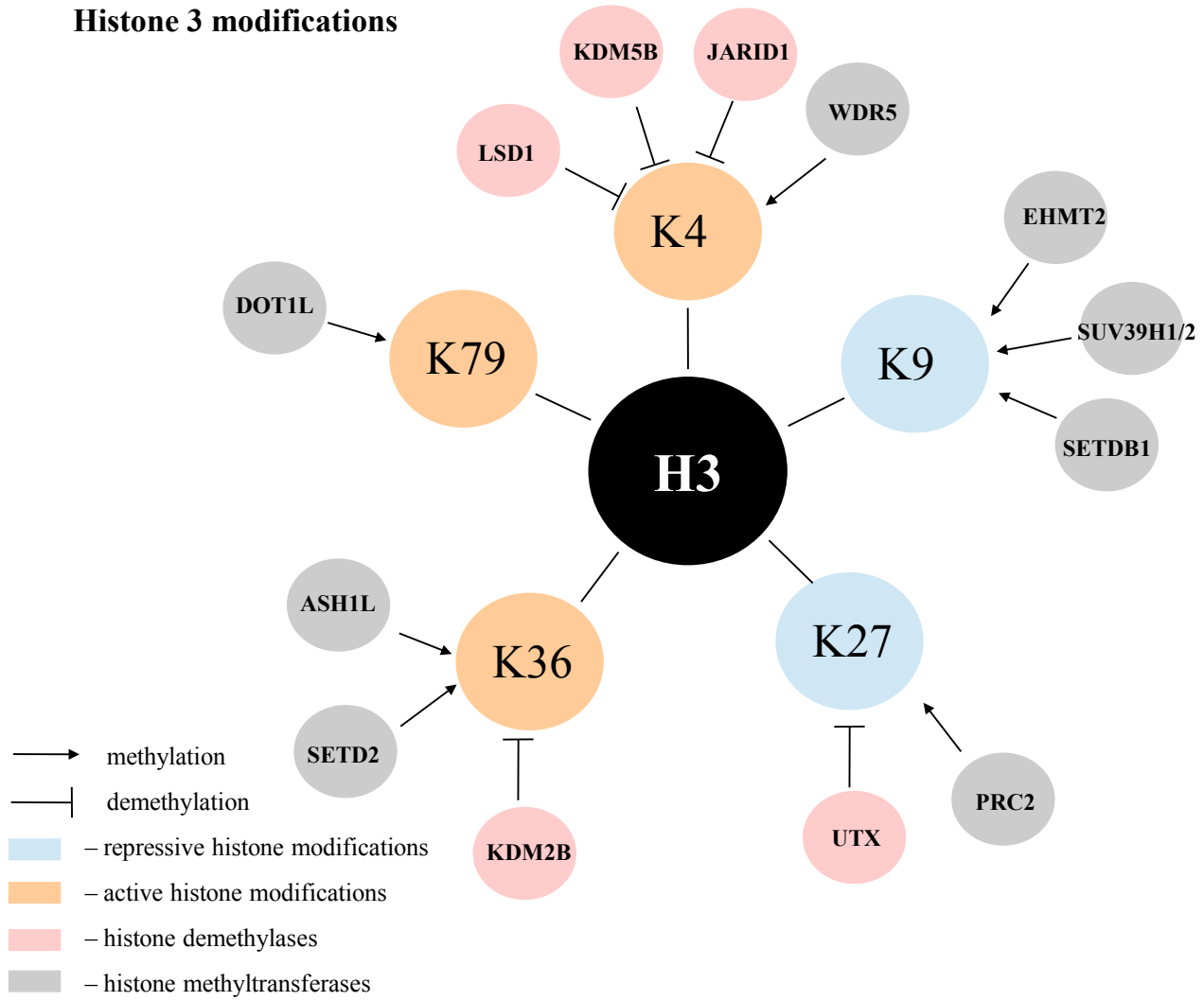

Figure 2. Schematic representation of H3 methylation modifications. Red indicates active and blues indicates repressive modifications. Writers (methyltransferases) and erasers (demethylases) for each modification are also shown.

that drive the decondensation or condensation of a given locus.

3.4.1. Histone acetylation

The histone acetylation profile is essential for adequate functioning of stem cells. Acetylation decreases the positive charge of histones, thus enabling acquisition and maintenance of the open chromatin structure characteristic for pluripotent cells (Kingston and Narlikar, 1999). Indeed, somatic cell reprogramming to iPSCs is greatly enhanced by the addition of HDAC inhibitors (e.g., valproic acid, trichostatin A, butyrate). It was shown that inhibition of HDACs can improve reprogramming efficiency even more than 100-fold (Huangfu et al., 2008; Mali et al., 2010). HDAC inhibition enhances the expression of pluripotency factors, even without the addition of exogenous c-Myc or Klf4 in the reprogramming cocktail (Mali et al., 2010). Most frequently, histone acetylation is associated with actively transcribed genes. Xie et al. (2009) demonstrated that in pluripotent stem cells H3K56Ac is deposited within the active, pluripotency-associated genes and miRNAs (Nanog, Sox2, Oct4, Lefty1, Dppa4, miR-302/367). Upon differentiation, H3K56Ac deposition shifts towards developmental genes. Nevertheless, in certain cases H3K56Ac might also mark repressed loci.

Additionally, acetylated lysines may alter protein binding sites, thus allowing for expanded protein association. Many transcription factors and chromatin modifiers contain a bromodomain, whose docking onto chromatin may be dependent on the acetylation status of chromatin (Yang, 2004). One such bromodomain-containing protein is Atad2, which associates with acetylated histones to maintain open chromatin and

to support active transcription of neighboring genes. Although loss of Atad2 has little effect on the self-renewal properties of stem cells, it reduces the proliferative potential of ESCs during differentiation and modulates the timing and intensity of expression of differentiation markers (Morozumi et al., 2015). Another protein with a bromodomain, Brd4, is a histone chaperone that is recruited by acetylated H4 to facilitate transcription. In stem cells, Brd4 is crucial to maintain the expression of Nanog and other pluripotency markers, while its inhibition leads to the spontaneous onset of differentiation (Horne et al., 2015; Gonzales-Cope et al., 2016).

3.4.2. H3K4 methylation

In human cells, the trimethylated histone H3 lysine 4 (H3K4me3) is deposited at both active and inactive promoters. However, when associated with RNA PolII, H3K4me3 marks actively transcribed genes. The presence of H3K4me3 within inactive regions may be partially explained by its frequent colocalization with the repressive H3K27me3 mark within so-called bivalent domains (Guenther et al., 2007). Although bivalent regions are present in all cell types, they occur particularly frequently in ESCs to maintain many differentiation-specific genes in a poised state (Bernstein et al., 2006; Mikkelsen et al., 2007). H3K4 methylation is deposited by several specific histone methyltransferases from the trithorax group (TrxG) and is removed by demethylases (e.g., Lsd1/Kdm1a and Jarid1/Kdm5a) (Figure 2). Appropriate H3K4me turnover and deposition is essential in developmental processes, and many H3K4 methylation modifiers have been shown to control self-renewal and lineage commitment (Gu and Lee, 2013). The H3K4 mono- and dimethyltransferases Mll4/Kmt2d bind to DNA in a pattern specific for a given differentiation stage and cell type. During cellular differentiation, Mll4 colocalizes with lineage-specific transcription factors on the enhancers involved in differentiation to contribute to their activation (Lee et al., 2013). Expression of the Wdr5 protein, which is a main subunit of the trithorax complex, was shown to be highly specific to pluripotent stem cells. Wdr5 physically interacts with Oct4, Nanog, and Sox2, which together cooperatively upregulate key self-renewal genes. It is highly likely that for this reason ESCs lacking Wdr5 lose their self-renewal capacity, whereas downregulation of Wdr5 decreases the generation of iPSC colonies (Ang et al., 2011).

Contrasting effects were observed in the case of the Jarid1/Kdm5b demethylase. Jarid1/Kdm5b colocalizes with H3K4me3 close to active gene promoters and enhancers in ESCs. Upon differentiation, Kdm5b catalyzes H3K4me3 removal to inhibit the expression of pluripotency-specific genes (Kidder et al., 2014). Another demethylase (Lsd1/Kdm1a) fine-tunes the balance between the H3K4me3 and H3K27me3 marks at bivalent regions of the developmental gene promoters targeted by Oct4 and Nanog in human ESCs. Adamo et al. (2011) demonstrated that the loss of Lsd1/Kdm1a resulted in the progressive differentiation of ESCs due to the accumulation of H3K4me3, which disrupted the poised status of lineage-specific genes and activated their expression.

3.4.3. H3K36 methylation

The H3K36me3 mark accumulates within the bodies of active genes and facilitates their transcriptional elongation (Mikkelsen et al., 2007). H3K36 methylation (Figure 2) is catalyzed by the Ash1l and Setd2 enzymes, whereas Kdm2b acts as a vitamin C-dependent H3K36me3 demethylase (Eram et al., 2015). The addition of vitamin C to the reprogramming media facilitates Kdm2b-induced H3K36me3 demethylation, thereby promoting iPSC generation. Moreover, Kdm2b increases reprogramming efficiency by suppression of the Ink4/Arf locus, thus overcoming cell senescence (Wang et al., 2011). Liang et al. (2012) also demonstrated that Kdm2b overexpression augmented somatic cell reprogramming, especially during its early phases. Kdm2b was shown to bind to the promoters of the genes activated early in the reprogramming and participate in their upregulation. The identified Kdm2b-affected genes are known to be involved in adhesion, morphology, epithelial phenotype, and pluripotency.

3.4.4. H3K79 methylation

Another marker of active chromatin deposited at transcribed gene bodies is H3K79me3. Deletion of the Dot1l gene, which serves as an H3K79 methyltransferase, leads to severe developmental defects and embryonic lethality. In in vitro culture, ESCs lacking Dot1l present reduced levels of heterochromatin modifications at telomeres and centromeres, which leads to aneuploidy, abnormal telomere elongation, and, as a consequence, impaired proliferation (Jones et al., 2008). Despite deleterious effects in ESCs, Dot1l inhibition was shown to improve the efficiency of reprogramming in the absence of Klf4 and c-Myc. Indeed, the H3K79 methylation level decreases at genes involved in the epithelial-to-mesenchymal transition during the early stages of reprogramming. Therefore, the reduction in Dot1l expression enables the demethylation of H3K79 from somatic genes, including the genes responsible for the mesenchymal state. This facilitates epigenetic silencing of these genes, promotes the mesenchymal-to-epithelial transition, and accelerates the reacquisition of pluripotency features (Onder et al., 2012).

3.4.5. H3K27 methylation

H3K27me3 is a histone modification that represses gene expression in multiple contexts. Trimethylation of H3K27 is mediated by the polycomb repressive complex 2 (Prc2) that contains Ezh2, Suz12, and Eed, whereas demethylation is catalyzed by Utx1 (Figure 2).

The H3K27me3 modification plays an important role in differentiation and dedifferentiation; however, it seems to have a lower impact on the maintenance of the pluripotent state (Pasini et al., 2007; Chamberlain et al., 2008; Marks et al., 2012; van Heeringen et al., 2014). The global level of H3K27me3 increases with the progress of differentiation: it is reduced in ESCs cultured in the presence of the Mek and Gsk3 inhibitors that force the cells into naïve pluripotency state (Marks et al., 2012), and it rises after the blastula stage (van Heeringen et al., 2014). Interestingly, ESCs lacking Pcr2 subunits, Eed or Suz12, demonstrate slightly elevated expression of Prc2 target developmental genes, but they preserve their self-renewal properties and can contribute to the three germ layers (Pasini et al., 2007; Chamberlain et al., 2008). Nevertheless, chimeras with Eed null cells show developmental postimplantation defects (Chamberlain et al., 2008). Additionally, ESCs devoid of Suz12 undergo improper differentiation that manifests as the abnormal morphology of embryonic bodies, retained expression of pluripotency genes, and limited activation of differentiation markers (Pasini et al., 2007). Appropriate H3K27me3 deposition is essential for successful iPSC generation. Multiple studies reported that knockdown of Ezh2, Suz12, and Eed subunits decreased reprogramming efficiency (Pereira et al., 2010; Onder et al., 2012; Ding et al., 2014). The H3K27me3 demethylase Utx is also required for appropriate reprogramming. Loss of Utx leads to a reduced number of iPSC colonies due to aberrant retention of the H3K27me3 mark at the OSK target genes, which prevents their activation (Mansour et al., 2012).

3.4.6. H3K9 methylation

Pluripotent cells harbor low amounts of H3K9me3, which is deposited mainly at the satellite, telomere, and retroelement repeats to maintain a repressed chromatin within these sequences (Mikkelsen et al., 2007). Its global level rises during differentiation, spreading along silenced regions and inducing cell type-specific heterochromatization (Wen et al., 2009). H3K9 methylation is catalyzed by the G9a/Ehmt2, Setdb1, and Suv39h1/2 methyltransferases and is removed by a number of demethylases, including Kdm3a, Kdm3b, Kdm4b, and Kdm4c (Figure 2). An appropriate H3K9me3 distribution is essential for the maintenance and reacquisition of pluripotency. In pluripotent stem cells, Oct4 induces the expression of the H3K9me2/3 demethylases Kdm3a and Kdm4c, which in turn positively regulate the expression of several other pluripotency factors by maintaining the demethylated status of H3K9 within their promoters (Loh et al., 2007). Conversely, the G9a/Ehmt2 methyltransferase is upregulated at the early stages of differentiation to drive the epigenetic inactivation of Oct4 and Nanog. Embryos lacking G9a/Ehmt2 expression fail to downregulate the core pluripotency factors, ultimately leading to impaired embryonic development and lethality (Yamamizu et al., 2012). Other histone methyltransferases (i.e. Setdb1 and Suv39h1/2) participate in the inhibition of retroelements in ESCs. It is highly likely that H3K9me3-mediated silencing prevents the potentially deleterious activation of retroelements during the global rearrangement of DNA methylation that occurs during the early stages of development (Matsui et al., 2010; Bulut-Karslioglu et al., 2014). Tight regulation of H3K9 modification was also shown to be an important event during somatic cells' dedifferentiation. The retention of H3K9me3 in cells undergoing reprogramming blocks the transition from the pre-iPSC to iPSC stage (Chen et al., 2013; Sridharan et al., 2013). Reduced H3K9me3 and subsequent chromatin decondensation obtained through the repression of the H3K9 methyltransferases (G9a/Ehmt2, Suv39h1/2, and Setdb1) enhances the formation of fully reprogrammed iPSC colonies (Soufi et al., 2012; Sridharan et al., 2013). In contrast, overexpression of the H3K9me3 demethylases facilitates reprogramming via OSKM induction (Chen et al., 2013) and somatic cell nuclear transfer (Matoba et al., 2014).

4. DNA modifications

4.1. DNA methylation

DNA methylation and demethylation are essential for mammalian development and adult homeostasis, and thus these processes need to be strictly regulated. DNA methylation is crucial for genome stability, X chromosome inactivation, repression of retroelements, and proper expression of imprinted genes (Messerschmidt et al., 2014). In the human genome DNA methylation predominantly occurs in a symmetrical CpG context. The regions of increased CpG density, so-called CpG islands, tend to be hypomethylated in contrast to hypermethylated dispersed CpG sites. CpG islands are often located within housekeeping gene promoters and genes involved in development (Deaton and Bird, 2011). Most of the germ cell-specific genes and pluripotency gene promoters contain intermediate CpG density. Hypermethylation of these promoters depends on lineage commitment and results in gene expression silencing during differentiation (Weber et al., 2007; Meissner et al., 2008).

Establishing the correct DNA methylation pattern is an important event during the first few days of embryogenesis. Upon fertilization, CpG methylation in the male pronucleus is erased. Then, after zygote formation, both the maternal and paternal DNA is progressively demethylated, and only parent-of-origin-specific imprinted regions preserve their methylation patterns during embryogenesis (Li, 2002). Low DNA methylation levels are associated with the open chromatin structure required for the transcriptional activity

of the zygotic genes. After implantation, a specific DNA methylation profile is reestablished in a lineage-specific manner by de novo DNA methyltransferases (Dnmt3a and Dnmt3b) and is maintained during subsequent cell divisions by Dnmt1. The precision of DNA methylation is critical for appropriate embryonic development because a deficiency in any of the Dnmts causes postimplantation or postnatal lethality (Li et al., 1992; Okano et al., 1999).

Defined methylation profiles are stable in normal somatic cells throughout life. Active gene promoters and enhancers are characterized by a low level of DNA methylation in contrast to repressed pluripotency-related genes (Figure 1), which tend to be hypermethylated (Polo et al., 2012). Thus, reprogramming of somatic cells to iPSCs requires massive changes in the DNA methylation profile. The global level of DNA methylation is higher in pluripotent cells compared to differentiated cells. In fact, pluripotent and somatic cells can be distinguished based on their DNA methylation signatures that include specific sets of genes characteristic for a given cell type (Bock et al., 2011). The demethylation of pluripotency-related genes occurs late in reprogramming and is thought to be a rate-limiting step in this process. The DNA methylation levels of Oct4 and Nanog promoters seem to be particularly important for establishing the proper pluripotent character of the obtained iPSCs (Takahashi and Yamanaka, 2006). Unsurprisingly, the efficiency of reprogramming is increased by the addition of DNA methyltransferase inhibitors (e.g., 5-azacytidine) (Mikkelsen et al., 2007; Hou et al., 2013). Both the demethylation of pluripotency-related genes and the methylation of lineage-specific genes seem to occur subsequent to histone modifications. This may explain why pre-iPSC colonies are unstable and require an adequate DNA methylation pattern to stabilize pluripotency (Koche et al., 2011).

4.2. DNA demethylation

As mentioned in the previous section, the DNA methylation pattern is reset during the early stages of embryogenesis. Demethylation has been proposed to proceed via active mechanisms utilizing Tet (ten-eleven translocation) enzymes independent of DNA replication. The Tet proteins belong to the family of dioxygenases that catalyze the hydroxylation of 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine) (Pastor et al., 2013). Hydroxymethylated cytosine may be further modified to an unmethylated form through the base excision repair mechanism or DNA replication (Bagci and Fisher, 2013). DNA in male gametes contains high levels of 5hmC. This phenomenon is not observed after Tet3 knockdown, suggesting the essential role of the enzyme in the hydroxylation of 5mC (Iqbal et al., 2011). Interestingly, the distribution of 5hmC was shown to be enriched at the promoters within the bivalent chromatin marked by H3K4me3 and H3K27me3. Thus, hydroxymethylation contributes to transcriptional repression but protects DNA from methylation and permanent inactivation (Pastor et al., 2013).

Tet factors also participate in the epigenetic reprogramming of iPSCs. The forced expression of OSKM factors in mouse embryonic fibroblasts upregulates Tet2 expression, whereas Tet2-deficient fibroblasts are incapable of forming iPSC colonies (Doege et al., 2012). Costa et al. (2013) demonstrated that overexpression of Tet1 and Tet2 augments reprogramming process, resulting in an increased number of iPSC colonies. Furthermore, they provided evidence that the interaction between Nanog and Tet1 increases hydroxylation within the promoters of other pluripotency-related genes, Oct4 and Esrrb, subsequently resulting in their activation. Tet1 not only enhances reprogramming by demethylating the Oct4 promoter, but also may act as a reprogramming factor. It has been shown that Tet1 can replace Oct4 in the reprogramming cocktail with comparable efficiency (Gao et al., 2013).

4.3. Epigenetic memory

Although iPSCs are highly similar to ESCs from the molecular, phenotypic, and functional points of view, iPSCs may maintain some of the methylation pattern of their cells of origin. This phenomenon (defined as epigenetic or somatic memory) may impair the differentiation capacity of iPSCs (Doi et al., 2009; Kim et al., 2010; Bock et al., 2011). It was shown, for example, that iPSCs generated from blood cells can be differentiated to blood cell lineages with much greater efficiency than iPSCs derived from fibroblasts (Kim et al., 2010). This problem may be partially overcome by the addition of DNA demethylating agents such as 5-azacytidine (Kim et al., 2010). Despite global similarities between the DNA methylation profiles of iPSCs and ESCs, several research groups identified differentially methylated regions (DMRs) that could be used to distinguish between these cell types. Bock et al. (2011) showed that each of the ESC and iPSC lines analyzed in their study could be characterized by a cell type-specific DNA methylation pattern that was stable during subsequent cell divisions. The DNA methylation patterns were more frequently variable among the iPSC than the ESC lines. In another study, Ruiz et al. (2012) identified a minimal reprogramming-associated epigenetic signature that could be used to discriminate iPSCs from ESCs. This signature comprised nine aberrantly methylated genes whose methylation profiles were sustained even after differentiation.

4.4. Genomic imprinting

Genomic imprinting is a regulatory mechanism that leads to the preferential expression of a subset of genes either from the maternal or paternal allele. This epigenetic process involves changes in the DNA methylation pattern

as well as histone modifications. Genomic imprinting provides a mechanism for the dosage regulation of genes with an essential role in embryonic growth and development (Plasschaert and Bartolomei, 2014). Aberrant imprinting of certain loci might initiate numerous pathologies, including transient neonatal diabetes or Beckwith–Wiedemann syndrome (Choufani et al., 2010; Mackay and Temple, 2010; Plasschaert and Bartolomei, 2014). Imprinted loci are marked with methylated cytosines in a uniparental fashion at the promoters of long noncoding RNAs (lncRNAs), thereby providing cis-acting transcriptional control of neighboring genes (Kanduri, 2016). The imprinting-associated methylation is removed during the development of primordial germ cells. During the course of gametogenesis, most of the DMRs adopt their monoallelic methylation, which is preserved during embryonic and somatic development. Interestingly, only placental tissue acquires a different imprinting pattern that includes sites frequently hypomethylated in germ cells and ESCs (Court et al., 2014). Thus, faithful maintenance of imprinting plays an important role in developmental biology, and also in the reprogramming of somatic cells into high-quality iPSCs. Because iPSCs may harbor methylation aberrations, imprinting sites may undergo hypo- or hypermethylation in some iPSC colonies (Hiura et al., 2013; Takikawa et al., 2013). The most frequently cited exception is the Dlk1-Dio3 cluster. Transcriptome analysis of low grade-iPSCs compared to ESCs showed repressed expression of the genes encoded by the Dlk1-Dio3 cluster due to DNA hypermethylation. Downregulation of the Dlk1-Dio3 cluster was also associated with a reduced ability of iPSCs to form chimeras (Stadtfeld et al., 2010). This phenotype could be reversed by the addition of vitamin C, which prevented hypermethylation and thus silencing of the Dlk1-Dio3 locus (Stadtfeld et al., 2012). The addition of vitamin C to reprogramming media leads to the early upregulation of the Dppa3 protein, which associates with the Dlk1-Dio3 locus and antagonizes Dnmt3a binding, thereby preventing Dnmt3a-mediated methylation within this region (Xu et al., 2015).

4.5. X activation/inactivation

In mammals, X chromosome inactivation (XCI) compensates for the dosage of X-linked gene expression between sexes through the silencing of one of the two X chromosomes in female cells. X reactivation in female cells is one of the hallmark features of pluripotency. The process occurs at the early stages of embryogenesis and as one of the latest steps during iPSCs generation, whereas differentiation induces XCI (Maherali et al., 2007; Stadtfeld et al., 2008; Barakat et al., 2015). Several lncRNAs, including Xist (X-inactive specific transcript) and Tsix (antisense lncRNA repressor of Xist), participate in the rearrangement of the X chromosome (Figure 3). Xist is transcribed from the inactivated X chromosome (Xi) and interacts with Prc2. Prc2 recruited to the X chromosome catalyzes the deposition of the repressive H3K27me3 mark and induces DNA methylation and macro-H2A incorporation within the Xist-coated Xi (Jeon et al., 2012). Pluripotency-related factors facilitate X inactivation. Oct4 was shown to regulate XCI through its direct interaction with Tsix and other XCI factors, and its deficiency led to the aberrant inactivation of both X chromosomes (Donohoe et al., 2009). During reprogramming, female iPSCs lose Xist expression and undergo X chromosome reactivation (XCR) (Maherali et al., 2007; Pasque et al., 2014). Upon XCR, stable Xi is converted to the active form (Xa) by the erasure of the Xi-heterochromatin marks. This event is observed late during reprograming after the activation of endogenous pluripotency genes (Pasque et al., 2014). Female mouse ESCs and iPSCs usually have both X chromosomes active. However, human pluripotent stem cells are highly heterogeneous in terms of their X status, which supports the notion that mouse pluripotent stem cells have a more naïve state in in vitro culture (Tchieu et al., 2010; Tomoda et al., 2012). Nevertheless, culture conditions and prolonged passaging of female iPSCs contributes to XCR (Pasque et al., 2014).

5. Long noncoding RNAs (lncRNAs)

Long noncoding RNAs are endogenous RNAs longer than 200 nucleotides. The molecular mode of action of lncRNAs is exerted through various mechanisms that are still insufficiently understood. Nevertheless, many lncRNAs were shown to play a crucial role in the gene expression control of the pluripotent and differentiated states. These noncoding transcripts were shown to act as scaffolds for chromatin remodeling complexes (e.g., Prc2), competitors of endogenous miRNAs, or guides targeting various molecules for degradation or to specific genomic/transcriptomic sequences (Flynn and Chang, 2014). As previously mentioned, lncRNAs participate in XCI (Jeon et al., 2012) and in the cis-regulation of imprinted genes (Kanduri, 2016), which are processes involved in normal stem cell biology and development. The expression of lncRNAs is tissue-type specific; thus, the induction of pluripotency evokes massive changes in the expression profile of lncRNAs (Kim et al., 2015). One of the frequently upregulated transcripts in iPSCs is Regulator of Reprogramming (lincRNA-RoR), which has been found to be critical for the reprogramming of human fibroblasts. Its upregulation improves and its downregulation hinders the reprogramming efficiency (Loewer et al., 2010). Wang et al. (2013) recently demonstrated that lncRNA-RoR acted as a competing endogenous RNA (ceRNA) for a subset of miRNAs (miR-145-5p, miR-181a-5p, and miR-99b-3p) by targeting core pluripotency factors (Oct4, Sox2, and

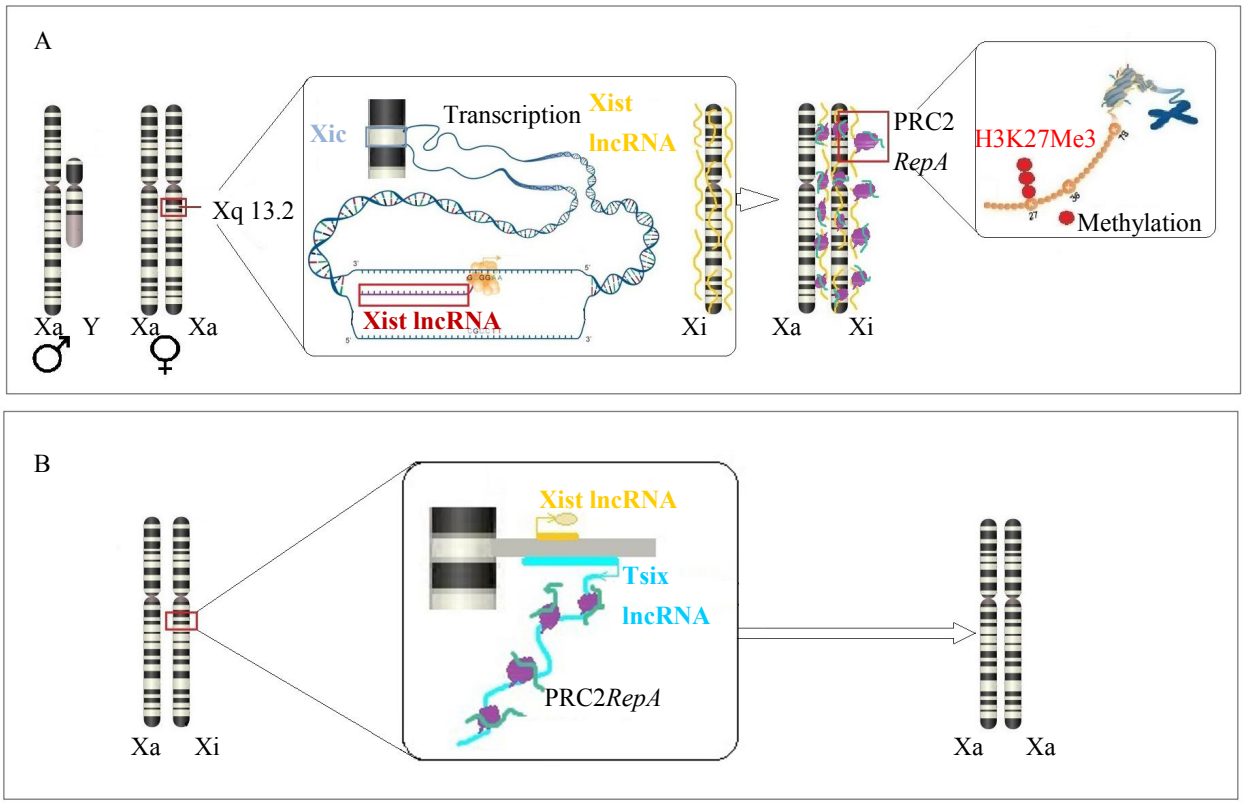

Figure 3. X chromosome inactivation and reactivation mechanisms. (A) During X chromosome inactivation (XCI), the X-inactivation center (Xic) on one of the female X chromosomes triggers the transcription of the Xist lncRNA. Transcribed Xist covers the X chromosome and recruits the Prc2 complex through RepA (repeat A transcript). Prc2 catalyzes the deposition of the repressive H3K27me3 mark that strengthens X chromosome inactivation. (B) X chromosome reactivation (XCR) occurs during the early stages of embryogenesis and during induction to pluripotency. The Xist antisense transcript lncRNA Tsix competitively binds to Prc2, thereby abolishing the interaction between Xist and Prc2 and inducing X chromosome reactivation.

Nanog) to prevent their miRNA-mediated decay. Linc-RoR creates a feedback loop with specific miRNAs and pluripotency-related transcription factors to govern stem cell identity. Downregulation of lncRNA-RoR reduced the self-renewal properties of ESCs, whereas overexpression sustained the high expression of pluripotency factors and the undifferentiated state in ESCs cultured without FGF.

6. miRNAs

MiRNAs are short, noncoding, 18–25-nucleotide RNAs that regulate gene expression in a sequence-specific manner via directing RISC (RNA-induced silencing complex) to degradation-targeted mRNAs. These tiny molecules play an enormous role in the posttranscriptional gene regulation of many pathways, including the pathways involved in pluripotency, self-renewal, and differentiation. Pluripotency-promoting miRNAs repress differentiation signals, whereas differentiation-promoting miRNAs suppress the pluripotency regulation network and reinforce differentiation and lineage commitment (Li and He, 2012). Profiling of miRNA expression revealed a signature that was characteristic for the ESC state that included the upregulated miR-290/295, miR-17/92, and miR-302/367 clusters and downregulation of the let-7 family, miR-210, and miR-145 (Melton et al., 2010; Li and He, 2012; Gao et al., 2015; Sen and Ghatak, 2015). MiRNA clusters belonging to the miR-290/295, miR-302a/367, and miR17/92 families function as cell cycle moderators and are known to be embryonic stem cell-specific cell cycle-regulating miRNAs (ESCCs) (Li and He, 2012). In ESCs, the miR290/295 cluster targets cell cycle inhibitors (e.g., the Wnt pathway inhibitor Dkk1), resulting in increased ESC proliferation due to the promoted transition from the G to S1 phase (Wang et al., 2008; Zovoilis et al., 2009). Moreover, this family (and specifically miR-294) was shown to improve the reprogramming efficiency of the OSK cocktail compared to OSK alone to a level similar to the OSKM-induced reprogramming by partial substitution of the c-Myc function (Judson et al., 2009). Similar effects in enhanced reprogramming by replacing c-Myc were obtained with the ectopic expression of miR-93, miR-106b, and the miR-302a/367 cluster (Anokye-Danso et al., 2011;

Li et al., 2011; Subramanyam et al., 2011). Interestingly, core pluripotency transcription factors bind to the promoter of the miR-290 cluster and induce its expression. The miR-290 cluster also acts as a functional antagonist of let-7 (known to be an inducer of differentiation) via the upregulation of Lin28, which is an RNA-binding protein that hinders the maturation of let-7 (Melton et al., 2010). Moreover, Gao et al. (2015) reported that the addition of vitamin C upregulated the expression of ESCC miRNAs and maintained their pluripotency-specific miRNA expression patterns.

7. Summary and future perspectives

The last few years have provided mounting evidence for the involvement of the epigenetic machinery in the maintenance of pluripotency, differentiation, and reprogramming of somatic cells into iPSCs. Pluripotent stem cells contain an open chromatin structure with multiple activating modifications. This type of architecture needs to be reestablished during reprogramming to enable the generation of high-quality iPSCs. The field exploring stem cell biology is still expanding, and many processes involved in stemness regulation need to be further investigated. The majority of studies have tested how a single or a small subset of epigenetic modifiers influences stem cell behavior. Moreover, a number of cellular transcription factors, signaling molecules, and metabolites were shown to affect epigenetic states. Nevertheless, the communication between these cellular entities has been insufficiently defined. With the growing accessibility to high-throughput and single cell assays, it will be possible to integrate various molecular profiles (e.g., DNA methylation, histone modifications, RNA and protein expression, metabolites) of cells at various developmental stages. This systematic approach will help to elucidate the exact interactions between epigenetic modifiers and other cellular factors implicated in the cell fate transition and the kinetics of developmental shifts. An improved understanding of the spatiotemporal molecular alterations driving differentiation and dedifferentiation will provide novel tools for the conscious manipulation of these cells and new insights into the pathological events associated with disordered development.

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