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Stem Cells and Epigenetic Reprogramming

Perla Cota, Mehdi Shafa and Derrick E. Rancourt

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1. Introduction

In general stem cells have to fulfill two characteristics: self-renewal and the ability to differentiate into different cell/tissue types. Depending on their limitations in differentiation (pluripotent vs. multipotent) stem cells can be divided in embryonic or adult stem cells, depending on their limitations in differentiation [1]. This chapter will focus only on embryonic stem cells (ESCs) and their cognate artificial derivatives known as induced pluripotent stem cells (iPSCs). Embryonic stem cells, have been the center of much attention because of their pluripotency or ability to differentiate into any cell type in the body [2,3]. Induced pluripotent stem cells (iPSCs) are reprogrammed into the pluripotent state by the introduction of exogenous factors. These factors change the potency state of terminally differentiated somatic cells to by interacting with cellular chromatin and protein/RNA networks with the somatic cell. Following reprogramming, the newly formed stem cell resembles the ESC [4]. The recent development of these artificial or "man-made" cells has delivered two key potential upsides: (a) the ability to avoid the ethical issues associated with embryo-derived cells, and (b) the ability to generate autologous (i.e. patient derived) cells for regenerative medicine, tissue engineering, and disease modeling purposes [4]. Compared to ESCs, which are derived from the limited resource of assisted fertility by-products, iPSCs can potentially provide an unlimited source of pluripotent cells.

One of the applications of iPSCs is the ability to model diseases for drug screening, toxicology testing, and cell therapy among others [4]. For basic biomedical research, cell culture has been a key element for every approach. However one drawback when studying human cells is that they have limited life span in culture. Many cell lines have not been faithfully adapted for growth *in vitro*. Hence the lack of accessible models of normal and pathologic tissue has left many important questions in human pathogenesis inaccessible [5]. In contrast due to their self-renewal and pluripotency patient derived iPSCs can be extremely useful for patient research and diagnostic purposes. Every iPSC that is compromised in disease can be restructured into

tissue in culture giving researchers an unlimited source of cells/tissue for the study of the disease [6, 4].
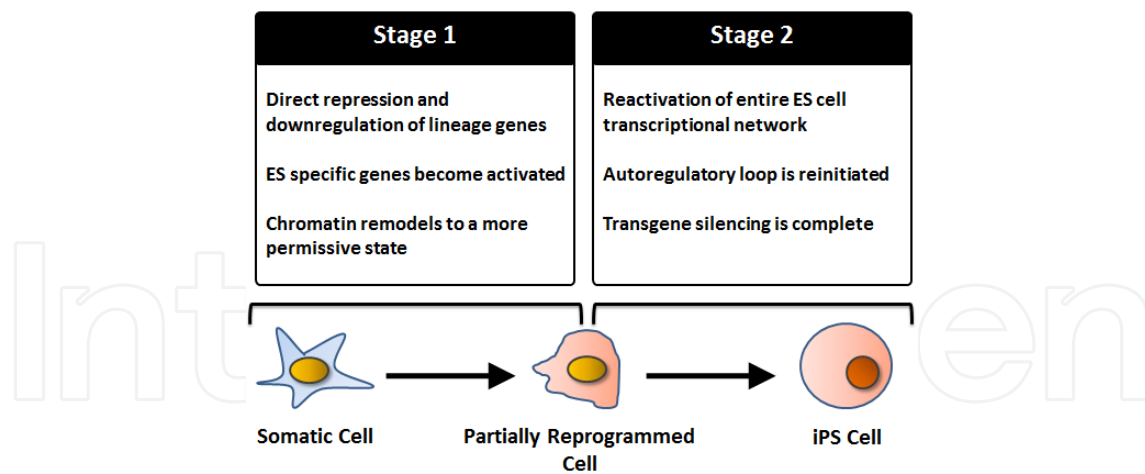
When applied to cell therapy, autologous iPSCs are differentiated into a chosen cell type and then transplanted to the damaged tissue with the advantage that immune rejection can be avoided. Furthermore iPSCs be used as a conduit for somatic gene therapy. For example a disease-causing mutation can be repaired in patient iPSCs by homologous DNA recombination prior to transplantation [4]. A study on engineered mice that suffer from human sickle cell anemia showed that when applying hematopoietic progenitor cells produced from autologous iPSCs, animals were rescued from systemic hematological symptoms. In this case, for the production of the hematopoietic progenitor cells, a biopsy of adult fibroblast was taken from the afflicted mouse and reprogrammed into iPSCs. Derived iPSCs were repaired by homologous recombination. These cells were then differentiated to hematopoietic progenitor cells *in vitro* and transplanted back into the affected mouse [7].

Despite the success in animals, there are still some drawbacks with using iPSCs for human benefit. Since the derivation of the iPSCs commonly involves integrating viral vectors for introducing reprogramming factor, this represents a risk to the human health. Moreover certain epigenetic abnormalities in the iPSCs including the epigenetic memory of their donor cells could lead to mutation in prolonged culture [4]. Such epigenetic differences are one reason why research has begun to focus on the epigenetics of cellular reprograming. Although iPSCs are the functional equivalent of ESCs, epigenetic differences have been noted, including differences in gene expression, DNA methylation, histone marks and telomere/telomerase status [8, 9]. Moreover, researchers have also recognized a role for chromatin remodeling during reprogramming and have recently applied small molecules to circumvent epigenetic blocks and enhance reprogramming efficiency [10,11].

Given that there is a huge interest in using iPSCs, mainly in regenerative medicine; researchers want to understand the exact mechanism of reprogramming, as any error in this process could cause tumor formation once applied to patients. Understanding the fundamentals of this reprogramming process by comparing it to the pluripotent state of ESCs will give us many tools to be able to manipulate the reprogramming process within a controlled environment.

Since iPSC are being compared at all times to ESCs, a basic concept that must be kept in mind is that ESCs rely on a complex network of interacting pluripotency transcription factors, and different "epigenetic landscapes" in order to maintain their "open" chromatin to regulate either self-renewal or differentiation [1]. Moreover, when a somatic cell is subjected to reprogramming, it suffers large-scale epigenetic alterations, carried on as if they were different multiple layers of epigenetic events that control the expression and accommodation of important pluripotency transcription factors [1].

In this chapter, a deeper explanation about iPSCs together with the basic concepts of epigenetics and the different levels of regulation will be provided. Insight into some of the recently discovered epigenetic events of cellular reprogramming will be discussed.

Stage 1	Stage 2
Direct repression and downregulation of lineage genes	Reactivation of entire ES cell transcriptional network
ES specific genes become activated	Autoregulatory loop is reinitiated
Chromatin remodels to a more permissive state	Transgene silencing is complete

Somatic Cell → Partially Reprogrammed Cell → iPS Cell

Figure 1. Two state process for reprogramming somatic cells (Adapted from 6)

2. Induced pluripotent stem cells

It was recently discovered that a terminally differentiated cell could be reprogrammed into an ESC-like cell using four transcription factors. Having pluripotent characteristics, these iPSCs are capable of becoming one of more than 200 cell types [12]. In order to be consider ESCs, they must fulfill certain criteria: (1) to express pluripotency factors such as Oct4, Sox2, Nanog, and SSEA1, however this criteria only apply for mouse ESCs, since in human ESCs SSEA3 and SSEA4 are expressed in stead however this criteria only apply for mouse ESCs, since in human ESCs SSEA3 and SSEA4 are expressed in stead (2) in female cells there must be the reactivation of the inactive X chromosome, (3) they should be able to differentiate into the three germ layers (ectoderm, endoderm and mesoderm) and in the case of mouse, be able to generate chimeras upon blastocyst implantation and pass through germline [13].

Takahashi and Yamanaka (2006) were the first that found a way to circumvent two of the most important drawbacks when using ESCs related to immune rejection and ethical. In their study they first hypothesized that the factors that play a role in maintaining ESC pluripotency could potentially turn somatic cells back into a pluripotent state. Starting with 24 candidate genes known to be involved in pluripotency and a herculean combinatorial effort they reduced this original 24 down to four factors: Oct3/4, Klf4, Sox2, and c-Myc able to reprogram mouse fibroblasts into an ESC-like state. Yamanaka and colleagues also reported the same results one year later for the human (2007) when using this same combination of factors [14].

After the four factor derivation of the iPSCs, much interest was focused on the process of somatic cell reprogramming. Although still not well understood, Scheper and Copray (2009) proposed one approach that divided reprogramming in two broad stages. First Oct4 and Sox2 repressed genes associated with the host cell lineage and reset the epigenome of the cell towards a permissive chromatin mode putting the cell in an embryonic-like state. The second stage allowed the reprogramming factors to reactivate the endogenous autoregulatory loop that triggers the pluripotency transcriptional network [6] (Figure 1).

After the first proposed cocktail to reprogram differentiated cells into iPSCs, many research-ers started to ask the question of how these factors were interacting in order to modify existing epigenetic marks and return to a pluripotent state. To date it has been reported that differentiated cells have been successfully reprogrammed by substituting some of the factors such as Klf4 or c-Myc with other transcription factors such as Nanog or Lin28 or molecules (valproic acid or Wnt ligand). It tells us that there are different pathways involved in this process and that epigenetic enzymes are being activated in every case [9] all to one end-point of pluripotency.

Waddington referred to epigenetics for the first time as genetic interactions that can affect the phenotype. Later, he proposed a model based on how cells followed a developmental differentiation path much like traveling down a series of canals that start from a fertilized totipotent embryo and ending up as a specific lineage committed cell [15]. In this model, cells committed to a specific lineage cannot be recommitted to another lineage or canal. Howev-er, with the recent milestone of iPSC generation, Yamanaka suggested that cells could be pushed back up the canal towards the pluripotent state. During reprogramming, cells can experience other events. They can be stopped by some epigenetic bump and remain incompletely reprogrammed. In this situation cells return to their specific lineage or transition to another lineage. Finally, instead of moving they can undergo apoptosis or cellular senescence. This model proposed by Yamanaka (2009) is known as the stochastic model of iPSC generation [16] (Figure 2).

Since the development of iPSCs, many researchers have focused their attention on the epigenetics changes that iPSCs acquire, together with the chromatin dynamics that occurs during cellular reprogramming. It has been already proposed that one way to ease cell destiny is by having less lineage epigenetic patterns [17]. The most used protocol for the production of iPSCs is the one that involves the application of the four transcription factors previously described by Yamanaka (2006) [15]. Thus initial studies have focused on how these four factors worked together to initiate the reprogramming cascade. In this regard it has been proposed that Oct4 and Sox2 are totally indispensable for reprogramming while Klf4 and c-Myc enhance the efficiency and alter the structure of the chromatin to enable Oct4 and Sox2 to target more genes that are important for pluripotency [15].

Finally, it is crucial to find the best method of reprogramming in order to approximate ESCs as much as possible. For this purpose, there are several variables that have to be taken into consideration in order to have reproducible and efficient reprogramming. First is the selection of reprogramming factors where the combination (and efficiency) can vary depending on the cell type. Second is the type of method used for factor delivery, be it viral vectors, RNA, protein, or small molecules, among others. Third is the selection of cell type, since the efficiency and kinetics reprogramming changes between cell types. Understanding how reprogramming factors coordinate the cascade to orchestrate reprogramming means it is important to know the right timing and stoichiometry for optimal reprogramming. Culture environment likewise is very important. Finally the selection of a method to identify and characterize iPSCs is very critical (Figure 3) [18].
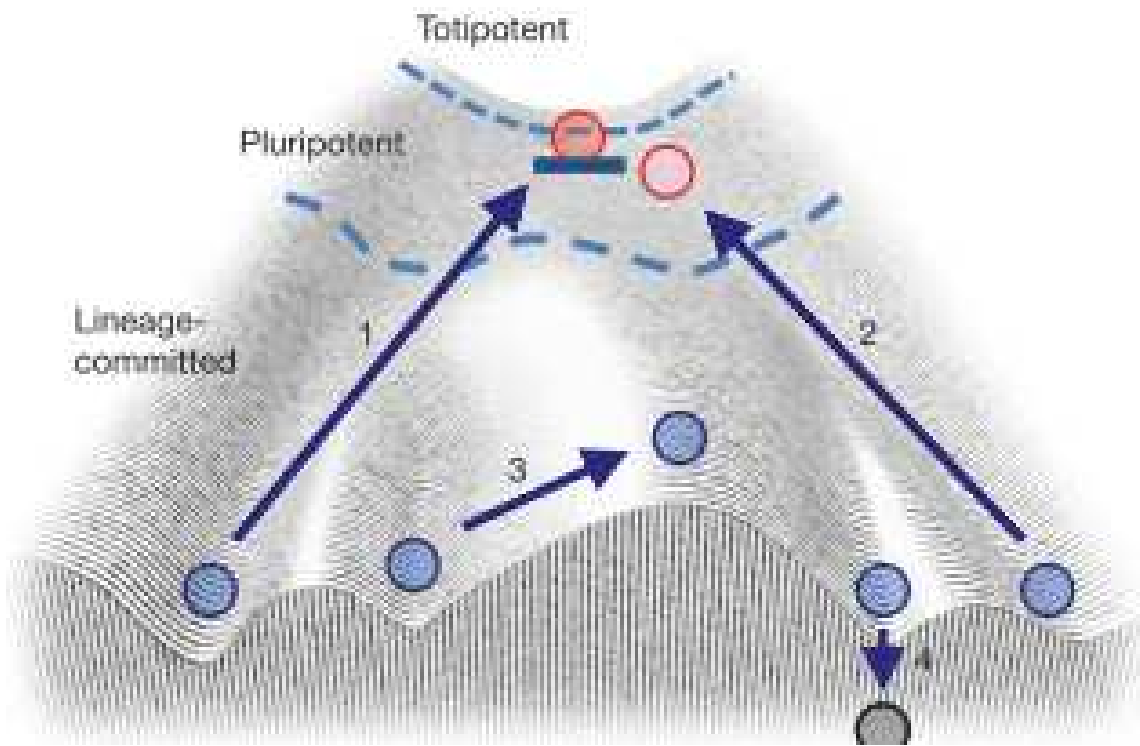
Figure 2. Stochastic model for iPSC generation. All of the cells initiate reprogramming, but only a few can achieve complete reprogramming. (Adapted from 16)

3. Epigenetics

Epigenetics is defined in general as heritable changes in gene expression that do not affect DNA-sequence [19, 20]. In the nucleus DNA is wrapped into a protein complex known as chromatin. This protein complex, known as the nucleosome, is formed by proteins called histones (H2A, H2B, H3, H4) (Figure 4) [21] into a structure resembling beads on a string. Histone H1 in turn play a role in assembling higher order chromatin structure by interacting with the "inter-bead" regions of chromation. Via changes in histone post-translational modifications (acetylation, methylation, ubiquitination, and phosphorylation), chromatin becomes very dynamic, controlling the expression or repression of specific genes in specific cells, as well as during the cell cycle or in response to environmental cues. These changes in histone via reversible post-translational marks (as well as reversible marks to primary DNA sequence) are considered to be epigenetic modifications. Additionally, changes in the nucleosome moving through DNA can be facilitated by chromatin remodeling enzymes [22, 21]. Histone modifications associated with active transcription, such as acetylation of histones 3 and 4 or di-/trimethylation of H3K4, are usually referred as euchromatin modifications. On the other hand there are the heterochromatin modifications which are characteristic to be on inactive regions such as H3K9me and H3k27me [23].
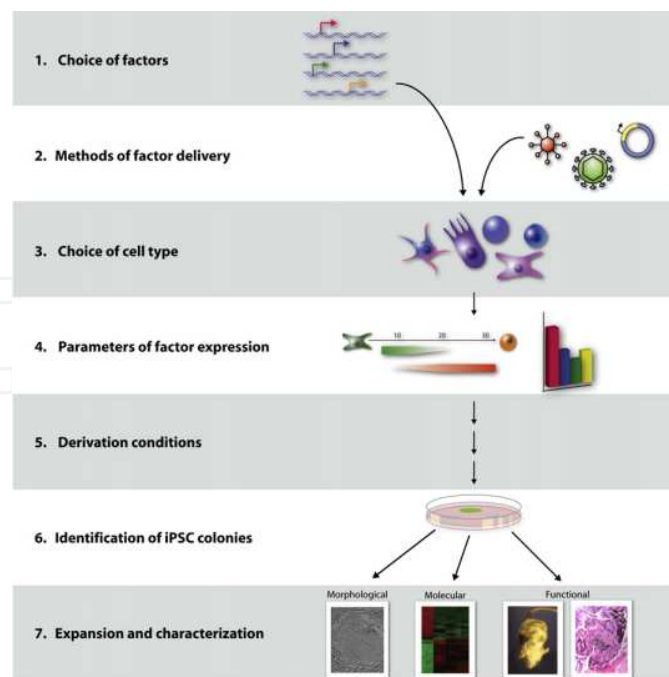
Figure 3. Overview of the iPSC Derivation Process (Adapted from 18)

4. Chromatin phases (epigenetic regulation)

• Chromatin Remodelling

Chromatin remodeling refers to the architectural change of chromatin by the movement of the nucleosomes along the DNA, giving rise to change the condensation of the chromatin. Protein complexes use ATP-hydrolysis to alter the histone DNA interaction, suggesting that there is a transient separation of the DNA from histone complexes, moving nucleosomes to a different position in the DNA or forming a DNA loop. These movements adjust the accessibility of DNA to transcription factors [23]. The many chromatin remodeling complexes are divided into families depending on their composition and biochemical activity. In this chapter, two of the most well studied ATP chromatin remodeling enzymes are discussed: SWI/SNF and CHD1 [24].

The basic assembling of the SWI/SNF chromatin remodeling enzyme in mammals (also known as BAF) is with the genes that code for the 9-12 subunits of the mammal SWI/SNF (mSWI/SNF) in combination with one catalytic ATPase subunit called brahma homolog (BRM or SMARCA2, BRM/SWI2-related gene 1[25]. mSWI/SNF uses the energy from ATPase hydrolysis to move along in the DNA. One way this works to move along is to bind the DNA into an internal site of the nucleosome, then pull it in order to weaken the nucleosome (Figure 5) [26].

One of the characteristics of mSWI/SNF chromatin remodelers is the subunit change during the transition from a pluripotent to a multipotent state and then from a multipotent state to differentiation. Ho and colleagues (2009) [27] did a whole genome study to observe the binding

of mSWI/SNF in mouse ESCs and observed that the majority of the binding does not occur in transcriptional start sites, but at distal enhancers and silencer sites. In another study of Ho and collaborators, they showed that mSWI/SNF complex binds to promoters/enhancers of pluripotency transcription factors such as Oct4 and Sox2, in accordance with studies that have shown enhanced reprogramming when they add mSWI/SNF together with reprogramming factors [28].
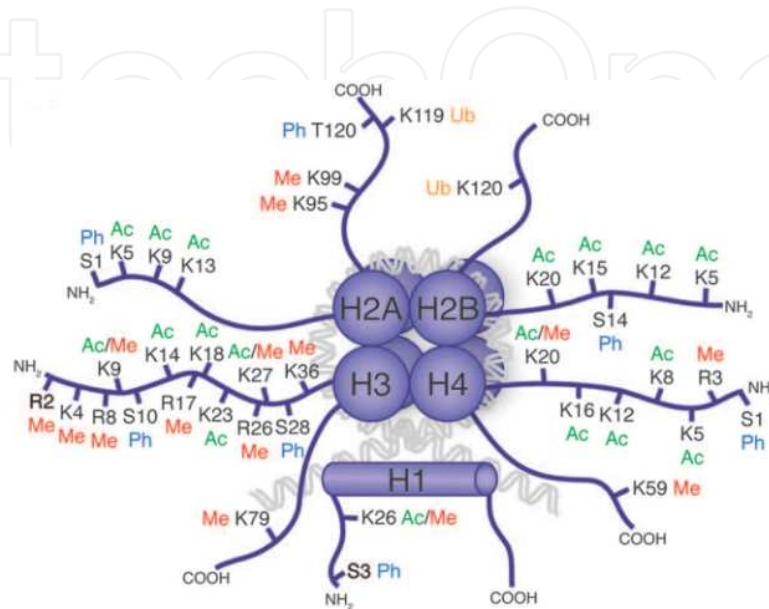

Figure 4. Nucleosome with histone posttranslational modifications (Adapted from 1)

The chromodomain helicase DNA binding protein 1 (CHD1) is well known for its remodeling activity in the maintenance of stemness. It also has main function in recognizing a substrate of transcription regulatory histone acetylation complex SAGA. CHD1 has been suggested to act as a molecular adaptor, which bring several epigenetic complexes together [29]. In ESCs, this adaptor has been suggested to be indispensable for the maintenance of pluripotent chromatin state where it is highly expressed when compared to differentiated cells. After knockdown of the CHD1 with RNAi, the pattern of diffuse ESCs heterochromatin disappears showing a higher amount of heterochromatin. In turn, CHD1 knockdown fibroblasts reprogrammed less efficiently [30]. The nature of CHD1 in pluripotent cells specifies that it can prevent the formation of heterochromatin foci [30]. CHD1 has also been reported to be one of the genes that activate Oct4, Sox2 and Nanog [31].

- Histone Modification

Histones are highly positively charged proteins that bind to DNA and have a major role in DNA packaging and gene expression. As mentioned earlier, they are subjected to a variety of post-translational modifications that alter the interaction of the histone protein with the bound DNA. These modifications include acetylation and methylation of the N-terminus tails as well as phosphorylation, poly ADP-ribosylation, ubiquitination and sumoylation. Differential modification of the core histones yields different chromatin structure. These patterns of modification form a kind of "histone code" that will ultimately govern gene expression [1].
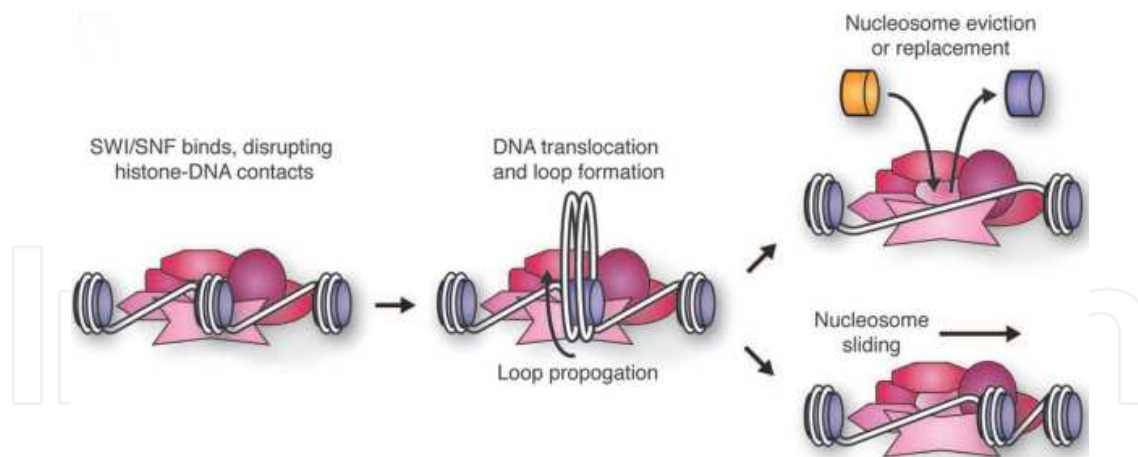
Figure 5. Chromatin Remodeling Enzyme SWI/SNFN (Adapted from 1)

Histone acetylation is the addition of acetyl moieties onto each of the histones of the nucleo-some and is regulated by the activity of histone acetyltransferases (HAT) and histone deace-tylases (HDAC). HATs and HDACs operate as coactivators and corepressors and together they dynamically change the activation and repression of genes in both a site specific as well as global manner. There are four families of HATs; Gcn5-related N-acetyltransferase (GNAT), MYST and p300/CBP. These HATs share highly similar motifs including an acetyl-CoA binding domain with the Arg/Gln-X-XGly-X-Gly/Ala sequence [32]. HAT activity and specificity are highly dependent on the complexes they form with other HATs and transcriptional co-activators. Lysine, found at the amino terminus of the histone, is the primary targeted site of acetylation. At physiological pH, lysine is positively charged and contributes to the overall positive charge of the histone. However, the amount of lysine acetylation is directly correlated with the accessibility of the amino terminus or "histone tail" [32]. More accessibility means greater degree of acetylation. Upon acetylation, the residue is neutralized, reducing the positive charge of the histone, decreasing the interaction with the negatively charged DNA and directly influencing chromatin structure. HDACs are broken down into two families; classical HDACs and NAD+ HDACs. Like their HAT counterparts, HDACs share a conserved active site [33] and also require the need to complex with co-repressors in order to function properly. Once bound, active HDACs serve to remove the acetyl moiety from the histone tail through a charge-relay system of residues found within the active site [33, 34]. Once removed, the histones bind tighter to the DNA as well as enabling tighter packing of adjacent histones leading to more transcriptional repression (Figure 6) [35].

Histone methylation is the addition of methyl groups onto lysine and arginine residues of histones in both transcriptionally active as well as silenced regions of the chromatin. The patterns of modification of lysine residues within histones are more defined as compared to arginine. Methylation is catalyzed by Histone methyltransferases (HMTs) and demethylation by histone demethylases (HDMs). Lysine residues can be methylated up to three times whereas arginine can only be methylated twice. Moreover, the symmetry of the methyl groups on each of the residues also plays an important role in the function of chromatin. HMT all share a common SET domain within their catalytic core [37]. Lysine HMTs are very well defined as
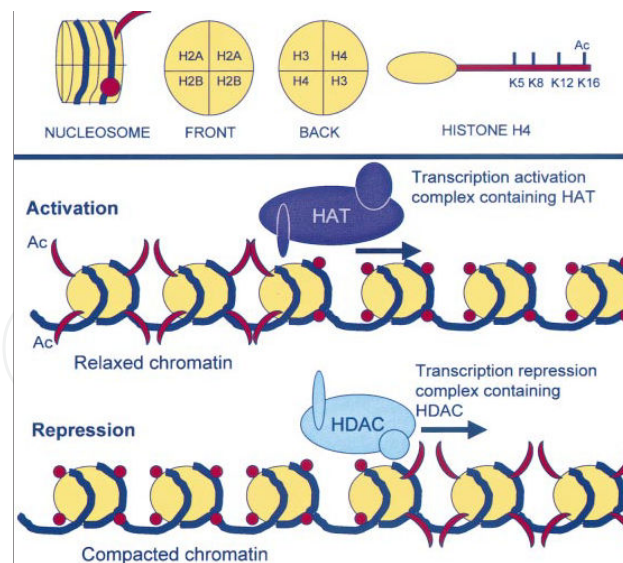
Figure 6. Histone acetylation and deacetylation mechanism (Adapted from 36)

compared to arginine HMT. Arginine N-methyltransferases (PRMT) are less defined in terms of the targeted sites of methylation, with multiple target residues in histone H3 and H4 [37].

The process of methylation does not alter the charge properties of the targeted residue, unlike those of acetylation. Instead, the addition of methyl groups serves as a recognition site for regulatory proteins to bind and elicit additional modification. This allows for a great deal of complexity depending on the target region of the MMT and the resultant recognition effector protein. In essence, it allows additional information to be encoded in the histones beyond just stearic and charge hindrance of acetylation and phosphorylation [38]. Thus, this places a great deal of importance on the proteins that interact with the methylated residue. There are a variety of motifs that are able to recognize both single and double methylated lysine residues and even one methylated lysine and methylated arginine. The basic conformation that recognizes single methylated lysine residues is a cage with polar and non-polar regions that envelops the methylated lysine residues [39]. A few of the common motifs include ankyrin repeats, chromodomain, MBT repeats, PHD finger, and double tudor [40]. An example of a motif that is able to recognize methylated lysine and arginine residues is RAG2-PHD. These varieties of recognition motifs underline the great deal of complexity behind methylated residues and even hints at potential cross talk between methylated lysine and arginine residues. There have been studies showing that the methylation of one residue, H3R2, precluded the recognition of a neighbouring methylated residue, H3K4me3 (Figure 7) [37].

Methylation of lysine and arginine residues has recently been discovered to also undergo demethylation via histone demehtylases (HDM). These enzymes are divided into two classes: amine oxidases, which are able to demethylate the first and second methyl lysine groups, and JmjC domain-containing proteins, which are able to demthylate all three methyl lysine groups. As well, it was also found recently that a JmjC domain-containing protein, JMJD6, was able to reverse arginine methylation [41]. HDM became a very key regulator of pluripotency after it was found that KDM3A and KMD4C are direct transcriptional targets of the pluripotency
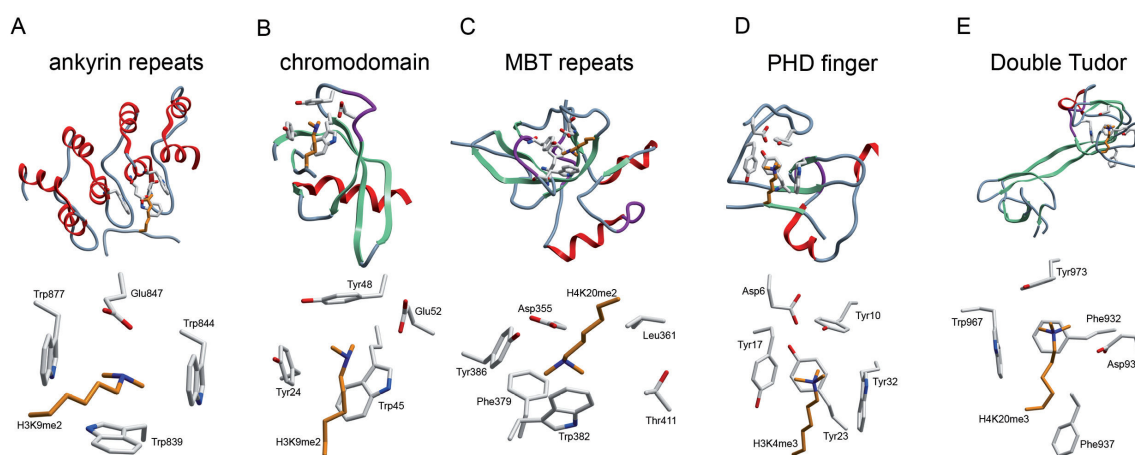
Figure 7. Methyl-lysine binding effector proteins (Adapted from 37)

promoting transcription factor Oct4 [42]. When these two enzymes were knocked down, the resulting cells lost the ability to self-renew as well as showed an altered morphology. Moreover, another high-ranking HDM JARID2 is highly expressed in ESCs but becomes rapidly downregulated upon differentiation [43]. The level of regulatory complexity of the genes, not only in ESCs or iPSCs, but also somatic cells, Hence, further examination is needed to elucidate how these enzymes contribute to the epigenetic regulation of genes.

- DNA methylation

DNA Methylation is the classical example of epigenetic regulation of gene expression. This process, catalyzed by DNA methyl transferase (DNMT) enzymes, involves the addition of a methyl group onto the carbon 5 position of cytosine residues within DNA, forming 5-methylcytosine. There are three main members of the DNMT family: Dmnt1, Dmnt3a and Dmnt3b. Dmnt1 is the best studied of the three and its primary role is to copy DNA methylation patterns during DNA synthesis as well as repair of DNA methylation patterns [44]. Dmnt3a and Dmnt3b are similar enzymes both in structure as well as function. These two DMNTs are capable of methylating native DNA, regardless of whether the DNA is in a replicative state or not [45]. Since they are able to write DNA methylation patterns onto "naked" DNA, they are termed *de novo* DMNTs.

Patterns of DNA methylation can be "read" through the recruitment of three different protein families: MBD, zinc-finger, and UHRF proteins. The most well-known are the MBD proteins, which interact with the DNA via a methyl-CpG-binding domain. Once the MBD proteins bind to the 5'site of the methylated cytosine, they repress transcription. Zinc finger proteins, like MBD proteins, also recognize and bind to methylcytosine, however they have a preference for consecutively methylated cytosine residues as well as non-methylated residues. Interestingly they are still able to repress transcription of DNA in a similar manner. Ubiquitin like containing PHD and RING finger Domain (UHRF) proteins use their intrinsic RING and SET DNA binding domains to interact with the methylated cytosine. However, the purpose of UHRF is not to repress transcription, but actually to aid DMNT, especially during DNA replication, in order to conserve and maintain the DNA methylation [46].
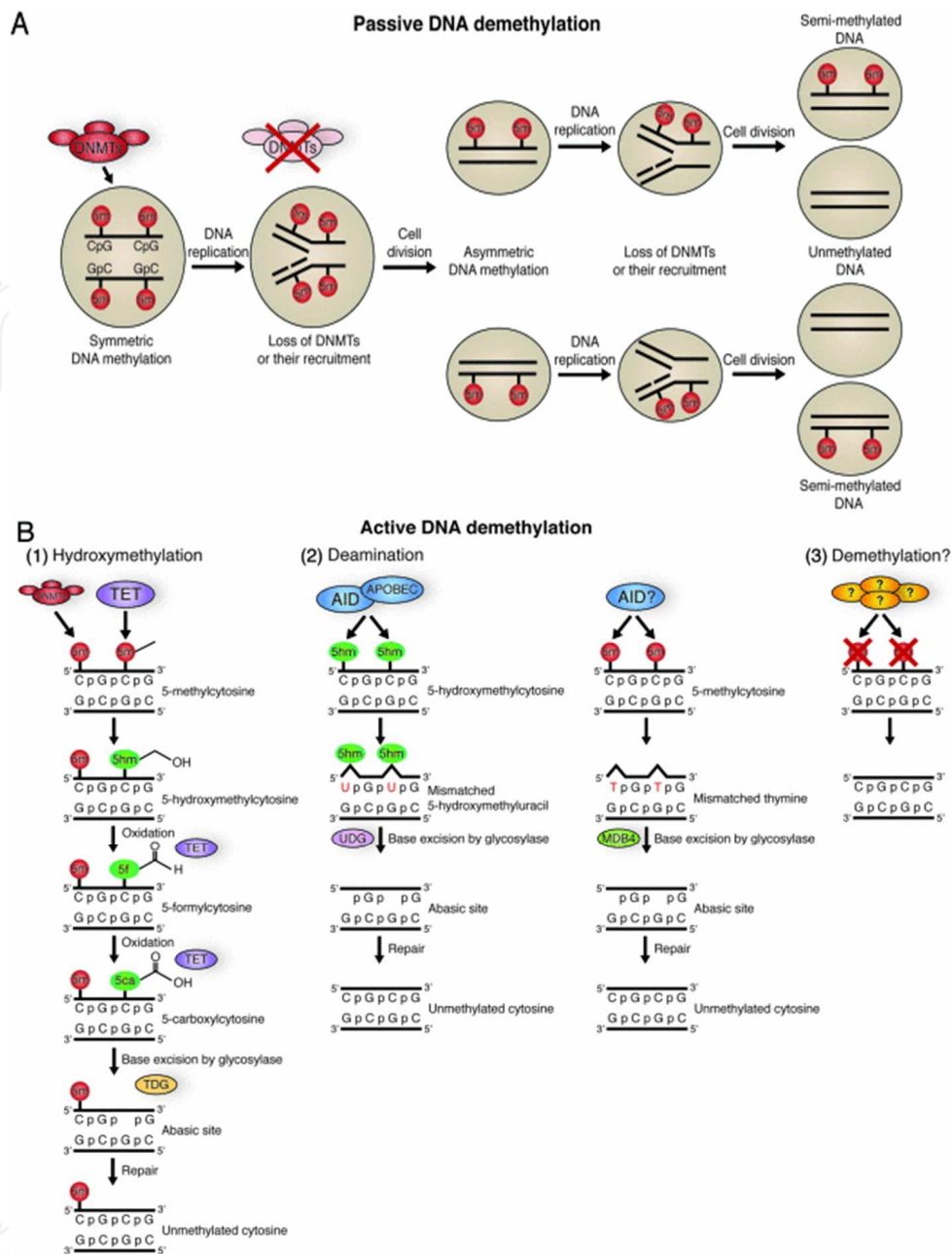
Figure 8. Active and passive DNA methylation mechanism (Adapted from 1)

DNA methylation can be achieved via two mechanisms, either actively or passively (Figure 8). Passive demethylation involves the inhibition of the DMNT protein during DNA replication and allows for newly synthesized cytosine to escape methyl imprinting from its parent DNA strand. This process usually occurs during cellular replication. Active demethylation can occur in both dividing and non-dividing cells [47]. Currently, there is no known enzyme that is able to remove the strong covalent bond of the methyl group from the cytosine residue. Instead, the methylated cytosine is thought to undergo a series of further modifications (AID/APOBEC) that ultimately change the 5mC into a thymine [48]. This elicits a base mismatch and activates the base excision repair pathway to replace the residue with a naked cytosine. Another

proposed demethylation pathway involves the use of the ten-eleven translocation (Tet) enzymes. This family of proteins are able to add a hydroxyl group onto the methyl moiety of 5mC to form 5hmC. Once in this state, 5hmC can return to an unmodified cytosine residue through either further oxidation by Tet enzymes or deamination by AID/APOBEC. Unlike the deacetylation and demethylation of histones, DNA demethylation is much more complex and involves a number of enzymatic processes which has contributed to the great deal of debate about which pathway is more dominant [50].

5. Epigenetic reprogramming

In order to understand the interactions and mechanisms involved in reprogramming a differentiated cell into an iPSC, a great effort has been made to study the ESC pluripotent state, in particular the means by which pluripotency transcription factors interact with each other or with other proteins such as chromatin remodeling enzymes and histone modifying enzymes. Moreover researchers have focused on finding the networks in iPSCs once the endogenous pluripotency factors have been activated by the exogenous Yamanaka factors [13]. Understanding the interactions between the core pluripotency transcription factors and the previously mentioned epigenetic enzymes will provide some advantages to the iPSC field. One such advantage is the possible discovery of new cocktails that enhance reprogramming. In addition, it could explain the chronology of the epigenetic events for reprogramming on a molecular level. This section will cover some of the known molecular interactions among the pluripotent transcription factors and some of the epigenetic enzymes.

5.1. Pluripotency gene networks

The first event toward transition from a differentiated to an iPSC state is the establishment of a proper chromatin state. Once the cells have found the correct chromatin state, the second event is to maintain and inherit it as they divide and proliferate [14]. The natural state of an ESC chromatin is known as "open", where the heterochromatin is disperse and dynamic, which at the same time reflects a hyperactive transcriptional status [49]. The molecular structure for ESC to maintain pluripotency requires an interconnection of transcription factors with epigenetic proteins that are also interacting with the DNA. Due to fact that iPSCs are like ESC, they have to sustain the same molecular structure. In addition, they have to overcome an epigenetic barrier during the reprogramming process. The reprogramming process involves a chain reaction involving transcription factors, chromatin modifying enzymes and other histone related enzymes.

An approach of how reprogramming occurs, suggests that the first step maybe interaction of transcription factors with the naked DNA, via histone modifiers or together with chromatin remodeling factors [14]. There are not time points or an order to follow for each specific transcription factor. Certain transcription factors are able to interact with DNA or with a chromatin remodeling enzyme depending what gene is activating. This molecular mechanism

is determined by the locus, the type of transcription factor and on the context [14]. Hence the function of the four Yamanaka factors, Oct4, Sox2, Klf4 and c-Myc, [13] is crucial.

It has been reported that Oct4 is indispensable in the reprogramming process. In some cases, such as what has been observed in neural stem cell reprogramming, the presence of Oct4 is sufficient for reprogramming in [51]. Moreover, as Oct4 can work alone, it also has a great effect in reprogramming when combined with Sox2. Oct4 and Sox2 form a heterodimer that interact with some promoters. In addition this heterodimer has been shown to interact with Nanog. Nanog is another transcription factor that participates in the ESCs regulatory circuitry together with Oct4 and Sox2 to maintain pluripotency [52]. In this context they activate transcription in a chromatin independent manner by interacting with transcriptional co-activators [53, 54]. Moreover, it has been shown in mouse ESCs that Oct4 and Nanog can repress gene expression through interaction with histone deacetylase such as Mta1 [55]. It has been elucidated that in the first stage of reprogramming, a cascade of differentiation genes are turned off, while pluripotency genes progressively become upregulated in order to push the differentiated cell toward an ESC-like state.

c-Myc is an important participant in recruiting multiple chromatin modifications, such as histone acetyltransferases (GCN5, p300) and histones deacetylases (HDACs). In this regard, c-Myc increases the methylation site H3K4me3 and the global acetylation [56]. In the reprogramming process c-Myc activates its target before other core pluripotency transcription factors are activated, facilitating the opening of the chromatin for other factors [57, 58]. An example of c-Myc's potential in opening chromatin is its association with Tip60-p400 complex, which acetylate and remodel nucleosomes respectively. p400 is a member of the Swi2/Snt2 family which is well known among the ATPase chromatin remodelling enzymes, exchanging histones H2AZ-H2B within nucleosomes [57]. It also functions to release paused RNA polymerase from about one-third of the genes that are being actively transcribed. This activity could enhance cellular reprogramming [59]. At the same time, the transcription factor Klf4, activates the transcription of Sox2 which participates in the pluripotency cascade [60].

5.2. Chromatin remodelling

Based on the Yamanaka's stochastic model (Figure 2), cells need to overcome the epigenetic barrier in order to become pluripotent [16, 17]. Nowadays, one of the major focuses in the iPSC field is to understand the epigenetic molecules that orchestrate chromatin remodelling in order to organize it into a pluripotent state similar to ESCs. While some somatic cell reprogramming mechanisms are being unveiled, many are still yet unknown [9].

• DNA methylation

Once the differentiated cells have been reprogrammed, the epigenetic marks in iPSCs resemble ESCs [61]. For a great amount of eukaryotic DNA methylation is a mark that serves to define different cellular functions such as X chromosome inactivation, aging, imprinting, genome stability, tissue specific gene regulation, and so on [63, 64]. DNA methylation is one of the epigenetic marks that is modified during reprogramming (Figure 9). In this case, the process of demethylation is most common taking the methyl group from the promoters of

some genes that are responsible for pluripotency which in turn allows them to return to a pluripotent state.

Currently there is not too much evidence about the process of demethylation and the enzymes that catalyze this event. However, DNA demethylation events have been classified as passive or active. Passive DNA demethylation occurs during the process of DNA replication when maintenance methytransferases are inactive, and thus they are not able to methylate newly released strands [62]. On the other hand for active DNA demethylation, the main protagonists are enzymes that work regardless of DNA replication [62].
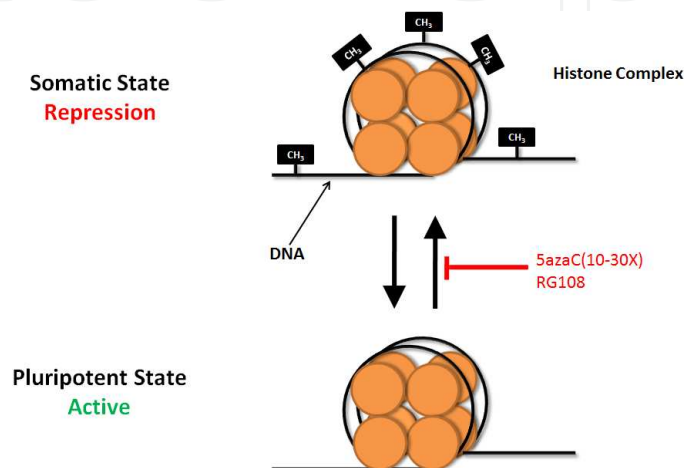
Figure 9. DNA demethylation as a reprogramming process when going to iPSC (Adapted from 13)

An example of an active DNA demethylation event is shown by Pereira and colleagues (2008), where they have studied the efficiency of reprogramming of human lymphocytes by fusing them with mouse ESCs. They found that one of the first events occurring was the demethylation of the Oct4 gene. They suggest that this event is a result of an active chromatin remodeling locus before its actual expression [65].

Two mechanisms have been proposed as candidates for the active DNA demethylation mechanism. The first one involves the deamination of 5-methylcytosine in DNA by an enzyme called activation-induce deaminase (AID) [62]. The other mechanism is based on the oxidization of the 5-methyl group ($-CH_3$) followed by conversion into 5-carboxylcytosine (-COOH). This conversion is catalyzed by the enzyme TET1 in a Fe(II) and α-ketoglutarate dependent reaction [66]. To date neither of these mechanisms has been proven *in vitro.* Hence there are still a number of unknown molecular mechanisms that govern the reprogramming process. Is there an active DNA demethylation? How are the DNS demethylating enzymes activated? Are they recruited by other processes of chromatin remodeling or do transcription factors initialize the process?

• Histone modifications

The most common marks in ESC and iPSCs are the active mark of H3k4me3 (histone 3, trimethylated at lysine 4) and the repressive mark H3k27me3 (histone 3, trimethylated at lysine

27). These marks in the histones occur by S-adenosylmethionine (SAM-dependent) protein methylation. Due to this bivalent mark in the histones, these cells have the capacity to activate or repress genes them in order to change their fate [67].

Since the histone-lysine N-methyltransferase (MLL) catalytic subunits are well known to introduce the H3k4me3 mark and activate transcription, this makes them potential regulators in reprogramming. An example of their activity is shown with Wdr5, a subunit in common with H3k4 methyltransferases. Wdr5 has been proposed to play an important role in maintaining pluripotency and has been proposed as one possible mechanism occurring during reprograming. Wdr5 is activated by exogenous Oct4 when mouse embryonic fibroblast are transfected with the four Yamanaka factors. Wdr5 directly binds to loci where self-renewal genes are encoded such as Oct4 and Nanog, in order to re-establish an H3k4 mark. Wdr5 is thus defined as an indispensable subunit which proportions H3k4 methylation [68]. Similarly, MLL interacts with some other chromatin remodeling enzymes, such as CHD1 and NURF, in order to achieve H3k4 methylation [29, 69].

It has been already mentioned that ESCs and iPSCs are known to have bivalent chromatin bearing both the active mark H3k4me3, and the repressive mark H3k27me3. One of the mechanism that controls the bivalency is through the activity of the polycomb proteins found in two major complexes PRC1 and PRC2 [70]. It is thought that PRC1 and PRC2 act as antagonists and are intrinsically involved in establishing the fate of ESC development. PRC2 is in charge of the H3k27me3 mark [71] and known to silence the HOX genes used and other regulators during ESC differentiation [72]. One of the basic mechanisms in ESC/iPSC differentiation is the demethylation of this H3k27me3 mark.

Utx demethylase has been reported to be a significant regulator of cellular reprogramming [73]. Utx is encoded by an X-chromosome gene and belongs to the small family of Jmjc proteins, mediating the demethylation of H3k27 tri- and di-methyl repressive chromatin marks. In this study, it was found that Utx was dispensable for the maintenance of pluripotency, since pluripotency marker expression was maintained in knockout ESC lines. However fibroblasts derived from Utx knockout mice failed to be reprogrammed. This result indicated that the absence of Utx prevented the demethylation of H3k27me3 marks needed to re-establish pluripotency *in vitro* [73].

H3K9me3 and H3k79me2 have also been reported to be important for the maintenance of pluripotency in ESCs. These marks are usually left by repressive methyltransferases via the identification of specific motifs in heterochromatin. Reprogramming has been facilitated by the inhibiting two methyltransferases (GLP and G9a) that methylate H3k9 [74].

Histone acetylation is another important histone mark that has usually been correlated with gene activation (Figure 10). This mark has been reported to transform chromatin during reprogramming. Little is known about histone acetyltransferases for pluripotency maintenance or reprogramming, however Tip60/p400 has been reported as a histone acetyltransferase important for maintaining the ESC state. Here Tip60/p400 also works as a chromatin remodeling enzyme, since it has a SWI2/SNF2 subunit [75].
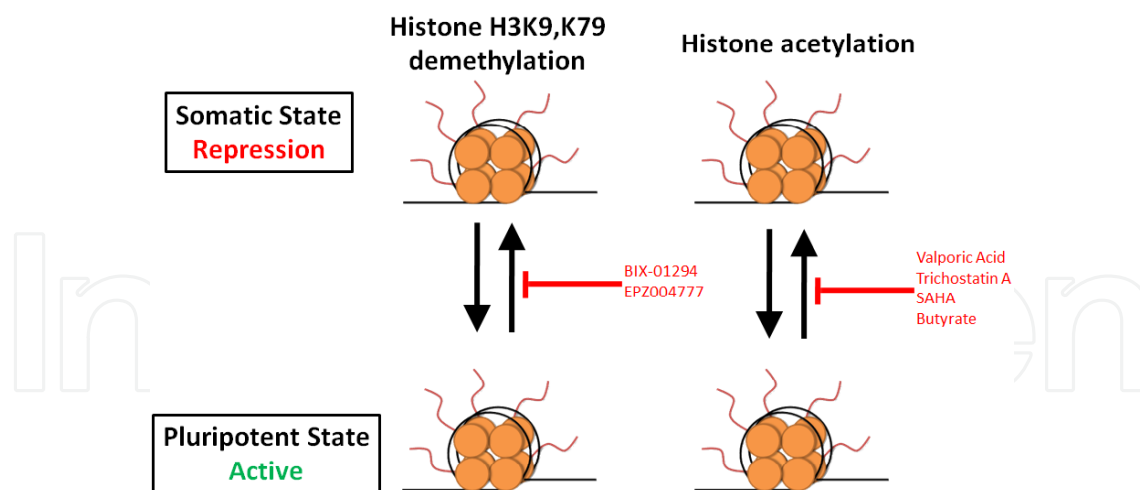
Figure 10. Histone acetylation as an activating mark for reprogramming. (Adapted from 13)

On the other hand the histone deacetylases (HDACs) are known to repress the expression of genes, therefore there is an increased interest on their inhibition. An example of their importance in reprogramming of somatic cells was shown by Hadas Hezroni and collaborators (2011) [76]. In this study they used hybrid cell lines by fusing mouse embryonic fibroblast with ESCs and found that low H3k9 acetylation correlated with low reprogramming capacity. When they tried to overcome this effect using histone deacetylase inhibitors, they found an increase in the reprogramming efficiency. They reported that genes involved in extracellular matrix (ECM) activity were enriched during reprograming and concluded that H3K9ac is a mark intrinsically related to pluripotency and that promoting its increase using HDACs inhibitors promote ECM activity, which co-relates positively affect pluripotency and self-renewal [76].

Most epigenetic reprogramming studies have focused on isolated chromatin marks, revealing the down regulation of somatic genes. However there are more than some marks that lead to an "open" dynamic chromatin. Anna Mattout and colleagues [77] presented a study where for the first time they showed chromatin dimensions as global changes occurring during reprogramming. They analyzed a battery of histone modifications (H3ac, H4ac, H4k5ac, H3k27ac, H3k4me3, H3k36me2, H3k9me3, and H3k27me3 also γH2AX, HP1α and lamin A, by immunofluorescence and biochemical fractionations comparing mouse ESCs to fully- and partially-reprogrammed mouse iPSCs. They first identified that H3k36me2, H4k5ac and H3k4me3 have the highest correlation with pluripotency. Later, they showed that most of the euchromatin/active marks (H3ac, H3k9ac, H3k27 ac, H4ac, H4k5ac, H3k4me3 and H3k36me2) are higher in the ESCs and fully reprogrammed iPSCs, whereas in partially reprogrammed cells these marks more closely resembled that of mouse embryonic fibroblasts. On the other hand they observed that the marks in heterochromatin, such as HP1α and H3k9me3 rearrange during reprogramming towards a more diffused pattern. This was seen in all of the cells lines including partially reprogrammed iPSCs. With these two phenomena they presented a time line suggesting that marks in heterochromatin start changing at a very early stage (by day 6 during reprogramming) compared to the histone changes occurring in the active euchromatin. They concluded that during reprogramming global histone heterochromatin defining marks start changing and

spreading at an early stage of reprogramming as a form of physical rearrangement prior to the euchromatin epigenetic alterations which occur after day 7 (Figure 11) [77].
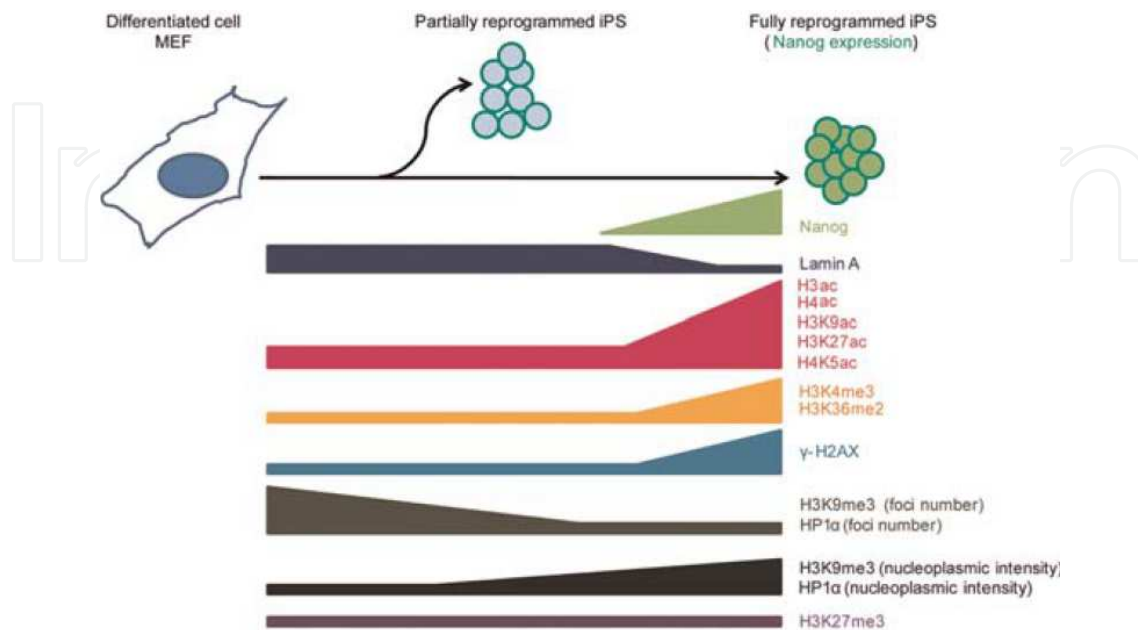
Figure 11. Global epigenetic changes in iPSCs (Adapted from 80).

• Chromatin remodeling

As previously mentioned, chromatin remodeling is caused by catalytic modification where ATPases use the energy from the ATP to move along in DNA. Thus they regulate gene expression by spacing nucleosome arrays, exchanging histone variants, disassembling or sliding the nucleosome [14]. One example of the importance of the chromatin remodeling enzymes is observed in a study of Brg, part of a family of DNA ATPases homologous to the catalytic subunit of yeast SWI2/SNT2 ATPase [78]. Brg is assembled to 11 other Brg/Brahma associated factors (BAFs). In ESCs, BAF complexes have an exclusive subunit which is called esBAF. The authors observed that esBAF facilitates STAT3 to access binding sites that will respond to LIF, which will further activate the pluripotency transcription factor Klf4 [78]. Therefore, it is thought that the LIF signaling pathway is dependent on prior chromatin remodeling [78]. A previous study showed that overexpressing of esBAF in addition to the four Yamanaka was able to acquire a euchromatic chromatin by increasing the kinetics of Oct4, Nanog and Rex1 promoter demethylation. This facilitated the accessibility of the reprogramming factors and hence the process was enhanced [28].

Moreover, Onder and colleagues (2012) [79] focused on the study of chromatin-modifying enzymes during the reprogramming process in iPSCs. They used a loss of function approach with shRNA where they selected 22 genes involved in DNA and histone methylation pathways. From their results, they found that inhibition of the histone methyltransferase DOT1L, reprogramming was enhanced resulting in more iPSCs colonies. DOT1L inhibition does not enhance the upregulation of the pluripotency gene network but can be used to substitute for

Klf4 and c-Myc during reprogramming. Inhibition of this molecule is associated with an increase of Nanog and Lin28, factors, which are necessary for reprogramming. Finally using ChIP-seq they found that the H3K79me2 mark was lost in genes that participate in epithelial mesenchymal transition. Among some of the mesenchymal regulators were SNAI1, SNAI2, ZEB1, ZEB2 and TGFB2, that at the same time where strongly repressed during reprogramming. Together with this, they also reported that epithelial genes such as CDH1 (E-cadherin) and OCLN were upregulated. The above was a clear example of how chromatin modifying enzymes are critical in the molecular process of reprogramming enhancing the cascade that begins with the four Yamanaka factors [79].

The above are just some of the studies of many that have been reported. They lead us to question whether remodeling enzymes are in charge of the major chromatin opening that occurs during reprogramming, or if histone marks lead the process.

5.3. MicroRNAs in reprogramming

MicroRNAs (miRs) are small RNAs involved in the inhibition of the gene expression by destabilizing target RNAs. They are usually formed by the proteins Dicer and Drosha with its cofactor Dgcr8. The importance of miRs arises from the observation that some miRs induce reprogramming of somatic cells into iPSCs [80]. Among some of the miRs found to positively regulate ESC pluripotency are: ESC cell-cycle regulating miR291a-3p, miR291b-3p, miR294, miR295 and miR302. Interestingly, miR302 has been reported regulate some of the epigenetic modifications that occur during reprogramming. miR302 is a family of four highly homologous microRNAs that are transcribed together and form a noncoding RNA cluster [81]. They are highly expressed in human ESCs and absent in differentiated cells. Lin and collaborators (2011) have focus in how the miR302 controls several enzymes that are involved in active demethylation [81]. MiR302 targets and represses AOF2/1 histone demethylases and MECP1/2 (methyl CpG binding proteins). At the same time it blocks cytosine methyltransferase 1 (DNMT1). During reprograming miR302 coordinates DNA demethylation, together with a histone methylation on the active mark H3k4 that will alter the chromatin structure and the gene activity. The example above highlights the critical role of miRs in founding and sustaining pluripotency in cells [82].

6. Conclusion

The biology of pluripotent stem cells is still in a very early stage; even understanding what is the best way to obtain a true embryonic stem cell remains unclear. During reprogramming a number of changes occur in the cell. These changes start usually by the stimulation of exogenous transcription factors that consequentially trigger a large number of other reactions: signalling, gene transcription, and most importantly epigenetic changes, including chromatin remodeling, histone modification, and DNA methylation [1, 12, 13].

During reprogramming, chromatin changes to an "open" dynamic configuration resembling the epigenetic landscape as in ESCs. In order to reach this configuration, the somatic cell has

to interconnect transcription factors, chromatin, and histone modifier enzymes. What is the kinetics of this process? This is one of the first questions that puzzle most researchers. While there is firm understanding that the exogenous transcription factors are first to prompt these changes, reprogramming is not an efficient process. From the extensive interest in making reprogramming an efficient process, there have been a lot of remarkable results using different type of molecules that target chromatin enzymes. Thus understanding of the reprogramming process, including the timing of chromatin remodeling, interactions with transcription factors, increase or decrease of histone acetylation and most important, the precise interconnection of factors that break the epigenetic barrier, will give us a base line to design a better protocol for the develop of iPSCs.

There will come a point where researchers will manipulate chromatin kinetics in order to promote the reprogramming of somatic cells into iPSCs. This achievement will bring a cell that reprograms efficiently; in a short period of time, which will have an epigenetic signature identical to ESCs. In the future, with a better understanding of chromatin not only in reprogramming but also in differentiation, the iPSC field could become an area of synthetic biology. In any case, the iPSC field still has a long way to go before it is fruitful. A clearer understanding of the epigenetics of the reprogramming must come forward before iPSCs can be fully accepted for regenerative medicine.

Author details

Perla Cota, Mehdi Shafa and Derrick E. Rancourt[*]

*Address all correspondence to: rancourt@ucalgary.ca

Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada

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