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## Mechanisms and models of somatic cell reprogramming

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### Abstract

Conversion of somatic cells to pluripotency by defined factors is a long and complex process that yields embryonic stem cell-like cells that vary in their developmental potential. To improve the quality of resulting induced pluripotent stem cells (iPSCs), which is important for potential therapeutic applications, and to address fundamental questions about control of cell identity, molecular mechanisms of the reprogramming process must be understood. Here we discuss recent discoveries regarding the role of reprogramming factors in remodeling the genome, including new insights into the function of c-Myc, and describe the different phases, markers and emerging models of reprogramming.

### Introduction

Resetting the epigenome of a somatic cell to a pluripotent state has been achieved by somatic cell nuclear transfer (SCNT), cell fusion, and ectopic expression of defined factors such as Oct4, Sox2, Klf4 and c-Myc (OSKM)<sup>1–3</sup>. Understanding the molecular mechanisms underlying somatic cell reprogramming to pluripotency is critical for the creation of high-quality pluripotent cells and may be useful for therapeutic applications. Moreover, gaining insight from *in vitro* reprogramming approaches may yield relevant information for SCNT or cell fusion-mediated reprogramming and may broaden our understanding of fundamental questions regarding cell plasticity, cell identity and cell fate decisions<sup>4–6</sup>.

Reprogramming by SCNT is rapid, is thought to be deterministic and yields embryonic stem cells (ESCs) from the cloned embryo that are similar to ESCs derived from the fertilized embryo<sup>7,8</sup>. However, the investigation of SCNT and cell fusion is difficult because oocytes and ESCs contain multiple gene products that may be involved in reprogramming. In contrast, in the transcription factor-mediated reprogramming method, the factors that initiate the process are known and can be easily modulated which makes examination of the process less complicated and easier to follow. However, the process is long, inefficient and generates induced pluripotent stem cells (iPSCs) that vary widely in their developmental potential<sup>1,2,9,10</sup>.

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In this review, we focus on recent studies and technologies aimed at understanding the molecular mechanisms of cellular reprogramming mediated by transcription factors. For example, insights have been gained from methods to study single cells as well as studies of populations of cells undergoing reprogramming. We describe current views of the phases of transcriptional and epigenetic changes that occur and discuss new concepts regarding the role of OSKM in driving the conversion to pluripotency. We then consider markers of cells progressing through reprogramming and emerging models of the process. Finally, we summarize criteria that allow assessment of iPSC quality.

## Phases of reprogramming

### Insights gained from population-based studies

After the first demonstration of reprogramming to pluripotency by defined factors<sup>11,12</sup>, many groups raced to study the reprogramming process by analyzing transcriptional and epigenetic changes in cell populations at different time points after factor induction. These are the most straightforward experiments to perform for unraveling the molecular mechanism of this complicated process. Most studies analyzing cellular changes during the reprogramming process were performed using populations of mouse embryonic fibroblasts (MEFs).

Microarray data at defined time points during the reprogramming process<sup>13</sup> showed that the immediate response to OSKM is characterized by de-differentiation of MEFs and upregulation of proliferation genes, consistent with the expression of c-Myc. Gene expression profiling and RNAi screening in fibroblasts revealed three phases of reprogramming termed initiation, maturation, and stabilization; the initiation phase marked by a mesenchymal-to-epithelial transition (MET)<sup>14,15</sup>. Also, BMP signaling has been shown to synergize with OSKM to stimulate a microRNA expression signature associated with MET-promoting progression through the initiation phase<sup>15</sup>.

The late maturation and stabilization phases have been studied by tracing clonally-derived cells<sup>16</sup>. This study showed that repression of the OSKM transgenes is required for the transition from maturation to the stabilization phase. By comparing the expression profiles of clones that could transit from the maturation to stabilization phase to those that could not, the authors found a unique signature associated with competency. Surprisingly, few pluripotency regulators played a role in the maturation-to-stabilization transition. Rather, genes that are associated with gonads, gametes, cytoskeletal dynamics and signaling pathway were upregulated during this phase<sup>16</sup> (Figure 1). The authors also found that genes that are induced upon transgene inhibition (for example, *Eras* and *Lefty2*) tend to be important for ESC maintenance, whereas genes that retain a similar expression level before and after transgene silencing (for example, *Arid3b* and *Sall1*) tend to be involved in regulating the maturation-stabilization transition. This study suggests that the transition to the stabilization phase upon transgene removal is dependent on regulatory pathways distinct from those controlling ESC pluripotency<sup>16</sup>.

Another study used genome-wide analyses to examine intermediate cell populations poised to become iPSCs<sup>17</sup>. This study revealed two distinct waves of major gene activity: the first

wave occurred between days 0 and 3; and the second wave started after day 9, which is toward the end of the process (day 12). The number of differentially expressed genes between progressing cells and cells that are refractory to reprogramming at each time point was gradually increased, reaching 1,500 genes by the end of the process<sup>17</sup>. The first wave was characterized by the activation of genes responsible for proliferation, metabolism, cytoskeleton organization, and downregulation of genes associated with development (Figure 1). This step occurred in the majority of cells and is equivalent to the initiation phase described above. Several early pluripotency-associated genes were upregulated gradually and some developmental and cell-type-specific genes were transiently regulated during the process. The second wave was characterized by the expression of genes responsible for embryonic development and stem cell maintenance. Genes from this step facilitate the activation of the core pluripotency network and mark the acquisition of a stable pluripotent state. In contrast, genes related to extracellular space or matrix, plasma membrane, retinoic acid binding, and immune response processes were aberrantly expressed in cells refractory to reprogramming<sup>17</sup>.

In agreement with these findings, quantitative proteomic analysis during the course of reprogramming of fibroblasts to iPSCs revealed a two-step resetting of the proteome during the first 3 days and last 3 days of reprogramming<sup>18</sup>. Proteins related to regulation of gene expression, RNA processing, chromatin organization, mitochondria, metabolism, cell cycle and DNA repair were strongly induced at an early stage and proteins related to the electron transport system were downregulated. In contrast to these processes, glycolytic enzymes exhibited slow increase in the intermediate phase, suggesting a gradual transformation of energy metabolism<sup>19</sup>. Proteins involved in vesicle-mediated transport, extracellular matrix, cell adhesion and EMT were downregulated in the early phase, retained low levels during the intermediate step and became up regulated in the final stage<sup>18</sup>. These data suggest that reprogramming is a multi-step process characterized by two waves of transcriptome and proteome resetting<sup>20</sup>.

### Insights gained from single-cell studies

Knowledge gained from population-based studies is essential for the understanding of the global changes that occur in cells during the reprogramming process. A challenge for gaining mechanistic insights of reprogramming by the analysis of cell populations is cell heterogeneity. Because only a small fraction of the induced cells become reprogrammed, gene expression profiles of cell populations at different time points after factor induction will not detect changes in rare cells destined to become iPSCs. In an attempt to overcome the problem of cell heterogeneity, reprogramming has been traced at single-cell resolution using time-lapse microscopy<sup>21,22</sup>. Single-cell tracking by real time microscopy has given insights into morphological changes during reprogramming but the approach has not provided information on molecular events driving the process at the single-cell level. These studies showed that the cells underwent a shift in their proliferation rate and reduction in cell size soon after factor induction. These events occurred within the first cell division and with the same kinetics in all cells that give rise to iPSCs.

As a complementary approach to the population-based studies, two single-cell techniques have been utilized to quantify gene expression in the rare cells that undergo reprogramming<sup>23</sup>: Fluidigm BioMark, which allows quantitative analysis of 48 genes in duplicate in 96 single cells<sup>24–27</sup>; and single-molecule-mRNA fluorescent *in situ* hybridization (sm-mRNA-FISH), which enables the quantification of mRNA transcripts of up to three genes in hundreds to thousands of cells<sup>28</sup>. The 48 genes in the BioMark system included those known to be involved in major events that occur during reprogramming (for example, proliferation, epigenetic modification, ESC supporting pathways, pluripotency markers and MEF markers). In the first six days after factor induction, there was high variation amongst cells in expression of the 48 genes<sup>23</sup>. This suggests that early in the reprogramming process OSKM induce stochastic gene expression changes in a subset of pluripotency genes, which are critical for instigation of the second phase (Figure 1). These stochastic changes are in addition to the alterations in expression of genes that control MET, proliferation and metabolism, which are global changes that must occur during reprogramming but are not restricted to cells that are destined to become iPSCs<sup>15–17</sup>. Single-cell analyses of clonally derived cell populations revealed that the stochastic gene expression phase is long and variable<sup>23</sup>. Although cells with an ESC-like morphology appear early, they must pass through a bottleneck - likely a rate-limiting stochastic event - before transiting into stable iPSCs<sup>23,29</sup>. At a later stage, when the cells start to express *Nanog*, the variation between individual cells decreases dramatically, consistent with a model in which the early stochastic phase of gene expression is followed by a deterministic or more “hierarchical” phase leading to activation of the pluripotency circuitry. This deterministic or hierarchical phase is discussed further below in the context of models of reprogramming.

## Epigenetic changes

The studies discussed above characterized phases of transcriptional changes during reprogramming, so what are the epigenetic alterations that underlie these changes and what might drive them? The epigenetic signature of the somatic cell must be erased during the conversion in order to adopt a stem cell-like epigenome. These changes include chromatin reorganization, DNA demethylation of promoter regions of pluripotency genes like *Nanog*, *Sox2* and *Oct4*, reactivation of the somatically silenced X chromosome, and genome-wide resetting of histone posttranslational modifications<sup>11,30–32</sup>. There are more than 100 different histone posttranslational modifications, with lysine methylation and acetylation being the ones studied most frequently<sup>33</sup>. Changes in histone marks and the role of various chromatin modifiers during reprogramming have been extensively reviewed elsewhere<sup>4,34,35</sup>, so here we briefly summarize the key points. The roles of the relevant histone marks and of chromatin modifiers are summarized in table 1 and table 2, respectively.

DNA demethylation and X reactivation occur late in the reprogramming process<sup>17</sup>, whereas changes in histone modifications can be seen immediately after factor induction<sup>36</sup>, suggesting that changes in histone marks are an early event that is associated with initiation of the reprogramming process. Immediately after factor induction, a peak of *de novo* deposition of H3K4me2 is observed at promoter and enhancer regions. At this time,

H3K4me2 accumulates at the promoters of many pluripotency genes, such as *Sall4* and *Fgf4*, which are enriched for Oct4 and Sox2 binding sites and lack H3K4me1 or H3K4me3 marks<sup>36</sup>. This stage is also associated with a gradual depletion of H3K27me3 and promoter hypomethylation in regions that are important for the conversion<sup>17</sup>. However, at early time points, H3K4me2 does not correlate with the transcription-associated histone mark H3K36me3, occupancy of RNA PolIII, or transcriptional activity suggesting that these loci have not completed chromatin remodeling at early time points and an additional step is required to achieve full activation of these genes<sup>36</sup>. At the beginning of the reprogramming process, changes in these modifications are restricted almost exclusively to CpG islands, as these regions are more responsive to transcription factor activity and permissive to changes<sup>37</sup>. In parallel, the promoters of somatic genes begin to lose H3K4me2, consistent with early down-regulation of MEF markers such as *Thy1* and *Postn*<sup>38,39</sup>. A large number of somatic gene enhancers also lose H3K4me2; this change leads to hypermethylation and silencing at later stages. Thus, epigenetic modifications of key MEF-identity factors and early pluripotency genes resulting in changes in their expression may represent one of the first steps in the conversion of somatic cells to a pluripotent state.

### Chromatin modifiers involved in reprogramming

Although histone marks are robustly modified during reprogramming, it is not clear which chromatin modifiers participate in reshaping the epigenomic landscape of the somatic cells and how they are targeted to genes whose altered expression is crucial for the conversion. It is reasonable to assume that OSKM binding sites throughout the genome mark regions that will be eventually epigenetically modified. Consistent with this notion is the finding that Oct4 interacts with the WD-repeat protein-5 (Wdr5), a core member of the mammalian Trithorax (*trxG*) complex, on pluripotency gene promoters and this maintains global and localized H3K4me3 distribution<sup>40</sup>. The H3K27 demethylase enzyme Utx physically interacts with OSK (Oct4, Sox2, Klf4) to remove the repressive mark H3K27me3 from early activated pluripotency genes such as *Fgf4*, *Sall4*, *Sall1* and *Utf1*<sup>41</sup>. Loss of Utx is associated with aberrant H3K27me3 distribution throughout the genome and with inhibition of reprogramming<sup>41</sup>. Tet1 and Tet2, two methylcytosine hydroxylase family members that are important for the early generation of 5-hydroxymethylcytosine (5hmC) during reprogramming, can be recruited by Nanog to enhance the expression of a subset of key reprogramming target genes such as *Nanog* itself, *Esrrb* and *Oct4*. Tet1 and Tet2 thus appear to be involved in the demethylation and reactivation of genes and regulatory regions that are important for pluripotency<sup>42–44</sup>. The poly (ADP-ribose) polymerase-1 (Parp1) has a complementary role in the establishment of early epigenetic marks during somatic cell reprogramming by regulating 5-methylcytosine (5mC) modification<sup>43</sup>. Brg1 and Baf155, two components of the BAF chromatin remodelling complex, enhance reprogramming by establishing a euchromatic chromatin state and enhancing binding of reprogramming factors to key reprogramming gene promoters<sup>45</sup>. Overexpression of Brg1 and Baf155 induces OSKM-mediated demethylation of pluripotency genes such as *Oct4*, *Nanog* and *Rex1* and enhances conversion to iPSCs.

Many other chromatin modifiers have been shown to play a role in resetting the epigenome of reprogrammable cells (summarized in table 2). For example, Kdm2a and Kdm2b, which

are H3K36me2 demethylases, cooperate with Oct4 and play a role in facilitating the reprogramming process by regulating H3K36me2 levels at the promoters of early-activated genes: mainly epithelial-associated genes, the microRNA 302/367 cluster and early pluripotency genes<sup>46,47</sup>. In the conversion of human fibroblasts to iPSCs, the H3K9 methyltransferases EHMT1 and SETDB1, and five components of the Polycomb repressive complexes (PRC) (BMI1, RING1 from PRC1, and EZH2, EED and SUZ12 from PRC2), are required to reset the epigenome of the somatic cells; loss of these genes significantly reduces iPSC formation<sup>48</sup>.

Another H3K9 methyltransferase, SUV39H, which contributes to heterochromatin formation<sup>49</sup>, hinders the reprogramming process. This suggests that loss of SUV39H may have a global effect on chromatin organization that leads to aberrant transcriptional regulation or that H3K9 methyltransferases have different specificities, with some targeting somatic state-associated genes and others targeting pluripotency-associated genes. Similarly, the histone H3 lysine 79 (H3K79me2) methyltransferase DOT1L inhibits the reprogramming process in the early to middle phase. Loss of DOT1L increases reprogramming efficiency by facilitating loss of H3K79me2 from fibroblast-associated genes such as the mesenchymal master regulators, *SNAI1*, *SNAI2*, *ZEB1*, and *TGFB2*. Silencing of these genes is essential for proper reprogramming and indirectly increases the expression of the pluripotency genes *NANOG* and *LIN28*<sup>48</sup>.

It will be interesting to explore whether specific combinations of chromatin modifiers are able to reset the epigenome of a somatic cell and to reprogram it to pluripotency in the absence of pluripotency factors. In addition, these data raise the question whether the four factors themselves act as pioneer factors that direct conversion by physical interaction with epigenetic and transcriptional regulators.

## Roles of the OSKM factors

### OSK as pioneer factors

Little is known about how ectopic expression of OSKM drives the conversion of somatic cells to the pluripotent state. It has been shown that the first transcriptional wave is mostly mediated by c-Myc and occurs in all cells whereas the second wave is more restricted to reprogrammable cells and involves a gradual increase in the expression of Oct4 and Sox2 targets, leading to the activation of other pluripotency genes that aid in the activation of the pluripotency network. Klf4 seems to support both phases by repressing somatic genes during the first phase and facilitating the expression of pluripotency genes in the second phase<sup>17</sup>.

In mouse or human fibroblasts, immediately after factor induction, OSKM occupy accessible chromatin, binding promoters of genes that are active or repressed<sup>34,36,38,50</sup>. In addition, OSK proteins become associated with distal elements of many genes throughout the genome that display minimal, if any, preexisting histone modifications or DNase I hypersensitivity (Figure 2)<sup>50</sup>. Thus, the multiple distal genomic sites initially occupied by OSK do not correspond to the distal genomic regions that are bound by these pluripotency factors in ESCs; we will refer to this atypical binding of ectopic OSK in somatic cells as “promiscuous binding” throughout the manuscript. Based on these observations it has been



suggested that OSK may act as “pioneer” factors that open chromatin regions and allow the activation of those genes that are essential for establishment and maintenance of the pluripotent state<sup>50</sup>, whereas c-Myc only facilitates this process (the mode of action by which c-Myc aids in the conversion is discussed extensively in the next section).

The initial promiscuous binding of OSKM, when expressed in fibroblasts, to target sequences present in many genomic regions raises the question of their molecular role in the conversion of somatic cells to pluripotent cells. Vector transduction-mediated or doxycycline-induced expression of the reprogramming factors in fibroblasts probably does not mimic the expression mode of the endogenous genes in ESCs, in terms of expression levels and factor stoichiometry. This may result in the widespread and seemingly promiscuous binding of OSKM to multiple regions in the genome, many of which are not occupied by these factors in ESCs. Possibly, OSKM can interact with the Mediator or Cohesin complexes or with RNA pol II elongation factor El13 and recruit them initially to atypical distal enhancers to aid in the opening of these “closed” regions<sup>51,52</sup>. Mediator bridges interactions between transcription factors at enhancers and the transcription initiation apparatus at core promoters and in combination with RNA polymerase II and TATA-binding protein (TBP) may gradually initiate transcription from those “blocked” regions<sup>51</sup>. Binding of the “pioneer” factors OSK to “super enhancers” and the recruitment of the Mediator complex may provide cell type specificity<sup>53</sup> at later stages in the reprogramming process. Supporting the notion that OSKM are capable of “loosening” chromatin and inducing cell plasticity early in reprogramming is the observation that transient expression of the factors is sufficient to open the chromatin and to induce transdifferentiation of fibroblasts to other somatic cells, such as cardiomyocytes and neural progenitor cells<sup>54,55</sup>.

Though the four factors often bind jointly to their targets, subsets and different combinations of the factors frequently occupy non-overlapping genomic regions. For example, Klf4 and c-Myc frequently bind jointly to promoters, whereas all the other OSKM combinations predominantly occupy distal elements, at sites conserved between human and mouse<sup>50</sup>. OSKM bind together at gene regions that initiate and support the conversion to pluripotency, such as *Glis1*, *mir-302/367* cluster, *Fbxo15*, *Fgf4*, *Sall4* and *Lin28*, and factors that promote mesenchymal to epithelial transition (MET)<sup>14,23,50,56–59</sup>. However, only half of the enhancers that acquire H3K4me2 in the induced cells are shared enhancers with ESCs<sup>36</sup> with the other half representing enhancers that are not ESC-specific, supporting the promiscuous binding of OSKM to various genomic regions that aid in the conversion process (Figure 2). Also, in addition to the four factors, activation of other genes early in the reprogramming process may affect the efficiency and specificity of OSKM binding. Binding of the “pioneer” factors OSK in combination with c-Myc to enhancer regions that are not ESC-specific results in ectopic gene expression. This may render the initial cells susceptible to other gene expression changes, such as activation of apoptotic genes, metabolic genes and MET-inducing genes, silencing of MEF specific genes and eventually activation of pluripotency genes<sup>17</sup> (Figure 2).



## Revisiting the function of c-Myc in reprogramming

Because c-Myc enhances the transcription of proliferation-associated genes<sup>60–62</sup>, its role in cellular reprogramming was initially attributed to its ability to promote proliferation and to activate a set of pluripotency genes and microRNAs. C-Myc is a basic helix loop helix (bHLH) transcription factor that at basal levels interacts with Max on actively transcribed genes via E-box sequences<sup>63</sup>. It has been shown to be dispensable for reprogramming but facilitates the emergence of rare reprogrammed cells<sup>64,65</sup>. Supporting this observation is the finding that c-Myc does not greatly contribute to the activation of pluripotency regulators in partially reprogrammed cells and that its expression is essential only for the first five days<sup>38</sup>. However, in ESCs, c-Myc augments the transcription elongation of many actively transcribed genes via their core promoter regions and by these means maintains pluripotency<sup>66</sup>.

Recently, the role of c-Myc during transcription has been revisited, and it has been demonstrated that c-Myc does not regulate a unique set of target genes but rather acts as a general amplifier of gene expression, increasing the transcription at all active promoters<sup>67,68</sup>. In contrast to many other transcription factors that activate genes in a binary switch way<sup>69</sup>, c-Myc binding resembles a continuous, analog process<sup>67</sup>: c-Myc binding to promoter regions is associated with open chromatin marks including H3K4me3 and H3K27ac and is correlated with the amount of RNA polymerase recruited at those promoters<sup>67,68</sup>. C-Myc recruits the pause release factor P-TEFb, increases transcriptional elongation and the transcription levels<sup>66,70,71</sup> and when overexpressed, its localization to the enhancers of active genes is increased substantially through binding to a variant E-box motif. When OSK are overexpressed together with c-Myc, OSK act as pioneer factors to enable c-Myc to bind to regions that are in inaccessible chromatin. In parallel, driven in part by a variant c-Myc binding site<sup>50</sup>, c-Myc also cooperatively enhances the initial OSK engagement with chromatin. Continuous binding of the factors to those “blocked” distal elements leads to binding at the promoters of genes that acquire a *de novo* H3K4me2, and eventually leads to the transcription of those genes.

It will be interesting to examine whether in cancer cells other pioneer factors recruit c-Myc to specific “blocked” regions through the variant E-box motif. Given this notion, c-Myc expression should enhance any given transdifferentiation or cellular reprogramming process. However, expression of c-Myc in combination with transcription factors that generate iPSCs but lack Oct4 (such as Sall4, Nanog, Esrrb and Lin28) only slightly enhanced the reprogramming process<sup>23</sup>, suggesting that different key factors have a different affinity for c-Myc. Future studies should address how different key factors cooperate with this master transcriptional amplifier.

## Factor stoichiometry

The number of proviruses in iPSCs differs widely among the factors, suggesting that reprogramming requires different expression levels of the individual factors<sup>23,31</sup>. Indeed, factor stoichiometry can profoundly influence the epigenetic and biological properties of iPSCs, as was demonstrated by comparing two genetically characterized doxycycline-inducible transgenic “reprogrammable” mouse strains<sup>72,73</sup>. The authors showed that,

although a high number of iPSC colonies could be obtained, about 95% exhibited aberrant methylation of the *Dlk1-Dio3* locus and were unable to generate mice derived entirely from iPSCs (“all-iPSC” mice) by tetraploid complementation, which is the most stringent test for pluripotency<sup>73</sup>. In contrast, another study using an almost identical “reprogrammable” transgenic donor mouse strain showed that the majority of iPSCs had retained normal imprinting at the *Dlk1-Dio3* locus and were competent to generate “all-iPSC” mice by tetraploid complementation<sup>72</sup>. The only difference between the two transgenic systems was a different stoichiometry of the reprogramming factors: high quality iPSCs resulted from the donor strain that generated 10 to 20 fold higher levels of Oct4 and Klf4 protein and lower levels of Sox2 and c-Myc<sup>72</sup> than the donor strain that produced low quality iPSCs<sup>73</sup>. Consistent with this notion, two other studies concluded that high levels of Oct4 and low levels of Sox2 are preferable for iPSC generation<sup>74,75</sup>.

The levels of transgene expression also play a role in the formation of partially reprogrammed iPSCs. It has been shown that partially reprogrammed colonies express a unique set of genes that are often bound by more reprogramming factors in the intermediate state than in ESCs<sup>38</sup> (for example, promoter or enhancer regions that are bound only by Oct4 and Sox2 in ESCs are bound by OSKM in the intermediate stage). In contrast, genes that are highly expressed in ESCs are bound by fewer reprogramming factors in the partially reprogrammed cells. Promoter regions bound by OSKM in partially reprogrammed cells often contain known DNA binding sites for the bound factors, indicating that the factors might bind those sites when the factors are present at high levels. These observations are consistent with the notion that excess levels of transgenes or different factor stoichiometry can cause binding of the four factors in a manner that differs from that seen in ESCs. Therefore, the promiscuous binding of OSKM may be influenced by the stoichiometry of the four factors and can either facilitate or block reprogramming.

Other parameters known to affect the characteristics of pluripotent cells are the culture conditions and supplements used to derive the cells<sup>76</sup>. For example, addition of small molecules and supplements such as vitamin C, valproic acid (VPA) and Tgf- $\beta$  inhibitors to the medium lead to more efficient derivation of iPSCs<sup>77–80</sup>. More importantly, derivation of iPSCs in the absence of serum and in the presence of vitamin C produced high quality tetraploid complementation-competent iPSCs even when a suboptimal factor stoichiometry was used for inducing pluripotency<sup>81,82</sup>. In addition, use of physiological oxygen levels during the isolation of human ESCs (hESCs) led to hESCs with two active X chromosomes, whereas X inactivation occurs if conventional conditions are used<sup>83</sup>. Thus, the available evidence suggests that factor stoichiometry as well as specific culture conditions strongly affect the quality and the efficiency of iPSC generation (summarized in Table 3).

## Markers of reprogramming

Ectopic expression of the reprogramming factors induces a heterogeneous population of cells with individual cells embarking on different fates such as cell death, cell cycle arrest (senescence), uncontrolled proliferation (malignant transformation), transdifferentiation and partial or full reprogramming (Figure 1). Although it is easy to differentiate between non-reprogrammed and reprogrammed cells, it is more challenging to distinguish partially from

fully reprogrammed cells. This is because partially reprogrammed cells can be morphologically identical to ESCs and can express many pluripotency genes<sup>23</sup>. Also, due to the stochastic nature of reprogramming<sup>29</sup>, no molecular markers have been identified that would predict whether a given cell early in the process will generate an iPSC daughter. Changes including loss of MEF markers, activation of the MET program or appearance of markers such as stage-specific embryonic antigen 1 (SSEA1) or alkaline phosphatase (AP) must occur in the reprogramming process, but these are not restricted to cells destined to become iPSCs<sup>23,18,59</sup>.

To define molecularly the various phases of the reprogramming process, global gene expression and proteomic patterns of clonal cell populations or enriched populations were established at different stages after factor induction<sup>15–18</sup>. These analyses suggested genes such as *Fbxo15*, *Fgf4*, *Sall1*, *Fut9*, *Chd7*, *Cdh1* mark the initiation phase, genes including *Sall4*, *Oct4*, *Nanog*, *Eras*, *Nodal*, *Sox2* and *Esrrb* are activated during the intermediate or maturation phase, and genes such as *Zfp42*, *Gdf3*, *Dppa2*, *Dppa3* and *Utf1* might define the late or stabilization phase. However, the information from gene expression or proteomic analyses of heterogeneous populations is limited because the rare cells destined to become iPSCs are masked.

Single-cell expression analyses of intermediate SSEA1-positive cells identified early, intermediate and late makers. These included the early epithelial cell adhesion molecule (EPCAM), the intermediate c-Kit receptor and the late platelet endothelial cell adhesion molecule (PECAM1)<sup>17</sup>. Sorting SSEA1-positive, EpCAM-positive early cells showed modest increase in reprogramming efficiency, but could not predict which cells would eventually reprogram<sup>17</sup>. Pluripotency genes such as *Utf1*, *Esrrb*, *Lin28* and *Dppa2* were identified as potential “predictive” indicators that were activated in a small subset of cells and might mark cells early in the process that are destined to become iPSCs<sup>23</sup>. Some of these markers were also detected in the population-based studies but, in contrast to single cell analyses, were only detected at late stages of the process and thus could not identify potential genes whose activation may constitute early markers for cells destined to become iPSCs. The question whether these genes execute a crucial role in the conversion to fully reprogrammed cells or only mark those rare cells is unresolved.

Endogenous copies of the key reprogramming factors *Oct4* and *Sall4* are activated early in rare cells but are also activated in partially reprogrammed cells and thus do not represent “predictive” early markers for iPSC generation<sup>23</sup>; this was confirmed in a study using an inducible *Oct4* lineage label<sup>84</sup>. In agreement with these observations, *Sall4* and endogenous *Oct4* have been found to be poor predictors of reprogramming competency<sup>16</sup>.

## Models of reprogramming

### Somatic stem cells vs. differentiated donor cells

Because the generation of cloned animals by SCNT is so inefficient, it was hypothesized that cloned animals like Dolly the sheep may not have been derived from differentiated cells as assumed but rather from rare somatic stem cells present in the heterogeneous donor cell population<sup>85</sup>. This issue was resolved when mature B and T cells were used as donors to

create monoclonal mice that carried in all tissues the immunoglobulin and T cell receptor rearrangements of the B and T cell donors, respectively, thus proving a terminally differentiated donor cell<sup>86</sup>. Similarly, because reprogramming by transcription factors is inefficient, it appeared possible that only a fraction of cells are able to generate iPSCs, consistent with an “elite model” in which only rare somatic stem cells present in the donor population could generate iPSCs whereas the differentiated cells would be refractory to reprogramming<sup>87,88</sup>. Several lines of evidence rule out the elite model and argue that all cells, including terminally differentiated cells, have the potential to generate iPSC daughters. Firstly, iPSC colonies have been derived from terminally differentiated cells such as B cells, T cells, liver and spleen cells<sup>82,89–91</sup>. As with SCNT, specific genomic rearrangement of the immunoglobulin locus or the T cell receptor in iPSC clones proved unambiguously that the cells were indeed derived from mature B or T cells and excluded the possibility of mesenchymal stem cell contamination<sup>90</sup>. Secondly, clonal analysis of single B cells indicated that >90% have the potential to generate daughter cells that at some point become iPSCs<sup>29</sup>.

### The stochastic and deterministic modes of reprogramming

In principle, reprogramming of somatic cells could occur by two mechanisms: a “stochastic” mode in which iPSCs appear with variable latencies; or a “deterministic” mode in which reprogrammed cells would be generated with a fixed latency. In the stochastic model it cannot be predicted whether or when a given cell would generate an iPSC daughter. Strong support for the stochastic model comes from single-cell cloning experiments demonstrating that sister cells from an early colony generate iPSCs with variable latency and with some sister cells never giving rise to iPSCs<sup>23,92</sup>. Though it cannot be predicted whether or when a given cell will generate an iPS daughter cell, activation of some genes such as *Esrrb* or *Utf1* (as discussed above), may mark rare early cells that are on their path to iPSCs (Figure 3). Activation of these genes early in the process suggests that their promoter regions are accessible for OSKM (Figure 2)<sup>15–17,23</sup>. In contrast, late activated loci are marked by H3K9me3 and are refractory to OSKM binding at early stages and activation of these loci appears to be a critical step for the proposed transition from a stochastic to a deterministic phase (Figure 1 and 3;<sup>50,93</sup>). Indeed, several essential pluripotency loci that are marked by H3K9me3, such as *Nanog*, *Dppa4*, *Gdf3* and *Sox2*, are activated later in reprogramming and are refractory to activation by the reprogramming factors during early stages<sup>13,15,16,23,38,50</sup> (Figure 1 and 2). Thus, the removal of H3K9me3 may represent another primary epigenetic barrier to complete reprogramming<sup>93</sup>.

The key event initiating the late hierarchical phase appears to involve activation of the endogenous *Sox2* gene, which then triggers a series of steps of gene activation that allow the cells to enter the pluripotent state<sup>23</sup> (Figure 1 and 3). *Sox2* represents one of a group of pluripotency initiating factors (PIFs) that are crucial and indispensable for the instigation of the deterministic phase<sup>16,23</sup>. The hierarchical network displayed in Figure 1 predicts that factors other than the canonical Yamanaka factors Oct4, Sox2, Klf4, c-Myc or Nanog should be able to induce pluripotency. Indeed, down-stream factors such as *Esrrb*, *Lin28*, *Dppa2* and *Sall4* were sufficient to induce iPSCs from MEFs<sup>23</sup>.

It has been suggested that the initial response to ectopic expression of OSKM in somatic cells may be an orchestrated and possibly deterministic response involving epigenetically definable events that activate loci critical for pluripotency<sup>17,22</sup>. Here we suggest an alternative view of the initial interaction of OSKM with the genome. As outlined in Figure 3, initial stochastic gene activation may render the cells susceptible to other gene expression changes (such as activation of apoptotic genes, metabolic genes, MET-inducing genes, silencing of MEF specific genes and eventually activation of pluripotency genes)<sup>17</sup>. During this initial phase, stochastic OSKM-genome interactions could also instigate the activation of early PIFs such as *Esrrb* or *Utf1*<sup>23</sup> in rare cells (Figure 3), and these would eventually lead to the expression of the late pluripotency genes *Sox2* and *Nanog* and stabilization of the core pluripotency circuitry. At this later stage, the endogenous pluripotency factors (*Oct4*, *Sox2* and *Nanog* (OSN)) will, in contrast to the exogenous OSKM factors, occupy only ESC-specific target regions<sup>94</sup>.

The initial promiscuous interaction of OSKM with the genome might be initiated by any factor that destabilizes the compacted chromatin typical of somatic cells. It is this destabilization that may render the somatic chromatin susceptible to becoming “hyperdynamic”, which is the hallmark of the ESC epigenetic state<sup>95,96</sup>. Consistent with this notion are the findings that general chromatin remodeling complexes such as BAF<sup>45,97</sup>, or global basal transcription machinery components like the transcription factor IID (TFIID) complex<sup>98</sup>, or exposure of cells to general DNA methyltransferase and histone deacetylase inhibitors like 5-azacytidine<sup>13</sup> and valporic acid<sup>78</sup>, can substantially enhance reprogramming in cooperation with OSKM. Also, in fibroblasts, down-regulation of the global chromatin organization modulator Lamin A, which is not expressed in ESCs<sup>99</sup>, has been reported to increase reprogramming efficiency<sup>100</sup>. Thus, although OSKM are highly efficient in inducing pluripotency, any chromatin remodeler or transcription factor - even those that do not normally function in ESCs - might be able to initiate the process leading to pluripotency, albeit with an efficiency that might be too low to be detected in standard reprogramming assays.

It has been suggested that reprogramming by SCNT or by somatic cell-ESC fusion is deterministic as it leads to activation of the somatic *Oct4* within two cell divisions (in the case of SCNT) or in the absence of DNA replication (in the case of fusion)<sup>1,2</sup>. However, to define pluripotency functionally in cloned embryos or in heterokaryons has been difficult, so it remains to be determined whether these methods activate the pluripotency circuitry by deterministic or stochastic mechanisms. Both types of mechanism might be involved in the various forms of reprogramming.

## How similar are ES and iPS cells?

Although ESCs and iPSCs are similar in morphology, age-affected cellular systems such as telomeres and mitochondria<sup>101,102</sup>, surface markers and overall gene expression, a number of studies have identified biological and epigenetic differences between ESCs and iPSCs as well as among individual ESC and iPSC lines<sup>103–115</sup>. For example, genetic alterations and differences in the transcriptome, proteome and epigenome were detected when ESCs and iPSCs were compared; these have raised concerns about the safety of iPSCs for therapeutic

applications. However, other studies have failed to find epigenetic and genetic abnormalities that consistently distinguish iPSCs from ESCs<sup>105,116–119</sup>. Rather, these data suggested that the extent of variations seen between ESCs and iPSCs were similar to variations seen within different ESC lines or within different iPSC lines<sup>120</sup>.

Recently, it has been suggested that the genetic abnormalities seen in iPSCs might be a result of oncogenic stress induced by the four reprogramming factors<sup>121</sup>. Significantly higher level of phosphorylated histone H2AX, one of the earliest cellular responses to double-strand breaks (DSBs) DNA, was detected in cells exposed to OSKM or OSK. The authors also linked the homologous recombination pathway, a pathway essential for error-free repair of DNA DSBs, to the reprogramming process and suggested a direct role for this pathway in maintaining genomic integrity<sup>121</sup>. In summary, the available evidence has not settled whether the alterations seen in iPSCs are the result of the reprogramming process *per se* or due to pre-existing genetic and epigenetic differences within individual parental fibroblasts<sup>119,122</sup>.

Much evidence indicates that the biological properties, such as *in vitro* differentiation, differ among individual ESC and iPSC lines, raising the concern that the unpredictable variation among cell lines could pose a potentially serious problem for iPS-based disease research. That is, a subtle phenotype seen between a disease-specific iPSC and a control iPSC line might not be relevant to the disease but rather reflect a system immanent difference<sup>123</sup>. Efforts have been directed towards defining experimental conditions of iPSC and ESC derivation that affect the developmental potential of the cells (summarized in Table 3).

## Perspective

The 2012 Nobel Prize in Physiology and Medicine was awarded to Shinya Yamanaka and John Gurdon for their discoveries on reprogramming somatic cells to pluripotency<sup>124</sup>. The seven years since Yamanaka's first demonstration of somatic reprogramming using defined factors<sup>12</sup> have witnessed much progress in understanding this complex process, and the most straightforward experiments have been done. However, many questions pertaining to the molecular mechanism of reprogramming remain unsolved. For example: how do OSKM convert chromatin to a “hyperdynamic” state; how does the promiscuous binding of OSKM in somatic cells contributed to the reprogramming process; what defines the rate-limiting step; what are the criteria for and the most effective methods for producing high quality iPSCs? Addressing these questions will be essential for a deeper understanding of reprogramming and will require the development of new technologies allowing genome wide epigenetic analyses of individual cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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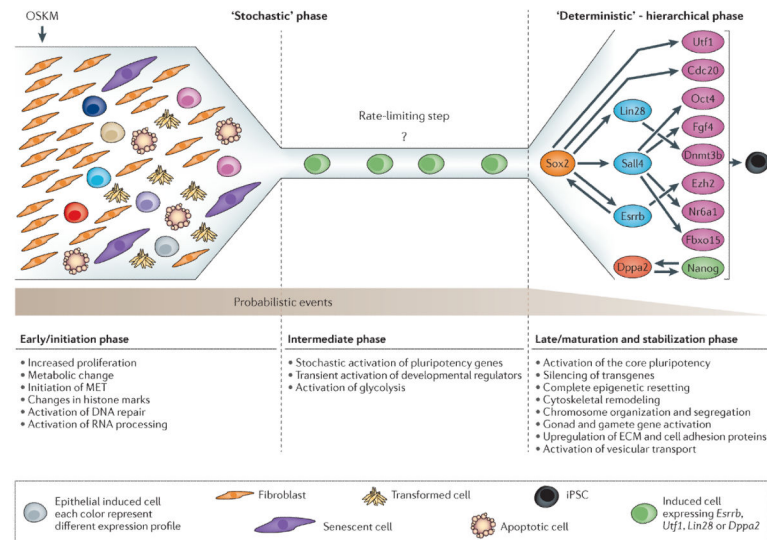
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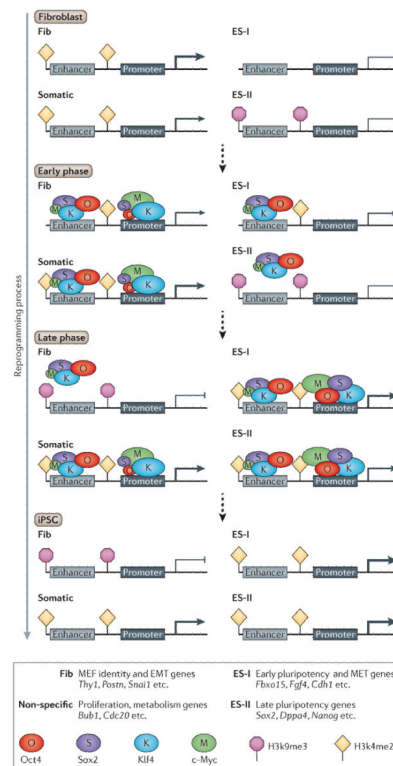
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**Figure 1. Phases of the reprogramming process**

In the model we discuss in this review, the reprogramming process can broadly be divided into two phases: firstly, a long 'stochastic' phase of gene activation; and secondly, a shorter hierarchical more 'deterministic' phase of gene activation that begins with the activation of the *Sox2* locus. After a fibroblast is induced with OSKM, it will initiate stochastic gene expression and assume one of several possible fates (such as, apoptosis, senescence, transformation, transdifferentiation or reprogramming). In the early phase, reprogrammable cells will increase proliferation, undergo changes in histone modifications at somatic genes, initiate mesenchymal to epithelial transition, and activate DNA repair and RNA processing. Then the reprogrammable cells will enter an intermediate phase with an unknown rate-limiting step that delays the conversion to iPSCs and contributes to the long latency of the process. In this phase, cells undergo a stochastic activation of pluripotency markers<sup>23</sup>, a transient activation of developmental regulators<sup>17</sup>, and activation of glycolysis<sup>18</sup>. In general the transcriptional changes in this phase are small. In some rare cases, the stochastic gene expression will lead to the activation of "predictive markers" such as *Utl1*, *Esrrb*, *Dppa2*, and *Lin28*, which then will instigate the second phase that starts with the activation of *Sox2*. Activation of *Sox2* by the "predictive markers" can be direct or indirect and will trigger a series of deterministic events that will lead to an iPSC. In this late phase, the cells eventually stabilize into the pluripotent state in which the transgenes are silenced, the cytoskeleton is remodeled to an ESC-like state, the epigenome is reset and the core pluripotency circuitry is activated<sup>16–18,23</sup>. In this model, probabilistic events decrease and hierarchical events increase as the cell progresses from a fibroblast to an iPSC.



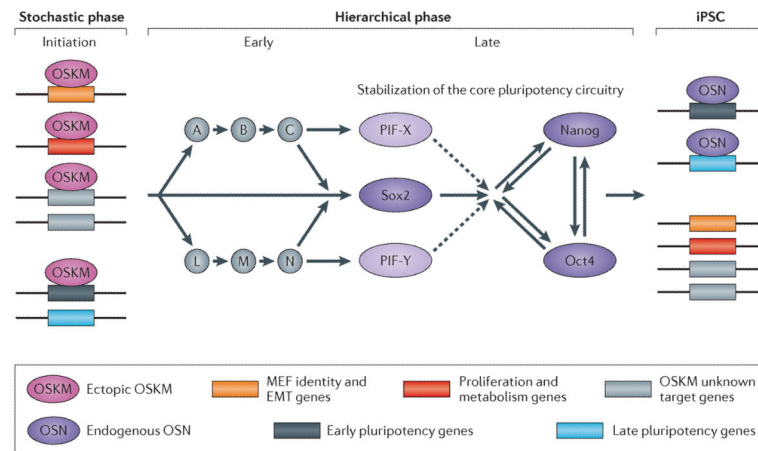


**Figure 2. OSKM as pioneer factors for remodeling the epigenome**

During reprogramming, exogenous OSKM bind enhancers and promoters of fibroblast and ESC genes along with regions that are not occupied by OSKM in ESCs and are not specific to fibroblasts (here called 'somatic'). The factors mark the loci that eventually will be epigenetically modified. In general, OSKM bind four different classes of genes. The first class (Fib) contains genes that are important for the identity of the fibroblasts such as *Thy1*, *Postn*, *Col5a2* and EMT genes like *Snail*, *Snai1* and *Twist1*. The second class (Somatic) contains genes that are bound by OSKM in somatic cells but not in ESCs and are not specific to fibroblasts. This includes apoptotic genes such as p53, genes that are important for proliferative cells, such as cell cycle genes (for example *Bub1*, *Cdc20* and *Cdc25c*), and metabolic genes such as *Pfkf* and *Gp*. The third class (ES-I) contains ESC genes that are activated early in the process such as *Fbxo15*, *Fgf4* and *Sall4*. The fourth class (ES-II) contains genes that are activated late in the reprogramming process such as *Sox2*, *Nanog* and *Dppa4*. During the early phase of reprogramming, OSKM occupy the enhancers of all classes except enhancers of ES-II genes that contain the heterochromatin mark H3K9me3 and are refractory to the four factors. c-Myc and Klf4 bind promoters of Fib genes and repress their activity while increasing the activation of genes from the Somatic class (shown by the weight of the arrow). As a result, enhancers and promoters from Fib start to lose H3K4me2 while genes from the Somatic class maintain high levels of H3K4me2. OSK act as pioneer factors and occupy the distal enhancer of ES-I genes, which gain *de novo* H3K4me2 and will initiate expression a few days later. The late phase is less well understood, but it can be speculated that Fib genes become heterochromatic and are silenced while the genes from the Somatic class are highly activated. ES-I genes are highly activated and contain high levels of H3K4me2 and ES-II genes start to lose the H3K9me3 mark, gain



H3K4me2 marks and initiate expression. It is reasonable to assume that more pluripotency late factors that are switched on late in reprogramming are needed to open those “blocked” regions. After the silencing of the exogenous factors, all groups are highly expressed except Fib, which remains silenced. The sizes of the ovals representing OSKM indicate their binding preference. For example, c-Myc is a global amplifier of gene expression increasing the transcription at all active promoters, therefore the oval “M” is larger on promoters.



**Figure 3. Model of molecular events that precede iPSC formation**

In the early phase, ectopic OSKM act as pioneer factors and occupy many genomic regions and help to generate a hyperdynamic chromatin state. OSKM will bind many regions throughout the genome of the fibroblast that are not OSKM targets in ESCs. Among these regions are: genes that determine the identity of the fibroblast, like extracellular components and EMT genes (orange box); genes that promote proliferation and increase metabolism (red box); unknown target genes that facilitate genomic fluidity, i.e., a state that allows rapid changes in transcription (gray box). In addition, OSKM will occupy distal regions of early pluripotency genes (black box); this binding will aid in activating those loci at later stages. A group of late pluripotency genes (blue box) is refractory to OSKM binding in this early phase. In the early hierarchical phase (which is more speculative), early pluripotency genes become activated in rare individual cells and either directly or in a hierarchical manner will instigate a more deterministic process that eventually leads to the activation of *Sox2*. *Sox2* represents one gene of a group of late pluripotency initiating factors (PIFs) that are essential for the activation of the core pluripotency circuitry. Once activated, the endogenous pluripotency proteins Oct4, *Sox2* and Nanog (OSN) occupy their target genes<sup>94</sup> and maintain the iPSC state in the absence of the exogenous factors.

**Table 1**

Roles of various histone marks during reprogramming

Histone mark	Function	Phase of reprogramming in which change occurs	Example of change
Histone H3 lysine 4 dimethylation (H3K4me2)	Marks promoters and enhancers	Early phase	Decrease at MEF and EMT genes. Increase at proliferation, metabolism, pluripotency and MET genes <sup>34,36,38,50</sup>
Histone H3 lysine 4 trimethylation (H3K4me3)	Marks active loci	Early phase	Increase at proliferation and metabolism genes <sup>34,36,38</sup>
Histone H3 lysine 27 trimethylation (H3K27me3)	Marks repressed loci	Early phase	Increase at MEF and EMT genes <sup>34,36,38</sup>
Histone H3 lysine 4 monomethylation (H3K4me1)	Marks enhancers	Early phase	Increase at proliferation and metabolism genes <sup>36</sup>
Histone H3 lysine 36 trimethylation (H3K36me3)	Marks transcriptionally active regions	Early to Middle phase	Increase at early and late pluripotency genes <sup>36</sup>
Histone H3 lysine 9 trimethylation (H3K9me3)	Marks heterochromatin regions	Late phase	Decrease at late pluripotency genes <sup>50,93</sup>
Histone H3 lysine 36 diimethylation (H3K36me2)	Marks potential regulatory regions (such as newly transcribed genes)	Early phase	Increase at early pluripotency genes <sup>46,47</sup>
Histone H3 lysine 79 diimethylation (H3K79me2)	Marks transcriptionally active regions	Early to middle phase	Decrease at MEF and EMT genes <sup>48</sup>
Histone H3 lysine 27 acetylation (H3K27ac)	Marks open chromatin and active enhancers	Unknown	Unknown

**Table 2**

Roles of example chromatin modifiers in reprogramming

Chromatin modifier factor	Enzymatic function	Role in reprogramming
Utx	H3K27 demethylase	Physically interacts with OSK to remove the repressive mark H3K27 from early pluripotency genes <sup>41</sup>
Kdm2a/2b	H3K36 demethylases	Initiation of the reprogramming process by regulating H3K36me2 levels at the promoters of early-activated genes <sup>46,47</sup>
Ehmt1, Setdb1	H3K9 methyltransferases	Required to reset the epigenome of somatic cells <sup>48</sup>
Bmi1, Ring1, Ezh2, Eed, Suz12	H3K27 methyltransferases	Involved in maintaining the transcriptional repressive state of genes <sup>48</sup>
Suv39h	H3K9 methyltransferase	Contributes to heterochromatin formation, hinders the reprogramming process <sup>48</sup>
Dot1l	H3K79 methyltransferase	Inhibits the reprogramming process in the early to middle phase by maintaining the expression of EMT genes such as SNAI1, SNAI2, ZEB1, and TGFB2 <sup>48</sup>
Parp1	Chromatin-associated enzyme, poly(ADP-ribose)transferase, which modifies various nuclear proteins by poly(ADP-ribosylation)	Functions in the regulation of 5mC, targets Nanog and Esrrb <sup>43</sup>
SWI/SNF (BAF) complex	Chromatin remodeling complex	Induce demethylation of pluripotency genes such as Oct4, Nanog and Rex1 <sup>45</sup>
Tet1 and Tet2	Methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine	Important for the early generation of 5hmc by oxidation of 5mC, targets Nanog, Esrrb and Oct4 through physical interaction with Nanog <sup>42-44</sup> .
Wdr5 complex	A core member of the mammalian Trithorax ( <i>trxG</i> ) complex. An “effector” of H3K4 methylation.	Interacts with Oct4 on pluripotency gene promoters and facilitates their activation <sup>40</sup> .

**Table 3**

Parameters that influence the quality of iPSCs

Parameter	Reprogramming cocktail or conditions	Effect on the quality of iPSCs
Stoichiometry	High Oct4, high Klf4, low Sox2, low c-Myc	Low reprogramming efficiency, normal <i>Dkl-Dio3</i> (A) methylation, no tumors in mice, improved efficiency to produce 4n mice <sup>72</sup>
	High Sox2, high c-Myc, low Oct4, low Klf4	High reprogramming efficiency, aberrant methylation of <i>Dkl-Dio3</i> , tumors in mice, low efficiency to produce 4n mice <sup>73</sup>
Other factors	Tbx3 (B), Zscan4 <sup>C</sup>	Improve reprogramming efficiency and/or improved efficiency to produce 4n mice <sup>125,126</sup>
Culture conditions	Knockout DMEM <sup>D</sup> , 20% KSR <sup>E</sup>	Efficient generation of iPSCs from MEFs and TTFs, improved efficiency to produce 4n mice <sup>127</sup>
	O <sub>2</sub> levels	Hypoxia conditions improve iPSC generation and aid X reactivation <sup>83</sup>
Supplement	Vitamin C	Activates <i>Dkl-Dio3</i> locus, improved efficiency to produce 4n mice <sup>82</sup>
	Histone deacetylase inhibitor	Activates <i>Dkl-Dio3</i> locus, improved efficiency to produce 4n mice. <sup>73</sup>
	2i/LIF <sup>F</sup>	Upregulation of Oct4 and Nanog, competence for somatic and germline chimerism <sup>128</sup>
	Protein arginine methyltransferase inhibitor AMI-5 and Tgf- $\beta$ <sup>G</sup> inhibitor A-83-01	Improved efficiency to produce 4n mice <sup>129</sup>
Genetic and epigenetic background	Not applicable	Unknown

4n mice: mice produced through tetraploid complementation

<sup>A</sup> Imprinted control domain that contains the paternally expressed imprinted genes DLK1, RTL1, and DIO3 and the maternally expressed imprinted genes MEG3 (Gtl2), MEG8 (RIAN), and antisense RTL1 (asRTL1). Reported to distinguish “good” (those that generate all-iPSC mice and contribute to chimeras) iPSCs from “bad” (those that do not generate all-iPSC mice and contribute to chimeras) iPSCs in Stadtfeld et al. Nature 2010. Carey et al. Cell Stem Cell 2011 found that loss of imprinting at the Dkl-Dio3 locus did not strictly correlate with reduced pluripotency.

<sup>B</sup> Tbx3 encodes a transcriptional repressor involved in developmental processes.

<sup>C</sup> Zscan4 encodes a protein involved in telomere maintenance, specifically aiding cell in escaping senescence. Also plays a role as a pluripotency factor.

<sup>D</sup> Dulbecco's Modified Eagle Medium

<sup>E</sup> KnockOut Serum Replacement

<sup>F</sup> Leukemia Inhibitory Factor

<sup>G</sup> Transforming Growth Factor Beta