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Mechanisms underlying the formation of induced pluripotent stem cells

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Abstract

Human pluripotent stem cells (hPSCs) offer unique opportunities for studying human biology, modeling diseases and for therapeutic applications. The simplest approach so far to generate human PSCs lines is through reprogramming of somatic cells from an individual by defined factors, referred to simply as reprogramming. Reprogramming circumvents the ethical issues associated with human embryonic stem cells (hESCs) and nuclear transfer hESCs (nt-hESCs), and the resulting induced pluripotent stem cells (hiPSCs) retain the same basic genetic makeup as the somatic cell used for reprogramming. Since the first report of iPSCs by Takahashi and Yamanaka, the molecular mechanisms of reprogramming have been extensively investigated. A better mechanistic understanding of reprogramming is fundamental not only to iPSC biology and improving the quality of iPSCs for therapeutic use, but also to our understanding of the molecular basis of cell identity, pluripotency and plasticity. Here we summarize the genetic, epigenetic and cellular events during reprogramming, and the roles of various factors identified thus far in the reprogramming process.

Introduction

Pluripotent stem cells (PSCs) can self-renew indefinitely in culture while maintaining the potential to differentiate into all cell lineages of an adult organism. Human PSCs (hPSCs) are relevant to a wide range of applications from basic biology to regenerative medicine. Aside from the promise of using hPSC-derived cells for cell replacement therapies, there is great potential of using hPSCs for modeling lineage decisions during differentiation and

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studying disease-relevant phenotypes that are manifested at the cellular level. Moreover, hPSCs also offer an attractive platform for drug efficacy and toxicity screening. Therefore, great efforts have been made to identify ways to generate PSCs, especially hPSCs. One approach is to derive PSCs through culturing various embryonic, adult or malignant cells with stem cell properties (Sidebar 1 and Figure 1). Among them, embryonic stem cells (ESCs) are the classic example of a PSC^{1–3} and they remain the gold standard to which newly derived PSC lines are typically compared molecularly, through expression and epigenetic profiling and functionally, by assessing their differentiation potential *in vitro* and *in vivo* (Table 1). Another approach is to reset a somatic cell to a pluripotent state by exposing its nucleus to exogenous transacting factors. This is currently achieved by three methods: somatic cell nuclear transfer (SCNT), cell fusion, and direct reprogramming by defined transcription factors. SCNT allows generating ESCs (ntESC) from cloned embryos obtained through injection of a somatic nucleus into an enucleated oocyte. NtESCs have been derived from different species, including mouse⁴ and more recently human somatic nuclei^{5,6} (Figure 1 (f)). SCNT and experiments involving fusions between PSCs and somatic cells (Figure 1 (g)) demonstrate that factors present in the egg and in PSCs have the ability to reset somatic nuclei to a pluripotent state⁷. Based on these observations, Yamanaka and colleagues screened 24 pluripotency transcription factors and demonstrated that over-expression of the reprogramming factors Oct4, Sox2, Klf4 and c-Myc (referred to as OSKM) is sufficient to create induced pluripotent stem cells (iPSCs) from mouse fibroblasts (Figure 1 (h))⁸. Soon after this groundbreaking discovery, iPSCs were generated from human fibroblasts using the same^{9–11} or a slightly different combination of reprogramming factors (OCT4, SOX2, NANOG and LIN28)¹². Use of hiPSCs circumvents the ethical controversies associated with hESCs or nt-hESCs, and as one can easily generate hiPSCs that match the genetic background of any individual, this offers an ideal platform for cell replacement therapy and disease modeling.

This review aims to provide a summary of current understanding of the molecular mechanisms of iPSC generation. We first describe the current models of reprogramming and experiments attempting to dissect the stochastic vs deterministic nature of this process. Next, we delineate the different phases of reprogramming focusing on the major transcriptional and epigenetic changes observed upon expression of the reprogramming factors OSKM. We then functionally dissect the reprogramming process by first describing the roles of OSKM as well as various pluripotency-associated or lineage-restricted transcription factors during reprogramming. Next, we provide an overview of the roles of epigenetic remodeling factors involved in reprogramming including histone post-translational modifiers, histone variants, nucleosome remodelers, chromatin topology and DNA methylation/demethylation enzymes. Finally we cover the roles of non-coding RNAs, including microRNAs and long non-coding RNAs (lncRNAs), and the role of signaling pathways, including developmental, stress-induced and metabolic pathways during iPSC formation. Because the reprogramming field has been extremely prolific during these years, it is beyond the scope of this review to give an exhaustive account of all mechanistic studies completed on reprogramming. We apologize in advance to those whose work couldn't be cited here.

I. Conceptual models of reprogramming

Reprogramming was initially described as an inefficient process requiring ectopic expression of OSKM for a period of time varying significantly between species. For instance using retroviral delivery, only ~ 0.5% of mouse embryonic fibroblasts (MEFs)¹³ and ~ 0.0002% of human adult dermal fibroblasts (HDFs)¹¹ form iPSC lines in ~ 12 and ~ 25 days respectively. Why reprogramming is only achieved in such a small fraction of donor cells and what controls the kinetics of reprogramming? The models describing reprogramming fall into two categories: deterministic and stochastic (Figure 2). Deterministic models predict that somatic donor cells give rise to iPSCs with a fixed latency, while stochastic models suggest variable latencies. Both models are further divided into sub-categories where “all” or only a subset of “elite” cells are permissive to reprogramming.

I. I. All versus elite

It is important to determine whether the low efficiency of reprogramming is due to the limitation of the reprogramming techniques or the intrinsic inability of a terminally differentiated cell to be reprogrammed. Most initial reprogramming studies were performed in MEFs through the use of integrative viral vectors. Thus it has been proposed that reprogramming may require additional genetic events triggered by random transgene insertional mutagenesis in rare cells (Figure 2 (c, f)). However, systematic analysis of transgene insertions in mouse fibroblasts does not support this hypothesis¹⁴. Random transgene integration is also known to cause heterogeneous transgene expression, and it is possible that optimal expression and stoichiometry of the reprogramming factors is only achieved in rare cells (Figure 2 (c, f)). Alternatively, it is possible that only a small fraction of elite somatic stem cells among the heterogeneous MEF culture can undergo reprogramming (Figure 2 (b, e)). Through the development of homogenous OSKM-delivery systems, up to 10% of MEFs can be reprogrammed. These systems include secondary reprogrammable MEFs¹⁵ or transgenic reprogrammable MEFs, in which a doxycycline (dox)-inducible polycistronic OSKM expression cassette is integrated into the *Collagen* locus^{16, 17}. While the efficiency achieved with these systems argues against reprogramming occurring only in a very rare stem cell population, the majority of MEFs still fail to be reprogrammed, raising questions as to what is preventing the remaining cells from being reprogrammed.

This question has also been addressed in more defined cell types. Terminally differentiated B and T cells can both form iPSCs, arguing against the idea that only “less” differentiated cells can be reprogrammed¹⁸. However, the developmental stage seems to affect the efficiency and kinetics of reprogramming, as hematopoietic stem and progenitor cells generate up to 300 times more iPSC colonies than terminally differentiated B and T cells¹⁸. It has also been shown, in pre-B cells at least, that almost all cells (>92%) can give rise to reprogrammed colonies if the reprogramming factors are expressed for enough time¹⁹. These observations argue that all cells, including terminally differentiated cells, can form iPSCs. They also highlight that reprogramming efficiency is influenced by the differentiation status, suggesting that the epigenetic and transcriptional state of the donor cell may affect its plasticity to become pluripotent.

I. II. Deterministic versus stochastic models

Reprogramming appears stochastic in many experimental settings. Most notably, single-cell cloning experiments show that the reprogramming kinetics varies even between individual clones of an apparently homogenous, lineage-committed cell population¹⁹. This stochasticity may reflect differences in cell cycle stage, cell-intrinsic fluctuation in gene expression and epigenetic status. Supporting this notion, p53 knockdown increases the kinetics of iPSC generation in a cell-division-rate-dependent manner¹⁹. Similarly, granulocyte monocyte progenitor (GMP) cells, which display an ultrafast cell cycle of 8 hours give rise to iPSC colonies almost synchronously in only four to five cell divisions upon OSKM expression²⁰. This study also highlights that one can achieve almost deterministic reprogramming using a privileged somatic cell population suggesting reprogramming models may be context-dependent. Along this line, a recent study suggests that chromatin de-repression through Mbd3 depletion allows rapid deterministic reprogramming of MEFs²¹, though this effect also depends on the reprogramming context²². In the next sections, we will discuss some of the mechanistic studies in more details, and we will also see that the identification of distinct reprogramming phases and the markers associated with them allows capturing subsets of cells in which reprogramming is much more likely to occur, if not absolutely deterministic. As such, at least part of the apparent stochastic nature of reprogramming may be attributed to our temporary partial understanding of the underlying mechanisms.

II. A molecular roadmap of reprogramming

Successful reprogramming requires the erasure of the somatic transcriptional and epigenetic signature in donor cells and the establishment and maintenance of the PSC transcriptome and epigenome in iPSCs. These two processes rely on the proper repression, activation and maintenance of different sets of transcription factors, non-coding RNAs, chromatin/DNA-modifying enzymes and signal-transduction pathways²³, which will be discussed in more details below. We would like to point out that most studies addressing the mechanism of reprogramming have been performed in MEFs using dox-inducible expression of OSKM. While this experimental setup has been extremely powerful to reveal the molecular events and the functions of many genes and pathways involved in reprogramming, the reprogramming process can be influenced by the species, the cell type, the reprogramming factors used and how the factors are introduced into donor cells. Therefore, much remains to be learned regarding divergent routes to pluripotency and alternative pluripotent states that share overlapping molecular and phenotypic features with ESCs.

II. I. Reprogramming phases

Experiments performed in Oct4-GFP or Nanog-GFP reporter MEFs using dox-inducible lentiviral vectors have shown that donor cells transition through a number of defined intermediates before reaching the pluripotent state. 1–2 days upon dox-induction of OSKM, the fibroblast-associated surface marker Thy1 is down-regulated coinciding with the up-regulation of the early pluripotency marker Alkaline phosphatase (*Alpl*) followed by embryonic antigen SSEA1, between day 3 and 5. Around day 8, reprogramming cells up-regulate the core pluripotency transcription factor *Oct4* followed by *Nanog*^{24, 25}. In this

setup, exogenous factor expression is required for at least 1.5 weeks to generate *bona fide* iPSC colonies that are transgene-independent^{24, 25} (Figure 3 (a)). Global transcript and protein profiling during reprogramming of bulk MEF cultures and analysis of single cells have identified three phases in reprogramming: initiation, maturation and stabilization^{26–29} (Figure 3(b)).

II. I. I. The initiation phase—During the initiation phase MEFs undergo a coordinated mesenchymal-epithelial transition (MET), change their proliferation rate and up-regulate early PSC markers^{26, 30}. Single cell time-lapse microscopy has shown that the majority of cells undergo a MET, increase proliferation during this period^{31–33} and single cell expression profiling of genes involved in reprogramming has uncovered stochastic variation in gene expression among cells during this phase²⁹. Transcriptional profiling of sorted Thy1⁺/SSEA1⁺ reprogramming intermediates between days 0 and 6 uncovers a first wave of global transcriptional changes involving mRNAs, miRNAs and lncRNAs, starting after OSKM expression and peaking around day 3. These changes affect cell proliferation, metabolism, cytoskeleton organization and developmental processes^{27, 34, 35}. Consistent with the hallmark MET and increased cell proliferation during the initiation phase, mesenchymal transcription factors are repressed (*Snai1/2*, *Zeb1/2*, *Slug*)^{25, 36, 37}, epithelial (*Cdh1*, *Epcam*, *Cldn3*, *-4*, *-7*, *-11*, *Ocln*, *Crb3*) and early pluripotency genes (*Alpl*, *Fbxo15*) are up-regulated^{26, 27, 30, 38} together with DNA replication (*Poli*, *Rfc4* and *Mcm5*), cell cycle progression (*Ccnd1* and *Ccnd2*) and stress-induced genes (*Cdkn1a* and *Cdkn2a*)³⁶. Similar changes in protein expression are observed through proteomic analysis, identifying proteins involved in the regulation of pluripotency (Jarid2, Rif1, Tcf3, Eed) as well as general transcription regulation (Mediator, TAF, RNA Pol II, Nurd)²⁸.

Although SSEA1 identifies MEFs primed for reprogramming, only a small subset of sorted SSEA1⁺ cells progress successfully towards pluripotency at early reprogramming stages. Through a functional surface marker screen, a recent study identified an earlier set of surface markers (CD73, CD49d, CD200) transiently expressed in MEFs poised for reprogramming. CD73⁺, CD49d⁺, CD200⁺ cells express the transcriptional regulators *Nr0b1* and *Etv5* prior to up-regulation of other pluripotency regulators such as *Rex1*, *Dppa2*, *Nanog* and *Sox2*³⁹.

II. I. II. The maturation phase—The maturation phase is characterized by an initial period of stasis followed by a second wave of extensive transcriptional^{26, 27, 38} and proteomic^{28, 38} changes in MEFs between days 9 and 12. These changes affect development/stem cell genes (*Oct4*, *Sall4*, *Nanog*, *Esrrb*, *Tcl1*, *Nr5a2*)^{27, 29, 38, 40} and hypoxia-responsive genes (*ALDOC*, *ENO3*, *PKM2*, *Pfkl*, *Gpi*, *Pgk1*)^{28, 41}, most of which are also up-regulated during the reprogramming of human fibroblasts. Moreover, proteins involved in vesicle-mediated transport, extracellular matrix, cell adhesion and EMT that were silenced during the initiation phase, are reactivated in reprogramming of MEFs between days 9 and 12²⁸.

The end of the maturation phase is marked by the up-regulation of *Sox2* and other pluripotency genes in both mouse and human fibroblasts, leading to the establishment of the endogenous transcriptional network that sustains pluripotency independent of transgene expression^{25–27, 29, 38}. Transgene independence is a critical event marking the completion

of the maturation phase. Interestingly, rather than classic pluripotency regulators (*Oct4*, *Nanog*, *Sall4*), a different set of genes seem to play a prominent role in this transition in MEFs, including pluripotency (*Esrrb*, *Utf1*, *Lin28* and *Dppa2*), germline (*Mnd1*, *Mutyh*, *Rad54b*), cytoskeletal (*Tuba3a*, *Pdzk1*, *Itgb7*, *Kirrel2*) and RET-signalling (*Nrtn*, *Kif26a*) genes^{29, 40}. All together, these observations point towards an early stochastic and late deterministic model of reprogramming using the OSKM factors^{42, 43}.

II. I. III. The stabilization phase—The stabilization phase starts when iPSCs become transgene independent. At this stage, telomeres are actively elongated soon reaching embryonic levels^{25, 44}, DNA undergoes important changes, including reactivation of both copies of the X chromosome in a fraction of female mouse iPSCs²⁵, and 5-methylcytosine (5mC) marks are remodeled correlating with the up-regulation of genes involved in the regulation of DNA methylation (*Aid*, *Tet* and *Dnmt* family members)²⁷ (Table 2 (a)).

II. II. Epigenetic changes during reprogramming

Bona fide iPSCs maintain pluripotency independent of transgene expression, suggesting the cells have acquired a different epigenetic state compared to the donor somatic cells and partially reprogrammed intermediates. The epigenetic modifications underlying the transcriptional changes observed during the initiation, maturation and stabilization phases of reprogramming involve genome-wide resetting of DNA methylation and histone post-translational modifications⁴⁵. Below we first provide a brief overview of commonly studied histone modifications.

Among the more than 100 known histone modifications, lysine methylation and acetylation are the most studied. In general, transcription start sites of actively transcribed genes are marked by trimethylated H3K4 (H3K4me3) and acetylated H3K27 (H3K27ac), and active enhancers are enriched with monomethylated H3K4 (H3K4me1) and H3K27ac. Gene bodies of actively transcribed genes are associated with trimethylated H3K36 (H3K36me3). Transcriptionally silenced heterochromatin is associated with trimethylated H3K9 (H3K9me3), whereas regulated gene repression is often associated with trimethylated H3K27 (H3K27me3). Notably, although H3K4 methylation marks active promoters in yeast, the presence of this mark at promoters and enhancers in mammalian cells does not necessarily correlate with gene expression. This difference results from the specific regulation of RNA polymerase II (Pol II) through pause and release at the promoter of mammalian cells and the presence of “bivalent promoters” containing both active (H3K4me3) and repressive (H3K27me3) marks in both PSC and terminally differentiated cells^{23, 46}.

II. II. I. Changes in histone marks—Unlike lineage-committed cells, which contain extensive regions of highly condensed heterochromatin, the chromatin of PSCs is more accessible, contains fewer heterochromatin foci, and correlates with a higher abundance of active- and a lower amount of repressive-histone post-translational modifications⁴⁷. Global reduction of H3K27me3 during the initiation phase of reprogramming is associated with the acquisition of a transient open/primed chromatin state and coincides with changes in RNA expression, loss of heterochromatin up to day 8 of reprogramming, followed by re-

establishment of novel H3K27me3 marks³⁸. In contrast, H3K4me3/H3K27me3 bivalent domains are established gradually, starting around day 3 and increasing progressively until stable iPSCs are formed^{27,38}. Unlike the H3K27me3 marks, the global H3K4me levels do not change significantly³⁸, but there are significant increases of monomethylated H3K4 (H3K4me1), H3K4me2 and H3K4me3 marks at proliferation and metabolism genes immediately upon OSKM induction, and H3K4me2 increases at MET and pluripotency genes and decreases at MEF-specific and EMT genes^{37,48–50}.

Reprogramming also involves additional histone modifications at enhancers and promoters of key epithelial, mesenchymal and pluripotency genes. H3K36me2, whose depletion at CpG islands (CGIs: clusters of CpG dinucleotides, in which a cytosine precedes a guanosine) is thought to contribute to a transcriptionally permissive state⁵¹, is removed in early responsive genes (*Cdh1*, *Epcam*) during the initiation phase⁵². Sequential loss of H3K36me2/3 at the *Ink4/Arf* locus and the promoter of the microRNA cluster 302/367 are later events occurring during the maturation phase⁵³. H3K79 methylation is associated with active transcription and is usually enriched at gene bodies⁵⁴. Mesenchymal gene down-regulation during the initiation and maturation phase correlates with a reduction of H3K79me2 marks. As such, H3K36 and H3K79 methylation is believed to constitute a barrier for the acquisition of an epithelial fate during reprogramming⁴⁷. Another barrier to reprogramming is H3K9 methylation, and its removal during the late maturation phase marks the transition from pre-iPSCs to iPSCs⁵⁵.

II. II. II. Changes in DNA methylation—DNA methylation is considered the most stable epigenetic modification associated with gene silencing in lineage-committed cells. In mammals, DNA methylation occurs exclusively at the C5 position of cytosine (5mC), predominantly in the context of CpG dinucleotides. CpG clusters, called CGIs are predominantly found in gene promoters and represent major targets of DNA methylation in mammalian somatic cells. Generally speaking, promoter methylation is inversely correlated with gene expression⁵⁶.

PSCs possess a unique DNA methylation signature compared to lineage-committed cells. In particular, *Oct4*, *Nanog* and other pluripotency promoters are largely unmethylated and a significant number of tissue-specific promoters are hypermethylated⁵⁷. Genome-wide methylation studies reveal that in contrast to gene expression and histone modifications, global changes in DNA methylation occur predominantly at the end of the reprogramming process, coinciding with the up-regulation of different enzymes regulating DNA methylation. Hence, genome-wide demethylation of a large set of pluripotency-associated promoters including *Nanog* and *Oct4* or *de novo* methylation of mesenchymal promoters such as *Hoxa10*, *Gja8* occur in the late maturation phase^{27,38}. Similarly, demethylation of the inactive X chromosome in female donor cells and down-regulation of the large noncoding RNA *Xist* (responsible for triggering X inactivation) occurs late after pluripotency genes are up-regulated and only in a fraction of iPSC lines^{58–60} (Table 2 (b)).

II.III. Reprogramming intermediates, partially reprogrammed cells and alternative pluripotent states

Using reprogrammable MEFs, cells undergoing successful reprogramming transit in a linear fashion from $\text{Thy1}^+/\text{SSEA1}^-$ to $\text{Thy1}^-/\text{SSEA1}^-$ and to $\text{Thy1}^-/\text{SSEA1}^+$ in the first 6 days of reprogramming and eventually to a $\text{SSEA1}^+/\text{Oct4}^+$ state by days 9–12²⁷. Unlike $\text{Thy1}^-/\text{SSEA1}^+$ permissive cells, $\text{Thy1}^-/\text{SSEA1}^-$ or refractory $\text{Thy1}^+/\text{SSEA1}^-$ cells fail to properly activate PSC genes and repress mesenchymal genes, though this refractory state can be reversed in $\text{Thy1}^+/\text{SSEA1}^-$ cells with an additional dose of OSKM. In addition, approximately 200 genes are aberrantly activated in refractory $\text{Thy1}^+/\text{SSEA1}^-$ cells compared to progressing $\text{Thy1}^-/\text{SSEA1}^+$ cells at day 6 of reprogramming, including extracellular space/matrix, plasma membrane, retinoic acid binding and immune response transcripts, which may prevent reprogramming.

Analysis of $\text{Thy1}^-/\text{SSEA1}^+$ cells also reveals transient up- or down-regulation of gene expression specific to these reprogramming intermediates^{26–28, 38}. For instance, a large subset of transiently up-regulated genes are epidermal genes (*Ehf*, *Elf5*, *Krt17*, *Krt6a*) not expressed in either fibroblasts or iPSCs⁶¹. A similar analysis used the expression of the surface antigen TRA-1–60 to identify intermediates during the reprogramming of human fibroblasts. This study has revealed transient up-regulation of primitive streak-like mesendoderm transcripts (*T*, *EOMES*, *FOXH1*) during reprogramming⁶².

Transient activation/repression of developmental regulators during the maturation phase may reflect a permissive stage, as expression or silencing of certain lineage genes not directly involved in the acquisition of pluripotency may not be under selective pressure. Alternatively, transient activation or repression of some developmental regulators may facilitate the transition towards iPSCs. For example, over-expression of the *FOXH1* gene mentioned above promotes OSKM-mediated HDF reprogramming. In either scenario, reprogramming intermediates may represent a transiently “plastic” state more amenable to cell-fate manipulation. This hypothesis has been supported by the successful derivation of epiblast stem cells⁶³, neural progenitors⁶⁴, endothelial cells⁶⁵ and cardiomyocytes⁶⁶ from fibroblasts transiently expressing OSKM and exposed to lineage-specific culture conditions. However, applying the same cardiac or neural transdifferentiation approach using a pluripotency-lineage tracing system suggests that the cells successfully transdifferentiating towards the cardiac or neural lineages acquire transiently a pluripotency state^{67, 68}. Although, one cannot exclude that some transiently “plastic” cells may be able to directly convert into an alternative lineage, these experiments strongly suggest that OSKM transdifferentiation (OSKM-TD) requires passage through a transient iPSC state.

The success and apparent ease of reprogramming also raises interesting conceptual questions such as whether there could be alternative pluripotent states, such as the F-class cells recently generated using a PiggyBac transposon-mediated dox-inducible secondary reprogramming system⁶⁹. These cells do not form typical ESC-like colonies and exhibit higher proliferative rates. Functionally, they differ from iPSC and ESCs in that although they can form teratomas containing tissue derivatives of the three germ layers, F-class cells don't contribute to embryonic development when injected into mouse blastocysts^{38, 69–72}.

Notably, this pluripotent state depends on high and constitutive OSKM expression. Sustained and high levels of OSKM result in rapid up-regulation and maintenance of *Nanog* and *Sall4* expression in F-class cells, suggesting similarities in pluripotency network with iPSCs and ESCs. However, there are also notable differences in global methylation and histone marks among others, indicating that F-class cells represent an alternative pluripotent state⁷³. From a conceptual point of view, the transgene-dependency of F-class cells also challenges the traditional criterion that a fully reprogrammed cell (including cells created through lineage reprogramming not discussed in this review) must be able to maintain the cellular phenotype independent of continuous transgene expression.

III. A functional dissection of the reprogramming process

The acquisition and the maintenance of pluripotency depend on the activity of a large set of transcription factors, chromatin- and DNA-modifying enzymes, non-coding RNAs and signal-transduction pathways as outlined below.

III. I. Roles of transcription factors

III. I. I. OSKM—The transcription factors Oct4, Nanog and Sox2 (OSN) play a central role in maintaining the molecular and phenotypic features of PSCs, and their inactivation leads to loss of pluripotency²³. OSN form an auto-regulatory loop in PSCs by binding to their own and each other's promoters. In addition, they co-occupy the promoters of hundreds of genes, half of them encoding genes actively expressed in PSCs, while the other half encoding developmental genes associated with lineage commitment that are silenced in PSCs²³.

cMyc is a facultative reprogramming factor that facilitates the emergence of reprogrammed cells^{74, 75}. Intersection of the transcriptional profiles identified at different stages of reprogramming with cMyc binding sites in PSCs reveals that the majority of cMyc targets are bound during the initiation phase. This is consistent with the finding that the main activity of cMyc is restricted to the initiation phase and its activity doesn't seem to be mediated by the activation of pluripotency genes³⁷. One probable role of cMyc in reprogramming is through regulating cell proliferation: cMyc can repress cell cycle checkpoint genes, inhibit Cyclin-dependent kinase (CDK) inhibitors and associate with pre-replication complexes to promote DNA synthesis⁷⁶. Increased proliferation, in turn, may increase the probability for epigenetic resetting in the presence of OSK.

Overall, the binding sites of OSK evolve considerably during reprogramming but coincide at the onset of the stabilization phase with a PSC-like binding profile. More dramatic expression changes are usually observed in genes co-occupied by OSK in PSCs where they act as transcriptional activators, suggesting OSK play similar roles in reprogramming^{36, 37, 77, 78}. During the initiation phase, OSK bind to promoters and enhancers of active and repressed genes prior to significant histone modifications^{37, 49, 50} including GLIS family zinc finger 1 (*Glis1*)⁷⁹, *miR-302–367* cluster^{80–82}, *Fbxo15*, *Fgf4*, *Sall4*, *Lin28* and *MET* genes^{29, 30, 50}. Interestingly, only half these enhancers are PSC-specific⁴⁹, the other half contains atypical distal elements suggesting OSK could be pioneer factors opening chromatin at critical pluripotency loci⁵⁰. Klf4 exerts a dual role during

reprogramming, early in somatic gene repression (*Tgfb1*, *Pdgfra*, *Col6a1*), and late in pluripotency gene activation (*Oct4*, *Tdgf1*, *Klf5*)²⁷.

Widespread and promiscuous binding of OSK may result from the ectopic and non-physiological expression levels of the reprogramming transgenes. It has been proposed that, OSK interaction with Mediator, Cohesin complexes or RNA Pol II elongation factor ELL3 initially recruits these factors to non-canonical enhancers and promote chromatin opening^{45, 83, 84}. Supporting this idea transient expression of OSKM allows transdifferentiation of fibroblasts to other somatic lineages^{63–66}. Recent studies also show that cMyc is a global amplifier of gene expression, increasing transcription at all active promoters^{85, 86}. When OSK are over-expressed with cMyc, OSK factors may act as pioneer factors to enable cMyc binding to previously inaccessible distal elements on chromatin, and cMyc in turn may cooperatively enhance OSK binding and transcriptional activation capacity⁵⁰.

III. I. II. Other pluripotency-associated transcription factors—A number of additional totipotency- and pluripotency-associated transcription factors have been shown to replace part of the OSKM set and/or increase reprogramming efficiency. For instance iPSCs can be generated with OCT4, SOX2, NANOG and LIN28 (referred to as OSNL) from HDFs¹², or Sall4, Nanog, Esrrb and Lin28 (SNEL) from MEFs⁴². Thus the pluripotent state can be achieved through different though likely convergent paths. On the other hand, differences have been observed in reprogramming using different factor combinations. For example, SNEL leads to significantly fewer iPSC colonies compared to OSKM, but the majority of iPSC lines established are of high quality based on the tetraploid complementation assay. In contrast, a combination of Oct4, Sox2, Sall4, Nanog and Esrrb (OSSNE) gives a much higher number of iPSC colonies but most lines exhibit poor developmental potential. These results suggest that the interplay between the reprogramming factors plays a critical role in fine-tuning the reprogramming process⁴². Below we describe the roles of a number of pluripotency-associated reprogramming transcription factors other than the classic OSKM.

Nanog and Lin28 are perhaps the best studied reprogramming factors other than the OSKM factors. Together with *Oct4* and *Sox2*, Nanog homeodomain transcription factor holds a central position in the pluripotency transcriptional network and is required for the maintenance of pluripotency in the epiblast and in ESCs⁸⁷. In OSNL-mediated reprogramming of human mesenchymal cells, *NANOG* is not required for the initial appearance of OCT4⁺ colonies, but improves the recovery of iPSC clones suggesting a role in the stabilization phase¹². Similarly, Nanog over-expression facilitates the transition from the maturation towards the stabilization phase during OKM-mediated reprogramming of mouse neural stem (NS) cells⁸⁸. The inclusion of Nanog in OSKM-mediated reprogramming of mouse B cells results in the formation of a similar numbers of iPSC colonies but with a significantly accelerated kinetics in a cell-division-rate-independent manner¹⁹.

Lin28 is not absolutely required in the OSNL set, though removal of Lin28 leads to an approximately 5-fold reduction in the number of ESC-like colonies obtained from human

mesenchymal cells¹². Lin28 may promote reprogramming through both miRNA-dependent and independent mechanisms⁸⁹. In ESCs, Lin28 binds and blocks the maturation of let-7 miRNA family members, which normally counteract the activity of cell-cycle-regulating miRNAs involved in ESC self-renewal. Lin28 can also regulate protein levels by controlling mRNA stability and has been shown to mediate post-transcriptional regulation of *OCT4* in hESCs and cell-cycle regulators in mESCs⁹⁰. Thus LIN28's function in the OSNL set may be analogous to that of cMYC (which is a let-7 target) in OSKM⁹¹

Additional pluripotency-associated transcription factors have been shown to play a role in reprogramming, including Glis1, Sall4, and the orphan nuclear receptor Esrrb. Glis1 is enriched in unfertilized oocytes and one-cell stage embryos, and it promotes OSKM or OSK-mediated reprogramming in mouse and human fibroblasts⁷⁹. Glis1 has been shown to physically interact with OSK during reprogramming, and it contributes to the activation of multiple pro-reprogramming genes (*Foxa2*, *Esrrb*, *Lin28a*, *Nanog*). Glis1 over-expression also increases the expression of Mycn and Mycl1, but suppresses the expression of cMyc, and this altered balance between Myc family genes may also contribute to the effect of Glis1 in reprogramming. *Sall4* is expressed in PSCs and its binding sites overlap with those of OSN^{92,93}. When knocked down, ESCs differentiate toward a trophoblast fate suggesting *Sall4* contributes to the maintenance of the pluripotent state^{94,95}. *Sall4* over-expression with OSK in MEFs leads to a 2-fold increase in Nanog-GFP⁺ iPSC colonies compared to OSK alone, whereas *Sall4* knockdown leads to a 2-fold reduction⁹⁶. *Esrrb* was identified through screening for factors to replace Klf4 in the OSKM cocktail, though OSEM exhibit a 2-fold decrease in efficiency compared to OSKM in reprogramming of MEFs. In ESCs, *Esrrb* targets many genes involved in self-renewal and pluripotency^{97,98} and its binding sites co-localize with Klf4, consistent with its capability to replace Klf4 in reprogramming⁹⁹.

III. I. III. Lineage transcription factors—A well-accepted model for the maintenance of pluripotency is that the core pluripotency factors prevent differentiation through binding and repression of key lineage specifiers in PSCs¹⁰⁰. In an interesting and not mutually-exclusive model, the pluripotency factors may act as early lineage specifiers and the balance of their antagonistic activities results in the maintenance of the undifferentiated state¹⁰¹. Supporting the second model, *Gata3* (a mesendoderm-lineage specifier) and *Gmnn* (an ectoderm-lineage specifier) can replace *Oct4* and *Sox2* respectively in OSKM-mediated reprogramming, and over-expression of *Gata3* and *Gmnn* together with KM but without OS is sufficient to reprogram MEFs into iPSCs¹⁰². A number of additional lineage transcription factors have been shown to work in similar ways as *Gata3* and *Gmnn* in both mouse and human cells^{102,103}. These results suggest a more dynamic and complex regulation of the induction, maintenance and exit of pluripotency than previously thought, though the exact mechanisms remain to be elucidated. By extension, one might also directly convert one somatic cell type into another (lineage reprogramming) through perturbing the balance between differentiation cues rather than over-expressing master transcription factors¹⁰⁴.

III. II. Roles of epigenetic remodeling factors

III. II. I. Histone post-translational modifiers—In PSCs, the promoters of most developmental genes regulated by Oct4, Sox2, and Nanog are co-occupied by Polycomb group (PcG) proteins, a family of epigenetic regulators responsible for the maintenance of transcriptional repression through H3K27 methylation¹⁰⁵. It is likely that reprogramming factors recruit, directly or indirectly, PcG proteins to silence developmental genes during the induction and maintenance of PSCs. Consistent with this notion, knockdown of H3K27 methyltransferases belonging to the PRC1 (BMI1, RING1) and PRC2 (EZH2, EED, SUZ12) PcG complexes substantially reduce the number of iPSC colonies obtained from human fibroblasts¹⁰⁶. Further studies have shown that *Ezh2* negatively regulates TGF β signaling components and pro-EMT miRNAs such as the miR-23a cluster through association with cMyc during the initiation phase of reprogramming in human fibroblasts¹⁰⁷.

Trithorax group (TrxG) complexes mediate H3K4 methylation, and H3K4me3 is enriched at actively transcribed promoters¹⁰⁸. During reprogramming, *Wdr5*, a core member of the mammalian TrxG complex, is up-regulated through direct binding of Oct4 to its promoter, and *Wdr5* in turn promotes reprogramming by interacting with Oct4¹⁰⁹. Oct4 targets *Wdr5* to pluripotency loci to re-establish H3K4me3 active marks leading to robust transcriptional activation. Consistent with this model, knockdown of *Wdr5* results in decreased occupancy of *Wdr5* on chromatin, global reduction of H3K4me3, locus-specific decrease of H3K4me3 at pluripotency-associated gene promoters including *Oct4* and *Nanog*, and decreased efficiency of reprogramming in MEFs.

Inhibition of the H3K27 demethylase *Utx* (also known as *Kdm6a*) and the H3K36 demethylases *Jhdm1a* and *Jhdm1b* (also known as *Kdm2a* and *Kdm2b*) also decrease reprogramming efficiency. *Utx* has been shown to promote reprogramming through physically interacting with OSK and removing H3K27me3 from early pluripotency genes such as *Fgf4*, *Sall4* or *Sall1*¹¹⁰. *Jhdm1b* plays a prominent role during the initiation phase of reprogramming when it enhances the activation of early responsive genes through binding and demethylating their promoters⁵². Moreover, *Jhdm1a* and *Jhdm1b* were also shown to mediate the enhancing effect of Vitamin C on reprogramming, by repressing the *Ink4/Arf* locus, increasing proliferation and suppressing senescence⁵³ (see chapter III. IV. III.).

H3K9 trimethylation (H3K9me3) is usually associated with repressed heterochromatin. During reprogramming, H3K9me3-containing regions have been shown to prevent OSKM binding in human fibroblasts⁵⁰ and their presence at pluripotency loci represents a major roadblock in the transition from pre- to fully reprogrammed mouse iPSCs^{55, 111}. Consistently, both the depletion of the heterochromatin protein-1 γ (*Cbx3*), a protein known to recognize H3K9me3, or the H3K9 methyltransferases *Ehmt1*, *Ehmt2* and *Setdb1* increase mouse iPSC formation at early and late stages of reprogramming¹¹¹. H3K9 methyltransferases seem to act downstream of BMPs in combination with their corresponding demethylases. They constitute a switch controlling the transition from pre-iPSC to iPSC by regulating H3K9 methylation levels at core pluripotency loci⁵⁵.

Supporting an inhibitory role of H3K79me2-mediated gene expression in reprogramming, inhibition of the H3K79me2 methyltransferase *DOTIL* promotes reprogramming and can

replace the reprogramming factors KLF4 and cMYC in OKSM¹⁰⁶. These effects are likely mediated through early up-regulation of *NANOG* and *LIN28* and loss of H3K79me2 at EMT genes (*SNAIL1*, *SNAIL2*, *ZEB1*, *TGFB2*)¹⁰⁶.

III. II. II. Histone variants—Another important level of chromatin regulation is the incorporation of histone variants, which are often expressed at lower levels than major histone isoforms. For example, the histone variant H2A contributes to 1%–10% of the nucleosomes in the mammalian genome and gets phosphorylated by the ATM/ATR kinases upon DNA damage¹¹². We and others have detected significant increase in H2A.X phosphorylation (γ -H2A.X) during the initiation phase of reprogramming^{44, 113, 114}, suggesting ectopic expression of the reprogramming factors may trigger DNA damage. Supporting this hypothesis, homologous recombination genes including *Brca1*, *Brca2* and *Rad51* are required for efficient reprogramming^{113, 115}. More recent studies show that H2A.X depletion leads to decreased reprogramming efficiency¹¹⁶ and that aberrant accumulation of H2A.X marks poor-quality iPSCs^{42, 116}.

MacroH2A histone variants are characterized by their large size and are generally associated with heterochromatin¹¹⁷. MacroH2A variants are enriched in both differentiated mouse and human somatic cells^{118–120}. In mouse fibroblasts macroH2A1 and macroH2A2, together with H3K27me3, co-occupy repressed pluripotency genes^{118, 119} and are highly enriched at Utx target genes, which are reactivated early during reprogramming¹¹⁹. Similarly macroH2A1 occupies pluripotency and bivalent genes in human keratinocytes¹²⁰. More efficient reprogramming has been observed in macroH2A1 and macroH2A2-depleted mouse NSCs¹¹⁸ and double knockout mouse fibroblasts¹¹⁹. In human keratinocytes, macroH2A1 but not macroH2A2 knock-down leads to increased reprogramming efficiency, while macroH2A1 overexpression inhibits reprogramming¹²⁰. Thus macroH2A-mediated repression constitutes another barrier of reprogramming.

Unlike MacroH2A, other histones variants have been shown to promote reprogramming. TH2A and TH2B, which are highly expressed in mouse oocytes and contribute to activation of the paternal genome after fertilization, stimulate iPSC generation when over-expressed with OSKM in MEFs¹²¹. Moreover, knock-down and overexpression studies of SF1A (a histone-remodeling chaperone enriched in human oocytes) suggest this histone chaperone promotes reprogramming of HDFs¹²².

III. II. III. Nucleosome remodelers—Chromatin structure is also regulated by ATP-dependent chromatin remodeling factors that influence DNA accessibility through insertion, replacement or removal of nucleosomes. Deletion of components of the BAF (mammalian SWI/SNF) chromatin remodeling complex leads to defects in ESC self-renewal and differentiation¹²³. Through a combined functional and quantitative proteomics screen, *BRG1* and BAF155, two components of BAF, were found enriched in reprogramming-competent cell extracts. They increase OSKM-mediated reprogramming of MEFs by promoting demethylation of the promoters of pluripotency genes such as *Oct4*, *Nanog* and *Rex1*¹²⁴.

Other chromatin-remodeling proteins including Chd1 and INO80 have been shown to be required for pluripotency and reprogramming. Chd1 associates with euchromatin in ESCs and preferentially targets genes involved in chromatin organization and transcription¹²⁵. INO80 co-occupies pluripotency gene promoters in an OCT4- and WDR5-dependent manner, maintaining open chromatin and facilitating the recruitment of Mediator and RNA Pol II for gene activation¹²⁶. Both Chd1 and INO80 are required for efficient reprogramming^{125, 126}.

Mbd3 is a core member of the nucleosome remodeling and deacetylation (NuRD) repressor complex, and its exact roles in reprogramming are still under debate. In MEFs, Mbd3 overexpression significantly reduces reprogramming and its knockdown using shRNAs leads to a 10-fold increase in reprogramming efficiency¹²⁷. Using optimized *Mbd3* genetic deletion, OSKM transgene delivery and 2i/LIF naive pluripotency culture conditions *Mbd3*^{-/-} MEFs can be reprogrammed with close to 95% efficiency²¹. During OSKM-mediated reprogramming of MEFs, the genes bound by Mbd3 and Chd4 (another NuRD component) are enriched for Klf4, Oct4, Sox2 and Esrrb targets. OSKM-mediated recruitment of Mbd3/NuRD repressor complex seems to act as a potent epigenetic barrier to reprogramming²¹. In contrast to these observations, Mbd3 ablation in neural stem cells reduces iPSC formation²², and its knockdown in human fibroblasts differentiated from hESCs has a negative effect on iPSC induction¹⁰⁶. These discrepancies may result from differences in experimental setups, and the roles of Mbd3 may be sensitive to the reprogramming context.

III. II. IV. Chromatin topology—Beside transcriptional and epigenetic regulation, chromatin topology constitutes an additional layer of gene regulation in vertebrate genomes. Differentiation of PSCs leads to relocation of pluripotency loci towards the nuclear lamina where they get silenced. It has also been shown that differentiation disrupts the physical interactions (looping) between promoters and enhancers at highly transcribed pluripotency loci. Such interactions rely on the proper binding of mediator and cohesin complexes to these regulatory sequences. These observations suggest that proper reorganization of chromatin architecture may also be required for successful reprogramming¹²⁸.

Different subunits of mediator and cohesin bind to the promoters and enhancers of *Oct4* and *Nanog* in ESCs^{83, 129}. During reprogramming, they have been shown to be recruited by the reprogramming factor Klf4 at the Oct4 locus¹³⁰ and their knockdown inhibits reprogramming¹²⁹. The re-establishment of proper long-range interactions characterizes pre-iPSCs, precedes transcriptional activation of both *Nanog* and *Oct4* loci and is required for successful reprogramming^{129–131}. These results strongly support a causal relationship between the acquisition of a pluripotency-specific 3D chromatin structure and the transcriptional activation of key pluripotency loci.

III. II. V. DNA methylation/demethylation enzymes—DNA methylation marks gene inactivation in general and is considered the most stable epigenetic modification. It is established by the *de novo* methyltransferases Dnmt3a and Dnmt3b and preserved through cell division by the maintenance methyltransferase Dnmt1¹³². Silencing of somatic genes and activation of pluripotent genes during reprogramming involves DNA methylation and

demethylation respectively. *DNMT3a* and *Dnmt3a/3b* knockdown in human and mouse fibroblasts have mild effects on reprogramming¹³³, suggesting that somatic gene silencing is mainly triggered by chromatin remodeling during reprogramming.

On the other hand, DNA demethylation of pluripotency loci is a critical event in reprogramming, and it involves both passive and active mechanisms¹³⁴. Down-regulation of *Dnmt1* promotes passive, replication-dependent DNA demethylation and facilitates the transition from the maturation to the stabilization phase during reprogramming³⁶. Alternatively, active DNA demethylation can be achieved through TET-mediated hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and the subsequent replacement of 5hmC with an unmethylated cytosine through TDG-mediated base excision repair¹³⁴. 5hmC levels increase and correlate with *TET1* activation in human fibroblasts undergoing reprogramming¹³⁵. Tet1 over-expression stimulates reprogramming and can replace exogenous Oct4 expression^{136, 137}, Tet1 or Tet2 over-expression with Nanog enhance reprogramming, whereas inhibition of Nanog or Tet2 compromises iPSC formation^{88, 138, 139}. Triple *Tet1*, 2, 3-null and *TDG*-null MEFs cannot be reprogrammed demonstrating an absolute requirement of oxidative demethylation for reprogramming¹⁴⁰. However, this requirement appears specific to mesenchymal cells, as keratinocytes and neural progenitor cells can be successfully reprogrammed without any *Tet* genes. At day 4 of reprogramming, two proteins, poly(ADP-ribose) polymerase-1 (Parp1, also known as Artd1) and Tet2, are recruited to pluripotency promoters¹³⁹. Nanog has been shown to target both Tet1 and Tet2 to many pluripotency loci¹³⁸, and Tet2 induces hydroxymethylation of key pluripotency loci (Nanog, *Oct4*, *Esrrb*)¹³⁹. Parp1 has a complementary role in the establishment of early epigenetic marks during somatic cell reprogramming by regulating 5mC modification¹³⁹. There is also evidence suggesting that Parp1-mediated poly(ADP-ribosylation) of Sox2 leads to the dissociation of Sox2 from the *Fgf4* enhancer and relieves the inhibitory effect of excessive Sox2 on *Fgf4* expression in PSCs and during reprogramming^{141, 142}.

In addition to the TET proteins, activation-induced cytidine deaminase (AID) is also suggested to mediate DNA demethylation during development through base excision repair¹⁴³. However, the exact roles of AID during reprogramming have been debated. AID has been shown to be required for active demethylation of somatic cell nuclei in somatic-pluripotent cell fusion experiments¹⁴⁴, but this finding has been challenged by a following report¹⁴⁵. In OSKM-mediated reprogramming, AID over-expression leads to a 2-fold increase in iPSC generation¹⁴⁶, but no effects have been observed in subsequent studies^{147–149}. Transient shRNA knockdown between day 0–2 of reprogramming has been shown to result in a 4–5 fold reduction in iPSC colony formation¹⁴⁶. However, a later study reports that *AID* null MEFs are initially hyper-responsive to OSKM-mediated reprogramming and give rise to six-fold more Nanog⁺ colonies compared to control MEFs¹⁴⁷. This study also shows that by 4 weeks, most *AID* null iPSC colonies have differentiated, suggesting a requirement of AID for the stabilization of the pluripotent phenotype¹⁴⁷, but this finding has not been replicated in recent OSK-reprogramming studies^{148, 149}. Therefore all studies so far agree that AID is not absolutely required for

reprogramming, but there is disagreement about whether there is a subtle requirement, suggesting the roles of AID is sensitive to the reprogramming context.

III. III. Role of non-coding RNAs

III. III. I. microRNAs—MicroRNAs (miRNAs) represent a class of non-coding RNAs that are ~22 nucleotides long. In mammals, miRNAs act as post-transcriptional repressors through mediating mRNA degradation or translation blockage. A subset of miRNAs is specifically expressed in PSCs and progressively silenced during differentiation. During reprogramming, miRNAs change their expression profile in a biphasic manner like mRNAs and proteins. One wave occurs during the initiation phase and the second happens at the end of the maturation phase. miRNAs up-regulation often correlates with the down-regulation of their target genes such as *Lats2*, *TgfbR2*, and *Akt1* targeted by *miR-294*, and *Lin28*, *N-Myc*, and *Sall4* targeted by *let-7c*²⁷. Mouse PSCs deficient for *Dicer* or *DGCR8*, two enzymes essential for miRNA biogenesis, are viable but show defects in both proliferation and differentiation. In MEFs, shRNA knockdown of key miRNA processing enzymes including *Dicer*, *Drosha* or *Ago2* results in a dramatic decrease in the number of iPSC colonies generated with OSKM¹⁵⁰ suggesting miRNAs are also required in the acquisition of pluripotency.

Inhibition of the MEF-enriched miRNAs *miR-21*, *miR-29a*, *let-7*^{151, 152} or deletion of *miR-34*¹⁵³ has a positive effect during the initiation phase of reprogramming. These miRNAs act as barriers to iPSC generation through modulating the activity of different pathways antagonizing reprogramming (*TGFβ*, MAP kinase, *ERK1/2* and *p53*)^{151–154}. Binding of the EMT factor *Snai1* to *let-7* promoter inhibits *let-7* expression and releases *Lin28* repression¹⁵⁵. Moreover, *Snai1* and *Nanog* have been shown to co-occupy the regulatory region and activate the transcription of several pluripotency-associated genes including *Lin28* and *miR-290–295*. Interestingly, *Snai1* and its homolog *Snai2* exert opposite effects on reprogramming: ectopic expression of *Snai1* or conversely *Snai2* depletion promotes this process¹⁵⁶. On the other hand, over-expression of *miR-93*, *miR-106b*¹⁵⁰, *miR-130/301/721*¹⁵⁷, *miR-29b*¹⁵⁸ or *miR-135b*¹⁵⁹ enhance iPSC generation. *miR-93*, *miR-130/301/721* and *miR-29b* modulate MET through targeting *TGF-β receptor II* (*miR-93*, *miR-106b*), *Meox2* (*miR-130/301/721*) and *Dnmt3a* and *Dnmt3b*. This results in the up-regulation of epithelial genes such as *E-cadherin*, *Epcam* or *Cldn3*. Moreover, *miR-135b* modulates *Wisp1*, a key regulator of several extra cellular matrix proteins,^{150, 157, 159}.

A family of ESC cell cycle promoting miRNAs, known as the ESCC family, targets multiple inhibitors of the CyclinE-Cdk2 pathway and is highly expressed in ESCs promoting their unique cell cycle program¹⁶⁰. Over-expression of *miR-290* cluster (*miR-291-3p*, *miR-294* and *miR-295*)¹⁶¹ or the closely related *miR-106* family^{81, 150} enhances reprogramming of MEFs. Similarly, ESCC *miR-302* and *miR-372* promote reprogramming of human fibroblasts through regulating cell cycle and blocking *TGFβ*-induced EMT⁸². Furthermore, the ESCC *miR-302/367* cluster is a direct target of *Oct4* and *Sox2* in PSCs¹⁶², and its over-expression increases OSK-mediated iPSC generation through targeting *TGFβ receptor II*⁸¹. Notably, over-expression of *miR302/367* is sufficient to reprogram both MEFs and human

fibroblasts, yielding respectively 100- and 2-fold more iPSC colonies than OSKM reprogramming with a faster kinetics. *Hdac* inhibition through valproic acid (VPA) treatment¹⁶³ is however required to achieve miRNA-mediated reprogramming in MEFs⁸⁰.

These results highlight the importance of miRNAs in reprogramming, and miRNAs may offer an easier alternative to OSKM reprogramming for rapid generation of therapeutically relevant human iPSCs.

III. III. II. Long non-coding RNAs (lncRNAs)—Long non-coding RNAs (lncRNAs) represent a class of non-coding RNAs of >200 nucleotides in length. lncRNAs can activate or repress transcription *in cis* or *in trans*. Several studies indicate that lncRNAs are members of the PSC self-renewal regulatory circuit and are required for proper differentiation¹⁶⁴. A subset of lncRNAs binds one or more chromatin modification complexes, including readers, writers, or erasers of repressive histone modifications including polycomb complexes¹⁶⁵.

Relatively few specific lncRNAs have been studied in details for their roles in reprogramming. The lncRNA “regulator of reprogramming” (lncRNA-RoR) was identified through comparing lncRNA expression profiles between human fibroblasts, iPSCs and ESCs. Among a group of 28 “iPSC-enriched” lncRNAs, lncRNA-RoR inhibition or over-expression either decreases or increases the respective efficiency of iPSC formation, providing the first demonstration for functional contribution of a lncRNA during reprogramming¹⁶⁶. However, loss-of-function of most lncRNAs up-regulated during reprogramming has no significant effect on reprogramming, although transcriptional analysis suggests some of them may play a role in suppressing lineage genes (Ladr49, Ladr83, Ladr317) or activating metabolic genes (Ladr86, Ladr91)¹⁶⁷.

III. IV. Roles of signal transduction pathways

III. IV. I. Developmental signaling pathways—Several signaling pathways central to the regulation of pluripotency in PSCs also play important roles in reprogramming likely because they regulate components of the pluripotency transcriptional network. Activation of the Jak-Stat3 pathway by the cytokine leukemia inhibitory factor (LIF) and BMP-SMAD signaling is essential for the maintenance of mESCs. Other secreted factors, including Wnt, activin/nodal, and bFGF contribute to pluripotency in various culture conditions of mESCs and hESCs¹⁶⁸. Dual chemical inhibition of MEK (Erk1/Erk2) and canonical Wnt/ β -catenin pathways (GSK-3) also known as the 2i condition is sufficient to sustain self-renewal of mouse ESCs in the absence of any extrinsic signal, a condition referred to as the ground state pluripotency¹⁶⁹. When applied at 7–9 days of reprogramming, MEK inhibition limits the growth of non-iPSC colonies and promotes the growth of reprogrammed iPSCs from neural progenitor cells (NPCs)¹⁷⁰. Moreover, Wnt3a-conditioned media increase MEF reprogramming with OSK¹⁷¹, and GSK-3 inhibition allows reprogramming MEFs with only two factors OS¹⁷².

Wnt seems to mediate its effect on reprogramming through repression of Tcf3, which binds and inhibit *Oct4*, *Nanog* and *Sox2* in mouse ESCs¹⁷³. Moreover, β -catenin is indirectly regulated by Nanog and is necessary for conversion of pre-iPSCs into iPSCs¹⁷⁴. Combining 2i and LIF promotes the transition of partially reprogrammed cells toward *bona fide*

iPSCs¹⁷⁵. Similarly, activation of the Jak-Stat3 pathway by LIF promotes reprogramming by facilitating the transition from partially reprogrammed cells toward fully reprogrammed iPSCs¹⁷⁶. The conventional hPSCs bear more similarities to primed mouse EpiSCs than the naïve mESCs. For a long time, it was unclear whether hPSCs could adopt a naïve pluripotent state. Recently, several studies have reported the generation of naïve hPSCs using different combinations of chemical compounds and cytokines^{177–182}. Naïve hPSCs, including naïve hESCs and hiPSCs, share numerous morphological and molecular similarities with naïve mESCs, suggesting conservation of a naïve pluripotency program *in vitro*.

Other developmental or disease-related pathways, including cyclic AMP¹⁸³, PI3K¹⁸⁴, Hippo/Yap¹⁸⁵, Src¹⁸⁶, Fgf¹⁴¹ and Notch pathways¹⁸⁷ have also been shown to modulate reprogramming efficiency or functionally replace some of the reprogramming factors. Notably, inhibition of TGF β using small molecule inhibitors allows reprogramming MEFs with only OK^{188, 189} likely through promoting MET³⁰. Building upon these and other findings on chemicals that enhance reprogramming¹⁹⁰, Hou and colleagues performed an extensive small-molecules screen and identified a combination of seven chemical compounds sufficient to reprogram mouse somatic cells with up to 0.2% efficiency. Thus rather than imposing the pluripotency program through transcription factor over-expression, reprogramming can be achieved through simply modulating molecular pathways not strictly related to pluripotency¹⁹¹.

Importantly, the effects of signaling molecules may depend on the donor cell type and the reprogramming stage. While more than 80% of MEFs reprogram through OSKM expression, TGF β inhibition, Wnt activation and ascorbic acid treatment, hepatoblast or blood progenitors only require TGF β inhibition or Wnt activation alone respectively to achieve similar reprogramming efficiencies with OSKM¹⁹². In reprogramming MEFs, BMP promotes MET in the early initiation phase²⁶ but blocks the late transition of pre-iPSC to iPSCs⁵⁵; whereas Wnt signaling has inhibitory effects early and a stimulatory role late in reprogramming¹⁹³.

III. IV. II. Stress-induced signaling pathways—The p53 tumor-suppressor processes a variety of extra- and intracellular stress signals (e.g. DNA damage, oxidative stress, ...) into different cellular responses including DNA repair, cell cycle arrest, senescence, and programmed cell death. An important module regulating p53 activity is the p53-p19 (ARF)-Mdm2 network: p53 transcriptionally represses p19 and activates Mdm2, Mdm2 blocks p53 transcriptional activation and promotes its polyubiquitination and proteasome-mediated degradation, p19 binds to Mdm2 and inhibits its ubiquitin ligase activity¹⁹⁴. Elevated p53 in response to stress signals or elevated p19 as a consequence of oncogenic signals or release of E2F, results in p53 stabilization and up-regulation of its targets genes including pro-apoptotic *Bcl2* family members and *p21* (*CIP1*), a CDK (cyclin-dependent kinase) inhibitor, leading to cell-cycle arrest in the G1 and G2 phases¹⁹⁵.

Several studies have shown that p53 deletion or depletion in MEFs leads to the formation of significantly higher numbers of iPSC colonies^{44, 196–198}. Contrary to Nanog over-expression however, p53 inhibition affects reprogramming kinetics and efficiency in a proliferation-dependent manner¹⁹. In wild-type fibroblasts, over-expression of the

reprogramming factors triggers DNA damage and oxidative stress, leading to a significant increase in the number of apoptotic and senescent cells^{44, 113, 197, 199}. These effects correlate with an increase in p53 level and activity, and up-regulation of p19, p21 and Mdm2^{196, 197, 199}. This p53-mediated response is particularly pronounced in cells harboring pre-existing DNA alterations, such as shorter telomeres, suggesting that p53 limits reprogramming through elimination of genetically abnormal cells²⁰⁰. Consistently, as observed for p53, down-regulation of *p19*, *Mdm2* and *p21* also increases reprogramming efficiency^{196–198}.

The *Ink4/Arf* locus encodes two important tumor suppressors: p16 (Ink4a) and p15 (Ink4b) in addition to p19. P16 and p15 bind and inhibit the cyclin-D-dependent kinases Cdk4 and Cdk6, which in turn are important to relieve the cell-cycle inhibitory activity of the Retinoblastoma (Rb) tumour suppressor, and Rb controls multiple cellular functions, including proliferation, survival, differentiation, metabolism, and genomic stability²⁰¹. In wild-type fibroblasts silencing of the *Ink4/Arf* locus accompanies the establishment of iPSCs, and single or combined deletion or depletion of the genes encoded by this locus promotes reprogramming^{198, 199, 202}. Rb null MEFs also reprogram with a higher efficiency. Considering the regulation of Rb by p15/p16, Rb may act through limiting proliferation during reprogramming as shown for p53. However, contrary to this prediction, Rb does not significantly affect cell proliferation, and instead acts predominantly through globally repressing the pluripotency network in somatic cells²⁰³.

Ataxia-telangiectasia mutated (Atm) has a critical role in the cellular response to DNA double-strand breaks (DSBs). Upon DNA DSBs, Atm is activated and phosphorylates p53 and H2AX. H2AX recruits Mbc1 at the site of the break and triggers DNA repair by recruiting the homologous recombination (HR) repair machinery including Brca1, Brca2, Rad51, 53BP. Supporting the central role of p53 in eliminating DNA-damaged cells during reprogramming, MEFs deficient in either Atm or 53BP1 show a decrease in reprogramming efficiency^{44, 204, 205}. Moreover, HR DNA DSB repair genes such as Brca1, Brca2, Rad51^{113, 115, 206}, Fanconi anemia genes involved in both DNA DSB repair and interstrand cross-link (ICLs) processing^{114, 207–210} and non-homologous end joining (NHEJ) DSB repair genes^{211, 212} have all been shown to be required for efficient reprogramming to a pluripotent state.

These observations suggest that reprogramming and malignant transformation shares common features. Similar to cancer, reprogramming occurs in a small fraction of cells and proceeds through a multi-step process involving global transcriptional, epigenetic, metabolic and cellular changes. Unlike somatic donor cells, iPSC are immortal in culture, and these cells can form benign tumors (teratomas) when transplanted into immunocompromised mice. Molecularly, OSKM have each been shown to act as oncogenes in different contexts, and many chromatin and DNA methylation regulators cooperating with OSKM during reprogramming are associated with tumorigenesis. These similarities suggest that cancer-initiating cells may need to overcome similar barriers as iPSCs during tumor development. This is exemplified by the silencing of the *Ink/Arf* locus, which is required for iPSC formation and also observed in a large number of tumors. A major difference however exists: *bona fide* iPSCs display a normal karyotype and support full development in

tetraploid complementation experiments, whereas most cancer cells are aneuploid and characterized by limited differentiation potential^{128, 213}.

III. IV. III. Metabolic signaling pathways—Normal somatic cells usually generate energy from mitochondrial oxidative phosphorylation (OXPHOS), whereas PSCs and cancer cells rely mainly on glycolysis for energy production. In both PSCs and cancer cells, glycolytic genes are up-regulated, mitochondrial activity is reduced, and lactate production is significantly increased. While ambient air has a concentration of ~21% O₂, the concentration is much lower in tissues, between ~0.7% – 7%, and the O₂ concentration experienced by blastocysts and many cancer cells *in vivo* is close to the lower end of this range. The dependency of PSCs on glycolysis to produce ATP may therefore reflect an adaptation of ICM cells to the low-oxygen niche in the blastocyst²¹⁴.

Lowering oxygen concentration in culture from normoxic (~21%) to hypoxic (~5%) conditions has been shown to enhance the generation of iPSCs from mouse and human somatic cells²¹⁵. Additionally, reprogramming efficiency can be increased through activation of glycolysis or blockade of mitochondrial OXPHOS using fructose 2,6-bisphosphate (PFK1 activator), 2,4-dinitrophenol (mitochondria decoupler), quercetin (HIF activator), or PS48 (PDK1 activator)²¹⁶. The cellular adaptation to hypoxic conditions is mainly mediated through the activation of the oxygen-sensitive transcription factors, hypoxia-inducible factors (HIFs). Interestingly, HIF1 α has been shown to activate Notch signaling and maintain the undifferentiated state of various stem and progenitor cell populations²¹⁷, and HIF2 α binds to Oct4 promoter and regulates its expression in PSCs²¹⁸. During reprogramming of human fibroblasts, knockdown of either HIF1 α or HIF2 α reduces the number of iPSC colonies formed, and over-expression of non-degradable forms of either proteins during early phases of reprogramming significantly improves reprogramming efficiency and accelerates the switch toward glycolytic metabolism⁴¹.

Treatment with Ascorbic Acid (Vitamin C, or Vc), a potent antioxidant, significantly improves the efficiencies of both OSK- and OSKM-mediated MEF reprogramming. This effect is mediated at least in part through a reduction of cell senescence as suggested by a significant reduction in p53 and p21 levels in Vc-treated reprogramming cells²¹⁹. Vc also improves iPSC quality through preventing aberrant silencing of the imprinted *Dlk1-Dio3* locus, suggesting a direct role of Vc in epigenetic remodeling²²⁰. Supporting this notion, Vc promotes H3K36me2/3 demethylation during reprogramming, an activity mediated by Vc-dependent demethylating dioxygenases Jhdm1a/1b. In addition, Jhdm1b accelerates cell cycle progression and suppresses cell senescence by repressing the *Ink4/Arf* locus, and cooperates with Oct4 to activate the miR-302/367 cluster⁵³. In addition to Jhdm1a/1b, Vc also modulates TET1 function and 5hmC formation at loci critical for MET during reprogramming¹³⁷.

Conclusion

Since Takahashi and Yamanaka's demonstration that reprogramming can be achieved through ectopic expression of a surprisingly small number of pluripotency-associated transcription factors, a large body of work has contributed to the improvement of the

reprogramming methods and a better understanding of the mechanisms underlying this process. Several recent studies have achieved almost deterministic reprogramming using privileged donor cells or through epigenetic manipulation, suggesting that the initial stochasticity attributed to the process is context-dependent. These advances are not only fundamental to our understanding of pluripotency and the nature of cell identity in general, but are also of critical importance to the therapeutic application of reprogramming.

One important consideration is the safety of the cells for therapy. For instance, initial studies have identified transgene integration as a major limitation of hiPSC in a therapeutic setup due to risks of tumorigenesis associated with random transgene insertional mutagenesis and potential reactivation of the reprogramming transgenes. This issue has been mitigated through the development of non-integrating reprogramming methods²²⁴. Independent of transgene insertion, several studies have identified different types of genetic alterations in hiPSCs. Some of these alterations may result from passaging, other mutations have been identified in rare donor cells, and some are acquired *de novo* during the reprogramming process. While these mutations highlight the importance of screening multiple patient-derived iPSC lines in order to select the safer line(s) for therapeutic purposes, the extent of these alterations compared to hESCs is not dramatic²²⁵.

Another area of intensive investigation is regarding the similarity between hiPSCs and hESCs, which remains an important question relevant to both basic stem cell biology and the therapeutic use of hPSCs. While initial reprogramming studies highlighted the strong similarity between hiPSCs and hESCs, further analysis revealed significant differences in their transcriptomes, epigenomes, *in vitro* and *in vivo* differentiation potentials. Some of these differences may reflect intrinsic variability in the molecular and functional properties of PSCs. Some may be inherent to hiPSC as a consequence of the reprogramming itself²²⁶. For instance, incomplete epigenetic remodeling, referred to as a memory of the cell-of-origin, has been described in hiPSC lines, and epigenetic memory has been suggested to be responsible for lineage-specific bias of hiPSCs during differentiation, though this bias diminishes through passaging and could be prevented by optimizing the reprogramming conditions²²⁷. Recently, nt-hESC lines have been established from human cells^{5, 6}. Two studies comparing isogenic nt-hESCs and hiPSC lines reveal that the two reprogramming methods induce comparable numbers of *de novo* coding mutations^{228, 229}. However, while one study suggests that the transcriptional and epigenetic profiles of nt-hESCs are closer to hESCs than hiPSCs²²⁹, the other study identified equal numbers of epigenetic alterations in nt-hESCs and hiPSCs, suggesting these differences are inherent to reprogramming regardless of the exact reprogramming approach²²⁸. It remains to be seen whether and how any differences among hiPSCs, nt-hESCs and hESCs affect the usage of the cells for disease modeling and cell replacement therapy. The answers to these questions may become clearer as we gain a better understanding of reprogramming.

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Sidebar**Culture-derived Pluripotent Stem Cell lines**

Embryonal carcinoma cells (ECCs): derived from mouse teratocarcinomas, these are the first PSC lines generated. ECCs self-renew and differentiate into multiple embryonic lineages *in vitro* and to some extent *in vivo*. Human ECCs show limited differentiation potential (Figure 1 (a)).

Embryonic stem cells (ESCs): derived from the inner cell mass (ICM) of pre-implantation mouse blastocysts, they differentiate into a wide range of cell types *in vitro*. Unlike ECCs, they also contribute to all lineages at a high frequency in chimera experiments. The first hESC lines were generated in 1998 (Figure 1 (b)).

Epiblast stem cells (EpiSCs) and region-selective pluripotent stem cells (rsPSC): derived from post-implantation mouse epiblasts using different culture conditions, they self-renew and differentiate into various cell types *in vitro* (Figure 1 (c)). Conventional hESCs are considered primed, as they share common features with primed mouse EpiSCs and are distinct from naïve mESCs. rsPSCs can be derived from primed hPSCs and contribute to post-implantation interspecies chimeric embryos ²²¹.

Embryonic germ cells (EGCs) and germ line stem cells (GSCs): derived respectively from mouse primordial germ cells (Figure 1 (d)) or germ line stem cells (GSCs) from neonatal and adult testis (Figure 1 (e)), they resemble ESCs but retain some epigenetic features of their cell of origin. Human EGCs show limited self-renewal capacity and the pluripotency of human GSCs has been seriously questioned ^{222, 223}.

Figure 1. Sources of pluripotent Stem Cells
Culture-derived pluripotent stem cells (PSC) are generated from different *in vivo* cell types. (a) Embryonal carcinoma cells (ECCs), derived from germline tumors (teratocarcinomas); (b) embryonic stem cells (ESCs), derived from the inner cell mass (ICM) of pre-implantation mouse and human embryos at mouse embryonic day 3.5 (mE3.5) or human embryonic day 5.5 (hE5.5); (c) epiblast stem cells (EpiSCs) and region-selective pluripotent stem cells (rsPSCs), obtained from early post-implantation mouse embryos at mE5.5–7.5; (d) embryonic germ cells (EGCs) retrieved from mouse and human primordial germ cells (PGCs) respectively at mE8.5–12.5 or between weeks 3 and 5 of human development (hW3-5); (e) and germline-derived PSCs (GSCs), derived from spermatogonial stem cells of mouse neonatal and adult testes. In each of the above columns, the cell of origin of the different pluripotent stem cell lines is labeled in blue. Alternatively, exposing the nuclei of somatic cells to exogenous reprogramming factors can induce PSCs. (f) Nuclear transfer embryonic stem cells (ntESC) are obtained by reprogramming somatic nuclei (pink) with factors contained in an enucleated oocyte (blue), and cultured to the blastocyst stage to derive ntESCs from the ICM; (g) Through a similar approach, fusion between a somatic cell (pink) and a PSC (blue) gives rise to cell-fusion-derived tetraploid (4N) hybrid ESC (cfESC) lines; (h) Alternatively, over-expression of the reprogramming transcription factors, Oct4 (O), Sox2 (S), Klf4 (K) and cMyc (M) in a somatic cell (pink) using viral delivery (blue) allows generating induced pluripotent stem cells (iPSC); (i) F-class cells are generated through high and constitutive expression of OSKM in a somatic cell (pink) using an inducible integrated transgene (blue). F-Class cells share molecular and phenotypic features with iPSCs and ESCs.

Figure 2. Models of reprogramming

Two types of models have been proposed to describe reprogramming: deterministic and stochastic. (a–c) Deterministic models predict that somatic donor cells give rise to iPSCs with a fixed latency (green arrows of same length). (d–f) Stochastic models predict that somatic donor cells give rise to iPSCs with variable latencies (green arrows of different lengths). In both models, either all (a, d) or only a subset of elite cells (brown) (b–c, e–f) is permissive to reprogramming and elite cells can be present in the donor population before reprogramming (predetermined) (b, e) or induced upon viral delivery (red dots) (c, f). The latency (green arrows) reflects the absolute time or the number of cell divisions required to produce an iPSC from the donor population.

Adapted from Bambrink et al., Cell Stem Cell 2008, Stadtfeld et al., Cell Stem Cell 2008, Samavarchi-Tehrani et al., Cell Stem Cell 2010 and Polo et al., Cell 2012.

Figure 3. Phases of Reprogramming

(a) Kinetics of pluripotency-marker appearance and definition of the reprogramming phases. Alkaline phosphatase and Stage Specific Embryonic Antigen 1 (SSEA1) positive cells appear around day 2 and 3, respectively after OSKM transduction, coinciding with the down-regulation of the mesenchymal marker Thy1. GFP expressed from the endogenous *Oct4* or *Nanog* loci is detected later, around day 9 and 10 respectively. The virally transduced factors need to be expressed for approximately 12 days to generate stable, transgene-independent iPSCs. (b) A schematic drawing illustrating the kinetics and the magnitude of the molecular changes observed during reprogramming. Permissive reprogramming cells (positive y axis) show a biphasic pattern of Protein, mRNA and microRNA (miRNA) expression changes following the kinetics of individual histone modifications. Bivalent domains are generated gradually after an initial burst and DNA methylation changes occur predominantly at the end of reprogramming. Refractory cells (negative y axis), undergo a similar wave of expression changes during the initiation phase but remain relatively stable afterwards. Forced expression of OKSM in refractory cells can rescue their ability to form iPSCs (Adapted from Polo et al., 2012).

Table 1

Molecular and functional assays to assess the developmental potential of PSCs.

Pluripotency assays	Methods	Limitations	Mouse	Human
Expression profiling	Immunostaining, qPCR, RNAseq to assess expression profile of pluripotency genes.	Gene expression is not a functional test.	YES	YES
Epigenetic profiling	Chromatin: ChIP qRT-PCR, ChIPseq of common chromatin marks, PolI I, ... DNA: Targeted or genomewide DNA methylation patterns through bisulfite sequencing.	Epigenetic signature is not a functional test.	YES	YES
<i>In vitro</i> differentiation	Directed differentiation in culture towards specific lineages or embryoid body formation and assayed for the expression of specific lineage markers.	Marker expression is not a functional test.	YES	YES
Teratoma	Injection of PSCs in immunocompromised mice and formation of teratocarcinomas to test the differentiation potential of cells to various lineages.	Does not test the contribution of cells to normal development.	YES	YES
2N Blastocyst complementation	Injection of PSCs into a diploid blastocyst to test the contribution of cells to normal development through formation of a chimeric mouse.	Host cells may complement non-autonomous cell defects of PSCs.	YES	YES/NO*
Germline contribution	Injection of PSCs into a diploid blastocyst and breeding of the resulting chimera to test the contribution of cells to the germline.	Complementation by host cells. Epigenetic defects of PSCs not revealed.	YES	NO
4N Blastocyst complementation	Injection of PSCs into a tetraploid blastocyst. Since 4N cells cannot contribute to somatic lineages, the embryo is 100% PSC-derived.	Most stringent assay for pluripotency. Does not test trophectoderm potential.	YES	NO

* This assay may be used to test the contribution of hPSCs at early stages of development but is limited at later stages for ethical reasons related with the formation of human-mouse chimeras.

Table 2
Transcriptional and epigenetic changes during reprogramming

(a) Expression changes (mRNA or protein). Genes in green are up-regulated and genes in red are down-regulated. (b) Epigenetic changes. Marks in green are generally associated with active loci and marks in red are generally associated with repressed loci. Arrows pointing up or down indicate respectively an increase or a decrease in the corresponding epigenetic mark. EMT, epithelial-to-mesenchymal transition; MEF, mouse embryonic fibroblast; MET, mesenchymal-to-epithelial transition. H3, histone H3; K4, K9, K27, K36, K79, Lysines 4, 9, 27, 36 and 79 of histone H3; me1, me2, me3, mono-, di- or tri-methylated respectively; DNA-5mC, 5-methyl cytosine DNA methylation.

(a) EXPRESSION CHANGES OCCURING DURING REPROGRAMMING		
initiation	maturation	stabilization
Mesenchymal genes: <i>Snai1/2, Zeb1/2, Slug</i>	Pluripotency genes: <i>Oct4, Sall4, Nanog, Sox2, Esrrb, Lin28, Dppa2, Tcf1, Nr5a2, Sox2 (late)</i>	Late pluripotency genes: <i>Utf1, ...</i>
Epithelial genes: <i>Cdh1, Epcam, Cldn3, -4, -7, -11, Oc1n, Crb3</i>	Germline genes: <i>Mnd1, Mutyh, Rad54b</i>	DNA methylation: AID, TET and Dnmt family members
Early pluripotency genes: <i>Alpl, Fbxo15</i>	Cytoskeletal genes: <i>Tuba3a, Pdzk1, Itgb7, Kirrel2</i>	Somatic genes: progressive erasure of remaining "epigenetic memory" of the cell-of-origin. May be partially or completely achieved depending on the time in culture.
Core regulatory circuit: <i>Jarid2, Rif1, Tcf3, Eed</i>	RET-signaling genes: <i>Nrtn, Kif26a</i>	
Transcription regulation: <i>Mediator, TAF, RNA Pol II, Nurd</i>	Hypoxia-responsive genes: <i>ALDOC, ENO3, PKM2, Pfkf, Gpi, Pgk1</i>	
Proliferation genes: <i>Poli, Rfc4, Mcm5, Ccnd1, Ccnd2</i>	epidermal genes: <i>Ehf, Elf5, Krt17, Krt6a (transient)</i>	
Stress genes: <i>Cdkn1a, Cdkn2a</i>	Lineage genes up or down (transient)	
SURFACE MARKERS: <i>SSEA1, CD73, CD49d, CD200</i>	REPORTER GENES: <i>Oct4-GFP or Nanog-GFP</i>	
SURFACE MARKERS: <i>THY1</i>		
(b) EPIGENETIC CHANGES OCCURING DURING REPROGRAMMING		
initiation	maturation	stabilization
H3K4me1 ↑ Proliferation, metabolism genes	H3K9me3 ↓ Late pluripotency genes	H3K9me3 ↓ Late pluripotency genes
H3K4me2 ↑ Proliferation, metabolism, pluripotency, MET genes	H3K36me3 ↑ Late pluripotency genes	H3K36me3 ↑ Late pluripotency genes
↓ MEF, EMT genes	H3K79me2 ↓ MEF, EMT genes	DNA-5mC ↓ Pluripotency genes
H3K4me3 ↑ Proliferation, metabolism genes		X chromosome in XX cells
H3K27me3 ↑ MEF, EMT genes		Retroviral transgenes
H3K27me3 ↓ Global reduction	H3K27me3 ↑ Gradual increase (bivalent domains)	DNA-5mC ↑ Mesenchymal genes
H3K36me2 ↑ Early pluripotency genes		
H3K36me3 ↑ Early pluripotency genes		
H3K79me2 ↓ MEF, EMT genes		