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Chromatin Dynamics during Cellular Reprogramming

Effie Apostolou^{1,2} and **Konrad Hochedlinger**^{1,2,#}

¹Massachusetts General Hospital Cancer Center and Center for Regenerative Medicine; Harvard Stem Cell Institute, 185 Cambridge Street, Boston, MA 02114, USA

²Howard Hughes Medical Institute and Department of Stem Cell and Regenerative Biology, Harvard University and Harvard Medical School, 7 Divinity Avenue, Cambridge, MA 02138, USA

Preface

Induced pluripotency is a powerful tool to derive patient-specific stem cells. In addition, it provides a unique assay to study the interplay between transcription factors and chromatin structure. Here, we review the latest insights into chromatin dynamics inherent to induced pluripotency. Moreover, we compare and contrast these events with other physiological and pathological processes involving changes in chromatin and cell state, including germ cell maturation and tumorigenesis. We propose that an integrated view of these seemingly diverse processes could provide mechanistic insights into cell fate transitions in general and might lead to novel approaches in regenerative medicine and cancer treatment.

Introduction

Reprogramming of somatic cells to pluripotency can be achieved by different approaches, including somatic cell nuclear transfer (SCNT) into oocytes, fusion between somatic and pluripotent cells and ectopic expression of defined transcription factors (TFs)^{1,2}. SCNT demonstrated that epigenetic rather than genetic changes are the basis for most differentiation processes during normal development. Cell fusion experiments documented that the pluripotent state is dominant over the somatic state in the context of hybrids. Together, these observations led to the seminal discovery that a small set of TFs, such as Oct4, Sox2, Klf4 and c-Myc (collectively called OKSM), are sufficient to convert differentiated cells into induced pluripotent stem cells (iPSCs)³. Importantly, induced pluripotency provides a biochemically and genetically tractable system to dissect the mechanisms underlying this remarkable cell fate change.

Recent progress in genome-wide technologies and the analysis of small cell numbers has allowed researchers to capture transcriptional and epigenetic snapshots of rare cell populations undergoing cell fate transitions in different biological contexts. These analyses yielded important insights into the type and sequence of molecular changes inherent to transcription factor-induced pluripotency, germ cell reprogramming and cellular transformation. A common theme emerging from these studies is that nascent iPSCs, developing germ cells and premalignant cells utilize different as well as overlapping

[#]Correspondence: khochedlinger@helix.mgh.harvard.edu.

mechanisms to alter cell identity. The aim of this review is to define those transcriptional, chromatin and epigenetic changes that endow specialized cells with pluripotency as well as the molecular barriers that resist cell fate change.

Mechanisms of Induced Pluripotency

Acquisition of induced pluripotency is a slow (~2 weeks) and inefficient (0.1-3%) process^{1,3}, indicating that TFs need to overcome a series of epigenetic barriers that have been gradually imposed on the genome during differentiation to stabilize cell identity and to prevent aberrant cell fate changes. Earlier work has shown that cell populations expressing OKSM pass through a sequence of distinct molecular and cellular events (Figure 1). Fibroblasts initially downregulate markers associated with the somatic state and subsequently activate genes associated with pluripotency, suggesting an ordered process^{4,5}. As soon as nascent iPSCs activate endogenous core pluripotency genes including *Oct4*, *Sox2* and *Nanog*, they acquire a self-sustaining pluripotent state and no longer require exogenous factor expression. The latter events also coincide with the activation of the silenced X chromosome in female somatic cells, the upregulation of telomerase and the acquisition of immortality, which are hallmarks of cultured pluripotent cells^{5,6}. In the following sections, we will review current knowledge of how overexpressed TFs engage with chromatin, collaborate with epigenetic regulators and integrate extracellular signals in order to reprogram cellular identity (Figure 2).

Transcription Factors Drive Cell Fate Change

The most commonly used combination of reprogramming TFs comprises OKSM, and we will therefore primarily focus on this set of factors³. Previous results suggested that c-Myc and OKS play distinct roles during the acquisition and maintenance of pluripotency⁷. Briefly, OKS are the minimally required set of factors for iPSC generation from many cell types under classic reprogramming conditions (i.e. in the presence of serum and the cytokine LIF). OKS cooperatively suppress lineage-specific genes and activate embryonic stem cell (ESC)-related genes, resulting in the establishment of a self-sustaining pluripotency network⁷. In contrast, ectopic c-Myc expression significantly enhances and accelerates reprogramming but is dispensable for iPSC formation^{8,9}. c-Myc expression functions early during reprogramming, presumably by stimulating cell proliferation and inducing a metabolic switch from an oxidative to a glycolytic state typical of pluripotent cells^{10,11}. More recent evidence suggests that c-Myc may also contribute to reprogramming by inducing pause release and promoter reloading of RNA polymerase, leading to transcriptional amplification of target genes^{12,13}.

It is worth noting that each of the original four reprogramming factors has been functionally replaced by either related TFs, upstream epigenetic modifiers, miRNAs or small compounds¹. Moreover, iPSCs have been derived with molecules that do not contain any of the original TFs^{14,15}, indicating a remarkable flexibility and redundancy among reprogramming factors (Figure 2). For example, a recent report demonstrated that the core pluripotency factors Oct4 and Sox2 can be substituted for by lineage specifiers such as Gata3 and Geminin¹⁶. These molecules have been associated with mesendodermal and ectodermal differentiation, respectively, and they mutually repress the respective other

lineage program, suggesting that the suppression of these major differentiation pathways is sufficient to trigger iPSC induction. This idea is consistent with the observation that many classic pluripotency factors, including Oct4, Sox2 and Nanog, participate in early lineage decisions and hence may also be considered lineage specifiers¹⁷. A prediction that follows from these results is that TFs that stabilize the somatic state should be inhibitory to OKSM-mediated reprogramming. Indeed, depletion of the B cell-specifying TF Pax5 or ectopic expression of its antagonist CEB/P-alpha enables the reprogramming of terminally differentiated B cells¹⁸. Conversely, forced expression of lineage-specific TFs, in combination with OKSM, significantly impairs iPSC formation by sustaining a somatic gene expression program and preventing activation of pluripotency genes¹⁹. Together, these findings demonstrate that reprogramming TFs have to achieve two key tasks, namely the extinction of the somatic program, which is maintained by counteracting TFs, and the induction of a stable pluripotent state typical of ESCs.

Different Types of Chromatin Targets

Developmental progression from pluripotent stem cells via progenitors to terminally differentiated cells is accompanied by a gradual deposition of repressive histone marks, followed by chromatin compaction²⁰⁻²². A key question is therefore how reprogramming TFs dismantle somatic chromatin and establish an epigenetic state that is compatible with pluripotency. Recent studies assessing OKSM occupancy and histone marks early during the reprogramming of mouse and human fibroblasts into iPSCs have provided first clues to this question^{10,23,24}. Combining these observations, one may categorize OKSM targets into three classes of loci based on (i) chromatin accessibility, (ii) the requirement for additional remodeling, and (iii) the kinetics of transcriptional activation (Figure 3a). Genes with an “open” chromatin state in somatic cells comprise the first group of targets, characterized by increased DNaseI hypersensitivity, active H3K4me2/3 marks and the ability to bind OKSM immediately. Downregulated somatic genes and genes linked to a mesenchymal-to-epithelial transition (MET), which specifies early stages of reprogramming²⁵, fall into this group²⁴.

A second class of early-bound OKSM targets includes distal regulatory elements, which appear to require additional chromatin remodeling for transcriptional activation²⁴. A subgroup of these elements carries the H3K4me1 mark and exhibits nucleosome depletion as well as DNase I hypersensitivity, which are chromatin features characteristic of “permissive enhancers”. Permissive enhancers typically bind TFs prior to their associated promoter regions and before transcriptional activation²⁶. The *MyoD* locus exemplifies this group of enhancers; ectopically expressed Oct4 initially binds to the *MyoD* enhancer, triggering crosstalk with its promoter and subsequent acquisition of a poised chromatin state²⁶. Another subset of distal regulatory elements comprises DNase I-resistant loci unable to bind c-Myc alone²⁴. Early pluripotency genes such as *Sall4* belong to this group. Interestingly, occupancy of these targets by OKS facilitates binding of c-Myc. This observation thus identifies OKS as “pioneer factors” for c-Myc, which defines the ability of TFs to bind closed somatic chromatin and enable chromatin remodeling as well as recruitment of other TFs and cofactors²⁴.

Broad heterochromatic regions enriched for the repressive H3K9me3 mark constitute a third set of OKSM targets. Genes within this category comprise core pluripotency genes, such as *Nanog* and *Sox2*²⁴. These regions are refractory to immediate OKSM binding and seem to require the most extensive chromatin remodeling for transcriptional activation. Of interest, refractory domains are reminiscent of broad chromatin regions containing extensive H3K9 dimethylation termed LOCKs (Large Organized Chromatin K9 modification)²⁷. LOCKs are generally underrepresented in pluripotent cells and associated with repression of lineage-specific genes in differentiated cells²⁷.

Bivalent genes constitute a separate group of chromatin targets that are marked by both active H3K4 methylation and repressive H3K27 methylation in iPSCs or ESCs²⁸. Genes within this category are transcriptionally silent in ESCs and iPSCs but poised for rapid activation upon lineage commitment. Although the actual number and relevance of bivalent promoters remain controversial²⁹, induced pluripotency gradually restores these domains to an ESC-like pattern^{10,23}. The transcription factor Utf1 has recently been implicated in the regulation of bivalency by a dual mechanisms that involves deposition of the repressive H3K27me3 mark and degradation of residual transcripts³⁰. Notably, Utf1 expression can substitute for some of the original reprogramming factors, providing indirect evidence that the establishment of bivalent promoters may be an important step during the acquisition of pluripotency¹⁴. Together, these results illustrate how the initial chromatin state of somatic and pluripotency-related genes determines if and when they become occupied and transcriptionally regulated by OKSM in the course of reprogramming. They further suggest that certain histone marks (e.g., H3K9 methylation) act as potent barriers that resist acquisition of pluripotency. We will therefore next focus on the enzymes that deposit or remove these marks and their impact on cellular reprogramming.

Role of Histone-modifying Enzymes

Histone modifications and chromatin structure are regulated by histone code “writers” such as histone methyltransferases (HMTs) and acetyltransferases (HATs) and “erasers” such as histone demethylases (HDMs) and deacetylases (HDACs) (Figure 2). These enzymes function as coactivators or corepressors of OKSM at different stages of reprogramming and can profoundly influence iPSC derivation. For example, recruitment of PRC2, which deposits the repressive H3K27me3 mark, and inhibition of Dot1L, which establishes the active H3K79me2/me3 marks, have been associated with the downregulation of somatic genes early in reprogramming^{31,32}. Accordingly, loss of PRC2 abrogates whereas loss of Dot1L enhances iPSC formation. Activation of the H3K36 HDMs *Jhdm1a/1b*³³ and suppression of the H3K27 HDM *Jmjd3* promote intermediate-to-late stages of iPSC generation by suppressing the *Ink4/Arf* locus³⁴, which is essential for the acquisition of immortality. An additional early role for *Jhdm1b* in epithelial gene activation was recently reported³⁵. In contrast, H3K9 HMTs maintain the abovementioned “refractory” heterochromatic state of somatic cells and thus act as major barriers of reprogramming. Consistent with this notion, knockdown of G9a (H3K9me2 HMT) or *Suv39h1/h2* and *Setd1* (H3K9me3 HMTs), or overexpression of H3K9 HDMs, increases TF accessibility and results in more efficient iPSC generation from somatic cells^{24,36,37}. Altogether, these results demonstrate that histone code writers and erasers are essential components of iPSC

formation by either maintaining the somatic state or assisting in the TF-induced establishment of pluripotency.

Reprogramming TFs have been reported to directly interact with histone-modifying enzymes, providing a mechanistic explanation for how they may alter chromatin and cell state during induced pluripotency. Examples include the H3K27 HDM Utx¹⁴ and the H3K4me3 “effector” Wdr5³⁸, which bind to Oct4 protein and co-occupy many genomic targets in ESCs, thus keeping them in a transcriptionally active state. Depletion of either molecule blunts iPSC formation due to a failure to activate pluripotency genes^{14,38}. Oct4 may play a very specific role in recruiting epigenetic regulators to target genes compared with Sox2, Klf4 and c-Myc, as it cannot be replaced by related family members during induced pluripotency⁸. In agreement, deletion of a linker domain on Oct4, which is absent on other POU domain family members, associates with chromatin remodelers implicated in reprogramming and its deletion abrogates iPSC formation³⁹. Intriguingly, some reprogramming-associated cofactors function in a chromatin-independent manner. For example, the H3K27 HDM Jmjd3 blocks reprogramming not only by activating the *Ink4a/Arf* locus but also by targeting the methyl-lysine effector protein Phf20 for ubiquitination; Phf20 is required to activate *Oct4* transcription in collaboration with Wdr5³⁴. Overall, these and other⁷ examples document the physical association of reprogramming TFs with a variety of histone modifiers and exemplify the diverse mechanisms by which they assist in reinstating pluripotency in somatic cells.

DNA Methylation: Safeguarding Cell Identity

DNA methylation is considered to be the most stable epigenetic modification, which confers permanent gene silencing during development and in the adult. Changes in histone modifications typically precede the removal or deposition of DNA methylation marks during differentiation²⁰. Similarly, DNA methylation changes almost exclusively occur at the end of the reprogramming process and after chromatin changes have taken place¹⁰, indicating a hierarchy of events that recapitulates normal development (Figure 1). DNA methylation is established by the *de novo* methyltransferases Dnmt3a and Dnmt3b and preserved by the maintenance methyltransferase Dnmt1⁴⁰. Although DNMT3a knockdown promotes iPSC formation in human cells³¹, the mouse Dnmt3a/b enzymes are dispensable for cellular reprogramming⁴¹. This surprising finding suggests that the silencing of lineage-specific genes is mainly achieved through alternative mechanisms such as deposition of repressive H3K27 methylation, which is consistent with PRC2's essential role in iPSC formation³².

In contrast to the dispensability of *de novo* methylation for iPSC formation, DNA demethylation of pluripotency genes appears to be critical for faithful reprogramming. Demethylation can occur by either active or passive mechanisms⁴⁰, both of which have been implicated in iPSC generation. Downregulation of Dnmt1 in reprogramming intermediates facilitates their transition towards authentic iPSCs, consistent with a supportive role of passive, replication-dependent demethylation in iPSC formation⁴². Enzymes associated with active DNA demethylation have been more directly linked with iPSC formation (Figure 1). Tet proteins catalyze the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which serves as a substrate for TDG-mediated base

excision repair into unmodified cytosines⁴⁰. Shortly after overexpression of OKSM, Tet2 induces hydroxymethylation of key pluripotency genes such as *Nanog* and *Esrrb*, priming them for subsequent demethylation and transcriptional activation (Figures 1 and 3b)⁴³. Interestingly, proteomic and genomic analyses revealed that Tet1/2 directly interact with Nanog and co-occupy many pluripotency targets in ESCs, implicating Nanog in the targeting of Tets⁴⁴. In agreement, simultaneous overexpression of Tet1/2 and Nanog significantly enhances, while depletion of either factor abrogates iPSC formation⁴³⁻⁴⁵. Moreover, Tet1 overexpression can compensate for exogenous Oct4 expression during cellular reprogramming, providing genetic evidence that Tet1 contributes to the activation of somatically silenced pluripotency genes⁴⁶. A role for activation-induced deaminase (AID) in DNA demethylation during TF-induced reprogramming has also been reported, although the underlying mechanisms are incompletely understood⁴⁰.

Inefficient DNA demethylation or remethylation has further been suggested to be the main reason for the “epigenetic memory” observed in many iPSC lines. This term describes cell type of origin-dependent transcriptional and epigenetic patterns that can influence the differentiation potential of iPSCs¹. Of note, genomic regions that fail to undergo DNA demethylation towards an ESC-like state in human iPSCs were shown to be decorated by broad H3K9me3 domains in somatic cells⁴⁷, and some of these areas overlap with the abovementioned “refractory domains” that are inaccessible to OKSM early in reprogramming²⁴. These findings therefore suggest that promiscuous OKSM binding or lack of OKSM binding to targets could explain some of the observed differences between ESCs and iPSCs. It should be interesting to assess whether manipulation of H3K9 HDMs or HMTs is sufficient to erase epigenetic memory in iPSCs.

3D Chromatin Architecture in Reprogramming

Accumulating evidence suggests that local and three-dimensional (3D) chromatin architecture provide additional levels of gene regulation in pluripotent stem cells⁴⁸. However, their roles in cellular reprogramming are incompletely understood. Local chromatin architecture defines the position and density of nucleosomes as well as the presence of histone variants (Figures 1 and 3c)⁴⁹. Histone variants usually modify the ability of nucleosomes to undergo remodeling and to accommodate active or repressive histone modifications. The histone variant macroH2A has previously been associated with resistance to efficient chromatin remodeling⁴⁹. In agreement, the presence of macroH2A potently inhibits TF-induced reprogramming of somatic cells to pluripotency by maintaining pluripotency loci in a repressed state⁵⁰⁻⁵².

Local chromatin architecture is regulated by diverse remodeling complexes, which also impact iPSC formation (Figure 1). Components of the SWI/SNF complex, such as Brg1, BAF155 and Brm, are directly recruited by Oct4 to targets in order to relax chromatin structure and facilitate binding of other TFs⁵³. This finding thus corroborates Oct4's role as a pioneer factor by influencing local chromatin structure at silenced targets. Similarly, the CHD remodeling factor Chd1 has been proposed to actively open chromatin during factor-induced reprogramming, and its knockdown impairs iPSC formation⁵⁴. In contrast, members of the repressive NURD complex including HDAC1 and Mdb3, which are critical for

heterochromatin compaction, inhibit reprogramming and their knockdown strongly increases the efficiency of iPSC generation⁵⁵. Interestingly, Mbd3 associates with loci enriched for Tet1 and 5hmC in ESCs and its expression is essential for global levels of 5hmC⁵⁶. The latter observation may explain the discrepancy between the observed early deposition of 5hmC at pluripotency loci⁴³ but its delayed conversion into unmodified cytosines during iPSC generation¹⁰; Mbd3 may be recruited to these hydroxymethylated pluripotency promoters and block immediate demethylation until unidentified coactivators relieve Mbd3-mediated gene repression.

In addition to local chromatin structure, 3D chromatin architecture has been implicated in pluripotency, differentiation and reprogramming (Figure 3d)⁴⁸. Differentiation of ESCs is accompanied by a repositioning of pluripotency genes from the nuclear center to the nuclear periphery⁵⁷ and a disruption of promoter-enhancer looping at key pluripotency loci such as *Oct4*^{58,59} and *Nanog*^{54,55}. This raises the question of whether and how 3D chromatin structure is restored in iPSCs. A recent study identified complex pluripotency-specific long-range interactions of the *Nanog* locus, which become rearranged during differentiation and are largely restored during reprogramming⁶⁰. The establishment and maintenance of this network is dependent on Mediator and Cohesin complexes, which are known to orchestrate long-range chromatin interactions⁵⁹. Interestingly, subunits of these complexes were found to directly interact with the reprogramming factors^{60,61} and their knockdown inhibited iPSC formation⁶⁰.

Extending these findings, long-range chromatin interactions around the *Oct4* promoter were recently implicated in the reprogramming of murine and human cells^{61,62}. Importantly, these interactions took place specifically in those rare cells that were poised to form iPSCs, and they preceded transcriptional activation, suggesting a causal effect of 3D chromatin structure on transcription. These results, together with previous studies documenting a role for Oct4 and Klf1 (highly similar to Klf4) in mediating long-range chromatin interactions^{63,64}, support the idea that reprogramming factors do not merely activate or silence genes but also function as chromatin organizers, which rearrange chromatin architecture from a somatic to a pluripotent state. This interpretation is further consistent with the recent discovery of “super-enhancers”, which are broad distal regulatory elements characterized by cooperative and excessive binding of Mediator components and cell-type specific transcription factors, such as Esrrb and Klf4, in ESCs⁶⁵. Given the documented role of super-enhancers in controlling the expression of master regulatory genes in different cell types, it is likely that the resetting of somatic-specific to pluripotency-specific super-enhancers constitutes another roadblock for iPSCs formation. The dynamics of this switch during induced pluripotency and the potential role of super-enhancers in 3D chromatin architecture certainly warrants further investigation.

Cell Signaling and Chromatin

External cues are critical to direct cells expressing OKSM towards a stable pluripotent state and to prevent acquisition of alternative cell fates⁷. Extracellular signals can either support or inhibit iPSC formation (Figure 2). For example, dual chemical inhibition of the Gsk3b and Erk1/2 pathways (“2i” condition) enhances the transition of partially reprogrammed

cells into iPSCs⁶⁶. Similarly, activation of the Jak/Stat3 pathway by the cytokine LIF is limiting for iPSC formation⁶⁷. By contrast, the TGF- β pathway negatively affects iPSC generation and its suppression by chemical inhibitors significantly increases the acquisition of pluripotency^{68,69}.

Recent data offer new mechanistic insights into how external signals communicate with chromatin structure in pluripotency and reprogramming. For instance, the downstream effector of LIF signaling, Stat3, requires the chromatin remodeler subunit Brg1 in order to keep targets accessible and prevent their repression by the PRC2 complex⁷⁰. Moreover, culture of ESCs in 2i endows cells with a so-called “naïve” or “ground” state that is characterized by altered H3K27me3 distribution and a decreased number of bivalent promoters as well as global DNA hypomethylation^{71,72}. Mechanistically, growth of ESCs in 2i induces activation of the TF Prdm14, which directly represses Dnmt3a/b and inhibits differentiation-inducing Fgfr signaling⁷¹. It is important to mention here that signaling molecules can also have opposite effects on different stages of reprogramming. BMPs promote an early MET in a miRNA-dependent manner²⁵, while they block the late conversion of partially reprogrammed cells into iPSCs by targeting the repressive H3K9 HMTs Suv39h1 and Setdb1³⁶. Conversely, Wnt/Tcf3 signaling is inhibitory early but stimulatory late in reprogramming⁷³.

Nutrients and cofactors present in the extracellular environment represent a final class of molecules that influence the epigenome and cellular reprogramming. A point in case is ascorbic acid (vitamin C), which has been shown to strongly enhance the efficiency and kinetics of reprogramming⁷⁴ and to increase the quality of mouse iPSCs by preventing aberrant hypermethylation⁷⁵. Ascorbic acid presumably functions both as an antioxidant and as a cofactor for specific epigenetic modifiers such as the H3K36 HDMs Jmjd1a/1b³³. Furthermore, ascorbic acid was suggested to be a cofactor for H3K9 HDMs and Tet enzymes according to recent studies, which reported a global decrease of the repressive H3K9me2/3 marks³⁶ and genome-wide DNA hypomethylation⁷⁶, respectively, in nascent iPSCs exposed to this compound. Together, these observations provide compelling new evidence for the tight communication between reprogramming-associated signaling molecules and TFs in order to rewire epigenetic regulatory circuits.

Sequence of Molecular Events

An unresolved question is whether the rare cells that successfully give rise to iPSCs undergo a defined sequence of transcriptional and epigenetic events. Different approaches have been used to resolve this issue. A number of reports utilized surface marker combinations to prospectively identify those rare cell populations that are destined to form iPSCs^{10,77-79}. One of these studies identified two major waves of gene expression changes that coincided with the early extinction of somatic genes and with the late activation of core pluripotency genes, respectively¹⁰. The lack of major transcriptional changes during the intermediate phase suggested that cells undergo gradual epigenetic alterations in order to prime the genome for transcriptional activation of pluripotency genes. In agreement with this notion is the observation that histone marks associated with pluripotency enhancers are established at early and intermediate stages of reprogramming, whereas DNA methylation changes occur

late, coinciding with transcriptional activation of associated genes¹⁰. Integrating observations from other studies, this intermediate period is also characterized by the establishment of pluripotency-specific long-range chromatin interactions^{60,61} and Tet-mediated conversion of 5mC into 5hmC at pluripotency promoters⁴³, further supporting the interpretation that cells undergo a series of chromatin changes in preparation for stable pluripotency.

An independent study analyzed transcriptional changes of 48 genes in single cells undergoing reprogramming¹⁵ and concluded that the initial response to reprogramming factor expression is quite heterogeneous and consistent with a stochastic process. However, later events, leading to the activation of pluripotency genes, occur in a hierarchical fashion. This analysis led to the inference that activation of the endogenous *Sox2* locus is a critical upstream event in cells that undergo successful reprogramming. A parallel study examined clonal populations of cells expressing OKSM and defined “maturation” and “stabilization” phases of reprogramming that were distinguished by differential expression of pluripotency genes⁸⁰. Unexpectedly, the authors discovered that the transition to the stabilization phase is dependent on a different set of factors (e.g., GDNF signaling and meiosis genes) from those that control maintenance of pluripotency.

Several groups reported transient upregulation of developmental regulators, such as epidermal, extraembryonic and epiblast-associated genes, at intermediate stages of reprogramming^{10,16,42,73,77}. While the molecular mechanisms underlying this observation remain elusive, it is tempting to speculate that reprogramming intermediates transiently pass through a state with increased developmental plasticity, which could represent stages of normal development⁷. Alternatively, these genes might be activated as a consequence of aberrant TF binding¹¹ or unspecific effects incurred by small compounds⁸¹. Regardless, recent studies showed that depletion of some of these transiently expressed genes impairs reprogramming into iPSCs, suggesting functional relevance^{78,81}.

In conclusion, while the overall gene expression trends are similar among different studies, variability related to the exact sequence of molecular events and the relative contribution of stochastic and deterministic events remain to be resolved. Another fundamental question that needs to be addressed is whether cells expressing alternative reprogramming factors pass through the same sequence of events, described here, and encounter the same barriers as cells expressing OKSM. This question is particularly relevant with respect to reprogramming approaches that involve small compounds⁸¹ or TFs that have not been directly implicated in pluripotency¹⁶.

Lessons from Other Reprogramming Systems

In this section, we will compare induced pluripotency with other processes involving epigenetic reprogramming such as SCNT, cell fusion and germline specification with the goal of identifying mechanistic similarities and differences (Table 1).

Somatic Cell Nuclear Transfer

Remarkably, the somatic gene expression program is downregulated within 22 hours of SCNT in mice (Dieter Egli, pers. comm.)⁸². This observation is reminiscent of iPSC formation and suggests that extinction of somatically expressed genes is rapid and efficient in both approaches. Somatic *Oct4* activation after SCNT occurs with a similar kinetics (24-36 hours)⁸² and was suggested to require Tet3⁸³, whereas it takes a minimum of 8 days to detect *Oct4* expression during induced pluripotency⁸² and this process appears to involve Tet2⁴³. This marked difference in pluripotency gene activation poses the key question of whether SCNT and induced pluripotency depend on the same TFs for reprogramming; both *Oct4* and *Sox2* proteins are in fact detectable in oocytes. Schöler and colleagues recently addressed this question by genetically depleting maternal *Oct4* protein from mouse oocytes prior to SCNT⁸⁴. Surprisingly, loss of *Oct4* did not abrogate the oocyte's ability to reprogram somatic nuclei, indicating compensation by other TFs. An alternative explanation is that SCNT may depend on an entirely different suite of reprogramming factors. The identification of the oocyte-enriched *Glis1* protein⁸⁵ capable of enhancing iPSC formation in the context of OXS expression indeed supports this hypothesis.

A key molecular event that distinguishes induced pluripotency from SCNT is the rapid histone exchange between somatic nucleus and oocyte, as was demonstrated in *Xenopus* SCNT experiments. Specifically, the somatic linker histone H1 is replaced within hours of SCNT for the oocyte-specific counterpart B4, and this process is essential for pluripotency gene activation in reconstructed oocytes⁸⁶. This particular exchange of histone types might contribute to reprogramming by depleting somatic chromatin from epigenetic repressors known to interact with histone H1, such as *Dnmt1/3b* and H3K9 methyltransferases^{87,88}. Concomitant with the replacement of “repressive” histone variants, incorporation of “active” histone variants including H3.3 and H2A.X into the somatic chromatin facilitates efficient chromatin remodeling of embryonic genes^{89,90}. Despite these effective mechanisms, some epigenetic marks including those of the silenced X chromosome (Xi) in female somatic nuclei appear to be more recalcitrant to remodeling by the oocyte compared with pluripotency genes, indicating differential susceptibility of some genomic loci to the oocyte's reprogramming machinery⁹¹. The relative resistance of X reactivation to efficient remodeling during *Xenopus* SCNT has been functionally linked to the repressive histone variant macroH2A, since its knockdown resulted in a more efficient reactivation of the Xi⁹¹. Thus, the eviction of macroH2A represents a rate-limiting step for successful reprogramming in the context of both SCNT and iPSC induction. Given the prominent role of “active” and “repressive” histone variants during SCNT, it should be informative to systematically test their function and that of associated chaperones⁴⁹ during iPSC generation.

Cell-Cell Fusion

Downregulation of somatic genes in ESC-somatic hybrids also occurs within the first 1-2 days of fusion⁹². When examining the same *Oct4*-GFP reporter that was used for SCNT and iPSC formation, *Oct4* reactivation in fusion hybrids took place with a similar kinetics as SCNT (24-48 hours)⁹² and this process was reported to involve Tet2⁹³. This finding suggests that ESCs, like oocytes, contain additional reprogramming factors that are limiting

during iPSC formation. Corroborating this point, Nanog overexpression promotes cell fusion reprogramming and drives maturation of iPSCs, whereas its absence blunts both types of reprogramming⁴⁵. Surprisingly, Oct4 protein is required, whereas Sox2 protein is dispensable for fusion-mediated reprogramming, identifying a notable difference to induced pluripotency⁹⁴. It is further important to mention that the reprogramming of hybrids is not complete upon activation of somatic pluripotency genes 2 days post fusion. Activation of the silenced X chromosome in female hybrids takes several days, which is reminiscent of the delayed X reactivation observed during *Xenopus* SCNT⁹⁵. Consistently, transcriptome-wide analysis of hybrid formation documented that some silenced ESC-associated genes are activated more rapidly than others⁹⁶. These results are in line with the sequential activation of pluripotency-associated genes during iPSC derivation^{4,5} and likely reflect different chromatin accessibility of associated genomic loci to ESC-derived reprogramming factors.

At the chromatin level, inhibition of the H3K9 HMT G9a or overexpression of the H3K9 HDM Jhdm2a increases cell fusion-directed reprogramming, which is in accordance with their supportive roles in iPSC generation⁹⁷. Conversely, depletion of PRC1 or PRC2 subunits decreases cell fusion-mediated reprogramming and induced pluripotency, underscoring the general importance of H3K27me3-mediated gene repression for the acquisition of pluripotency⁹⁸. Collectively, these findings support the interpretation that iPSC formation and cell fusion-directed reprogramming face similar epigenetic barriers and are stimulated by the same TFs, consistent with the fact that iPSC factors were initially identified in ESCs³. Based on the accelerated kinetics of pluripotency activation in hybrids compared with nascent iPSCs, it should be feasible to devise genetic screens in order to identify other Nanog-like molecules that are limiting for efficient TF-induced reprogramming.

Primordial Germ Cell Reprogramming

PGC maturation represents yet another type of reprogramming, which occurs naturally and encompasses major epigenetic remodeling events that prepare the developing germ line for totipotency^{99,100}. Remarkably, PGCs that have completed reprogramming exhibit two active X chromosomes in females¹⁰¹, ESC-like transcriptional patterns and bivalent domains¹⁰². This includes expression of potent reprogramming factors, such as Oct4, Sox2, Nanog and Prdm14^{100,103}. Each of these factors is essential for PGC formation in vivo, although their precise roles in PGC reprogramming remain elusive. Importantly, PGCs are unipotent in vivo (i.e., they can only produce oocyte or sperm) but they have the unique potential to give rise to pluripotent stem cells, coined “embryonic germ cells” (EGCs), upon explantation in culture¹. Expression of these ESC-associated TFs might thus endow PGCs with the latent ability to acquire pluripotency upon isolation from the gonads and exposure to appropriate extracellular cues. Because germ cell reversion into EGCs rarely occurs in vivo, except in cases of spontaneous teratocarcinomas (i.e., pluripotent tumors), potent mechanisms must be in place to preserve the PGC state. Blimp1 is a possible candidate molecule owing to its role as a repressor of *c-Myc* and *Klf4* expression in PGCs¹⁰³. It should be possible to test this hypothesis by assessing whether acute loss of Blimp1 is sufficient to convert PGCs into EGCs in vitro and to cause teratocarcinomas in vivo. Notably, Blimp1's putative role in suppressing the acquisition of a pluripotent state in PGCs might be taken over by Dmrt1 at

subsequent stages of male germ line development. Male mice lacking this TF in the germline aberrantly express pluripotency factors and develop testicular teratomas with almost full penetrance¹⁰⁴. The notion that Blimp1 and Drmt1 might actively resist acquisition of pluripotency is analogous to the inhibitory effect that somatic TFs have during iPSC formation¹⁹.

With respect to chromatin dynamics, global loss of H3K9 methylation is among the most striking changes in developing PGCs⁹⁹. Notably, downregulation of H3K9 HMTs appears to be critical for efficient reprogramming in the context of PGC specification¹⁰⁰, cell fusion⁹⁷ and iPSC formation^{24,36,37}. This particular chromatin alteration may therefore represent a general requirement for cellular reprogramming in these very different cellular contexts. Similarly, loss of inhibitory H3K27 methylation at pluripotency loci through catalysis by Utx seems to be another required step during both PGC reprogramming and iPSC formation¹⁴. Lastly, Tet1 and Tet2 enzymes have been functionally associated with DNA demethylation in *in vitro*-derived PGCs¹⁰⁵, revealing similarities with SCNT⁸³ and cell fusion⁹³. However, it is noteworthy that genetic loss of both Tet1 and Tet2 is not essential for viability and fertility *in vivo*¹⁰⁶, suggesting compensation by other mechanisms. Indeed, passive demethylation was recently suggested to contribute to PGC reprogramming through downregulation of the *de novo* methyltransferases Dnmt3a/b and the Dnmt1 cofactor Uhrf1¹⁰⁷. Combined with the enhancing effects of Tet1/2 overexpression⁴⁴ and Dnmt1/Dnmt3a depletion^{31,42} on iPSC formation, these data show that the erasure of somatic DNA methylation patterns is a general roadblock for successful epigenetic reprogramming in different cellular settings. Cells utilize a combination of “passive” and “active” DNA demethylation strategies to overcome this barrier, although their relative contribution varies depending on the reprogramming context.

Induced Pluripotency and Tumorigenesis

Several lines of evidence support the idea that induced pluripotency and transformation are related processes at a cellular level (see Box). Reprogramming, like cancer, is a rare, multi-step process that ultimately leads to the formation of a small population of immortal cells with tumorigenic potential; iPSCs, like ESCs, have the ability to give rise to teratomas (benign tumors containing derivatives of the three germ layers) upon transplantation under the skin of mice¹. Another similarity between reprogramming and some types of cancer is the observation that somatic stem and progenitor cells are more susceptible to both iPSC formation^{108,109} and tumorigenesis^{110,111} compared to mature cells. This observation may indicate that the epigenetic state of the starting cell provides a permissive environment for both oncogenic and reprogramming factors. Further, TF-mediated reprogramming induces a metabolic switch from an oxidative to a glycolytic state typical of most cancer cells¹¹². Lastly, teratocarcinomas represent a special type of cancer that originates from transformed PGCs and contains pluripotent cells, documenting a rare example of spontaneous reprogramming of committed (germ) cells into pluripotent malignant cells¹. An important distinction, however, between these examples of cancer and induced pluripotency is the fact that iPSCs are normal diploid cells that support development when re-introduced into embryos, whereas most cancer cells are aneuploid and characterized by aberrant differentiation patterns. It should thus be informative to study those chromatin and

epigenetic events that transiently endow iPSCs with immortality and tumorigenic properties in addition to increased differentiation potential, as this might lead to new strategies to reverse malignancy.

Molecular data support the cellular commonalities between reprogramming and malignancy. First, each of the four classic reprogramming factors has been shown to be oncogenic in mice, and some of these genes are amplified or mutated in human cancer¹¹³, suggesting that they might destabilize cell state in tumors akin to their role in reprogramming. Chromatin regulators that cooperate with OKSM during reprogramming, such as *Jhdm1b* and *macroH2A*, have also been associated with tumorigenesis. *Jhdm1b* expression has been causally linked with myeloid transformation of hematopoietic progenitors through silencing of the *ink4b* gene¹¹⁴ and with pancreatic adenocarcinoma formation through silencing of developmental genes in collaboration with *PRC2*¹¹⁵. In contrast to the cancer- and reprogramming-promoting role of *Jhdm1b*, expression of *macroH2A* provides a barrier for both iPSC formation and the malignant progression of melanoma cells. *MacroH2A*'s promoting effect on melanoma invasion is in part exerted through upregulation of the cell cycle regulator *CDK8*, which differs from the pluripotency genes targeted by this histone variant during induced pluripotency¹¹⁶. These and several other examples¹¹³ hence document that premalignant cells and nascent iPSCs target some of the same chromatin regulators to manipulate cell identity, although their targets may vary.

Both cellular reprogramming and cancer are also characterized by similar global changes in chromatin structure and DNA methylation. Cancer cells, like ESCs, are devoid of LOCKs compared to normal differentiated cells²⁷. Given the observation that H3K9 methylation is a major barrier for iPSC formation^{24,36,37}, this finding suggests that many cancers have acquired a developmentally more primitive epigenetic state that might be required for the maintenance of malignancy. Another hallmark of most cancer genomes are altered methylation patterns, which can manifest as aberrant hypermethylation or hypomethylation. It is interesting to mention in this regard that reduced methylation levels, induced by hypomorphic expression of *Dnmt1*, causes T cell lymphomas in mice¹¹⁷ and promotes iPSC formation in vitro⁴². Likewise, mutations in *Dnmt3A* have been observed in AML¹¹³, and knock-down of this enzyme facilitates human reprogramming into iPSCs³¹. These similarities between reprogramming and tumorigenesis are thus further consistent with the view that cancer cells need to override some of the same somatic barriers as iPSCs to alter cellular states.

Of note, the correlation between reprogramming and cancer is not absolute. In fact, some epigenetic regulators and histone modifications have been shown to play opposite roles during reprogramming and malignancy. For example, loss of *Tet2* causes myeloid transformation in mice¹¹⁸, consistent with a tumor suppressor function, whereas depletion of *Tet2* protein abrogates reprogramming⁴³. Similarly, the H3K79 methyltransferase *Dot1L* promotes leukemia formation induced by *MLL-AF9* translocations¹¹⁹ although it prevents iPSC formation³¹. Components of the *SWI/SNF* complex, which facilitates reprogramming, act as potent tumor-suppressors and are frequently mutated in cancer¹²⁰, indicating opposite functions. Lastly, genome-wide methylation analyses of somatic cells and iPSCs identified a set of reprogramming-specific differentially methylated regions (*R-DMRs*), which showed

significant overlap with DMRs that changed during the transformation of normal into malignant cells¹²¹. However, R-DMRs that become hypomethylated and bivalent during iPSC generation are typically hypermethylated in cancer, whereas R-DMRs that become hypermethylated in iPSCs lack bivalent marks and are usually hypomethylated in cancer. Given the importance of bivalently marked genes in multilineage differentiation, their methylation silencing in cancer may be a secure way to keep cells in a self-perpetuating undifferentiated state while this change would be detrimental for pluripotency in iPSCs. In summary, the abovementioned discrepancies between tumorigenesis and reprogramming are likely explained by strongly stage and cell-context dependent roles these enzymes and their modifications play during tumorigenesis and reprogramming.

A testable prediction following the abovementioned observations is that some cancer cells should be susceptible to epigenetic reprogramming into a non-malignant state. Indeed, both SCNT and iPSC experiments demonstrated that malignant cells such as melanoma¹²² and medulloblastoma¹²³ can be reprogrammed into a pluripotent state that supports differentiation into a number of normal cell types. Thus, some cancers are not irreversibly locked in a tumorigenic state but instead amenable to epigenetic reversion into a phenotypically normal state.

Outlook

Extensive functional genomics and screening approaches over the past few years have provided important insights into the epigenetic mechanisms occurring during normal, induced and pathological examples of cell fate change. While the drivers of cell fate change may be quite different in distinct contexts (e.g., OKSM in reprogramming, unidentified factors during SCNT and oncogenes in tumorigenesis), the resultant chromatin and epigenetic changes leading to altered cell identities are often conserved. This observation probably underlies the fact that different reprogramming approaches face some of the same molecular barriers that have been established during development and terminal differentiation to resist aberrant cell fate changes. We therefore conclude that TF-induced reprogramming provides a powerful tool to interrogate those chromatin and epigenetic mechanisms that stabilize cell fates during development and that become corrupted in cancer. These analyses have implications for both regenerative medicine and cancer biology. A better understanding of the molecular steps leading to pluripotency and the roadblocks resisting cell fate change in different contexts have already enabled researchers to interfere in a rationalized way with defined molecules or pathways to promote or prevent desired cell fate changes^{50-52,91}. An interesting future challenge will be to isolate and stabilize intermediate stages of reprogramming, which might represent natural or artificial cellular states with increased differentiation potential. Dissecting the molecular roadblocks of reprogramming has also relevance for studying and treating cancer. Given that premalignant cells utilize some of the same epigenetic mechanisms as nascent iPSCs to change cell identity, their manipulation may lead to novel strategies that reverse malignancy by altering cellular state rather than cell survival. While the concept of reprogramming cancer cells to pluripotency has already been demonstrated, additional work is needed to develop more specific approaches that reverse malignant cells into a non-pluripotent state by targeting defined transcription factors or epigenetic regulators. Recent work on the conversion of

leukemia and lymphoma cells into non-tumorigenic, quiescent macrophages by a single TF¹²⁴ is a promising step in this direction.

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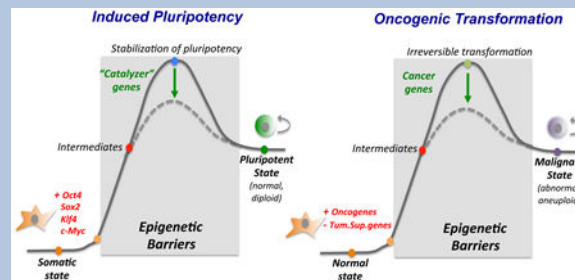
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Similarities between induced pluripotency and malignant transformation



Reprogramming of somatic cells to pluripotency and transformation of normal cells into malignant cells are illustrated as biochemical reactions with defined reactants (somatic cells) and products (iPSCs or cancer cells). Reprogramming is initiated by the overexpression of OKSM, while cellular transformation begins with the activation of oncogenes and/or repression of tumor suppressor genes. Intermediates of each reaction remain largely elusive. Both processes can be enhanced by modulation of additional genes, which can be considered “catalyzers” of the reaction. At a cellular level, both induced pluripotency and tumorigenesis are multi-step processes that require proliferation and result in a change of cell identity or differentiation potential. The “end product” is in both cases an immortal cell with tumorigenic potential. However, cancer cells almost always acquire genetic aberrations and become aneuploid while iPSCs retain a normal diploid genome. At a molecular level, many cancer cells have, like iPSCs, reduced levels of H3K9 methylation and altered DNA methylation patterns compared with differentiated cells. Overall, these findings suggest that nascent iPSCs and premalignant cells face some of the same epigenetic barriers to alter cell identity. This notion may explain why the same epigenetic regulators are involved in both processes, such as Utx, macroH2A, Jhdm1b, Ezh2, Tet2 or Dnmts. This idea is further consistent with the finding that certain somatic progenitor stem and cells are more susceptible to tumorigenesis and reprogramming than differentiated cells, indicating a more permissive epigenetic environment. A prediction that follows is that the malignant state should be reversible upon exposure of cancer cells to reprogramming factors, which is indeed consistent with recent results (see text for details).

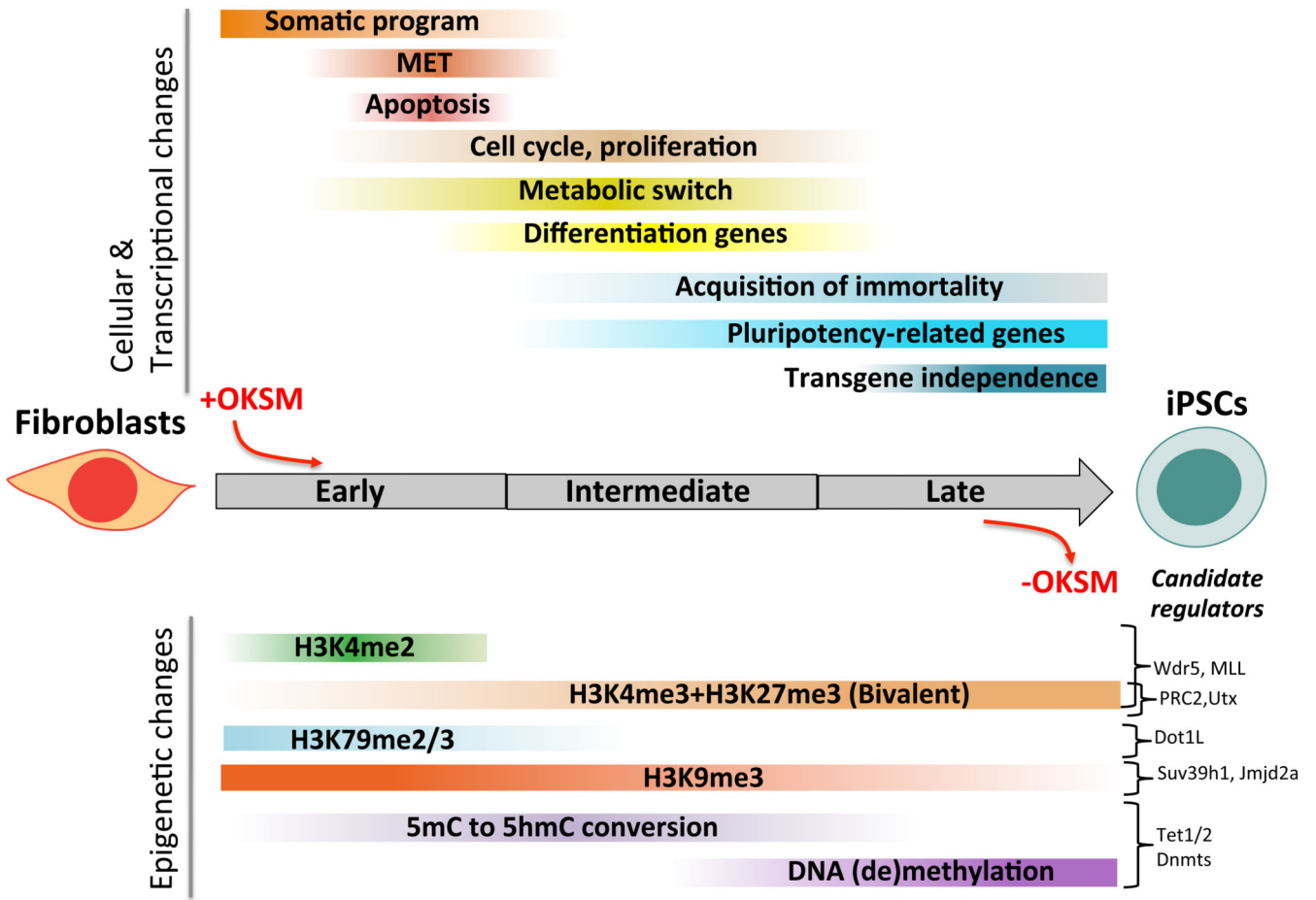


Figure 1. Dynamics of key epigenetic and transcriptional changes during direct reprogramming
 Colored bars represent different cellular and transcriptional events (top panel) or epigenetic modifications (bottom panel) that change in dynamic patterns during iPSC formation. Examples of candidate enzymes that have been associated with these epigenetic marks in the context of direct reprogramming are given on the right. **OKSM**: Oct4, Klf4, Sox2, cMyc; **5mC**: 5'-methyl-Cytosine; **5hmC**: 5'-hydroxy-methyl-Cytosine; **MET**: Mesenchymal-to-epithelial-transition.

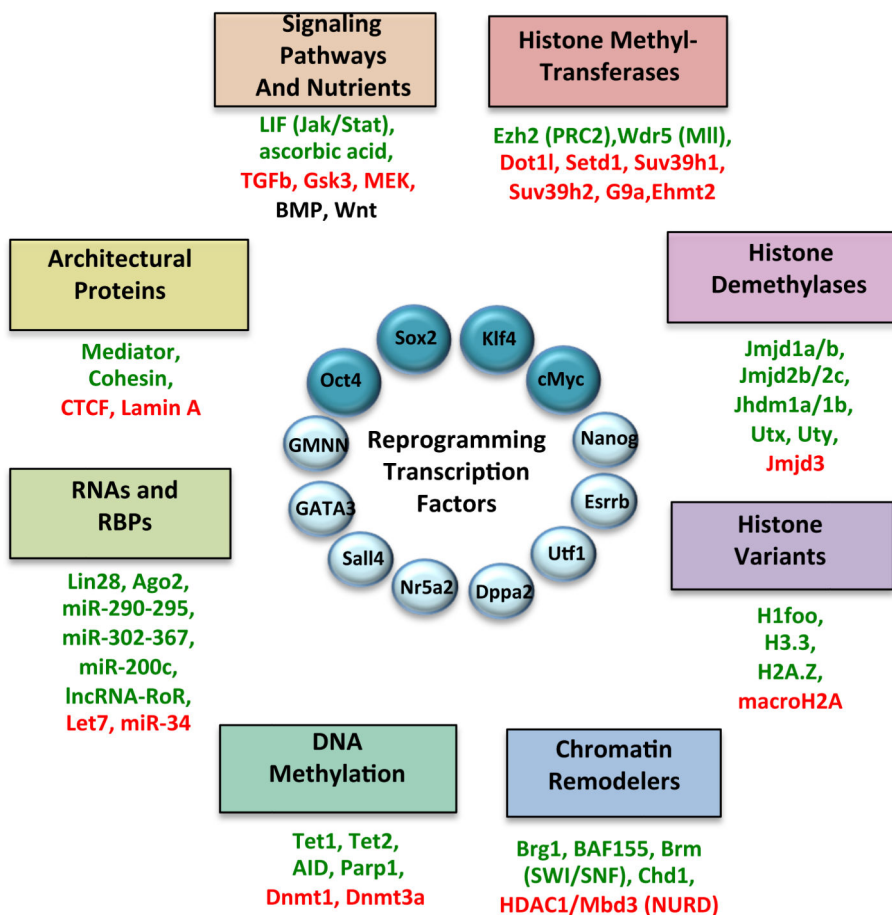


Figure 2. Interplay between reprogramming factors and molecules influencing chromatin state
 Shown are different transcription factors that have been shown to trigger induced pluripotency, with the classical combination (Oct4, Sox2, Klf4, c-Myc) highlighted. Below each category are examples of molecules that have been shown to facilitate (green) or inhibit (red) reprogramming. BMPs (Bone Morphogenetic Factors) and Wnts have stage-dependent enhancing or suppressive roles during iPSC formation (black, see text for details). **RBPs:** RNA Binding Proteins.

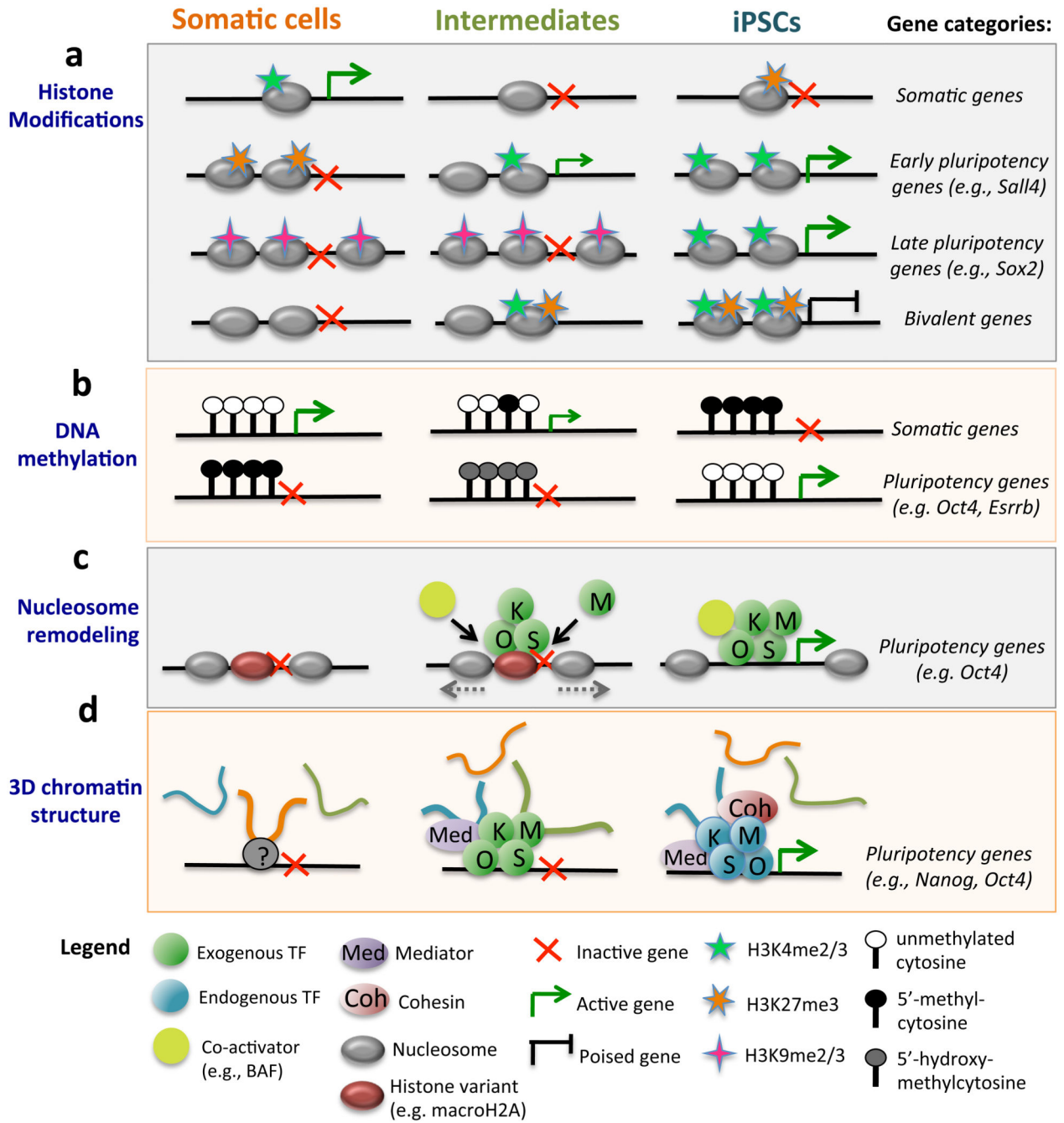


Figure 3. Levels of epigenetic gene regulation during induced pluripotency

a. Depicted are four categories of genes with associated histone modifications and their transcriptional response during reprogramming. Examples of genes within each category are shown to the right. **b.** Gain and loss of DNA methylation occurs late in reprogramming, while the acquisition of hydroxymethylation at pluripotency genes takes place at early-to-mid stages of iPSC formation. **c.** Oct4 (O), Klf4 (K) and Sox2 (S) function as “pioneer factors” that bind to high-density nucleosome regions, enabling chromatin remodeling and the recruitment of other factors including c-Myc (M). **d.** Changes in long-range chromatin

interactions around the *Nanog* locus during iPSC formation. iPSC formation re-establishes a 3D chromatin network typical of ESCs, and this process depends on Mediator and Cohesin. Colored loops represent somatic-specific (orange), intermediate-specific (green) and pluripotency-specific (blue) *Nanog*-interacting loci.

Table 1
Comparison of different types of cellular reprogramming

Key molecular and cellular events during different reprogramming strategies with examples of implicated molecules. SCNT, somatic cell nuclear transfer; PGC, primordial germ cell; EGC, embryonic germ cell; NPC, neural progenitor cell; ESC, embryonic stem cell; TF, transcription factor. Note that some references refer to review articles covering the discussed molecule.

Types of Reprogramming	Extinction of cell-of-origin expression program	Activation of pluripotency genes	X chromosome reactivation	Histone variants (chaperones)	Histone modifiers	DNA methylation factors	Requirement for endogenous TFs	Relevant signaling pathways
Direct Reprogramming (OKSM factors)	1-2 days (e.g., <i>Thy1</i> and <i>Snai1</i> during fibroblast reprogramming)(5)	8-12 days (Oct4-GFP reporter) (4,5)	~12 days (X-GFP reporter) (5)	macroH2A (50-52)	PRC1/2 (32) Utx (14) Dot1L (31) HDACs (1) (see also Figure 1)	Dnmt1 (42) Tet1/2 (43,44) AID (40)	Nanog (45) Stat3 (67)	Wnt (1,73) LIF (67) BMP (25,36) ERK/GSK inhibition ("2i") (66)
SCNT	22-24 hrs (2-cell embryo; global silencing of somatic genes/activation of zygotic genes)(82)	<36h hrs (4-cell embryo; Oct4-GFP reporter)(82)	~3 days (morula embryo; X-GFP reporter)	H1/B4 (86) H3.3 (89) H2A.X (90) H2A.Z (90) macroH2A (91)	HDACs (91)	Dnmt1 Tet3 (83)	(Oct4 not required)(84)	N/A
Cell Fusion	24-48 hrs (e.g., <i>Nestin</i> , <i>Glur6</i> in NPC-ESC hybrids)(92)	24-48 hrs (Oct4-GFP reporter) (92)	~9 days (94)	N/A	G9a (96) Jhd2a (96) PRC1/2 (97)	Tet1/2 (93) AID (2)	Nanog (45) Oct4 (2) (Sox2 not required)(2)	LIF Wnt
PGC maturation in vivo	~2 days, btw. E6.25-E8.5 (e.g., <i>Hox</i> genes) (100)	1-2 days, btw. E7.25-E8.5 (e.g., <i>Sox2</i> , <i>Dppa3</i> , <i>Nanog</i>)(100)	~3 days, btw. E11.5-E13.5 (X-GFP reporter)(101)	H1 (99) H2A.Z (99) (Nap-1 Hira)(99)	Glp (100) Utx (14) Prmt5 (103)	Dnmt3ab/L (107) Uhrf1 (107) Tet1/2 (105) AID (1,40)	Blimp1 (100) Prdm14 (100) Tcfap2c (100) Nanog (100) Oct4 (100) Sox2 (100)	BMP (100)
PGC-to-EGC conversion in vitro	1-2 days (e.g., <i>Blimp1</i>)(103)	3-4 days (e.g., <i>Klf4</i> , <i>Stat3</i>)(103)	3-4 days (X-GFP reporter) (101)	N/A	N/A	N/A	N/A	LIF/Stat3+FGF+ SCF or ERK/GSK inhibition ("2i") (71)