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Cellular trajectories and molecular mechanisms of iPSC reprogramming

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Abstract

The discovery of induced pluripotent stem cells (iPSCs) has solidified the concept of transcription factors as major players in controlling cell identity and provided a tractable tool to study how somatic cell identity can be dismantled and pluripotency established. A number of landmark studies have established hallmarks and roadmaps of iPSC formation by describing relative kinetics of transcriptional, protein and epigenetic changes, including alterations in DNA methylation and histone modifications. Recently, technological advancements such as single-cell analyses, high-resolution genome-wide chromatin assays and more efficient reprogramming systems have been used to challenge and refine our understanding of the reprogramming process. Here, we will outline novel insights into the molecular mechanisms underlying iPSC formation, focusing on how the core reprogramming factors OCT4, KLF4, SOX2 and MYC (OKSM) drive changes in gene expression, chromatin state and 3D genome topology. In addition, we will discuss unexpected consequences of reprogramming factor expression in *in vitro* and *in vivo* systems that may point towards new applications of iPSC technology.

Keywords

iPS cells; reprogramming; OKSM; pluripotency; chromatin organization; epigenetic remodeling; transcriptional activation; transcriptional silencing

Introduction

An identical set of transcription factors (TFs) – the now famous “Yamanaka factors” OCT4, KLF4, SOX2 and MYC (OKSM) – can convert mouse and human fibroblasts into embryonic stem cell (ESC)-like induced pluripotent stem cells (iPSCs) with species-specific properties [1,2]. This breakthrough discovery suggested the existence of shared molecular

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mechanisms that can establish pluripotency in somatic cells. Indeed, the universal applicability of OKSM to induce pluripotency has been demonstrated by successful reprogramming of fibroblastic cells from multiple species and of diverse adult cell types [3]. iPSC derivation has also been achieved by alternative TF combinations, containing some or none of the original Yamanaka factors [4,5] and, strikingly, by prolonged exposure to a mixture of chemical compounds that alter the activity of signaling pathways and chromatin modifying enzymes [6]. Nevertheless, OKSM reprogramming is currently more efficient and applicable to a wider range of cell types than alternative approaches to induce pluripotency and is consequently most widely used to derive patient-specific cell lines and study the mechanisms of iPSC formation.

A number of recent technological advances have facilitated monitoring iPSC formation driven by OKSM. For example, novel panels of surface antigens have been identified that dynamically change early or late during mouse [7–9] and human [10,11] cell reprogramming. This has allowed for the isolation and subsequent molecular dissection of better defined intermediate cell populations. In parallel, different strategies that reduce the inherent stochasticity of OKSM-mediated reprogramming have been described, including expression of additional TFs [12], interference with repressive chromatin-modifying complexes in specific media environments [13] or combined modulation of cellular signaling pathways [14–16], thereby enabling high-resolution, genome-wide approaches for the dissection of reprogramming mechanisms. In the following, we will describe recent advances in our understanding of general reprogramming trajectories, before discussing the molecular roles of OKSM factors in inducing pluripotency.

Reprogramming trajectories and alternative consequences of OKSM expression

Early work in mouse fibroblasts suggested that iPSC formation proceeds via an ordered sequence of events, each of which represents a bottleneck that aspiring iPSCs have to pass [17,18]. This provided the empirical basis for the current understanding of reprogramming as a – with respect to its outcome at the single cell level – stochastic process [19] that can be facilitated experimentally [20]. In addition to refining the “classic” reprogramming hallmarks of early down-regulation of somatic genes, epithelialization and ultimate reactivation of pluripotency-associated genes, research in recent years has also identified surprising intermediate stages of iPSC formation. For example, before embarking on the essential mesenchymal-to-epithelial transition (MET) [21,22], fibroblasts undergoing reprogramming transiently become more mesenchymal [23]. Accordingly, transcription factors promoting an epithelial-to-mesenchymal transition (EMT) such as SNAI1 are paradoxically enhancing iPSC formation [24,25]. The timing of EMT during fibroblast reprogramming correlates with transient metabolic changes, including hyperactivation of oxidative phosphorylation [26,27] and increase in reactive oxygen species signaling [28], that occur shortly after OKSM induction. This highlights the need for a better understanding of the links between changes in metabolism and other aspects of reprogramming [29].

It is becoming increasing clear that reprogramming trajectories can be influenced by species as well as, to a surprisingly strong degree, by the somatic cell of origin. For example, human fibroblasts seem to undergo MET not after but coinciding with reactivation of pluripotency loci such as *LIN28A*, *NANOG* and *TET1* [10]. In the mouse, reactivation of endogenous loci encoding core pluripotency regulators is a comparatively late event during iPSC derivation from fibroblasts and indicative of successful reprogramming [8,18]. However, blood cells reactivate *Pou5F1* (the locus encoding OCT4) earlier than the low-stringency marker SSEA1 and before becoming independent of transgenic OKSM expression [30], while neural stem cells and astrocytes activate *Nanog* before expressing E-CADHERIN and undergoing MET [31]. Interestingly, astrocytes but not fibroblasts expressing OKSM transiently upregulate the neural progenitor marker *Sox1*, which functionally appears to be redundant with exogenous *Sox2* expression [32]. These observations illustrate the complex interactions between OKSM and cell intrinsic factors and point towards using reprogramming technology to probe the molecular basis of cell-type specific pathogenesis, with possible implications for human disease [33]. For example, the possibility to greatly facilitate iPSC derivation from mouse blood progenitor cells by Wnt activation [14,15] is reminiscent of the preeminent role of this pathway in blood cancers and might suggest shared molecular mechanisms between blood cell reprogramming and leukemic transformation.

Aside from successfully inducing pluripotency, expression of Yamanaka factors can result in apoptosis triggered by MYC overexpression [34], senescence as a consequence of activation of the p53 and INK4/ARF pathways [35,36] (Figure 1) or a partial reprogrammed state due to unresolved chromatin barriers such as H3K9 [37] or DNA methylation [38] domains. In addition, several recent studies suggest the activation of alternative developmental programs during or in parallel to iPSC formation. Thus, both mouse [30] and human [10,39] fibroblasts undergoing reprogramming transiently express regulators of later developmental stages including the primitive streak. While the functional relevance of this observation is debated [40], recent observations in a highly efficient mouse reprogramming system suggest that transient upregulation of transcription factors associated with the post-implantation epiblast coincides with acquisition of functional pluripotency as measured by the ability to give rise to entirely iPSC-derived animals [41]. The observation that intermediates of iPSC derivation express pluripotency regulators yet are prone to differentiation might reconcile studies claiming OKSM factors can trigger direct lineage conversions [42–44] with subsequent work demonstrating that such conversions – at least in mouse – entail transient acquisition of pluripotency features such as *Nanog* expression and X chromosome reactivation [45,46]. As transient reactivation of a primitive streak program does not appear to occur during granulocyte and keratinocyte reprogramming [30], it would be interesting to determine if this affects molecular or functional properties of resultant iPSCs. It should also be noted that iPSC reprogramming, even when initiated from the same somatic cell of origin, is significantly impacted by experimental conditions and may not always proceed via identical molecular checkpoints. This is demonstrated by the role of TET1, which turns from a facilitator to a repressor of iPSC formation in presence of ascorbic acid [47] and the observation that two different KLF4 variants in a context-dependent manner can affect factor stoichiometry and consequently OKSM-driven MET [48,49].

OKSM expression in fibroblasts has also been shown to yield extraembryonic endoderm stem (XEN) cells in parallel with iPSCs [50]. This observation is in accordance with high-resolution analysis of fibroblast reprogramming by Mass Cytometry, which revealed appearance of a PDGFRA+NANOG- population late in reprogramming [51]. On the other hand, single cell RNA-sequencing analysis suggest that cells with placenta-like or neuronal-like signatures represent an alternative reprogramming outcome (Schiebinger *et al.*, bioRxiv, doi:10.1101/191056) (Figure 1A). It remains to be determined, for example by analysis of clonal reprogramming cultures, whether these observations reflect increased developmental plasticity due to reprogramming factor expression or are due to the intrinsic heterogeneity of starting fibroblast cultures.

Of note, *in vivo* expression of OKSM in context of some transgenic mouse models [52] but not others [53,54] has been reported to yield iPSCs with the capacity to give rise to trophectodermal cells. The specific aspect of *in vivo* reprogramming responsible for endowing iPSCs with these developmental features remains to determined, but it is worth noting that recently described *in vitro* culture conditions could overcome the inability of mouse and human pluripotent stem cells to differentiate into extraembryonic lineages [55–57] (Figure 1). Intriguingly, *in vivo* reprogramming is in part driven by secretion of the cytokine IL-6 as part of an OKSM-induced senescence program in a subset of cells [58]. This suggests a dynamic interplay between cells on two different trajectories following OKSM induction (initiation of senescence vs initiation of reprogramming) (Figure 1), which also appears to be relevant for *in vitro* reprogramming [58,59].

Reprogramming factor induction *in vivo* – in part depending on the length of expression intervals – can also have developmental outcomes distinct from pluripotency and subsequent teratoma formation. These include dysplasia and generation of partially reprogrammed, transplantable progenitor cell tumors [54] and tissue rejuvenation/regeneration in aged and injured animals [60] (Figure 1C–D). These observations suggest that *in vivo* OKSM expression has the potential to uncover unexpected aspects of iPSC formation and might represent a versatile tool to study physiological and pathological processes not directly related to pluripotency.

OKSM binding and activities

iPSC generation requires global and dramatic OKSM-driven molecular changes that will successfully erase the somatic identity and establish a stable pluripotent program. Recently, extensive genome-wide chromatin assays combined with transcriptomics and epigenomics datasets from early or later stages of iPSC generation offered new mechanistic insights into the distinct or synergistic properties and effects of OKSM binding. Here, we focus on the direct and indirect effects of OKSM binding on (i) the transcriptional erasure of the somatic identity, (ii) the activation of pluripotency program and (iii) the reorganization of chromatin architecture during reprogramming (Figure 2), highlighting novel findings and critical rate-limiting steps and cofactors.

Silencing of the somatic program

The necessity of early and robust silencing of the somatic program has been described in many reprogramming systems and is strongly supported by recent chromatin studies that demonstrate an extensive loss of chromatin accessibility around somatic loci and a strong decommissioning of somatic enhancers during the first phase of reprogramming [61–63]. Earlier papers studying the transcriptional effects of individual TF expression proposed an early repressive role of c-MYC and KLF4 [8,64], although evidence for a direct involvement of these factors was missing. Recent studies characterizing OKSM binding at early stages of reprogramming provide to some degree conflicting evidence. Chronis et al describe extensive OKSM co-binding on previously accessible and active fibroblast enhancers in mouse cells [65]. This suggests for the first time an active role of OKSM in somatic gene repression, for example by recruitment of co-repressors such as HDAC1 that directly interact with OCT4 or by displacement of somatic TFs through antagonism with OKSM (Figure 2) [65]. In support of these findings, another paper reported an early OCT4 binding on permissive/active chromatin that correlated with subsequent somatic gene silencing of bound regions in murine fibroblasts [63]. In contrast, a number of other studies using different human or mouse reprogramming systems and/or different time points, reported that Yamanaka factors bind mostly on previously inaccessible regions and do not directly associate with repressed somatic enhancers [61,62,66,67] (Zviran *et al.*, bioRxiv, doi: 10.1101/184135), arguing for an indirect role of OKSM in the erasure of somatic identity. A recently proposed indirect mechanism entails redistribution of somatic TFs from somatic enhancers to newly accessible sites generated by OKSM binding (Figure 2) [65]. It is argued that this would lead to decreased expression of critical somatic TFs, thereby triggering a negative feedback loop and culminating in silencing of the somatic program. In agreement, depletion of somatic TFs, such as c-Jun and Fra1 increases iPSC generation, while their overexpression inhibits reprogramming [61,62,65].

In addition to the role of TFs in the maintenance or repression of the somatic program during iPSC formation, recent studies highlighted novel roles of epigenetic modulators (Figure 2). OKSM-induced early activation of *Sap30*, encoding a SIN3A corepressor, was critical for efficient closing of the chromatin around somatic genes. In agreement, early depletion of SAP30 compromised iPSC generation, while this protein was dispensable at later stages [61]. Similarly, LSD1/HDAC1 were shown to be critical for the silencing of the B lymphocyte program prior to a very efficient and synchronized activation of the pluripotency network [68]. In this system, LSD1 was post-transcriptionally induced not by OSKM, but by CEBP/A, a specifier of the granulocyte-macrophage lineage, which was ectopically expressed prior to OKSM activation. On the other hand, the coactivator BRD4 has been shown to act as a barrier in early stages of reprogramming by bookmarking and preserving somatic gene activity [69], while also being essential for establishment and maintenance of pluripotency [68, 69]. Of note, erasure of the somatic identity during iPSC reprogramming is not always complete, usually due to inefficient resetting of the epigenetic landscape, an observation made initially in transgenic mouse models and recently confirmed for brain-derived human iPSCs using DNA methylation analysis of isogenic cell lines [70].

Activation of the stem-cell program

In contrast with the early and relatively efficient overwriting of the somatic signature, the establishment of the pluripotency program is considered a stochastic and rate-limiting process, involving step-wise selection and activation of pluripotency-related enhancers. Major roadblocks in this process include the inability of OKSM to access “refractory” genomic regions and the insufficiency of OKSM binding to induce gene activation in the absence of critical co-factors (Figure 2). As an example of the first type of barrier, repressive heterochromatin regions enriched in H3K9 methylation have been shown to be overall refractory to OKSM binding in early stages of reprogramming [66]. Critical stem cell regulators reside within these refractory regions, highlighting the necessity for chromatin remodeling and relaxation. Indeed, depletion of H3K9 methyltransferases (G9A, GLP, SETDB1, SUV39H1 and SUV39H2) promotes reprogramming of human and mouse fibroblasts [37,66,71]. Similarly, knock-down of CAF-1 complex, which normally deposits nucleosome in a replication-dependent manner, enables early relaxation of the somatic chromatin and increased accessibility and SOX2 binding at critical pluripotency-related enhancers and superenhancers [72]. High levels of repressive DNA methylation have been also associated with delayed and inefficient reprogramming, and in concordance, proteins involved in DNA demethylation, such as TET proteins, are strong albeit context-dependent [47] (Zviran *et al.*, bioRxiv doi:10.1101/184135) facilitators of reprogramming.

Despite the exclusion of reprogramming TFs from selective refractory regions, a number of recent studies strongly support that early binding of OKS –and to a lesser degree of MYC- occurs predominantly on “inaccessible”, nucleosomal and even DNA methylated regulatory elements and induces accessibility for other TFs and cofactors (Figure 2)[62,66] (Zviran *et al.*, bioRxiv doi:10.1101/184135). This early binding depends on (i) the ability of OKS to act as pioneer factors capable of partial motif recognition and binding on nucleosomal chromatin [66,73], (ii) their high affinity for DNA methylated targets [74] and (iii) their strong synergy among each other [65,66]. Importantly, although reprogramming TFs have been well-documented to function as trans-activators in established ESCs, their co-binding early during reprogramming does not guarantee immediate activation of target gene loci, suggesting context-dependent confinements of their activity. In fact, factors involved in chromatin remodeling (eg SWI/SNF subunits), epigenetic modulation (eg. BRD4 and MLL) (reviewed in Ref.31) and release of paused polymerase (eg. CDK9, P-TEFb) [68,75] have been described as critical cofactors for OKSM activity. Synergy between OKSM and other pluripotency-related transcription factors, such as ESRRB and NANOG, which are only induced later during reprogramming is another rate-limiting step that can be eliminated by enforced expression of these TFs during early reprogramming [20,65].

Re-organization of chromatin architecture

Increasing evidence supports that 3D chromatin architecture is cell-type specific and needs to be reorganized during cell fate transitions. Accordingly, chromatin topology differs between somatic and pluripotent cells and is largely reset during iPSC reprogramming [76–81]. However, evidence for the extent of this reorganization, the underlying mechanisms and the relationship to transcriptional and epigenetic changes has only begun to emerge. Previous studies have provided insights in to the chromatin rewiring around select

pluripotency-associated loci during reprogramming, reporting that the establishment of chromatin loops usually precedes or coincides with transcriptional change [76,77]. A recent study that characterized global topological changes at various stages of B cell reprogramming [82] further supported and refined that chromatin reorganization often occurs concomitantly or prior to gene expression changes, supporting a potential causal role. In addition, the degree of architectural rewiring around critical pluripotency genes appears to correlate with the timing and probability of their activation. For example, the major topological changes observed at the *Nanog* and *Sox2* loci correlate with their slower reactivation compared to the early-activated *Oct4* locus, which does not undergo major conformation changes in this system. These data suggest that architectural constraints in addition to other epigenetic barriers may act as bottlenecks for efficient establishment of the pluripotent program.

Although all the aforementioned topological changes are triggered by ectopic expression of OKSM, the direct involvement of these factors remains unexplored. Evidence for the potential architectural roles of OKSM include (i) bioinformatics analysis showing that OKS binding is enriched around topologically reorganized domains in the course of reprogramming [76–79,82], (ii) knock-down experiments that disrupted predicted KLF4-mediated loops in established ESCs [77] and (iii) protein-protein interactions of OKS with known architectural factors such as Cohesin and Mediator [76,77]. On the other hand, the recent evidence that changes in histone modifications, such as H3K4me2 [82], precede conformational alterations, may support an indirect architectural role of OKSM due to co-factor recruitment and/or epigenetic remodeling around their target sites. In that case, topological alterations may be directly induced and maintained by epigenetic modulators, which may also function as architectural factors -as has been reported for PRC2 [83]. Alternatively, deposition or removal of defined epigenetic marks may change the biophysical properties of the local chromatin contributing to new self-organized and phase-separated multimolecular assemblies due to high affinity with other genomic regions with similar properties [84]. Future experiments will be critical to determine the OKSM- dependent and -independent mechanisms of local and global topological reorganization during iPSC formation, potential rate-limiting steps and interconnections with epigenetic and transcriptional changes.

Conclusions

The study of reprogramming mechanisms has revealed common molecular themes, but also unexpected complexities. The realization that important aspects of OKSM activity are influenced by variables such as starting somatic cell type, culture conditions and factor stoichiometry demonstrates that there likely is no single trajectory of iPSC formation. Rather than being a shortcoming, the context-dependent nature of reprogramming, if applied correctly, will provide novel opportunities to study complex interactions between molecular regulators during controlled experimental cell fate change. As indicated by the wide range of intra- and intercellular processes operational during iPSC formation, such studies will likely provide insight into physiological and pathological events extending far beyond pluripotency.

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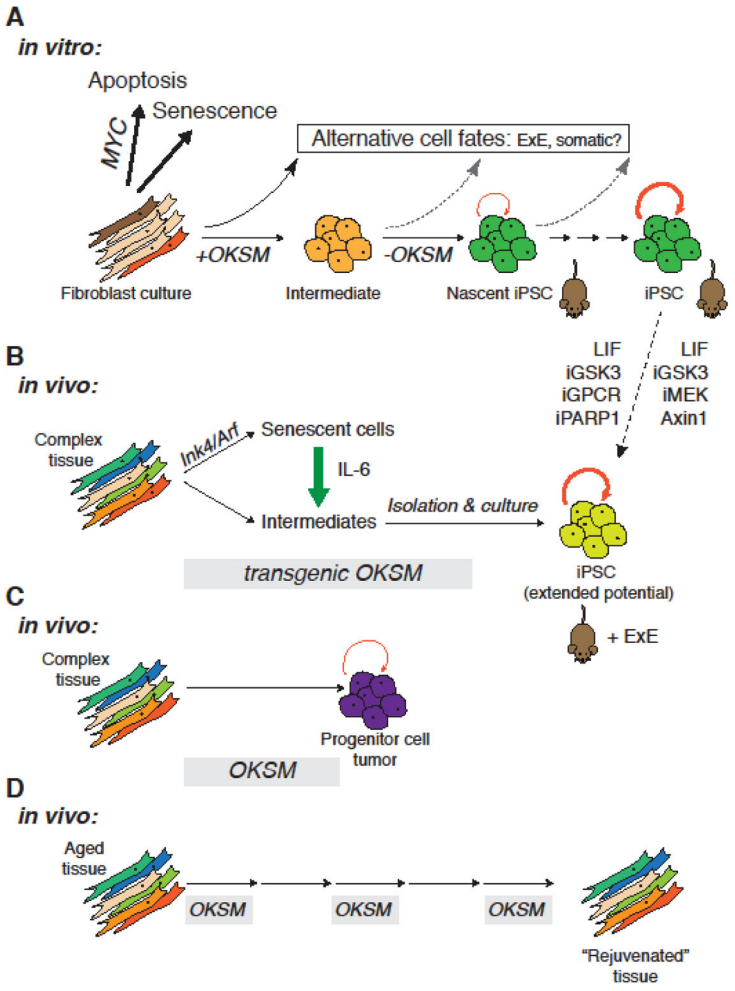

Figure 1. Developmental fates triggered by reprogramming factors

A) In cultured fibroblasts, OKSM leads to initiation of senescence or controlled cell death in the majority of cells. A small subset of cells gives rise to unstable reprogramming intermediates that still require exogenous reprogramming factors before giving rise to nascent iPSCs upon factor withdrawal and to established iPSCs upon passaging. Fibroblasts expressing OKSM can also give rise to alternative cell fates, which was most convincingly shown for extraembryonic endoderm, but evidence suggest that intermediates and nascent iPSCs might also be more prone to differentiate into other cell lineages than established iPSCs. Nascent murine iPSCs have less pronounced self-renewal capacity than established iPSCs, but both can efficiently give rise to all somatic cell types upon blastocyst injection. Established iPSC can be endowed with the ability to give rise to extraembryonic (ExE) tissues by culture in two alternative approaches targeting indicated molecules. B) Prolonged OKSM expression in adult transgenic mice yields intermediates as well as senescent cells that support reprogramming by secretion of factors such as IL-6. Culture of circulation-derived intermediates and ex vivo culture yields iPSCs that not only can give rise to extraembryonic and embryonic tissues. C) An intermediate interval of OKSM expression in vivo triggers partial epigenetic remodeling and in tissue such as kidney the emergence of transplantable tumors with molecular features of tissue-specific embryonic progenitor cells.

D) Repeated short intervals of OKSM expression in vivo lead to reversal of molecular features of aging and functional restoration of tissue function in aged animals.

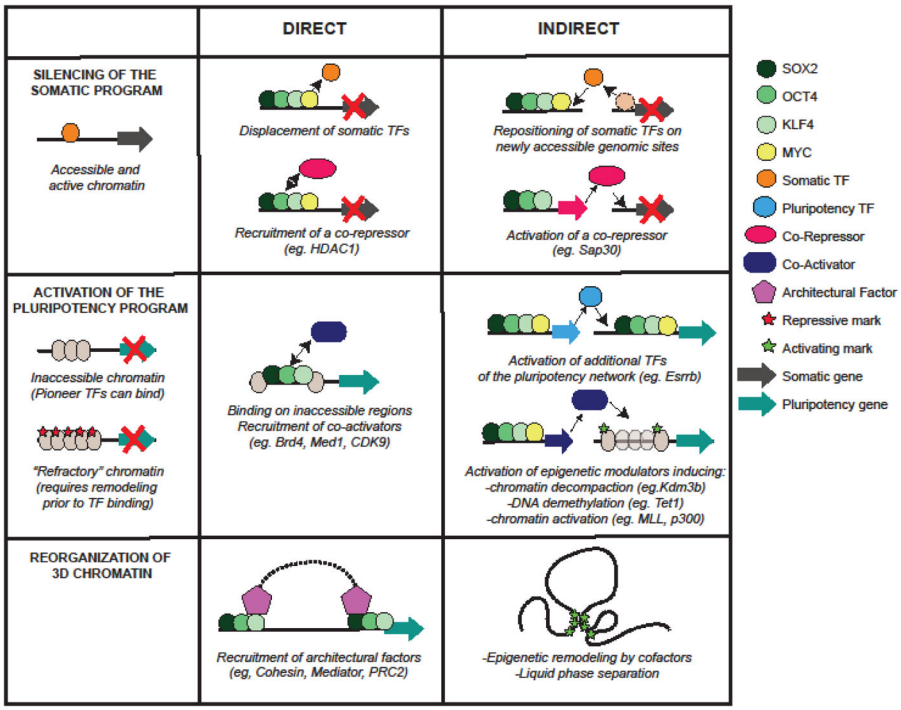

Figure 2. Mechanisms and molecular consequences of OKSM binding

OKSM impact on the silencing of the somatic program, activation of pluripotency program and topological reorganization of the chromatin are illustrated. Direct mechanisms assume that OSK(M) binding is sufficient to induce transcriptional or topological changes in *cis* by recruiting the necessary cofactors that are already available in the nuclear milieu. Indirect mechanisms rely on epigenetic and transcriptional changes that occur at different genomic sites in an OKSM-dependent or independent manner and result in the activation of critical co-factors (co-activators or co-repressors) that mediate the silencing or activation of OKSM target genes.