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REVIEW

Phases of reprogramming

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Abstract Despite advances in the field of somatic cell reprogramming, an understanding and exploration of the underlying mechanisms governing this process are only recently emerging. It is now increasingly apparent that key sequential events correlate with the reprogramming process; a process previously thought to be random and unpredictable is now looking, to a greater extent, defined and controlled. Herein, we will review the key cellular and molecular events associated with the reprogramming process, giving an integrative and conciliatory view of the different studies addressing the mechanism of nuclear reprogramming.
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Introduction

Since the discovery that somatic cells could be reprogrammed to induced pluripotent stem cells (iPSCs) (Takahashi and

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Yamanaka, 2006), many different pathways have been created based on Waddington's adaptation of the "epigenetic landscape", the model used to illustrate cell differentiation during development (Waddington, 1954). The somatic cell reprogramming and the process of transdifferentiation, further expanded the boundaries of cell plasticity giving rise, for example, to a non-hierarchical model of cell fate transition, represented by an "epigenetic disk" in which the ball of cell fate could assume any cell fate, provided that the master transcription factors were sufficiently expressed (Ladewig et al., 2013). While there is little doubt that such cell fate conversions are reproducible, a major hurdle that precludes further study of the reprogramming process is their low efficiency (Ho et al., 2011; Stadtfeld and Hochedlinger, 2010). To overcome this, secondary systems were implemented, and the resultant transgenic fibroblast could be reprogrammed through inducible expression of Oct4, Klf4, Myc and Sox2 (OKMS) (Carey et al., 2010; Maherali et al., 2008; Nagy, 2013; Stadtfeld et al., 2010; Woltjen et al., 2009). These improved reprogramming systems usually utilize doxycycline-inducible reprogramming factors. This allows temporally-controlled induction of expression of the reprogramming factors as well as a higher degree of homogeneity. As will be discussed during this review, the majority of the studies addressing the mechanism of reprogramming have made use of a secondary system.

First milestone: iPS cells are equivalent to ESC and can be obtained from the reprogramming of any adult cell

The first and more important questions that needed to be addressed were whether iPSCs were in fact identical to ESCs, and whether all cells were amenable to reprogramming. It was evident that iPSCs were not only morphologically and functionally equivalent to ESCs, but were also similar both transcriptionally and epigenetically (Maherali et al., 2007; Mikkelsen et al., 2008; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). While some studies found differences between ESCs and iPSCs, others that investigated a broader array of samples showed that the heterogeneity between ESC and iPSC lines was mainly due to the method used to derive them (Yamanaka, 2012).

Subsequently, the "reprogramming technology" needed to prove that iPSCs were the result of reprogrammed cells, and not the selection of novel uncharacterized tissue-specific pluripotent cells. This was achieved by reprogramming cells with specific traceable genetic characteristics, such as the albumin promoter in hepatocytes, insulin promoter in pancreatic beta cells or the recombined immunoglobulin locus of B lymphocytes (Aoi et al., 2008; Hanna et al., 2008; Stadtfeld et al., 2008a). Indeed, Hanna and colleagues demonstrated that iPSCs could emerge from daughter cells from any given cell of a starting population, provided that the cells were still viable and the four reprogramming factors could maintain expression for extended periods (Hanna et al., 2009).

The finding that the timing of faithful reprogramming varies widely among cells, suggests that at least one event driving the reprogramming process is likely to be stochastic. A priori the steps leading to successful reprogramming may involve one or several stochastic events and could be divided by: a) the nature of the molecular events taking place during this process, which raises the question of whether reprogramming can be achieved through different molecular pathways (Fig. 1A) and b) the order of these key events: is there a hierarchy or can they be acquired independently? (Fig. 1B). Finally, if these events transpire in an orderly fashion we will be able to unveil them. If on the contrary, these events were acquired "accidently" in nature and timing, their time of occurrence will remain largely unknown and highly susceptible to variability (Fig. 1C).

In the subsequent sections we attempt to consolidate and discuss recent findings that have emerged from the study of the reprogramming process. Primarily, this is composed of three phases: initiation, maturation and stabilization and are discussed in greater detail below (Fig. 2).

Second milestone: unveiling the reprogramming pathway

Early events — initiation phase — first wave

Different molecular transitions during reprogramming were first documented by the laboratories of R. Jaenisch and K. Hochedlinger in 2008, when they described distinct molecular events occurring at defined times during the reprogramming process (Brambrink et al., 2008; Stadtfeld et al., 2008b). These events ranged from downregulation of fibroblast-specific surface markers and the concomitant upregulation of genes associated with the pluripotency network, as well as reactivation of telomerase activity. Based on an extensive transcriptomic profiling time course during reprogramming of fibroblasts in bulk cultures, the reprogramming process was subsequently grouped in to three phases by the laboratory of J. Wrana: the initiation phase, maturation phase and stabilization phase (Samavarchi-Tehrani et al., 2010). This early period also correlated with changes in morphology, such that fibroblast cells (the main somatic cell model used for reprogramming studies) undergo a mesenchymal-to-epithelial transition (MET). Molecularly, this is characterized by a loss of the somatic cell signature, for example the loss of the transcription factors Snai1/2 or Zeb1/2, which was also described in previous studies (Mikkelsen et al., 2008; Sridharan et al., 2009; Stadtfeld et al., 2008b), and the gain of an epithelial signature, such as the expression of Cdh1, Epcam or epithelial-associated miRNA-200 family (Li et al., 2010; Samavarchi-Tehrani et al., 2010). The importance of these cellular changes was further highlighted by the demonstration that the cell shape itself can trigger epigenetic modifications regulating reprogramming (Downing et al., 2013). In this study, MEFs seeded on microgrooved surfaces for 3 days that entered a MET, therefore enhancing the reprogramming efficiency (Downing et al., 2013). Accordingly, in a kinase shRNA screen attempting to lift barriers of mouse reprogramming, top hits were 2 kinases blocking cytoskeletal rearrangement: TESK1 and LIMK2 (Sakurai et al., 2014). Interestingly, TESK1 siRNA led to enhanced reprogramming in human fibroblasts as well (Sakurai et al., 2014). Aside from MET-associated changes,

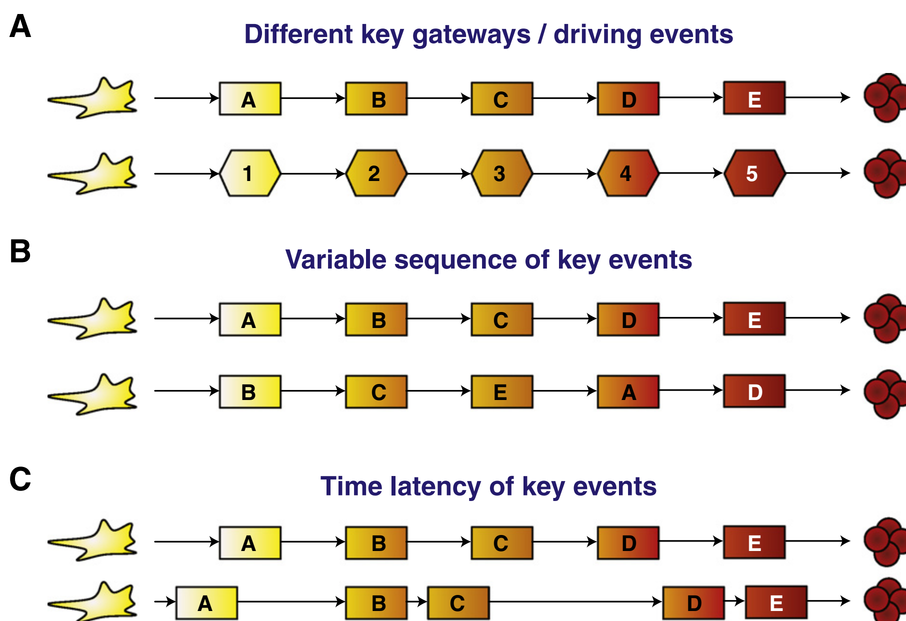

Figure 1 Different hypotheses regarding the sequence of event leading to reprogramming of somatic cells. A. There are multiple roads to reprogramming; the "driving" factors have totally different identities. B. The key events are always the same but their order of acquisition is highly variable. The cells will be reprogrammed once they have passed all the gateways, in any order. C. There is a defined sequence of events, but their time of acquisition is variable among different cells, due to unlikely statistical events.

multiple groups showed that the acquisition of ESC-like proliferation and the resistance to apoptosis and senescence cascades, induced by the reprogramming protocol, were important early events of reprogramming (Hong et al., 2009; Marion et al., 2009a; Mikkelsen et al., 2008; Utikal et al., 2009).

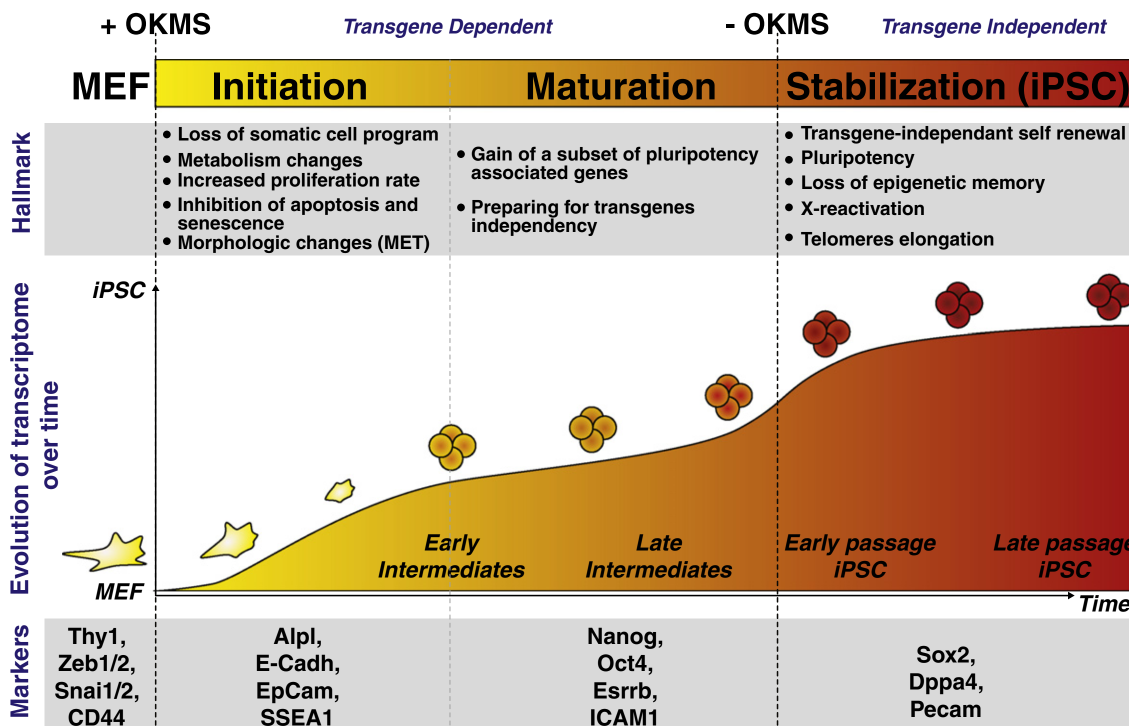

Figure 2 Sequential events occurring during somatic cell reprogramming. Markers and specific hallmarks of each phase of reprogramming are indicated. The iPSC transcriptome is acquired in two major steps.

In order to shed light on the cellular events occurring during reprogramming, several groups used multiple surface and genomic markers to monitor the progression of cells as they transit through the reprogramming process. These studies revealed that during the initiation phase, markers such as Thy1 and CD44 are lost, while the pluripotency markers alkaline phosphatase or SSEA1 are gained (Fig. 2) (Brambrink et al., 2008; Hansson et al., 2012; Mikkelsen et al., 2008; O'Malley et al., 2013; Polo et al., 2012; Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008b). Polo and collaborators were able to confirm and expand the initial work of the Wrana lab by further unveiling the two waves of molecular changes that transpire during reprogramming (Polo et al., 2012). A first wave, which occurs almost in every cell in culture and coincides with the initiation phase described by Wrana lab, is followed by gradual molecular changes, until a second wave emerges at the end of the process. The initiation phase was defined as the commencement of the reprogramming process until the first pluripotency-associated genes were expressed, whereas the first wave encompasses only the high transcriptional turnover section during the initiation phase. Events taking place during the initiation phase were validated at the single cell level by time lapse microscopy, backtracking faithful events that would lead to a Nanog- or Oct4-expressing colony: every cell was found to go through an increase in proliferation and a MET (Araki et al., 2010; Megyola et al., 2013; Smith et al., 2010).

The initiation phase of reprogramming has been most widely documented to date. While technical reasons may account for this, the success of those studies resided in the fact that the vast majority of cells were able to initiate reprogramming (Polo et al., 2012). Interestingly, the majority of the cells then became refractory to reprogramming, with few cells subsequently proceeding to the next steps of reprogramming. A possible explanation could be the "innate immunity" triggering protein degradation, which might explain why even in secondary systems not all cells reprogram, but can be rescued by additional overexpression of Yamanaka factors (Buckley et al., 2012; Polo et al., 2012).

The observed changes in gene and protein expression correlate with a hierarchical sequence of events that reflect a deeper interaction between OKSM, co-factors and the chromatin, which together will ultimately dictate the epigenetic state. For example, for a gene to be re-expressed, the region of chromatin needs to be de-condensed, inactive histone markers need to be removed, the DNA needs to be demethylated and active histone markers have to be added (Pasque et al., 2011). All or several of these events have to happen at each gene that needs to be re-expressed, while at the same time, the opposite phenomenon has to happen at lineage-specific genes in order to be repressed. How these epigenetic events impact on the kinetics of gene transcription during reprogramming is still poorly understood. Nevertheless, a few studies have shed some light into how those changes are orchestrated. Focusing on the initial days of human reprogramming and therefore underscoring the events occurring in the initiation phase or first wave, Soufi and collaborators suggested that high levels of reprogramming factors cause OKS to bind to many more genes than they would do physiologically (Soufi et al., 2012). Moreover, recent studies redefined Myc function as a transcriptional response amplifier, mostly binding core promoters rather than distant enhancers (Lin et al., 2012; Nie et al., 2012). This is in accordance with a previous mouse study showing that Myc served a different function during reprogramming than OKS (Sridharan et al., 2009). These findings showed that during the early stages of reprogramming, Myc binds to accessible DNA regions, while OKS can bind inactive DNA regions (Soufi et al., 2012). In addition, these studies suggested that Myc is responsible for the loss of somatic cell identity of MEFs and the induction of MET, while OKS mostly acts as pioneer (Soufi et al., 2012; Sridharan et al., 2009). At the chromatin level, in MEFs, this phase is characterized by changes in histone modifications and not DNA methylation (Polo et al., 2012). Even though, gain and loss of active and repressive markers happen during the first wave, it seems to be biased to genes with active chromatin markers (Koche et al., 2011). Furthermore, a small scale shRNA screen performed during reprogramming of human fibroblasts showed that the histone methyltransferase DOT1L had an inhibitory effect on reprogramming and a block in KLF4 induction of MET (Onder et al., 2012). Finally, the importance of epigenetic regulators during the initiation phase has been recently underscored by a paper showing that Tet1, 2, 3 triple knock-out failed to initiate reprogramming, specifically because Tets are required to activate the miR-200 family, which in turn is necessary for MET to occur (Hu et al., 2014). More information regarding epigenetic regulation of pluripotency and reprogramming can be found in recent reviews (Apostolou and Hochedlinger, 2013; Buganim et al., 2013; Liang and Zhang, 2013; Papp and Plath, 2013).

In conclusion, every cell undergoing reprogramming will transit through the initiation phase (Polo et al., 2012). This may reflect an overall non-stochastic phase. However, the clear sequence of events driving the initiation phase has not been completely defined yet. Moreover single cell analysis during this early phase suggested that the overall noise in the signature may reflect a stochastic process in which the path taken does not matter, as long as all the initiation phase-associated modifications are acquired (Fig. 1B) (Buganim et al., 2012).

The subsequent transition from the initiation to maturation phase is a major bottleneck for reprogramming. This has been highlighted during human reprogramming as well, through the systematic isolation of Tra-1–60 positive cells (the equivalent of SSEA1 in mouse) which showed that most of these cells could not progress to the maturation phase of reprogramming (Tanabe et al., 2013).

Intermediate events — maturation phase — second wave

The maturation phase coincides with the second wave of major transcriptional changes and is marked by the onset of the first pluripotency-associated genes (Hansson et al., 2012; Polo et al., 2012; Samavarchi-Tehrani et al., 2010). This period is more protracted compared to the initiation phase, and it can be further divided into different intermediate states. During this phase, some pluripotency-associated genes are gradually activated; some of the first markers to be detected are Fbxo15, Sall4 and endogenous

Oct4, followed by Nanog and Esrrb. At the very end of the maturation phase, on the cusp of the stabilization stage, it is possible to detect Sox2 or Dppa4, as cells can self-renew independently of transgenes, (Buganim et al., 2012; Golipour et al., 2012; Polo et al., 2012; Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008b). On this note, the pluripotency-associated markers have played a pivotal role from the start in the induced pluripotency field. In fact, the first selection marker used as a predictive marker of reprogramming by Takahashi and Yamanaka was Fbxo15. Curiously, Fbox15 was a poor predictor of reprogramming as it was also expressed in partially reprogrammed cells (Takahashi and Yamanaka, 2006). Nevertheless, other substitute markers such as Nanog and Oct4 were then used in replacement (Maherali et al., 2007; Okita et al., 2007), resulting in a more reliable assessment of achieving reprogramming.

The functional relevance of early pluripotency markers, or maturation genes, such as Nanog or endogenous-Oct4, is reaffirmed by the virtue that some of these factors enhance the reprogramming efficiency, and even substitute for some of the Yamanaka factors. These candidates include Nr5a2, Tbx3, Nanog or Esrrb (Buganim et al., 2012; Han et al., 2010; Hanna et al., 2009; Heng et al., 2010). It is worthy to note that while the maturation genes are good indicators of reprogramming, their sole acquisition does not guarantee complete reprogramming of cells (Buganim et al., 2012; Golipour et al., 2012; Polo et al., 2012). This clearly indicates that a necessary sequence of events must occur late during reprogramming, with a hierarchical relationship between those events.

As mentioned previously, through the use of clonal approaches, single cell analysis and parallel multi-culture techniques, multiple groups assessed the sequential events occurring in the cells committed to reprogramming and were able to properly investigate these late events. Two groups utilized single cell transcriptomics of genes previously identified in reprogramming populations (Buganim et al., 2012; Polo et al., 2012). One group coupled this technology with a clonal analysis (secondary cells originally seeded at one cell per well after the initiation phase) and validated the result by FISH (Buganim et al., 2012). The other groups coupled this technology with systematic cell sorting at different time points using previously described sequential markers of cells undergoing reprogramming (Thy1−, SSEA1+, Oct4+) (Polo et al., 2012). Although with some small differences, both studies showed that cells acquire pluripotency markers in a sequential way, with some being expressed during the maturation phase (i.e. Nanog, Esrrb) and others not expressed until the next late stabilization phase (i.e. Sox2, Pecam), validating the hypothesis based on previous transcriptomic studies or reporter genes (Samavarchi-Tehrani et al., 2010; Stadtfeld et al., 2008b). These findings have been further refined and confirmed through the use of a cell surface marker, ICAM1, allowing for committed-cell populations to be enriched during the reprogramming process (O'Malley et al., 2013).

An important feature of the late reprogramming event is transgene silencing, or in other words, the ability of iPSC to self-renew independently of the Yamanaka factors. Taking advantage of a mouse secondary system in which the last step does not occur in the presence of the transgenes, Golipour and collaborators studied the determinants of transgene independency. Using clonal analysis and the ability to survive transgene suppression, this group revealed a signature associated with the competency to survive transgene suppression and transit to the stabilization phase (Golipour et al., 2012). The functional validity of this stabilization competency signature was validated by a siRNA screen performed on cells undergoing transgene suppression, compared to the same siRNA screen repeated in iPSC. While the universal pluripotency-associated factors were found to be necessary for pluripotency in iPSCs, a distinct regulatory network was found to be important for survival upon transgene suppression. This demonstrated the importance of events occurring after the acquisition of pluripotency factors such as Oct4 or Nanog and how they prepare the cells to self-renew in the absence of the transgenes. Those events involved converting inactive DNA regions coding for a subset of pluripotency associated factors into poised regions, which correlated with the observation that DNA methylation changes occur mostly at the end of the reprogramming process (Polo et al., 2012).

The cellular and molecular events underpinning the maturation phase are characterized by their hierarchical and slow reactivation. However, how this happens remains poorly understood. One hypothesis is that specific complexes are required to activate each of the pluripotency genes, therefore, acquisition of factors one after another is necessary and explains the sequence of events. Potential regulators have been recently identified as playing a dual role by inhibition of the maturation phase program through BMP signaling, which is present in the serum and feeders. The recruitment of the polycomb group, the recruitment of Utx, and the loss of Mbd3 subunit of the NuRD complex have also been identified (Chen et al., 2013; Luo et al., 2013; Mansour et al., 2012; Rais et al., 2013; Singhal et al., 2010; Welstead et al., 2012). It remains to be shown how all these findings are interconnected and what regulates the redistribution of OKSM to those genes during the maturation phase.

Finally, the importance of lifting the epigenetic barriers during this period has recently been the center of a study focused on B cell reprogramming, using mouse secondary B cells as a model (Di Stefano et al., 2014). The authors found out that overexpressing C/EBPα triggers the expression of Tet2 in B cells, greatly improving reprogramming speed (Di Stefano et al., 2014). Interestingly, in this other cell type, upon overexpression of C/EBPα, two kinetics of pluripotency markers were observed: a first early induced group of Oct4, Lin28a and GDF3, and a later induced group with Nanog Sox2 and Esrrb. This highlights differential re-activation of pluripotency genes, with some similarities in the sequence (early Oct4, late Sox2) compared to MEF reprogramming. Such a study has to be repeated with other cell types in order to understand if there are multiple and different pathways to reprogramming.

Final events — stabilization phase

The stabilization phase encompasses the changes that occur in iPSCs after they have acquired pluripotency (Ho et al., 2011; Stadtfeld and Hochedlinger, 2010).

Once the cells undergoing reprogramming express the first subset of pluripotency-associated genes (maturation-phase genes) and remain poised for the second subset (stabilization-phase genes), the cells are able to successfully transit to the stabilization phase and acquire the full pluripotency signature, a pluripotent state that can be sustained independently of ectopic reprogramming factor expression (Brambrink et al., 2008; Maherali et al., 2007; Okita et al., 2007; Stadtfeld et al., 2008b; Wernig et al., 2007). During this phase, the iPSCs will be expanded and pluripotency will be assessed (i.e. by blastocyst injection). Based on chimeric aggregation, cells become pluripotent very shortly after suppression of transgene expression. In human iPSCs, extensive passaging is also necessary to adapt the cells to hESC growth conditions and amplify them sufficiently before these can be applied in an experimental setting. It also normalizes the minor differences in expression profile that might exist between early hiPSCs and hESCs (Chin et al., 2009).

While iPSCs are pluripotent at this point, numerous epigenetic changes are occurring. An example in mouse is telomere elongation back to an embryonic level (Marion et al., 2009b; Stadtfeld et al., 2008b). The DNA is also subject to important rearrangements, such as reactivation of the inactive X chromosome in female mouse iPSCs (Stadtfeld et al., 2008b). Understanding the mechanisms linking X reactivation to the induction of pluripotency is particularly relevant for human reprogramming, which often leads to hiPCS with an inactive X chromosome, and disease modeling (Papp and Plath, 2013). Other epigenetic rearrangements can be assigned to the stabilization phase, such as the resetting of epigenetic memory found in mouse and human iPS cells derived from different somatic cell types. This phenomenon of "epigenetic memory" has been shown to bias iPS cells towards their donor somatic cell type of origin. Interestingly, in accordance with the stabilization phase, passaging the cells or treating them with 5-aza, a DNA methylase inhibitor, helped to reset the epigenome to prevent differentiation biases (Kim et al., 2011; Ohi et al., 2011; Polo et al., 2010). Clearly, changes in DNA methylation profiles that started in the late maturation phase continue throughout the stabilization phase and are likely to be responsible for the events associated with this phase. One likely explanation is the reactivation of regulators of DNA methylation, such as AID, TET family and DMNTs during the late maturation/stabilization phase (Polo et al., 2012). AID has been shown to be actively promoting this epigenetic reset (Bhutani et al., 2010; Kumar et al., 2013). Further study of the epigenetic rearrangements taking place late during reprogramming is another area of great importance to ensure the production of high quality iPSCs.

Discussion

While the roadmap to reprogramming is now taking shape, further refinement of this process is still needed. Indeed, recent advances have shed light on different road blocks, such as an improved OKSM stoichiometry (Carey et al., 2011) and understanding that cells can become refractory to reprogramming even by shutting down transgenic expression systems (Polo et al., 2012). There is a molecular connectivity or cascade of expression changes in specific genes during the reprogramming steps which have allowed the use of different surface and genetic markers during the study of the mechanism of reprogramming. As discussed, the initiation stage is marked by Cdh1, alkaline phosphatase, and SSEA1 followed by the maturation phase markers (e.g. Oct4, Nanog) and the stabilization phase markers (e.g. Pecam1, Sox2) which results in the production of bona fide iPSCs (Buganim et al., 2012; Golipour et al., 2012; O'Malley et al., 2013; Polo et al., 2012).

As the transcriptional signatures are now quite defined, it seems that for a given starting cell type, the sequence of events is highly conserved in nature and order. The last question, the time latency issue, points out epigenetic barriers such as, what makes a gene easier to reactivate than others, especially among the pluripotency associated genes? Bypassing the epigenetic barrier(s) is therefore a major challenge and solving this issue will require an in depth analysis of epigenetic regulatory events during reprogramming, including 3D structure of the genome studies, as performed recently with the Nanog promoter (Apostolou et al., 2013). This should yield factor-based or chemically-based reprogramming approaches matching the speed and faithfulness of somatic-cell nuclear transfer (SCNT) reprogramming. This is particularly relevant as human ESCs derived by SCNT, thereby mimicking embryo-derived ESCs, have just been successfully obtained (Tachibana et al., 2013). Importantly, alternative cocktails of reprogramming factors, for example by including Esrrb or knocking-down Mbd3, have demonstrated that overcoming this barrier is possible (Buganim et al., 2012; Luo et al., 2013; Rais et al., 2013). It remains to be shown whether those alternative or accelerated reprogramming strategies are changing the sequence of events, and if they change the faithfulness of reprogramming.

While most of the knowledge on the mechanisms of reprogramming has been acquired using mouse models, many of those mechanisms have also held true during human reprogramming. This extraordinary rapid progress in our understanding of nuclear reprogramming increases our hope that in the foreseeable future, somatic cell reprogramming will yield "perfect" iPSCs with high throughput and reproducibility. Clinical applications and clinical trials using this technology are already starting to appear and the development of more reliable reprogramming protocols based on mechanistic studies will help propel advancements in this field.

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