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Sequential addition of reprogramming factors improves efficiency

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Abstract

Addition of a specific set of transcription factors reprograms somatic cell nuclei to a pluripotent state. Sequential addition of these factors, rather than the simultaneous exposure used in standard protocols, improves reprogramming efficiency. This sequential method favors a transition through a state with enhanced mesenchymal characteristics before driving an epithelial transformation on the way to the pluripotent state.

Since it was demonstrated that fibroblasts can be converted to pluripotent cells by induction of a cocktail of transcription factors (originally Oct4, Sox2, Klf4 and c-Myc; known as OSKM)¹, this protocol has been extensively used by researchers to analyze the pluripotent state and to study development and disease *in vitro*. Essentially, these factors are introduced through transduction of either DNA or RNA, the cells are placed into medium that promotes the pluripotent state, and a couple of weeks later, pluripotent clones can be isolated. Despite its widespread use, the efficiency of this process is still low (0.1% in mouse; 0.01% in human). Although many of the experimental design elements have been altered to improve efficiency — from the number and identity of transcription factors to various medium components — most published work so far has described the addition of the reprogramming factors *en masse* as the first step of the protocol. Pei and colleagues suggest that adding the factors sequentially is a better strategy to increase the number of pluripotent cells obtained². They found that for both murine and human repro-gramming, adding the factors as a sequence improves reprogramming efficiency by 300%. This method of delivery of the reprogramming factors also had an additive effect in the presence of vitamin C, which is already known to increase reprogramming efficiency³. These data suggest that moving through particular transitions as well as the original state of the target is key in determining the response to a particular reprogramming factor and the final outcome. This result is particularly interesting in light of recent results from other groups^{4–7}.

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Several recent efforts to understand transcription-factor-mediated reprogramming have merged whole-genome and transcriptome techniques with single-cell analysis. Although it is still difficult to investigate every epigenetic mark or transcript in a single cell, these studies have shed significant light on what was once known as the ‘black box’ of reprogramming⁸. Given the low reprogramming efficiency (<10%) even when all cells receive all the factors transgenically⁹, there is much speculation as to how these factors act and whether there are subsets of somatic cells in the culture that are more susceptible to reprogramming (referred to as the elite model¹⁰), or whether a series of unexplained epigenetic events randomly combine to remove barriers to the pluripotent state (the stochastic model¹⁰). Analysis of cells at several time points along the path to the pluripotent state are beginning to shed light on which of these two models can best explain the process.

Based on the results from ChIP-Seq to study chromatin-bound factors, RNA-Seq to obtain an overview of the gene expression program, and DNA methylation analyses over time and in single cells, it seems that fibroblasts undergoing the reprogramming process pass through several stages that can be defined morphologically and molecularly on their way to the pluripotent state⁴⁻⁷. Several papers separately described stages that go by different names but share much in common. Jaenisch and colleagues suggest that there are two main phases: an early stochastic stage, followed by a late, deterministic one^{4, 5, 7}. Wrana and co-workers define the stages succession as initiation, maturation, and stabilization phases⁵. The early stage is most easily characterized by a loss of mesenchymal nature (when starting with fibroblasts). The middle stage is noted for the first evidence of expression of the endogenous versions of the reprogramming factors. The last stage is marked by pluripotent gene expression, X chromosome activation (in murine models), and acquisition of pluripotency. Each of these studies implicated similar types of molecular processes at each stage; which was to be expected, as they all used simultaneous introduction of the factors and all ended with cells in the same state.

Pei and colleagues² approached the introduction of the reprogramming factors in a distinct way. They hypothesized that cells might respond more efficiently to the factors if some of them were added at different stages of the early phase of reprogramming. After trying dozens of sequential combinations of addition for the four reprogramming factors (many of which increased reprogramming efficiency over synchronous OSKM addition), the authors finally settled on a protocol that first introduces Oct4 and Klf4, then c-Myc, and finally Sox2. Several groups, including this one, have clearly demonstrated that mesenchymal cells such as fibroblasts require a mesenchymal-to-epithelial transition (MET) to proceed towards pluripotency^{11, 12}. This same group now provides evidence that before the MET, mesenchymal cells seem to undergo transition into an even more pronounced mesenchymal state by upregulation of hallmark genes such as Slug (Snail2), decreased expression of epithelial adhesion molecules, and an increase in mesenchymal-type motility in a functional assay. The authors propose a model whereby this hyper-mesenchymal state delays the eventual MET to facilitate certain aspects of reprogramming. The authors argue that the MET delay and the generation of a more homogenous pool of fibroblasts are key for the increased efficiency of the eventual transition to pluripotency (Fig. 1). It is also conceivable that by adding Oct4 first, this protocol generates a population of immature fibroblasts that are more amenable to eventual reprogramming by Sox2 (Fig. 1).

Several lines of evidence, including in the report by Pei and colleagues², suggest that Oct4 can induce Slug expression in a variety of settings, whereas Sox2 has been shown to promote the epithelial state by suppressing Slug expression¹². Therefore, Pei and colleagues argue that addition of Oct4 first and Sox2 later is able to promote epithelial-to-mesenchymal transition (EMT) and delay MET long enough for some transcriptional and epigenetic events to occur before acquisition of the epithelial and, ultimately, pluripotent state. Moreover, they show that synchronized OSKM induction does not lead to EMT or delayed MET, suggesting that the timing of factor addition determines the path these cells will follow (Fig. 1). It is tempting to speculate that during this period, the bulk of erasing and re-writing of the epigenome occurs, and that it could be critical to carry out this reorganization before induction of the endogenous pluripotency factors to avoid being side-tracked to various dead ends of reprogramming. The fact that defined levels of expression of key pluripotency factors such as Oct4 and Nanog are essential to maintain the pluripotent state, and that embryonic stem cells are surprisingly sensitive to perturbations of these genes, supports this idea^{13, 14}.

These findings call for a recalibration of our understanding of the mechanisms of reprogramming, as all previous work defining the nature of the process and the actions of the reprogramming factors was performed in cells receiving all the factors simultaneously. Recent studies have mapped OSKM binding by ChIP-Seq after just 48 hours, and one report showed that OSK bind in tandem at many loci¹⁵. When these factors are added sequentially, do they have the same binding sites? Do they bind in clusters? Does their synchronous expression in fact inhibit binding or activity of one of more members? Answering these questions will certainly shed light on reprogramming, but more importantly will also lead to more efficient protocols that produce higher quality iPSCs.

Pei and colleagues also show that sequential addition of OSKM improves reprogramming in human cells², suggesting that the mechanism is broadly applicable. This finding is important as human reprogramming is still generally 10-fold less efficient than murine reprogramming — for unknown reasons. From the data presented in the paper, it is possible that human reprogramming could be plagued by the same problem faced by researchers for murine cells — namely, that we do not yet fully understand which characteristics of target cells predict their responses to the overexpression of a reprogramming factor. Presumably, human cells pass through similar stages of reprogramming as murine cells^{4, 5, 7}, so it will be interesting to determine how the early (or stochastic) and late (deterministic) stages coincide with EMT and MET when the factors are introduced sequentially into human cells. Furthermore, although fibroblasts were the original target cell described by Yamanaka¹, it seems that almost any cell in the body can be reprogrammed to a pluripotent state, including epithelial cells. It is unclear at this point how the work of Pei and colleagues² will translate to epithelial cell reprogramming, which obviously does not need to pass through MET on the way to the pluripotent state, and therefore may not benefit from sequential factor addition, or may simply prefer a different sequence.

The work by Pei and colleagues² did not demonstrate that human iPSCs made by sequential factor addition were of higher quality, mostly because the field lacks a functional assay for human iPSCs that can distinguish lines that are closest to hESCs from those that are not

reprogrammed completely. Instead, the field relies on epigenetic analyses as a proxy for quality control among lines that can at least perform in a teratoma assay. Therefore, considerable effort is required to determine whether sequential addition of the factors leads to higher-quality hiPSC lines, which is of paramount importance for clinical application of this exciting new technology.

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Figure 1.

Heterogeneous fibroblast cultures often serve as the target population for reprogramming to the pluripotent state. Pei and colleagues show that sequential addition of the factors takes the cells through an enhanced mesenchymal state before a mesenchymal-to-epithelial transition on the way to the pluripotent state. They provide data to suggest that although this progression through specific transitions is a longer process, it results in a much higher efficiency of conversion.