Illuminating the Black Box of Reprogramming

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Yamanaka and colleagues, in a *Science* article currently published online, have generated induced pluripotent stem (iPS) cells from liver and stomach cells, suggesting that transcription factor-induced reprogramming is not restricted to particular cell types (Aoi et al., 2008). These results also provide important insight into the mechanistic basis of reprogramming.

The reprogramming of somatic cells into induced pluripotent stem (iPS) cells was a breakthrough discovery of 2006 (Takahashi and Yamanaka, 2006). Yamanaka and colleagues showed that murine fibroblast populations can be reprogrammed by overexpressing four transcription factors: Oct4, Sox2, Klf4, and c-Myc. Fibroblast-derived iPS cells are molecularly and functionally indistinguishable from embryonic stem (ES) cells (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). The same approach has since been shown to work with human fibroblasts (Takahashi et al., 2007; Yu et al., 2007), providing an elegant method to obtain human ES-like, pluripotent populations that hold great promise for the study and treatment of human diseases.

Although reprogramming fibroblasts to an ES-like state is now clearly feasible, typically only one in a thousand recipient cells is reprogrammed. Several explanations may account for this low efficiency (Figure 1). The integration of retro- or lentiviruses used to deliver the four factors may fortuitously modify the expression of an essential, unknown factor in a small subset of cells. Alternatively, the heterogeneous fibroblast population could harbor cell types that are predisposed to reprogramming. It is even possible that only rare adult stem cells rather than differentiated cells are the source of the reprogrammed cells. Can only fibroblast cultures be reprogrammed? In their latest study, Yamanaka and colleagues (Aoi et al., 2008), in combination with recent papers from the Jaenisch and Hochedlinger labs (Brambrink et al., 2008; Stadtfeld et al., 2008), begin to address these questions of inefficiency, donor cell type, and the mechanism(s) responsible for reprogramming (Figure 1).

To test whether the same set of transcription factors could induce reprogramming of epithelial cell types, Yamanaka and colleagues retrovirally transduced primary murine cells from liver and stomach tissues (Aoi et al., 2008). Selection for expression of the ES cell markers Fbx15 and Nanog 7 days after viral infection yielded iPS cells that (1) had activated ES cell-specific transcription, (2) were demethylated at the endogenous Oct4 and Nanog promoters, and (3) gave rise to germline-competent chimeric mice, the gold standard for demonstrating pluripotency (Table 1). Thus, reprogramming is not only restricted to cells of mesodermal origin, such as fibroblasts, but also works on endodermal cell types.

Of note, the kinetics of reprogramming appears to vary when target populations from different tissues are used. Specifically, when selection for Fbx15 expression was applied as early as 3 days after viral transduction, fibroblast-derived iPS cells were only partially reset to the ES-like state (Takahashi and Yamanaka, 2006), whereas populations of infected liver cells yield completely reprogrammed iPS colonies when selected at this early time point (Aoi et al., 2008) (Table 1). Why might liver cells be reprogrammed faster than fibroblasts? Gene expression in epithelial cells may be more similar to ES cells than patterns observed in fibroblasts (Aoi et al., 2008). In addition, a loss of hepatocyte-specific gene expression is characteristic of explanted hepatocytes (Elaut et al., 2006). Hence these cells may be more amenable to induced alterations and perhaps require fewer transcriptional changes to reach a reprogrammed state.

Two recent papers used inducible lentiviruses to assess the temporal requirements for factor expression and defined intermediate stages of the reprogramming process (Brambrink et al., 2008; Stadtfeld et al., 2008). Both groups found a gradual activation of ES cell maker genes, including upregulation of the stage-specific embryonic antigen 1 (SSEA1) that preceded the increase in endogenous Nanog, Oct4, and Sox2 expression. SSEA1-positive cells arose from a subpopulation of cells that had lost the fibroblast surface marker Thy1 and activated the stem cell marker alkaline phosphatase. Importantly, Hochedlinger's group isolated Thy1-negative or Thy1-negative/SSEA1-positive cells, which yielded cell populations enriched for precursors of fully reprogrammed iPS cells. In these "primed" subpopulations, retrovirally encoded factors were gradually silenced while endogenous, ES cell-specific transcription took over (Stadtfeld et al., 2008). Performing a similar analysis during liver iPS cell formation will be a first step toward explaining the differences in reprogramming kinetics observed in various target populations.

Two surprising observations distinguish liver and stomach iPS cells from their fibroblast-derived counterparts (Aoi et al., 2008) (Table 1), even though the cells appear completely reprogrammed to the ES-like state. First, the majority of chimeric mice generated from stomach and liver iPS cells die postnatally, which is not the case for fibroblast iPS chimera. Second, while 30% of chimeric mice

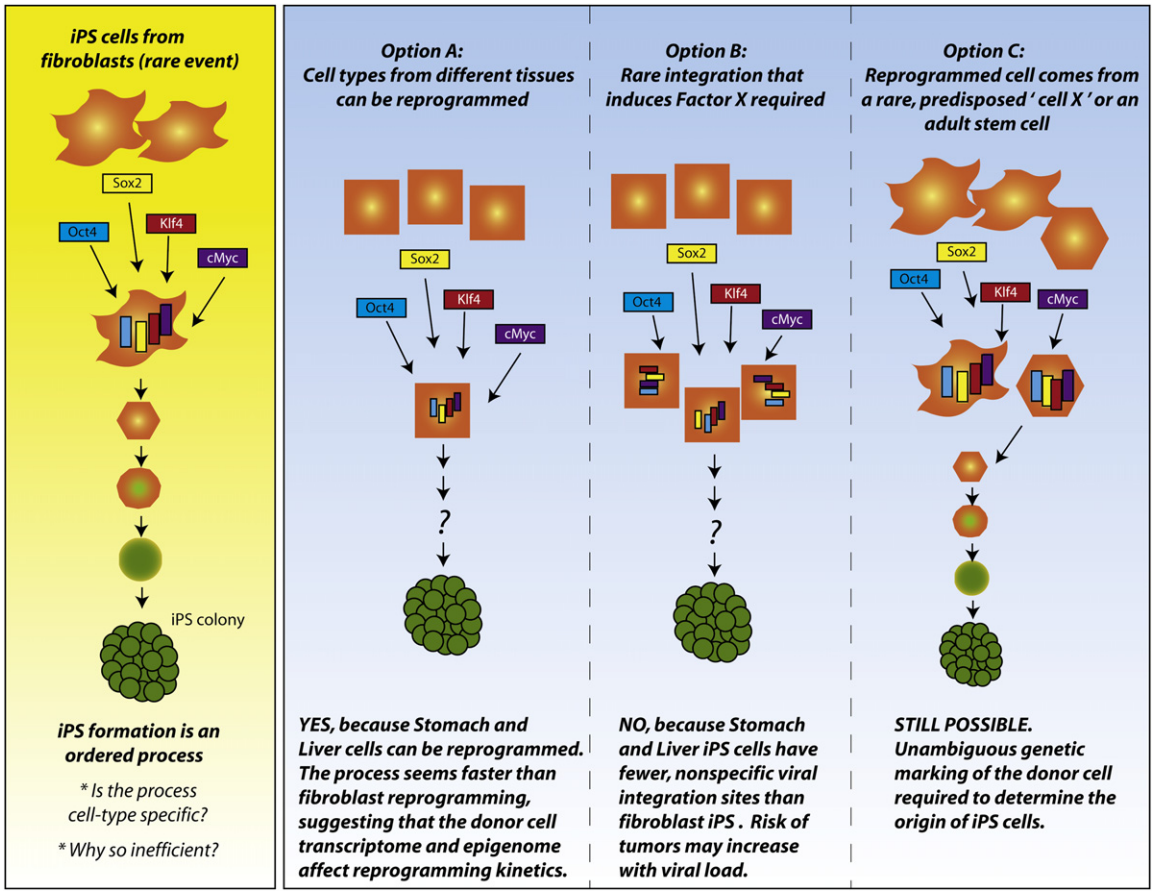

Figure 1. Reprogramming Is an Ordered, yet Inefficient, Process
Data presented by Aoi et al. (2008) and the Jaenisch and Hochedlinger laboratories (Brambrink et al., 2008; Stadtfeld et al., 2008) are integrated and begin to open the "black box" of events that take place during somatic cell reprogramming and iPS colony generation. Some points are clarified, but questions remain.

derived from fibroblasts develop tumors, adult mice derived from liver or stomach iPS cells did not. Of note, Aoi et al. (2008) also observed that liver and stomach iPS cells had 2- to 3-fold fewer viral integration events than fibroblast iPS cells. Thus, epithelial iPS cells might be less likely to have integration-induced oncogene expression, or the reduced tumor incidence could be due to more stable silencing of the retroviral transgenes, especially of transgenic c-Myc. Perinatal death, on the other hand, might reflect differences in genomic stability of the donor cell population. iPS cells derived from liver or stomach cells could have acquired more mutations or have unbalanced imprinted gene expression compared to fibroblasts, a phenomena that has been an issue in cloned animals (Yang et al., 2007). A detailed analysis of the transcriptome, chromatin state, and ploidy of fibroblast and epithelial cell-derived iPS cells will be required to explain these differences in iPS cell properties.

In addition to the reduction in epithelial iPS viral integrations, Aoi et al. failed to detect any common retroviral integration sites in iPS cells. This finding argues against the model that reprogramming depends on the activation or repression of a specific, as yet unknown, essential factor (Figure 1). Furthermore, the Jaenisch and Hochedlinger groups reveal that fibroblast reprogramming requires ectopic transcription factor expression for only

Table 1. Properties of iPS Cells Originating from Fibroblast or Epithelial Cell Populations

	Timing of iPS Selection	Fibroblast iPS	Liver iPS	Stomach iPS
Reprogramming of ES cell marker gene expression	Early (day 3)	Partial	Complete	ND
Generation of adult chimera	Early (day 3)	No	Yes	ND
Reprogramming of ES cell-specific transcription of marker genes	Late (at or after day7)	Complete	Complete	Complete
Generation of adult chimera	Late (at or after day 7)	Yes	Yes	Yes
Perinatal death of chimera		No	Yes	Yes
Tumors observed in chimera		Yes	No	No
Number of integration sites per virus		10–12	<4	<4
Enhancement of iPS production by c-Myc		10-fold	<2-fold	ND

a transient period (8–12 days: Brambrink et al., 2008; Stadtfeld et al., 2008). Together, these findings suggest that nonintegrating reprogramming methods may be developed to provide transient overexpression of the transcription factors, which will positively impact the ability to translate iPS technology into therapies.

To begin to determine the origin of the cell that gives rise to iPS colonies, Aoi et al. (2008) used a lineage-tracing strategy that identifies cells that, at some time, have expressed the hepatic gene albumin. Their results indicate that liver-derived iPS cells were almost all positive for this reporter, suggesting that lineage-committed cells can be reprogrammed to an ES-like state. However, although albumin is expressed in mature hepatocytes and liver progenitors, it could conceivably have been activated during the reprogramming process in vitro. To conclusively demonstrate that iPS cells arise from terminally differentiated cells and not rare stem cells, populations with differentiation-associated genomic rearrangements, such as lymphocytes, will need to be examined.

Although the hypothesis that iPS cells arise from a rare stem cell remains possible until an unambiguously genetically marked cell can be reprogrammed to indicate the differentiation state of the donor cell, Yamanaka's latest studies suggest that the low efficiency of reprogramming is not a result of directed insertional mutagenesis and that factor-induced reprogramming is a universal process that is not restricted to particular cell types. Given that the overall efficiency of reverting early reprogramming intermediates into iPS cells is still low (Brambrink et al., 2008; Stadtfeld et al., 2008), transcription factor-induced reprogramming must require rare stochastic, likely epigenetic, events. Analyzing subpopulations of iPS intermediates from multiple tissues via genome-wide approaches for factor binding and chromatin changes should reveal important molecular events that occur during this cascade. Such insights may lead to safer, more efficient reprogramming methods that will be necessary to translate iPS cells into therapeutic tools.

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Cancer: Inappropriate Expression of Stem Cell Programs?

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Cancer stem cells (CSCs) are a subpopulation of cancer cells that possess characteristics, including self-renewal, associated with normal stem cells. In this issue of *Cell Stem Cell*, Wong et al. (2008) define a core embryonic stem cell (ESC)-like gene expression program that may be important for CSC function in multiple epithelial cancers.

Increasing evidence suggests that pathways and properties associated with normal stem cells are important for cancer development. The link between genes important for normal stem cell development and cancer is most clearly established in hematopoietic malignancies, based largely on the study of chromosomal translocations identified in leukemias and lymphomas. An extensive body of work has demonstrated that these translocations often involve genes that play critical roles in normal hematopoietic stem cell (HSC) biology (reviewed in Orkin and Zon, 2008). Human acute myelogenous