iPS Cells: Insights into Basic Biology

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The discovery that adult somatic cells can be induced to become pluripotent by overexpression of a few key transcription factors provides an exciting new window into the basic biology of pluripotency and differentiation.

The discovery 3 years ago that adult somatic cells can be induced to become pluripotent stem cells, so-called iPS cells, by overexpression of a few key transcription factors (Takahashi and Yamanaka, 2006) generated much excitement because of the potential therapeutic applications of these cells (reviewed in Yamanaka, 2009). Efforts are underway in many laboratories to apply iPS cell technology to modeling diseases in vitro and to developing tissue-replacement therapies. To fulfill the therapeutic promise of iPS cells, a major research focus has been to improve the efficiency and completeness of reprogramming back to a pluripotent state. Other research avenues include finding ways to deliver the genes encoding the reprogramming factors without using viruses, thus avoiding the need for genomic integration, and compiling panels of iPS cells derived from patients with different diseases in order to drive their in vitro differentiation into the cell type affected in the disease. The generation of iPS cells, however, may also be valuable for gaining insights into the basic biology of pluripotency and differentiation.

The mechanisms by which somatic cells are reprogrammed back to a pluripotent state are largely unknown. During the reprogramming process, cells often get "trapped" in partially reprogrammed states due to inefficient DNA demethylation or incomplete repression/ectopic expression of lineage-specific transcription factors (Mikkelsen et al., 2008). The contribution of each reprogramming factor is not well understood, but c-Myc is thought to act early to repress somatic cell genes (Sridharan et al., 2009). Binding of the other three reprogramming factors—Oct4, Sox2, and Klf4—to pluripotency genes may be a later, rate-limiting step in the progression to complete reprogramming (Sridharan et al., 2009). These and other studies are beginning to reveal the mechanisms that underlie the induction of pluripotency (reviewed in Hochedlinger and Plath, 2009). But can iPS cells provide insights into basic biology?

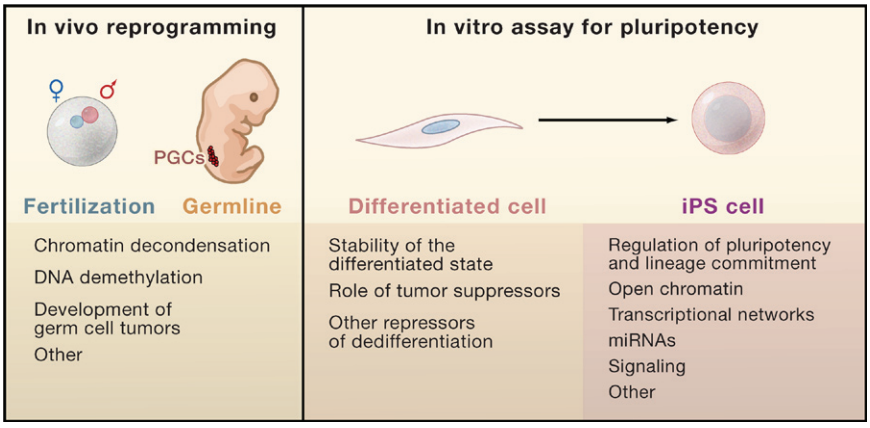

Figure 1. The iPS Cell Assay
The epigenetic reprogramming that occurs in vivo in the fertilized egg or in primordial germ cells (PGCs) may have a parallel with the molecular events underpinning iPS cell generation in vitro. The derivation of iPS cells may provide a gain-of-function assay for analyzing and quantifying pluripotency. The iPS cell assay is reproducible, quantitative, and can be modulated by various conditions. This assay may provide new insights into the roles of chromatin factors, transcriptional networks, and microRNAs in pluripotency and in lineage commitment decisions during differentiation. The iPS cell assay can also be used to identify the mechanisms that "lock-in" the state of differentiated cells and prevent dedifferentiation.

Modeling In Vivo Reprogramming

Cells developing in vivo progress from undifferentiated states with broad cell-fate potential to committed states with restricted potential. Arguably, the generation of iPS cells represents an artificial experimental manipulation that "plays the development tape backwards" and therefore may not have a parallel in vivo. However, iPS cell generation may involve molecular processes that have parallels with fundamental events during mammalian development (Figure 1). One such event is the reprogramming of the gamete pronuclei at fertilization, which leads to initiation of the embryonic program. The DNA in the sperm pronucleus is highly compacted and undergoes decondensation and demethylation under the influence of oocyte intracellular factors. Some of these same factors, most of which are unknown, presumably also are involved in the reprogramming of adult cell nuclei by somatic cell nuclear transfer (SCNT). The mechanisms underlying SCNT have proven difficult to dissect, mostly because of the complexity and low reproducibility of the assay.

Later in development, primordial germ cells (PGCs) also undergo a process of reprogramming that involves genome-wide demethylation of DNA and modification of histones. The generation of iPS

cells from adult somatic cells involves extensive epigenetic reprogramming that includes chromatin decondensation and DNA demethylation. Epigenetic reprogramming during the generation of iPS cells may well be mediated by mechanisms very different from those that operate during in vivo reprogramming in either oocytes or PGCs, and this will need to be carefully assessed. However, should there be some molecular parallels between epigenetic reprogramming in vivo and iPS cell generation in vitro, then the latter may provide a particularly tractable genetic and biochemical system to dissect the underlying mechanisms. Unlike oocytes or PGCs, which exist in very limited numbers in vivo, large numbers of cells can be reprogrammed, in a quantitative and reproducible manner, to become iPS cells in vitro.

The derivation of iPS cells also may be used to explore the molecular underpinnings of germ cell tumor development. The transcriptional profile of PGCs is similar to that of embryonic stem (ES) cells and includes expression of Oct4, Sox2, Nanog, and other pluripotency-associated factors. PGCs do not express c-Myc but do express high levels of another Myc family member, n-Myc, and n-Myc can substitute for c-Myc in the generation of iPS cells (Blelloch et al., 2007). PGCs do not express Klf4, which is activated during conversion of PGCs to pluripotent stem cells in vitro. It will be interesting to determine whether acquisition of Klf4 expression, or other molecular events that occur during iPS cell generation, play any role in the transformation of PGCs and the development of germ cell tumors.

A Gain-of-Pluripotency Assay

The derivation of iPS cells may be viewed as a biochemical assay for pluripotency. It is reproducible, quantitative, and can be modulated by various conditions including the addition of cofactors (Figure 1). Genetic manipulation of the iPS cell assay—such as knockdown or overexpression of candidate genes in addition to or substituting for the four reprogramming factors—may allow questions to be asked about the molecular mechanisms that underlie pluripotency during normal embryonic development.

The initial report of iPS cell generation (Takahashi and Yamanaka, 2006) provided an important basic insight into the transcriptional networks of pluripotency. Of the four factors used by Takahashi and Yamanaka, Klf4 was the most unexpected because it is not required for pluripotency. It is now known that a redundant network composed of Klf2, Klf4, and Klf5 is essential for ES cell pluripotency and regulates many of the same target genes as Nanog (Jiang et al., 2008). Also, an orphan nuclear receptor (estrogen-related receptor-β) required for ES cell self-renewal can substitute for c-Myc and Klf4 during the induction of pluripotency (Feng et al., 2009). Thus, an interplay between research on iPS cells and ES cells may accelerate our understanding of the basic biology of pluripotency.

Another area in which the iPS cell assay may provide fundamental insights is in microRNA (miRNA) biology. The miRNAs miR-291-3p, miR-294, and miR-295, which are expressed specifically in ES cells and regulate progression of ES cells through the cell cycle, enhance the efficiency of pluripotency induction (Judson et al., 2009). Although it remains unclear whether these miRNAs have overlapping targets in ES cells and during reprogramming, the iPS cell assay provides a complementary approach for studying the biology of miRNAs associated with pluripotency.

Recent data from our laboratory suggest that some of the mechanisms that maintain the open chromatin state of ES cells may also operate during the generation of iPS cells. Chd1, a chromatin-remodeling factor required for maintenance of open chromatin and pluripotency of ES cells, is also needed for the induction of pluripotency (Gaspar-Maia et al., 2009). Therefore, the iPS cell assay can provide opportunities to gain insights into the functions of transcription factors, miRNAs, and chromatin-remodeling factors in pluripotent cells. Analogous questions may be asked regarding the roles of signaling proteins, metabolic pathways, cell-cycle regulators, and cytoskeletal proteins in pluripotency.

The iPS cell assay may be particularly useful as a gain-of-function assay in cases where loss of function of particular genes in early embryos or ES cells would provide only limited insight. For example, the cell-cycle and metabolic pathways of early embryos and ES cells have unique characteristics. Although loss-of-function approaches to analyze these characteristics may lead to lethality, the iPS cell assay enables their investigation during the reprogramming of somatic cells to the pluripotent state.

Beyond addressing these questions, the iPS cell assay provides an opportunity for structure-function studies to identify the relevant protein domains or amino acids of a particular transcription factor, chromatin-remodeling protein, or signaling protein that may be involved in pluripotency. It is not that such studies are impossible in early embryos or in ES cells, but rather that the iPS cell assay provides a simple complementary approach with which to accelerate the mechanistic dissection of pluripotency.

Finally, it may be possible to use variations of the iPS cell assay to gain insight into lineage commitment decisions. In a restricted number of cases, it is possible to convert one cell type into another by manipulating the expression of lineage-specific transcription factors (reviewed in Zhou and Melton, 2008). Knowledge gained about how cells change states during the generation of iPS cells—for example, how they alter chromatin accessibility or reprogram epigenetic marks—may contribute to a better understanding of lineage commitment and cell-fate switching.

Dissecting the Stability of the Differentiated State

In addition to providing new insights into the regulation of the pluripotent state, the iPS cell assay may be a new tool to probe the molecular mechanisms that underlie the stability of the differentiated state (Figure 1). Developmental biologists have long pondered whether a terminally differentiated state is reversible and what locks that state in place. Indeed, this was the motivation behind the original SCNT experiments in amphibians (Gurdon, 1962). Many decades later, we still know little about the process of dedifferentiation, which may have important consequences for our understanding of cellular transformation and tumor development. Transformed cells may in some

cases arise by dedifferentiation, that is, by losing markers of terminally differentiated cells and acquiring stem cell-like properties. The iPS cell assay may shed light on the genes and pathways that "lock-in" cells in the differentiated state enabling them to resist dedifferentiation.

Inhibition of the tumor suppressor protein p53 facilitates the generation of iPS cells, suggesting that it represses dedifferentiation (Zhao et al., 2008). The iPS cell assay can be used to study how p53 modulates the stability of the differentiated state. Other tumor suppressors, such as Rb or Pten, are also candidate repressors of dedifferentiation that can be investigated using the iPS cell assay. Lineage-specific transcription factors and epigenetic modifications such as DNA methylation or lack of H3K4 methylation at certain loci also may repress dedifferentiation (Mikkelsen et al., 2008). It will be interesting to test whether inhibition of miRNAs expressed in differentiated cells but not in stem cells, such as let-7, facilitates reprogramming.

More broadly, it may be possible to apply the iPS cell assay to discover new genes that act as repressors of dedifferentiation in an unbiased way using, for example, large-scale RNAi screens. Screening for repressors of dedifferentiation using induction of pluripotency in different adult somatic cell types may reveal some genes that act universally to repress dedifferentiation, and others that regulate the stability of specific cell types. The transcriptional similarities between pluripotent stem cells and cancer cells (Wong et al., 2008) suggest that the iPS cell assay may provide a new quantitative approach for investigating regulation of the differentiated cell state and how it may be subverted in cancer.

iPS Cells in Basic Biology: Caveats

Some of these research avenues make the assumption that the same mechanisms that implement pluripotency in vivo or in ES cells operate during the generation of iPS cells in vitro. This may not necessarily be so, as exemplified by the case of Mbd3, a subunit of the NuRD transcriptional repressor complex. Mouse blastocysts lacking the *Mbd3* gene cannot give rise to ES cells but, when both alleles are mutated by gene targeting in preexisting ES cells, Mbd3 is not required for ES cell maintenance and propagation (Kaji et al., 2006). That is to say, Mbd3 is required for reprogramming of the inner cell mass of the embryo to the ES cell state, but not for maintenance of ES cells. It will be interesting to see if analogous examples of differences between reprogramming and pluripotency can be identified using the iPS cell assay. Presumably, there will be mechanistic similarities between the two phenomena.

A further caveat is that often reprogramming during iPS cell generation is incomplete and may not faithfully reproduce reprogramming in vivo or the pluripotent state of ES cells. The presence of incompletely reprogrammed iPS cell colonies, coupled with the low efficiency of iPS cell generation, means that there is significant cell heterogeneity during reprogramming, with properly reprogrammed iPS cells constituting a small minority of the somatic cells treated with reprogramming factors. This is further complicated by the time it takes to observe iPS cell colonies after induction of pluripotency, which varies from 7 to 14 days in mouse cells and 14 to 28 days in human cells. These complications constitute a "black box" in which the intermediate stages of reprogramming are difficult to access. Nevertheless, current reprogramming efficiencies allow the use of end-point data, that is, the number of iPS cells properly reprogrammed can be assayed through the expression of reporter genes in a quantitative manner. Further improvements in the extent and efficiency of pluripotency induction (Yamanaka, 2009 and references therein) will facilitate the use of iPS cells in basic biology studies. In addition, the development of readouts for early stages of reprogramming may allow the process of iPS cell generation to be visualized and studied in real-time.

In summary, knowledge of the basic biology of pluripotency in early embryos and ES cells paved the way for the discovery of iPS cells. The interplay between iPS and ES cell research extends beyond technical improvements to the method of iPS cell generation and is providing new insights into the regulation of pluripotency. The iPS cell assay is a new quantitative tool to study the basic biology of reprogramming in vivo and to dissect the regulation of both the pluripotent and the differentiated cell state. The iPS cell assay should make important contributions to developmental biology, regenerative medicine, and cancer biology.

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