Pluripotency and Cellular Reprogramming: Facts, Hypotheses, Unresolved Issues

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Direct reprogramming of somatic cells to induced pluripotent stem cells by ectopic expression of defined transcription factors has raised fundamental questions regarding the epigenetic stability of the differentiated cell state. In addition, evidence has accumulated that distinct states of pluripotency can interconvert through the modulation of both cell-intrinsic and exogenous factors. To fully realize the potential of in vitro reprogrammed cells, we need to understand the molecular and epigenetic determinants that convert one cell type into another. Here we review recent advances in this rapidly moving field and emphasize unresolved and controversial questions.

Introduction

Epigenetic changes, such as modifications to DNA and histones, alter gene expression patterns and regulate cell identity (Goldberg et al., 2007). Global epigenetic states must be tightly regulated during development to allow for the proper transitions between cellular states. However, cell fates during development are neither restrictive nor irreversible. The generation of animals by the nuclear transplantation of somatic nuclei into eggs (Gurdon, 1962) demonstrated that indeed the epigenome of differentiated cells can be reset to a pluripotent state.

Derived from cells at various embryonic and postnatal stages, stem cells are characterized by self-renewal and the capacity for differentiation (Jaenisch and Young, 2008). Pluripotent cells have the ability to form all somatic lineages, and the first pluripotent cells were derived from a type of germline tumor, called teratocarcinoma. When explanted in tissue culture, the teratocarcinoma cells generated embryonal carcinoma cells, demonstrating that cancer cells can be reprogrammed to pluripotent cells (Hogan, 1976). The next breakthrough in the field came when researchers isolated embryonic stem cells (ESCs) from normal mouse embryos, creating a platform for the genetic engineering of animals (Evans and Kaufman, 1981). The generation of ESCs from human embryos came less than a decade later (Thomson et al., 1998), and this technology, combined with the direct reprogramming of somatic cells to pluripotent cells (Takahashi and Yamanaka, 2006), is now paving the way for "personalized" regenerative medicine (Hanna et al., 2007).

This Review focuses on mechanisms that control the transition of cells between different states of pluripotency and differentiation. We will emphasize new concepts and unresolved questions in mammalian systems while concentrating on three aspects of epigenetic reprogramming: (1) the molecular definition of different pluripotent states and strategies to convert one cell state into another; (2) molecular concepts of somatic cell reprogramming to pluripotency; and (3) direct transdifferentiation between somatic cell states.

Distinct Pluripotent Cells Derived during Development

Development proceeds from a state of totipotency, characteristic of the zygote and blastomeres during the early cleavage of the embryo, to cells that are restricted in their potential for development. It is from these later stages that pluripotent cells can be derived. At the 16-cell stage, the outer cells of the mouse embryo are allocated to two lineages: the trophoblast lineage, which will form part of the placenta; and the bipotential inner cell mass, which generates the epiblast and the hyphoblast. The epiblast and hyphoblast will form the embryo and the yolk sac, respectively. Cells of the epiblast lineage are termed pluripotent because they are the origin of all somatic cells and germline cells of the developing embryo. Primordial germ cells emerge at gastrulation and, in male embryos, give rise to spermatogonial stem cells. Pluripotent cells have been derived from all of these cell types by explanting the cells from embryos at different stages of development (Figure 1). As outlined below, the state of the donor cells, as well as the culture conditions, have a profound effect on the characteristics of the derived cells. We focus on pluripotent cells that have unrestricted developmental potential and thus can give rise to all cell types in the developing embryo or in the culture dish.

A. Embryonic Stem Cells

ESCs were the first pluripotent cells isolated from normal embryos. They were created by explanting the inner cell mass of the embryos from a strain of mice called "129" (Evans and Kaufman, 1981). Mouse ESCs recapitulate full developmental potential when injected into mouse blastocysts, contributing cells to the three germ layers and to the germline of chimeric animals. Consistent with their origin from the inner cell mass, ESCs express key pluripotency genes, such as *Oct4*, *Sox2*,
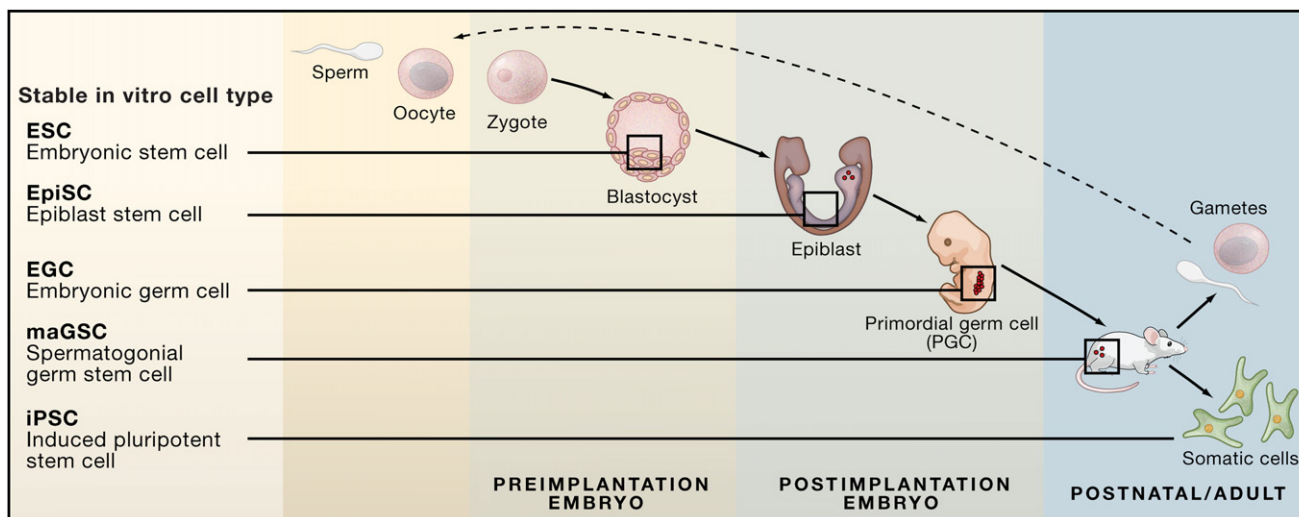
Figure 1. Developmental Origins of Pluripotent Stem Cells
Different types of pluripotent cells can be derived by explanting cells at various stages of early embryonic development. Induced pluripotent stem cells (iPSCs) are derived by direct reprogramming of somatic cells in vitro.

and *Nanog*, and they exist in a pre-X-inactivation state with both X chromosomes active in female cells (Nichols and Smith, 2009). However, distinct biological and molecular characteristics distinguish ESCs from their in vivo counterparts of the inner cell mass. For example, cells of the inner cell mass are not self-renewing, and they are characterized by a genome that is globally hypomethylated (Santos et al., 2002). In contrast, ESCs have unlimited proliferation potential, and their genome is highly methylated (Meissner et al., 2008).

Maintaining the pluripotent state of ESCs depends on key molecular signaling pathways (Table 1). Initially, researchers established ESCs in the presence of fetal bovine serum and other undefined factors secreted from irradiated mouse embryonic feeder cells. Studies identified the leukemia inhibitory factor (LIF) as an important mediator that supports maintenance of the pluripotency of mouse ESCs by signaling predominantly through the Stat3 pathway (Ying et al., 2008). Cultivating the cells in defined medium containing bone morphogenetic protein 4 (BMP4) allowed propagation of ESCs in the absence of feeders and serum. BMP4 acts in concert with LIF and supports stabilization of mouse ESCs by inducing inhibitors of differentiation (Id) genes. LIF and small-molecule inhibitors (termed "2i") of protein kinases ERK1/2 and GSK3β, which stimulates the WNT pathway, can replace the serum and thus allow the maintenance of ESCs in fully defined medium without embryonic feeder cells (Ying et al., 2008).

A core set of transcription factors consisting of Oct4, Nanog, Sox2, and Tcf3 maintains the pluripotent state of ESCs (Boyer et al., 2005; Loh et al., 2006; Marson et al., 2008). Oct4 is expressed throughout early mammalian development and is essential for formation of the pluripotent inner cell mass and for maintaining ESCs (Nichols et al., 1998). Nanog is another important pluripotency regulator that is activated at the 8-cell stage. However, Nanog is also expressed later in a subset of inner cell mass cells and cooperates with other factors in X chromosome reactivation (Silva

et al., 2009). Oct4, Sox2, and Nanog induce and cross-regulate their own expression. Thus, Oct4-Sox2-Nanog constitutes a core transcriptional circuit wired in a feed-forward type of regulation (Figure 4D). These factors also coactivate redundant target genes and cooperate with secondary transcription factors that provide further stability to the ESC state (Chen et al., 2008).

B. Epiblast Stem Cells
Pluripotent cells can also be derived from the epiblast of the implanted embryo. The epiblast is a single layer of epithelial cells and originates from the inner cell mass after implantation of the embryo. Independent pluripotent lines, called "EpiSCs," were established by explanting the epiblast from embryonic day 5.5–7.5 of postimplantation mouse embryos in growth conditions supplemented with FGF2 (basic fibroblast growth factor) and Activin (Brons et al., 2007; Tesar et al., 2007). EpiSCs express pluripotency markers and are pluripotent by a number of criteria, such as multilineage differentiation into embryoid bodies and teratomas. EpiSCs display flat colony morphology and grow poorly as single-cell clones following treatment with the protease trypsin (i.e., single-cell dissociation by trypsinization). Although these cells are termed pluripotent, they have more limited developmental potential than ESCs; they are highly inefficient in generating chimeras, have already undergone X chromosome inactivation, and demonstrate heterogeneous expression of early lineage-commitment markers. In contrast to ESCs, the growth of EpiSCs depends on signaling by Activin, FGF2, ERK1/2, and TGF-β; their growth is inhibited by BMP4 but is independent of LIF/Stat3 activity (Table 1). Molecularly, EpiSCs share a gene expression program reminiscent of the postimplantation epiblast, rather than that of the inner cell mass. EpiSCs exhibit reduced expression levels of the transcription factors Nanog, Rex1, and Klf, but differentiation markers, such as FGF5 and major histocompatibility complex (MHC) class I, are readily expressed in these cells (Tesar et al., 2007).

Table 1. Characteristics of Mouse Pluripotent States

Characteristic	Naive Mouse Pluripotent State	Primed Mouse Pluripotent State
Alternative names	Preimplantation inner cell mass-like; mouse ESC-like	Post-implantation epiblast-like; mouse epiblast stem cell-like
Stem cell types	Embryonic stem cells (ESCs); embryonic germ cells (EGCs); spermatogonial germ stem cell (maGSC); induced pluripotent stem cell (iPSC)	Epiblast stem cell (EpiSCs)
Developmental Potential		
Embryonic body formation	Yes	Yes
Teratoma	Yes	Yes
Blastocyst chimera	Yes	No
Susceptibility for primordial germ cell specification	Low	High
Growth properties		
Single-cell clonogenicity	High	Low
Ability to grow independent of feeders and/or in defined medium	Yes	Yes
Doubling time in vitro	10–14 hr	14–16 hr
Morphology	Domed	Flattened
Regulation by exogenous signaling pathways		
Positive regulators of the state	LIF/Stat3 BMP4 WNT IGF	TGF-β Activin FGF2 ERK1/2 WNT IGF
Negative regulators of the state	TGF-β Activin FGF2 ERK1/2	BMP4
Gene and marker expression signatures		
SSEA1, alkaline phosphatase	+	+
TRA1-60, TRA1-81, SSEA3/4	−	−
Oct4, Sox2	+	+
Nanog, Klf2, Klf4, Rex1, Stella	High ++	Low/absent +/−
Lineage specification markers (e.g., FGF5, Blimp1, Cer1)	Absent	Positive with heterogeneous expression pattern
MHC class I	Nearly absent	Present
Oscillatory gene patterns	Nanog, Rex1	Blimp1
Epigenetic state		
Oct4 enhancer activity	Distal element	Proximal element
XX status	XaXa	XaXi

C. Embryonic Germ Cells and Adult Germline Stem Cells

Pluripotent cells have also been derived from cells of the germline lineage. When cultivated in adequate growth conditions, primordial germ cells isolated from embryonic day 8.5 embryos generated ES-like cells, termed embryonic germ cells (Surani, 1999). These cells were pluripotent, capable of generating teratomas and chimeras. Interestingly, in contrast to ESCs, when embryonic germ cells are fused with somatic cells, they can induce demethylation of the somatic imprinted genes, reflecting an enzymatic activity that resets the imprints during the development of primordial germ cells (Surani, 1999). Embryonic germ cells have also been derived from human fetuses, but their characteristics are not as well defined (Shamblott et al., 1998).

Spermatogonial stem cells from newborn and adult male gonads also generate ES-like cells, although at very low efficiency and after an extended time when these cells are explanted in vitro. Called male germ stem cells, these ES-like cells can be propagated in serum and LIF (Kanatsu-Shinohara et al., 2004). They carry a male-specific imprinting pattern, and they can induce teratomas and contribute to chimeras. Nevertheless, embryonic germ cells and male germ stem cells are pluripotent and share defining features with ESCs. Adult germline stem cells

was highlighted by the failure to derive naive ESCs from certain mouse strains, other rodents, and other species, including humans.

have also been isolated from adult human testicular tissues (Conrad et al., 2008), but the identity of these cells has been questioned (Ko et al., 2010).

Molecular Definition of Distinct Pluripotent States

Much work has been devoted to establish molecular signatures that define pluripotency. As outlined above, ESCs are derived from the inner cell mass whereas EpiSCs are derived from the epiblast. Both display distinct biological characteristics that are reminiscent of their developmental origin, with some adaptations that occur upon explantation in vitro and during growth selection. It is becoming increasingly evident that different pluripotent cell types can be classified into two fundamentally distinct states of pluripotency: (1) the inner cell mass-like (ICM-like) pluripotent state, which is typical for ESCs derived from the inner mass cells, as well as embryonic germ cells and male germ stem cells derived from primordial germ cells or spermatogonial stem cells; and (2) the postimplantation epiblast-like state, characteristic of EpiSCs.

The two pluripotent states, which are stabilized in vitro by different growth conditions, exhibit distinct molecular signatures and in vitro growth properties. In addition, the two states depend on signaling pathways that often antagonize each other (Table 1). BMP4 signaling stabilizes ESCs in conjunction with LIF, but it induces differentiation of EpiSCs; TGF-β and FGF2 support renewal of EpiSCs, but they induce differentiation of ESCs; and, EpiSCs require signaling of the ERK1/2 pathway whereas the self-renewal of mouse ESCs is enhanced by inhibition of ERK1/2 signaling.

Nichols and Smith (2009) designated the ICM-like state of ESCs as the "naive" state and that of the epiblast-derived EpiSCs as the "primed" pluripotent state. This definition implies that the primed state is prone to differentiate whereas the naive ESCs correspond to a more immature state of pluripotency. This difference is, for example, reflected in the state of X chromosome inactivation. Naive ESCs are in the preinactivation state with both X chromosomes active (XaXa) in female cells; in contrast, primed EpiSCs have already undergone X chromosome inactivation. In addition, primed EpiSCs are poised to generate precursors of primordial germ cells in response to BMP4 signaling. This is consistent with the similarity of EpiSCs to the postimplantation epiblast state whereas BMP4 sustains the undifferentiated state of ESCs (Tesar et al., 2007).

A recent report described an EpiSC-like cell type, termed FAB-SCs (i.e., FGF2, Activin, and BIO-derived stem cells) (Chou et al., 2008), which share expression markers with EpiSCs but are unable to differentiate. These results lead to the suggestion that FAB-SCs may represent a novel state of pluripotency. However, the nature and relevance of these cells is unknown, given that the FAB-SCs are not pluripotent and that they lack differentiation potential unless exposed to LIF/BMP4.

Stability of and Transitions between Pluripotent States

The molecular and biological definitions of naive and primed pluripotent cells raised the question of whether these epigenetic states are derived from independent coexisting progenitors or whether they reflect distinct pluripotent states characteristic of embryonic cells at successive developmental stages. This issue

Naive ESCs are readily isolated from only a limited number of "permissive" mouse strains, such as 129, C57BL/6, and BALB/C. Explanted blastocysts from rats and "nonpermissive" mouse strains, such as nonobese diabetic (NOD) mice, yielded exclusively EpiSC-like pluripotent cells (Buehr et al., 2008; Hanna et al., 2009a). These results mistakenly suggested that the primed state is the only or "default" state of pluripotency in "nonpermissive" donor strains or species. The restriction of isolating naive cells from only particular strains or species is odd and difficult to explain, but most importantly, it has triggered researchers to determine how the two pluripotent states are related and whether it would be possible to switch one state into the other.

Both in vitro and in vivo experiments have recently defined the relationships between the two distinct types of pluripotent states. Whereas naive pluripotent cells can differentiate into a primed EpiSC-like state in vitro by promoting the signaling of TGF-β, Activin, and FGF2, EpiSCs can epigenetically revert back to naive pluripotency by a variety of genetic manipulations and culture conditions (Figure 2) (Bao et al., 2009; Guo et al., 2009; Hanna et al., 2009a). Exposure to LIF/Stat3 signaling reverts EpiSCs from permissive strains to naive mouse ESC-like cells. This conversion can be boosted by cultivating the cells in LIF and "2i" conditions (2i: GSK3β inhibitor and ERK1/2 inhibitor or Kenpaullone) or by the transient expression of pluripotency factors, including Klf4, Klf2, Nanog, or c-Myc, with different latencies and efficiencies (Figure 2A). These observations suggest that the naive and primed pluripotent states in the permissive genetic background are interconvertible and can be stabilized by appropriate culture conditions.

In contrast to deriving cells from blastocysts of the permissive 129 strain, when NOD mouse blastocysts were explanted under standard ESC conditions in LIF and on feeders, they generated only EpiSC-like cells, not ESCs (Hanna et al., 2009a). This is consistent with the observation that LIF/Stat3 signaling alone is not sufficient to generate ESCs from NOD mouse blastocysts or to facilitate epigenetic reversion of NOD EpiSCs. However, cultivation of the NOD embryos in LIF and 2i conditions generated naive ESCs that were dependent on the continuous presence of additional exogenous factors that promote naive pluripotency (Figure 2B). Hence, in contrast to ESCs generated from the 129 strain, which can be maintained in LIF and serum, stabilization of pluripotent naive cells from the NOD strain requires additional factors in the medium. This property underscores the inherent "bistability" (Figure 5) of pluripotency that allows the naive and primed states to interconvert. Notably, in both mice and humans, naive and primed states have *not* been observed to coexist stably in the same culture conditions. Thus, the transitions between primed and naive states are distinct from cell-to-cell differences that coexist during the culturing of mammalian ESCs (Figure 5), including oscillation in the expression of pluripotency markers Nanog and Stella (Hayashi et al., 2008).

In summary, pluripotent cells isolated in different mammalian species can exist as distinct pluripotent states, known as the naive and the primed states, and specific extrinsic and intrinsic
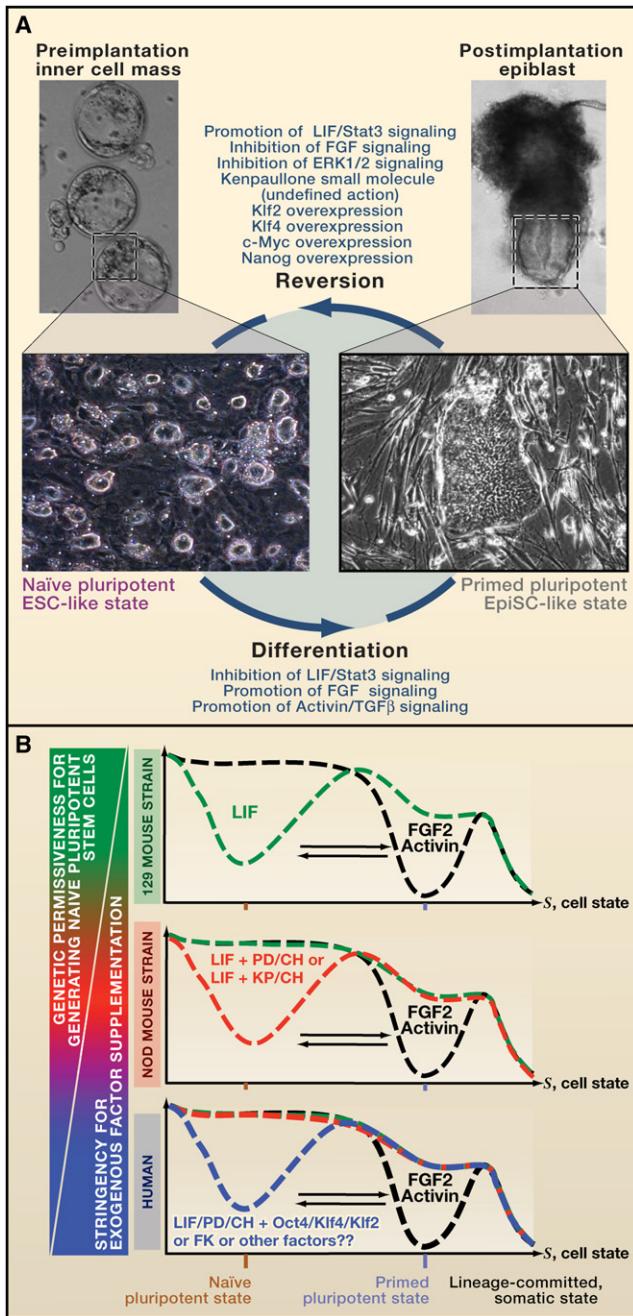
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Figure 2. Transitions between Naive and Primed Pluripotent States
(A) Naive and primed cell types represent distinct gene expression states, corresponding to those observed in the preimplantation inner cell mass and postimplantation epiblast, respectively. To stabilize the primed state in vitro, supplementation with basic fibroblast growth factor (FGF2) and Activin support the core transcriptional circuitry governing this state. In contrast, the naive state has distinct active signaling pathways and thus requires different exogenous signals to induce and stabilize this state in vitro. Depending on the genetic background, combinations of these perturbations promote reversion to naive pluripotency and differentiation into the primed pluripotent state. (B) In this illustration, stabilization of the naive and primed states in vitro by leukemia inhibitory factor (LIF) and FGF2/Activin signaling, respectively, is depicted as creating a well in a landscape. These factors can promote or antagonize interconversion between the states. LIF signaling promotes transfer of primed cells to the naive state and continuously prevents differentiation
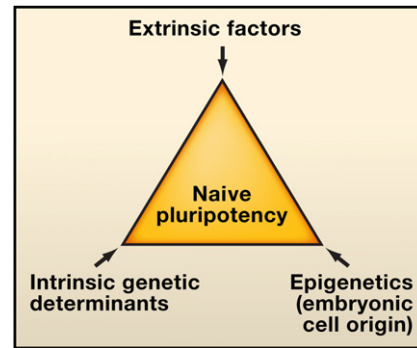

Figure 3. Three Parameters that Impact Pluripotency
Exogenous factors, genetic background, and epigenetics of the tissue origin of cells.

factors can induce transitions between the states (Figure 3). The genetic background of the permissive 129 mouse strain has the least requirements for naive pluripotency (LIF in Figure 2B) whereas the nonpermissive NOD background (or rat cells) requires additional factors that support the naive pluripotent state, such as the 2i conditions. The intrinsic genetic determinants of human cells are even less permissive because additional extrinsic factors are necessary for stabilizing the naive state (see next section). If the minimal growth requirements are not maintained, the cells lose the naive pluripotent state and may differentiate or convert into the primed state. In addition, the tissue origin of the cells may play a key role in establishing a particular state of pluripotency (Figure 3). For example, induced pluripotent stem cells (iPSCs) and ESCs derived from the NOD mouse strain require supplementation of 2i in addition to LIF whereas NOD germline stem cells derived from adult male gonads need only LIF for stabilization of the naive pluripotent state (Ohta et al., 2009). The molecular basis underlying this observation is still undefined.

Human Embryonic Stem Cells
Like mouse ESCs, human ESCs are isolated from explanted blastocysts before implantation (Thomson et al., 1998). Nevertheless, human ESCs share multiple defining features with mouse EpiSCs rather than mouse ESCs. These characteristics include flat morphology, dependence on FGF2/Activin signaling, propensity for X chromosome inactivation, and reduced tolerance to single-cell dissociation by trypsinization (Table 1). These molecular and biological similarities with mouse EpiSCs suggest

of the naive state. Shielding FGF2/Activin signaling can further enhance conversion into naive cells, as this signaling pathway is inhibitory to the naive state. In the nonpermissive NOD mouse strain, LIF signaling alone is not sufficient to maintain the naive state in vitro, as pluripotent cells derived from the inner cell mass or by in vitro reprogramming assume a primed state that can be stabilized by FGF2/Activin. However, the modulation of additional pathways, which are known to promote the naive state and prevent differentiation, allowed derivation of naive pluripotent cells from the NOD strain. This was achieved by altering the culture conditions, either with small molecules or by adding LIF, increasing Wnt signaling (small molecule "CH"), and inhibiting ERK1/2 (small molecule "PD"). The human genetic background is less permissive, as it required modulation of additional signaling pathways to induce the naive state.

that human ESCs correspond, at least partially, to the primed pluripotent state rather than to the naive state of mouse ESCs.

For pluripotent cells from mice, isolation and culture conditions, as well as genetic differences of the blastocysts from "permissive" and "nonpermissive" mouse strains, profoundly affect the cell's state of pluripotency (Figure 2). In fact, the interconversion between pluripotent states of mouse cells (Hanna et al., 2009a) raised the possibility that the appropriate culture conditions would allow isolation of naive human stem cells. Consistent with this possibility is that the isolation and culture conditions of human ESCs profoundly influence the state of X chromosome inactivation. Conventional human ESCs, isolated at atmospheric oxygen concentrations, have undergone X chromosome inactivation (XiXa), similar to mouse EpiSCs (Silva et al., 2008). However, human ESCs isolated and propagated under physiological oxygen conditions (5% O_2), display a pre-X-inactivation status (XaXa) and, similar to mouse ESCs, initiate random inactivation upon differentiation (Lengner et al., 2010). This result argues that human blastocysts contain pre-X-inactivation cells and that oxidative stress upon culture of the embryos in atmospheric oxygen accelerates precocious X inactivation. Thus, suboptimal culture conditions interfere with the in vitro capturing of the more immature XaXa state of human inner cell mass cells.

These results suggest that the proper conditions have not yet been devised for isolating human pluripotent cells with features similar to mouse naive ESCs or for converting human ESCs and iPSCs (which are similar to NOD EpiSCs) to a naive pluripotent state. Indeed, human ESCs and iPSCs were recently converted to a naive pluripotent state by propagating the cells in LIF and 2i conditions (i.e., the addition of PD/CH inhibitors) and simultaneously overexpressing Oct4/Klf4 or addition of Forskolin to the medium (Figure 2B) (Hanna et al., 2010). These naive human ESCs and iPSCs corresponded to naive mouse ESCs by several criteria. They reactivated the inactive X chromosome, resulting in a pre-X-inactivation status (XaXa in female cells). They exhibited a high efficiency of single-cell cloning and were dependent on LIF/Stat3 signaling instead of FGF/Activin signaling. They could routinely be passaged as single cells and showed a gene expression pattern that resembled that of naive mouse ESCs. Furthermore, the XaXa state of these naive ESCs was observed when the cells were cultured under 20% oxygen in contrast to the labile pre-X-inactivation state of primed ESCs isolated from human embryos under physiological oxygen conditions (Lengner et al., 2010).

These results provide the first direct evidence for a validated naive state of pluripotency in humans that is highly similar to that of mouse ESCs but that conventional isolation conditions of ESCs failed to capture. Nevertheless, this naive state could be maintained only for limited passages (Hanna et al., 2010) even when cells were cultivated in the presence of Forskolin, which allows propagation of human embryonic germ cells (Shamblott et al., 1998). Thus, a crucial challenge will be to define growth conditions that allow robust, long-term maintenance of the "naïve ground state" in genetically unmodified human cells.

Conventional human ESCs are impractical for use in disease-related research because of the laborious culture conditions required for their maintenance, their low efficiencies of gene-targeting by homologous recombination, and the dramatic heterogeneity in differentiation potential among different human ESC lines (Osafune et al., 2008). Recently, novel gene targeting strategies using zinc-finger nucleases have overcome some of these limitations (Hockemeyer et al., 2009; Zou et al., 2009). Nevertheless, it will be interesting to determine whether genetic manipulation by homologous recombination is as efficient in the new naive human pluripotent cells as in mouse ESCs.

A recent report (Buecker et al., 2010) described a cell state termed "hLR5" generated by the ectopic expression of the five different transcription factors Oct4, Sox2, c-Myc, Klf4, and Nanog in human fibroblasts. The cells were amenable to gene targeting of the HPRT (hypoxanthine-guanine phosphoribosyltransferase) locus by homologous recombination. However, the targeting efficiency at this particular locus was 2-fold lower than that reported previously for conventional human ESCs (Zwaka and Thomson, 2003). Although designated as "murine-ESC-like cells" and iPSCs, the hLR5 cells were not pluripotent because they failed to activate the endogenous pluripotency genes, lacked any informative expression of pluripotency markers, and were unable to differentiate (Buecker et al., 2010). Therefore, these cells likely represent transformed or partially reprogrammed cells (Mikkelsen et al., 2008).

Direct Reprogramming of Somatic Cells to Pluripotency

Epigenetic reprogramming of somatic cells to a pluripotent state has been achieved by nuclear transplantation, cell fusion, and direct reprogramming by expression of transcription factors. We focus here on unresolved or controversial issues of direct reprogramming because nuclear transfer and cell fusion approaches have been extensively reviewed elsewhere (Jaenisch and Young, 2008; Yamanaka and Blau, 2010).

Takahashi and Yamanaka (2006) achieved a breakthrough in this field by demonstrating that the overexpression of four transcription factors, Oct4, Sox2, Klf4, and c-Myc, can convert somatic fibroblasts to pluripotent cells (iPSCs) that can contribute to the germline in chimeric mice (Okita et al., 2007; Wernig et al., 2007). These factors initiate poorly defined events that lead eventually to the reactivation of the endogenous pluripotency genes encoding Oct4, Nanog, and Sox2 and to the activation of the autoregulatory loop that maintains the pluripotent state independent of the transgenes (Figure 4D). Murine iPSCs share all defining features with naive mouse ESCs, including expression of pluripotency markers, reactivation of both X chromosomes, and the ability to generate chimeras and all-iPSC mice following tetraploid complementation (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009).

Reprogramming can be induced not only by Oct4, Sox2, Klf4, and c-Myc but also by alternative combinations that employ Nanog, Lin28, ESRRB, NR5A2, and other genes that promote the establishment of the core transcriptional circuitry of stem cells (Ichida et al., 2009; Yu et al., 2007). The redundancy and cooperative action of reprogramming factors in establishing iPSCs likely results from the highly interconnected DNA-binding properties of the pluripotency factors that form the regulatory circuitry and that allow the transduced transcription factors to reestablish the autoregulatory and feed-forward loops by activating the endogenous pluripotency genes (Boyer et al., 2005).

Furthermore, given that the transcriptional circuit of pluripotency is positively or negatively regulated by cytokines or small molecules added to the medium that activate or suppress specific signaling cascades, it is not surprising that the same pathways influence the establishment and maintenance of both ESCs and iPSCs. For example, WNT signaling upregulates c-Myc expression and promotes naive pluripotency, but it also increases iPSC formation (Marson et al., 2008). Furthermore, the effect a small molecule has on iPSC formation depends on whether the small molecule affects a pathway that also stabilizes the given pluripotent state. For example, conventional human ESCs rely on TGF-β signaling, and as expected, addition of TGF-β inhibitors impedes human iPSC formation because these inhibitors are detrimental to the primed pluripotent state (Maherali and Hochedlinger, 2009). In contrast, inhibitors of this pathway enhance mouse iPSC formation because TGF-β destabilizes the naive pluripotent state of mouse ESCs.

Reprogramming of somatic cells to pluripotency is accompanied by extensive remodeling of epigenetic marks, including DNA demethylation of key pluripotency genes such as *Oct4* and *Nanog*. In somatic cells, the promoters of *Oct4* and *Nanog* are highly methylated, reflecting their transcriptionally repressed state. The formation of iPSCs involves activation of these genes, and their demethylation is widely used to monitor successful reprogramming (Mikkelsen et al., 2008). In principle, demethylation can occur by a passive mechanism, such as the inhibition of DNA methyltransferase 1 (Dnmt1) during DNA replication, or by an active mechanism in which the methylated base is removed from nonreplicating DNA.

Active demethylation is convincingly demonstrated only in cells that do not replicate their DNA. For example, global demethylation of the paternal genome occurs after fertilization and prior to the onset of DNA replication. In addition, it has been suggested that global demethylation of the genome in primordial germ cells may also occur by an active mechanism involving methyl-cytosine deamination by AID (activation-induced cytidine deaminase) (Popp et al., 2010). However, the expression levels of the enzymes in the AID pathway are low in primordial germ cells and the zygote, and a recent report suggests that enzymes of the base excision repair pathway rather than AID may catalyze this global demethylation in both types of cells (Hajkova et al., 2010).

Because the pluripotent state is dominant over the somatic state, one strategy for reprogramming somatic cells is to fuse them with ESCs. It has been suggested that AID is involved in active demethylation of the somatic *Oct4* gene at 48 to 72 hr after fusion but before the onset of DNA replication (Bhutani et al., 2009). Although the *Oct4* promoter was found to be partially demethylated, the only evidence for reprogramming was a residual level of *Oct4* expression (100-fold lower than the levels in ESCs). This is reminiscent of partially reprogrammed cells that express low levels of the endogenous *Oct4* gene without being demethylated (Mikkelsen et al., 2008). Although this fusion approach may allow the dissection of the initial events taking place at the beginning of direct reprogramming, it is still unknown whether demethylation of the *Oct4* gene detected in the early ES-somatic cell heterokaryons, combined with the very low levels of *Oct4* expression, do actually reflect events relevant for iPSC formation. A previous study, which fused B cells with ESCs, concluded that high *Oct4* expression occurred only later after DNA replication and cell proliferation of the hybrid cells had occurred (Pereira et al., 2008). Cell proliferation and DNA replication precede iPSC formation. Thus, it is likely that the activation of the somatic pluripotency genes in somatic cells fused with ESCs occurs, at least in part, by a passive mechanism involving inhibition of Dnmt1. This is consistent with the observation that inhibition of Dnmt1 increases the efficiency of reprogramming (Mikkelsen et al., 2008). Clearly, much remains to be learned about the nature of the biochemical machinery involved in facilitating chromatin modification during iPSC formation (Singhal et al., 2010).

Dynamics and Heterogeneity of Direct Reprogramming

Even when different strategies are used to induce reprogramming, a consistent finding is that only a small fraction of donor cells will become iPSCs, with the first ones appearing no earlier than 5–10 days after expression of the reprogramming factors (Jaenisch and Young, 2008). Thus, the reprogramming efficiency appears to be quite low. Parameters thought to restrict the reprogramming efficiency include the possibility that only rare somatic stem cells may be susceptible to reprogramming or that activation of additional genes by insertional mutagenesis might be crucial. However, iPSCs from mouse (Hanna et al., 2008) and human (Loh et al., 2010; Seki et al., 2010; Staerk et al., 2010) were recently generated from lymphocytes with high efficiency, providing conclusive evidence that terminally differentiated cells can be reprogrammed to pluripotency. In contrast to previous claims (Eminli et al., 2009), control of in vitro cell plating efficiency, growth expansion, and gene delivery demonstrated that iPSCs need not preferentially arise from less differentiated somatic cells (Hanna et al., 2009a, 2009b) and that differentiation progression is not accompanied with an intrinsic decrease in reprogramming amenability. Furthermore, the generation of genetically unmodified iPSCs argues that insertional mutagenesis is not an essential step in the process (Okita et al., 2008; Stadtfeld et al., 2008).

Determination of "reprogramming efficiency" and kinetics of in vitro reprogramming is typically based upon the appearance of iPSC colonies at a single and arbitrarily chosen time point (typically 3–6 weeks) after polyclonal somatic cell populations are transduced with reprogramming factors and plated. Efficiency is calculated by the fraction of reprogrammed cells (indicated by expression of pluripotency markers or reporter genes) divided by the total number of plated cells.

Although such measurements can be informative, they provide limited mechanistic insights because it is difficult to quantify the extensive expansion and/or apoptosis of the original cells, which can be an immediate technical consequence of factor expression. The mouse embryo fibroblasts used in most studies represent a heterogeneous population of cells that are highly variable in their predisposition for immortalization, senescence, and tolerance to ectopic expression of exogenous factors (such as Klf4 and c-Myc), which affect the survival of single cells. Further, pluripotency markers, such as alkaline phosphatase and SSEA1 or SSEA4, are unspecific, and only a small fraction of cells with these markers will develop later

into genuine iPSCs (Jaenisch and Young, 2008). Moreover, the possibility that individual iPSC colonies may be sister clones from the same infected cell has also been ignored hitherto. Thus, although p53 inhibition was reported to variably increase reprogramming efficiency by 4- to 100-fold (Krizhanovsky and Lowe, 2009), this variation may be due to technical parameters of somatic survival, senescence, and apoptosis after expression of genes, such as *c-Myc* and *Klf4*.

Because viral infections are usually used to induce reprogramming, heterogeneity in the expression of the reprogramming factors may be quite large, and thus, transgenic approaches were recently developed to overcome this experimental factor. Transgenic mice carrying a defined set of drug-inducible (doxycycline, DOX) proviruses, transposons, or a polycistronic construct encoding the reprogramming factors inserted into a single expression locus generated "secondary" somatic cells that could be reprogrammed by mere addition of DOX to the medium (Hanna et al., 2008; Woltjen et al., 2009). The use of these secondary cells avoided the need for a new virus infection to induce reprogramming and increased the reprogramming efficiency. However, still no more than 5%–10% of the cells eventually became reprogrammed, with a latency of 7–10 days before the first iPSCs appeared. This delay and the low efficiency are consistent with stochastic mechanisms involved in inducing reprogramming (Hanna et al., 2009b). Moreover, although the secondary cells after the initiation phases are genetically homogenous, cells at intermediate stages of reprogramming represent highly heterogeneous cell populations, and only a minority of these cells will ever become iPSCs. Thus, gene expression or epigenetic analyses of such heterogeneous cell populations may not be informative for characterizing those few cells that eventually form an iPSC.

Long-term analyses of clonal cell populations derived from Pro/Pre-B cells were conducted to determine the dynamics of reprogramming and to assess the fraction of donor cells that are susceptible to reprogramming (Hanna et al., 2009b). In comparison to mouse embryonic fibroblasts, Pro/Pre-B cells have a higher cloning efficiency for single cells and better tolerance of reprogramming factors. Plus, these cells represent a well-defined lineage-committed population with the rearrangement of the IgH locus allowing for the unambiguous retrospective identification of the donor cell.

In these experiments, reprogramming efficiency was defined as the *potential* of a donor cell to generate an iPSC daughter at some point. This experiment demonstrated that, with a balanced reprogramming factor stoichiometry, nearly *every* cell was able to generate iPSCs. These results argue that differentiation does not restrict the ability of somatic cells to be reprogrammed although the efficiency of a given daughter cell to become an iPSC is exceedingly small (Figure 4A). The kinetics of reprogramming displayed a broad distribution for the time before iPSCs appear, spanning 2–18 weeks, consistent with the notion that the process involves stochastic and rate-limiting epigenetic event(s).

Additional inhibition of the p53/p21 pathway or ectopic expression of Lin28, while controlling for the same growth conditions, accelerated conversion into iPSCs and was directly proportional to the increase in cell division rate. Thus, p53 inhibition did not increase the fraction of cells that could be reprogrammed but rather accelerated the formation of iPSCs in time, which appeared after a similar number of cell divisions. In contrast, ectopic expression of Nanog accelerated reprogramming in a manner that was independent of the rate of cell division (Figure 4B).

These observations suggest that the cell cycle is a key parameter in iPSC generation and that reprogramming may be driven in a mode that is either dependent or predominately independent of cell division (Hanna et al., 2009b). Two possibilities could explain why increased cell proliferation accelerates the kinetics of iPSC formation. Accelerated cell division could amplify the number of target cells in which each daughter cell has an independent probability of becoming an iPSC, or DNA replication may be the prerequisite for permitting the epigenetic changes, such as DNA and histone modifications, to occur that allow the transitions to pluripotency.

Several lines of evidence suggest that an important rate-limiting epigenetic event for reprogramming may be the reactivation of the key endogenous, autoregulatory circuitry that maintains the ESC state (Jaenisch and Young, 2008). The inability to generate iPSCs from somatic cells in which Nanog is disrupted (Silva et al., 2009) and the observation that ectopic expression of Nanog induces iPSCs in fewer cell divisions (Hanna et al., 2009b) suggest that activation of the endogenous *Nanog* gene is a required event in the establishment of pluripotency. In addition, a recent gene expression analysis of secondary populations of mouse embryonic fibroblasts indicated that irreversible commitment to reprogramming coincides with endogenous activation of *Nanog* (Samavarchi-Tehrani et al., 2010). Further, partially reprogrammed cell lines may represent stable intermediate stages in the reprogramming process that depend on the continuous expression of exogenous reprogramming factors (Mikkelsen et al., 2008; Sridharan et al., 2009). However, these cells can be induced to give rise to fully reprogrammed iPSCs upon additional manipulations that lead to the activation of the autoregulatory circuitry. Although these observations indicate that the reactivation of the endogenous autoregulatory circuitry is important and that the process is accompanied by many epigenetic changes, it is still unknown how many of these required epigenetic events are rate limiting for reprogramming.

Modeling Direct Reprogramming to Pluripotency

The transitions between lineage-committed and pluripotent cell states can be tracked quantitatively in terms of the fraction of cells in a given culture environment that change state over time. Mathematical modeling using experimental data of transition rates is consistent with the conclusion that a *single* epigenetic event is rate limiting. Both simulations and experiments indicated a single peak in the reprogramming latency distribution when reprogramming was sampled daily over 14 days in polyclonal populations of mouse embryonic fibroblasts (Smith et al., 2010) and every week over an extended 18 week culture in clonal pre-B cell and monocytes populations (Hanna et al., 2009b).

In these simulations, when somatic cells express key reprogramming transcription factors, they can transition to the iPSC state with a particular probability (Figure 5). This probability
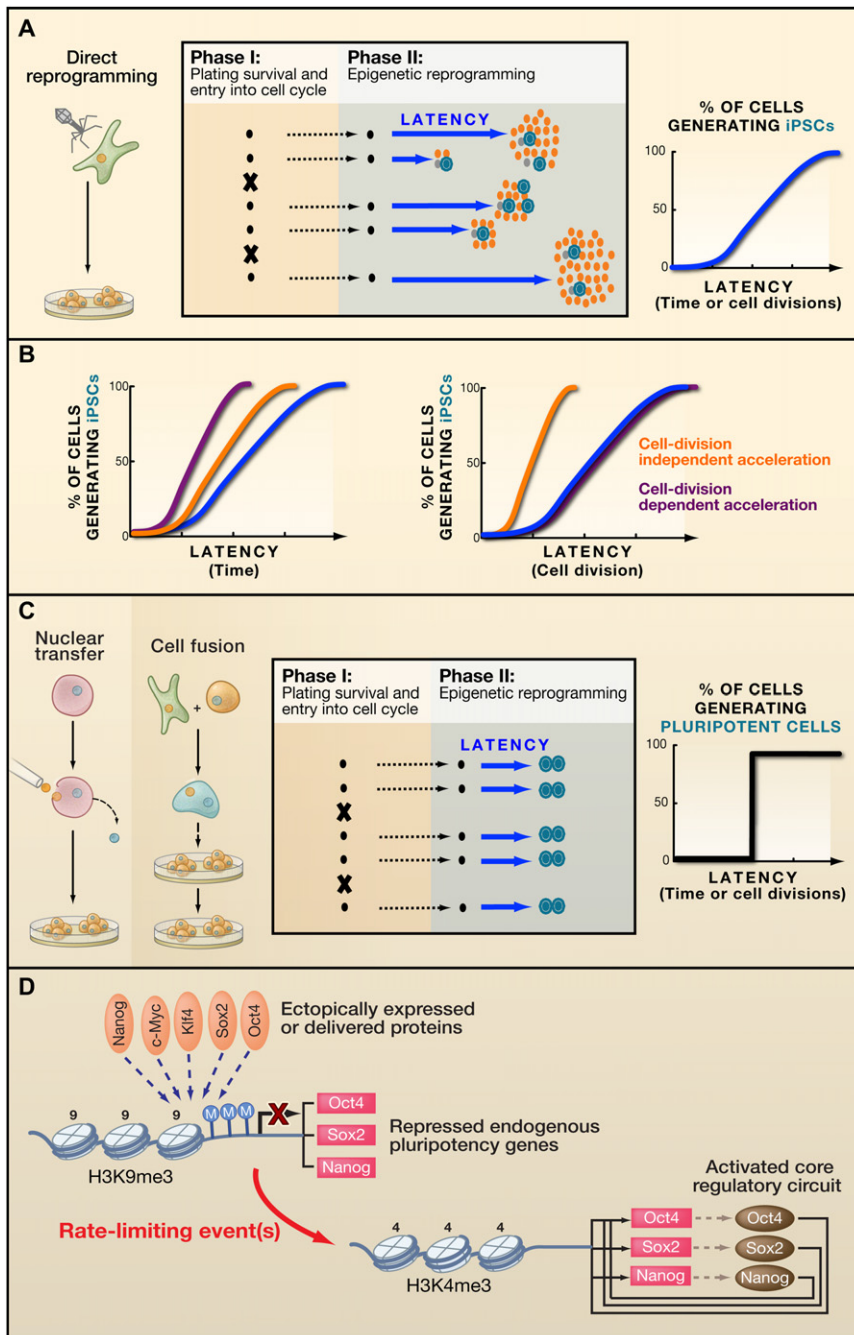
(A) In direct reprogramming, the progression of clonal populations to a reprogrammed state first involves an initial technical phase (I), which depends on the survival of plated cells and their entry into the cell cycle. Once cell division occurs, the most critical phase begins. During this second phase (II), direct reprogramming involves a stochastic event because clonal populations do not give rise to iPSCs at the same time after phase I. This variation in latency is represented by the blue line.
(B) Phase II can be accelerated by two mechanisms involving cell division (purple) or mechanisms independent of cell division (orange).
(C) As with direct reprogramming, the progression of reprogramming by nuclear transfer and cell fusion involves two phases. However, compared to direct reprogramming, much less heterogeneity is observed with nuclear transfer and cell fusion. For one, partially reprogrammed lines are not observed with nuclear transfer or fusion, and the reprogramming is hypothesized to progress in a more deterministic manner. This suggests that the current protocols of direct reprogramming are not optimal and may be accelerated by the supplementing with more factors to eliminate the stochasticity and achieve the deterministic conversion observed in fusion and nuclear transfer.
(D) One key rate-limiting step during direct reprogramming may be reactivation of the core pluripotency regulatory circuitry.

the experimentally observed kinetics of iPSC generation. Furthermore, simulations with multiple slow epigenetic events did *not* fit the experimental data better when the increase in model complexity was taken into account. Although this modeling suggests a single rate-limiting event, these results do not exclude the existence of other initial events that are not rate limiting but that occur with high probability and thus would not register in current models.

Detailed tracking of cell division in simulations indicates that the rate of cell division is a key parameter controlling the kinetics of reprogramming. Simulations of several different reprogramming conditions showed that ectopic expression of Nanog could accelerate reprogramming largely independent of changing

reflects the randomness in cell population size, arising from stochasticity in cell division times, fluctuations in the number of cells from apoptosis, and potential loss of iPSCs during passaging and cell culture. Such randomness gives rise to variability among genetically identical cells in populations undergoing reprogramming and is typically ignored in more conventional quantitative analyses that depend on a population-averaged doubling time (e.g., Figure 4B). Simulations incorporating a single rate-limiting stochastic event were able to recapitulate

the cell division rate or cell population size (Hanna et al., 2009b). Nanog is a component of the core autoregulatory loop controlling pluripotency (Figure 4D), but it is not in the original reprogramming cocktail. Thus, ectopic expression of Nanog could provide a key missing core component required for the rate-limiting event to occur. Although ectopic Nanog expression accelerates reprogramming, the process remains stochastic and inefficient even when Nanog, Oct4, and Sox2 proteins are supplied ectopically. Current modeling approaches combine
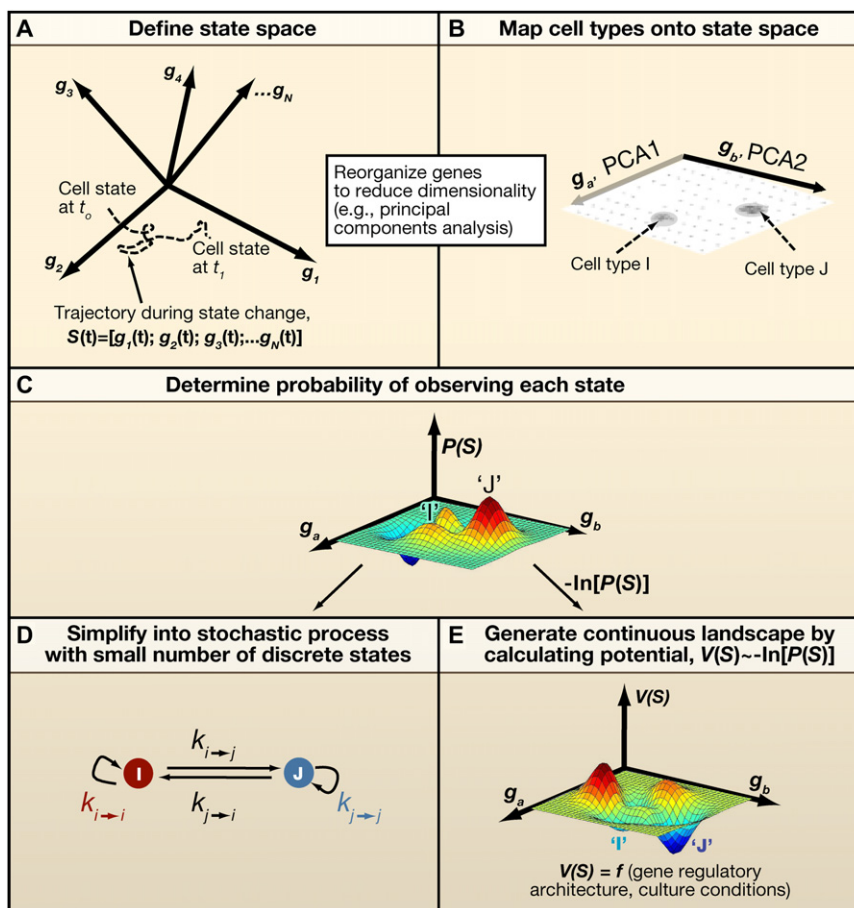
Figure 5. Developing Probabilistic Descriptions of Cell State

(A) First, a set of characteristics must be identified as being informative for the transition between two cell states of interest. These characteristics usually consist of levels of gene expression or levels of epigenetic methylation or acetylation marks on DNA or chromatin. For simplicity, we show here a state space generated by the level of N different genes, g_1 to g_N, where each arrow represents an axis corresponding to the expression level of that particular gene transcript ($N \sim 10^4$ for mammalian cells). A cell at any time t exists in a point in this space, and its state can change with time as a result of noise, reprogramming, or differentiation. One trajectory is plotted, and the vector Ŝ, which consists of gene expression levels changing with time, fully describes the cell state transition during this time.

(B) N can be reduced to a more manageable 2–3 dimensions through statistical techniques, such as principal component analysis (PCA). Shown here is a two-dimensional representation of the state space in (A), where each axis (g_a and g_b from PCA) is a linear combination of particular genes. Stable cells in vitro exist at particular points on this graph. By mapping quantitatively the gene expression levels of several single stable cells in the same space, regions with high densities of spots define observable cell types in vitro (as type "I" and "J").

(C) When a large sample of single cells is mapped in (B), the probability of occupying each point in this space can be calculated and plotted in a continuous fashion.

(D) The continuous probabilistic description of cell state in (C) can be simplified into a discrete representation, with a small number of discrete states that transition at particular rates, k. These rates, k, represent an average of all possible trajectories from region i to region j.

(E) Alternatively, a continuous description of the probabilities of staying at a particular point in state space can be represented as a landscape, by calculating $-\ln[P(Ŝ)]$ from (C) at each point. This landscape, V(Ŝ), represents "energy barriers" between transitions involving any two states and thus may provide a more thorough description of transitions than the description in (D).

and average several sources of biochemical noise (Figure 5) over the entire cell population and over the course of a week. Therefore, more detailed characterization through frequent single-cell tracking of gene expression, signaling activation, and the epigenetic state will probably provide more insights into the key parameters and required changes in gene expression controlling the kinetics of reprogramming.

In contrast to current protocols for direct reprogramming, nuclear transfer appears to reprogram the somatic nucleus in a single event, as suggested by the activation of Oct4 in the four-cell stage cloned embryo (Boiani et al., 2002). Moreover, only fully reprogrammed ES-like cells have been derived by cell fusion or following nuclear transfer, with no evidence for partially reprogrammed cells found in the iPSC approach (Hasegawa et al., 2010). This might suggest that reprogramming by nuclear transfer or cell fusion, in contrast to factor-induced reprogramming, may follow a more synchronized trajectory during reprogramming and progresses in a deterministic pattern (Figure 4C). It is important to devise approaches that could achieve synchronized and deterministic reprogramming for direct in vitro reprogramming as well. For the interested reader, we summarize in the next section approaches that are being used to model cell state transitions.

Emerging Quantitative Models of Cell State Transitions

Various approaches are being used to model transitions between different cell states. Depending on the differentiation pathway or the transitions between two states of interest, one may select a particular set of characteristics to describe quantitatively the cell state (Huang, 2009). Both gene expression and epigenetic changes on the timescale of days to weeks distinguish stable, functional cell types described by developmental biologists. Thus, the cell state can consequently be parameterized as a vector of molecular characteristics, Ŝ, which can be either a set of gene expression levels (Figure 5, $Ŝ = [g_1; g_2; g_3; \ldots g_N]$) or a set of epigenetic marks, such as DNA methylation and histone acetylation (not shown in Figure 5). Although such molecular characteristics clearly can correlate with one another, they are not necessarily correlated in the same fashion (Lu et al., 2009). During differentiation or noisy gene expression in a particular culture condition, the cell state can vary in time, t (i.e., Ŝ is a function of time Ŝ(t)).

The species or genetic background defines the architecture of the state space, meaning that particular gene-gene relationships, interaction modalities, and integrating transfer functions are "hard-wired" by the genome. Such state space provides a means to organize quantitatively and visualize different states of a cell with a fixed genome. Often cells cluster in particular regions of the state space, and developmental biologists typically describe these regions as stable cell types that express particular markers. There also may be regions where no cells are found, corresponding to cell states that are somehow not stable for the given genome of the cell in the extracellular environment considered.

Using standard statistical techniques, dimensionality reduction can transform this space into lower dimensions and greatly simplify the system (Figure 5B). For example, using principal components analysis, cells of the mouse embryo from the 8-cell stage to the blastocyst were mapped to two dimensions, where each dimension was a linear combination of genes g_1 to g_n (Tang et al., 2010). We can assign probabilities to each point in state space if we assume that we have reasonably sampled the entire space considered (Figure 5C). This continuous probability space can also be simplified further into a discrete number of observed states with particular kinetics for their transitions, allowing easier analysis with fewer variables (Figure 5D).

This probabilistic framework allows us to predict transition rates between two states when particular parameters are perturbed. Parameters incorporated in this framework include the genetic background, species differences, expression of ectopic transcription factors, culture conditions, and biochemical noise. Sources of biochemical noise among genetically identical cells include transcriptional noise in factor expression, biochemical noise in signaling processes, and biochemical noise in epigenetic modifications (Raj and van Oudenaarden, 2008). For example, noise in signaling pathways could arise as a consequence of the inherent stochastic nature of molecular binding events of ligands to receptors, receptors to secondary messengers, or secondary messengers to transcription factors. In addition, variable cell-cell contact in juxtacrine signaling can also create noise in signaling pathways. Finally, biochemical noise can occur when epigenetic modifiers bind to particular genomic loci.

Whereas a probabilistic framework can comprehensively characterize transitions among cell states in many contexts, generating a "landscape" has also been a popular way to summarize all possible transitions that a single cell can make in a particular culture condition. Such summaries enable predictions on transition rates when several parameters are perturbed over a continuous state space. For example, quantitative models for directed evolution (Bloom et al., 2005) have already described noisy searches in configured landscape and in sequence space instead of state space. Similar models are helping to guide experimentation by attempting to describe cellular differentiation states in terms of gene expression patterns.

To generate a landscape for a transition process driven by noise, one can estimate the rate of transitions from state i to j as proportional to exp(−height of energy barrier$_{ij}$/noise), using Kramers' escape-rate theory. To convert the landscape to a more intuitive plot in which "low energy (i.e., stability) = high probability," an inverse function of the probability $P(\hat{S})$ for each cell state can be used to plot an elevation, $V(\hat{S})$, which then generates a quasi-potential energy landscape (Figure 5E). The height of the barrier$_{ij}$ between states i and j is then $-N_o * \ln(k_{i \rightarrow j})$, where $k_{i \rightarrow j}$ is the measured transition rate from state i to j, and N_o is a constant. Each well or local minimum in such a landscape is a "probable" or stable state (i.e., a stable attractor) that is analogous to a "low-energy state." In contrast, hills are unstable states that are less likely ("improbable") to be occupied and correspond to a "high-energy state." Note that the elevation $V(\hat{S})$ on the z axis in Figure 5E does not constitute a true "potential energy" in the classical sense as proposed for systems like protein folding because the system equations for the regulatory networks are not integrable and constitute a non-equilibrium system (Huang, 2009).

"Bistability" describes a landscape in which several stable states may coexist. For example, several blue wells contain stable coexisting states in Figure 5E, and these states may interconvert with one another at a particular rate (the corresponding discrete description is shown in Figure 5D). In contrast, "metastability" describes observed states that exist transiently and are highly sensitive to particular culture conditions or genetic determinants. These states do not sit at the bottom of stable wells in the landscape; for example, in Figure 5E, metastable states do not exist in blue wells but rather in the more yellow or green areas of the landscape.

In practice, a metastable state can be defined as an observable state lasting at least an order of magnitude larger than its doubling time (e.g., >10^2 hr for mammalian cells). Mathematically, however, a metastable state in state space is time-invariant of their state-describing $\hat{S}$ parameters, with long lifetimes lasting many times longer (e.g., 100-fold longer) than the shortest lived state. From a thermodynamics perspective, all cell states are metastable because cells do not operate at thermodynamic equilibrium. However in the biological context, metastability emphasizes the transient lifetime of cell states in contrast to stable self-renewing stem cells and terminally differentiated cells. In Figure 2, this framework is applied to describe the multiple pluripotency states observed in vitro. Ultimately, single-cell experiments coupled with numerical simulations could refine such landscapes to guide future experimentation aimed at dissecting the mechanisms of cell transitions.

iPSCs versus ESCs: Are They Equivalent?

A complex and unresolved question in the field is whether iPSCs are equivalent to ESCs. This is an important issue as genetic or epigenetic abnormalities may influence iPSCs during differentiation and/or transplantation, generating cells with molecular profiles and biological characteristics that are different from ESC-derived cells. The criteria used to compare iPSCs and ESCs include biological assays that test for developmental potency and molecular assays that compare gene expression and epigenetic characteristics. Assays for developmental potency are considered to be crucial for concluding that an iPSC is pluripotent. In mice, chimera formation and germline contribution are routinely used to assess the developmental potential of iPSCs. The production of "all-iPSC mice" by tetraploid complementation recently demonstrated that iPSCs

can indeed have the same developmental potential as ESCs (Zhao et al., 2009). In the human system, however, researchers are restricted to using less stringent functional assays, such as in vitro differentiation and teratoma formation. Although teratoma formation is considered to be a prerequisite for designating human cells as pluripotent, it should be acknowledged that this is a qualitative test that does not allow for easy quantification of differentiation.

Compared to functional tests for pluripotency, molecular analyses allow for more quantitative comparisons of iPSCs and ESCs. Numerous studies indicate that, at least for some clones, iPSCs are similar if not indistinguishable from ESCs derived from embryo or nuclear transfer experiments. These include profiling of global gene expression, modifications of histone tails, the state of X chromosome inactivation, and profiles of DNA methylation (Mikkelsen et al., 2008). However, some studies using global expression analyses concluded that iPSCs are a unique subtype of pluripotent cells that retain a consistent gene expression signature distinguishable from ESCs, even after extended passaging (Chin et al., 2009). Yet, the reanalysis of a large collection of gene expression and histone modification data lead to the conclusion that small variations between human iPSCs and ESCs in chromatin structure and global gene expression may constitute experimental "noise" and do not reflect a consistent signature that distinguishes iPSCs from ESCs (Guenther et al., 2010; Newman and Cooper, 2010).

Two recent studies concluded that the only distinguishable difference between ESCs and the vast majority of iPSCs was the abnormal reduction in the expression of the maternally imprinted *Dlk1-Dio3* locus and that this expression difference was the underlying cause for the inability to generate all-iPS mice by tetraploid complementation (4n). In contrast, 4n-competent iPSC lines showed normal allelic imprinting at this locus (Liu et al., 2010; Stadtfeld et al., 2010). Abnormal expression of this cluster was not observed in human iPSCs (Stadtfeld et al., 2010). Although these studies represent interesting correlations, the conclusions need to be reconciled with the observation that mice with bi-allelic deletion in components of the *Dlk1-Dio3* locus (e.g., *Gtl2*) are viable (Takahashi et al., 2009). Moreover, iPSC clones derived from the same transgenic donor mouse system (Stadtfeld et al., 2010) were later reported to display additional global perturbations in transcriptional patterns, depending on the cell of origin (Polo et al., 2010). It remains to be clarified whether such specific gene expression signatures observed in early passage iPSC lines (Polo et al., 2010) represent "epigenetic memory" or simply result from residual transgene induction levels that are specific to the cell of origin and that induce perturbations in gene expression (Soldner et al., 2009), which may subside with silencing of the transgenes upon extended cell passaging.

Another study compared the patterns of global DNA methylation and in vitro differentiation of early passage iPSCs derived from B lymphocytes or fibroblasts with those of ESCs. This study concluded that reprogramming with transcription factors can leave an epigenetic memory mark in iPSCs reminiscent of the donor cell type (Kim et al., 2010). In contrast, such patterns were not seen in ESCs derived after nuclear transfer, suggesting that nuclear transfer might reset the epigenetic characteristics of

somatic cells more effectively than reprogramming in vitro with transcription factors. However, another explanation for these results is that the nuclear transfer-derived ESCs used in this study (Kim et al., 2010), but not iPSCs, were obtained in the presence of ERK inhibitors, which can facilitate complete reprogramming (Ying et al., 2008; Silva et al., 2009). Further, the fibroblast-derived iPSC lines used in this study showed only partial demethylation of the endogenous Nanog promoter, consistent with incomplete reprogramming (Kim et al., 2010).

Finally, iPSCs derived from patients with fragile X syndrome exhibited a phenotype that was not recapitulated in ESCs carrying the same mutation (Urbach et al., 2010). Fragile X syndrome is a common form of inherited mental retardation caused by an expansion of CGG-triplet repeats in the 5′ untranslated region of the *FMR1* gene, which leads to its transcriptional silencing. Interestingly, in fragile X-ESCs derived from blastocysts, the full expansion of the CGG-triplet repeat did not inactivate the *FMR1* gene and silencing occurred only after differentiation. However, upon in vitro reprogramming of fragile X fibroblasts, the *FMR1* locus remained inactive and was not reset to the transcriptional active state, demonstrating that in vitro reprogramming does not always faithfully reset the epigenetic state of the somatic cell to that of ESCs. It would be interesting to investigate whether this methylation pattern is lost with extended passaging of the iPSC lines.

Many of the studies summarized above suggested that somatic cells can be reprogrammed to a pluripotent state, which is molecularly and biologically indistinguishable from that of ESCs and compatible with the generation of all-iPSC mice by tetraploid complementation. However, in some circumstances subtle differences, which are inconsistent and often transient, can also be observed. Evaluating the frequency and origin of altered expression patterns in iPSCs is of biological and clinical importance. Unfortunately, a number of methodological limitations, which are known to affect the state of pluripotency, complicate a meaningful comparison of iPSCs and ESCs, and they must be simultaneously controlled for. As summarized in Table 2, such parameters include the presence and incomplete silencing of transgenes, different combinations of reprogramming factors used to induce iPSCs, natural heterogeneity that exists between different pluripotent ESC lines, incomplete reprogramming in early passage cell lines, and the genetic background of the cells. Also, even low levels of basal vector expression have been shown to significantly affect the global gene expression pattern of undifferentiated cells (Soldner et al., 2009). This is important because for some iPSCs derived from patients, a disease-specific in vitro phenotype has been reported (Carvajal-Vergara et al., 2010; Ebert et al., 2009). These iPSCs were generated with constitutively expressed lentivirus- or Moloney virus-based vectors. Therefore, variable and undefined levels of basal vector expression may have influenced the phenotype.

An unresolved question is whether the subtle epigenetic attributes resulting from incomplete reprogramming of the somatic donor nucleus have a meaningful and functional significance for the developmental potential of iPSCs. Commonly referred to as transient epigenetic memory, these technical attributes of incomplete reprogramming can be erased following additional

Table 2. Parameters that May Affect Gene Expression and Biological Characteristics of Pluripotent Stem Cells

Parameter	Factors Influencing the Properties of Induced Pluripotent Cell States	Considerations for Experimental Design
Transgene-containing iPSCs	The presence and incomplete silencing of reprogramming transgenes commonly used in generating iPSCs can perturb the identity and functionality of the induced cells.	Generation of vector-free reprogrammed cells.
Genetic background	The gene expression pattern and in vivo developmental competency can vary between different mouse strains (e.g., 129 strain versus the nonobese diabetic strain).	Comparison of ESCs and iPSCs from an identical genetic background.
Incomplete reprogramming	Direct reprogramming involves several cell divisions.	Analysis of fully reprogrammed lines that have achieved ample cell divisions after transduction of the reprogramming factors.
In vitro molecular heterogeneity among ESCs	Culture adaptation of cell lines may be the source for heterogeneity; ESC clones generated from identical genetic backgrounds can display interclonal variability.	Comparison of several independent ESC and iPSC lines from genetically identical backgrounds grown in the same growth conditions.
Reprogramming factor combinations	iPSCs can be generated by transduction of different combinations of reprogramming factors or small molecules, and this may affect the epigenetic characteristics of the iPSCs.	Inclusion of cell lines derived through different combinations of reprogramming factor or in "2i" conditions.

A number of constraints may affect the epigenetic state and biology of iPSCs; controlling for these parameters may facilitate the comparison between iPSCs and ESCs and result in more reliable characterization of the pluripotent state.

cell division or by supplementing other exogenous factors during reprogramming (Silva et al., 2009; Polo et al., 2010; Kim et al., 2010). Further, aberrant and variable imprinting are evident in cloned mice and in ESC lines derived from embryos (Humpherys et al., 2001), indicating that even substantial deregulation of genes still allows development to birth and beyond. Thus, differentiation to functional cells may be rather tolerant of epigenetic aberrations of the genome and subtle abnormalities in gene expression, and clearly, it will be important to establish criteria and minimal requirements that define the safety of iPSCs for clinical applications and disease research.

Transdifferentiation of Somatic Cells

Reprogramming of somatic cells into iPSCs requires the resetting of the epigenetic state from a somatic to a pluripotent embryonic state, which can be achieved even without using drug resistance to select for the activation of marked pluripotency genes. Thus, the pluripotent epigenetic state may represent a default cellular state easily captured in tissue culture. One explanation for this observation is that iPSCs may have a growth advantage over the somatic cells. However, another possibility is that the gene expression circuitry of pluripotency is a "ground" or default state that is the most stable state after erasing the somatic cell identity and thus reflects a state with a "minimal" epigenetic dominance of somatic programs.

Such hypotheses raise the question of whether cells can be induced to "transdifferentiate" directly into another state of differentiation. Here transdifferentiation is defined as the direct conversion of one somatic cell type into another type without first reprogramming into pluripotent cells and then differentiating into functional somatic cells (Graf and Enver, 2009). As outlined in Table 3, it is useful to define the extent of the transdifferentiation but also to distinguish between transdifferentiation within a lineage with those between lineages of different germ layer origins.

One of the first examples of transdifferentiation within the same germ layer was the conversion of fibroblasts into muscle cells by overexpressing *MyoD* (Weintraub et al., 1989). However, activation of the endogenous *MyoD* gene was not detected in the muscle cells, suggesting that conversion was incomplete and that the maintenance of the myogenic phenotype depended on the transgene. Recently, cardiac fibroblasts were elegantly converted into cardiomyocyte-like cells by the ectopic expression of *Gata4, Mef2C, and Tbx5* (Ieda et al., 2010). The reprogrammed cells did not depend on expression of the transgenes but exhibited transcriptional and functional differences from neonatal cardiomyocytes, particularly when the cells were derived from dermal fibroblasts.

Transdifferentiation has also been achieved within the hematopoietic lineage. Ectopic expression of the transcription factor C/EBPα converted lymphocytes to macrophage-like cells (Xie et al., 2004). However, the induced macrophage-like cells continued to express markers specific to Pro-B cells and failed to activate several macrophage-specific markers.

Similarly, in vitro transdifferentiation between different germ layers was achieved by the ectopic expression of *MITF* (microphthalmia-associated transcription factor), which converted fibroblasts into melanocyte-like cells that synthesized melanin through the activation of direct downstream targets of MITF, such as tyrosinase (Tachibana et al., 1996). However, the

Table 3. Transdifferentiation between Somatic Cell States

Somatic Cell Conversion (*with Germ Layer*)	Exogenous Reprogramming Factors	Experimental Setting	Complete Molecular Epigenetic Reprogramming	The Dependency of the New State on the Transgene	Ref
Fibroblasts (*mesoderm*) Converted to myocyte-like cells (*mesoderm*)	MyoD + 5-AzaC	–in vitro –intralineage conversion	No: Failed to reactivate endogenous MyoD and other myogenic markers	Dependent	[1]
B cells, T cells, and fibroblasts (*mesoderm*) Converted to macrophage-like cells (*mesoderm*)	C/EBPα ±PU.1	–in vitro –intralineage conversion	No: Failed to reactivate several macrophage-expressed genes; failed to suppress some donor cell somatic markers	Independent	[2]
Cardiac fibroblasts (*mesoderm*) Converted to induced cardiac myocte-like cells (*mesoderm*)	Gata4, Mef2c, and Tbx5	–in vitro –intralineage conversion	No: Gene expression signature distinguishable from neonatal cardiomyocytes; Induced cardiac myocyte-like cells derived from dermal fibroblast have significantly limited functionality	Independent	[3]
Fibroblasts (*mesoderm*) Converted to Melanocyte-like cells (*ectoderm*)	MITF	–in vitro –cross-lineage conversion	No: Failed to reactivate several melanocyte-expressed genes; failed to suppress donor cell markers	Not determined	[4]
Fibroblasts (*mesoderm*) Converted to induced Neuron-like (iN) cells (*ectoderm*)	Ascl1, Brn2, and Mytl1	–in vitro –cross-lineage conversion	Not determined	Not determined	[5]
B cells (*mesoderm*) Converted to common lymphoid progenitors, macrophages, and T cells (*mesoderm*)	Pax5 deletion	–in vivo (and in vitro) –intralineage conversion	Not determined; Functional in vivo hematopoietic reconstitution	Independent	[6]
Pancreas exocrine cells (*endoderm*) Converted to endocrine-like cells (*endoderm*)	Ngn3, Pdx1, and MafA	–in vivo –intralineage conversion	Not determined; Functional insulin-producing cells capable of improving hyperglycemia	Independent	[7]
Granulosa and Theca cells (*mesoderm*) Converted to Sertoli and Leydig cells (*mesoderm*)	Foxl2 deletion	–in vivo –intralineage conversion	Not determined; Functional male hormonal production	Independent	[8]

References: [1] Weintraub et al., 1989; [2] Xie et al., 2004; [3] Ieda et al., 2010; [4] Tachibana et al., 1996; [5] Vierbuchen et al., 2010; [6] Cobaleda et al., 2007; [7] Zhou et al., 2008; [8] Uhlenhaut et al., 2009.

resultant cells retained expression of fibroblast markers and had reduced expression of most melanocyte markers, suggesting that the partial transdifferentiation predominantly resulted from activation of direct targets of MITF.

In a more recent study (Vierbuchen et al., 2010), particular neural transcription factors converted mouse fibroblasts into neuron-like cells. The induced neural cells shared essential features with functional neurons, including morphological characteristics, expression of cortical markers, the generation of action potentials, and the formation of synapses. However, it has not been resolved whether the induced neural cells have silenced fibroblast-specific genes and maintain their newly acquired state independent of the expression of the transgenes.

Overall, several of these directed transdifferentiation experiments in vitro provide strong evidence that the induced cells exhibit aberrant gene expression patterns and incomplete reprogramming into the new lineage. Although such in vitro derived cells may prove valuable in the future, further

investigation is needed to determine whether a somatic gene expression program can be extensively and completely reinstated on another somatic program from a different lineage without first going through the ground state of pluripotency.

The deletion of the *Pax5* transcription factor induced dedifferentiation of B cells into common lymphoid progenitor cells. Indeed, these cells were able to reconstitute the entire lineage of T lymphocytes in mice (Cobaleda et al., 2007). Finally, inducible deletion of *Foxl2* in adult ovarian follicles led to the upregulation of testis-specific genes, resulting in reprogramming of granulosa and theca cells into functional Sertoli-like and Leydig-like cells, respectively, which were capable of expressing normal testosterone levels (Uhlenhaut et al., 2009).

Researchers have demonstrated that the expression of key transcription factors can convert one cell type into a developmentally related cell type inside an animal even in the absence of cell proliferation. This was most clearly demonstrated by the conversion of exocrine into endocrine pancreatic cells (Zhou et al., 2008). Transdifferentiation in the absence of DNA replication may be consistent with the notion that changes in the expression of transcription factors predominantly drives the conversion between cells states that are within the same lineage and closely related developmentally. Thus, these types of conversions may require only a limited amount of resetting of DNA or chromatin modifications. This appears to be different from direct reprogramming of somatic cells to a pluripotent state, which entails extensive epigenetic resetting and requires multiple rounds of DNA replication.

Finally, it should be mentioned that multiple studies have claimed that somatic cells, such as bone marrow cells, can be transdifferentiated into cells of other lineages merely by culturing the cells under specific conditions. As discussed elsewhere, the evidence for such claims has so far been unconvincing (Wagers and Weissman, 2004).

Concluding Remarks

Although rapid progress in our understanding of pluripotency and stem cells has been made, a number of important questions and technical hurdles remain. These include the stabilization of the various pluripotent states in cell cultures derived from different species. It is particularly important to define culture conditions that robustly and stably maintain the naive pluripotent state and then use this technology to derive and fully characterize naive iPSCs and ESCs from human embryos. Current direct reprogramming approaches are inefficient and involve stochastic changes occurring in highly heterogeneous cell populations. Because only a small fraction of cells will ever form an iPSC, the information gained from molecular analysis of the heterogeneous intermediate cell populations is limited. Such analyses cannot distinguish between the rate-limiting and non-rate-limiting epigenetic changes in those cells. To understand the complex epigenetic remodeling that precedes iPSC formation, we need to establish new experimental and theoretical approaches that allow for molecular analyses at the single-cell level.

It is likely that the generation of patient-specific iPSCs will have a significant impact on the study of human diseases and on regenerative medicine. However, a number of technical issues need to be resolved before the technology can be used in a clinical setting (Saha and Jaenisch, 2009). These include the establishment of efficient reprogramming strategies that do not result in genetically modified cells. Although the approaches currently available for generating genetically unmodified iPSC are inefficient, we expect that these technical hurdles will be resolved soon. In addition, one of the key challenges for translating these new technologies to the clinic is devising robust protocols for differentiating ESCs or iPSCs to self-renewing adult stem cells and lineage-committed cells. Armed with such protocols, researchers can then begin to define experimental conditions that allow the development and detection of relevant in vitro phenotypes for a given human disease, putting "personalized" regenerative medicine just over our horizon.

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