Guidelines and Techniques for the Generation of Induced Pluripotent Stem Cells

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Direct reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) provides an invaluable resource for regenerative medicine, enabling the generation of patient-specific cells of any lineage without the use of embryonic material. A variety of methods exist for iPSC derivation, all reliant upon manipulation of a select group of transcription factors. We compare the currently reported protocols, identify essential steps common to these methods, and suggest minimal criteria for defining fully reprogrammed cells. In addition, specific procedures aimed to optimize reproducible iPSC derivation are presented, with an emphasis on standardization of certain parameters for accurate comparison between independent experiments.

The generation of pluripotent cells from differentiated adult cells has vast therapeutic implications, particularly in the context of in vitro disease modeling, pharmaceutical screening, and cellular replacement therapies. In addition, the ability to revert somatic cells to an embryonic state provides a unique tool to dissect the molecular events that permit the conversion of one cell type to another.

Previously devised strategies to induce pluripotency, such as somatic cell nuclear transfer (cloning) or fusion of somatic cells with embryonic stem cells (ESCs) (Hochedlinger and Jaenisch, 2006) are fraught with technical, ethical, and logistical barriers that impede the use of the resulting pluripotent cells in both research and therapy. Thus, the direct generation of pluripotent cells without the use of embryonic material has been deemed a more suitable approach that lends itself well to mechanistic analysis and has fewer ethical implications.

The direct reprogramming of somatic cells to pluripotency was accomplished in 2006, when Takahashi and Yamanaka converted adult mouse fibroblasts to iPSCs through ectopic expression of a select group of transcription factors. Subsequent reports optimized this technique, demonstrating that iPSCs were indeed highly similar to ESCs when tested across a rigorous set of assays (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). In 2007, direct reprogramming was achieved in human cells (Takahashi et al., 2007b; Yu et al., 2007), providing an invaluable contribution to the field of regenerative medicine.

While the establishment of iPSC lines is conceptually and technically simple, direct reprogramming is a slow and inefficient process consisting of largely unknown events. Several variables must be considered in order to reproducibly obtain iPSCs, which include (1) the choice of factors used to reprogram cells; (2) the methods used to deliver these factors; (3) the choice of target cell type; (4) the parameters of factor expression, such as timing and levels; (5) the culture conditions used to derive iPSCs; and the methods of (6) identifying and (7) characterizing reprogrammed cells. This review addresses each of these steps in detail and is summarized as an overview in Figure 1. A discussion on the

efficiency of reprogramming has also been included to promote standardization in the calculation and reporting of efficiencies.

Choice of Reprogramming Factors
Direct reprogramming was initially performed in mouse fibroblasts through retroviral transduction of 24 candidate genes that were all implicated in the establishment and maintenance of the pluripotent state. This pool of 24 genes was ultimately narrowed down to four transcription factors, Oct4 (Pou5f1), Sox2, c-Myc, and Klf4, that were sufficient to mediate reprogramming (Takahashi and Yamanaka, 2006). This core set of factors has been shown to work across a multitude of mouse cell types (Aoi et al., 2008; Eminli et al., 2008; Hanna et al., 2008; Kim et al., 2008; Stadtfeld et al., 2008a, 2008c; Wernig et al., 2008a), as well as rhesus monkey (Liu et al., 2008) and human cells (Park et al., 2008a; Takahashi et al., 2007b; Lowry et al., 2008).

Variations on the four-factor cocktail have been used to successfully reprogram cells. In mouse fibroblasts, Sox1 and Sox3 can replace Sox2, albeit with a decrease in reprogramming efficiency; Klf2 can replace Klf4, and L-Myc and N-Myc can replace c-Myc (Blelloch et al., 2007; Nakagawa et al., 2008). It has also been reported that a partially different set of factors, OCT4, SOX2, NANOG, and LIN28, is sufficient to reprogram human fibroblasts (Yu et al., 2007).

The endogenous expression of certain reprogramming factors in different cell types has permitted their exclusion from the factor cocktail. For example, fibroblasts express c-Myc and Klf4, and it has been demonstrated that exogenous c-Myc is not necessary for the reprogramming of mouse and human fibroblasts, although the efficiency is much lower and reprogramming requires more time (Nakagawa et al., 2008; Wernig et al., 2008b). Neural progenitor cells, which express Sox2 and c-Myc at levels higher than in ESCs, have been reprogrammed using only Oct4/Klf4 or Oct4/c-Myc, though at lower efficiency than with four factors (Kim et al., 2008).

While the original suite of four factors remains the standard for direct reprogramming, a handful of small molecules and
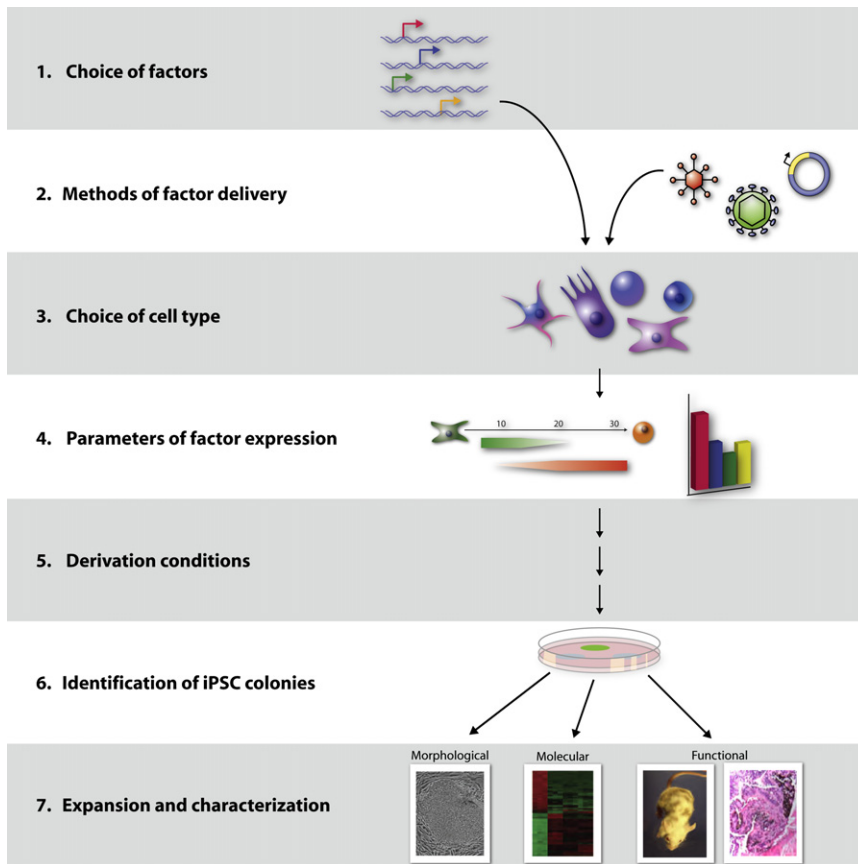
1. **Choice of factors**

2. **Methods of factor delivery**

3. **Choice of cell type**

4. **Parameters of factor expression**

5. **Derivation conditions**

6. **Identification of iPSC colonies**

7. **Expansion and characterization**

Morphological Molecular Functional

lentiviruses (Brambrink et al., 2008; Hockemeyer et al., 2008; Maherali et al., 2008; Stadtfeld et al., 2008b). These viral systems, however, have been criticized for their permanent integration into the genome, and endeavors to make iPSCs more therapeutically applicable have led to the pursuit of nonintegrating approaches (Table 2). Two such approaches, adenoviral delivery and transient transfection, have been successfully used in the reprogramming of mouse cells (Okita et al., 2008; Stadtfeld et al., 2008c), lending promise to the eventual use of transient delivery methods in human iPSC derivation.

The first attempts at direct reprogramming employed Moloney-based retroviral vectors that are known to undergo silencing in the ESC state (Jahner et al., 1982; Wolf and Goff, 2007); this self-silencing property provided an advantage for initial attempts as the temporal requirement of factor expression was undefined. However, several drawbacks in addition to genome integration preclude the use of these retroviruses: (1) their infectivity is limited to dividing cells (Miller et al., 1990), thus restricting the range of cell types that can be reprogrammed; (2) silencing occurs gradually during the course of iPSC induction, resulting in a lowered efficiency of conversion compared to nonsilencing viral methods (Stadtfeld et al., 2008b); and (3) iPSCs made with retroviruses often maintain viral gene expression (Dimos et al., 2008; Park et al., 2008c), thus limiting their utility. While lentiviruses permit the transduction of nondividing cell types with high expression levels (Naldini et al., 1996), they are poorly silenced in the pluripotent state (Lois et al., 2002), making the constitutive versions less suitable for reprogramming attempts. Although iPSCs made with constitutive lentiviruses have been reported (Blelloch et al., 2007; Yu et al., 2007), it is unclear how differentiation proceeds during continued transgene expression (Brambrink et al., 2008).

Drug-inducible lentiviruses have provided a more attractive approach, as they permit temporal control over factor expression. Although these viruses also integrate in the host genome, they are particularly useful for conducting mechanistic analyses (Brambrink et al., 2008; Stadtfeld et al., 2008b). For instance, the use of such viruses has led to the establishment of "secondary systems," whereby iPSC-derived differentiated cells harbor the proviral integrations in the same pattern that enabled primary iPSC induction. Upon reinduction, the viral transgenes are homogeneously reactivated, leading to a >100-fold increase in secondary iPSC production (Hockemeyer et al., 2008; Maherali et al., 2008; Wernig et al., 2008a). Such systems provide

additional factors have been reported to enhance the reprogramming process and/or functionally replace the role of some of the transcription factors (Table 1). The identification of such mediators is beginning to yield insight into the mechanisms by which reprogramming occurs, and many similar studies are likely to follow. The use of small molecules and soluble factors is particularly appealing given their ease of use and lack of permanent genome modification that constrains the use of retro- and lentiviruses (further described in the following section); however, it is currently unknown whether small molecules alone can recapitulate the series of transcriptional and epigenetic changes brought about by the four transcription factors. An important caveat to the increasing use of epigenetic modifiers is that their broad and nonspecific effects may elicit an overall dysregulation of gene expression. For instance, 5-azacytidine is mutagenic (Jackson-Grusby et al., 1997), and mice with global alterations in DNA methylation levels develop tumors at a high frequency (Gaudet et al., 2003). In general, the use of alternative and adjunct factors is growing in popularity, and newer methods should be subject to rigorous testing to ensure quality of the resulting iPSC lines.

Methods of Factor Delivery

The production of iPSCs has so far been achieved through nucleic-acid-based delivery of the reprogramming factors. Initial generations of mouse and human iPSCs employed retroviral vectors (Takahashi et al., 2007b; Takahashi and Yamanaka, 2006) and constitutive lentiviruses (Blelloch et al., 2007; Yu et al., 2007), while later generations were produced using inducible

Table 1. Small Molecules and Other Factors Used to Enhance the Reprogramming Process

Molecule/Factor	Target/Mode of Action	Role in Reprogramming	References
Valproic Acid	histone deacetylase inhibitor	enhances reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Huangfu et al., 2008a, 2008b
		restores reprogramming efficiency in mouse fibroblasts without c-Myc (O/S/K only)	
		permits reprogramming of human fibroblasts treated with OCT4 and SOX2, though at extremely low efficiency	
5-azacytidine; shRNA against Dnmt1	DNA demethylating agent	~4-fold enhancement of reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Mikkelsen et al., 2008
		no effect if applied too early; toxic to differentiated cells	
BIX01294	histone methyltransferase inhibitor	restores reprogramming efficiency in mouse neural progenitor cells with Oct4/Klf4 to four-factor level (O/S/M/K)	Shi et al., 2008b
		permits reprogramming of mouse neural progenitor cells in the absence of Oct4, though at extremely low efficiency and requires the presence of the other three factors (S/M/K)	
BayK8644	L-type calcium channel agonist	cooperates with BIX01294 to enable reprogramming of mouse embryonic fibroblasts with Oct4/Klf4	Shi et al., 2008a
Wnt3a	cell signaling molecule; transcriptional activation of multiple downstream targets, including c-Myc	1.2-fold enhancement of reprogramming efficiency with four factors (O/S/M/K) in mouse fibroblasts	Marson et al., 2008
		~20-fold enhancement with three factors (O/S/K); partially restores efficiency in the absence of c-Myc	
siRNA against p53 and Utf1 cDNA	tumor suppressor; ESC-specific cofactor, respectively	modest increase in four-factor (O/S/M/K) reprogramming efficiency of human fibroblasts with individual factors (4 + p53siRNA or 4 + UTF1)	Zhao et al., 2008
		~100-fold enhancement with the combined factors (O/S/M/K + p53siRNA + UTF1); further slight enhancement by the exclusion of c-MYC (O/S/K + p53siRNA/UTF1)	

O, Oct4; S, Sox2; M, c-Myc; K, Klf4; Dnmt1, DNA methyltransferase 1.

a powerful tool to aid in chemical and genetic screening for factors that enhance reprogramming, as well as the optimization of iPSC derivation conditions. Newer techniques that build on this approach may include the targeting of reprogramming factor DNA to known regions of the genome (Hochedlinger et al., 2005), thus preventing positional effects caused by random integration; methods to link all four reprogramming factors on one transcript may facilitate such targeting efforts (Okita et al., 2008).

The use of integrating viruses for iPSC induction has represented a major roadblock in the pursuit of clinically relevant applications, as genomic insertion has been shown to alter gene function (Kustikova et al., 2005), and viral transgene reactivation in iPSC-derived chimeric mice has been implicated in tumorigenesis (Nakagawa et al., 2008). Analysis of integration sites in iPSCs yielded no common targets or pathways, indicating that genomic integration is not necessary for reprogramming (Aoi et al., 2008; Varas et al., 2008). The derivation of mouse iPSCs

Table 2. Methods of Factor Delivery

Method	Advantages	Disadvantages	References
Moloney-based retrovirus	silenced in pluripotent cells	genomic integration; risk of insertional mutagenesis	Takahashi and Yamanaka, 2006
	self-silencing eliminates need for timed factor withdrawal	limited to dividing cells	
		expression often maintained in iPSCs; increased tumor incidence in chimeric mice due to transgene reactivation	
HIV-based lentivirus	Constitutive		
	transduction of both dividing and nondividing cells	genomic integration; risk of insertional mutagenesis	Brambrink et al., 2008; Blelloch et al., 2007; Yu et al., 2007
		lack of silencing in pluripotent state	
	Inducible		
	temporal control over factor expression	genomic integration; risk of insertional mutagenesis	Stadtfeld et al., 2008b; Brambrink et al., 2008
		possibility of leaky expression	
	Integrase-Deficient		
	low frequency of genomic integration	lower expression levels than integrated form	Nightingale et al., 2006
		integration provides selective advantage and necessitates clone screening	
		not yet reported for iPSC production	
Transient transfection	no viral components	multiple rounds of transfection are required	Okita et al., 2008
	low frequency of genomic integration	lower levels of expression than when integrated	
	technically simple procedure	delayed kinetics of reprogramming	
		integration provides selective advantage and necessitates clone screening	
Adenovirus	low frequency of genomic integration	repeated infection required for certain cell types	Stadtfeld et al., 2008c
		delayed kinetics of reprogramming	
		some generation of tetraploid cells	
Small molecules	transient controllable activity	issue of toxicity versus efficacy	Huangfu et al., 2008a, 2008b; Mikkelsen et al., 2008
	technically easy to work with	undefined/nonspecific effects	
		not yet reported for iPSC production	
Protein transduction	direct delivery of transcription factors avoids complications of nucleic-acid-based delivery	short half-life; multiple applications required	Gump and Dowdy, 2007; Bosnali and Edenhofer, 2008
		some proteins difficult to purify	
		not yet reported for iPSC production	

using transient delivery approaches has confirmed this notion (Okita et al., 2008; Stadtfeld et al., 2008c) and has provided a solid foundation on which such techniques might be optimized for human cells.

Choice of Cell Type

For the first reprogramming attempts in both mouse and human, fibroblasts were used as the starting cell population. Adult fibroblasts have been previously shown to be amenable to reprog-

ramming by nuclear transfer in mouse (Wakayama et al., 1998) and cell fusion in both mouse and human (Cowan et al., 2005; Tada et al., 2001). Further, the derivation of fibroblasts is technically simple (Nagy et al., 2003; Park et al., 2008b), and disease-specific human fibroblasts are readily available through cell repositories such as Coriell. Fibroblasts are also compatible with ESC culture conditions and are used as feeder layers for ESC growth, making them a feasible starting candidate for direct reprogramming efforts.

Since the success of fibroblast reprogramming, a multitude of mouse cell types, including stomach cells (Aoi et al., 2008), liver cells (Aoi et al., 2008; Stadtfeld et al., 2008c), pancreatic β cells (Stadtfeld et al., 2008a), lymphocytes (Hanna et al., 2008), and neural progenitor cells (Eminli et al., 2008; Kim et al., 2008), as well as human keratinocytes (Aasen et al., 2008; Maherali et al., 2008), have been reprogrammed. Many of these experiments employed genetic labeling or other techniques to confirm the identity of the donor cell, ruling out the possibility of contaminating resident fibroblasts as the cell of origin.

What has emerged from these studies is that there is a strong influence of cell type on reprogrammability, including the efficiency and kinetics of the process as well as the ease at which reprogramming factors can be delivered. For example, mouse stomach and liver cells showed reactivation of the ESC-specific Fbx15 gene during reprogramming much faster than fibroblasts and contained fewer viral integrations (Aoi et al., 2008), and human keratinocytes reprogrammed faster and more efficiently than human fibroblasts (Aasen et al., 2008; Maherali et al., 2008). The effective delivery of factors has also played a role in the reprogramming of a given cell type. For instance, the reprogramming of mouse fibroblasts with adenoviral vectors required 100- to 200-fold higher titers than that of liver cells (Stadtfeld et al., 2008c).

Several factors must therefore be considered in determining the optimal cell type for a given application: (1) the ease at which reprogramming factors can be introduced, which varies both by cell type and delivery approach; (2) the availability and ease of derivation of the given cell type; and (3) the age and source of the cell. Older cells or those that have undergone several passages in culture may harbor genetic lesions that undermine the therapeutic potential of the resulting iPSCs; similarly, cells obtained from organs that are more likely to have acquired DNA damage, such as skin cells that may have accumulated UV-induced mutations, would be less suitable for clinical applications. Thus, while fibroblasts are likely to remain the choice cell type in basic research efforts to mechanistically dissect the reprogramming process, iPSCs derived for therapeutic purposes will require the donor cells to be easily attainable, less likely to contain genetic aberrations, and easy to reprogram with transient approaches.

Parameters of Factor Expression

The shift toward nonintegrating delivery approaches has necessitated a better understanding of how the factors coordinate their efforts to orchestrate reprogramming. To improve the process of iPSC derivation, it is important to define the temporal requirement of factor expression, as well as the optimal factor levels and stoichiometry. Additionally, the quantification of factor delivery is important for exploiting such knowledge and can ensure reproducibility as well as allow proper comparison between independent experiments.

The length of time required for cells to become independent of factor expression has been addressed using doxycycline-inducible systems. The kinetics of factor requirements has been quantified in mouse fibroblasts, which require at least 8–12 days of factor exposure (Brambrink et al., 2008; Stadtfeld et al., 2008b), and in human keratinocytes, which require ~10 days (Maherali et al., 2008). Although prolonged exogenous factor expression beyond the minimal amount of time results in enhanced colony recovery (Brambrink et al., 2008; Stadtfeld et al., 2008b; Wernig et al., 2008a), persistent expression in the pluripotent state may be detrimental (Mikkelsen et al., 2008). Thus, a general guideline to follow is that exogenous factor expression should be discontinued as soon as genuine iPSCs become apparent (further discussed in the section "Methods to Identify Reprogrammed Cells"). While the kinetics of reprogramming is highly influenced by the starting cell type, in all instances reprogramming requires several days to proceed. The wide temporal ranges reported for each cell type suggest that aspects in addition to cell identity can influence the kinetics, which can most likely be attributed to differential factor delivery (levels and stoichiometry), but may also reflect cell-intrinsic differences such as cell cycle stage, differentiation status, and passage number.

The precise expression levels and stoichiometry required for reprogramming have been difficult to examine. Variance in factor delivery, coupled with the low efficiency of conversion, has made it impossible to retrospectively analyze the individual contribution of each factor that causes a single differentiated cell to acquire a pluripotent state. However, achieving optimal expression levels is indeed important for the reprogramming process; for example, the conversion of neural progenitor cells into iPSCs proceeds more efficiently when Sox2 is omitted from the four-factor cocktail, indicating that transgene-driven Sox2 expression in addition to high endogenous Sox2 levels is detrimental (Eminli et al., 2008; Silva et al., 2008). The establishment of secondary systems in which each clone has a distinct but reliable pattern of factor reactivation offers a more reliable tool to dissect the precise contribution of individual factors (Hockemeyer et al., 2008; Maherali et al., 2008; Wernig et al., 2008a).

Though the optimal expression levels and stoichiometry of factors still remain poorly defined, ensuring that cells receive all factors is an important step in achieving reprogramming, and quantification of individual factor expression within the chosen delivery system is critical for reproducibly obtaining iPSCs. No reports of iPSC generation have thoroughly quantified factor delivery; in most instances this has been addressed indirectly through the use of separately delivered reporter constructs, such as GFP-encoding vectors. However, the use of such surrogate markers provides an inaccurate readout. For example, reporter proteins are quite stable with long half-lives and are not subject to the same cellular handling experienced by transcription factors; this difference is particularly relevant to transient delivery methods where multiple applications of factors are required (Okita et al., 2008; Stadtfeld et al., 2008c), and reapplication depends on the length of time each factor is expressed. For viral-based methods, titers are influenced by the gene of interest, as the gene product is expressed at high levels during packaging and can potentially alter the function of the packaging cells (Tiscornia et al., 2006). Thus, even with constant transfection parameters, viral titer is highly variable. The best method for quantification is a direct analysis of expression in the cell type of interest; this assessment can be accomplished by using a reporter-linked construct, such as IRES-GFP, or through immunostaining, which permits analysis at a single-cell level. For a more accurate measure of factor delivery, one can also assess coinfectivity to determine the percentage of cells receiving all factors. While testing the expression of each factor in every batch

produced requires a considerable amount of work, there is a high payoff in reproducibility, and controlling for factor input facilitates the transition between different delivery methods.

As viral-based gene delivery methods remain popular for iPSC derivation, the ability to produce high-titer virus is of great value. Detailed protocols specifically written for iPSC derivation provide a good starting resource (Park et al., 2008b; Takahashi et al., 2007a); though limited to retroviruses, these basic protocols are highly adaptive and can be tailored to optimize factor delivery in different contexts. More extensive reviews covering a broad scope of viral methods serve as excellent references (Ramezani and Hawley, 2002; Rosenzweig, 2007; Tiscornia et al., 2006). An alternative is to have viruses produced commercially, which is more suitable for applications that solely require iPSCs for downstream analysis and do not require flexibility of manipulation during the derivation process.

Culture and Derivation Conditions

Both mouse and human iPSC derivation proceed under the same culture conditions used for ESC maintenance (Akutsu et al., 2006; Cowan et al., 2004; Lerou et al., 2008; Nagy et al., 2003), and it is important to ensure that the selected conditions support ESC growth. While alternative conditions for reprogramming have not yet been reported, there will indeed be a push toward creating defined and xeno-free cultures in efforts to establish iPSCs that will be more suitable for clinical applications.

As ESC conditions are sufficient to obtain iPSCs from most cell types, it has been speculated that conditions used to facilitate ESC derivation may also enhance iPSC derivation. For instance, the use of knockout serum replacement instead of fetal bovine serum greatly facilitates mouse ESC (mESC) derivation (Cheng et al., 2004), and it has also been reported to improve the reprogramming of mouse fibroblasts (Blelloch et al., 2007). The use of knockout serum replacement provides an alternative culture condition for the reprogramming of various cell types for which standard serum is unsuitable. An important point to note, however, is that the use of undefined media components such as serum introduces batch-to-batch variability and may not elicit reproducible effects. Thus, it is important to screen individual batches for ESC supportive capacity.

ESCs rely on fibroblast-derived factors to support their growth, particularly human ESCs (hESCs). mESCs can be derived and cultured on gelatin in the absence of feeders and additional growth factors (Ying et al., 2008), and similarly, mouse iPSCs can be derived under feeder-free conditions (Stadtfeld et al., 2008b; Wernig et al., 2008a). While defined culture conditions for hESCs have also been established (Amit and Itskovitz-Eldor, 2006), the derivation of human iPSCs without feeder cells has not yet been reported, though it remains a clinically relevant goal to avoid the use of animal products.

A key aspect for creating favorable derivation conditions is to achieve an optimal cell density. Cells seeded at very low densities may senesce and be less amenable to reprogramming, while cells seeded at high density can quickly become overconfluent, hindering the growth of new colonies and posing the risk of the cell layer lifting, particularly after the prolonged culture times required for reprogramming. This risk has been illustrated in secondary systems where reprogramming occurs at high efficiency; at high cell densities, the frequency of colony formation drops despite identical expression of the reprogramming factors (Maherali et al., 2008; Wernig et al., 2008a), indicating a nonlinear relationship between cell density and reprogramming efficiency. While the optimal cell density must be experimentally determined, a general guideline to follow is to seed the infected target cells at ~10% confluence and to use a feeder density less than 2.5 to 5 × 10^4 cells/cm^2.

Determining appropriate culture conditions for the reprogramming of nonfibroblast cell types presents a specialized case that must be tailored to satisfy the needs of both the donor cell and the arising iPSC. Accordingly, the reprogramming factors are typically introduced into the donor cells under their native conditions and then switched to ESC conditions during the course of reprogramming, the timing of which must be experimentally determined. For instance, the reprogramming of mouse neural progenitor cells requires a switch from serum-free conditions to serum-containing ESC conditions; if switched too early, no iPSCs are obtained (Wernig et al., 2008a). In some instances it is possible to employ cultures that support the growth of both the donor cell and iPSC; for example, in the reprogramming of lymphocytes, a combination of B lineage growth factors and LIF was used, making the culture environment suitable for both hematopoietic cells and iPSCs, respectively (Hanna et al., 2008).

Human iPSC (hiPSC) derivation also represents a unique case, as the cells are more sensitive than their mouse counterparts to the conditions under which they are grown. For example, hiPSCs/hESCs display some sensitivity to doxycycline exposure (Maherali et al., 2008), which must be accounted for when using such inducible systems. hiPSCs/hESCs also exhibit poor survival when grown as single cells; accordingly, the addition of small molecules that enhance single-cell survival in established hiPSC/hESC cultures, such as the Rho-associated kinase (ROCK) inhibitor (Watanabe et al., 2007) have been suggested to facilitate hiPSC derivation (Park et al., 2008b), although their use is not required for successful reprogramming.

Methods to Identify Reprogrammed Cells

In the first attempts at reprogramming, it was anticipated that the introduction of multiple factors would yield multiple cell fates, thus necessitating the use of a selection system whereby only cells that reactivated ESC-specific genes could survive (Takahashi and Yamanaka, 2006). Such systems, however, entailed permanent genetic modification through the introduction of reporter alleles, rendering the system unfeasible for reprogramming in a clinical setting. Several advances have since been made, leading to effective and therapeutically compatible methods to identify and obtain reprogrammed cells.

The first generation of mouse iPSCs was obtained via selection for the ESC-specific, but nonessential, gene Fbx15 (Takahashi and Yamanaka, 2006). While the resulting cells demonstrated pluripotency in the context of teratoma formation, they were not fully reprogrammed, as they could not generate chimeric mice, and their gene expression profiles and DNA methylation status were distinct from ESCs. It was later found that selection for the *essential* ESC-specific genes, Nanog and Oct4, permitted the generation of iPSCs that were much more similar to ESCs (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). With this finding also came the result that delayed onset of
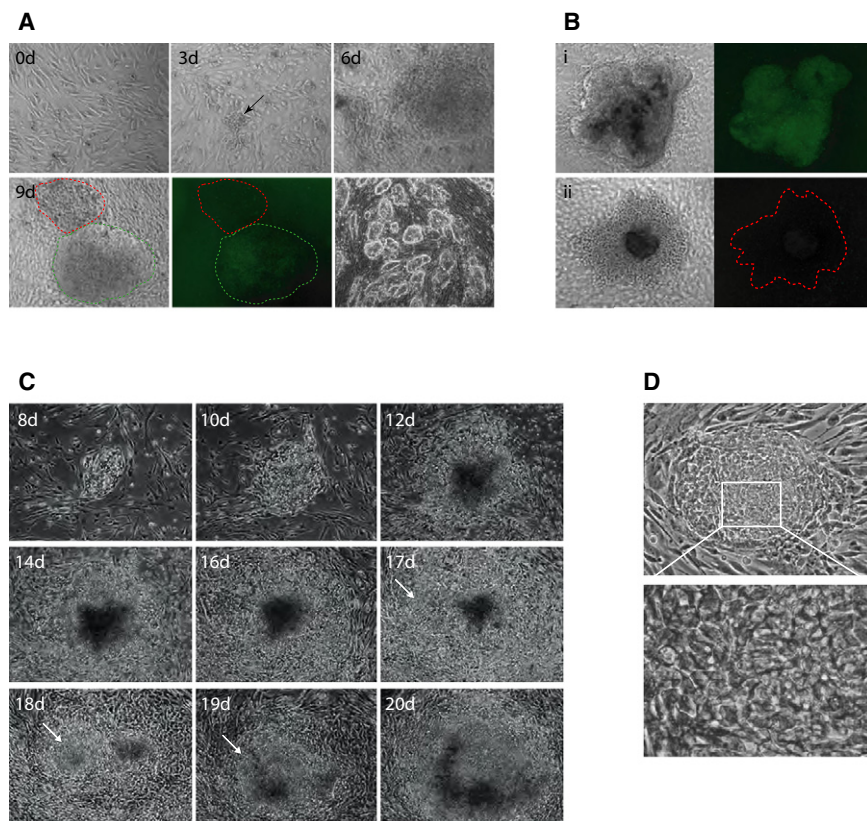
A

B

C

D

Figure 2. Progressive Formation of iPSC Colonies

(A) Time course of reprogramming in primary infected mouse fibroblasts. The arrow at 3d indicates a nascent colony. At day 9, two adjacent colonies are shown; only one has reactivated the endogenous Oct4-GFP reporter allele, representing a true iPSC colony. Bottom right image depicts iPSC colonies from an established line (Stadtfeld et al., 2008b).

(B) Two representative colonies derived from primary infected mouse fibroblasts. (Bi) iPSC colony, characterized by a tight well-defined border and Oct4-GFP expression; (Bii) differentiated colony, consisting of loosely packed cells and lacking expression of Oct4-GFP (Stadtfeld et al., 2008b).

(C) Colony tracking during the reprogramming of secondary human fibroblasts with a doxycycline-inducible system. After doxycycline withdrawal at 14 days, colony regression occurs, and at day 17, a hESC-like colony is seen to emerge (indicated by arrow). This colony continued to develop (denoted by arrows) and, by day 20, showed signs of differentiation typical for hESCs (Maherali et al., 2008).

(D) Primary human iPSC colony, depicting the flat colony structure with cobblestone morphology (top panel). Lower panel illustrates the characteristic morphology with pronounced individual cell borders.

selection was key to generating fully reprogrammed cells, ultimately leading to the discovery that selection methods were unnecessary and actually counterproductive (Blelloch et al., 2007; Maherali et al., 2007; Meissner et al., 2007).

The identification of iPSC colonies based solely upon morphological criteria requires a considerable degree of ESC expertise. In general, mouse ESC colonies can be distinguished by their refractive, or "shiny," appearance and tight, well-defined borders, while human ESC colonies display a cobblestone appearance with prominent nucleoli and pronounced individual cell borders. The stepwise morphological changes that occur during reprogramming have been depicted in both systems (Figure 2). Of importance to note is that morphologically similar but non-iPSC colonies also arise during the course of human fibroblast reprogramming. These colonies are often mistaken for iPSCs, particularly by novices in the field, but are distinguishable from iPSCs becuase they are loose and granular in appearance, contain phase-bright cells, and appear earlier in the process (after 1–2 weeks) (Lowry et al., 2008; Takahashi et al., 2007b).

Additional methods to identify iPSCs have been described; these techniques become useful when one is dealing with cell types that provide a high background of non-iPSC colonies (for example, those formed during human fibroblast reprogramming), or when ESC expertise is lacking. Two such methods have used ESC-specific surface antigen expression and loss of transgene dependence as strategies to identify reprogrammed cells. For example, isolation of the Thy-1-SSEA-1+ population during the course of mouse fibroblast reprogramming greatly enriches for cells poised to become iPSCs (Stadtfeld et al., 2008b), and live staining of cultures for the hESC-specific surface antigen Tra-1-81 has aided in the identification of genuine hiPSC colonies derived from human fibroblasts (Lowry et al., 2008). The loss of transgene dependence, which correlates with full reprogramming, can be assessed through the use of reporters that are silenced in the pluripotent state (Stadtfeld et al., 2008b; Zhao et al., 2008), or through factor withdrawal, which requires an inducible or transient delivery system. Upon withdrawal, cells that rely on continued factor expression are eliminated, thus permitting selective expansion of fully reprogrammed cells (Maherali et al., 2008).

Expansion and Characterization of Cells

The steps involved in taking a new colony to a fully established iPSC line are identical to those for ESC derivation, which have been described in detail elsewhere (Akutsu et al., 2006; Lerou et al., 2008; Nagy et al., 2003). Of particular importance to note, however, are the methods used to passage mouse and human iPSCs, as well as those used to ensure purity of the resulting iPSC lines.

Mouse iPSCs/ESCs can withstand single-cell dissociation, and newly derived colonies can be immediately subjected to enzymatic passaging, thus facilitating their quick expansion into lines. Human iPSCs/ESCs, however, survive poorly as single cells, and initial passaging of new colonies must be done mechanically; several passages (approximately five to ten) are required before the cells can be adapted to enzymatic dissociation (Lerou et al., 2008). As hiPSCs/ESCs are highly prone to differentiation, especially within the first few passages, it is important to continually remove differentiated structures to prevent
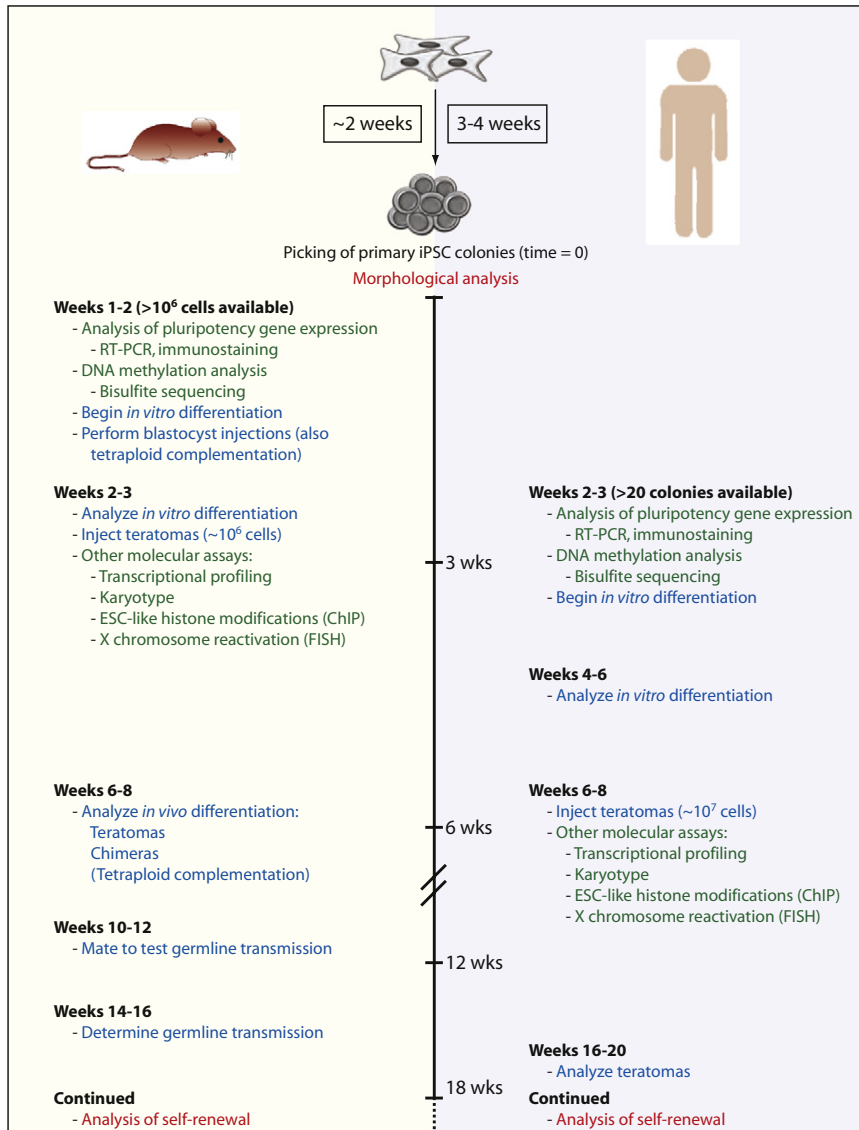
Figure 3. Timeline for the Characterization of Mouse and Human iPSC Lines
Suggested time points for key assays are indicated, providing a rough outline for a general characterization of iPSCs. Red text indicates morphological assays, green indicates molecular assays, and blue indicates functional characterization. ChIP, chromatin immunoprecipitation; FISH, fluorescent in situ hybridization.

On a molecular level, iPSCs must display gene expression profiles that are indistinguishable from ESCs, which extends to the display of other associated features, including (1) protein-level expression of key pluripotency factors (e.g., Oct4, Nanog) and ESC-specific surface antigens (e.g., SSEA-1 in mouse; SSEA-3/-4, Tra-1-60/-81 in human); (2) functional telomerase expression; and (3) expression of genes involved in retroviral silencing, such as de novo methyltransferases and Trim28 (Lei et al., 1996; Wolf and Goff, 2007). Accordingly, genuine iPSCs must be independent of transgene expression and thus lack expression of the delivered factors. iPSCs must also be epigenetically similar to ESCs, demonstrating DNA demethylation at the promoters of pluripotency genes, X chromosome reactivation in female cells (Maherali et al., 2007; Rideout et al., 2001), and the presence of bivalent domains at developmental genes, consisting of overlapping histone modifications that have opposing roles (Bernstein et al., 2006; Maherali et al., 2007; Wernig et al., 2007).

At a functional level, iPSCs must demonstrate the ability to differentiate into lineages from all three embryonic germ layers. A hierarchy of criteria has been put forth, and in order of increasing levels of stringency, these include: (1) in vitro differentiation, (2) teratoma formation, (3) chimera contribution, (4) germline transmission, and (5) tetraploid complementation (direct generation of entirely ESC/iPSC-derived mice) (Jaenisch and Young, 2008).

As performing all available assays for the demonstration of pluripotency is infeasible, a suggested minimal set of criteria should be fulfilled in order to ascertain that a genuine iPSC has been obtained. Accordingly, these include (1) all morphological attributes, including unlimited self-renewal; (2) expression of key pluripotency genes with a concomitant downregulation of lineage-specific genes associated with the cell of origin; (3) transgene independence; and (4) proof of functional differentiation through the highest-stringency test acceptable.

In mouse, the accepted standard is germline transmission, demonstrating the competence of iPSCs to contribute to all lineages including germ cells and ultimately giving rise to offspring. While tetraploid complementation remains the most stringent

them from being carried forward in the expansion. While use of a ROCK inhibitor can greatly facilitate hiPSC/hESC line expansion (Watanabe et al., 2007), it is not known to safeguard against differentiation and may result in carryover of differentiated cells; thus, its use is left to the discretion of the experimenter. Both mouse and human iPSC cultures can harbor initial contamination with improperly reprogrammed or differentiated cells, and subcloning may be necessary to ensure the quality of newly derived lines (Maherali et al., 2007; Wernig et al., 2007). This has been of particular importance in iPSC clones that maintain transgene expression, for instance, in partially reprogrammed cells (Mikkelsen et al., 2008).

Several criteria have been set forth to ascertain whether a fully reprogrammed state has been achieved, which include an array of unique features associated with pluripotency, encompassing morphological, molecular, and functional attributes (Figure 3). Morphologically, iPSCs must appear identical to ESCs and demonstrate unlimited self-renewal.

functional assay, iPSCs, unlike ESCs, have not been reported to autonomously generate full-term mice. It is not yet clear whether this reflects a fundamental issue in reprogramming, such as an inability to fully reset the epigenome of a somatic cell, or whether it is a technical issue that remains to be addressed, such as the presence of proviral integrations, the identity of the starting cell type, or the passage number of the iPSCs. Further experiments are therefore needed to truly ascertain whether iPSCs are capable of fulfilling this criterion.

For a functional assessment of pluripotency in human cells, teratoma formation should be demonstrated and include both histological and immunohistochemical analysis to confirm the presence of structures derived from all three germ layers (Gertow et al., 2007). While the ability to form teratomas is considered the most stringent assay for human cells, it is not as rigorous as the assays available for mouse cells. For instance, first-generation mouse iPSCs, though able to form teratomas, could not give rise to live-born chimeras (Takahashi and Yamanaka, 2006), indicating that additional assays would be beneficial in testing the functional differentiation capacity of hiPSCs. As such, directed differentiation efforts have introduced robust in vitro assays coupled with transplant models to assess the function of specific cell types derived in vitro from hiPSCs/hESCs (Kroon et al., 2008; Lee et al., 2007; Mummery et al., 2003).

In addition to the demonstration of pluripotency, it is crucial to ensure that the resulting iPSCs are free from genetic aberrations. Cells cultured for long periods of time can become genetically unstable, particularly human ESCs in that they have a tendency to acquire abnormal karyotypes (Draper et al., 2004; Lerou et al., 2008). Thus, testing iPSC lines periodically for genetic lesions is important for proper maintenance of the line.

Calculation and Reporting of Reprogramming Efficiencies

An accurate assessment of reprogramming efficiency is critical for proper comparison between individual experiments, particularly in the interpretation of optimization procedures and the reprogramming of different cell types. Most iPSC derivations have reported the frequency of colony formation, which is an indication of the number of colonies formed per number of cells seeded, yet the large range in reported values indicates that this measure portrays an incomplete view of derivation efficiency, thus requiring other variables to be factored into the calculations.

Differences in factor delivery strongly contribute to discrepancies between independent experiments. For example, variability in viral titer leads to quantitative differences in factor delivery, thus altering the proportion of cells that receive all factors and ultimately leading to a change in the frequency of colony formation. Other contributing factors include the plating efficiency of cells, cell survival (encompassing proliferation and cell death/apoptosis), and the counting of sister clones (multiple iPSC colonies derived from an individual cell).

The specific methods used to quantify colony number often differ between groups, which also strongly contributes to variation in the reported efficiencies. Such methods include morphology-based counts, alkaline phosphatase (AP) activity, immunostaining, transgenic and knockin reporter allele expression, and

the ability of a colony to form an iPSC line; all methods vary in their accuracy to reflect genuine iPSC colonies. While the ability to form an iPSC line is the most stringent quantification method, it is laborious and not feasible for large colony numbers. The use of knockin reporter alleles or immunostaining for endogenous pluripotency gene expression are suitable surrogate methods; however, morphology-based identification or AP activity is not sufficient for denoting true pluripotent cell colonies (Brambrink et al., 2008). Transgene-based reporter methods should also be used cautiously, as expression of such alleles is not subject to the same regulation as endogenous knockin reporter alleles. This has been exemplified by the kinetics of Oct4 reporter gene reactivation during cell fusion, which occurs much faster with a transgenic allele than an endogenous knockin allele (Do and Scholer, 2004; Maherali et al., 2007).

A number of steps can be taken to reduce variability in the reported frequencies and gain the most accurate and reliable estimate of the true reprogramming efficiency. These include (1) controlling for factor delivery through assessment of expression and coinfectivity and to report values as a fraction of the cells expressing all factors, rather than the total number of input cells; (2) calculation of plating efficiency, which can be done via cell counts or single-cell plating; (3) eliminating the count of sister clones through single-cell plating or retrospective analysis of integration patterns; and (4) use of a reliable and stringent method to identify and quantify iPSC colonies. Such standardization between methods will greatly facilitate the interpretation and comparison of independent experiments and, in turn, accelerate progress in the field.

Concluding Remarks

The generation of iPSCs represents a major advance in the field of regenerative medicine and provides a powerful tool for the study of cell-fate transitions. While new techniques and insights are continually unveiled, the foundation of iPSC derivation rests upon successful manipulation of a core set of transcription factors. The key steps involved in this process consist of the choice of factors and molecules used, their delivery method, and the choice of target cell type, as well as the parameters of factor expression, culture conditions, methods to identify cells, and the assays used to verify pluripotency. To fully exploit the abundance of new information requires a standardization of certain parameters of the reprogramming process, such as the calculation of reprogramming efficiency and qualification of the pluripotent state. As such, this review has attempted to present a comprehensive comparison of the currently available technologies for iPSC derivation and put forth standards to minimize variability between independent experiments, thus providing a framework to aid in the designing and conducting of future experiments, as well in the evaluation of existing iPSC literature.

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