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Induced Pluripotent Stem Cells: What Lies Beyond the Paradigm Shift

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Abstract

The discovery that somatic cells can be reprogrammed to become induced pluripotent stem (iPS) cells has ushered in a new and exiting era in regenerative medicine. Since the seminal discovery of somatic cell reprogramming by Takahashi and Yamanaka in 2006, there has been remarkable progress in the characterization of iPS cells and the protocols used to generate them. The new information generated during the past year alone has vastly expanded our understanding of these cells. Accordingly, this review provides a basic overview of the different strategies used to generate iPS cells and focuses on recent developments in the field of iPS cells. In the final section, we discuss three broad, unanswered questions related to somatic cell reprogramming, that are just starting to be addressed.

Keywords

induced pluripotent stem cells; iPS cells; reprogramming; Oct4; Sox2; Nanog; DNA methylation; epigenetic; chromatin remodeling; mi-RNA; X chromosome inactivation; cell cycle

INTRODUCTION

In 2006, Takahashi and Yamanaka reported the astonishing discovery that somatic cells could be reprogrammed back to pluripotent stem cells (1). This discovery truly represents a paradigm shift in stem cell biology, and it was recently recognized by this year's Albert Lasker Basic Medical Research Award. After minor refinements in the initial reprogramming protocol, induced pluripotent stem (iPS) iPS cells were shown to be germline-competent and capable of forming any cell type of the body. The unanticipated discovery of somatic cell reprogramming generated considerable excitement, because iPS cells were produced by introducing just a few genes into somatic cells that typically exhibit little or no capacity to differentiate. Although the study of iPS cells has already provided a wealth of new information, many questions surrounding iPS cells remain unanswered. For stem cell biologists, the study of iPS cells offers the exciting prospect of dissecting the molecular mechanisms responsible for rewiring the gene regulatory networks active in somatic cells to those required to support the self-renewal and pluripotency of iPS cells. This alone would warrant the excitement surrounding iPS cells. However, iPS cells have generated even greater interest in the medical community, as well as in the public at large, because many believe that iPS cells could help pave the way for unprecedented advances in new therapies for a wide range of debilitating human diseases. Importantly, iPS cells not

only offer the prospect of producing patient-specific cells, they circumvent the ethical conundrum that surrounds the isolation of human ES cells from early embryos.

In just three years since the discovery of iPS cells, we have witnessed the publication of a large and diverse body of work dealing with the reprogramming of somatic cells. To assist those interested in iPS cells, this review provides an overview of iPS cells and discusses an array of new developments in this rapidly expanding field of research. Specifically, this review focuses on: 1) the basic parameters of somatic cell reprogramming, 2) reprogramming with and without viral delivery of reprogramming factors, 3) reprogramming of different somatic cells and early work using iPS cells in preclinical models, 4) the need for correct levels and ratios of reprogramming factors for efficient reprogramming, and 5) the timing of critical events that take place during reprogramming. Underlying several of these topics is the question why is somatic cell reprogramming so slow and inefficient? In the final section of this review, we discuss three of the larger unanswered questions surrounding somatic cell reprogramming and iPS cells.

REPROGRAMMING OF SOMATIC CELLS: BASIC APPROACHES AND RECENT ADVANCES

Production and Characterization of iPS Cells

The seminal work of Takahashi and Yamanaka demonstrated that it was possible to reprogram somatic cells to a pluripotent stem cell state by retroviral delivery of just four genes (1). Of the 24 genes tested, Sox2, Oct4, Klf4 and c-Myc (SOKM) were sufficient for reprogramming to occur. However, reprogramming was neither efficient nor rapid. In this initial study, reprogrammed somatic cells were identified by selecting for the reactivation of the Fbx-15 locus just three days after initial retroviral transduction. Although the cells were able to form teratomas composed of cells representative of three germ-layers (pluripotent), they were not germline-competent. Subsequent refinements in reprogramming protocols delayed selection to at least seven days before assessing reactivation of gene loci known to be critical for ES cell maintenance (2,3). Selection for reactivation of endogenous genes, such as Oct4, which are necessary for the maintenance of ES cells, allowed for the isolation of iPS cell clones that were germline-competent (2,3). Other reports demonstrated that iPS cells capable of contributing to chimeric mice could be isolated solely based upon morphology, thus eliminating the need for drug selection of reprogrammed cells (4,5).

Properly reprogrammed iPS cells exhibit a wide range of properties, which include: loss of somatic cell-specific markers, expression of the appropriate stage-specific embryonic antigens (see below), telomerase activity, X chromosome reactivation (in female cells), reactivation of endogenous genes essential for pluripotency and self-renewal (e.g. Sox2, Oct4 and Nanog), and silencing of exogenous factors used to initiate reprogramming. Establishment of endogenous pluripotent gene expression requires epigenetic remodeling of the somatic cell genes, including the demethylation of critical gene promoters. Fully reprogrammed mouse iPS cells are germline-competent (Figure 1). Success in an even more rigorous test of full reprogramming has been reported recently (6,7). In two reports, mouse iPS cells transplanted into tetraploid embryos were able to generate live mice. In this stringent test, the cells of tetraploid blastocysts are capable of contributing only to extraembryonic tissues during development (8). Thus, for embryos to develop into viable adult mice, the inner cell mass must be reconstituted fully from the injected diploid pluripotent cells.

Given the challenge of generating iPS cells that are fully reprogrammed, there are concerns regarding how best to assess the reprogramming status of human iPS cells. For obvious ethical reasons, human iPS cells cannot be characterized as robustly (chimera formation) as

mouse iPS cells. Additionally, human ES/iPS cells are not identical to mouse ES/iPS cells in terms of their developmental status and their cell culture requirements. Mouse ES/iPS cells are more closely related to cells of the preimplantation epiblast (9) and require LIF, but not endogenously expressed FGF4, to maintain their self-renewal and pluripotency *in vitro* (10). Human ES/iPS cells appear to be closely related to cells in the late epiblast and require an FGF, such as FGF2, but not LIF, to maintain their pluripotency and self-renewal in culture (11). Additionally, mouse ES/iPS cells express SSEA-1, whereas human ES/iPS cells express SSEA-3 and SSEA-4, but not SSEA-1. Thus, different markers of reprogramming are utilized to assess the extent to which human somatic cells have been reprogrammed.

The quality and/or stage of development of different human ES cells can also be assessed by the status of the second X chromosome of female cells. Female human ES cells can be grouped into two broad categories based upon the status of their X chromosomes. Although less common, some human ES cell lines have two active X chromosomes, one of which undergoes X chromosome inactivation (XCI) after differentiation. However, most of the female human ES cell lines examined had already undergone XCI (12). If the X chromosome status of human pluripotent stem cells is a reflection of the quality of the cells, this would be another important parameter to assess when characterizing human iPS cells (12).

Importantly, gene expression profiling has revealed subtle differences between human embryonic stem cell lines and induced pluripotent stem cells (13), arguing there are subtle differences between embryonic and induced stem cells, despite the ability of both cell types to self-renew and contribute to all three germ layers during teratoma formation. Interestingly, passage of human iPS cells leads to gene expression profiles that more closely reflect the gene expression profile of human embryonic pluripotent cells. Further study will be necessary to determine if these subtle differences in gene expression influence the self-renewal, pluripotency and, ultimately, the clinical use of human iPS cells.

Commonly Used Reprogramming Factors

Since the first report of reprogramming, it has become evident that there is some flexibility in the factors needed to reprogram somatic cells. Protocols that employ exogenous expression of SOKM are frequently used to reprogram mouse somatic cells. c-Myc is not strictly required for the reprogramming of somatic cells. However, when c-Myc is omitted from the SOKM protocol, the time required for reprogramming is dramatically longer, and there is a significant drop in reprogramming efficiency (14). Interestingly, exogenous expression of downstream miRNA products of c-Myc (miR-291-3p, miR-294 and miR-295, all expressed in ES cells) can recover some of the lost reprogramming efficiency when only three factors (SOK) are used (15). Human fibroblasts have also been reprogrammed using the SOKM combination (16), as well as the Sox2, Oct4, Lin28 and Nanog (SOLN) combination (17). Additionally, certain reprogramming factors can be replaced by transcription factors belonging to the same family. Sox1 or Sox3 can substitute for Sox2 in reprogramming, L-Myc or NMyC can substitute for c-Myc, and Klf2 or Klf5 can substitute for Klf4 (18,19). Exogenous expression of genes (e.g. Sall4) that are essential in early development can also be beneficial to the reprogramming process (20). As discussed in a later section of this review, the specific factors needed for reprogramming depend upon the type of somatic cells being reprogrammed.

Viral Integration: Neither Needed Nor Desirable

Many reprogramming protocols rely on retroviruses or lentiviruses to integrate into the genome, to express the exogenous reprogramming factors. However, continued expression or later reactivation of the exogenously supplied reprogramming factors poses serious

problems. For example, Okita *et al.* determined that reprogrammed cells could generate chimeric mice. However, some of the chimeric mice developed tumors, apparently due to reactivation of the c-Myc transgene [also see (17,21)]. Additionally, several studies have shown that incomplete transgene silencing compromises or biases the differentiation of iPS cells. For example, iPS cells exhibiting constitutive transgene expression formed tumors devoid of differentiated cells; whereas, iPS cells, in which transgene expression has been silenced, form highly complex differentiated tumors (22). Hence, residual transgene expression or transgene reactivation would not only be highly risky after transplantation in a clinical setting, but also run the likely risk of biasing the direction of iPS cell differentiation.

The use of retroviral and lentiviral vectors to reprogram somatic cells raised other important issues. One dealt with the question of whether reprogramming required viral insertion into specific gene loci. Soon after the first reports of reprogramming, it was established that this was not the case (23). Of greater concern, is the obvious risk of insertional mutagenesis, which would raise serious doubts about the clinical use of cells derived from human iPS cells. Therefore, the development of reprogramming protocols that eliminate permanent genomic integration or which do not depend on viral vectors is a critical goal of regenerative medicine.

To minimize the risk of chromosomal disruption, reprogramming protocols were developed in which genetic material would not permanently integrate into the genome. Several groups developed reprogramming protocols in which the genetic material is first integrated into the genome, and later removed once pluripotency has been established, thereby eliminating the potential of SOKM gene reactivation. For example, Soldner *et al.* described a Cre-lox recombination protocol for reprogramming of somatic cells from Parkinson's patients, in which the reprogramming factor within each lentiviral construct was flanked by loxP sites (24). Following viral integration and reprogramming, exogenous expression of Cre-recombinase was used to remove the reprogramming factors from the genome. However, residual viral inserts still remained in the genome of these reprogrammed cells, and thus, cells were still at risk for insertional mutagenesis. Another Cre-lox recombination-based reprogramming system described by Kaji *et al.* employed transient transfection of a single multiple protein expression plasmid into mouse fibroblasts already containing loxP sites (25). Interestingly, this protocol utilized an expression vector which included an inducible polycistronic transgene flanked by loxP sites. [Within the polycistronic element, coding sequences for the four reprogramming factors (OSKM) were separated by the 2A self-cleaving peptide of the foot and mouth disease virus.] After reprogramming had occurred, Cre-recombinase was used to remove the transgene, restoring the genomic sequence at the loxP sites to that of the starting fibroblast population.

A more refined system in which reprogramming factors could be delivered into the host genome and removed without any residual elements was developed recently (26). This protocol utilized the piggyBac transposon/transposase system coupled with the polycistronic transgene described above. The piggyBac transposon/transposase system does not require sites previously engineered into the starting cell population, as piggyBac transposon elements can integrate into sites already present in the mammalian genome. Specifically, the inducible polycistronic transgene is flanked by piggyBac terminal repeats, which allow the transgenes to insert into the host genome. Once pluripotency has been established and the transgenes are no longer required, expression of piggyBac transposase removes the transgenes, including the piggyBac terminal repeats. This leaves no residual trace of genomic integration. Importantly, these transposon/transposase based systems were also able to reprogram mouse fibroblasts at a rate significantly higher than most other protocols.

Other investigators took a different approach to avoid viral integration into the genome altogether. Stadtfeld *et al.* used adenoviral vectors to deliver the reprogramming factor genes, SOKM (27). Adenoviruses do not integrate into the host genome and integration of adenoviral sequences into the genome of the reprogrammed cells was not detected. In yet another approach, it was demonstrated that it was possible to repeatedly transiently transfect cells with expression plasmids (encoding the necessary reprogramming factors) and generate iPS cells (21). However, reprogramming efficiencies using adenoviral vectors or expression plasmids were dramatically lower for most cell types compared to the standard retroviral/lentiviral mediated reprogramming.

Non-nucleic Acid Based Reprogramming

Recent studies argue that the use of genetic materials is not required for reprogramming of somatic cells. Kim *et al.* reported the reprogramming of human fetal fibroblasts by culturing them in ES cell media supplemented with extracts prepared from HEK293 cells engineered to express reprogramming factors (SOKM), each of which were fused to a transducing peptide from human immunodeficiency virus-transactivator of transcription (HIV-TAT) (28). [The HIV-TAT peptide (amino acids 48-60) allows the fused protein to cross cellular membranes, enabling the reprogramming factors added to the culture media to penetrate into somatic cells.] In this study, human iPS cells were generated after 3-4 cycles of HEK293 cell extract exposure, though the rate of formation took approximately twice as long when compared to retroviral reprogramming. Recently, Zhou *et al.* further refined reprogramming protocols using purified recombinant proteins (SOKM) containing a polyarginine protein transduction domain fused to their C-termini (29). iPS cells were isolated 30 days after the initiation of reprogramming, which involved treatment of the somatic cell with four cycles of recombinant protein plus the histone deacetylase inhibitor, valproic acid. The iPS-like cells generated in this study were able to generate chimeric embryos.

Small molecules have also been shown to eliminate the need for certain reprogramming factors. The most notable example is reprogramming using only Sox2, Oct4 and valproic acid (30). Additionally, 5-aza-cytidine (AZA) has been shown to drive partially reprogrammed cells to fully reprogrammed iPS cells (31). AZA is a methyltransferase inhibitor shown to impede the maintenance of CpG methylation at promoters of essential pluripotency genes, such as Oct4 (31). As discussed below demethylation of these promoters is a key step in generating fully reprogrammed iPS cells. However, AZA is known to be mutagenic, and is capable of promoting tumors in animal models (32). Thus, it remains to be determined whether a combination of small molecules and recombinant proteins will be sufficient to fully reprogram somatic cells (without genetic damage) to pluripotent stem cells that are germline-competent.

Modifications to the culture conditions, not specific to somatic cell reprogramming, may also increase the efficiency and rate in which somatic cells are reprogrammed. Hypoxic culture conditions are thought to help maintain the pluripotency of ES cells (33). Furthermore, fibroblasts cultured in hypoxic conditions have been reported to up-regulate Oct4, Sox2 and Nanog (34), and mouse embryonic fibroblasts down-regulate the p53 pathway in low oxygen conditions (4%) (35). Yoshida *et al.* recently demonstrated that fibroblasts can be reprogrammed more efficiently (3 to 7-fold increase) under hypoxic (5% oxygen) conditions (36). As described below, up-regulation of the p53 pathway hinders somatic cells reprogramming. Other modifications of culture conditions may also help to supplement the reprogramming protocols described to date. The Rho-kinase (ROCK) inhibitor, Y-27632, has been shown to enhance the survival and growth of hES cells at low density (37), and substantially increase the recovery of cryopreserved human ES cells and human iPS cells (38). Moreover, Park *et al.* 2008 utilized ES cell media supplemented with ROCK inhibitor during their report of human somatic cell reprogramming (39). However,

the specific influence of the ROCK inhibitor on reprogramming was not discussed in that study. Additional details regarding the use of small molecules in reprogramming protocols are provided in a comprehensive review by Feng *et al.* (40).

Reprogramming By Altering Host Cell Machinery

In 2008, Zhao *et al.* reported that disruption of the p53 pathway by siRNA and UTF1 expression enhances the efficiency of reprogramming up to 100-fold (41). Recent reports extend this interesting finding and demonstrate, using four different approaches, that the p53-p21 pathway serves to impede the reprogramming process. More specifically, reprogramming efficiency of both mouse and human cells was found to increase (by ~25-fold) when using p53 null cells, up-regulating a negative regulator of p53, knocking down p53 or knocking down its targets (p21) (42,43). Furthermore, decreased levels of p53 allowed Sox2 and Oct4 alone to reprogram mouse fibroblasts into cells capable of generating germline-competent adult chimeric mice. However, p53 can serve as a checkpoint to prevent cells with significant DNA damage and chromosomal abrogation from undergoing reprogramming (44). iPS cell lines generated by depletion of p53 would therefore be prone to DNA damage, and, thus, would not be suitable for clinical use.

The role of p53 during reprogramming raises an important question. How does p53 act? More specifically, does p53 knockdown prevent a subpopulation of cells from undergoing apoptosis, and/or does p53 knockdown enhance the processes of reprogramming itself? miRNA-145 is essential for ES cell differentiation, and it exerts its effects by interfering with the translation of the critical pluripotency factors Sox2, Oct4 and Klf4, by binding to the 3'-UTR of their mRNA transcripts (45). Interestingly, p53 promotes the maturation of precursor microRNA-145 to mature miRNA-145 (46). Moreover, it has been reported that miRNA-145 can also target and disrupt regulators of cell cycle and cell proliferation pathways, such as CDK6, known to be important in the G1/S cell cycle transition (46). Thus, a potential mechanism by which p53 interferes with reprogramming may involve its ability to drive maturation of miRNA-145 (Figure 2): down-regulation of mature miR-145, due to disruption of p53, may allow endogenous gene transcripts that are essential for self-renewal and pluripotency to be translated into functional proteins. The appropriate expression of these factors could allow the cell to enter a pluripotent state, independent of exogenous gene expression, more readily when compared to cells that must overcome the effects of p53 and miR-145. Consequently, it is tempting to speculate that knockdown of miR-145 would lead to improvement in the reprogramming efficiency. If that is true, knockdown of p53 targets, such as miR-145, instead of p53 itself, may enhance reprogramming efficiency without dramatically disrupting DNA repair machinery. Interestingly, other miRNAs that are likely to influence reprogramming are the miR-17-92 and the miR-302 clusters. Both clusters are highly expressed in ES cells. miR-17-92 is a target of p53 under hypoxic conditions (47), and forced expression of miR-302 has been shown to reprogram tumor cells to an iPS-like state (48). Other findings mentioned above also point to the value of manipulating miRNAs regulated by c-Myc (15).

A Wide Range of Cell Types Can Be Reprogrammed

Multiple cell types have been used for reprogramming, including: embryonic fibroblasts, adult fibroblasts, keratinocytes, neural progenitor cells, hepatocytes, stomach epithelial cells, pancreatic β cells, and intestinal epithelial cells (Reviewed (49)). In most studies, the efficiency of reprogramming is reported to be very low, typically in the range of 0.01 to 0.5% of the starting somatic cell population. Although the values reported are somewhat dependent on the way in which reprogramming efficiencies are calculated, intrinsic properties of the starting cell population and the specific protocol used have a much greater impact on the efficiency of reprogramming. For instance, human keratinocytes are

reprogrammed more rapidly and with greater efficiency than human fibroblasts (100-fold more efficient) (50,51), quite possibly because keratinocytes express c-Myc at higher levels (52). It has also been reported that mouse stomach and liver cells reprogram faster than fibroblasts (23), and hepatocytes are more amenable to adenoviral-mediated reprogramming than fibroblasts, requiring 100-fold lower titers of the adenoviral vectors (27). Given the diversity of cell types that can generate iPS cells, it remains to be determined whether one particular cell type is better suited for generating patient-specific iPS cells. In this regard, it would not be surprising to find that subtly different iPS cells are generated when starting with different somatic cell types.

To further tease out somatic cell types that are amenable to reprogramming, secondary iPS cell systems have been developed (51,53). For these studies, primary iPS cells were generated using inducible lentiviral vectors to express the reprogramming factors. These primary iPS cells were then implanted into blastocysts, and chimeric animals and tissues were generated. Somatic cells from embryos or adult mouse tissue were then harvested and the expression of reprogramming factors was re-induced, forming a 'secondary' iPS cell population. By generating secondary iPS cells, a variety of tissue types can be assessed for their reprogramming potential. For example, iPS cells were more readily generated from intestinal crypt epithelial cells compared to cells of villus origin (53). The use of secondary iPS cell systems can also be used to assess how reprogramming efficiency is affected by the differentiation status of the starting cell population. Eminli *et al.* determined that adult progenitor cells from the hematopoietic lineage were reprogrammed up to 300 times more efficiently than terminally differentiated hematopoietic cells; over 25% of myeloid progenitors were reprogrammed compared to 0.05% of B-cells (54).

Preclinical Models Developed Using iPS Cells

One of the most promising aspects of somatic cell reprogramming is the ability to generate patient-specific pluripotent stem cells. Disease therapy, disease modeling and patient-specific toxicology studies have already been demonstrated in various reports as proof of principle. A recent report described the generation of iPS cells from human fibroblasts and subsequent differentiation of the cells into insulin-producing islet cells (55). Mouse iPS cells have been differentiated into cells of cardiovascular and hematopoietic lineages (56,57), and more specifically, into rhythmically contracting cardiomyocytes (58). Functional retinal pigmented epithelial cells have also been generated from human iPS cells (59). Hanna *et al.* demonstrated that gene targeting could be conducted with iPS cells to repair gene defects (60). Disease modeling using iPS cells derived from patients affected with genetic diseases is also being studied to better understand the underlying pathophysiology. Specifically, iPS cell lines have been derived from patients with Parkinson's, Huntington, type I diabetes mellitus and amyotrophic lateral sclerosis patients (39,61). In a study by Ebert *et al.*, iPS cells generated from a spinal muscular atrophy patient (iPS-SMA cells) demonstrated the characteristic loss of the SMN1 gene (62). Additionally, to further emphasize the role of iPS cells in drug screening and toxicity, Yokoo *et al.* demonstrated it was possible to elicit the same drug response (contractility and beat frequency) from iPS cell-derived cardiomyocytes, as observed in clinical studies (63).

Effects of Varying the Levels of Reprogramming Factors

The low level of reprogramming efficiency typically observed is not only influenced by the cell type being reprogrammed, but also the levels and ratios of the reprogramming factors expressed in a given cell. The finding that secondary iPS cells are reprogrammed at far greater frequency (>100-fold), when the expression of their transgenes are reactivated, argues strongly for a critical role played by the levels and/or the ratio of the reprogramming factors (51,53). In this regard, Papapetrou *et al.* systematically evaluated the importance of

generating the correct ratio of the reprogramming factors (64). Using a lentiviral reprogramming system where four separate viruses were used to express Oct4, Sox2, Klf4 and c-Myc, it was initially determined that simultaneously increasing the multiplicity of infection (MOI) of each virus by the same amount (at viral titers that achieve significant reprogramming) did not significantly alter the efficiency of reprogramming. However, reprogramming efficiency was significantly affected when the ratios of the four reprogramming factors were changed by increasing or decreasing the MOI of one virus, while holding the MOI the other three viruses constant. Most notably, a variable, but significant, increase in reprogramming efficiency (~2-fold) was observed when the MOI of Oct4 was increased 3-fold. Conversely, when the MOI of Oct4 was decreased by approximately 3-fold, reprogramming efficiency dropped off precipitously (<10-fold). Changing the ratios of the other reprogramming factors also influenced the frequency of reprogramming. Increasing the MOI of Sox2, Klf4 or c-Myc individually, while holding MOI of each of the other viruses constant, caused a pronounced decrease in reprogramming efficiency. However, in contrast to elevating their levels, lowering the MOI of Sox2, Klf4 or c-Myc approximately 3-fold had little, if any, effect on the efficiency of reprogramming.

Although it is evident that efficient reprogramming for a given cell type requires the optimal ratio of the reprogramming factors, different ratios are likely to be needed for other cell types. A good case in point is the reprogramming of neural progenitor cells. These cells already express Sox2 and can be reprogrammed without exogenous expression of Sox2. In fact, excessive levels of Sox2 decrease reprogramming efficiency of neural progenitor cells (65). This is not surprising given that elevated levels of Sox2 have been shown to trigger the differentiation of ES cells (66). The same is true for Oct4 (67). For both Sox2 and Oct4, elevating their levels 2-fold, or even less, is sufficient to promote the differentiation of ES cells. Nanog and Lin28 levels can also be expected to influence reprogramming and the properties of the reprogrammed cells. Overexpression of Lin28 in human ES cells enhances their differentiation when plated at clonal cell densities (68), and elevating the levels of Nanog in mouse ES cells eliminates their need for LIF (69). Thus, when viewed in the larger context, there appears to be a strong relationship between the efficiency of reprogramming and the expression levels and ratios of the reprogramming factors. We propose that this explains, at least in part, the finding that reprogramming efficiencies are low. More specifically, given the stochastic expression of reprogramming factors in each cell within the starting somatic cell population, it is not surprising that reprogramming efficiency is relatively low, because of the low probability of achieving the correct levels and ratio of reprogramming factors in any given cell.

Early and Late Reprogramming Events

As noted above, reprogramming of somatic cells is a slow process. The reasons for this remain unclear. Several studies have focused on the timing of key events that occur during reprogramming, and progress is being made in understanding the interrelationship between early and late events. The minimal time for full reprogramming of mouse somatic cells (between 8 and 12 days) has been defined most clearly using drug-inducible lentiviral vectors (22,70). The use of drug-inducible lentiviral vectors is also beginning to provide insights into some of the most basic mechanistic questions surrounding somatic cell reprogramming. Notably, does full reprogramming typically follow a common sequence of events or does reprogramming largely follow a stochastic path that varies even within a given cell type? Although this question has not been fully resolved, several studies have provided evidence that there is sequential activation of different pluripotency markers. For example, the pluripotency markers SSEA-1, which is expressed by mouse pluripotent stem cells, and alkaline phosphatase (AP), which is expressed by pluripotent stem cells, appear in a fraction of the cells during the early stages of reprogramming (22,70). Moreover, both AP

and SSEA-1 appear long before the cells undergoing reprogramming become independent of virally expressed reprogramming factors (Figure 3).

As reprogramming progresses, additional properties of pluripotent stem cells begin to appear. Reactivation of the inactive X chromosome, and reactivation of telomerase activity occur during the later stages of reprogramming, near the time that viral expression of the reprogramming factors is no longer required (70). Recently, the critical role of telomerase activity has been demonstrated using telomerase null mouse embryonic fibroblasts (71). As expected, the frequency of generating iPS cells decreases as the generation (G1, G2, etc.) from which the telomerase null MEFS were isolated increases. Other critical pluripotency markers, in particular reactivation of the endogenous Oct4, Nanog and Sox2 genes, also occur late in the reprogramming process. An important question that will require further study is the temporal relationship between these late stage events and whether one depends on the other.

Interestingly, recent studies from two groups have directly implicated Oct4, Sox2 and Nanog in the regulation of XCI. Previous work has shown that female mouse ES cells not only shut off Oct4, Sox2 and Nanog expression when they undergo differentiation, but they also inactivate one of their X chromosomes. XCI is regulated by the X-inactivation center, a locus that contains at least three genes, Xist, Tsix and Xite, which produce non-coding regulatory RNAs. Expression of Xist plays a key role in XCI, whereas expression of the Tsix gene, which overlaps the Xist gene, codes for a non-coding antisense RNA that helps repress Xist expression. Recently, Donohoe *et al.* demonstrated Oct4 and Sox2 binding to the Tsix gene and the Xite gene by chromatin immunoprecipitation (72). They also identified an Oct4 binding site in the Tsix gene, as well as adjacent binding sites for Sox2 and Oct4 in the Xite gene. This is significant, because adjacent binding sites for Sox2 and Oct4 (known as HMG/POU cassettes) have been found in multiple genes that are tightly regulated by Sox2 and Oct4, and many of these genes play key roles during embryogenesis, as well as in the self-renewal and pluripotency of ES cells (73).

The connection between the regulation of X-inactivation and Oct4, Sox2, and Nanog appears to be even tighter. An earlier report by Navarro *et al.*, using chromatin immunoprecipitation, demonstrated that Oct4, Sox2 and Nanog are bound to intron 1 of the Xist gene prior to its activation (74). When female ES cells undergo differentiation, XCI occurs and the binding of Oct4, Sox2 and Nanog to the Xist gene is depleted. These and other results, discussed more fully elsewhere (75), have led to the proposal that Oct4 and Sox2, in the absence of Nanog, contribute to the repression of Xist, whereas Nanog plays a key role in initiating the silencing of Xist and reactivation of the inactive X-chromosome. If this model is correct, especially regarding the initiating role of Nanog, it could help explain why X-chromosome reactivation occurs during the later stages of somatic cell reprogramming, especially when using reprogramming protocols that employ the cocktail of Oct4, Sox2, Klf4 and c-Myc. Using this set of four reprogramming factors, reactivation of the endogenous Nanog gene and X-chromosome reactivation both occur late during reprogramming (70). Thus, it would be interesting to determine whether X-chromosome reactivation, or at the very least repression of Xist, would occur much earlier if one were to use the Oct4, Sox2, Nanog and Lin28 reprogramming protocol.

Another important event that appears to occur relatively late during the process of reprogramming is remodeling of the machinery that regulates cell cycle control, in particular the G1 checkpoint. It has long been known that mouse ES cells and embryonal carcinoma cells, unlike somatic cells, lack an important G1 checkpoint. However, when these cells differentiate, the cell cycle structure is altered and the G1 checkpoint is established. Consequently, the cell cycle structure present in stem cells needs to be reestablished during

reprogramming of somatic cells. Interestingly, it has been argued that remodeling of the cell cycle structure has not occurred in partially reprogrammed cells (52). Thus far, the mechanisms responsible for remodeling of the cell cycle structure have not been defined. However, the levels of c-Myc and the phosphorylation status of Rb are likely to be involved. Clearly, more work on this important issue is warranted. Readers interested in a more in-depth discussion of this topic are referred to a recent review by Singh and Dalton (52).

In an effort to understand the timing of key events, it will also be important to determine when, and eventually how, the relatively open chromatin structure of pluripotent stem cells is established. An open chromatin structure (euchromatin) is a hallmark of pluripotent stem cells, which allows transcription factors to activate a large battery of genes needed to maintain pluripotency or to prepare genes (e.g. bivalent genes) for rapid activation when stem cells differentiate into specific developmental lineages. As stem cells commit to various differentiated fates, unnecessary chromatin regions are closed (heterochromatin), and the genes within these regions are inactivated. Open chromatin is associated with a variety of histone markers, including histone 3, lysine 4 di- or tri-methylation (H3K4me2, H3K4me3, respectively). Chromatin remodeling proteins, such as Chd1, associate with these histone markers and maintain chromatin in an open, accessible state by interfering with histone modifying complexes (e.g. NuRD complex and histone demethylases, respectively) (76-78). Interestingly, Gaspar-Maia *et al.* demonstrated that knockdown of Chd1 by RNAi, decreases the efficiency of somatic cell reprogramming to iPS cells (79). As discussed below, the remodeling of chromatin and the demethylation of pluripotency genes (e.g. Oct4 and Nanog) do not appear to occur at a single time point during reprogramming.

Partially Reprogrammed/Intermediate Cell Populations

Other events that occur during the later stages of reprogramming can be deduced from the analysis of partially reprogrammed cells. Mikkelsen *et al.* performed a detailed analysis on several partially reprogrammed clonal cell lines (e.g. MCV8) (31). MCV8 cells express some pluripotency markers (e.g. 20-30% of the cells express SSEA-1); however, critical endogenous genes (Oct4, Sox2 and Nanog) remain inactive. Analysis of the histone modifications associated with specific genes indicates that chromatin remodeling has occurred on some genes (e.g. FGF4) in the partially reprogrammed cells, whereas other genes (e.g. Nanog) have not been remodeled (31). Thus, chromatin remodeling appears to be a gradual process that takes place over an extended period of time. This also appears to be true for the remodeling of DNA methylation. Analysis of the methylation status of the Oct4 and Nanog genes (their promoters are methylated in somatic cells and demethylated in iPS cells and ES cells) indicates that these promoters remain largely methylated in the partially reprogrammed cells. Interestingly, a number of other genes, which are demethylated in iPS cells and ES cells (UTF1, Dppa5, and Gdf3), remain methylated in the partially reprogrammed cells; whereas, the FGF4 gene, which is demethylated in iPS cells and ES cells, is also demethylated in partially reprogrammed cells. These findings lead to the obvious conclusion that demethylation of critical pluripotency genes occurs during the later stages of reprogramming. What remains to be determined, is how remodeling of these genes occurs. Recent work linking Oct4 and Dnmt3b offers some promising leads (80).

The fact that clonal lines of partially reprogrammed cells can be isolated raises several interesting questions. In particular, does full reprogramming of somatic cells normally proceed through intermediate cell populations, or do these stable intermediates represent a non-productive endpoint? The early expression of some, but not other, pluripotency markers suggests that reprogramming proceeds through cellular intermediates. This possibility is further supported by the effects of the DNA methyltransferase inhibitor, AZA. Specifically, partially reprogrammed cells exposed to AZA have been shown to lead to reactivation of the endogenous Oct4 gene and to the generation of cells that are capable of producing teratomas

containing cells from each of the three embryonic germ layers. Importantly, if in fact reprogramming proceeds through several intermediate stages, this would account for the slow time course for full reprogramming. More specifically, if each stage of reprogramming takes a day or more, which seems very likely, it is not surprising that full reprogramming requires at least a week to occur.

FUTURE PERSPECTIVES

Progress in our understanding of iPS cells has generated as many questions as answers. We suggest that the answers to three broad questions will heavily influence the long-term impact of iPS cell on regenerative medicine. First, how do reprogramming factors act mechanistically? Obtaining this information could lead to significant improvement in the efficiency of generating iPS cells. More importantly, this information could provide insights into how to avoid damage to the cells and how to best assess the quality of iPS cells. Thus far, few studies have focused on the mechanisms involved in reprogramming. However, rapid progress on this question is likely to be forthcoming in the very near future. During the past decade, work in ES cells has provided extensive knowledge of the roles played by reprogramming factors in maintaining pluripotency. For example, the target genes of Oct4, Sox2 and Nanog in ES cells have been identified by ChIP-chip and ChIP-seq analyses (81-83). This, in turn, has produced testable hypotheses regarding key gene regulatory networks regulated by these transcription factors. Furthermore, there is a growing body of proteomic data collected from ES cells, which is beginning to identify a wide range of nuclear proteins that associate with Oct4, Sox2 and Nanog (84,85). Undertaking parallel studies with somatic cells during the early stages of reprogramming will help dissect the molecular mechanisms responsible for rewiring the gene regulatory networks active in somatic cells into those required to support the self-renewal and pluripotency of iPS cells.

A second important question is whether human iPS cells can be directed effectively to cell types that are suitable for cell replacement therapy? A great deal of effort is underway to use iPS cells to develop preclinical models, in particular those that seek to direct the differentiation of iPS cells to clinically useful cell types. However, there are significant obstacles to overcome. Besides the obvious need to ensure that all iPS cells have been eliminated from any cells to be transplanted, it will be crucial to determine that iPS-derived cells have not been genetically damaged either during the process of generating patient-specific iPS cells or during the process of directing their differentiation to suitable cell types. In addition to the possibility of genetic damage, the epigenetic status of cells, such as DNA methylation and chromatin structure, will need to be assessed, since this could affect both the safety and/or function of the transplanted cells. If the process of directing iPS cells to desired cell types involves many steps, which at best can only partially mimic the steps that occur *in vivo*, it may be challenging to produce the correct epigenetic state of iPS-derived cells. In this regard, it will be difficult to assess whether the correct epigenetic state of iPS-derived cells has been achieved, unless the epigenetic status of their normal *in vivo* counterparts has already been established.

A third important question to ask as the field of reprogramming moves forward is whether it is necessary, in all cases, to reprogram cells back to a pluripotent stem cell state. Specifically, would it be sufficient, in certain cases, to reprogram to an adult stem cell population more closely related to the cells needed in cell replacement therapies? This could have important advantages. For example, reducing the extent to which the cells need to be reprogrammed could reduce the potential for genetic damage. While this is a very attractive idea, and there is evidence to support it (86), it may be far from simple to achieve. For starters, we will need to determine how to reprogram the cells to appropriate cell populations. The strategy of Takahashi and Yamanaka (2006) provides a conceptual

blueprint for how to proceed. However, to do this will also require that we know the characteristics of the desired cell population. In the case of iPS cells, we already had extensive knowledge of the desired endpoint—for iPS cells the gold standard was ES cells. Unfortunately, for many clinically interesting cell populations, knowledge of a desired endpoint is fragmentary at best. Thus, unless care is taken, the cell generated may have some, but not all, of the properties needed for successful clinical outcomes. The potential for generating imperfect cellular look-alikes (cellular mimicry) is the subject of a recent review (87).

In conclusion, the discovery that somatic cells can be reprogrammed to a pluripotent stem cell state has ushered in new and exciting opportunities in the field of regenerative medicine. Given the high level of interest in iPS cells and the large number of laboratories around the world that are now studying these cells, we can anticipate new developments at an even faster pace than that of the past three years. Perhaps the most exciting question is: what's next? More specifically, what will be the next paradigm shift brought about by studying somatic cell reprogramming and iPS cells?

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Figure 1.

Reprogramming of somatic cell to iPS cells. Examples of reprogramming factors are provided, along with characteristics of a typical starting somatic cell and those of an iPS cell.

Figure 2.

Model of reprogramming inhibition mediated by p53 and miR-145. p53 drives the processing of immature miR-145 to mature, functional miR-145. miR-145 is known to impede the expression of endogenous reprogramming factors Sox, Oct4 and Klf4. Inefficient production of endogenous reprogramming factors interferes with the formation of iPS cells.

Figure 3.

Sequence of progression of molecular and cellular events that occur during the reprogramming of somatic cells to iPS cells. The sequence of events shown, especially those that take place during the latter stages of reprogramming, have not been firmly established.