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Inducible pluripotent stem cells: Not quite ready for prime time?

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Abstract

Purpose of review—Inducible pluripotent stem (iPS) cells derived from somatic cells represent a novel renewable source of tissue precursors. The potential of iPS cells is considered to be at least equivalent to that of human embryonic stem (hES) cells, facilitating the treatment or cure of diseases such as diabetes mellitus, spinal cord injuries, cardiovascular disease, and neurodegenerative diseases, but with the potential added benefit of evading the adaptive immune response that otherwise limits allogeneic cell-based therapies. This review discusses recent advances in pluripotency induction and the use of iPS cells to produce differentiated cells, while highlighting roadblocks to the widespread use of this technology in the clinical arena.

Recent findings—Whereas ethical and safety issues surrounding the use of human ES cells for the treatment of disease continue to be debated, use of iPS cells may be viewed as a more widely acceptable compromise. Since the first descriptions of inducible pluripotency from somatic cells, multiple laboratories have collectively made tremendous strides both in developing alternative, more clinically acceptable, induction strategies and in demonstrating the proof-of-principle that iPS cells can be differentiated into a variety of cell types to reverse mouse models of human disease.

Summary—Although the prospect of using patient-specific iPS cells has much appeal from an ethical and immunologic perspective, the limitations of the technology from the standpoint of reprogramming efficiency and therapeutic safety necessitate much more in-depth research before the initiation of human clinical trials.

Keywords

Induced pluripotency; stem cells; somatic reprogramming; regenerative medicine; tissue engineering

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Introduction

The long-standing goal of regenerative medicine is to replace diseased cells and organs using a renewable source of pluripotent cells. Cells derived from the human blastocyst (embryonic stem, or ES, cells) or somatic cell nuclear transfer (SCNT) (“therapeutic cloning”) offer great potential in this regard, but are also mired in a vociferous ethical public debate that hampers ongoing funding and scientific progress. Nonetheless, significant strides have been made in the ability to convert both rodent and human ES cells into a variety of differentiated cell types with the ability to mitigate or even reverse animal models of disease [1]. However, assuming that the current ethical and scientific barriers to the use of ES cells are surmountable, their eventual utility would be dependent upon effective therapies to overcome adaptive immune responses, since such cells are of allogeneic origin. Alternatives to the use of pluripotent ES cells have also been actively studied in recent years. Notably, pluripotency may not be a prerequisite for the generation of certain differentiated cell types, and therefore autologously derived cells of non-embryonic origin (such as endothelial colony forming cells or adipose stromal cells) may serve as adequate sources for regenerative therapy. The accompanying articles in this *Stem Cells Special Issue* address the use of both ES and non-ES cells in a variety of disease states, including cardiovascular disease, diabetes, bone disease, and wound healing, thereby highlighting the clinical potential of these cell types.

Because all cells of an organism derive from a common pluripotent stem cell, the strategy of taking a pluripotent cell *in vitro* and directing its conversion into a specific differentiated cell fate represents a rational and ongoing approach in regenerative medicine. Although incomplete, our improved understanding of the phenotypes and markers that represent pluripotency has challenged researchers to find ways to essentially *reverse* the differentiation process, thereby allowing the reprogramming of somatic cells to their pluripotent origins. These so-called induced pluripotent stem (iPS) cells would have profound implications for both basic research and clinical therapeutics by providing a patient-specific model system to study the pathogenesis of disease and test the effectiveness of pharmacological agents, as well as by providing an ample source of autologous cells that could be used for transplantation (Fig. 1). In this review, we summarize the current strategies for pluripotency induction, discuss the current state of iPS cell differentiation, and consider both the promise and limitations of this technology for treating human disease.

Methods for cellular reprogramming

The field of iPS cells has seen a number of major milestones in the last few years. In 2006, Takahashi and Yamanaka systematically screened 24 different genes believed to be essential for the maintenance of pluripotency and self-renewal in ES cells, and reported the landmark discovery that somatic cells could be reprogrammed to a pluripotent state using retroviral transduction of only 4 of these genes and subsequent selection using the promoter of the ES cell specific, but non-essential, pluripotency gene *Fbx15* [2]. These 4 genes, termed the “Yamanaka factors,” include *Sox2* (Sex determining region Y-box 2), *Oct4/Pou5f1* (Octamer binding transcription factor 4/POU class 5 homeobox 1), *c-Myc* (cellular myelocytomatosis oncogene), and *Klf4* (Krüppel-like factor 4). Although these cells exhibited phenotypic characteristics of ES cells and formed teratomas when injected subcutaneously into nude mice (and therefore capable of differentiating into all three germ layers), they exhibited significant genetic/epigenetic differences compared to murine ES cells and failed to produce germline transmissible chimeric mice [2]. Interestingly, these differences compared to true ES cells appeared to result from the use of *Fbx15* as a selection marker for pluripotency. When promoters of alternative, essential, pluripotency genes were used as selection markers (*Nanog* or *Oct4*), several groups were able to generate iPS cells

more closely resembling ES cells in their capacity for teratoma formation and germline transmissible chimera formation from mouse somatic cells [3–6] and teratoma formation from human somatic cells [7–10].

Whereas these original cellular transduction approaches using retroviral and/or lentiviral vectors established proof-of-principle for induced pluripotency, at least two safety concerns limit the clinical utility of these approaches. First, virus-mediated transgene integration may lead to mutagenesis at the insertion site or to the altered expression of surrounding genes such that cellular dysfunction or tumorigenesis may result. To address this concern, non-integrative approaches, such as use of adenoviruses [11] or repeated transfections with plasmid vectors [12], were attempted with some success. In another approach, Kim *et al.* [13] generated fusion proteins in which each of the 4 reprogramming factors was fused to a cell-penetrating peptide (nonameric Arg) sequence, thereby enabling it to cross the cellular membrane within as little as 8 hours. However, these methods may require repeated rounds of treatment and suffer from reprogramming efficiencies that are up to 1000-fold lower than with retroviral vectors.

A second safety concern lies in the potential oncogenicity arising from the continued expression of the exogenous genes introduced for reprogramming. For example, the high frequency of tumor formation in chimeric mice derived from iPS cells is thought to arise from the persistent expression of the *c-Myc* gene in the reprogrammed cell population [4]. Given that the reprogramming process involves the activation of endogenous pluripotency genes and eventual silencing of exogenous genes, one may achieve successful reprogramming simply via transient expression. More recently, therefore, there has been a distinct push towards non-viral delivery methods that involve only transient expression of genes in a non-integrative manner, which generate iPS cells free of the exogenous genes used in the reprogramming process. Using a moth-derived transposon system (*piggyBac*) that allowed for transposase-mediated excision of exogenous genes, two separate groups have generated iPS cells from mouse fibroblasts that were void of the exogenous factors [14, 15]. The use of either a single doxycycline inducible [14] or constitutively active [15] self-cleaving polycistronic plasmid containing all four transgenes (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) simplified the delivery method and, more importantly, resulted in an induction efficiency equivalent to that seen with retroviral delivery. Using a similar polycistronic approach and the Cre/LoxP excision system, mouse [16] and human fibroblasts [17] have been reprogrammed and complete removal of the exogenous factors has been reported. Researchers have also sought ways to eliminate one or more of the reprogramming factors by substitution with small molecules [18], or by elimination of *c-Myc* (a pro-tumorigenic factor) from the vector cocktail [19–22]. Such strategies hold great promise, but clearly require further optimization to enhance reprogramming efficiency.

Much of the aforementioned work on improving pluripotency induction has been conducted in either murine or human embryonic fibroblasts, which are mesoderm-derived. In principle, however, iPS cell induction should apply to cell sources regardless of their embryonic origin. Table 1 lists other cell sources that have been used for iPS cell induction, and suggests that sources from all three embryonic layers (mesoderm, endoderm, and ectoderm) can give rise to iPS cells using the same or similar cocktail of reprogramming factors.

Possible mechanisms of iPS induction by transcription factors

Although all four Yamanaka transcription factors reprogram somatic cells to iPS cells with higher efficiency than any one factor alone, they may not be absolutely required for reprogramming [7, 19, 20]. For example, *Klf4* and *c-Myc* can be substituted by *NANOG* and *LIN28* in the reprogramming of human fibroblasts to iPS cells [7]. This finding and

others suggest either that distinct molecular pathways governed by each transcription factor converge to activate a common reprogramming mechanism or that some of the factors have functions that can be mimicked by others. Interestingly, in support of the latter hypothesis, both *c-Myc* and *LIN28* have been reported to repress transcription of certain microRNAs, especially members of the let-7 family [32, 33]. Thus, it is possible that negative regulation of certain microRNAs, and even positive regulation of others (e.g. miR-291-3p, miR-294 and miR-295) [34] could contribute to cellular reprogramming by transcription factors. Emerging evidence suggests that factors such as c-Myc and Klf4 may function to regulate the balance between proliferation and senescence/apoptosis during the cellular reprogramming process [35–37], thus creating an optimal environment for Sox2 and Oct4 to activate and/or suppress the appropriate genes to execute cellular reprogramming.

Use of small molecule epigenetic modulators for efficient cellular reprogramming

To ensure correct reprogramming, a somatic cell has to undergo appropriate changes to its chromatin structure (i.e. “epigenetic” changes) to mimic the state of the ES cell “epigenome” [35, 38, 39]. Completely reprogrammed cells, among other things, should demonstrate DNA demethylation of the *Oct4* and *NANOG* promoter regions, exhibit reactivation of somatically inactivated X-chromosome in female cells, and display global patterns of histone modifications within developmental genes. In this regard, prior studies of somatic cell nuclear transfer-mediated cellular reprogramming revealed a modest improvement in reprogramming efficiency by altering the state of DNA methylation and by use of histone deacetylase (HDAC) inhibitors [40–42]. These findings therefore prompted the use of small-molecule inhibitors in transcription factor-induced reprogramming. Indeed, compounds that induce chromatin modifications by affecting either DNA methylation or histone acetylation greatly improve reprogramming efficiencies of both mouse and human somatic cells [18, 43]. Notably, the HDAC inhibitor valproic acid (VPA) not only improves reprogramming efficiency by 50 to 100-fold in the presence of either three or four of the transcription factors both in mouse and human cells [18, 43], but it also allows for efficient reprogramming of human somatic cells to iPS cells with only two of the four Yamanaka factors, *Oct4* and *Sox2* [18]. Given the fact that VPA is already FDA approved for use in patients with epilepsy, use of this agent to enhance reprogramming efficiency with fewer exogenous factors may prove helpful in finding more clinically acceptable protocols for generating patient-specific iPS cells [44]. In addition to the use of small molecules as epigenetic modulators, there may be a role for small molecules that specifically inhibit cellular differentiation-associated pathways. Recently, use of inhibitors of the extracellular signal-related kinase (ERK) and glycogen synthase kinase 3 (GSK3) proteins have been shown to facilitate efficient reprogramming of neural stem cells into iPS cells [45].

Directed differentiation of iPS cells

Although much attention has focused on how to improve ease, safety, and efficiency of pluripotency induction, there has also been significant progress in the directed differentiation of iPS cells to specific cell types. Much of the research in directed differentiation attempts have relied on protocols that have proven successful in ES cells, thereby underscoring the importance of ongoing research in stem cells of embryonic origin. The majority of these studies utilized iPS cells generated from embryonic fibroblasts using retro- or lentiviral delivery of either the Yamanaka factors (*Sox2*, *Klf4*, *Oct4*, and *c-Myc*) or a slightly modified version described by Thomson and colleagues (*Oct4*, *Sox2*, *NANOG*, and *LIN28*) [7]. A particularly promising example of directed differentiation was described by Zhang and colleagues, who showed it was possible to generate glucose responsive pancreatic islet-like cell clusters from human skin fibroblast-derived iPS cells [46]; this

study provides optimism that autologous somatic cells could one day be used to treat individuals with diabetes. In another landmark study, Dimos *et al.* [47] demonstrated that skin cells from patients with amyotrophic lateral sclerosis (ALS) could be reprogrammed and subsequently differentiated into disease-free motor neurons. The finding that such reprogramming could be achieved using cells from elderly individuals assuaged concerns over whether cellular ageing and long-term environmental exposure hindered the induction and directed differentiation processes. Also of note, Ebert and colleagues [48] were the first to demonstrate a preserved patient-specific disease phenotype in motor neurons generated from primary fibroblast-iPS cells obtained from a patient with spinal muscular atrophy. Treatment of these cells *in vitro* with valproic acid and tobramycin led to an upregulation of survival motor neuron protein synthesis, as expected, and displayed selective deficits when compared to normal motor neurons. This finding suggests that patient-specific iPS cells can be used to study patient-specific disease processes *in vitro*, prior to initiation of specific drug therapies. Other studies have recently appeared, describing the directed differentiation of iPS cells into a diversity of functional cell types, as detailed in Table 2.

Reversal of disease: Use of iPS cells to treat mouse models of disease

Several recent studies have tested the hypothesis that differentiated iPS cells can be used to rescue organ function in mouse disease models. In a seminal study, Hanna, *et al.* [61] demonstrated that autologous somatic cells could be used to cure disease in a humanized mouse model of sickle cell anemia. In this study, the authors reprogrammed autologous fibroblasts to iPS cells, genetically corrected the defective β -globin locus, and then differentiated the iPS cells into disease-free transplantable hematopoietic cells, which corrected the underlying disease in mice. More recently, several other groups have demonstrated the therapeutic potential of iPS cells, both alone and in combination with genetic corrective therapy. These include the generation of patient-specific, disease-free hematopoietic progenitors from keratinocytes obtained from patients with Fanconi anemia [62], the correction of hemophilia in mice using iPS cell-derived endothelial progenitors [63], the correction of Parkinson disease in rats using iPS cell-derived dopaminergic cells [50], and the multi-lineage functional repair of diseased heart tissue in immunocompetent mice using undifferentiated iPS cells [69].

Conclusions: The future of induced pluripotency in regenerative medicine and organ transplantation

The field of iPS cell research has seen dramatic advances since the original description of the Yamanaka factors in 2006. To date, iPS cells appear to satisfy virtually all criteria of true pluripotent cells, including unique cellular morphology, gene expression profiles similar to that of ES cells, the ability to form teratomas *in vivo*, and the capacity to form germline transmissible chimeras upon injection into blastocysts. Most recently, completely iPS cell-derived viable mouse embryos were generated through tetraploid complementation [66–68], with one group reporting the development of fertile adult mice [68]. Compared to ES cells, the primary advantage of iPS cells is that they can be derived from the same individual for whom disease treatment is being sought (Fig. 1). Thus, iPS cells are not subject to the same ethical issues that have plagued ES cell research, and moreover, their use may obviate the need for immunosuppression upon transplantation. However, several limitations remain that raise some concerns for their potential to be translated into a cell-based therapy. First and foremost, safety concerns persist. Aside from the worries over the use of viral vectors for cellular transduction, there are also concerns that the factors required for reprogramming may lead to tumorigenicity (possibly malignant) of the iPS-derived cells upon transplantation, or that incomplete/aberrant reprogramming may result in teratoma formation at later stages. In this regard, even a small number of undifferentiated iPS cells present in the

differentiated population can form teratomas in the transplanted tissues [70] (similar to ES cell concerns). This concern raises the need for a robust assay system to ensure that no undifferentiated iPS cells are present in the final cell population before transplantation. Studies by Wernig, *et al.* showed that addition of selection protocols to deplete differentiated populations of pluripotent precursors could significantly reduce teratoma formation [50]. Cellular immortalization, a process that would augment iPS cell propagation, can be greatly enhanced by disrupting the p53 pathway [71–73]—a strategy that increases even more the concern for tumor formation by cells derived from iPS cells induced in this manner. Other limitations to iPS cell technology include the poor efficiency of iPS cell formation and the concern that the underlying genetic abnormalities that led to disease in the first place may still be inherent in the iPS-derived cell types (particularly relevant to autoimmune diseases such as diabetes). Thus, the question arises whether iPS cell research can or should supplant ES cell research. Notwithstanding the great achievements in iPS cell research over the past 3 years, it remains in its infancy and much better strategies for pluripotency induction are required before viable therapeutic strategies are foreseeable.

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Figure 1. Use of autologously derived inducible pluripotent stem (iPS) cells to reverse human disease

The figure describes how iPS cells could be derived from somatic cells of an individual with disease, then be used to generate a desired functional cell type that would be transplanted back into the same individual to correct the underlying disease.

Table 1

Various sources for inducible pluripotent stem cell generation.

Cellular Sources	Method for Pluripotency Induction
Murine Islets of Langerhans [23]	Lentiviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i>
Murine adult neural stem cells[24, 25]	Retroviral delivery of either 4 factors (<i>Oct4</i> , <i>Sox2</i> , <i>c-Myc</i> , <i>Klf4</i>), various combinations of 3 factors (<i>Oct4</i> , <i>Klf4</i> , and <i>c-Myc</i> , <i>Oct4</i> , <i>Klf4</i> and <i>c-Myc</i> , and <i>Oct4</i> , <i>c-Myc</i> , and <i>Sox2</i>), and even 2 factors (<i>Oct4</i> and <i>c-Myc</i> or <i>Oct4</i> and <i>Klf4</i>)
Murine adult liver cells [26]	Retroviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i>
Murine adult stomach cells [26]	Retroviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i>
Adult rhesus monkey fibroblasts [27]	Retroviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i>
CD34+ hematopoietic progenitor cells [28]	Retroviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i>
Porcine embryonic fibroblasts [29]	Retroviral delivery of both human and mouse <i>Sox2</i> , <i>Klf4</i> , <i>c-Myc</i> , <i>Oct4</i>
Porcine fetal fibroblasts [30]	Lentiviral delivery of human <i>Sox2</i> , <i>Klf4</i> , <i>c-Myc</i> , <i>Oct4</i>
Immature B lymphocytes[31]	Lentiviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , <i>c-Myc</i>
Mature B lymphocytes[31]	Lentiviral delivery of <i>Oct4</i> , <i>Sox2</i> , <i>Klf4</i> , and <i>c-Myc</i> followed by overexpression of <i>C/EBPα</i> or knockdown of <i>Pax5</i>

Table 2

The differentiation capacity of iPS cells.

Successful Directed Differentiation
Insulin-secreting Islet-like clusters [46]
Motor neurons [47–49]
Dopaminergic neurons [50]
Auditory spiral ganglion neurons[51]
Smooth muscle cells [52, 53]
Vascular Endothelial cells [53]
Cardiomyocytes [22, 54–57]
Dendritic Cells and Macrophages [58]
Adipocytes [59, 60]
Osteoblasts [60]
Hematopoietic cells [61, 62]
Endothelial progenitor cells[63]
Retinal Progenitors and Photoreceptors [21, 64]
Retinal pigmented epithelium [21, 65]
Viable Mice [66–68]