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Review

# Nuclear Reprogramming by Defined Factors: Quantity Versus Quality

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**The generation of induced pluripotent stem cells (iPSCs) and directly converted cells holds great promise in regenerative medicine. However, after in-depth studies of the murine system, we know that the current methodologies to produce these cells are not ideal and mostly yield cells of poor quality that might hold a risk in therapeutic applications. In this review we address the duality found in the literature regarding the use of 'quality' as a criterion for the clinic. We discuss the elements that influence reprogramming quality, and provide evidence that safety and functionality are directly linked to cell quality. Finally, because most of the available data come from murine systems, we speculate about what aspects can be applied to human cells.**

## iPSCs and Directly Converted Cells in Regenerative Medicine

Cell and organ transplantation is the conventional medical treatment for lost/damaged cells or tissues and for end-stage organ failure. However, the field of regenerative medicine is redefining how transplantation occurs, by growing cells, tissues, and organs in the laboratory and implanting them into patients [1]. One of the most attractive cell types for regenerative medicine is embryonic stem cells (ESCs) because they are capable of long-term growth, self-renewal, and can give rise to every cell type [2]. However, two major bottlenecks to realizing such potential are allogenic immune rejection of ESC-derived cells by recipients and ethical issues involving the destruction of a 'live' embryo.

The discovery that murine and human fibroblasts can be converted into stable and fully functional embryonic stem-like cells, termed **induced pluripotent stem cells** (iPSCs, see Glossary), by the ectopic expression of **key master regulators** Oct4, Sox2, Klf4, and Myc (OSKM, also known as Yamanaka factors) [3,4] has encouraged scientists to look beyond ESCs for regenerative medicine, as well as to re-evaluate the terminology 'terminally differentiated state' and the notion of cellular plasticity [5]. Since their discovery, researchers have attempted to directly convert various adult cells to different cell types, by avoiding the pluripotent state, using a unique combination of cell type-specific key master regulators [6–8]. Several medically-relevant cell types have been generated, including hematopoietic cells [9,10], different neuronal cells [11–13], cardiomyocytes [14], hepatocytes [15,16], embryonic Sertoli cells [17], endothelial cells [18], neural crest cells [19], and pancreatic β cells [20]. Furthermore, the first clinical trials using iPSC technology have been launched [21,22]. However, despite remarkable progress in characterizing the reprogramming process and the resulting iPSCs and directly converted cells [23–27], it remains to be seen if these converted cells are safe and of sufficiently high quality to warrant their immediate use in the clinic.

Theoretically, iPSCs and **directly converted** cells are ideal for regenerative medicine and for disease modeling [28–31]. In contrast to ESCs, their use does not involve ethical issues and,

## Trends

Despite the great promise that iPSCs and directly converted cells hold for regenerative medicine, concerns regarding the safety and functionality of these cells currently hold back their use in the clinic.

Many criteria affect the quality of the converted cells, such as genome integrity, complete somatic epigenetic erasure, histone deposition, and expression of long terminal repeats of endogenous retroviruses.

While the quality of murine iPSCs can best be assessed by their ability to form 'all iPSC' mice, this assay is not practicable for human iPSCs, thus highlighting the need for other quality control measures.

The choice of reprogramming factors and their stoichiometry, the use of non-integrating agents, and specific culture conditions provide routes by which iPSC quality may be improved.

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because they can be derived from patients, they should not be rejected by the host [32]. However, rigorous functional assays in the mouse system show that, unlike ESCs – which are relatively uniform in their differentiation capacity – the quality of iPSCs varies widely between different colonies [33]. Some colonies can contribute to chimeras but are unable to generate a healthy 'all-iPSC' mouse using the stringent pluripotency assay, **tetraploid complementation** (4N), while others may differentiate to the three germ layers *in vitro* but do not contribute to the embryo *in vivo* [34–37]. These experimental differences clearly demonstrate a significant gap in the quality of the various iPSC colonies in mice. Furthermore, many mouse assays such as the 4N test and **chimera contribution** are not applicable with human iPSCs (hiPSCs), highlighting the need for alternative and reliable quality measures for testing hiPSCs.

Assessing quality in direct conversion models is more problematic because many of them do not reach a stable and complete reprogramming state [8,38,39]. While iPSCs can grow independently of exogenous factors and are almost indistinguishable in their epigenome and transcriptome to their ESC counterparts, in the vast majority of direct conversion models the converted cells express only a fraction of the relevant markers and are dependent either fully or partially on their transgenes (Table 1) [8,38,39]. This observation raised the possibility that a high nuclear resetting state can be achieved only in stem cell populations. However, an incomplete reprogramming process was also noted in the generation of neuronal stem-like cells [40,41] and hepatic stem-like cells [42]. Taken together, these data suggest that the currently prevailing technology to reset the somatic nucleus by a defined number of factors is not ideal and mostly yields cells of poor quality. This is not surprising given that a normal reprogramming process (i.e., the reprogramming of a sperm nucleus by an egg) involves a large number of proteins that are present within the cytoplasm of a fertilized oocyte [43] as well as a unique nuclear chromatin condensation and epigenetic state of the sperm that make it adequate for early embryonic development [44]. Nevertheless, does cell quality really matter? After all, we are not trying to clone a human being, but instead to generate safe and functional cells for therapeutic applications.

In this review we focus on the current ability to analyze and compare the quality of murine iPSCs and directly converted cells, their validity, and the elements that affect the reprogramming quality. We address the duality found in the literature regarding the use of 'quality' as a parameter for the clinic and provide evidence that quality is directly linked to safety, stability, and functionality of the cells. Finally, we touch upon the limitations in assessing the quality of human cells and possible solutions.

Table 1. Properties of High-Quality iPSCs and Directly Converted Cell Types

| Criterion | iPSCs (Related to High-Quality Cells) | Directly Converted Cells (Related to Studies Described) |
|---|---|---|
| Transgene dependency | No | Yes (either fully or partially) |
| Activation of the entire endogenous circuitry | Yes | No or only partially |
| Functionality | Fully equivalent to ESCs | Partial |
| Show full rescue in mouse model | Yes | Partial |
| *De novo* mutations/copy-number variation | Yes, but still debatable | N/D[a] |
| 'Epigenetic memory' | Yes, very few loci | Yes, many loci |
| Transcriptome | Highly similar | Partially similar |
| Superenhancer activation | Yes | N/D |

[a]N/D, not determined.

## Glossary

**Chimera contribution:** an assay for pluripotency evaluation. ESCs/iPSCs are injected into blastocysts that are then transferred into pseudopregnant females. Developing embryos, pups, and mature mice are analyzed for the contribution of the cells. High-grade chimeras represent high-quality ESCs/iPSCs.

**Directly converted cell:** adult cell that uses a unique combination of cell type-specific key master regulators to undergo a specific nuclear reprogramming process to acquire the identity of a different cell type. The process avoids the pluripotent state.

**Epigenomic assembly:** a process that includes complete erasure of the epigenomic landscape of the donor cell followed by the acquisition of a new epigenome, including histone acetylation, methylation, and chromatin organization, that is similar to the targeted cell.

**Ground state:** the basal proliferative state of ESCs, which is free of epigenetic restriction and has minimal requirements for extrinsic stimuli. Cells in ground state are fully pluripotent and can generate an entire embryo.

**Hyperdynamic chromatin state:** a state of dynamic chromatin characterized by hypermobility of chromatin-associated proteins in pluripotent cells.

**Induced pluripotent stem cells (iPSCs):** somatic cells that underwent a nuclear reprogramming process to resemble ESCs, by introduction of a defined transcription factor combination such as Oct4, Sox2, Klf4, and Myc (OSKM); or Sall4, Nanog, Esrrb, and Lin28 (SNEL).

**Key master regulators:** potent cell type-specific transcription regulators that, when highly expressed in a parallel cell, can initiate a cellular program that alters cell fate.

**Somatic cell conversion models:** this term relates to all conversion experiments including directly converted cells and iPSCs.

**Somatic memory:** remnants of epigenome and transcriptome marks of the donor cells.

**Teratoma assay:** a teratoma is a nonmalignant tumor comprised of cells from all three embryonic germ-layers. In the teratoma assay, ESCs/iPSCs are implanted under the skin

## Elements Affecting Reprogramming Quality

Successful nuclear reprogramming depends on multiple components that together define the quality of the resulting cells. We discuss here several aspects regarding the reprogramming process that influence the resulting cells (Table 2). Because many of the directly converted cells represent a partially reprogrammed state, as indicated by transcriptome analyses [38,39], we primarily focus our attention on iPSCs, representing a relatively high degree of nuclear resetting.

### Level of Reprogramming Factors

Given the pro-cancerous role of Myc in malignant transformation [45], mouse and human cells have been reprogrammed in the absence of Myc (OSK-iPSCs) [46–48]. These mouse OSK-iPSCs have been extensively characterized and compared to their OSKM-iPSCs counterparts. However, depending on the method used to introduce the reprogramming factors, several groups have reported conflicting findings. Using non-integrating plasmids that produce relatively low levels of factors revealed that Myc is crucial for the generation of high-quality iPSCs as measured by chimeric formation and germline transmission [49]. Application of a retroviral system that yields intermediate levels revealed a decrease in the production of 'all-iPSC' mice in the absence of Myc [50], and a third study employed a doxycycline (dox)-inducible lentiviral system that generates a relatively high level of transgenes, and this gave a significant improvement in the quality of iPSCs that were generated by OSK, in comparison to OSKM-iPSCs, using the 4N assay [51]. High levels of reprogramming factors have been shown to rescue mouse pluripotency-refractory cells such as late reprogrammable Thy1-positive cells, Nanog knockout somatic cells, and pre-iPSCs [52–54]. Moreover, high expression of Oct4 and Klf4 combined with lower expression of Myc and Sox2 produce a low number of iPSCs, the majority of which generate 'all-iPSC' mice [55]. Because Myc is considered to be a global gene amplifier [56], it is tempting to speculate that the addition of Myc during the conversion process using non-integrating plasmids or retroviruses can facilitate complete reprogramming but induces genomic instability in systems that produce high levels of reprogramming factors. Indeed, in the dox-inducible lentiviral system, a unique chromosomal abnormality of chromosome 8 was detected in poor-quality iPSCs [51]. Recently, a unique, but artificial, pluripotency state has

of an immune-compromised mouse where they may proliferate and differentiate to form a teratoma, indicating pluripotency.

**Tet proteins:** a family of three members, Tet1, Tet2, and Tet3, that all have the capacity to convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), leading to DNA demethylation, and are therefore important regulators of cellular identity.

**Tetraploid complementation assay (4N):** a stringent assay in the murine model that tests ESCs/iPSCs quality. In this test ESCs/iPSCs are injected into tetraploid blastocysts generated by the fusion of two-cell embryos, producing cells with four sets of chromosomes (tetraploid cells) that can generate only the extraembryonic tissues but not the embryo itself. High-quality ESCs/iPSCs will integrate into the defective tetraploid inner cell mass (ICM) and will produce the entire mouse.

Table 2. Elements that Influence the Quality of the Reprogramming Process

| Elements Affecting Reprogramming | Elements Improving the Quality of Murine iPSCs | Ref. |
|---|---|---|
| *Culture conditions* | | |
| Medium | Knockout DMEM, 20% KSR | [109] |
| Oxygen level | Hypoxia | [110] |
| Supplements | Vitamin C (ascorbic acid) | [111] |
| | Histone deacetylase inhibitors | [65] |
| | Ascorbic acid and GSK3-β inhibitor (AGi) | [73] |
| | Inhibition of TGF-β together with activation of Wnt signaling in the presence of ascorbic acid | [112] |
| | Protein arginine methyltransferase inhibitor AMI5 and TGF-β inhibitor A-83-01 | [113] |
| *Reprogramming factors* | | |
| Levels | High Oct4, high Klf4, low Sox2, low Myc | [55] |
| Factor combination | Sall4, Nanog, Esrrb, Lin28 (SNEL) | [51] |
| | Tet1, Oct4 | [67] |
| | Oct4, Sox2, Klf4 | [50] |
| | Oct4, Sox2, Klf4, Myc, Zscan4 | [63] |
| | Oct4, Sox2, Klf4, Myc, Dppa3 | [64] |
| *Cell origin* | Unknown | |

been described that generates fuzzy pluripotent cells with limited developmental potential following constitutive high levels of reprogramming factors [57]. Widespread loss of histone H3 lysine 27 trimethylation (H3K27me3), representing a general opening of the chromatin state at the beginning of the process, was followed by reacquisition of H3K27me3 and a stable pluripotent state. These studies suggest that the use of suboptimal levels and stoichiometry of the reprogramming factors is more prone to produce a unique or incomplete/aberrant reprogramming process.

### The Identity and Interplay Between Reprogramming Factors

Many different combinations of factors have been demonstrated to generate mouse and human iPSCs (Table 2). Various chromatin modulators, such as Ezh2 [58], Tet1 [59], and loss of DOT1L [60], miRNAs [61], and different combinations of lineage-specifier genes [62], have been shown to facilitate reprogramming and to replace some or all of the Yamanaka factors [26]. These data suggest that the pluripotency state can be achieved via multiple molecular conduits, and raises the possibility that some pathways might be superior to others. The fact that OSKM can lead to a DNA damage response (DDR) early in the reprogramming process suggests that factors involved in oocyte-induced DNA repair may stabilize the somatic genome during reprogramming and improve the quality of the resulting iPSCs. Indeed, Zscan4, in combination with OSKM, not only reduced the DDR but also markedly promoted the efficiency of iPSC generation, enhanced telomere lengthening as early as 3 days post-infection, and significantly increased the percentage of iPSC lines that gave rise to 'all-iPSC' mice as determined by 4N complementation [63]. In addition, forced expression of the germ cell marker, Dppa3, together with OSKM enhances reprogramming kinetics and generates mostly high-grade iPSCs, as evidenced by the production of iPSC clones with intact *Dlk1–Dio3* imprinted locus [64], a locus that when hypermethylated is associated with poor-quality iPSCs [65]. Moreover, the quality of iPSCs is dramatically affected by the specific choice of reprogramming factors [51]. Reprogramming with OSKM results in a high number of iPSC colonies, but the majority exhibit low developmental potential, while reprogramming by Sall4, Nanog, Esrrb, and Lin28 (SNEL) generates a low number of iPSC colonies that are primarily of high quality as defined by their capacity to produce healthy 'all-iPSC' mice. Surprisingly, in the absence of the oncogenes Myc and Lin28, introduction of Oct4, Sox2, Sall4, Nanog, and Esrrb (OSSNE) yielded the highest number of poor-quality iPSCs, suggesting that the interplay between reprogramming factors is a crucial parameter of cell quality and a determinant of successful reprogramming [51]. Given the fundamental role that **Tet proteins** play in DNA methylation and during epigenetic reprogramming [59,66], the DNA dioxygenase Tet1 can replace multiple pluripotency transcription factors and can generate high-quality iPSCs with Oct4 alone, although with reduced efficiency [67].

### Origin of Starting Cells and Culture Conditions

The ability to reset the epigenome of somatic cells is one of the major roadblocks of the reprogramming process [68], thus major efforts have been focused on identifying small molecules and culture conditions that can aid in facilitating the reprogramming process by modulating the epigenome of cells. The identification of two small-molecule kinase [MEK (mitogen-activated protein kinase kinase 1) and GSK3-β (glycogen synthase kinase 3β)] inhibitors ('2i', PD0325901 and CHIR99021) demonstrates the importance of defining the culture conditions that support a **ground state** [69,70]. The characterization of the optimal culture conditions [i.e., hypoxia and the addition of 20% knockout DMEM (Dulbecco's modified Eagle medium), 20% knockout serum replacement (KSR)] and the identification of various small molecules [i.e., ascorbic acid, a GSK3-β inhibitor, and a transforming growth factor (TGF)-β inhibitor] have been extensively reviewed elsewhere (Table 2) [23,71–73].

The selection of the starting population is also thought to be a major determinant because cells with a shared epigenetic landscape to ESCs should reprogram more efficiently and quickly,

which should aid in eliminating genetic mutations or epigenetic aberrations [25]. Indeed, Oct4 alone is sufficient to reprogram neuronal progenitor cells, which express high levels of Sox2 [68]. Other studies show that various starting cell populations have a higher tendency to retain a specific signature of '**somatic memory**', suggesting that the Yamanaka factors might have a limited affinity for various regions in the genome, and thus some epigenetic landscapes will be more adequate than others [74–76]. Indeed, subsets of hematopoietic progenitors are privileged, as defined by the observation that some progeny cells predominantly adopt a pluripotent fate with the activation of an endogenous *Oct4* locus after 4–5 divisions in reprogramming conditions [77]. However, the observation that, although a large number of pluripotent genes are shared between trophoblast stem cells (TSCs) and ESCs, such as Sox2, Sall4, Utf1, and Esrrb, the conversion of TSCs to iPSCs is less efficient and longer than for other somatic cells [78] challenges the concept that cells with shared epigenetic landscape will reprogram more efficiently, suggesting that opposing pathways executed by TSC key master regulators such as Cdx2 and Elf5 counteract the pluripotency state. In addition, three independent studies also demonstrated an inverse correlation between reprogramming efficiency and cell quality by using stringent pluripotency tests [51,55,59]. Thus, the current data suggest that parameters other than reprogramming efficiency and epigenetic landscape similarity to the target cells (Table 2) should be taken into consideration when attempting to achieve high-quality reprogrammed cells.

## Association Between Reprogramming Quality, Cell Safety, and Functionality

Genomic stability and **epigenomic assembly** are two of the most influential parameters that determine cell quality [33,79,80]. While complete nuclear resetting and an intact genome are essential in yielding functional and safe cells, an aberrant epigenomic landscape or genetic mutations might lead to unstable and dysfunctional cells that hold a high tumorigenic potential.

### Linking Reprogramming Quality to Safety

The molecular mechanisms that underlie the nuclear reprogramming process are remarkably similar to those that are deregulated during malignant transformation [81–83]. In both cases, abnormal expression of key master regulator/s induces oncogenic stress that leads to DNA damage and genomic instability [84,85] and, in parallel, initiates a series of events that alter the epigenome of the cell. Interestingly, while several studies have identified genetic and epigenetic differences between ESCs and iPSCs, even in lines that passed the 4N complementation test [86], others have failed to detect abnormalities that consistently distinguish iPSCs from ESCs, when considering the mutation load and the genetic background of the starting cells [87–89]. Because every cell undergoes a unique reprogramming event that is influenced by many stochastic elements [23], such as levels of transgenes, phase in cell cycle, transcriptional burst, and **hyperdynamic chromatin state**, it is not surprising that those later studies could not identify common aberrations among different iPSC colonies. Whole genome and epigenome analyses at the single cell level are ultimately required to resolve this issue because acquisition of a single *de novo* mutation or one aberrant epigenetic locus, in even one cell, is sufficient to impair safety. For example, a hot-spot mutation or aberrant DNA methylation in a regulatory element or within the coding region of an oncogene or tumor suppressor may render the cell more susceptible to malignant transformation. Indeed, transient expression of reprogramming factors *in vivo* resulted in global changes in DNA methylation in cells from various tissues that predisposed the mouse to tumor development, indicating that incomplete conversion or poor reprogramming quality can facilitate malignancy [90]. However, unlike somatic cells, stable pluripotent cells are presumed to have robust DNA repair pathways to ensure genome stability [91] and a hyperdynamic chromatin state [92]. Moreover, it is important to note that a high incidence of tumors was observed only in chimeric mice and 'all-iPSC' mice that were produced from iPSCs generated by leaky or reactivated viruses [55]. Thus, the question of whether stable iPSCs hold a higher malignant potential than ESCs is still debatable and requires further investigation using non-integrating agents. Nevertheless, because only high-quality cells can contribute to

high-grade chimeras and 'all-iPSC' mice, it is plausible to assume that their genome and epigenome are relatively normal, and that those that are more prone to malignant transformation are also functionally defective and thus cannot contribute to chimeras or to 'all-iPSC' mice.

### Linking Reprogramming Quality to Functionality

In murine systems, functionality can be assessed at best using stringent assays such as complete rescue experiments for directly converted cells or 4N test for iPSCs. However, the vast majority of iPSCs have failed to pass the 4N test, and none of the directly converted cells have been able to completely rescue organ failure to the same extent as their endogenous counterparts. The first indication for the correlation between epigenetic reprogramming state and the function of the converted cells came from the identification of a specific imprinted *Dlk1–Dio3* gene cluster on chromosome 12qF1, which could discriminate between 4N-competent to 4N-incompetent iPSCs [65]. Since then, many genomic loci have been suggested to mark poor-quality iPSCs that are distinguishable from ESCs. For example, a comparison between '4N-ON' to '4N-OFF' iPSC lines by high-throughput sequencing, core histone modifications, and DNA methylation revealed all iPSC lines to be globally similar. The only parameter that correlated with '4N-OFF' iPSC lines was loss of methylation at the imprinted locus *Zrsr1* [93]. Besides loci with aberrant methylation, multiple transcriptional signatures including extraembryonic genes [94] and developmental genes [51] have been proposed to discriminate between fully functional and dysfunctional cells. However, the controversy regarding epigenetic and genetic differences found between iPSCs and ESCs raises doubts regarding the robustness of these approaches. Thus, scientists have used other methods to identify high-quality cells (Figure 1, Key Figure). Indeed, analysis of the deposition of histone variant H2A.X (H2A histone family, member X) following the completion of the reprogramming process revealed that H2A.X organization is a functional marker that could distinguish between cells with high or poor developmental potential. ESC-specific H2A.X deposition patterns have been found to be faithfully recapitulated in iPSCs that fully support the development of 'all-iPSC' animals. By contrast, iPSCs that failed to support 'all-iPSC' embryonic development showed aberrant H2A.X deposition and a predisposition to extraembryonic differentiation [94]. Furthermore, two histone variants, TH2A and TH2B, which are expressed at high levels in oocytes and contribute to the activation of the paternal genome after fertilization, facilitate OSKM-dependent generation of iPSCs and generate iPSCs in the absence of Myc and Sox2 [95]. These studies provide some insight regarding the role of histone variants during the reprogramming process.

### Lessons Learned From Human Cells

Because cell replacement therapy requires fully functional cells, it is of paramount importance to distinguish high-quality cells from the entire population of induced cells. Because the 4N test is not applicable for hiPSCs, the current methodologies to assess their quality are *in vitro* differentiation, **teratoma assay**, transcriptional profile, and karyotyping analysis. However, these techniques are not sufficiently stringent, and the vast majority of iPSCs pass these tests. Thus, defining a test or a criterion that will faithfully identify high-quality cells is particularly crucial for human cells.

Similarly to mouse cells, hiPSCs and hESCs show changes in DNA methylation. A recent study identified a panel of 82 CpG methylation sites that could distinguish poor-quality hiPSCs from hESCs with high accuracy [96]. In accordance, a clear somatic memory was observed showing that lineage-specific marks emerge upon differentiation induction of hiPSCs that correlate to the cell of origin [97]. However, paralleling studies using the mouse system, different studies found comparable frequencies of coding mutations and loss of imprinting in human pluripotent cells derived by either nuclear transfer or defined factors introduced by non-integrating agents [98]. These observations raise a fundamental question in the reprogramming field: is somatic memory an intrinsic and integral part of the reprogramming process by defined factors, or is it only a

## Key Figure

### Quality Control Criteria for Mouse and Human iPSCs

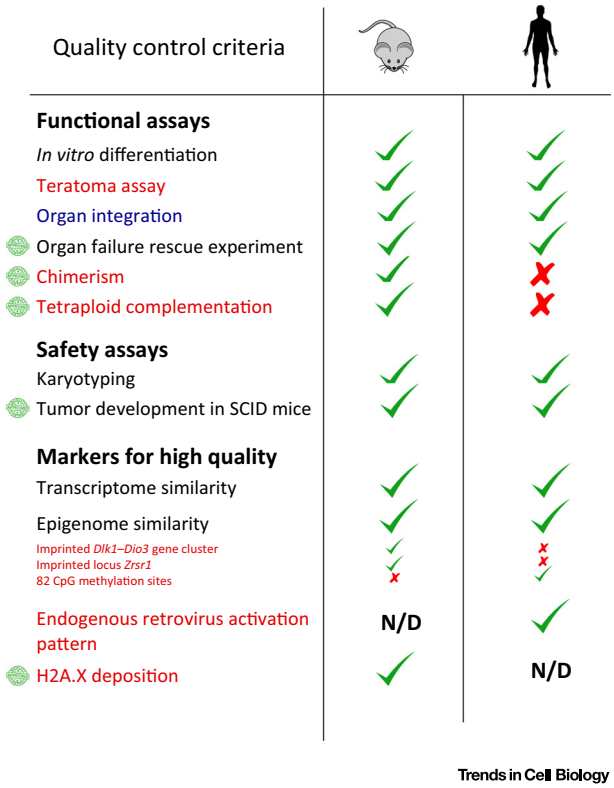


Figure 1. A table comparing the quality control criteria for iPSCs and the various directly converted cell types between mouse and human. Red indicates relevant to iPSCs, blue indicates relevant to directly converted cells, and black indicates relevant for both. The quality control logo indicates a stringent parameter. Abbreviations: H2A.X, H2A histone family, member X; iPSCs, induced pluripotent stem cells; N/D, not determined; SCID, severe combined immunodeficiency.

reflection of a suboptimal reprogramming system that generates incomplete reprogrammable cells?

In support of the importance of histones as major players in the reprogramming process and markers for high-quality iPSCs, the histone-remodeling chaperone ASF1A was found to be required to achieve hiPSCs. ASF1A is specifically enriched in metaphase II (MII) of human oocytes and during reprogramming of human adult dermal fibroblasts (hADFs) to hiPSCs. Ectopic expression of ASF1A and OCT4 alone in hADFs exposed to the oocyte-specific paracrine growth factor GDF9 reprogrammed hADFs into pluripotent cells [99]. Moreover, seven of 40 hiPSC clones tested were found to retain a significant number of undifferentiated cells after neural differentiation, and these formed teratomas when transplanted into mouse brains [100]. These differentiation-defective hiPSC clones were marked by higher expression levels of long terminal repeats of specific human endogenous retroviruses (HERVs) [100].

The dynamic regulation of HERVs is important for proper reprogramming and differentiation potential. Aberrant expression of the long terminal repeats of HERV type-H (LTR7s), or lincRNA-RoR, a HERV-H-driven long noncoding RNA, early in reprogramming markedly reduced

reprogramming quality, as measured by iPSC colony number and differentiation capacity [101]. In accordance, HERVH, a primate-specific endogenous retrovirus, have been identified within a subpopulation of hESCs and hiPSCs with characteristics similar to those of the mouse naïve pluripotent cells [102]. These latter studies emphasize the need for other measures to assess quality in human cells (Figure 1).

## Concluding Remarks

Quality control is defined as a procedure intended to ensure that a manufactured product adheres to a defined set of quality criteria and meets specific requirements. Two of the most crucial criteria for cells to be used in the clinic are safety and functionality. The vast majority of iPSCs and directly converted cells do not meet these criteria, and the fact that a robust and reliable predictor for high-quality cells has not yet been found suggests that converted cells currently hold high risk when considered for therapeutic applications.

Stringent pluripotency tests are the best way to assess the functionality and safety of cells; however, these assays are currently not applicable for human cells. Recently, a major progression in stem cell technology has allowed the conversion and derivation of human ESCs with some naïve characteristics [103–105]. In addition, a new embryonic stem-cell type with unique spatial characteristics, designated region-selective pluripotent stem cells (rsPSCs), was isolated from mouse embryos and primate pluripotent stem cells, including humans [106]. These cells might hold the potential of generating interspecific chimera that could be employed as a stringent developmental test which would be useful in assessing the quality of human pluripotent cells.

Abnormal chromatin resetting and genomic instability are common features in reprogramming with OSKM, but the fact that mutual genetic or epigenetic aberrations have not been found between multiple systems suggests that numerous elements affect the quality of the resulting cells following a reprogramming process. Therefore, other approaches to measure quality of hiPSCs must be developed. Some encouraging approaches to consider are faithful histone-remodeling deposition and expression levels of long terminal repeats of specific HERVs.

There are three main approaches [i.e., somatic cell nuclear transfer (SCNT), cell fusion, and introduction of defined transcription factors] to reprogramming a somatic nucleus. These approaches differ in their technical difficulty, speed of reprogramming, efficiency of inducing pluripotency, and cell yield [107]. One of the most intriguing questions in the reprogramming field is how the oocyte can reprogram a somatic cell in less than 3 days while retaining an intact genome and epigenome. It is believed that a complex reprogramming protein interactome and a robust and efficient DNA repair system are responsible for this intact reprogramming event. Therefore, investigating unfertilized MII oocytes as a means to understand the molecular pathways governing somatic cell reprogramming is one, albeit daunting, way to determine the important factors and conditions necessary to reprogram a somatic nucleus. Another way to improve the current protocols is by using elements that reduce the risk of genomic instability and at the same time aid in resetting the epigenome. Using non-integrating agents [108] to produce iPSCs and directly converted cells in conjunction with a unique combination of small molecules that facilitate nuclear resetting [71,72] might yield high-quality cells. The selection of the reprogramming factor combination is an issue that should receive more attention from the stem cell community because the interplay between reprogramming factors is essential for proper reprogramming events. Yamanaka factors generate iPSCs relatively efficiently, but tend to 'leave' somatic memory and yield a high number of poor-quality cells. Other combinations of factors produce better-quality iPSCs [51,63,67] but with low efficiency. Because one high-quality iPSC colony is sufficient to perform all therapeutic applications, generating a low number of colonies with better quality may be more beneficial for the clinic. Two notions might explain why low efficiency is associated with high-quality cells. First, high levels of Myc or a specific

## Outstanding Questions

Stringent pluripotency tests are definitely the best way to assess the functionality and safety of iPSCs. Major progress in stem cell technology has recently allowed conversion and derivation of human ESCs with some naïve characteristics. In addition, a new stem-cell type with unique spatial characteristics, designated region-selective pluripotent stem cells (rsPSCs), was obtained from mouse embryos and primate pluripotent stem cells, including humans. Can these cells serve as a platform for the generation of interspecific chimera that can be used as a stringent developmental test?

Two of the most crucial criteria for cells to be used in the clinic are safety and functionality. Because these criteria are highly linked, are they sufficient to define high-quality cells?

In contrast to iPSCs that can grow independently of exogenous factors, and are almost indistinguishable in their epigenome, transcriptome, and function to their ESC counterparts, in the vast majority of cases of the direct conversion models the converted cells express only a fraction of the relevant markers and are dependent either fully or partially on their transgenes. This raises the question: can a high degree of nuclear reprogramming be attained in cells undergoing reprogramming to non-pluripotent cells?

Do stable and integration-free iPSCs hold higher potential for malignancy than ESCs?

Is somatic memory an intrinsic and integral part of the reprogramming process, or is it only a reflection of a suboptimal reprogramming system that generates incompletely reprogrammable cells?

interplay between the reprogramming factors induces rapid conversion of cells, but 'leaves' many genetic and epigenetic mistakes in a large number of cells. By contrast, combinations of factors without Myc, or with a specifically accurate stoichiometry, tend to retain a relatively normal genome and epigenome but the reprogramming process is longer and complete reprogramming can be attained only in a small number of cells. Second, these low-efficiency combinations of factors can target only a small fraction of elite cells within the starting cell population that is more susceptible and adequate to reprogramming.

In summary, iPSCs and directly converted cells hold great promise in the clinic for transplantation therapy, but the quality of the converted cells is still a major roadblock for clinical applications (see Outstanding Questions). Thus, major efforts should be focused on defining alternative and stringent quality-control measures, such as histone variant deposition analysis, as well as on the identification of ideal reprogramming factor combinations, culture conditions, starting cell population, and small molecules that will allow the production of high-quality cells. Understanding the elements that affect the reprogramming process will bring us closer to the ultimate goal; the production of high-quality iPSCs and directly converted cells.

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