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Does transcription factor induced pluripotency accurately mimic embryo derived pluripotency?

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Abstract

When Takahashi and Yamanaka first demonstrated that just four transcription factors could reprogram a fibroblast to a pluripotent stem cell, the first wave of data to emerge focused on how similar these induced pluripotent stem cells (iPSCs) were to embryo-derived pluripotent stem cells (ESCs) [1]. The next wave of data focused on determining the degree of difference between iPSCs and ESCs [2]. Now the focus is on tweaking the process to generate iPSCs that are more similar to ESCs [3, 4]. Because transcription factor based reprogramming allows for nearly any type of cell to be created from any donor cell, there is obviously enormous interest in this technique as a tool for both basic developmental biology and for clinical applications. In this review, I will attempt to summarize the data that serve to distinguish these types of pluripotent stem cells and speculate on any ramifications of the differences.

Introduction

For those not indoctrinated, iPSCs are generated by forced expression of transcription factors, known to be highly expressed in pluripotent stem cells, into somatic cells [1]. This forced expression appears to recapitulate the type of nuclear reprogramming previously only accomplished by somatic cell nuclear transfer [5–7]. The relative ease with which somatic cells can be reprogrammed has led to the widespread adoption of this technology for a variety of applications requiring patient specific pluripotent stem cells. It is important to point out that reprogramming is not simply the adoption of an alternative cell fate, but also suppression of the previous fate. Current evidence suggests that the cocktail of reprogramming factors appear to possess the ability to drive both processes [8]. It is thought that suppression of the somatic cell fate is the first step of the process in tandem with epigenetic rearrangement, and subsequent induction of pluripotency [8] (Fig 1). Significant effort is currently underway to precisely define the role of the reprogramming factors on a temporal basis throughout reprogramming, some of which will be reviewed elsewhere in this issue (Meissner, Scholer etc). Clearly, many cells get lost along the way, as the efficiency is

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very low, despite significant improvements over the original protocols. Even in cases where all cells in the culture receive all the reprogramming factors, efficiency hovers around 10% [9], leading to theories of stochastic and/or elite mechanisms playing a role in this process (reviewed in [10]). For those few cells that do successfully navigate up Waddington's epigenetic hills, it would seem surprising that they could ever be identical to pluripotent stem cells derived from an epiblast or inner cell mass of a pre-implantation embryo considering the difficulty of their journey.

From epigenome, to genome, transcriptome, proteome and metabolome analyses, a wealth of new data has led to a consensus that iPSCs and ESCs are much more similar to each other than any other type of cell (Fig 1). Furthermore, many argue that iPSCs can be generated that are indistinguishable from ESCs [3, 11]. However, there are also compelling reasons to think that human iPSCs harbor a molecular memory of their past as somatic cells [2, 12–17]. Because of the vast molecular chasm between somatic and pluripotent cells, it is difficult to even fathom how just a few transcription factors can impart such a dramatic cell fate change. Nevertheless, it appears as though iPSCs possess all the functional hallmarks of embryo derived ESCs, justifying the enormous attention paid to them. Here, I will attempt to review what has been described thus far both at the molecular level and speculate on the consequences of any differences.

iPSC vs ESC

The first wave of iPSC papers used microarray gene expression profiling to demonstrate both that iPSCs were similar to ESCs and also that they were quite different from the fibroblasts from which they were derived [1, 18–20]. Upon closer inspection, nearly every molecular analysis performed showed that iPSCs clustered separately from ESCs indicating that they were somewhat different. In 2009, our group and others suggested that perhaps this separation was not random [2, 21]. In fact, when looking at genes that were differentially expressed between iPSCs and ESCs from several independent groups, there was significant overlap that could not be accounted for by batch variation [22]. We showed that at the transcriptional level, hiPSCs expressed a group of genes at a different level than in hESCs, and that much of that difference disappeared as the hiPSCs were passaged continuously [2, 22]. Many of these differences seemed to be fibroblast-specific genes that were not appropriately reset during reprogramming [2]. Subsequently, other groups showed similar gene expression differences between iPSCs and ESCs in both human and murine settings [4, 14–17, 21, 23–27], and have argued that transcription factor reprogramming leaves a molecular memory of the cell type of origin that can be read out at the RNA and/or protein level. Studies conducted in the following two years have aimed to understand how any molecular memory could be retained in these cells, whether there are any consequences, and whether these memories can be erased.

The obvious answer to this question is that the process of reprogramming certainly requires some degree of erasure and re-writing of the epigenome, so perhaps incomplete epigenetic re-wiring is to blame for differences in gene expression. As a result, iPSCs have been analyzed by nearly every described molecular method and compared to ESCs. Several groups have looked at differences in DNA methylation in both genic and non-genic regions of the genome and identified numerous differentially methylated regions (DMRs), some of which also correlated with gene expression differences [12–15, 23, 28–30]. Some of these differences appear to even be enriched at specific locations on chromosomes [12]. In our original work, we showed that two histone modifications (H3K4me and H3K27me) were indistinguishable between hiPSCs and hESCs, even for those genes that were differentially expressed [2]. This finding would prove to be myopic as others looked at a variety of epigenetic marks. Hawkins et al found that H3K9me3 was differently applied at promoters

between hiPSCs and hESCs and also correlated with previously described gene expression differences [31].

Another obvious explanation for any molecular differences between hiPSCs and hESCs is the fact that the reprogramming process itself induces genomic mutations, some of which could be read out at the gene expression level. Numerous reports have addressed this issue and come to somewhat contradictory conclusions [32–35]. Array Comparative Genomic Hybridization, SNP Array, and high throughput sequencing have all been applied to iPSCs generated with different methods [32, 34, 36]. For the most part, all iPSC lines generated have detected genomic abnormalities. While some have argued that iPSCs only have mutations because of routine passaging, most have shown that the reprogramming process itself induces additional mutations that cannot be explained by routine culture. The difficulty with interpreting these data is that it is nearly impossible to isolate cells immediately after reprogramming and sequence their genomes prior to subsequent divisions during which mutations can occur. One study sequenced the genome of three hiPSC lines that were produced without genomic integration (episomal delivery) and found that these lines did not show a higher mutation rate than would be expected by culture [35]. This suggests that reprogramming itself is not mutagenic, but approaches that rely on genomic integration of the factors could be. Despite the low incidence of mutations following episomal delivery of the Yamanaka factors, hiPSC lines generated with this method have still be shown to have expression differences with hESCs at the RNA and protein levels [2, 22, 37, 38]. Taken together, it would appear that that epigenetic memory of the somatic cell of origin has more consequences to iPSCs than genetic abnormalities.

Some groups have instead argued that there are no inherent differences between ESCs and iPSCs [3, 11]. No one disagrees with the fact that, within each cell type, there is a broad diversity at the molecular if not the functional level. The question instead is whether there are consistent differences between ESCs and iPSCs that arise as a result of the reprogramming process itself. Several studies have demonstrated that the culture conditions can have an influence on their similarity with ESCs. Hochedlinger and colleagues first demonstrated that aberrant epigenetic silencing at a particular imprinted locus (*Dlk1-Dio3*) could predict the quality of reprogramming as read out by pluripotency assays (contribution to chimeric animals, tetraploid aggregation assay) [24]. They further showed that addition of histone deacetylase (HDAC) inhibitors to relieve this silencing correlated with success in tetraploid aggregation, or the generation of all-iPSC derived mice [24]. Another group argued instead that activity at this locus did not correlate with the quality of the iPSCs derived, but instead the stoichiometry of the reprogramming factors was key to generating iPSCs that could undergo tetraploid aggregation [3]. However, Hochedlinger then showed that simply adding Vitamin C to the culture or extended passaging created iPSCs with active *Dlk1-Dio3* [4]. Together, these studies suggested that perhaps iPSCs are not necessarily different from ESCs, but instead suggest that small changes to the culture conditions or reprogramming cocktail can not only affect the epigenetic quality of reprogrammed cells, but also their pluripotency. Consistently, continued passaging drives iPSCs closer to ESCs [2, 17], but it is still not clear if this is due to further reprogramming. There are intriguing data suggesting that continued passaging drives further lengthening of telomeres as part of the reprogramming process [39], and many have shown that telomere length is strongly correlated with the degree of pluripotency [40]. Alternatively, it is still possible that standard Yamanaka reprogramming drives a small number of cells to a completely pluripotent state and that these cells are selected for over time in culture. Regardless, it is likely that improvements to reprogramming technology will bring iPSCs ever closer to ESCs to a point where current molecular biology techniques can no longer detect differences.

If typical reprogramming is inherently deficient in recreating a state identical to embryonic stem cells, then gene expression or epigenetic changes would be expected to be conserved across all lines generated. There appears to be some debate as to whether any consistent differences across lines are found (reviewed in [41]). A survey of the literature uncovers at least two genes have come up differentially expressed in nearly every study. *TCERG1L* and *TMEM132D* [2, 12, 14]. These were also shown to be differently abundant at the protein level [38]. Furthermore, these two genes were identified by Lister et al as typical examples of loci within DMRs between hESCs and hiPSCs [12]. Is activity at these loci a landmark of inherent difference between hiPSCs and hESCs? Are these genes the equivalent of the *Dlk-Dio* locus that correlates with pristine pluripotency in murine iPSCs [4, 24]? Is there a consequence to differential expression of these genes? Until functional analyses are performed, the relevance of these markers will remain a mystery.

Another unresolved issue is whether murine reprogramming is more complete than the human equivalent. There is more evidence in the literature for significant differences between hiPSCs and hESCs than for murine counterparts. Is the human epigenome more difficult to rewrite? Is the traditional Yamanaka cocktail, which was originally identified for murine reprogramming, less than ideal for the human process?. Studies of X chromosome inactivation (XCI) after reprogramming of female murine iPSCs clearly show that the X chromosome is reactivated [18], whereas human iPSCs retain XCI [42–44]. This clearly has significant consequences for the study of X-linked disorders [43]. However, continued culture appears to make XCI disappear, further demonstrating that epigenetic states are potentially evolving over time [43, 44].

A structural impediment to understanding the functional consequences of any differences between hiPSCs and hESCs is that the most stringent assay to define pluripotency for human cells is the teratoma assay. This assay sets a rather low bar for pluripotency as even murine epiblast stem cells (EpiSCs) can succeed in making teratoma while failing at contribution to chimeric embryos. So, instead of quantifying the degree of pluripotency or reprogramming with a functional assay, we are more or less resigned to simply compare them at the molecular level

Recent attempts to resolve this issue have relied on analyses of more and more cell lines to facilitate comparisons despite the inherent variability amongst both hiPSCs and hESCs. The most complete examples of this work to date use dozens of cell lines and compared them at both the epigenetic and transcriptional levels to define the molecular variability both within each group and across both groups [44, 45]. In the end, hiPSCs and hESCs were found to occupy overlapping clouds of variability, and that some lines in each category were indistinguishable [45]. On the other hand, only a small portion of these clouds overlapped further suggesting that, on average, hESCs and hiPSCs are distinct.

The biggest hurdle to answering questions of equivalency between hiPSCs and hESCs is the fact that it is not trivial for a single investigator to culture and analyze many different cell lines simultaneously. Because iPSCs and ESCs are *so* similar and the molecular methods to analyze them *so* precise, slight differences in reprogramming strategies, cell culture techniques, or reagents can significantly influence the molecular differences between them. In addition, it is impossible to discern from most studies as to whether all cell lines analyzed were grown simultaneously by the same investigators with the same methods or whether data sets analyzed were compiled over time in different settings. To my knowledge, just a few studies that analyzed large numbers of hiPSC and hESC lines that were cultured simultaneously by the same practitioner [14, 16, 46]. These studies still identified consistent differences between the hiPSCs and hESCs, suggesting that hiPSCs and hESCs, as currently produced and cultured, represent slightly different types of cell lines [14, 46]. In studies

where more than a dozen lines of each type are used [44, 45], it is not practical to expect all lines to be cultured simultaneously. Therefore, despite the increased power inherent in the study of many cell lines, small variation in culture conditions, preparation of DNA/RNA, and molecular analysis can obscure the detection of consistent differences between classes of cell lines.

hiPSCs ≠ hESCs, but does it matter?

Assuming there are small differences between iPSCs and ESCs, and that those differences are caused by either a molecular memory of the cell of origin or an inconsistency in methodologies, is there a consequence to these small differences? Is one type of pluripotent stem cell somehow better than the other? By definition, both types of cell are pluripotent and can self-renew. While there are a few reports that hiPSCs have more variable differentiation efficiency to particular cell types, the findings are less consistent across a number of studies [47, 48]. For now, it is thought that some lines will prove to be superior for particular applications based on their relative propensities to make particular cell types through directed differentiation. It is very difficult to predict a priori which lines are more proficient at making particular cell types, though some progress has been made for particular lineages [46].

Less understood, but perhaps even more relevant, is whether the progeny of hiPSCs and hESCs share gene expression or functional inconsistencies as every hiPSC line can make any type of cell to some degree. We took hiPSCs and hESCs and differentiated them down various lineages, purified and profiled them [49]. We found that, despite observing transcriptional differences between the hiPSCs and hESCs in the undifferentiated state, the differentiated progeny of these pluripotent cells were essentially identical, except for just a handful of genes [49]. While others have shown that the molecular memory of the original somatic state can be read out as transcriptional changes in the progeny of iPSCs [14], it is not clear if there is any functional significance to these differences. This work suggested that, even if hiPSCs and hESCs are inherently different in the undifferentiated state, perhaps these discrepancies are inconsequential if one is interested in using the progeny of these cells for studies of basic development, disease modeling, or for clinical application.

Instead, the discrepancies between hiPSCs and hESCs are probably most informative in the study of the reprogramming process itself. The studies that outlined small numbers of DMRs highlight the possibility that either the erasing or re-writing of the epigenome is the main stumbling block to efficient, complete reprogramming [13–15, 23, 28, 50]. Given the fact that the Yamanaka factors are transcription factors and not epigenetic modifiers, perhaps these discrepancies are not surprising. Of course, most assume that part of the role the Yamanaka factors play is to recruit the activities of epigenetic modifiers. As a testament to this, one study looked at molecular changes that occur within a day or two of delivery of the Yamanaka factors and found that one of the first detectable events was epigenetic, application of a histone mark associated with active gene expression [51]. This even preceded transcriptional changes at these loci, but did mark promoters that would eventually be induced. This work, and that of others suggest that transcription factor based reprogramming is nearly sufficient for complete reprogramming and perhaps culture conditions or pharmacological manipulation of the epigenome will soon reproducibly generate hiPSCs that are indistinguishable from hESCs. Towards this end, much will be gained through the identification of new methods to manipulate the induction of pluripotency, as those that prove to be most effective will also provide insights to the induction and the maintenance of the pluripotent state.

Conclusion

To summarize, it appears as though iPSCs are on average slightly different from ESCs on various molecular levels. At this point it is unclear if there are significant functional consequences to these differences that should preclude the use of iPSCs instead of ESCs in studies of basic development, disease modeling or even clinical applications. Instead, these differences should be exploited to uncover the landmarks and barriers to reprogramming that still to this day prevent reprogramming in more than 10% of cells under ideal conditions. Eventually, it seems likely that based on our growing knowledge of the reprogramming process and improving reprogramming methods, somatic cells will be reprogrammed to a state of bioequivalence with ESCs.

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Figure.

Transcription factor based reprogramming drives somatic cells through a long molecular rearrangement to iPSCs (Top arrow). Recent work has shown that modification of the original reprogramming conditions can drive somatic cells to a state ever closer to ESCs, which represent the gold standard for pluripotency (Bottom arrow). Additionally, simply allowing the cells to divide over time also appears to bring iPSCs closer to ESCs (Right arrow).