Cell Stem Cell
Perspective

Induced Pluripotent Stem Cells: Past, Present, and Future

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The development of iPSCs reflected the merging of three major scientific streams and has in turn led to additional new branches of investigation. However, there is still debate about whether iPSCs are functionally equivalent to ESCs. This question should be answered only by science, not by politics or business.

Introduction

In 2006, we showed that stem cells with properties similar to ESCs could be generated from mouse fibroblasts by simultaneously introducing four genes (Takahashi and Yamanaka, 2006). We designated these cells iPSCs. In 2007, we reported that a similar approach was applicable for human fibroblasts and that by introducing a handful of factors, human iPSCs can be generated (Takahashi et al., 2007). On the same day, James Thomson's group also reported the generation of human iPSC using a different combination of factors (Yu et al., 2007).

The Merging of Three Scientific Streams Led to the Production of iPSCs

Like any other scientific advance, iPSC technology was established on the basis of numerous findings by past and current scientists in related fields. There were three major streams of research that led us to the production of iPSCs (Figure 1). The first stream was reprogramming by nuclear transfer. In 1962, John Gurdon reported that his laboratory had generated tadpoles from unfertilized eggs that had received a nucleus from the intestinal cells of adult frogs (Gurdon, 1962). More than three decades later, Ian Wilmut and colleagues reported the birth of Dolly, the first mammal generated by somatic cloning of mammary epithelial cells (Wilmut et al., 1997). These successes in somatic cloning demonstrated that even differentiated cells contain all of the genetic information that is required for the development of entire organisms, and that oocytes contain factors that can reprogram somatic cell nuclei. In 2001, Takashi Tada's group showed that ESCs also contain factors that can reprogram somatic cells (Tada et al., 2001).

The second stream was the discovery of "master" transcription factors. In 1987, a *Drosophila* transcription factor, Antennapedia, was shown to induce the formation of legs instead of antennae when ectopically expressed (Schneuwly et al., 1987). In the same year, a mammalian transcription factor, MyoD, was shown to convert fibroblasts into myocytes (Davis et al., 1987). These results led to the concept of a "master regulator," a transcription factor that determines and induces the fate of a given lineage. Many researchers began to search for single master regulators for various lineages. The attempts failed, with a few exceptions (Yamanaka and Blau, 2010).

The third, and equally important, stream of research is that involving ESCs. Since the first generation of mouse ESCs in 1981 (Evans and Kaufman, 1981; Martin, 1981), Austin Smith and others have established culture conditions that enable the long-term maintenance of pluripotency (Smith et al., 1988). A key factor for maintenance of mouse ESCs was leukemia inhibitory factor (LIF). Likewise, since the first generation of human ESCs (Thomson et al., 1998), optimal culture conditions with basic fibroblast growth factor (bFGF) have been established.

Combining the first two streams of research led us to hypothesize that it is a combination of multiple factors in oocytes or ESCs that reprogram somatic cells back into the embryonic state and to design experiments to identify that combination. Using information about the culture conditions that are needed to culture pluripotent cells, we were then able to identify four factors that can generate iPSCs.

Maturation and Understanding of iPSC Technology

Soon after our initial report of mouse iPSCs, other groups recapitulated the factor-based reprogramming both in mice (Maherali et al., 2007; Wernig et al., 2007) and humans (Lowry et al., 2008; Park et al., 2008b). One of the advantages of iPSC technology is its simplicity and reproducibility. Many laboratories began to explore the underlying mechanisms and to modify the procedures.

Although iPSCs can be generated reproducibly, the efficiency of the process remains low: typically less than 1% of transfected fibroblasts become iPSCs. This low efficiency initially raised the possibility that iPSCs are derived from rare stem or undifferentiated cells coexisting in fibroblast cultures (Yamanaka, 2009a). Subsequent studies showed, however, that iPSCs can be derived from terminally differentiated lymphocytes (Loh et al., 2009) and postmitotic neurons (Kim et al., 2011a). Thus, most, if not all, somatic cells have a potential to become iPSCs, albeit with different efficiencies.

How then can just a small set of factors induce reprogramming of somatic cells? It is beyond the scope of this essay to provide an overview of the many studies that have addressed this important question. From my perspective, the consensus of many scientists seems to be that the reprogramming factors initiate the reprogramming process in many more than 1% of transfected cells but that the process is not completed in most of the cells. Poorly understood stochastic events seem to be required for full reprogramming to take place (Hanna et al., 2009; Yamanaka, 2009a). As I discuss below, culture conditions

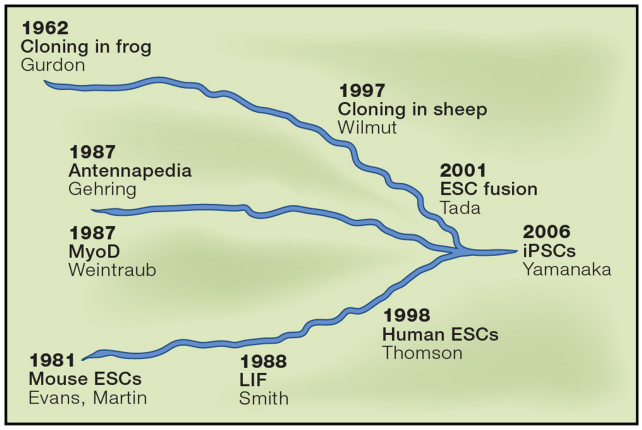


Figure 1. Three Scientific Streams that Led to the Development of iPSCs

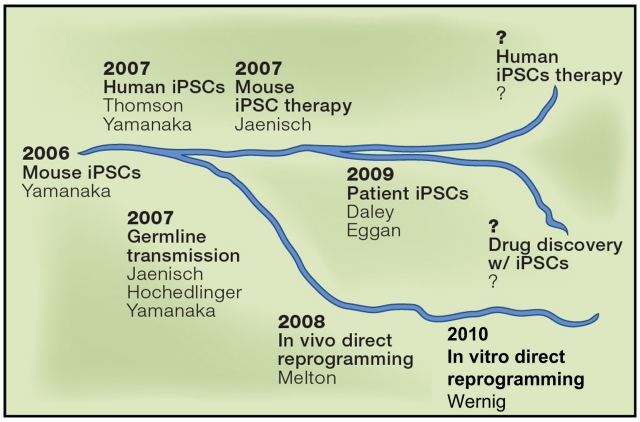


Figure 2. New Scientific Streams that Emerged from the Development of iPSCs

seem to function as a driving force that can help promote full reprogramming.

Initially iPSCs were generated using either retroviruses or lentiviruses, which might cause insertional mutagenesis and thus would pose a risk for translational application and could perhaps even lead to adverse effects like those seen in some attempts at gene therapy (Hacein-Bey-Abina et al., 2003). Mice derived from retrovirally derived iPSCs are apparently normal, as long as expression of the c-Myc transgene is repressed (Aoi et al., 2008; Nakagawa et al., 2008). However, the long-term safety of human iPSCs cannot be guaranteed through mouse studies alone. In addition, retroviruses may make iPSCs immunogenic (Zhao et al., 2011). Thus, for the purpose of cell transplantation therapy, we will need to avoid induction methods that involve vector integration into the host genome.

Many ways to generate integration-free iPSCs have been reported. These methods include plasmid (Okita et al., 2011a; Okita et al., 2008), Sendai virus (Fusaki et al., 2009), adenovirus (Stadtfeld et al., 2008), synthesized RNAs (Warren et al., 2010), and proteins (Kim et al., 2009). In addition, attempts have been made to induce reprogramming by small molecules. Among these, plasmids and Sendai viruses are now routinely used in many laboratories. In the Center for iPS Cell Research and Application, Kyoto University, our favored methods are to use episomal plasmids for regenerative medicine and either retroviruses or episomal plasmids for in vitro studies. We prefer these methods because of their simplicity and reproducibility. Scientists are now largely shifting their efforts from technology development per se to applications.

New Scientific Streams Have Emerged from iPSC Technology

Streams in science never cease (Figure 2). After the seminal work in mice by Rudolf Jaenisch's laboratory (Hanna et al., 2007), scientists are now making progress toward using iPSCs in regenerative medicine, for example for the treatment of Parkinson's disease (Kriks et al., 2011), platelet deficiency (Takayama et al., 2010), spinal cord injury (Nori et al., 2011; Tsuji et al., 2010), and macular degeneration (Okamoto and Takahashi, 2011). Patient-derived iPSCs have been shown to be useful for modeling diseases and screening drug candidate libraries. Starting with the seminal studies by the groups led by George Daley (Park et al., 2008a), and Kevin Eggan (Dimos et al., 2008), more than 100 reports published in the past three years use disease-specific iPSCs. I was surprised that patient-specific iPSCs can be used to recapitulate phenotypes of not only monogenic diseases but also late-onset polygenic diseases, such as Parkinson's disease (Devine et al., 2011), Alzheimer's disease (Israel et al., 2012; Yagi et al., 2011; Yahata et al., 2011), and schizophrenia (Brennand et al., 2011). Excitement surrounds the potential for application of these cells to both analysis of disease mechanisms and investigation of potential new treatments. Somatic cells derived from iPSCs, particularly cardiac myocytes and hepatocytes, could also be used for toxicology testing as an alternative to existing approaches (Yamanaka, 2009b).

In addition, to these medical applications, iPSCs can be used in animal biotechnology. Monkey (Liu et al., 2008), porcine (West et al., 2010), and canine (Shimada et al., 2010) iPSCs can be used for genetic engineering in these animals, allowing for the generation of disease models and the production in larger animals of useful substances, such as enzymes, that are deficient in patients with genetic diseases. The technology might potentially be useful in the future for preserving endangered animals as well (Ben-Nun et al., 2011), although many challenges would need to be overcome. One of the most striking applications of iPSCs was reported by Nakauchi and colleagues, who generated a rat pancreas in a mouse, by microinjecting rat iPSCs into mouse blastocysts deficient in a gene essential for pancreas development (Kobayashi et al., 2010). In the future, it might become possible to generate organs for human transplantation using a similar strategy.

Another scientific stream that emerged from iPSC technology is "direct reprogramming" from one somatic lineage to another. As mentioned above, attempts to identify a single "master" transcription factor have failed for most somatic lineages. However, in light of the success of iPSC reprogramming, scientists switched from searching for a single factor to looking for a combination. Melton and colleagues reported the conversion of exocrine cells to endocrine cells in the mouse pancreas by using a combination of three transcription factors (Zhou et al., 2008).

Table 1. Number of ESC and iPSC Clones Analyzed in Published Studies

Conclusion about the Relationship between ESCs and iPSCs	First Author	Year	Clone Numbers ESC	iPSC
It is difficult to distinguish between them	A.M. Newman	2010	23	68
	M.G. Guenther	2010	36	54
	C. Bock	2011	20	12
There are notable differences	M. Chin	2009	3	5
	C.M. Marchetto	2009	2	2
	J. Deng	2009	3	4
	Z. Ghosh	2010	6	4
	A. Doi	2011	3	9
	Y. Ohi	2011	3	9
	K. Kim	2011	6	12
	R. Lister	2011	2	5

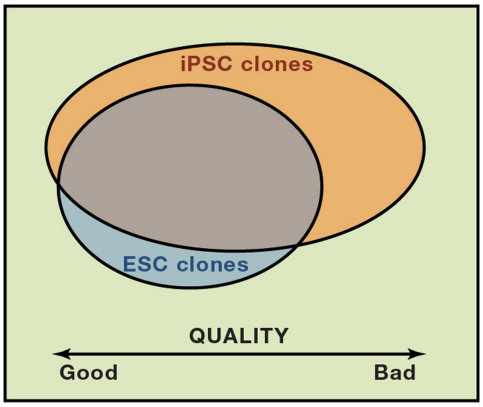


Figure 3. Overlapping Variations Present in iPSC and ESC Clones
Measurement of a range of properties of iPSCs and ESCs, including gene expression, DNA methylation, differentiation propensity, and (for mouse cells) complementation activity in embryos has led to the realization that the properties of both ESC and iPSC lines vary. However, as analysis of significant numbers of clones from multiple laboratories has accumulated, it has become clear that there is considerable overlap in terms of the properties of ESC and iPSC lines and, at a general level, these two cell types are difficult to distinguish.

Their seminal work was soon followed by many in vitro examples of converting fibroblasts to various somatic cells, such as neural cells (Vierbuchen et al., 2010), hepatocytes (Huang et al., 2011), cardiac myocytes (Ieda et al., 2010), and hematopoietic progenitor cells (Szabo et al., 2010). Direct reprogramming is straightforward and rapid. One hurdle that remains is how to obtain a sufficient amount of target cells for downstream applications. The best usage of this new technology may be in situ direct reprogramming (Qian et al., 2012).

The Big Question: Are iPSCs Different from ESCs?

One of the most important questions regarding iPSCs is whether they are different from ESCs and, if so, whether any differences that do exist are functionally relevant. During the first few years of our studies of iPSCs, we were amazed by their remarkable similarity to ESCs. Starting in 2009, however, scientists started reporting differences between iPSCs and ESCs. For example, Chin et al. (2009) compared three human ESC lines and five iPSC lines by expression microarrays and identified hundreds of genes that were differentially expressed (Chin et al., 2009). They concluded that iPSCs should be considered a unique subtype of pluripotent cells. Two other studies also compared the global gene expression between ESCs and iPSCs and identified persistent donor cell gene expression in iPSCs (Ghosh et al., 2010; Marchetto et al., 2009).

It was Deng et al. (2009) who first reported that there were differences in DNA methylation between the two types of pluripotent stem cell lines after they performed the targeted bisulfite sequencing of three human ESC clones and four iPSCs lines. Doi et al. (2009) also reported that there were differentially methylated genes, such as BMP3, between ESCs and iPSCs. Subsequently, three studies reported epigenetic memories of donor cells in human induced pluripotent cells (Kim et al., 2011b; Lister et al., 2011; Ohi et al., 2011).

However, other studies have concluded that it is difficult to distinguish iPSCs from ESCs by gene expression or DNA methylation. Two reports showed that both iPSC clones and ESC clones have overlapping variations in gene expression and thus that the two types of pluripotent stem cells are clustered together by these analyses (Guenther et al., 2010; Newman and Cooper, 2010). They argued that these variations are, at least in part, derived from the different induction and culture conditions used by each laboratory. Bock et al. (2011) demonstrated that iPSCs and ESCs are very similar in their gene expression and DNA methylation and that some iPSC clones cannot be distinguished from ESCs. By examining how many iPS and ES clones were compared, we observed a clear tendency (Table 1). Studies that reported differences either in gene expression or DNA methylation compared relatively small numbers (generally fewer than 10) for each group), whereas those that found it difficult to distinguish iPSCs from ESCs analyzed many more clones, and clones from multiple laboratories.

Another major point of discussion has been the ability of the cells to differentiate and whether iPSCs are functionally different from ESCs in this respect. Hu et al. (2010) performed in vitro directed neural differentiation of five human ESC clones and 12 iPSCs clones. They showed that all of the ESC clones differentiated into Pax6 positive cells, with more than 90% efficacy, but the iPSC clones showed poorer differentiation, with ~10% to 50% efficacy. However, Boulting et al. (2011) examined 16 human iPSC clones for their ability to differentiate into motor neurons and found that 13 of these iPSC clones differentiated with comparable efficacies to ESCs. So, again, there are conflicting conclusions regarding the similarity between iPSCs and ESCs.

Taken together, these studies showed that iPSC clones and ESC clones have overlapping degrees of variation (Figure 3). It should be noted that variations among ESC clones have been well documented (Osafune et al., 2008; Ward et al., 2004). Although it is possible that iPSC clones show greater variation, and that some clones differ from ESCs in their gene expression, DNA methylation, or differentiation ability (Miura et al., 2009), it appears that at least some iPSC clones are indistinguishable from ESC clones.

It is interesting to consider what brings about such variation between the iPSC clones. We learned an important lesson from two related reports regarding mouse iPSCs (Carey et al., 2011; Stadtfeld et al., 2010). These two studies, conducted in the Hochedlinger lab and the Jaenisch lab, used very similar secondary induction systems to generate mouse iPSCs. However, the properties of the iPSC clones were very different between the two laboratories. Most of the iPSC clones generated in Hochedlinger lab could not be successfully used to generate germline competent chimeras by microinjection or "all-iPSC" mice by tetraploid complementation, the most stringent criterion to evaluate pluripotency. They showed that the loss of imprinting of the Dlk1-Dio3 gene cluster predicts these poor iPSC abilities. In sharp contrast, most of the iPSC clones generated in the Jaenisch laboratory had normal imprinting of the *Dl1-Dios3* cluster and were capable of generating high quality chimeras and viable all-iPSC mice.

The only notable difference between the two laboratories' methods was the order of the reprogramming factors in the expression cassettes, and this difference resulted in higher expression levels of Oct4 and Klf4 in the cells generated by the Jaenisch laboratory. By increasing the expression of Oct4 and Klf4 (Carey et al., 2011), or by supplementing with ascorbic acid (Stadtfeld et al., 2012), the quality of the iPSCs generated by an otherwise very similar method was enhanced. Thus, the level and stoichiometry of the reprogramming factors, as well as culture conditions, during iPSC generation can contribute significantly to the variation seen in the epigenetic state and pluripotent potential of the resulting iPSCs.

These data demonstrated that incomplete or imperfect reprogramming is not a fundamental problem associated with iPSCs. Instead, differences in the quality of iPSC clones seem to be largely due to technical variables, such as the factor combinations, gene delivery methods, and culture conditions. In addition, some variation between iPSC clones can be attributed to stochastic events during reprogramming, which cannot be controlled. Thus, evaluation and selection will be essential for identifying iPSC clones that are suitable for medical applications.

Is There a "Dark Side" to Induced Pluripotency?

Several reports have suggested that, in addition to variation in gene expression, DNA methylation, and pluripotent potential, there are other potential abnormalities in iPSCs, including somatic mutations (Gore et al., 2011), copy number variations (Hussein et al., 2011), and immunogenicity (Zhao et al., 2011). In some of these reports, the negative aspects of iPSCs were, in my opinion, overstated. The media overreacted, as did accompanying scientific commentaries with alarming words in their titles, such as "dark side," "under attack," "flaw," "troublesome," and "growing pains" (Apostolou and Hochedlinger, 2011; Dolgin, 2011; Hayden, 2011; Pera, 2011; Zwaka, 2010).

However, despite these doomsday headlines, subsequent analyses have indicated that many of the genetic differences found in iPSCs seem to have pre-existed in the original somatic cells, and therefore arose independently of the reprogramming process itself (Cheng et al., 2012; Young et al., 2012). Reprogramming to form iPSCs is inherently clonal, and therefore variations that exist at a low frequency within the starting cell population can become more apparent when analyzing individual clones derived from it and comparing them to the parental cell population as a whole.

Another study showed that a set of iPSC clones that are capable of generating all-iPSC mice have very few genetic alterations relative to their parental cells (Quinlan et al., 2011). The chimeric and progeny mice derived from iPSCs that are devoid of the Myc transgene appear to be normal, indicating that these iPSCs do not contain detrimental genetic alterations that have a negative impact on function (Nakagawa et al., 2008; Nakagawa et al., 2010). With regard to immunogenicity, it is not clear whether the reported weak immune reaction to transgene-free iPSCs is significant (Okita et al., 2011b) because the most prominent study that reported the immunogenicity of the cells examined undifferentiated iPSCs (Zhao et al., 2011), which will never be used in cell transplantation therapy. We have to understand all of these results and consider them in context to have a balanced view of iPSCs.

Why Are ESCs and iPSCs So Remarkably Similar?

Although there may be some differences between iPSCs and ESCs, they are, nevertheless, remarkably similar. If anything, we should perhaps be wondering why iPSCs and ESCs are in fact so similar despite their different origins and generation methods. No other examples of this level of similarity between man-made cells and naturally-existing cells exist. Several types of somatic cells, such as neural cells and cardiac myocytes, have been generated from ESCs/iPSCs or directly from fibroblasts. These man-made somatic cells have some of the characteristics of their normal counterparts that exist in vivo, but they are still very different from natural neural cells and cardiac myocytes. The similarity between ESCs and iPSCs is therefore in many ways exceptional.

One potential explanation is that ESCs are in fact also man-made. It is possible that ESCs do not exist under physiological conditions and instead are selected and established by cultivating the cells of the inner cell mass (ICM) under specific culture conditions. ESCs are different from the majority of cells in the ICM in many respects. For example, although cells in the ICM possess a low degree of global DNA methylation (Reik et al., 2001), ESCs have a higher level of methylation (Li et al., 1992). A Ras family gene, ERas, is highly expressed in mouse ESCs but not in embryos (Takahashi et al., 2003). ESCs also have longer telomeres than are seen in embryos (Varela et al., 2011). Thus, we may be discussing the relationship between two types of man-made cells rather than between man-made cells and naturally existing cells.

Through many researchers' efforts, the field has established culture conditions that enable the generation and long-term maintenance of both mouse and human ESCs. It is likely that these culture conditions select for cells with certain properties, and this selection would also contribute to making ESCs and iPSCs appear as similar as they do.

Concluding Thoughts

If we accept the idea that ESCs and iPSCs are both artificial cells types generated in the laboratory, we move on to another important question: do ESCs truly represent an ultimate control or gold standard for iPSCs? I think the answer is probably no. Instead, future studies should focus on the capacity of iPSCs themselves

to form new tissues, organs, and model organisms, as a stream that exists in parallel to that of ESCs as a branch of the same overall experimental river.

I believe that iPSC technology is now ready for many applications, including stem cell therapies. From each induction procedure, multiple iPSC clones of various qualities emerged (Figure 3). It is thus essential to select good clones for medical applications. We may be able to narrow down candidates for good clones by marker gene expressions. However, we have to confirm in vitro differentiation propensities and genome and epigenome integrities. For wide-spread use, it might be necessary to establish in advance stocks of qualified iPSC clones from healthy volunteers or from cord-blood stocks. Immunorejection could be decreased by generating iPSCs from HLA homozygous donors (Okita et al., 2011a).

iPSC technology will likely have a substantial impact not only on science but also on business and politics. However, iPSCs should be evaluated based strictly on the scientific data, and all such data should be thoroughly considered for its relevance to potential clinical applications of the cells. Scientists should focus on research, and politicians and businesses should rely on the hard evidence generated from scientific studies to inform future directions rather than on the opinions of those who do not fully understand the field.

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