Review Article

Induced pluripotent stem (iPS) cells offer a powerful new tool for the life sciences

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Abstract

Stem cell biology started with the analysis of somatic stem cells that function to maintain the adult body. We now know that the body is maintained by regeneration of a wide range of cell types, such as skin cells, blood cells and gastrointestinal mucous cells, from somatic stem cells. This regenerative activity is essential for survival. Regenerative medicine was initiated to identify therapies that support and/or accelerate this natural regenerative ability. For example, bone marrow transplantation is a therapy for reconstituting hematopoiesis from the hematopoietic stem cells present in the donor bone marrow. The successful development of a protocol for obtaining human embryonic stem (ES) cells prompted medical scientists to utilize human ES cells for regenerative medicine. However, use of these cells raises ethical issues as they are derived from human embryos. An alternative approach using ES-like pluripotent stem cells has the considerable advantage that it does not necessitate use of human embryos. Pluripotent stem cells can be induced from terminally differentiated somatic cells by the introduction of only four defined factors. The products of this method are termed “induced pluripotent stem (iPS)” cells. iPS cells have considerable promise as a substitute for ES cells not only for regenerative medicine but also in many other fields. For example, liver and heart cells derived from iPS cells can be used in pharmaceutical research. In addition, iPS cell technology opens new avenues of disease research, for example, by construction of so-called “disease-specific iPS cells” from a patient's somatic cells.

Key words: ES cells, iPS cells, Regenerative medicine, Disease specific iPS cells
Prologue

The development of technologies such as PCR and gene knock-out that enable the manipulation of an organism's genetic material contributed tremendously to progress in the life sciences in the final decades of the last century. This century looks to continue this progress through the development of further new technologies such as that described here, the production and manipulation of induced pluripotent stem (iPS) cells.

Discovery of plasticity in terminally differentiated cells

It was believed for a long time that epigenetic modifications in differentiated somatic cells were irreversible. This meant that terminally differentiated cells could never return to being immature cells. However, in 1962 it was reported that the nuclei of somatic cells of an amphibian (frog) were reprogrammed following transfer into enucleated unfertilized eggs. Following transfer of a somatic cell nucleus, the egg could undergo cell division and differentiate to produce an adult frog. This result clearly indicated that epigenetic modifications in terminally differentiated somatic cells were reversible. Dr. John Gurdon, who performed this groundbreaking study, received the Albert Lasker Basic Medical Research Award in 2009.

Initially, many biologists believed that this reversibility of epigenetic modifications in terminally differentiated cells was restricted to amphibian somatic cells and did not occur in mammalian somatic cells. However, in 1997, a nuclear transfer experiment in sheep in which somatic nuclei were transferred into unfertilized eggs showed that epigenetic modifications in terminally differentiated mammalian somatic cells were also reversible. This experiment famously resulted in the birth of the first live cloned sheep, named “Dolly”.

The methodology for isolating and culturing mouse embryonic stem (ES) cells was first developed in 1981 and has aided research in a wide range of biological studies. Dr. Martin Evans, who developed the technology for establishing mouse ES cell lines, was awarded a Nobel Prize in 2007 together with Dr. Mario Capecchi and Dr. Oliver Smithies, who developed homologous recombination technology in mouse ES cells. As a result of these technical advances, functional analysis of genes has progressed considerably using mice with gene knock-outs or other genetic modifications.

It is well known that mouse cells can be immortalized simply by continuous *in vitro* culture, for example, using the so-called “3T3 protocol”. One widely exploited example of an immortalized cell line is NIH3T3, which continues to be used in a wide range of experiments. In contrast, it is not possible to immortalize human somatic cells in a similar manner and this difficulty gave rise to the widespread assumption that it would not be possible to establish human ES cell lines. However, in 1998, 17 years after the first establishment of mouse ES cell lines, it was reported that human ES cell lines could also be produced by continuous *in vitro* culture.

Therapeutic cloning

The ability to reprogram mammalian somatic cells by nuclear transfer and to establish human ES cell lines stimulated medical scientists to investigate the creation of ES cell lines using nuclear transfer as a potential means of achieving "therapeutic cloning". If this technology could be established as a viable therapy, then patients who would benefit from somatic cell transplantation could be treated with nuclear-transferred ES cells produced using their own somatic cells, which would avoid the possibility of transplant
rejection as the cells possess the same major histocompatibility (MHC) antigens as host tissue.

Although an earlier report of successful therapeutic cloning by a group in Korea proved false, it was recently reported that primate ES cell lines have been established by nuclear transfer technology\(^5\). Since unfertilized primate eggs are much more fragile than those of rodents, it may still take some time to establish the technology for use in human therapeutic cloning. However, the prospect of using such therapy no longer seems to be so distant.

**A search for alternative technologies to therapeutic cloning**

An important limitation to the use of therapeutic cloning is that it requires unfertilized eggs. Human eggs are very difficult to obtain and, moreover, their use for this purpose also raises serious ethical issues. For these reasons, a search has been initiated for alternative methodologies that avoid nuclear transfer. One approach has been to search for factors in unfertilized eggs that may be required for the reprogramming of transferred somatic nuclei. Another avenue of research has been to elucidate which genes specifically function in ES cells, since these genes may maintain the undifferentiated state of ES cells and thus might be able to induce reprogramming of nuclei in terminally differentiated somatic cells.

The research group led by Dr. Shinya Yamanaka reported the first success in the latter approach. They were able to induce differentiated mouse somatic cells to become pluripotent stem cells by application of four defined factors\(^6\). The enforced expression of the transcription factors Oct3/4, Sox2, Klf4 and c-Myc in terminally differentiated somatic cells induced cellular reprogramming and changed the cells into ES-like pluripotent stem cells. These reprogrammed cells were named “induced pluripotent stem (iPS) cells”. Subsequently, in the year after establishment of human iPS cell lines was first reported, several other groups also succeeded with this methodology\(^7\)\(^-\)\(^10\). Dr. Shinya Yamanaka, who developed the method, was given the Albert Lasker Basic Medical Research Award in 2009 together with Dr. John Gurdon.

The mechanisms underlying the reprogramming of terminally differentiated somatic cells following the enforced expression of the four factors remain to be elucidated. It is now known that expression of these factors after exogenous introduction is completely suppressed in established iPS cells. Thus, the factors seem to be required only for the reprogramming process but not for maintenance of pluripotency. Regardless of the mechanisms involved, this discovery clearly indicated that terminally differentiated somatic cells could be reprogrammed without nuclear transfer into unfertilized eggs and opened a new dawn for therapeutic cloning\(^11\)\(-\)\(^13\).

A mouse iPS cell line, iPS-MEF-Ng-20D-17, established by Dr. Yamanaka’s group, that can differentiate into germ line cells. In this cell line, GFP has been knocked-in under control of the Nanog promoter and thus the undifferentiated cells express GFP.

**Progress in methods to establish iPS cells**

Although the first mouse iPS cell lines could not differentiate into germ line cells, the next generation of iPS cell lines was able to undergo this differentiation process (Figure 1)\(^14\). It has also been reported that c-Myc is not necessary for establishing iPS cells, although the efficiency of reprogramming was reduced...
in its absence\textsuperscript{15}. Use of c-Myc will probably not be necessary in future as methodological improvements have continuously increased the efficiency of cellular reprogramming\textsuperscript{16-24}.

The first method for establishing iPS cells utilized a retroviral vector to introduce the four factors. Since retroviral vectors integrate into the genome, it is possible that they might affect the function of endogenous genes and thus it might be inappropriate to make use of such cells in the clinic. The group of Dr. Yamanaka developed an alternative approach that avoided the use of retroviral vectors by employing ordinary plasmid vectors\textsuperscript{25}. Yu \textit{et al.} also reported a method for establishing iPS cell lines that obviated the need for vector and transgene sequences\textsuperscript{26}.

Other approaches for producing iPS cell lines have recently been described\textsuperscript{16-24}. One of the most promising is that utilizing the Sendai virus vector. As the Sendai virus does not integrate into the host genome, it is possible to create iPS cell lines that do not carry the exogenous reprogramming factors\textsuperscript{27}. Of course, since the mechanisms involved in reprogramming somatic cells remain uncertain, we cannot be certain that iPS cells produced with the Sendai virus vector possess identical characteristics to ES cells. However, it is highly likely that they will be more suitable for clinical applications than cells with integration of the exogenous genes.

**Standardization of iPS cells**

The characteristics of ES cells vary among different cell lines. Indeed, even those of mouse ES cell lines derived from an inbred mouse strain show significant differences; for example, some cell lines can differentiate into germ line cells whereas others cannot. It is therefore unsurprising that the characteristics of human ES cell lines also differ among cell lines. Additionally, the genomic backgrounds of human ES cell lines differ among cell lines. As mentioned above, the mechanisms of reprogramming are uncertain and, therefore, standardization of human iPS cell lines is currently a very difficult process. It is clear that conventional analyses, such as detection of molecular markers of the undifferentiated state or observation of teratoma formation in immunodeficient mice, are insufficient to characterize iPS cells and that more objective and quantitative analyses are required. Gene expression profiling analysis and epigenetic modification analysis are candidate methods for characterizing iPS cell lines. Given the diversity in human genetic backgrounds, characterization of human iPS cell lines will require objective and quantitative analyses of at least a few hundred cell lines.

 Needless to say, fundamental quality controls of the cell lines are also essential. First, the cell lines should be free of microbial contamination. Bacterial or fungal infections are less important as these are readily detected. However, mycoplasmal infections are less easy to identify without examination of the cultures since the majority of cell lines will survive the presence of mycoplasma. Second, misidentification of cell lines should be eliminated. Profiling of short tandem repeat (STR) polymorphisms is a very useful means of ensuring the correct identification of cell lines and is now routinely performed in major cell banks around the world\textsuperscript{28, 29}.

**Clinical grade stem cells**

Cells derived from ES or iPS cell cultures have applications in many fields of medical science. For example, liver or heart cells derived from stem cells can be used for pharmaceutical drug screening. Needless to say, however, the most attractive application is for clinical therapies. Such applications require so-called clinical grade stem cells. What are the characteristics of clinical grade stem cells?

There are many risks involved in using long-term cultured cell lines in the clinic\textsuperscript{30}. The most critical of these risks is tumorigenicity. Since both ES and iPS cells are immortalized, in one sense they are very close in nature to tumor cells. Indeed, they can give rise to
teratomas when transplanted into immunodeficient mice. Although these teratomas are not malignant teratocarcinomas, they are nevertheless tumors.

One way to make use of stem cell-derived cells is to induce post-mitotic cells that are unable to proliferate. Terminally differentiated neural and heart cells are possible candidates for post-mitotic cells. Enucleated cells (cells that do not possess nuclei), such as red blood cells and platelets, are absolutely free of risk of tumorigenicity and may therefore be close to use for clinical applications, although systems for mass production of such cells need to be established prior to their application.

**Disease-specific iPS cells**

The ability to produce iPS cell lines will benefit both regenerative medicine and also research into a variety of diseases. For example, it is currently not possible to obtain neural cells from patients suffering from neural diseases. However, iPS cell lines could be established from their somatic cells, such as skin fibroblasts, and neural cells could then be derived in culture. Such iPS-derived neural cells could be subjected to a range of research analyses, such as investigation of the underlying mechanisms of disease or for drug discovery for disease therapy.

There are still many diseases for which the causes are unknown and therapies have not been developed. Thus, the value of disease-specific iPS cells should increase tremendously in the near future. Human cancer cells are exceptional in that they can be immortalized simply by continuous in vitro culture, and many cancer cell lines are currently used for experimental analyses. Of note, however, low malignancy and benign tumor cells cannot be immortalized in a similar fashion to normal human somatic cells. Therefore, iPS cells derived from low malignancy or benign tumor cells may also be useful for the study of such diseases.

**Establishment of progenitor cell lines from iPS cells**

Immortalized cell lines that possess the ability to differentiate are very valuable not only for analyzing the mechanisms of differentiation but also for the provision of abundant differentiated and mature cells. For example, immediate progenitor cell lines of red blood cells have been established from mouse ES cells, and these cell lines can produce mature enucleated red blood cells in vitro (Figure 2). If similar progenitor cell lines can be established from human ES or iPS cells, then they may be of use for the in vitro production of transfusable red blood cells.

**Figure 2**

Mouse ES cell-derived erythroid progenitor (MEDEP) cells before (left) and after (right) in vitro differentiation. Arrowheads in the right photograph indicate enucleated red blood cells.

With regard to mature cells such as liver or heart cells, immediate progenitor cell lines for these cells will be invaluable. Such progenitor cell lines could be established using human ES or iPS cells in a similar manner as for the establishment of red blood cell progenitor cell lines from mouse ES cells.

**New insights into cell plasticity**

It is possible that the reprogramming of somatic cells is incomplete, that is, that the cells are not fully reprogrammed. Such partially reprogrammed cells might be termed “pseudo iPS cells”. What characteristics might distinguish such cells? As they are highly...
likely to be progenitor cells for specific somatic cells, we might be able to obtain progenitor cell lines using these “pseudo iPS cells” without recourse to genuine iPS cells that have been fully reprogrammed. In this context, a highly instructive paper was published recently. Vierbuchen et al. reported that they had succeeded in directly converting fibroblasts into functional neurons by induced expression of three transcriptional factors. By utilizing cell plasticity, it may be feasible to obtain various types of somatic cells without making genuine iPS cells.

Epilogue

We now have an excellent technology for the relatively simple production of ES-like pluripotent stem (iPS) cells from somatic cells. A recent report indicates that iPS cell lines can be established even from peripheral blood cells. Since obtaining a blood sample is a commonplace clinical activity, development of iPS cell lines will undoubtedly spread rapidly through use of peripheral blood cells, particularly for preparation of disease-specific iPS cell lines.

In future, it is possible that every person will have their own iPS cell lines, prepared when they were still healthy, for future applications in clinical examination and/or therapy. Is this just science fiction? I believe not.

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