

## **Superman flew away too soon: how to make embryonic stem cells from adult cells**

- **Pranay Sinha**

The horse changed its mind about jumping over the fence and the burly rider was thrown violently to the ground. The helmet saved his head, but the impact shattered the first and the second vertebrae; his spinal cord was disconnected from his brain. Unlike skin cells that are replaced everyday cells of the nervous system don't mend themselves once damaged. Superman doesn't fly off-screen, after all.

Christopher Reeves went on to campaign for Human Embryonic Stem Cell research. His death in 2004 didn't decelerate the race to develop stem cell technology to a point where a miracle cure for people like him couldn't exist. In addition, stem cell mediated regenerative therapies have frequently been considered the way to cure degenerative diseases such as Alzheimer's and Parkinson's disease.

Recently, some exciting events have taken place in the world of stem cells. This review paper recounts some of the recent advances in this marvelously promising, but frightfully controversial, field of science. Indeed, *The Economist* has dubbed this field "the most operatic in science." However, it is imperative to establish some basic facts regarding stem cells first.

Stem cells are pluripotent cells: undifferentiated cells that have the potential to become any cell type. They can be classified into two types: adult stem cells and embryonic stem cells. Adult stem cells are used to repair and replace cells that are lost during wear and

tear. These can be found in minute quantities within the bone marrow and the umbilical cord. Embryonic stem (ES) cells have the potential to mature into any cell type. They are collected from the inner cell mass of embryos in the blastocyst or morula stage of development. ES cells have significant potential in regenerative therapies.

However, it is distinctly possible for the body of a stem cell recipient to reject it due to histo-compatibility issues. Therefore, it was proposed to prepare embryonic stem cells using the DNA of the recipients themselves, thereby reducing the probability of a rejection. Hence, a technique called Somatic Cell Nuclear Transfer (SCNT) was developed. In this procedure, the nucleus from a somatic cell (like a skin cell) is transferred to an enucleated egg cell. Next, cell division is simulated using an electric shock. The cell now develops into an embryo that can be harvested for stem cells. The stem cells are extracted and grown in separate Petri-dishes to keep them away from chemical signals from other cell types that might prompt their differentiation, robbing them of their pluripotent status. These isolated stem cells divide to form “cell lines” and can be used for research or therapy.

Unfortunately, stem cells can't be extracted from embryos without destroying the latter. This aspect of stem cell research offends the moral sensibilities of those who oppose it. Political ideologues have deftly entangled the stem cell extraction process with the abortion controversy. Consequently, the moral outrage of a minority of tax-payers has brought stem cell research in the USA to a shuddering halt. Today, a limited number of stem cell lines are available for research purposes in America. These lines are not only

contaminated, but possibly contain mutations due to repeated division. Many scientists question why the destruction of embryos during the extraction of stem cells draws such vociferous protests when fertility clinics routinely dispose of extra embryos as medical waste. Stringent laws have resulted in countries like Japan and South Korea racing past the US in stem cell technology. However, there may be light at the end of the tunnel (and it's not an LIRR train).

The success of SCNT and the subsequent cloning of Dolly<sup>4</sup> made the scientific community aware of the existence of cytoplasmic trans-acting factors that were capable of reprogramming somatic cells into reverting to an embryonic stem cell like state. This realization prompted a hunt for these transcription factors.

In November 2007 --10 years after Wilmut's critical breakthrough-- Takahashi et al. reported their success in synthesising pluripotent cells without using the controversial SCNT protocol. They reported that induced pluripotent cells (iPS) could be produced from human dermal fibroblasts (HDF) by transducing four transcription factors- Oct3/4, Sox2, Klf4, and c-Myc.<sup>1</sup>

It seems almost amazing that the transduction of four transcription factors can lead to such an astounding transition from an HDF to a pluripotent cell. Prior to Takahashi et al., Wernig et al. had shown that Oct3/4, Sox2, Klf4, and c-Myc can epigenetically reprogramme a mouse somatic cell into an embryonic stem cell like state<sup>2</sup>. These seemed to be some of the aforementioned trans-acting factors involved in converting somatic cells into embryonic stem cells. Oct3/4 and Sox2 have been shown by previous studies to

act as the central pluripotency generating transcription factors. Takahashi et al. speculate that c-Myc and Klf4 make the chromatin structure more conducive to the binding of Oct3/4 and Sox2. This speculation is strengthened by the fact that Klf4 is known to regulate the acetylation of histones<sup>1</sup>.

Wernig et al. transduced the four transcription factors into mouse fibroblast cells. iPS cells were selected for by looking for the activation of a gene downstream of Oct4-Fbx15. However, the cells, though pluripotent, were dissimilar to ES cells in some regards. For instance, some differences exist in the methylation patterns and gene expressions of ES cells and iPS cells selected using Fbx15. Furthermore, these iPS cells do not contribute to viable chimaeras<sup>2</sup>.

Okita et al. showed that selecting for Nanog instead of Fbx 15 produces cells that resemble the ES cells even more with regard to epigenetics and gene expression<sup>3</sup>. Nanog is more closely associated with pluripotency than Fbx 15, since it is known that disrupting Nanog in mice results in a loss of epiblast pluripotency. Conversely, disrupting Fbx15 doesn't produce a readily observable effect. The researchers isolated a bacterial artificial chromosome (BAC) containing the mouse Nanog gene and inserted a GFP-internal ribosomal entry site(IRES)- puromycin resistance gene (Puro<sup>r</sup>) cassette into the 5' untranslated region. These BACs were introduced within ES cells. The ES cells that incorporated the modified BAC were GFP-positive. However, they became negative for GFP when they differentiated because the Nanog gene is highly expressed in pluripotent cells, but not in differentiated cells. Thus, this cassette enabled the researchers to spot

cells that expressed Nanog strongly and to select for such cells using puromycin.

The ES cells that stably incorporated the GFP were then introduced into mouse blastocysts to obtain chimaeric mice that were used to produce transgenic mice containing the aforementioned reporter construct (Nanog-GFP-IRES-Puro<sub>r</sub>). The blastocysts from the transgenic mice contained the reporter in the inner cell mass. The primordial germ cells were found to be GFP-positive 9.5 days post coitum (d.p.c.) and by the 13.5 d.p.c, the genital ridges were also GFP positive.

Okita et al. extracted MEF (mouse embryonic fibroblasts) cells (which were GFP negative) and introduced the aforementioned transcription factors into the mouse cells using retroviral vectors. The researchers used a mutant of c-Myc (T58A) instead of the wt gene. The MEF cells were cultured on feeder cells. After giving the cells time to heal from the retroviral infection, puromycin selection was commenced. Twelve days post retroviral infection, hundreds of GFP positive colonies were observed. Some GFP negative colonies were also present. The researchers are unsure about the reason for their occurrence. By contrast, no GFP positive colonies were observed when mock DNA was used instead of the four transcription factors. Remarkably, raising the puromycin concentration during the selection process lowered the number of GFP-negative colonies. Significantly, if any of the four transcription factors were not added, GFP-positive colonies weren't produced. This shows that all four transcription factors are necessary for the production of iPS cells.

Bisulfite sequencing was used to show a congruence between Nanog selected iPS cells and ES cells in their methylation patterns. Methylation patterns reveal the pattern of gene silencing in a genome. DNA methyltransferases mark cytosines with methyl groups. This results in the formation of 5-methylcytosine that has normal base-pairing abilities, but has an altered structure. Methylation results in the formation of heterochromatin which forms DNA that cannot be transcribed. Treating DNA with bisulphite results in the conversion of normal cytosine bases into uracil. However, the existing methylcytosine is not altered. The conversion of cytosine to uracil is almost like inducing single nucleotide polymorphisms (SNPs) within the DNA. These SNPs can then be studied to produce a high resolution picture of the DNA methylation in the cells using numerous sequencing techniques.

Furthermore, these iPS cells resembled ES cells in their morphology (ES cells have flat colonies), teratoma formation, and proliferation pattern. It was shown that Nanog gets downregulated when a cell begins to differentiate, thus further strengthening the case for using Nanog as a selection criterion for pluripotent cells. However, the induction efficiency was very low. Now that the induction of pluripotency using these four transcription factors has been established, it would be profitable to scrutinize the work of Takahashi et al. in greater detail.

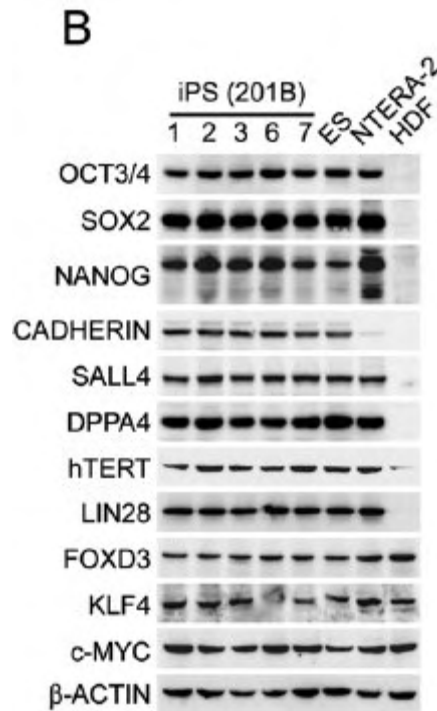
The first step taken was to increase the transduction efficiency of the HDFs they were working with. This was achieved by the introduction of a mouse retroviral receptor (Slc7a1) using a lentivirus as a vector. These HDF-Slc7a1 cells had increased



It was found that the human iPS cells resemble human ES cells in numerous ways. Takashi et al. carried out reverse transcriptase polymerase chain reaction analysis (RT-PCR) to show that the human iPS cells expressed many genes normally found in undifferentiated ES cells. The list included OCT3/4, SOX2, NANOG, GDF3 (growth and differentiation factor 3), REX1 (reduced expression 1), FGF4 (fibroblast growth factor 4) etc. RT-PCR is an excellent method of assaying transcription levels. It contains two steps: first strand reaction and second stand reaction. Essentially, the first strand reaction involves the treatment of mRNA with reverse transcriptase to convert it into cDNA. The primers are targeted to the 3' polyadenylated sequences (Poly A tail) of the mRNAs being processed. Subsequently, the cDNA is amplified using DNA polymerase. This is the second stand reaction. The RT-PCR results can be seen in figure 1.

Moreover, western blot analysis reported that the iPS cells resembled ES cells in their OCT3/4, SOX2, NANOG, SALL4, E-CAD-HERIN, and hTERT protein levels. Western blot is a technique used to assay the expression of a specific protein. Essentially, the protein is extracted and electrophoresed. After the electrophoresis, the proteins are transferred onto a nitrocellulose (or PVDF) membrane where they are probed with antibodies. Subsequently, a secondary antibody is added. This antibody attaches to the primary antibody. Usually, the secondary antibody is fluorescent or chemo-luminescent. By monitoring the abundance of the secondary antibody, it is possible to estimate the level of protein expression. By comparing the displacement of the band to a molecular marker, it becomes possible to identify the protein on the basis of its mass. Figure 2

shows the western blot analysis off iPS cells.



*Figure 2: This figure shows that iPS cells contain comparable level of proteins with respect to ES cells. Indeed, a striking resemblance is observed in terms of protein expression. Note that HDF cells have no or almost null expression of OCT3/4, SOX2, NANOG, E-CADHERIN, SALL4, SPPA4, hTERT, and LIN28. However, the iPS cells derived from HDF cells express these proteins exactly like ES cells.*

Furthermore, DNA microarray analysis confirmed that iPS cells and ES cells are similar (though not exactly equal) in terms of gene expression. When compared with HDF cells, it was found that HDF cells had methylated loci for OCT3/4, NANOG and REX1 while iPS cells had unmethylated loci for the same genes (probably since these are required for maintaining pluripotency). This was confirmed using the chromatin immunoprecipitation (ChIP) analysis.

Proteinaceous trans-acting factors bind to specific DNA domains. However, they don't bind to all possible domains at the same time. It is possible to infer DNA methylation and gene silencing patterns based on information regarding the DNA sequences that a particular regulatory is binding to in a cell. This can be done using ChIP analysis. Basically, the DNA is extracted and broken down mechanically. Subsequently, the broken up DNA is probed with the antibody of the regulatory protein. This yields a collection of DNA sites that the regulatory protein was bound to. This methodology can be, and was, employed to detect histones modified by methylation. The ChIP analysis showed that the histones in the promoter sites of Oct 3/4, Sox2, and Nanog were demethylated (unlike the HDF cells that were highly methylated at these loci).

In addition, iPS resembles ES cells in their high telomerase activity, exponential proliferation, and teratoma formation. (this is not the first time you mentioned teratoma formation and proliferation patterns, if that matters) Furthermore, Takahashi et al. were also able to demonstrate the ability of the iPS cells to differentiate into dopaminergic neurons by co culturing them with PA6 cells. They demonstrated using PCR analysis that OCT3/4, SOX2 and NANOG were downregulated (showing reduction of pluripotency) while dopaminergic neuron markers (assumption of dopaminergic neuron properties) were upregulated: showing that the cells were capable of differentiation. Perhaps bisulfite sequencing could have been done to ascertain whether the pluripotency related genes were methylated and the neuron genes were unmethylated in the iPS cells co-cultured with PA6 cells. Alternatively, a serial analysis of gene expression (SAGE) could have

been done as a confirmatory to monitor the transcription levels of the known dopaminergic neuron genes within the cocultured iPS cells.

This characteristic of the iPS cells creates hope for people suffering from degenerative diseases such as Parkinson's and Alzheimer's. Even more phenomenally, the iPS cells were induced, using Activin A and bone morphogenic protein (BMP), to turn (in-vitro) into cardiomyocytes. Within 12 days of induction, the cells began beating. RT-PCR showed the presence of cardiomyocyte markers. Clearly, the iPS cell technology may, once perfected and fine tuned, be the much-awaited knight-in-shining-armour of the field of regenerative medicine, especially since histo-compatibility issues can be swept away by using somatic cells from the patients themselves.

In addition to the HDF cells (taken from a 36 year old Caucasian woman) that were converted into iPS, human-fibroblast-like-synovocytes (HFSL) from the synovial joint of a 69 year old man were also successfully turned into iPS cells. For an encore, Takahashi et al. also turned BJ cells from neonate fibroblasts into iPS cells. All the iPS cells resembled the HDF iPS cells in the aforementioned ways.

There is however a small drawback to this procedure. It was found that each iPS clone receives three to six retroviral integrations per transcription factor. This increases the probability of tumour formation. Indeed, 20% of mice derived from iPS cells possessed tumours. This has been blamed on the reactivation of the c-Myc retrovirus<sup>1</sup>. c-Myc is a known oncogene. Indubitably, the utilization of c-Myc is a risky proposition. The usage

of retroviruses is controversial as well and the authors propose to either begin using adenoviruses instead, or to find molecules small enough to induce gene transfer sans gene transfer.

However, it seems the somewhat hazardous transduction of  $c\text{-Myc}$  is not imperative for the creation of iPS cells. Yu et al. made a list of genes that were enriched in ES cells relative to myeloid precursors. OCT 4 is known to be expressed greatly in pluripotent cells and is also known to confer geneticin resistance to cells. Hence, geneticin selection was used to isolate pluripotent cells. Initially, a combination of 14 genes was used to reprogramme CD45<sup>+</sup> haematopoietic cells into OCT4 positive, geneticin resistant cells that resembled ES cells in the morphology and cells surface markers. Re-testing was carried out. Finally, using this approach, Yu et al. showed that OCT4, SOX2, NANOG, and LIN28 genes were ample for making iPS cells. Once again, DNA microarray analysis showed that the gene expression in the reprogrammed cells resembled ES-cell gene expression. Furthermore, these cells were shown to form teratomas like ES-cells. Additional tests verified the similarities between ES cells and the iPS cells. The STR (short tandem repeat) pattern of the iPS cells was checked to ensure that they were reprogrammed cells and not contaminating ES-cells. These results, by eliminating the use of the dangerous  $c\text{-Myc}$  transcription factor, enhance the prospects of the usage of stem cells in regenerative medicine.

In other news, Byrne et al. report the production of primate ES-cells using a modified SCNT protocol. Byrne had previously reported that the removal of lamin A/C was

essential for the remodeling of the oocyte into an ES cell. This required the functioning of a maturation-promoting factor (MPF), which might have been degraded in the previous protocol (due to the usage of Hoechst stains and UV light), resulting in non-removal of lamin A/C. The old protocol also tended to damage mitochondrial DNA, thereby reducing the success rate. In their new protocol, Byrne et al. used a system called the oosight imaging system that utilises polarized light to view the chromosomes; allowing them to manipulate the oocyte without damaging MPF. The researchers attribute much of their success to this system. In addition, they modified calcium and magnesium levels in the oocyte media to decelerate the degradation of MPF. Whatever they did obviously worked! Indeed, the “most operative field in science” seems to be turning into the most dynamic one as well.

Why should we care about all this? Stem cells are a fascinating resource. They can be used extensively for research purposes. For instance, if it is difficult to obtain diseased tissues to study, patient-specific cells can be concocted to study the disease (using ES cells) since these cells are identical to the diseased cells. Drug trials could be expedited with the use of these iPS cells as well. Obviously, regenerative medicine is one of the most touted, as well as the most famous, applications of stem cells.

Some concerns do exist about the long time delays and the difficulties in producing the differentiated treatments required for regenerative medicine. However, one thing that has been established by these recent developments in the stem cell world is that technology and science are sprinting rapidly. It is very likely that in a very short amount of time,

regenerative medicine will go from just being a distinct theoretical possibility to a routine medical procedure. As this paper was being finished, the media announced that a putative stem cell treatment for Duchenne's muscular dystrophy has been developed. Excitingly, the DMD treatment technique can find applications in other genetic diseases as well.

Things like neuronal regeneration are right around the corner. Superman flew away too soon.

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