Overview of cellular reprogramming

The discovery of induced pluripotent stem (iPS) cells emerged from the synthesis of two scientific technologies:

1. Somatic cell nuclear transfer (SCNT): the finding that differentiated cells can be reprogrammed into early embryonic stem cells, and
2. Transcription factor technology: our improved understanding of the roles transcription factors play in cellular differentiation.

In 2001, President George Bush signed into law a ban that severely restricted federal funds to support human embryonic stem cell research because this research called for the destruction of the embryo. This stimulated scientists to develop alternative means to generate stem cells. One could even speculate that the development of induced pluripotent stem cells (iPS) in 1996 was a direct result of this ban (Loike and Fischbach 2009).

Induced Pluripotent State (iPS)

Recent successes in transforming adult mouse fibroblasts into pluripotent stem cells (iPS), and the use of lineage reprogramming (see Module 6), represent prime examples in which technology has been developed to defuse the intense ethical and political debate concerning embryonic stem cell research. While these successes are exciting, a great deal of work nonetheless remains to establish whether stem cells obtained using iPS cell technology are as safe, plastic, as easily obtainable, and as pluripotent as the classical stem cells obtained from the inner cell mass of an embryo (human or animal). For the foreseeable future, stem cells from the early embryo will remain the gold standard in stem cell research, against which we will need to compare and contrast all other technologies.
Induced pluripotent stem cells technology was first developed with mouse fibroblasts as reported by Shinya Yamanaka and his colleagues. The researchers demonstrated that the addition of four transcription factors could induce pluripotency, thereby transforming a differentiated cell into a stem cell (Mitsui, Tokuzawa et al. 2003).

- Octamer-binding transcription factor-3/4 (OCT3/4),
- SRY-related high-mobility-group (HMG)-box protein-2 (SOX2),
- MYC
- Kruppel-like factor-4 (KLF4)

To induce pluripotency, researchers packaged the genetic material coding for these four transcription factors into a retrovirus, which they then used to introduce the transcription factors in host cells. Once the validity of this method was established, the protocol was applied to other types of mouse cells and eventually to human somatic cells. Today there are a variety of viral or plasmid delivery systems including retroviruses and lentiviruses, adenovirus transduction, and plasmid transfection – all of which can be employed to introduce specific transcription factors into cells.

It is interesting to recount how Yamanaka and his colleagues developed iPS cell technology. To identify
transcriptional regulators that can reprogram adult cells into pluripotent cells, Dr. Yamanaka and his team of scientists (Takahashi and Yamanaka 2006) devised an elegant screen for factors within a pool of 24 pluripotency-associated candidate genes that they believed together or separately would activate a dormant drug-resistant allele integrated into the ESC-specific Fbxo15 locus. They then screened these 24 factors for those factors that activated Fbxo15 and induced the formation of drug-resistant colonies with characteristic ESC morphology. Their research led them to identify four genes, Klf4, Sox2, c-Myc, and Oct4, that were required to transform a terminally differentiated cell into a pluripotent stem cell. Although the original gene set for reprogramming the parent cell comprised of Oct3/4, Sox2, Myc, and Klf4, recent studies have shown that other combinations of transcription factors can substitute for MYC and KLF4 and produce iPS cells.

In 2006 Yamanaka and his colleagues published their report on induced pluripotent stem (iPS) cells in mouse cells (Takahashi and Yamanaka 2006) and within a year they demonstrated iPS in human cells. Shinya Yamanaka and his colleagues (see Module 1 for more on Dr. Yamanaka) used the same methods that they had described in their publication regarding iPS in mouse cells and using the same four pivotal genes: Oct3/4, Sox2, Klf4, and c-Myc with a retroviral system (Takahashi, Tanabe et al. 2007). In November 2007, another independent research teams reported generating human stem cells via iPS. James Thomson and colleagues (Yu, et al., 2007) (for more on Dr. Thomson see Module 1) used the following transcription factors - OCT4, SOX2, NANOG, and LIN28 - in a lentiviral system to generate iPS cells. Recall that in 1998, Dr. James Thomson published the first article describing how human stem cells can be obtained from the inner cell mass of human embryos.

**Biological properties of iPS cell transcription factors**

- **Oct-3/4 (Pou5f1):** Oct-3/4 is a member of the octamer ("Oct") transcription factors, and plays a crucial role in maintaining pluripotency.
- **Sox family:** The Sox family of genes is associated with maintaining pluripotency similar to Oct-3/4, but is associated with both multipotent and unipotent stem cells. Oct-3/4, in contrast, is exclusively expressed in pluripotent stem cells.
- **Klf family:** Klf4 of the Klf family of genes was initially identified by Yamanaka et al. (2006,2007) and confirmed by Welstead, et al. (2008) as a factor for the generation of mouse iPS. Yu et al., (2007) however, reported that Klf4 was unnecessary for generation of human iPS cells and, in fact, failed to generate human iPS cells.
- **Myc family:** The Myc family of genes are proto-oncogenes implicated in cancer. Usage of the "myc" family of genes in the induction of iPS cells creates possible causes for concern regarding the eventuality of using iPS cells in clinical therapies. One finding suggests that 25% of mice transplanted with c-myc-induced iPS cells developed lethal teratomas.
- **Nanog:** In embryonic stem cells, Nanog, along with Oct-3/4 and Sox2, is necessary in promoting pluripotency.
- **LIN28:** LIN28 is an mRNA binding protein expressed in embryonic stem cells and embryonic carcinoma cells associated with differentiation and proliferation.

The first generation of iPS cells did not generate viable chimeras (organisms composed of two genetically
different cells- see module 7) when injected into blastocysts, in part because the endogenous Oct4 promoter was still methylated. It was only after subsequent experimentation that researchers realized that in order to generate fully reprogrammed iPS cells following the introduction of the four factors (Oct4, Sox2, Klf4, and cMyc), they had to select for endogenous expression of Oct4 and Nanog. Ultimately, the fully reprogrammed iPS cells were unmethylated at their Oct4 and Nanog promoters, which allowed for their contribution to the chimera germline (Okita, Ichisaka et al. 2007).

Problems with iPS cells

One problem with transfecting these genes into adult cells is that c-Myc is an oncogene with the potential to transform a normal cell into a cancerous-like cell. In fact, over 25% of embryonic stem cells injected into mice form tumors. Thus, considerable efforts have been made to circumvent the potentially harmful effects of leaky transgene expression and insertional mutagenesis. This is particularly important when considering iPS cells technology in a therapeutic setting.

Two main approaches have been employed to prevent tumor formation. Methods have been developed:

1. to use gene vectors that do not integrate into the host cell genome,
2. to integrate vectors in the genome with the capacity to subsequently remove these vectors from the genome.

It is difficult to predict which one of these methods will translate into a safe and effective application of iPS cells in therapy.

In April 2009, Sheng Ding and his colleagues at the Salk Institute in La Jolla, California, (Zhou, Wu et al. 2009) demonstrated that it is possible to generate iPS cells without any genetic alteration of the adult cell. A repeated treatment of the cells with certain proteins channeled into the cells via poly-arginine anchors was sufficient to induce pluripotency. The acronym given for those iPS cells is piPSCs (protein-induced pluripotent stem cells) (Zhou, Wu et al. 2009). Since 2009, there have been very few papers to further describe this method to generate iPS like stem cells (see Higuchi, 2015 for review).

Sheng Ding

Sheng Ding and his colleagues use combinatorial chemistry to develop large libraries of small molecules and screen them for their ability to influence cell fate in a variety of ways. Their methods include holding stem cells in a state of self-renewal, reprogramming cells to an earlier developmental state, or precisely directing differentiation of stem cells to desired lineages. By using chemical tools, Ding and his colleagues are succeeding in influencing the course of stem cell biology.
Plasticity of iPS cells versus embryonic stem cells (ESC)

All stem cells do not show the same degree of plasticity as ESCs. For example, iPS cells from skin fibroblasts are not as easily converted into blood cells as ESCs or bone marrow-derived stem cells. However, reprogrammed fibroblasts were better at making bone cells than bone marrow-derived ESCs. The reason may be that reprogrammed cells still retain some of the epigenetic molecular tags that block access to some genes in adult cells. The fact that SCNT-generated stem cells were more flexible than induced pluripotent stem cells indicates that SCNT might be more successful in completely reprograming cells than iPS technologies.

Another question associated with iPS cell technology is whether iPS cells and blastocyst-derived ESCs are molecularly and functionally equivalent. At the molecular level, gene-specific and global differences in DNA methylation and in the expression of mRNAs and miRNAs have been reported to be different between both mouse and human ESCs and iPSCs. In addition, genetically identical clones selected from somatic cells infected with the basic pluripotency factors display heterogeneity in their ability to generate iPSCs. These reports indicate that from both a genetic and functional perspective, ESC and iPS stem cells are not exactly equivalent (See Table 1, below). Whether and how these differences may or may not be relevant to the many stem cell applications being developed for very diverse therapeutic purposes remains to be established in future studies.

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<th>iPSC</th>
<th>ESC</th>
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<tr>
<td>Morphology</td>
<td>Round shape, large nucleolus, and scant cytoplasm</td>
<td>Round shape, large nucleolus, and scant cytoplasm</td>
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<td>Growth properties</td>
<td>Equal rate</td>
<td>Equal rate</td>
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<td>Stem cell surface markers</td>
<td>iPSCs expressed same cell surface antigenic markers expressed on ESCs. Human iPSCs expressed the markers specific to hESC, including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, and Nanog.</td>
<td>Mouse mESCs expressed SSEA-1 but not SSEA-3 nor SSEA-4, similarly to iPSC's</td>
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<td>Stem cell genes</td>
<td>iPSCs expressed genes expressed</td>
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in undifferentiated ESCs, including Oct-3/4, Sox2, Nanog, GDF3, REX1, FGF4, ESG1, DPPA2, DPPA4, and hTERT

**Telomerase activity**

Exhibit high telomerase activity (Marion and Blasco 2010) Exhibit high telomerase activity (Marion and Blasco 2010)

**Table 1.** Comparison of iPS cells (iPSC) and embryonic stem cells (ESC) according to various parameters

In practical terms, a major challenge of reprogramming is the extremely low efficiency (0.01–0.1%) of iPSC generation. Overcoming the epigenetic repression of pluripotency genes in differentiated cells is difficult, and evidence suggests that the few cells that do overcome these barriers do so due to stochastic events (i.e., random events). Additionally, reprogramming of cells using iPS technology is a very gradual process that takes several weeks (Hochedlinger and Plath 2009). For a recent review see de Lázaro, et al., 2014.

**Ethical considerations of iPS cell technology**

As mentioned at the beginning of this module, the creation of iPS cells raises numerous ethical issues. One major issue in deriving embryonic stem cells is whether it is ethically justifiable to destroy a pre-implanted human embryo in order to obtain these cells? At first, one could argue that induced pluripotent stem cells (iPS) do not require the creation of an embryo to generate a stem cell line. However, if iPS-derived cells are totipotent then theoretically they could be used to generate an embryo to be implanted into a female surrogate.

Another issue is that iPS can be used to create human gametes. As of 20115, there are no reports documenting that iPS-generated human sperm or eggs have been produced that function like sperm or eggs to generate an embryo. Will the fact of their generation, or of any potential use in human fertilization, create ethical concerns?

**Thought question**

What Would You Do?

Reproductive Scientists were recently able to manipulate cells in order to create a mouse embryo from two genetic fathers. First, the scientists created an iPS stem cell line from the cells of a male (XY) mouse fetus. Certain cells of this line randomly lost their Y chromosome, transforming into XO cells.

Next, scientists injected the XO cells into embryos from donor female mice and transplanted these cells into surrogate mouse moms. The resulting mice born were then mated with other males. Their offspring, contained genetic material that only came from the two fathers.

- Would it be ethical to develop this technology for humans?
- How could this technology be of use to same-sex couples?

Source: [http://community.acs.org/chembiol/?TabId=66](http://community.acs.org/chembiol/?TabId=66)
Some bioethicists, for example Kazuto Kato of Kyoto University, and Jan Helge Solbakk of the University of Oslo, Norway, believe that questions surrounding the possibility of using iPS to clone human beings mandates immediate bioethical attention. Questions about the moral status of human cells, and longstanding moral prohibitions against any contemplation of human cloning, are immediate and weighty. While human cloning in the context of iPS is still “very hypothetical” according to bioethicist Kato, he cites the three teams that have cloned mice using iPS cells, as of 2010. Thousands of infertile couples, in hopes of having their own genetic children, would surely be very interested in the possibility of creating gametes from their own more differentiated cells using iPS. The field of fertility medicine is likely to seek to answers to these ethical questions in the near future, suggesting that bioethical guidelines need to be developed starting very soon (see: Lehrman 2010).

**Conclusion**

There is still much to be learned about stem cells generated via iPS if obtained from the inner mass of embryos. While scientists have highlighted differences in the genetics or functionality of these stem cells, it remains to be determined whether these changes are subtle or significant. Only then can the applications of these stem cells begin toward the development of new therapies, and only then can the complex

References