“Generation of Germline-Competent Induced Pluripotent Stem Cells,” by Okita et al.

In the July 2007 issue of Nature, Keisuke Okita, Tomoko Ichisaka, and Shinya Yamanaka added to the new work on induced pluripotent stem cells (iPSCs) with their “Generation of Germline-Competent Induced Pluripotent Stem Cells” (henceforth abbreviated “Generation”). The authors begin the paper by noting their desire to find a method for inducing somatic cells of patients to return to a pluripotent state, a state from which the cell can differentiate into any type of tissue but cannot form an entire organism. If this is made possible, the authors claim, the ethical controversy surrounding the use of embryonic stem cells (ES cells) and the dangers of patient rejection of donated ES cells could be bypassed completely.

Okita and colleagues introduce their work by pointing to a previous experiment. They reference “ Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors,” published in Cell in 2006, in which Kazutoshi Takahashi and Shinya Yamanaka demonstrate that it is possible to influence cells to return to a state of pluripotency by introducing four transcription factors—Oct3/4, Sox2, c-myc, and Klf4—and selecting for Fbx15 expression. Referring to the resulting cells as Fbx15 iPS cells, and observing that these induced pluripotent stem (iPS) cells were unable to contribute to adult chimeras, Okita et al. decided to introduce the same transcription factors via retrovirus to mouse embryonic fibroblasts (MEFs), but instead of selecting for Fbx15 expression, they selected for Nanog expression, referring to such cells as Nanog iPS cells. The authors list several reasons why Nanog expression is connected to pluripotency, and examine the various ways in which Fbx15 iPS cells compare to Nanog iPS cells in terms of their similarities to ES cells. Their results indicate that when selecting for Nanog expression it is possible to isolate iPS cells that can contribute to adult chimeras, which in turn are able to pass genes from the iPS cells on to their offspring (hence the term “germline-competent” in the title of the article).

The writers list four main reasons for their view that Nanog expression is tightly linked to pluripotency. They note that when Nanog expression is disrupted in mice the pluripotent epiblast, part of the inner cell mass of an embryo, is lost. Second, “Generation” notes that ES cells that don’t express Nanog tend to differentiate randomly. Okita and colleagues also point out that leukemia inhibitory factor (LIF) can be used to allow mouse embryonic stem cells to perpetuate self-renewal, but in cells that express Nanog, LIF is not needed for the cells to maintain self-renewal. Finally, authors write that forcing expression of Nanog gives ES cells greater reprogramming efficiency when they’re combined with somatic cells.

Convinced that selection for Nanog would give them iPS cells more similar to ES cells that selection for Fbx15 could offer, the authors needed a way to select for Nanog expression as well as a method to test the germline competence of their Nanog iPS cells. To accomplish this they engineered a bacterial artificial chromosome (BAC) containing the Nanog gene. They inserted a green fluorescent protein (GFP)–internal ribosome entry site (IRES)–puromycin resistance gene ($Puro^r$) cassette into the 5’ untranslated region of the BAC. This way, the authors could select for resistance to the antibiotic puromycin or look for fluorescence and know that cells expressing these traits were also expressing the Nanog gene.

The researchers incorporated their BAC containing the GFP-IRES-$Puro^r$ cassette into mouse ES cells, and introduced these cells to blastocysts to create transgenic mouse blastocysts. These blastocysts were allowed to mature to the 13.5 days post-coitum stage, after which mouse embryonic fibroblasts (MEFs) were taken from the male embryos and cultured. The researchers introduced the four retroviral factors, Oct3/4, Sox2, c-myc, and Klf4, to these MEFs. After allowing the MEFs

to grow in culture for a few days, the experimenters then added the antibiotic puromycin. The researchers did note that out of the colonies that survived the puromycin selection, only about 5% tested positive for GFP. They were unable to explain this, but added that they obtained fewer GFP-negative colonies when they increased the concentration of puromycin.

The GFP-positive colonies were cultured, and showed morphological similarity to ES cells. In addition to this they divided at a rate similar to, though slightly slower than that of ES cells. When cells from these GFP+ colonies were transplanted into nude mice, the mice developed tumors in all three germ layers, demonstrating that the cells were indeed pluripotent. The authors had succeeded in introducing a Nanog GFP-IRES-Puro^r gene construct into mouse ES cells, in subsequently inducing differentiation in those stem cells, in isolating the differentiated, non-pluripotent embryonic fibroblasts containing the gene Nanog GFP-IRES-Puro^r construct, and in exposing those fibroblasts to four retroviral factors with the intent of inducing plurpotency in the fibroblasts. Since the Nanog gene in all of these fibroblasts was connected to a puromycin resistance gene, the researchers were able to expose these cells to the antibiotic and be left with only cells that expressed Nanog. The researchers demonstrated that these cells are pluripotent, and termed them Nanog iPS cells.

Before considering whether or not Nanog iPS cells were germline competent, the authors compared them to Fbx15 iPS cells in terms of the similarity of each to ES cells. In the paper, they note that by testing gene expression with reverse transcription polymerase chain reaction (RT-PCR), they found that although Nanog iPS cells did not match ES cells perfectly, they did outperform Fbx15 iPS cells. The authors also mention that it is normal for ES cells to silence the genes activated by the retroviral factors used to induce pluripotency. They observe that Nanog iPS cells expressed the four transgenes, Oct3/4, Sox2, Klf4, and c-myc, with much lower frequency than Fbx15 iPS cells. In this regard, Nanog iPS cells are shown to be more similar to ES cells than Fbx15 iPS cells, since ES cells typically silence retroviral genes.

In addition to this, Okita and his colleagues observe that Nanog iPS cells were more similar to ES cells than Fbx15 iPS cells in terms of DNA methylation, expression of ES cell marker genes after multiple passages through culture while exposed to a selection drug, and in terms of their behavior in the presence of two factors, LIF (which typically inhibits differentiation in ES cells) and retinoic acid (which typically induces differentiation). In the presence of LIF, note the authors, Fbx15 iPS cells differentiate despite the presence of the inhibiting factor, while Nanog iPS cells do not. In addition to this Fbx15 cells tend to form what the authors call compact colonies in the presence of retinoic acid, while Nanog iPS cells–like ES cells–tend to differentiate when exposed to the factor.

However, the authors note that selection for Nanog expression is a more exclusive process than selection for Fbx15 expression. They report that after transfection with retroviral factors, about 0.001–0.003% of MEFs tested GFP-positive. On the other hand, Fbx15 iPS cells have an induction efficiency of 0.01–0.5%, about ten times more efficient than selection for Nanog expression.

Having completed their comparison of Nanog iPS cells to Fbx15 iPS cells, Okita and co-workers turn their attention towards the germline competence of the Nanog iPS cells. To test this, they injected Nanog iPS cells into mouse blastocysts, which were then transplanted into the uteri of mice. Seven adult chimeras were obtained. Within them, Nanog iPS cells differentiated to help form what the authors called various organs, although they did not specify which organs were formed. The paper reports chimerism in the organs at levels ranging from 10% to 90%.

The paper then explains that the three adult chimeras displaying the greatest level of Nanog iPS cells contribution in the testes were crossed with females. The resulting generation contained integration of the four retroviral transcription factors, and about half of the offspring also contained the GFP-IRES-Puro^r gene construct. The fact that half contained this construct suggests that the genes in these iPS cells can be passed on through sperm, that is to say the authors succeeded in confirming the germline competence of the Nanog iPS cells.

Okita and colleagues also report that a large number of the offspring produced by the aforementioned chimeras (24 out of 121) expired because of wheezing, weakness, or paralysis. The reason for this was that tumors formed in the mice. The researchers found that the reactivated retroviral expression of the transcription factor c-myc was to blame (but retroviral expression of Oct3/4, Sox2, and Klf4 had not been reactivated).

The authors conclude “Generation” by noting that Nanog iPS cells display numerous similarities to ES cells, suggesting Nanog expression is significant in determining whether or not a cell is pluripotent. They also write that in another study they discovered that germline-competent iPS cells could be obtained from adult somatic cells of mice. However, they remind the reader, when inducing pluripotency with retroviral factors there is still the danger that reactivation of the c-myc retrovirus could lead to tumor formation.

Okita et al. speculate that retroviral factors may only be needed to induce pluripotency, not to maintain it. Therefore, they conclude, it might be possible to use an adenovirus transfer system to induce plurpotency, as such a system would only express necessary factors for a limited period of time. They also suggest that if the molecules formed by the expression of the retroviral factors can be identified, these molecules can be used to directly induce pluripotency, which would eliminate the need for viruses entirely. In May 2009, Hongyan Zhou and colleagues reported success in inducing pluripotency in murine (mouse or rat) cells using such molecules in “ Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins” in Cell Stem Cell.

The low efficiency (less than 0.1%) in obtaining Nanog iPS cells from MEF cultures is considered as well. The writers speculate that the low efficiency might actually show that the Nanog iPS cells originate from a very small number of stem cells living alongside the MEF cells. The also consider the possibility that the low efficiency of their method for generating iPS cells highlights the need to identify more transcription factors that will help efficiently induce pluripotency in cells. Okita, Ichisaka, and Yamanaka showed that it is possible to generate germline-competent pluripotent stem cells. They look to the possibilities of using adenoviruses and the possibility of eliminating the use of viruses entirely when inducing pluripotency as avenues that might be used to advance their research into the realm of clinical relevance, but rightly point out that elimination of risk for tumor formation is necessary before such research can be applied to regenerative medicine.

Sources

1. Okita, Keisuke, Tomoko Ichisaka, and Shinya Yamanaka. “Generation of Germline-Competent Induced Pluripotent Stem Cells.” Nature 448 (2007): 313–17.
2. Takahashi, Kazutoshi, and Shinya Yamanaka. “Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors.” Cell 126 (2006): 663–76.
3. Zhou, Hongyan, Shili Wu, Jin Young Joo, Saiyong Zhu, Dong Wook Han, Tongxiang Lin, Sunia Trauger, Geoffery Bien, Susan Yao, Yong Zhu, Gary Siuzdak, Hans R. Schöler, Lingxun Duan, and Sheng Ding. “Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins.” Cell Stem Cell 4 (2009): 381–84.