"Generation of Induced Pluripotent Stem Cells without Myc from Mouse and Human Fibroblasts" (2007), by Masato Nakagawa et al. [1]

By: Philbrick, Samuel Keywords: Mice [2] Chimeras [3] Stem cells [4] Pluripotency [5]

In November 2007, Masato Nakagawa, along with a number of other researchers including Kazutoshi Takahashi [6], Keisuke Okita, and Shinya Yamanaka [7], published "Generation of Induced Pluripotent Stem Cells without Myc from Mouse and Human Fibroblasts" (abbreviated "Generation") in *Nature* [publishedIn]. In "Generation," the authors point to dedifferentiation of somatic cells as an avenue for generating pluripotent stem cells [8] [isBackgroundTo] useful for treating specific patients and diseases. They provide background to their research by observing that previous attempts to reprogram somatic cells to a state of greater differentiability with retroviral factors [9] *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4* had succeeded in producing induced pluripotent stem (iPS) cells that contributed to viable [10] adult chimeras [11] and possessed germline competency [hasRelevence]. However, as they note, the *c-Myc* retrovirus [12] contributes to tumors in generated chimeras [11], rendering iPS cells produced with *c-Myc* useless for clinical applications. The authors attempt to overcome this problem by modifying the standard protocol for producing iPS cells in mice in such a way that the *c-Myc* retrovirus [12] is removed. They identify problems and benefits associated with this method, but most importantly note that their method generated iPS cells that did not cause tumors in chimeric mice. Nakagawa and colleagues also report that they successfully reprogrammed adult dermal fibroblasts to return to a pluripotent state without *c-Myc*.

The writers open the discussion of their experiment by describing the beginning of their investigation, which originally was aimed at determining whether other family proteins of the four transcription factors, *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*, could be used to induce mouse embryonic fibroblasts [13] (MEFs) to return to a pluripotent state. The authors tested *Oct1*, *Oct6*, *Sox1*, *Sox3*, *Sox7*, *Sox15*, *Sox17*, *Sox18*, *Klf1*, *Klf2*, *Klf5*, *N-Myc*, and *L-Myc*. They discovered that some of these family proteins could successfully reprogram MEFs to become iPS cells, but some could not.

To test whether these proteins cause the MEFs to dedifferentiate to a pluripotent state the experimenters used the *Nanog* GFP-IRES-Puro^r cassette described in a paper published by Keisuke Okita, Tomoko Ichisaka, and Shinya Yamanaka [7], " Generation of Germline-Competent Induced Pluripotent Stem Cells" [isBackgroundTo] (abbreviated "Germline-Competent"). This gene construct allowed Nakagawa's group to test for green fluorescent protein [14] (GFP) expression. GFP expression would imply that the cell was also expressing the *Nanog* gene. Researchers took Nango expression as a clear sign that the cell had dedifferentiated to a pluripotent state.

As the authors of "Germline-Competent" reported in the previous year, no colonies of MEFs tested positive for GFP expression without modification by the *c-Myc* retrovirus [12]. In "Germline-Competent" Okita et al. report that selection for puromycin resistance (connected with the *Nanog* GFP-IRES-Puro^r cassette to *Nanog* expression) began just seven days after exposure to retroviral factors [9]. Nakagawa's team confirmed that no GFP expressing colonies were obtained after exposing MEFs to only *Oct3/4*, *Sox2*, and *Klf4*, and selecting for puromycin resistance after seven days. However, they found that they could obtain GFP+ colonies without *c-Myc* when they began selecting for puromycin resistance fourteen or twenty-one days after exposing MEFs to the other three retroviruses. The authors tested the viability [15] of these cells generated without *c-Myc* by injecting them into mouse [16] blastocysts, which gave rise to adult chimeras [11], confirming that Nakagawa and co-workers had successfully generated iPS cells.

The experimenters also described the results of an experiment done to see whether high-quality iPS cells could be generated without a Myc retrovirus [12] using a method that selected for *Fbxo15* expression instead of *Nanog* expression. Previous experiments had shown that iPS cells obtained using the four standard retroviral factors [9] and the method selecting for Fbxo15 expression differed from embryonic stem (ES) cells in gene expression and in viability [15] for producing adult chimeras [11]. However, as the paper reports, iPS cells produced without the use of a *Myc* retrovirus [12] via selection for *Fbxo15* express ES cell marker genes [17] on levels similar to actual ES cells. Furthermore, these iPS cells are able to contribute to adult chimeras [11]. This is significant because it shows that omitting the *Myc* retrovirus [12] actually raises the quality of the obtained iPS cells.

Having generated iPS cells from MEFs, both by selecting for *Nanog* and *Fbxo15* expression, and in each case with and without *Myc*, the authors tested the *Myc*– iPS cells to see if they would cause tumors in chimeras [11]. They reported that six of the thirty-seven chimeras [11] generated from iPS cells produced with all four retroviral factors [9] died because of tumors. In contrast, the authors write that all twenty-six chimeras [11] generated without *Myc* survived a 100-day time-span, and that they need to continue studying the *Myc*– mice to see whether they will develop tumors or not.

All iPS cells described thus far, whether *Nanog* or *Fbxo15* was selected for, and whether *Myc* was used or not, were selected for using antibiotics. The later portion of "Generation…" describes tests on mouse [16] adult tail tip fibroblasts (TTFs) containing the the *Nanog* GFP-IRES-Puro^r cassette. These tests sought to determine whether omitting *Myc* makes selection for antibiotic resistance unnecessary in the process of isolating iPS cells. To test this Nakagawa et al. tested TTFs transfected by all four retroviral factors [9], and by the three retroviral factors [9] minus the *c-Myc* retrovirus [12]. They also included a DsRed retroviral factor that, thanks to its red fluorescence, allowed the researchers to see which cells had been transfected by the retrovirus [12]. In the cells to be tested, since GFP expression would signal *Nanog* expression, which would signify that the cells had returned to a pluripotent state, researchers did not expect to see any GFP expessing cells also expressing DsRed. This is because the cells were expected to silence retroviral factors [9] upon return to pluripotency [18]. So the researchers expected to see three kinds of cells post exposure to retroviral factors [9]: non-fluorescent cells, which would not have been transduced by the retroviral factors [9] at all, DsRed+ cells, which would have been transfected but would not be pluripotent, and GFP+ cells, which would have been transfected, and then would have returned to a pluripotent state in which they expressed GFP but silenced *Nanog*.

Nakgawa et al. report that the group of TTFs tranducted by all four retroviral factors [9] resulted in many colonies of cells that were mostly GFP negative (though many expressed DsRed), and in addition to this there were many background cells. As expected, in the fraction of colonies that did express GFP, DsRed was silenced.

On the other hand, the group of TTFs transfected with *Oct3/4*, *Sox2*, and *Klf4*, but not a *Myc* retroviral factor, produced few background cells and much fewer colonies than the TTFs transfected with four retroviral factors [9]. About half of the colonies, however, expressed GFP in what the authors call a patchy manner. DsRed was not expressed in most of these colonies, which indicates that it is largely silenced in transducted cells. And, as the authors reported, most of the colonies that did express DsRed actually silence DsRed and begin expressing GFP after the second passage.

The experimenters then did tests to find out if iPS cells could be generated from TTFs devoid of selection markers (but possessing a DsRed transgene); that is, they wanted to do an experiment to see if they could generate iPS cells from TTFs not containing the *Nanog* GFP-IRES-Puro^r cassette. The researchers tested cultures of TTFs exposed to all four retroviral factors [9], and to three retroviral factors [9] but not *Myc*. They also introduced GFP retroviruses to their TTF cultures so they would be able to easily determine if and when the cells started silencing retroviruses, signaling return to a pluripotent state. In the cultures exposed to all four retroviral factors [9] retroviral silencing did not occur. However, the TTF cells exposed to only the three retroviral factors [9], but not *Myc*, express ES cell marker genes [17] at similar levels to actual ES cells. Moreover, when these cells were implanted into blastocysts adult chimeras [11] resulted, proving that viable [10] iPS cells can be obtained without *Myc* or drug selection.

The authors then mention that they succeeded in generating colonies similar to human embryonic stem cells [19] [hasRelevance] (hESC) without using *Myc* using the same methods reported in the paper by Takahashi et al., " Induction of Pluripotent Stem Cells from Human Fibroblasts by Defined Factors." [isBackgroundTo] Takahashi et al. had reported that no hESC-like colonies were obtained without the use of *Myc*. However, the authors of "Generation" used ten times more human dermal fibroblasts (hDFs) than Takahashi et al. used. This higher concentration allowed Nakagawa et al. to obtain what they numbered as 0-5 hESC-like colonies. They were able to grow these colonies and found that they expressed similar marker genes [17] to hESCs.

"Generation" closes by noting that the research reported in this paper does not show that *Myc* is unimportant in generating iPS cells. Rather, they posit, *Myc* is important but can possibly be recruited by other factors (such as *Oct3/4*, *Sox2*, and *Klf4*). Such a possibility is consistent with the finding in their paper, which proves that both mouse [16] and human cells can be programmed to return to a pluripotent state without the *Myc* retrovirus [12]. This has clinical significance because the iPS cells generated without a *Myc* retrovirus [12] do not appear to cause tumor formation in chimeras [11]. This is encouraging, but as the authors point out, removing the *Myc* retrovirus [12] greatly reduces the efficiency of adult cells in producing pluripotent stem cells [8]. Therefore, conclude the authors, if iPS stem cells [8] are to become a viable [10] tool in medicine further research is needed to find factors or molecules that can increase the efficiency of iPS cell generation without the *Myc* retrovirus [12].

Sources

1. Nakagawa, Masato, Michiyo Koyanagi, Koji Tanabe, Kuztoshi Takahashi, Tomoko Ichisaka, Takashi Aoi, Keisuke Okita, Yuji Mochiduki, Nanako Takizawa, and Shinya Yamanaka [7]. "Generation of Induced Pluripotent Stem Cells without Myc from Mouse and Human Fibroblasts." *Nature Biotechnology* 26 (2008): 101–106.
2. Okita, Keisuke, Tomoko Ichisaka, and Shinya Yamanaka [7]. "Generation of Germline-Competent Induced Pluripotent Stem Cells." *Nature* 448 (2007): 313–17.
3. Takahashi, Kazutoshi, Koji Tanabe, Mari Ohnuki, Megumi Narita, Tomoko Ichisaka, Kiichiro Tomoda, and Shinya Yamanaka [7]. "Induction of Pluripotent Stem Cells form Adult Human Fibroblasts by Defined Factors."*Cell* 131 (2007): 1–12.

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