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


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] 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 experimenter used the *Nanog* GFP-IREs-Puro 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?ts 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 expressing 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 transduced but would not be pluripotent, and GFP+ cells, which would have been transfeced, and then would have returned to a pluripotent state in which they expressed GFP but silenced Nanog.

Nakagawa et al. report that the group of TTFs transducted 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 transduced 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' 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] (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
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