

# Induced Pluripotent Stem Cell Experiments by Kazutoshi Takahashi and Shinya Yamanaka in 2006 and 2007

In 2006, Kazutoshi Takahashi and Shinya Yamanaka reprogrammed mice fibroblast cells, which can produce only other fibroblast cells, to become pluripotent stem cells, which have the capacity to produce many different types of cells. Takahashi and Yamanaka also experimented with human cell cultures in 2007. Each worked at Kyoto University in Kyoto, Japan. They called the pluripotent stem cells that they produced induced pluripotent stem cells (iPSCs) because they had induced the adult cells, called differentiated cells, to become pluripotent stem cells through genetic manipulation. Yamanaka received the Nobel Prize in Physiology or Medicine in 2012, along with John Gurdon, as their work showed scientists how to reprogram mature cells to become pluripotent. Takahashi and Yamanaka's 2006 and 2007 experiments showed that scientists can prompt adult body cells to dedifferentiate, or lose specialized characteristics, and behave similarly to embryonic stem cells (ESCs).

Takahashi and Yamanaka worked together at Kyoto University. Takahashi was a post-doctoral researcher who had earned a graduate degree in biology at the Nara Institute of Science and Technology in Ikoma, Japan. Yamanaka had earned an MD from Kobe University in Kobe, Japan in 1987. In 2004, Yamanaka began working at Kyoto University as a professor, where he studied factors that help an organism fend off retroviruses, which are single-stranded RNA viruses that can incorporate their genetic material into the DNA of a host cell. Yamanaka and others hypothesized that retroviruses could influence somatic cells to become stem cells. Yamanaka worked to find new ways to acquire embryonic stem cells to avoid the social and ethical controversies surrounding the use of human embryos in stem cell research during the late twentieth and early twenty-first centuries. Yamanaka studied the work of John Gurdon, a researcher who had experimented with *Xenopus* frogs at the University of Oxford in Oxford, United Kingdom. Yamanaka claimed that Gurdon's work in reprogramming mature cells in frogs (*Xenopus*) in 1962 influenced his own work in reprogramming differentiated cells.

Yamanaka also noted that experiments in cloning Dolly the sheep in 1996, conducted by Ian Wilmut, Angelica Schnieke, Jim McWhir, Alex Kind, and Keith Campbell at the Roslin Institute in Roslin, Scotland, influenced his work. The Dolly experiment showed that scientists could reprogram the nucleus of somatic cells by transferring the contents of the nucleus into oocytes that have had their nuclei removed, a technique called somatic cell nuclear transfer (SCNT). Other research groups such as Masako Tada's group in Japan in 2001 and Chad Cowan's group in Massachusetts in 2005 combined embryonic stem cells with somatic cells to produce pluripotent cells. After these experiments with somatic cells, Takahashi and Yamanaka hypothesized that there were common factors, genes in particular, which caused somatic cells to become pluripotent stem cells.

In 2006, Takahashi and Yamanaka selected twenty-four candidate genes as factors that they hypothesized could possibly induce somatic cells to become pluripotent, and they began to test them one at a time. They used retroviruses to insert each of the twenty-four genes into the chromosomes of differentiated mouse embryonic fibroblasts. Each gene was inserted near the mouse *Fbx15* gene, a gene that embryonic stem cells express during development in mice. The newly inserted gene endowed mice with resistance to an antibiotic named G418. The researchers labeled the resulting retroviruses mixed with host DNA as retroviral factors. Takahashi and Yamanaka placed the retrovirus-infected cells into cell culture with G418 antibiotic and cells to provide nourishment, called feeder cells. If one of the infected cells showed G418 resistance, then the scientists would know that one of the twenty-four genes influenced the cell to become an embryonic stem cell-like

cell. However, none of the cells showed a resistance to G418, so Takahashi and Yamanaka reworked their approach.

Next, Takahashi tried to insert into a fibroblast cell multiple retroviral factors instead of one at a time. The researchers added all of the twenty-four retroviral factors at the same time into mouse fibroblast cells. This time, there were twenty-two cell colonies that showed a resistance to G418, meaning that there were colonies in which the cells exhibited embryonic stem cell properties. After examination, Takahashi and Yamanaka concluded that the cells were similar to embryonic stem cells and duplicated themselves in similar periods of as embryonic stem cells. They named the cells iPS-MEF24, signifying pluripotent stem cells induced from mouse embryonic fibroblasts by twenty-four factors.

The next experiments aimed to identify specific factors responsible for the generation of iPS cells. To isolate these specific factors, the researchers removed retroviral factors one at a time from the original twenty-four, and each time they removed a factor, they repeated their cell colony procedures. If the researchers removed a factor and the resultant cell colony wasn't resistant to antibiotics, they knew that the missing factor somehow influenced the generation of iPS cells. Takahashi and Yamanaka repeated their procedure until they found ten genes that, when combined together in cells, yielded colonies of cells with G418 resistance. They named those cells with the ten genes as iPS-MEF10 cells. Takahashi and Yamanaka found that of the ten genes, when they combined four genes in particular (Oct3/4, Klf4, Sox2, and c-Myc), they produced the most cells that were like embryonic stem cells. The scientists named the cells iPS-MEF4. Takahashi and Yamanaka deemed those four genes important in the role of iPS cell generation. They concluded that iPSCs are similar, but not identical to embryonic stem cells.

To determine how embryonic stem cells were different from iPSCs, Takahashi and Yamanaka used primers, or strands of nucleic acid that help to start the process of DNA synthesis, to promote replication of genes found in normal embryonic stem cells. If an iPSC had a normal embryonic stem cell gene, the primer would prompt the normal gene to replicate, and the scientists could then see that the iPSC had a normal gene.

Takahashi and Yamanaka continued their experiments and injected the iPSC samples into mice that had no body hair. These nude mice were a variation of the common mouse (*Mus musculus*), but they had an inhibited immune system and lacked the *Fox1* gene. When the researchers injected iPSCs into the mice, teratomas, which are tumors with germ layer components, formed. The teratomas resulting from iPS-MEF4 injections differentiated into all three germ layers (ectoderm, endoderm, and mesoderm), including neural and muscular tissues, cartilage, and epithelium. These tissue types formed aggregates of pluripotent stem cells called embryoid bodies. From the teratomas, Takahashi and Yamanaka took some cell samples and cloned them. They inserted the cloned cells into blastocysts by microinjection and obtained four different embryos.

After analysis, Yamanaka and Takahashi found that the four embryos contained iPS cells that contributed to all three germ layers, providing further evidence that the four genes (Oct3/4, Klf4, Sox2, and c-Myc) helped produce cells that were the most like embryonic stem cells. Takahashi and Yamanaka observed that the iPS-MEF4 cells continued to be more similar to embryonic stem cells than to other iPS cells. After further experimentation, they concluded that the iPS cells they generated were pluripotent in mice, and therefore provided the possibility of repeating a similar experiment in humans. Takashi and Yamanaka published the results of their experiment in 2006.

After their mouse experiments, in 2007 Takahashi and Yamanaka published the results of another experiment that detailed methods and results used to produce iPS cells with human cells. They used the same four genes from humans that were used in mice. Another group led by James Thomson at the University of Wisconsin in Madison, Wisconsin, published their findings on iPS in humans. They found that four genes—Oct4, Sox2, NanoG, and Lin28—were sufficient to reprogram human somatic cells into pluripotent stem cells. Independent confirmation of Takahashi and Yamanaka's previous experiments with mice supported the hypothesis that scientists can generate and use induced pluripotent stem cells in a similar manner as embryonic stem cells. Scientists later used iPSCs in regenerative medicine to research treatments for various human diseases such as Parkinson's disease, platelet deficiency, spinal cord injury, and macular degeneration.

In June 2012, Yamanaka reported that experiments showed many epigenetic differences, as well as gene expression differences, between iPSCs and embryonic stem cells. Yamanaka cited as an example the chemical addition of methyl groups (methylation) of the gene that makes bone morphogenic protein 3 (BMP3), which partly causes bone and cartilage development in humans. He also mentioned that there are other examples of high amounts of variation and mutations in iPSCs, which alarmed some scientists. Yamanaka said that many of the variations in the genes and epigenetics are likely from the original somatic cell from which the iPSCs are descended, and that the negative reports on iPSCs were often overstated.

In 2012, Yamanaka and John Gurdon received the Nobel Prize in Physiology or Medicine their discovery that differentiated cells can be reprogrammed to become pluripotent. The Nobel Prize committee stated that Takahashi and Yamanaka's experiments with mice iPSCs contributed to Yamanaka's award. Scientists in the second decade of the twenty-first century experimented with iPSCs to generate cells similar to embryonic stem cells without destroying or manipulating embryos.

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