

# “Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins,” by Zhou et al.

Induced pluripotent stem cells (iPSCs) are studied carefully by scientists not just because they are a potential source of stem cells that circumvents ethical controversy involved with experimentation on human embryos, but also because of their unique potential to advance the field of regenerative medicine. First generated in a lab by Kazutoshi Takahashi and Shinya Yamanaka in 2006, iPSCs have the ability to differentiate into cells of all types. If scientists discover how to induce differentiated cells to return to a pluripotent state using a method that leaves the iPSCs safe for transplantation, then patients could receive stem cell transplants with cells containing their own DNA. This would presumably remove the danger of transplant rejection that comes with foreign cell transplantation.

In “Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins” (abbreviated “Generation of”), Hongyan Zhou and colleagues begin with a summary of important work on iPSCs. They first note that in 2006 Takahashi and Yamanaka published “Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors” in *Cell*. In this article Takahashi and Yamanaka report how their team successfully induced murine (mouse or rat) somatic cells to return to a pluripotent state using retroviral factors. The four retroviral factors genes, Oct4, Klf4, Sox2, and c-Myc, were also used to successfully generate germline-competent (having the ability to be passed to the next generation through germ cells) iPSCs. In 2007 Takahashi et al. published “Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors” in *Cell* and Junying Yu et al. published “Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells” in *Science*. Both articles demonstrated that human iPSCs could be generated.

However, iPSCs derived using methods described in the aforementioned papers often form teratomas, tumors containing cells from all three germ layers. The authors of “Generation of” indicate that they think this is because the retroviral factors modify the genomes of target cells. Research has been done on methods of obtaining pluripotent stem cells that might reduce the risk of teratoma formation. For example, in 2007 Nakagawa and a group of researchers including Kazutoshi Takahashi and Shinya Yamanaka published “Generation of Induced Pluripotent Stem Cells without Myc from Mouse and Human Fibroblasts” in *Nature Biotechnology*. However, as Zhou et al. point out, all such research on “reduced-risk” methods still requires genetic modification of target cells. Such modification of target cells’ genomes could be viewed as problematic because such modification is associated with teratoma formation.

In “Generation of,” Zhou and colleagues record their attempt to leave behind the risks associated with changing target cells’ genomes as they induced mouse embryonic fibroblasts (MEFs) to return to a pluripotent state using only proteins. The rationale for this is simple; in numerous previous experiments researchers had modified the genomes of target cells with retroviral factors introducing Oct4, Klf4, Sox2, and c-Myc. Genes code for proteins, and the proteins for which the genes coded helped induce cells to return to a pluripotent state. So Zhou et al. created an experiment that skips the modification of the target cells’ genomes, and instead pumps proteins into target cells which, had the cell been genetically modified, would have been created thanks to Oct4, Klf4, Sox2, and c-Myc. The authors discuss how the formation of protein-induced pluripotent stem cells (piPSCs) is enabled by use of recombinant cell-penetrating programming proteins.

This statement can be confusing because recombinant usually refers to non-natural, modified DNA. In this case, recombinant describes the nature of the proteins, not the action they had on target cells. The researchers needed a lot of proteins from Oct4, Klf4, Sox2, and c-Myc, so *E. coli* was genetically modified to produce the proteins of the reprogramming factors. The *E. coli* had the

recombinant DNA, and that DNA coded for factors that had been introduced directly to target cells via retroviruses in previous research.

The word reprogramming also deserves clarification. The authors did not mean that the protein physically changes a target cell's genome; rather, it induces a change in the genes the target cell expresses. The proteins do not change the genome of the cell, but they do reprogram it in that they tell the cell to behave differently.

The proteins produced by *E. coli* had to be able to get into target cells, so the bacteria were modified to add code next to each of the four reprogramming factors that would produce a protein capable of moving the reprogramming factor across the cell membrane. To assess the efficacy of these carrier proteins the researchers exposed MEFs to the proteins and reported that proteins penetrated target cells and entered their nuclei inside of six hours. Furthermore, they wrote that the carrier protein remained stable for up to 48 hours inside the cell.

Having established that their carrier proteins could get proteins from the four reprogramming factors into target cells, Zhou et al. continually exposed target cells to the reprogramming proteins over the course of four 48-hour periods. Four cycles were used because the researchers knew the proteins remained viable for about two days, and previous experiments indicated that seven to ten days of protein activity were necessary for inducing cells to return to a pluripotent state. Researchers used their proteins on MEF cells that were modified to express green fluorescent protein (GFP) along with Oct4. This allowed researchers to test for Oct4 expression (and implicitly for pluripotency) by looking for fluorescence in the MEF cells.

Some of the MEFs were also treated with valproic acid (VPA), a histone deacetylase inhibitor (HDAC). This acid pulls binding proteins off DNA that are involved in gene silencing. The goal of adding this acid is to make the DNA of the target cells open to regulation by the proteins that were introduced by the researchers.

After exposure to the reprogramming proteins for four cycles, the cells were left in mouse embryonic stem cell (mESC) growth media for 30 to 35 days. In MEF cultures treated with all four proteins and VPA, researchers found three GFP-expressing colonies per 50,000 cells. Some cells were treated with proteins from just three factors (the c-Myc protein was omitted) and VPA, and some cultures were not treated with VPA. Cells treated without c-Myc and VPA still produced one GFP-expressing colony per 50,000 cells, but cultures not treated with VPA failed to produce any GFP-expressing colonies.

Zhou et al. record that they passaged their piPSCs over thirty times. They also report that their cells were morphologically indistinguishable from regular mESCs. Furthermore, the piPSCs expressed proteins such as Oct4 and nanog, which are usually found in pluripotent cells.

Not only were they similar in protein expression, but they also matched mESCs more closely than the MEFs from which they were derived in terms of DNA methylation. As Zhou et al. note, Oct4 and Nanog promoters are generally highly methylated in MEFs, but in classic mESCs and in the obtained piPSCs, the promoter regions were demethylated. Analysis of overall gene expression in piPSCs showed they had greater similarity to classic mESCs than MEFs (with Pearson correlation values of 0.969 and 0.895, respectively).

Similarity to classic embryonic stem cells in terms of gene expression and DNA regulation was not sufficient to demonstrate that the protein-induced PSCs were definitely pluripotent. So Zhou et al. performed two different tests to see if piPSCs can differentiate into different cell types. The first experiment tested the piPSCs' ability to differentiate in vitro. They were able to form embryonic bodies (EBs) in vitro, forming cells in all three germ layers including, as researchers highlighted, mature, beating heart muscle cells. The second experiment was even more significant, as "Generation of" reports that the piPSCs were viable in vivo. After the piPSCs were successfully integrated with the inner cell masses of blastocysts, these chimeric blastocysts were transplanted into mice. The produced fetuses possessed what researchers called apparent germline-contribution. They observed contribution from the piPSCs by testing all three germ layers in the produced fetuses for GFP, and "Generation of" reports that three out of seventeen fetuses expressed GFP in gonad tissue.

Zhou et al. conclude "Generation of" by writing that easily producible iPSCs would present amazing

possibilities for biomedical research, and that for human cells, using iPSCs circumvents ethical controversies surrounding ESCs. They also point out that in order for such possibilities to be realized, major difficulties need to be overcome. Potential for teratoma formation makes iPSCs unsafe for use in regenerative medicine, and current methods for getting iPSCs have very low efficiency. Zhou et al. acknowledge that others have made progress in overcoming these problems by developing techniques to obtain iPSCs more efficiently. Experiments have been designed to reduce the risk of teratoma formation in iPSCs by using adenoviruses or temporary transfection by reprogramming plasmids rather than retroviruses (both of these techniques avoid the permanent genetic modification that comes with the use of retroviruses).

However, “Generation of” is quick to remind readers that none of these studies generated iPSCs without using genetic material. Zhou et al. say that their study is the first to show that iPSCs can be generated from somatic cells using only proteins. As a result, they argue, their method is superior to previously developed techniques.

Zhou et al. write that their technique removes the risk of modifying the target cell genome. As Takahashi observed a month prior to publication of “Generation of,” in “A Fresh Look at iPS Cells,” published in *Cell*, “the exact mechanisms that cause aberrant reprogramming are not yet fully understood.” Importantly therefore, removing the risk that comes causally from modifying the target cell genome (because the target cell genome is not modified at all) is not the same thing as eliminating all risk associated with modification of the target cell genome. Nevertheless, the authors argue, and perhaps fairly, that their method is safer because it eliminates introduction of genetic material from an outside source into target cells’ genomes. If such a method could eliminate or greatly reduce incidence of teratoma formation, which some suspect is linked to modification of target cells’ genomes, it would be a tremendous step forward for iPSC technology.

Finally, the paper expounds on the practical applicability of the method described in the research. Zhou et al. express confidence that their method is simpler and faster than other advanced methods, pointing out that other methods for obtaining iPSCs necessitate extensive selection processes to obtain desired cells. Finally, the writers claim that high volume recombinant protein production is readily available, which they argue should enable further application of their proposed techniques.

The research done by Zhou and company as described in “Generation of” is significant because scientists have had concerns about the effects of retroviral factors introducing Oct4, Klf4, Sox2, and c-Myc because of teratoma formation in iPSCs. If the technique developed by Zhou et al. does lead to efficient techniques for generating piPSCs that do not have increased risk of teratoma formation, it would be a tremendous step forward for regenerative medicine. It would also open many doors in the field of biomedical research in general, because a free-flowing supply of piPSCs could allow researchers to better understand embryological development and stem cell differentiation.

## Sources

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