



# Reprogramming somatic cells without fusion or ethical confusion

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**Evaluation of:** Takahashi K and Yamanaka S: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676 (2006) [1]. Somatic cell nuclear transfer experiments in the past 10 years demonstrate that mammalian cells can be reprogrammed, since the nucleus of a differentiated somatic cell, when injected into an enucleated oocyte, can, upon transfer to a surrogate mother, give rise to an entire organism. Cell fusion of differentiated cells with mouse and human embryonic stem cells also results in hybrids (although in tetraploids) that have many unique features of pluripotent embryonic stem cells. Furthermore, exposure of somatic cells or nuclei to cell extracts from oocytes or embryonic stem cells can lead to reprogramming to a more multipotent state. While these experiments highlight that nuclear reprogramming is indeed possible through several means, the underlying molecular details and necessary gene expression have not yet been elucidated. In a recent *Cell* report, Takahashi and Yamanaka take a significant step in this direction. They demonstrate, for the first time, that adult and embryonic mouse fibroblasts can be reprogrammed into embryonic stem cell-like pluripotent cells by the overexpression of four genes. Could this achievement be reproduced in human somatic cells, and if so, how?

In the past 10 years, several animals (from sheep to cow) have been cloned [2,3]. Typically, the nucleus of a terminally differentiated somatic cell, when injected into an enucleated oocyte, can, upon transfer to a surrogate mother, give rise to an entire organism or, upon explantation in culture, give rise to embryonic stem cells (ESCs). To date, ESCs are the only stem cells that have proven to proliferate extensively in culture while maintaining a unique pluripotency to differentiate into a spectrum of cells, generating the three (somatic) germ layers and all types of somatic cells therein. Thus far, generating ESC lines from somatic cells after nuclear transfer (NT) into enucleated oocytes remains low, even in extensively studied mice. Similar approaches are being pursued with somatic cells of humans and other nonhuman primates. Although generating patient-specific human ESC lines could be achieved in the future, this approach has inherent limitations, including that efficiency will likely be low (a few percent) at least initially, thus a great number of human oocytes are needed and there is ethical controversy relating to oocyte donation and creating blastocysts for ESC derivation or chimeras if animal oocytes are used. Therefore, many scientists are attempting to generate ESC-like pluripotent cell lines from somatic cells without the use of oocytes. It appears that the factors required for

reprogramming are not restricted to those residing in oocytes. In fact, cell fusion of differentiated somatic cells with ESCs also results in hybrids that have many unique features of pluripotent ESCs [4,5]. The disadvantage of the cell fusion is that the resulting hybrids are tetraploids and not suitable for cell transplantations. More recently, it has been found that exposure of somatic cells or nuclei to cell extracts from ESCs or embryonal carcinoma (EC) cell lines (roughly a tumor-version of ESCs) can lead to reprogramming to a ESC-like or EC-like multipotent state [6,7]. Together, these experiments highlight that nuclear reprogramming is indeed possible through several means and oocytes or cell fusion are not absolutely necessary. However, the precise identity and nature of the underlying players for somatic cell reprogramming have not yet been elucidated.

## Results

The basic premise of Takahashi's and Yamanaka's attempt to reprogram embryonic mouse fibroblasts into ESC-like pluripotent cells was to up-regulate genes that are expressed at a high level in ESCs but silenced or reduced in somatic cells. By doing so, they hoped to start the necessary ES-like transcriptional circuitry in the cells as most of these genes reside or modulate key nodes of the known regulatory network.

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They used a set of 24 genes that were delivered and overexpressed by the MX type of retroviral vectors. Some of the genes, such as *Oct3/4*, *Nanog* and *Sox2*, are known transcription factors that are expressed uniquely in, and required for, sustaining undifferentiated mouse and human ESCs. Other genes also encode key transcriptional factors, such as *Stat3* and *c-Myc*, which are also known to be critical for undifferentiated proliferation of mouse ESCs, although they are more ubiquitously expressed and associated with upregulated cell proliferation and tumor transformation in other cell types. The third type in their set of 24 genes were less studied for their roles in undifferentiated ESCs, and were selected largely based on their favorable expression profiles and/or unpublished functional data familiar to the authors.

To test the candidate genes for reprogramming ability, they introduced them into mouse embryonic fibroblasts (MEFs) through retroviral transduction. To assay for successful reprogramming, they developed an elegant reporter system whereby induction of pluripotency translated to a gain in drug resistance. This was established by inserting a bifunctional (selection-reporter) cassette into the mouse *Fbx15* gene, which is expressed specifically in mouse ESCs. Thus, growth of drug-resistant colonies with an ESC-like morphology implied reprogramming of these otherwise differentiated cells to an ESC-like state.

Their initial experiments involving transduction of just individual genes failed to activate the *Fbx15* locus, thus implying there was no single master inducer of pluripotency in the set. However, surprisingly, transduction of all 24 candidates did yield drug-resistant colonies possessing ESC-like properties, specifically, similar morphology, proliferation rates and also expression of ESC-specific markers. This implied that successful reprogramming had indeed occurred.

Next, elegant experiments were conducted where the effect of withdrawal of individual factors from this pool was studied. Transductions that yielded no or few colonies implied that the excluded factor was critical. They thereby narrowed their starting set to ten genes. In fact, transduction of the MEFs by these ten factors alone produced more ESC-like colonies than transduction by all 24. Proceeding further in this fashion, they were able to narrow their selection to just four essential genes: *Oct3/4*, *Sox2*, *c-Myc* and *Klf4*. Transduction of adult tail-tip fibroblasts with these four selected factors also yielded similar successful induction of reprogramming. Similar to mouse ESCs, the reprogrammed clones require

feeder cells and leukemia inhibitory factor (LIF) for their undifferentiated growth. However, the authors noted that only a small percentage (~0.02%) of the transduced cells by the vectors were reprogrammed to a pluripotent state.

A further characterization of their induced cells showed that several formed teratomas in nude mice bearing differentiated cells of all three germ layers. Furthermore, the induced cells also formed embryoid bodies in suspension, which, when grown in tissue culture dishes, underwent differentiation on attachment to all three germ layers. Also, these cells, when injected into blastocysts, contributed widely to diverse tissues in chimeric embryos until as late as day 13.5, but not to newborns.

Reverse transcriptase (RT)-PCR analysis of the induced clones revealed that they expressed a majority of ESC markers, but the expression levels across clones were not uniform. Distinctly from normal mouse ESCs, *Ecat1* was not expressed in any line. In fact, real-time PCR confirmed that endogenous expression of *Oct3/4* and *Sox2* was lower compared with ESCs. Similar results held for *Nanog* and *E-Ras*, while *p21* (which is regulated by the fourth gene, *Klf4*) had expression levels that varied between clones. However, the total protein levels of the four factors, *Oct3/4*, *Sox2*, *c-Myc* and *Klf4*, were comparable with those in ESCs, but those of *Nanog*, *E-Ras* and *p53* showed variability. Global gene-expression profiles of the clones also clustered them closer to ESCs than MEFs and fibroblasts; however, their signature was still distinct from ESCs.

The authors also examined epigenetic marks of these reprogrammed clones. Chromatin immunoprecipitation analyses showed that the promoters of *Oct3/4* and *Nanog* had increased acetylation of histone H3 and decreased histone H3 lysine 9 dimethylation. Also, the CpG dinucleotides in these promoters remained partially methylated. Thus, these results implied only a partial epigenetic reprogramming of the promoters.

In conclusion, the authors succeeded in inducing adult differentiated somatic cells to a pluripotent state through the upregulation of just four genes encoding known transcriptional factors with an efficiency of 0.02%.

### Significance

Takahashi and Yamanaka's work represents the first defined approach to nuclear reprogramming and provides insights into the molecular aspects of reprogramming and a starting point for generating patient-specific stem cells without use of human oocytes or embryos. It is certainly one of

the most important studies of stem cell research this year. In fact, Dr Yamanaka's presentation in June was considered a major highlight in the International Society for Stem Cell Research (ISSCR) annual meeting [8]. Since its publication in August, several comments have been published on its significance and implications [9,10]. Its impact on stem cell biology and regenerative biology/medicine will be more evident in months or years to come.

### Future perspective

This work is far from being a complete recipe for induction of pluripotency. Additional factors, especially those associated with the epigenetic machinery (histone remodeling and modifications as well as DNA methylation), will likely play a key role in achieving a pluripotent state efficiently and fully. We are particularly intrigued by two questions: could this approach be translated into human cell systems, and if so, how? If the overexpression of *c-Myc* and *Klf4* in mouse fibroblasts is critical for their reprogramming, are both proteins required constantly for the pluripotent and undifferentiated state without inference of (terminal) differentiation?

While the roles of *Oct3/4* and *Sox2* in maintenance of self-renewal are well documented, the absence of *Nanog* from the four required factors and the presence of the two oncogenes, *c-Myc* and *Klf4*, was interesting. *Oct3/4* and *Sox2* have adjacent binding sites within the *Nanog* promoter region and their binding may be able to turn the gene on and account for its expression in the induced cells. In fact, *Oct3/4*, *Sox2* and *Nanog* coregulate expression of many of their target genes and also themselves [11]. *c-Myc* was rationalized by the authors to be significant since it is known to increase the expression of a number of genes (in particular telomerase), has a large number of

genome binding sites and associates with histone acetyltransferase complexes and, thus, could potentially induce global histone acetylation. This would allow increased accessibility of transcription factors to the genome and, in particular, of *Oct3/4* and *Sox2* themselves, to their target sites. *Klf4* may play a role as a transcription factor to control predominantly the downregulation of *p53* or *p21* transcription. *p53* regulates *Nanog* expression and *c-Myc*-induced apoptosis. And, although the extent of reprogramming by these four factors was neither uniform nor completely consistent with the ES cell gene expression profiles and epigenetic signature, extensive changes were successfully and reproducibly induced.

The authors had not yet tried the technique with human cells, when they were interviewed in *Science* in June [10]. According to Dr Yamanaka, owing to differences in human and mouse embryo development, it is possible that a different set of genes would be required to reprogram human cells. If the four genes used in the current work are insufficient to reprogram human somatic cells, such as adult fibroblasts, what other genes should we consider? Is the vector system used in the mouse study adequate for human cells? Also, if the selection was based on the activation of a locus that was essential to ES cell pluripotency, would the degree of reprogramming of the selected clones be greater? In particular, if endogenous *Oct3/4* gene activation was the selection assay, would the epigenetic profile of the selected clones be closer to that of ESCs? Furthermore, does this study imply that it is *Oct3/4* and its cotranscriptional factors *Sox2*, *Nanog* and *Klf4*, and their access to their target genes that ultimately establishes and maintains the ESC pluripotent state? We expect this will be an active research area in months and years to come, thanks to this landmark paper.

### Executive summary

- Induction of pluripotency in mouse embryonic and adult fibroblasts was achieved by the introduction of just four defined transcription factors: *Oct3/4*, *Sox2*, *c-Myc* and *Klf4*.
- Pluripotency of the induced cells was assayed through reverse transcriptase-PCR and protein expression analyses of induced embryonic stem cell markers, by teratoma formation in nude mice, by *in vitro* embryoid body formation and differentiation, through contribution to diverse tissues in chimeric embryos upon blastocyst injection of these cells and through global gene-expression analyses by DNA microarrays.
- Both the epigenetic reprogramming and expression of stem cell markers was incomplete and nonuniform across the various induced clones.
- Of note were the observations that the efficiency of the process was approximately 0.02%. When injected into blastocysts, the reprogrammed cells developed in embryos proper, but died around 13.4 days and did not yield newborns.
- This work will have ramifications in the field of patient-derived stem cells for regenerative medicine, and also in the better understanding of the underlying molecular mechanisms that determine the unique transcriptional regulatory circuitry of embryonic stem cells.

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