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The magic of four: induction of pluripotent stem cells from somatic cells by Oct4, Sox2, Myc and Klf4

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The developmental process from a fertilized egg to a grown adult is programmed with remarkable accuracy. While the genetic information of the fertilized egg and its descendent somatic cells are the same, it is the selective expressions of the same genome that give rise to the 200 or so different cell types in an adult. The differentiated states of these adult cells are maintained epigenetically, presumably through the modification of chromatins and the associated histones. In higher mammals, it was thought that the differentiation process is irreversible until the successful cloning of Dolly [1]. By transferring a nucleus from a fully differentiated cell in the mammary gland, Wilmut and colleagues were able to generate an exact replica of a higher mammal, the Dolly [1]. This work not only demonstrated that the genome of differentiated cells can be reprogrammed into an embryonic state and then to resume a full-fledged developmental process to generate a normal adult, but also rejuvenated the field of animal cloning. The prospect that a somatic cell from a patient may be reprogrammed to the embryonic state and subsequently induced to generate a specific cell type suitable for transplantation therapy has encouraged a new research direction called "therapeutic cloning" [2]. Yet, technical difficulties and ethical concerns associated with "therapeutic cloning" have cast doubts over its eventual success in the clinic.

A promising alternative to therapeutic cloning was published last year by Yamanaka and colleagues, demonstrating for the first time that the reprogramming process can be achieved without going through a nuclear transfer into the eggs, but with the introduction of four transcription factors into fibroblasts [3]. The expression of four transcription factors, Oct4/Sox2/cMyc/Klf4, was able to convert somatic cells from a terminally differentiated state to an embryonic one. While this work was well received with excitement, there were also doubts about the utility of the so-called iPS or induced Pluripotent Stem cells generated by this approach in human therapy. One of such doubts is whether iPS cells are the true equivalent of embryonic stem cells. Now, this concern was answered by three independent groups who succeeded in demonstrating that similarly derived iPS cells are the functional equivalent to embryonic stem cells in mice [4-6]. Specifically, iPS cells possess similar gene expression profiles and epigenetic properties as ES cells (embryo stem cells). When these iPS cells were introduced into the blastocysts, live chimeras were generated and germline transmission was achieved. Thus the iPS cells possess the same developmental potential as ES cells.

To generate and identify potential ES-like iPS cells, Yamanaka and colleagues initially placed dual selection markers of $_{\beta}$*Geo* (*neo* for G418 resistance and $_{\beta}$*Gal* for visualization) into the *FBX15* locus such that the expression of these markers was under the control of endogenous *FBX15* promoter [3]. Then, 24 candidate genes were introduced into MEF (mouse embryo fibroblasts) derived from *FBX15*$^{\beta geo/\beta geo}$ mice, and the cells were subjected to G418 selection. Since FBX15 is only expressed in pluripotent cells, but not in differentiated ones, the expression of $_{\beta}$*Gal* would indicate a pluripotent state achieved by the reprogramming of somatic states into embryonic ones. This was a simple, but critical, assumption and it worked magically. Initially, no single factor was able to generate any clones, suggesting that reprogramming is not governed by a single gene or single pathway. However, when all 24 factors were introduced into the fibroblasts, ES-like cells emerged from a lawn of fibroblasts after a period of G418 selection. Through a subtraction process, the number of genes required for reprogramming was narrowed down to the magic four, *Oct3/4, Sox2, c-Myc* and *Klf4*. The result-

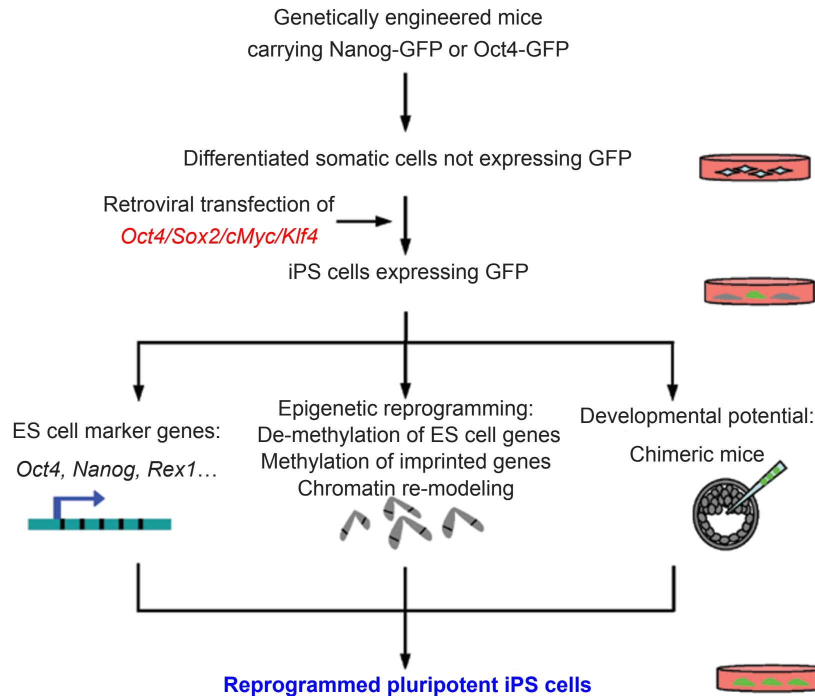

Figure 1 Generation of iPS cells by retroviral transfection of four transcription factors: several groups used the depicted strategy to generate pluripotent stem cells from terminally differentiated somatic cells. As shown in the figure, mice carrying Nanog-GFP or Oct4-GFP were first generated. Embryonic or somatic fibroblast cells were isolated from these mice and cultured *in vitro*. When transfected with the four transcription factors, *Oct4/Sox2/cMyc/Klf4*, some of the differentiated fibroblast cells can be reprogrammed back to the embryonic state and thus start to express Nanog or Oct4 in conjunction with GFP. GFP can then be used as a selection marker to purify the single cell colonies which were expanded into iPS cell clones. These selected iPS cells were characterized for their ES-like properties via different methods, such as the expression of ES cell marker genes, their epigenetic properties and their developmental potentials. The results showed that the four transcription factors could indeed reprogram terminally differentiated cells into pluripotent stem cells.

ing iPS cells showed similar morphology, proliferation and teratoma forming ability in immuno-deficient mice when compared to ES cells. However, when the authors injected these iPS cells into mouse blastocyst, embryos failed to develop beyond mid-gestation stage [3].

The assumption in the original strategy of the Yamanaka study proved to be correct and the design was brilliant. However, the choice of *FBX15* could be debated. One could have argued that better markers such as the pluripotency genes *Oct4* and *Nanog* should have been used instead of the nonessential *FBX15*. This is exactly the strategy used in the three latest articles [4-6]. The Yamanaka group used a more stringent condition to select ES-like iPS cells, namely the expression of *Nanog*, one of the ES cell marker genes downstream of *Oct4/Sox2* [4]. They first generated a transgenic mouse line using a BAC (bacteria artificial chromosome) clone carrying a cassette of GFP and the puromycin resistance gene inserted at the 5′ untranslated region of *Nanog*. Embryonic fibroblast cells were then isolated from these mice and cultured *in vitro*. The subsequent transfection with retroviral vectors containing the four transcription factors reversed the differentiated fibroblast cells back into *Nanog* expressing stage (ES cell stage), allowing the simultaneous expression of GFP (Figure 1). GFP-expressing colonies not only allowed for the selection of ES-like cells but also enabled the proliferation of these cells in a feeder cell independent manner (a characteristic of ES cells when over-expressing *Nanog*). Two other groups [5, 6] took a similar strategy to obtain differentiated fibroblast cells from mice carrying GFP-neo or GFP-puro marker at either *Oct4* or *Nanog* locus (generated through homologous recombination), respectively. Both embryonic and mouse tail-tip fibroblast cells were isolated, cultured and subsequently transfected with the four transcription factors. Stable iPS cell colonies were then selected by G418 or puromycin resistance and GFP expression. All the iPS cells from these three groups have been shown to be functionally equivalent to ES cells, including chimera

formation and germline transmission [4-6].

The key improvement here is the selection of iPS cells using *Oct4* or *Nanog* expression as an indicator of pluripotency. Both genes are required for ES cells to maintain pluripotency, while *FBX15* is not, which may help explain the difference of these two approaches. iPS cells selected this way showed remarkable similarities to ES cells in many aspects. First, ES cell marker genes (such as *Nanog*, *Fbx15*, *Rex1*), including the endogenous genes for these four transcription factors, were turned on to a similar level as compared to control ES cells. The expression of these genes was not detectable in differentiated fibroblast cells before retroviral transfection, suggesting that the cells' differentiated state was reversed during the induction of iPS cells [4-6]. Secondly, the epigenetic properties of the iPS cells were similar if not identical to that of ES cells. Using bisulfite genomic sequencing analysis and Southern blots, they showed that the global gene methylation patterns in iPS cells were also similar to ES cells. While imprinted genes, such as *H19* and *Igf2r* were only partially methylated, promoters of *Oct4*, *Nanog* and *Fbx15* became largely unmethylated. Two important histone methylation patterns were also analyzed. H3K4 trimethylation and H3K27 trimethylation, which are known to be associated with transcribed genes and silenced genes, respectively, were comparable to that of ES cells. In addition, Maherali, *et al*. also showed that the iPS cells generated from female fibroblast cells possessed similar X chromosome inactivation pattern as that of female ES cells [6]. These results demonstrated the ability of the four transcription factors to epigenetically reprogram the differentiated somatic cells into pluripotent stem cells. Lastly, the developmental potential of these iPS cells was examined by their ability to generate adult chimeras. When injected into mouse blastocysts, these cells contributed to wide varieties of adult tissues (such as cartilage, liver, heart and lung) and transmitted into germline as well. In one study, the chimerism ranged from 10% to 90%. In addition, when these iPS cells were injected into 4N blastocysts, "all iPS embryos" were generated beyond E14.5, further confirmed that the reprogrammed iPS cells possessed the developmental properties of the "true" ES cells.

Stem cells in bone marrow and cord blood are of therapeutic value for several blood disorders. By analogy, other stem cells, especially embryonic stem cells, may possess therapeutic potentials for broader ranges of diseases. To this end, one would argue that iPS cells from patient themselves could overcome problems such as immuno-rejection after cell (or organ) transplantation and also avoid the use of human embryos to generate ES cells through somatic nuclear transfer. However, there are many technical difficulties that remain to be addressed. First, no iPS cells have been derived from human yet. Given the conservation of functions associated with these four genes, one could argue that these four factors should be able to generate iPS cells from human fibroblasts. The difficulty here is the lack of similar selection systems for human fibroblasts as those demonstrated in mouse [4-6]. It may take considerable time to generate the Nanog- or Oct4- selection systems as reported for mouse. It is also conceivable that human fibroblasts may need different set of transcription factors for reprogramming. Second, the reprogramming process is inefficient. Although the iPS cells selected by using *Oct4* or *Nanog* were much closer to ES cells by all means used to characterize them, the efficiency of generating the stable iPS clones was still low (0.03% when using *Nanog* and 0.08% when using *Oct4*). Nanog-iPS and Fbx15-iPS cells also showed slightly longer doubling time when compared to ES cells. The epigenetic properties of iPS cells were not completely identical to those of ES cells, which could cause problems for animals' later development. Third, there is a concern of safety as *Oct4/Sox2/Myc/Klf4* are oncogenic or potentially oncogenic. Indeed, about 20% of the F1 mice in one study developed neck cancer, probably due to the reactivation of *c-Myc*. In addition, retroviral vector insertion may activate endogenous oncogenes and result in cancer. These problems must be resolved before this new approach of iPS cell generation can be applied to human disease therapeutics.

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