

Review Article

Recent advance in induced pluripotent stem cells

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We have previously shown that embryonic stem (ES)-like cells can be induced from mouse fibroblasts, hepatocytes and stomach epithelial cells by introducing four factors (Oct3/4, Sox2, c-Myc, and Klf4). The cells are similar in morphology, proliferation and gene expression profile to those of ES cells, and are called induced pluripotent stem (iPS) cells. When the iPS cells are transferred into blastocyst, they can contribute to adult chimeric mice and transmit through germline to the next generation. Therefore iPS cells have almost same differentiation potential as ES cells. In 2007, we and others reported the establishment of iPS cells from human somatic cells and showed their pluripotency.

These iPS cells would supply patient-specific pluripotent stem cells for cell transplantation therapies. However, iPS cells still have several problems to be overcome, especially tumorigenicity owing to the use of oncogenes and retrovirus. Recent studies revealed that c-Myc is not a crucial factor for iPS induction, albeit it greatly increases the efficiency. The improvement of reprogramming efficiency was reported with soluble factor, Wnt3a, and several small molecules that influence epigenetic modification, such as BIX-01294 and VPA. Induction of mouse iPS cells without virus vector has been reported. Through the basic researches on iPS and ES cells, molecular mechanisms underlying the reprogramming process were gradually being uncovered. Here we try to summarize current studies on iPS cells. The iPS cells will contribute to the fields of elucidation of pathogenesis, drug discovery, toxicology study, and cell transplantation therapy in the future.

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Introduction

The regenerative medicine with stem cells has recently been attracting a lot of attention as they may lead to new treatment for several incurable diseases such as Parkinson's disease and dia-

betes. The most well-known stem cell is embryonic stem (ES) cell. Mouse ES cells were established from early developmental embryo in 1981¹⁾, and human ES cells have been established in 1998²⁾. Since ES cell can differentiate into various types of

cells in the body under an appropriate culture condition, they are thought to be a suitable source for cell transplantation therapies. In fact, mouse ES cells can treat disease model mice^{3,4}. However, there are still several problems to be solved before clinical use. ES cells would induce immunological rejection after transplantation into patients, because they are not derived from the patient's own cells. They also face ethical problem with the use of embryonic tissues. One solution is to make ES-like pluripotent stem cells from patient's cells.

Establishment of mouse iPS cells

Reprogramming of somatic cells into pluripotent cells has been studied for more than 50 years. For example, Dr. J. Gurdon and his colleagues showed that frog somatic cells can be reprogrammed after the fusion of enucleated oocyte, and that they develop into feeding tadpole⁵. After 30 years, reprogramming in vertebrates was also proved by the creation of clone animals from sheep⁶ and mouse⁷ somatic cells fused with enucleated oocyte. In addition to oocyte, human and mouse ES cells also can reprogram somatic cells into ES cell-like state after cell fusion or exposing their cell extracts^{8,9}. These results indicate that somatic cells can become pluripotent state by certain reprogramming factor(s) within oocytes and ES cells.

Hence, we'd tried to find these reprogramming factor(s) and then selected 24 genes as candidates¹⁰. Some of them play important roles in the maintenance of pluripotency or cell growth of ES cells, and some showed specific expression in ES cells. To evaluate their reprogramming activity, fibroblasts obtained from Fbx15 knock-in mice were used (Fig.1A)¹¹. Fbx15 is specifically expressed in ES cells, and is controlled by Oct3/4 and Sox2. However, Fbx15 seemed to be dispensable for mouse, because its disruption in mice and ES cells hardly showed abnormal phenotype. We made knock-in mice by inserting a β -geo cassette (a fusion of β -galactosidase and the neomycin-resistant gene) into the locus. Fibroblasts from the knock-in mice do not activate Fbx15 promoter, and are sensitive for G418, a neomycin derivative, but ES cells are resistant to extremely high concentration of G418. Thus the reprogramming activity of the candidate factors could be translated into the resistance to the drug. Fbx15 fibroblast were infected with retrovirus vectors encoding the candidate genes, and were selected with G418 for two weeks. Although we did not obtain drug resistant colony with any single factor, we found drug resistant colonies with all 24 factor mix. After several round of screening to narrow down the factors, it was found that the combination of 4 transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) is sufficient for the reprogramming, re-

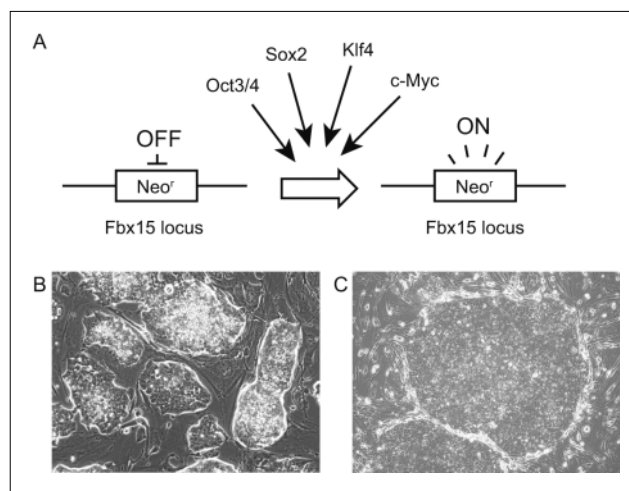


Fig.1 Establishment of iPS cells

(A) Schematic diagram of reprogramming factor screening. The reporter gene at the Fbx15 locus is not active in fibroblasts (left). After the retroviral transduction of the four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4), a small portion of the infected cells are reprogrammed and become to express the reporter (right). Morphology of mouse (B) and human (C) iPS cells are shown.

sulting in generation of ES-like cells from mouse fibroblasts (Fig.1B). We named this established ES-like cells iPS (induced pluripotent stem) cells¹⁰.

iPS cells selected for Fbx15 expression (hereafter described as Fbx15-iPS cells) showed ES-like morphology, and expressed several ES cell marker genes, such as Oct3/4, ERas¹², and Esg1. When injected subcutaneously into immuno-deficient mice, they can differentiate into variety of cell types in all three germ layers, such as gut-like epithelium, cartilage, skeletal muscle, and neuronal tissues. Fbx15-iPS cells can contribute to embryonic chimera mice, however they fail to develop into adult chimeric mice. DNA methylation status of Oct3/4 and Nanog promoter region were not fully demethylated as known in ES cells. Hence Fbx15-iPS cells were reprogrammed by 4 factors but it would be incomplete.

To obtain better iPS cells, we and other groups used other selection markers, Nanog and Oct3/4, both of which specifically express in ES cells and are functionally important to the pluripotency, and establish more ES-like iPS cells¹³⁻¹⁵. This high-quality iPS cells are comparable to ES cells in morphology, proliferation, gene expression, and epigenetic modifications. They can make teratoma and more importantly they can contribute to adult chimeric mice. iPS cells exist in several tissues of chimeric mice including brain, lung, liver, and stomach. Some

iPS clones contribute to gonads and transmitted to the next generation. These data suggest that mouse somatic cells become pluripotent state by defined four factors, and differentiate the cells which can compensate the function of the body.

Establishment of human iPS cells

Human and mouse ES cells have many properties in common, but many difference are also reported. For instance, mouse ES cells form tightly packed round-up colonies while human ES cells form flat colonies. LIF is an important factor to maintain mouse ES cells in undifferentiated state. On the other hand, human ES cells need bFGF but not LIF for the culture. Therefore it was not certain whether reprogramming of human somatic cells occur by introduction of the same 4 factors used in mice. Human foreskin fibroblasts were infected by retrovirus encoding 4 transcription factors, and cultured in the condition for human ES cells¹⁶. A month later, a few flat colonies look like human ES cells appeared, then we selected some of the colonies for expansion. These human iPS cells proliferated more than half year and expressed various ES cell markers, such as NANOG, SALL-4, and ESG1 (Fig. 1C). Global gene expression analysis with microarray demonstrated that human iPS cells are similar to human ES cells. Their epigenetic modifications of promoter region of OCT3/4 and NANOG were also comparable to ES cells. To confirm their differentiation ability, human iPS cells were injected into immuno-deficient mice. After three months, they developed several cell types in all three germ layers. They also differentiated into β III-tubulin positive neuron and cardiac muscle *in vitro*. The data clearly indicate that human iPS cells are pluripotent stem cells. Human iPS cells were also established with slightly different factors, OCT3/4, SOX2, NANOG, and LIN-28¹⁷.

The origin of iPS cells

As described above, iPS cells were first established from primary mouse fibroblast culture. Since the efficiency of iPS cell induction was very low (less than 0.1%), their origin was thought to be some tissue stem cells included in the culture. Dr. T. Aoi et al. showed mouse iPS cells can be established from mouse hepatocytes and stomach epithelial cells and that most hepatocyte-derived iPS cells were from albumin-positive cells by lineage tracing experiments¹⁸. Mouse iPS cells were also established from pancreatic islet β cells¹⁹. Therefore the origin of iPS cells is not only stem cells but also differentiated somatic cells.

Dr. R. Jaenisch and his colleagues generated iPS cells from mouse fibroblast by lentivirus vectors, which encode the 4 factors under the control of doxycyclin (Dox)-dependent expression

system²⁰. Then the iPS cells were used for the generation of chimeric mice. Primary cells were collected from various organs of the chimeric mice, and were treated again with Dox to express reprogramming factors. The establishment of "secondary" iPS cells was observed from adrenal gland, muscle, and intestinal epithelium^{20,21}. iPS cells were also established from neural progenitor cells by only 2 factors (Oct3/4 and Klf4)^{22,23}. It may be because neural progenitor cells highly express Sox2. Considering these results, iPS cells can be induced from various cell types, and the reprogramming factors and manipulation steps can be reduced by choosing appropriate source. To make human iPS cells, this knowledge would be useful to select the starting cells for reprogramming.

Safety concerns for medical application

As described above, establishment of human and mouse iPS cells were achieved by transduction with transcription factors by retrovirus or lentivirus vectors. After the infection, these viral vectors integrate encoding transgenes into the host genome. It enables to carry out stable gene expressions in mother cells and in daughter cells. During reprogramming process, the expressions of transgenes were gradually suppressed maybe by the epigenetic modifications, such as DNA methylation. However these exogenous sequences still remained in the genome of iPS cells. Integrations of transgenes, especially c-Myc, a well-known proto-oncogene, were concerned as a safety problem. In fact, we found tumor formation in around 20% of the chimera mice and offspring of mouse iPS cells within 4 months¹⁴. All tumors we examined showed reactivation of retroviral c-Myc. Subsequent studies revealed that c-Myc is not a crucial factor for iPS induction, albeit it greatly increases the efficiency²⁴. The Myc-free iPS cells have pluripotency and its chimeric mice did not show tumor formation for 4 months. The number of retrovirus integration would also affect a possible tumor formation. iPS cells derived from hepatocyte have less integration sites than the ones derived from fibroblast¹⁸. Comparing their chimera mice, the former showed less tumor formation even their iPS cells were established with 4 factors. This result would be because c-Myc was rarely reactivated in the chimera mice. Therefore we would be able to reduce the risk of tumor formation by avoiding the use of c-Myc and by inducing iPS cells with a few integrations. However, there are reports that Oct3/4²⁵ and Klf4²⁶ are involved in tumor development, and that various human tumor express OCT3/4, SOX2 and KLF4. Furthermore, the retroviral insertion to the genome itself may disturb endogenous gene structure and increase tumor risks²⁷.

To avoid these risks, induction methods without integrated virus vectors should be developed. Dr. M. Stadtfeld et al. generated mouse iPS cells using non-integrating adenoviral vectors²⁸, while we tried to establish iPS cells without viral vectors²⁹. By repeated transfection of a single plasmid containing the cDNAs of Oct3/4, Sox2, and Klf4, together with a c-Myc expression plasmid, into mouse embryonic fibroblasts resulted in iPS cells. These adenovirus- and plasmid-iPS cells showed teratoma formation and contributed to adult chimeric mice indicating their pluripotency. These iPS cells did not show evidence of genomic integration by PCR and southern blot analysis. Although integrations of short fragments derived from transgene were not fully ruled out, these iPS cells are most likely free from the integration. These studies would be an important step toward development of safe iPS cells for future clinical use.

Molecular mechanisms in iPS cell induction

From the studies on mouse and human ES cells, the transcriptional network maintaining pluripotent state has gradually been proved. The important factors in the network are Oct3/4, Sox2, and Nanog^{30,31}, and they also reported to interact with Klf4^{32,33}. These transcription factors have many common targets including themselves and regulate them simultaneously in both activation and repression manner. While any single transcription factor could not induce iPS cells, it is suggested that their unique target genes are also important for the reprogramming process. It is also supported by the result that the combination of all 6 factors (Oct3/4, Sox2, Klf4, c-Myc, Nanog, and Lin28) increased the efficiency of human iPS cell establishment³⁴. There are family genes for Oct3/4, Sox2, and Klf4, and some of them can induce iPS cells²⁴. For example, Klf2 can replace Klf4. Comparison of the target genes among reprogramming factors and the family genes might be useful to understand molecular mechanisms underlying iPS cell formation.

Lin28 is a RNA binding protein expressing in ES cells, which inhibits the maturation of let-7 family microRNAs by the 3' terminal uridylation^{35,36}. During ES cell differentiation, Lin28 gradually decreases and mature let-7 family microRNAs become detectable with inverse correlation. let-7 is reported to promote differentiation of breast cancer cells and inhibit their proliferation³⁷. Taking these results together, Lin28 would facilitate the induction of iPS cells by suppressing their differentiation through the inhibition of let-7 family microRNA, but further analysis is needed to understand the detailed mechanism.

What happened during the induction process? According to

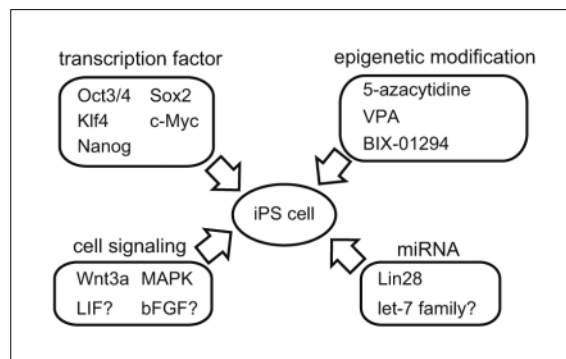


Fig.2 Factors which influence on iPS cell induction

the studies using Dox-dependent expression system, first step of the mouse fibroblast reprogramming is downregulation of differentiation marker genes, Thy1, following upregulation of ES cell markers, such as SSEA-1 expression and alkaline phosphatase activity^{38,39}. Since the cells at this step could not become iPS cells by Dox withdrawal, their reprogramming would still be incomplete. More than 10 days of Dox addition led to the upregulation of endogenous Oct3/4 and Sox2 expression and telomerase activity, and iPS cell establishment. Therefore iPS cell generation requires continuous transgene expression and the process contains activation and suppression of various genes. The gene expressions are regulated not only by transcription factors but also by epigenetic modifications, such as DNA methylation and methylation/acetylation of histones. Promoter regions of Oct3/4 and Nanog in fibroblasts are highly DNA methylated and inactive, while these regions are demethylated and active in iPS cells. There are several studies that the efficiency of iPS cell induction was increased by the treatment with epigenetic modification drugs, 5-azacytidine⁴⁰, valproic acid (VPA)^{40,41}, and BIX-01294²³. Hence the alteration of epigenetic modifications would be also important for iPS cell induction.

iPS cells are cultured in medium for ES cells. As described above, LIF and bFGF are important factor for mouse and human ES cell maintenance, respectively. However the roles of these cytokines in the induction process are still unclear. The promotions of mouse iPS cell establishment were reported by the addition of Wnt3a and inhibitor of glycogen synthase kinase-3 (GSK3)^{42,43}. Inhibition of MAP kinase pathway, which induces differentiation of mouse ES cells, also increased mouse iPS cell induction⁴³. Cytokine signals, including Wnt/ β -catenin and MAP kinase pathway, may affect the reprogramming.

iPS cell induction takes at least one week in mouse and two weeks in human. On the other hand, the reprogramming by fu-

sion of ES cells occurred very rapidly. Activation of endogenous Oct3/4 promoter of somatic cell nuclei is observed within two days⁸). Although transgene expression in iPS cells need a few days, because of virus vector infection followed by integration into host genome, the reprogramming of iPS cells takes much more time than that of cell fusion. There would be other factor(s) in ES cells that facilitate the reprogramming.

Conclusion

The establishment of iPS cells would apply not only to medical field but also to the elucidation of the control mechanisms of the stem cells and then for the development of efficient differentiation protocol. However, there are still many safety concerns which must be overcome before clinical use in cell transplantation therapies. On the other hand, basic medical research has already been launched using iPS cells from patients with a variety of genetic diseases, including adenosine deaminase deficiency-related severe combined immunodeficiency, Duchenne and Becker muscular dystrophy, and amyotrophic lateral sclerosis^{44,45}). It has been reported that in vitro differentiation of mouse iPS cells into neuron⁴⁶), hematopoietic cells and cardiomyocytes⁴⁷⁻⁴⁹). Treatment of humanized sickle cell anemia mouse model with mouse iPS cells was also reported⁵⁰). Further studies will improve the current human iPS induction procedure, and supply safer iPS cells to all in need.

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