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The Past, Present and Future of Induced Pluripotent Stem Cells

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1. Introduction

Our body is derived from only one cell, a fertilized egg. At the birth, the body consists of 220 kinds of somatic cells. The fertilized egg divides many times during development. The resulting cells differentiate into many kinds of somatic cells, and a fertilized egg can differentiate into all of the different types of cells, including intraembryonic and extraembryonic tissues. This ability is called totipotency. Fertilized eggs differentiate into various kinds of somatic cells. However, somatic cells do not divide and differentiate into other types of somatic cells after differentiation in a disorderly manner. There are two possibilities to explain this. First, somatic cells can completely lose their potential to differentiate into other kinds of cells during development, or second, they may retain their potential, but such potential may be suppressed after development. The studies to elucidate how these processes occur were the origin of reprogramming science and regenerative medicine.

In 1938, Spemann was the first person to carry out nuclear transplantation, but the experiment failed (Spemann, 1938). In 1952, Briggs and King transplanted the nucleus of a frog blastula into enucleated unfertilized eggs. The eggs developed into tadpoles. This was the first cloned animal with nuclear transplantation, and the origin of the cloning technique. An interesting discovery was that the later the nucleuses were taken during the developmental stage, the lower the efficiency of generating clone frogs. It was impossible to produce a cloned frog using the nucleus from a stage later than the development of a tailbud. At that time, they thought the information in the nucleus changed during development (Brigge & King, 1952). However, Gurdon arrived at a different conclusion from Briggs and King. He transplanted the nuclei of small intestinal epithelial cells into enucleated unfertilized eggs and obtained tadpoles (Gurdon, 1962). His data suggested that the nucleus of a somatic cell could be reprogrammed, and thereby regain the ability to differentiate into many kinds of cells. In 1997, the cloning of a sheep demonstrated that mammalian somatic cells could also be reprogrammed (Wilmut, 1997). These data suggested that the information in the nucleus did not change irreversibly during development, and indicated that somatic cells have the potentially ability to differentiate into other kinds of cells after development.

2. What are ES cells?

It was necessary for the growth of developmental engineering and reprogramming science to make cells that can easily expand and maintain the ability to differentiate into many kinds

of cells in a cell culture system. Embryonic stem (ES) cells fulfilled these characteristics. ES cells have two important abilities, self-renewal and pluripotency. The ability for self-renewal allows these cells to grow semipermanently. Pluripotency, as described above, is the potential for differentiation into many kinds of cells which make up the body, such as muscle cells, neural cells and so on. Mouse ES cells injected into mouse blastocysts contribute to the formation of all tissues in the body. The mice generated from embryos injected with ES cells are called chimeric mice. These abilities of the ES cells made it possible to generate a large number of any type of cells that is desired.

ES cells are established from fertilized eggs. The inner cell mass of blastocyst-stage embryos are transformed into ES cells. The fertilized egg is first cultured on feeder cells, which provide several necessary factors to the egg and ES cells. A few days later, the cells of the inner cell mass start to grow under culture conditions. ES cells are established from these growing cells. In 1981, mouse ES cells were established (Evans & Kaufman, 1981), and human ES cells were established in 1998 (Thomson et al 1998). Interestingly, the characteristics of human ES cells are different from those of mouse ES cells. The morphology of human ES cells was more like that of cynomolgus ES cells, which had been established several years before the mouse ES cells. Moreover, the optimal culture conditions differ between human ES cells and mouse ES cells. For example, leukemia inhibitory factor (LIF) and bone morphogenic protein (BMP) are important for maintaining the abilities of mouse ES cells in vitro. On the other hand, basic fibroblast growth factor (bFGF) and Activin A are required for the maintenance of human ES cells (Boiani & Schöler 2005).

Mouse ES cells are commonly used as a tool to generate transgenic and gene-targeted animals. These animal models have controbuted to the progress made in basic and medical sciences. Human pluripotent stem cells, including ES cells, are expected to be a good source of regenerative medicine, because of their outstanding capacities such as self-renewal and pluripotency.

3. Why generate artificial pluripotent stem cells?

There are several outstanding issues surrounding the use of ES cells for clinical applications. One of them is immunological rejection. ES cells are generated from fertilized eggs, which can have different immunizing antigens from the recipient who received the regenerative medicine developed using these cells. If the somatic cells from ES cells are transplanted into recipients, then the cells are rejected by the patient's own immune system. To overcome this problem, a new technique was developed. In this technique, the nuclei of an individual's somatic cells are transplanted into enucleated unfertilized eggs. The eggs can then be used to make ES cells expressing the individual's own immunizing antigens. These ES cells were called nuclear transfer ES cells (ntES cell), and the somatic cells derived from ntES cells are not rejected by the recipient after transplantation. These ntES cells have been established from not only mouse, but also from monkey cells (Rideout et al., 2000; Byrne et al., 2007). However, there have been no reports of human ntES cells. A likely reason for this is that the efficiency of generating ntES cells is very low, thus requiring a lot of embryos. This is not only a technical challange but also poses ethical problems. Generating human embryos for research is questionable and in case human nuclear transplanted embryos are implanted in a uterus, a cloned human would be generated.

Another ethical problem is the use of ES cells for clinical applications. To generate ES cells, it is necessary to either injure or break up embryos, which are the origin of human life. To

avoid these problems, ES cells were generated from embryos which arrested their own development or from poor-quality embryos generated for in vitro fertilization treatments. The embryos which would be discarded as of low quality for fertilization treatment are used to make ES cells. Moreover, ES cells can be generated from single blastomers of embryos, and the biopsied embryos still can grow normally (Chung et al., 2006; Klimanskaya et al., 2006; Chung et al., 2008). That is, ES cells can be generated without embryonic destruction. However, there are still discussions ongoing about how these "origins of human life" are handled by humans, and many countries have legislation preventing the development and use of ES cells. Therefore, it is necessary to be able to make pluripotent stem cells artificially for clinical applications. One of the major goals of nuclear reprogramming research is to generate ES-like cells by the conversion of somatic cells.

4. The search for reprogramming factors

One of the most difficult points for finding ways to convert somatic cells to pluripotent stem cell was to identify reprogramming factors. There had been hints based on the previous research on ES cells. For example, it was known that ES cells could induce pluripotency in somatic cells. When mouse somatic cells were hybridized with mouse ES cells, the nuclei of the somatic cells were reprogrammed. The genes which were normally expressed specifically in ES cells started to be expressed from the genome of somatic cells after hybridization. These hybridized somatic cells could differentiate into all three germ layers. When human ES cells were used for hybridization, the nuclei of human somatic cells were also reprogrammed. These data demonstrated that there were factors (reprogramming factors) which could induce pluripotency in somatic cells that were present not only in oocytes, but also in ES cells.

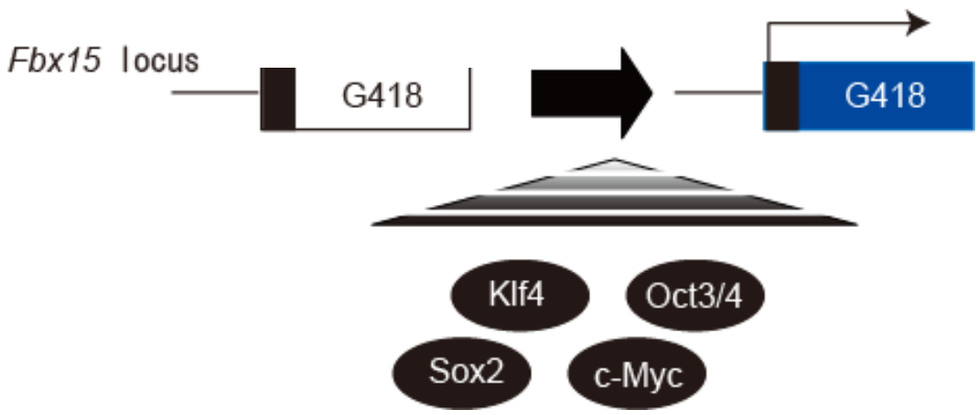
It was hypothesized that tsuch reprogramming factors would be important factors for maintaining pluripotency in ES cells, and that the identification of the factors required by ES cells would also indicate the factors required for the reprogramming of somatic cells. These factors would be expressed highly and specifically in ES cells. Next, Then gene expression pattern was compared between ES cells and somatic cells to narrow down the candidates of reprogramming factors using a computer database. The selected genes were named ES cell associated transcripts (ECATs). These ECATs were expressed highly and specifically in ES cells. They also were shown to play important roles in maintaining the properties of ES cells. For example, Nanog is one of the ECATs. In the absence of Nanog, mouse ES cells differentiate into visceral or parietal endoderm, and do not maintain the properties of ES cells. An overexpression of Nanog also maintains the self-renewal of ES cells, independent of LIF (Mitsui, et al., 2003; Chambers, et al., 2003).

In the 1990s, the transcription network involved in maintaining the pluripotency and self-renewal of ES cells gradually started to become clearer. Oct3/4 was discovered to be one of key factors that make ES cells unique. Oct3/4 is expressed in ES cells, germ cells and also differentiated cells. However, the expression level of Oct3/4 is strictly regulated strictly by the transcription network in ES cells. A mere 1.5-fold increase in the expression of Oct3/4 induces the differentiation of ES cells to primitive endoderm. A reduction in the expression of Oct3/4 by half leads ES cells to generate trophectoderm (Niwa et al, 2000).

Moreover, several oncogenes were also shown to be important for maintaining ES cells. The Myc family of genes plays an important role in maintaining ES cells. Max is an important partner required for the functions of Myc. If max is knocked out, Myc family genes such as

c-Myc, L-Myc and N-Myc cannot exert their effects. Max knockout ES cells cannot survive. Several genes were selected from the ECATs, important transcripts for ES cells and oncogenes as candidates of reprogramming factors. It was necessary to create an assay system which evaluated candidates reprogramming factors for their ability to reprogram somatic cells. Fbx15 is another of the ECATs. That is, Fbx15 is expressed specifically in ES cells, and not in somatic cells. Fibroblasts with a G418 antibiotic resistance gene in the Fbx15 locus were used for the assay system. Normal cells cannot survive in the presence of G418. If the fibroblasts are reprogrammed by the candidates, their Fbx15 locus is activated, the G418 resistance genes are expressed, and the fibroblasts are resistant to G418. The cells that were reprogrammed cells by the candidate could then be selected with G418 (Fig1.A).

A)



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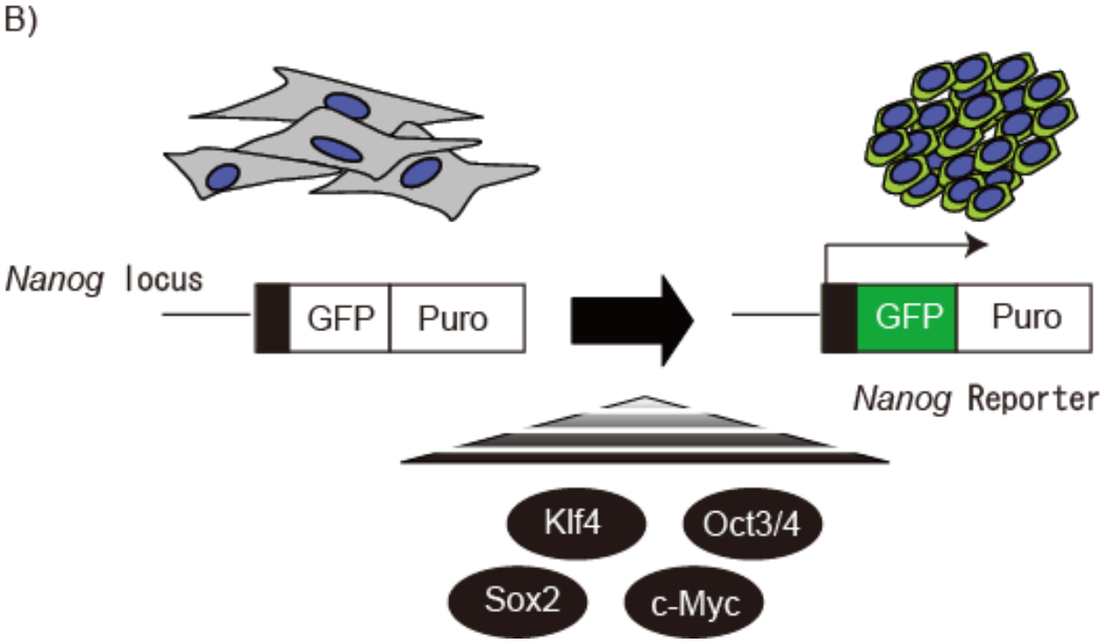
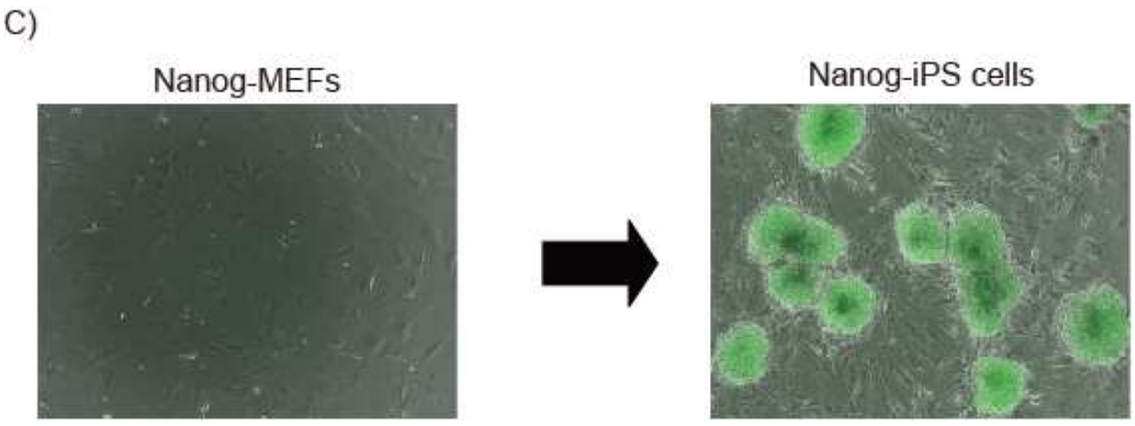




A - An illustration of the Fbx15 reporter system. Fbx15 is a marker gene of ES cells which is specifically expressed in ES cells. Fbx15 is not expressed in fibroblasts. If fibroblasts are reprogrammed by reprogramming factors, then the Fbx15 locus is activated in the cells. These reprogrammed cells thereafter demonstrate resistance for G418; a tocix antibiotic to mammalian cells.
B - An illustration of the Nanog reporter system. The Nanog locus is inactivated in somatic cells. On the other hand, the Nanog locus is activated in reprogrammed cells. The reprogrammed cells are positive for GFP (green fluorescent protein) and also show resistance for Puromycin ,
C- MEFs and iPS cells carrying the Nanog reporter system. The iPS cells are positive for GFP driven by the Nanog reporter system.

Fig. 1. Reporter system

5. Creating the world's first iPS cells

Candidate reprogramming genes were introduced into mouse embryonic fibroblasts (MEF) carrying a Fbx15 reporter system. When 24 candidates were introduced into MEF at the same time, G418 resistant mouse ES-like colonies appeared about 2 weeks later. The 24 candidates were narrowed down to just 4 factors: Oct3/4, Sox2, klf4 and c-Myc. The cells reprogrammed from somatic cells by these four factors were named "induced pluripotent stem cells" (iPS cells). Their global gene expression patterns were similar to those of mouse ES cells. The proliferation of iPS cells was also similar to ES cells. The iPS cells can differentiate into all 3 germ layers in vitro and in vivo. The iPS cells generated using the Fbx15 reporter system could also contribute to mouse embryos, but the chimeric embryos did not survive until birth (Takahashi & Yamanaka, 2006). These data indicated that these first iPS cells had many features like ES cells, but were not completely ES cell like. These iPS cells were considered to be only partially reprogrammed, so the reporter system was improved to facilitate the development of completely reprogrammed iPS cells. Nanog and Oct3/4 are more tightly associated with pluripotency in ES cells than Fbx15. The iPS cells established using a Nanog or Oct3/4 reporter system (Fig1.B,C) contributed to chimeric mouse embryos which survived beyond birth, and these improved iPS cells contribute to the germline of chimeric mice (Okita et al.,2007; Wernig et al.,2007; Maherali et al., 2007) (Fig,2). Moreover, it was reported that cloned live pups could be generated using iPS cells by tetraploid complementation (Kang et al., 2009). These studies strongly suggest that mouse iPS cells are substantially comparable to mouse ES cells, at least in terms of their differentiation potential.

A - iPS cells are injected into blastocysts to make chimera mice. iPS cells are injected into mouse blastocysts (middle) using a micro manipulation system consisting of a holding- (left) and a transfer pipette (right)
B, C - Mouse iPS cells expressing red fluorescent proteins are injected into mouse blastocysts. The mouse iPS cells contribute to all tissues in the mice bodies. The right mouse pup is the chimera.

Fig. 2. Contribution of iPS cell to all tissues in chimera mice

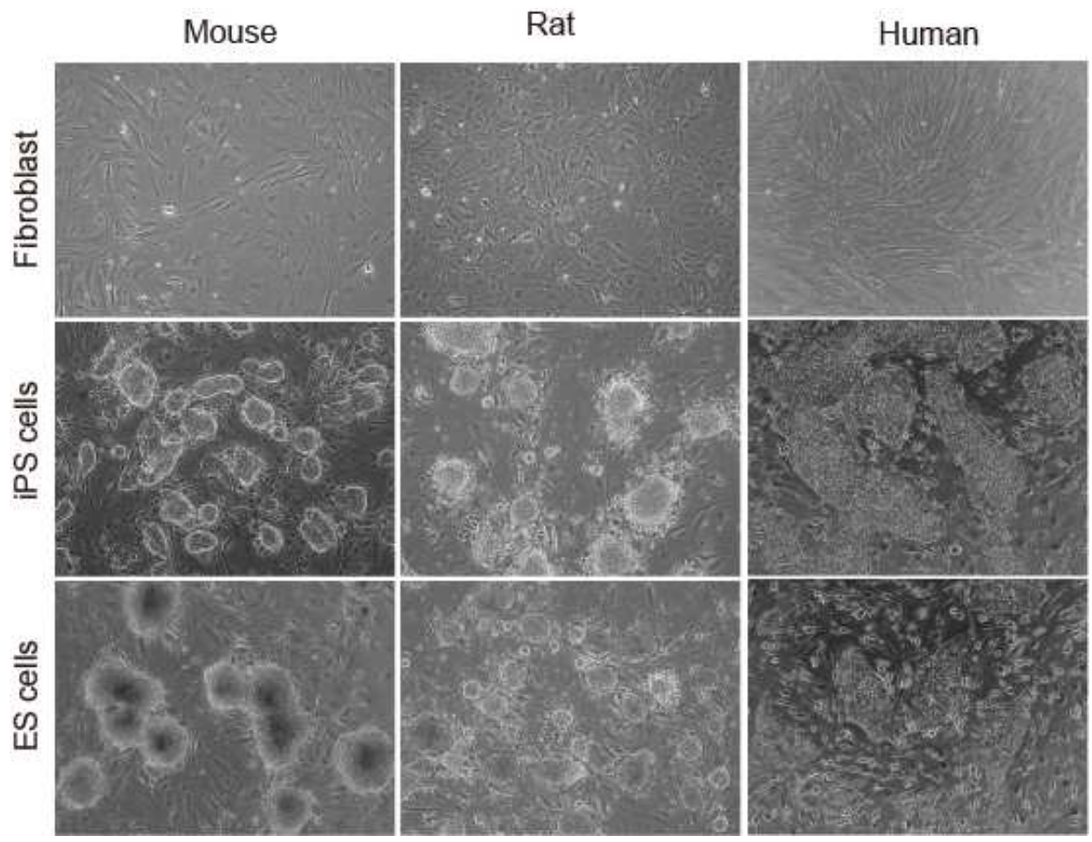
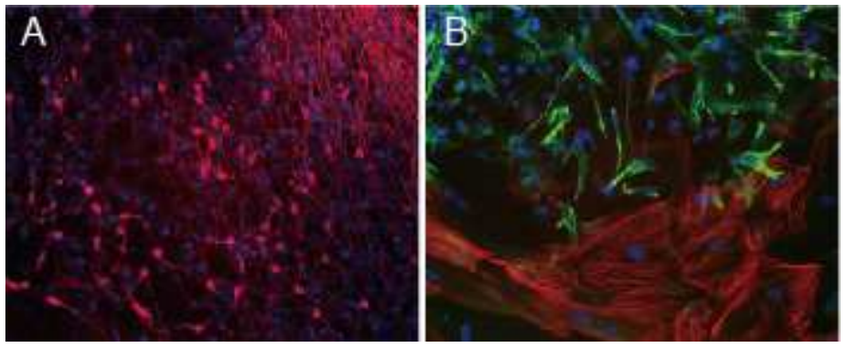


Fig. 3. Comparing the morphologies among the mouse, rat and human.
The mouse, rat and human morphologies of fibroblasts, iPS cells and ES cells. Mouse, rat and human iPS cell colonies are morphologically very similar to ES cell colonies. Mouse and at iPS and ES cell colonies are round shaped. On the other hand, human iPS and ES cell colonies are flat shaped and different for many properties compared to mouse and rat.

In 2007, Human iPS cells were established (Takahashi et al., 2007; Yu et al., 2007). This was only one year after the establishment of mouse iPS cells, which is remarkable considering that it took about 15 years to establish human ES cells after establishing mouse ES cells. The establishment of the human iPS cells was the result of the accumulation of knowledge regarding human ES cells and mouse iPS cell induction. Human iPS cells were established using two different combinations of reprogramming factors. Our group used Oct3/4, Sox2, and Klf4 with or without c-Myc. Another group used Oct3/4, Sox2, and Nanog, with or without Lin28 (Takahashi et al., 2007; Yu et al., 2007). Therefore, Oct3/4 and Sox2 were common between our combinations and the other group's combinations. Human iPS cells can differentiate into all 3 germ layers in vitro (Fig.4) and in vivo (Fig.5). Up to now, rat, monkey, pig, dog and rabbit iPS cells have been established, however, the germline transmission of these iPS cells has not yet been reported (Liu et al., 2008; Jing et al., 2008; Wenlin etal., 2008; Esteban et al.,2009; Zhao et al.,2009; Shimada et al,. 2009; Wu et al., 2010; Honda et al., 2010).



A - In vitro differentiation of human iPS cells into neurons (Red).
B - In vitro differentiation of human iPS cells into smooth (Red) and striated muscles (Green).
Fig. 4. Human iPS cells were differentiated in vitro.

Fig. 5. In vivo differentiation of human iPS cells.

Human iPS cells are transplanted into testes of immunocompromised mice. After about 10 weeks, human iPS cells form teratomas. The teratomas are analysed histologically with haematoxylin and eosin staining. iPS cells are differentiated into all three germ layers.

6. Increasing the efficiency of generating iPS cells

In the beginning of iPS cell research, the generation efficiency was very low, when Oct3/4, Sox2 and klf4 were used. It was found that the addition of c-Myc increased the efficiency more than 100-fold. Although c-Myc is not essential for iPS cell induction, it is a very effective factor for increasing the efficiency. The stability of c-Myc is regulated by the glycogen synthase kinase 3 (GSK3), which negatively regulates the Wnt pathway. Phosphorylated c-Myc is rapidly degraded by the ubiquitin-proteosome pathway. Therefore, Wnt may enhance the generation efficiency of iPS cells with Oct3/4, Sox2 and klf4 (Marson et al., 2008). However, precisely role of c-Myc during iPS cell induction is still unclear. Even when iPS cells were induced with c-Myc, the overall efficiency calculated from the number of potentially reprogrammable cells was less than 1%. Therefore, further improvements in the reprogramming efficiency were needed. There are three main ways to increase this efficiency: inducing iPS cells with the help of chemicals, adding more reprogramming factors, and changing the combination of reprogramming factors.

6.1 Increasing the efficiency with chemical compounds

6.1.1 Chemicals affecting DNA and histone modifications

DNA and histone modifications regulate the gene expression patterns in cells. These modifications stably maintain the gene expression pattern to ensure the proper characteristics of the cells. During iPS cell generation, these modifications are changed dramatically (Deng et al., 2009). One of DNA methyltransferase inhibitors, 5-azacytidine, improved the efficiency of reprogramming by nuclear transfer. Several inhibitors of DNA methyltransferase, such as 5-azacytidine, BIX-01294, RG108, etc. improved the efficiency of iPS cell generation. Inhibitors of histone deacetylases, for example valproic acid (VPA), butyrate, and trichostatin A, also increased the iPS cell generation efficiency. iPS cells could be induced using just Oct3/4 and Sox2 with VPA (Xu et al., 2008; Shi et al., 2008; Huangfu et al., 2008; Danwei et al., 2008; Mali et al., 2010; Zhou et al., 2010). It was thought that the effects of these chemicals which change DNA and histone modifications was due to inhibition of genes expressed in somatic cells and the induction of those expressed in ES cells. However, these chemicals have low specificity. They change the global DNA and histone modifications. Therefore, they inhibit not only the expression of genes which define somatic cells, but also those which are important for ES and iPS cells. As a result, if the concentration of these chemicals, the length of the treatment, or the original somatic cells are different, these chemicals may either have no effect or may even decrease the efficiency of iPS cell generation.

6.1.2 Chemicals affecting molecular signaling pathways

The inhibition of the Tgf-β (transforming growth factor- β) pathway increases the efficiency of mouse iPS cell generation. This inhibition is effective during the early stage of iPS cell induction. It is thought that the mechanism of Tgf- β inhibition is as follows: Fibroblasts are mesenchymal cells, while iPS cells are epithelial cells. Fibroblasts need to be converted to epithelial cells during iPS cell induction (Payman et al., 2010). The Tgf- β pathway accelerates the epithelial-to-mesenchymal transition. Therefore, the inhibition of the Tgf- β pathway improves the iPS cell generation efficiency by accelerating the mesenchymal-to-epithelial transition (Maherali et al., 2009). The combined inhibition of the MAPK pathway and the Tgf- β pathway has a synergistic effect (Tongxiang et al., 2009) to generate human iPS cells. Moreover, using just an Oct3/4 transgene, mouse iPS cells can be generated from

neonatal human epithelial keratinocytes with a combination of compounds including sodium butyrate (a histone deacetylase inhibitor), PS48 (an activator of 3′-phosphoinositide-dependent kinase-1), A-83-01 (a TGF-β inhibitor) and PD0325901 (a MAPK inhibitor) (Zhou et al., 2011). The use of 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP), a cyclic AMP analog, also improves human iPS cell induction efficiency (Wang & Adjaye, 2010). It is thought that 8-Br-cAMP exerts its pro-induction effect by decreasing the expression of p53 and increasing the expression of Cyclins.

6.2 Promoting the efficiency by adding more reprogramming factors

Suppressing p53 gene (TP53) expression enhances the efficiency of generating both mouse and human iPS cells (Kawamura et al., 2009; Rowland et al., 2009; Hong et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal Banito et al., 2009). The p21 gene is one of the p53 downstream targets. The p21 protein binds and inactivates the G1/S-cyclin dependent kinase (cdk) and S-Cdk complexes to stop the cell cycle. Overexpression of p21 negated the amplifying effect of p53 suppression during iPS cell transduction. Inhibition of the retinoblastoma protein (RB) also improves the efficiency of iPS cell generation. RB inhibits E2F, which accelerates the transcription of S-phase genes such as Cyclin E and Cyclin A. The complex of G1-cdk and Cyclin D1 phosphorylate RB. Phosphorylated RB cannot bind and inhibit E2F, allowing the cell cycle to progress from the G1 phase to the S phase. Cyclin D1 also increases the iPS cell generation efficiency. Rem2 GTPase is one of the suppressors of the p53 pathway. Rem2 is an important player to maintain human ES cells. Rem2 enhances the reprogramming by regulating p53 and cyclinD1 (Edel et al., 2010). These data suggest that accelerating cell proliferation enhances the iPS cell generation efficiency. Promoting cell proliferation accelerates the stochastic process of reprogramming. However it is thought that the amplifying effect of p53 inhibition does not only result from the acceleration of cell cycle. It is known that p53 directly binds to the promoter region of the Nanog gene and suppresses its expression in mouse ES cells (Sabapathy et al., 1997; Qin et al., 2007). There is a possibility that p53 directly regulates the gene expression pattern during iPS cell generation.

Lin28 is also effective for increasing the efficiency of reprogramming. Lin28 was used to generate some of the first human iPS cells. Like c-Myc, Lin28 is effective, but not essential, for the generation of human iPS cells. Lin28 is also effective in combination with Oct3/4, Sox2, Klf4 and c-Myc. Lin28 can interfere with the maturation of miRNA and promote their degradation by uridylation of miRNA (Heo et all., 2008 2009). Let-7 is one of the Lin28-associated miRNAs, and regulates the translation of several genes including c-Myc, K-Ras, Cyclin D1 and Hmga2. However, the mechanism(s) underlying the effects of Lin28 are still unclear (Kim et al., 2009; Viswanathan et al., 2009).

Tbx3 also improves the efficiency of mouse iPS cell generation. The association between Tbx3, Nanog and Tcf3 is important for pluripotency and self-renew of ES cells. Moreover, the efficiency of germline transmission of mouse iPS cells with Tbx-3 is higher than that with just Oct3/4, Sox2 and Klf4 (Jianyong et al ., 2010).

E-Cadherin also enhanced the mouse reprogramming efficiency in combination with Oct3/4, Sox2 and Klf4 or Oct3/4, Sox2, Klf4 and c-Myc. E-Cadherin is a molecule that mediates cell-cell interactions, and is upregulated during iPS cell induction. An antibody against the extracellular domain of E-cadherin reduced the efficiency of iPS cell generation. These data indicated that the cell-cell contact mediated by E-cadherin plays an important role in reprogramming (Chen et al., 2010).

In addition, some micro RNAs also enhance the efficiency of iPS cell generation. The mir-290 cluster is highly expressed in mouse ES cells. The efficiency of mouse iPS cell generation with Oct3/4, Sox2 and klf4 was improved by miR-291-3p, miR-294, miR-295, which are included in the cluster of mir-290. However, they are not effective with c-Myc. While c-Myc binds to the promoter of the mir-290 cluster, introducing c-Myc could not induce the expression of the mir-290 cluster in fibroblasts. The promoter of the mir-290 cluster is regulated negatively by histone modifications in fibroblasts. These data suggest that the mir-290 cluster is one of targets which are regulated by histone modification (Robert et al., 2009).

6.3 Promoting the efficiency by using different combinations of reprogramming factors

L-Myc is one of Myc family members. L-Myc is more effective for iPS cell induction than c-Myc. Moreover, mouse iPS cells established with L-Myc contribute to the germline more efficiently than iPS cells with c-Myc (Nakagawa et al., 2010).

Utf1 also improves the efficiency of mouse iPS cell generation. The number of mouse iPS colonies generated using a combination of Oct3/4, Sox2, klf4 and Utf1 was 10 times higher than that with Oct3/4, Sox2, Klf4 and c-Myc (Zhao et al., 2008).

Recently, both human and mouse iPS cells were established using just the miR-302/367 cluster in the absence of any other reprogramming factors. The miR-302/367 cluster is highly expressed in ES and iPS cells, and is one of the target of Oct3/4 and Sox2. The use of just the miR302/367 cluster reprogrammed both human and mouse cells more efficiently and rapidly than the combination of Oct3/4, Sox2, Klf4 and c-Myc (Anokye-Danso et al., 2011).

7. The methods for generating iPS cells

There are two main methods to generate iPS cells. These are the genomic integration method and the genomic integration-free method.

7.1 The Genomic integration method

7.1.1 Retrovirus systems

Retrovirus systems were used to generate the world's first mouse and human iPS cells (Takahashi et al., 2006, 2007). The reprogramming factors introduced by a retrovirus system are strongly and stably expressed in somatic cells. The retrovirus system can efficiently introduce several reprogramming factors into cells at the same time. For these reasons, a retrovirus system efficiently generates iPS cells. Therefore, retrovirus systems are suitable for investigating the mechanism of iPS cell induction. Moreover, reprogramming factors introduced into somatic cells by the retrovirus system are gradually silenced during the reprogramming progress (Okita et al., 2007). This is good for iPS cell generation, because the expression of reprogramming factors in reprogrammed cells sometimes induces differentiation and cell death. Moreover, expressing transgenic reprogramming factors into reprogrammed cells can induce tumorigenicity.

7.1.2 Lentivirus systems

Lentiviral vectors were also used to generate some of the first human iPS cells (Yu et al., 2007). Lentivirus can infect not only the dividing cells, but also non-dividing cells. Lentivirus infection therefore occurs independent of cell division. The reprogramming factors introduced by lentiviruses are stably expressed and less silenced than those

introduced by retroviruses. Thus, drug-inducible transgene expression systems were made because of these characteristics of lentiviral vectors to investigate the mechanism of iPS cell induction (Hockemeyer et al., 2008; Maherali et al., 2008).

7.2 Genomic integration-free method

The genes introduced by either retroviral or lentiviral vectors permanently integrate into the genome. Such integration increases the risk of tumorigenicity for two reasons: the first reason is that the integration can interrupt genes and gene promoters; the second reason is that the integrated factors could be reactivated unexpectedly by nearby promoters. For clinical applications, these issues will need to be overcome. Recently, two principal ways to generate iPS cells without genomic integration were developed. One of them is removing the genomic integration after establishing iPS cells. The other is establishing iPS cells without integration vectors.

7.2.1 Removing genomic integration after establishing iPS cells

Cre-mediated recombination can be used to remove transgenes from the iPS cell genome. Human iPS cells have been established using lentiviral constructs including loxP sequences in their long terminal repeat (LTR). Established iPS cells can be treated with Cre recombinase in order to excise the lentiviral cassettes. However, the LTR sequence still remains in the genome (Soldner et al., 2009).

A "piggybac" transposon vector system can also solve this problem. Using this system, the integrated reprogramming factor can be removed seamlessly. Transposase has activities for both the insertion and excision of transposon vectors by recognizing the TTAA tetra-nucleotide sequence in the host genome (Kaji et al., 2009 Woltjen et al., 2009 Yusa et al., 2009).

7.2.2 Generating iPS cells without genomic integration of reprogramming factors

The first integration-free iPS cells were generated from mouse somatic cells with adenoviral vectors or conventional expression vectors (Okita et al, 2008; Stadtfeld et al, 2008). Recently, episomal vectors were used to generate human iPS cells (Yu et al., 2009). Episomal vectors consist of the replication origin and an Epstein-Barr nuclear antigen (EBNA). The EBNA vector can self-replicate and maintain the expression of transgenic reprogramming factors without genomic integration. However, the efficiency of reprogramming with episomal vectors was 10 times less than that with integration vectors. However, the efficiency was recently improved using episomal vectors encoding Oct3/4, Sox2, Klf4, L-Myc, Lin28 and a short hairpin RNA against p53 (Okita et al., 2011). This method is very promising for clinical application because the possibility of episomal vector genomic integration is very low, although it is still not zero. There is a little possibility that this vector may accidentally integrate into the genome accidentally. This possibility should be kept in mind when planning trial for clinical application.

The Sendai RNA virus is also a promising vector that can be used to generate clinical-grade iPS cells. This virus does not enter into the nucleus for replication, transcription or translation. Therefore, there are no risks of insertion of reprogramming factors introduced by this virus. The transduction efficiency using this virus is comparable to that using retrovirus system. iPS cells were also established from less than 1 ml of peripheral blood using this system(Seki et al. 2010). If deficient sendai viral vectors are used for iPS induction, the vectors can be removed by siRNA (Nishimura et al., 2011).

There are also other ways to establish iPS cells virus-free. In one study, the Oct3/4, Sox2, Klf4 and c-Myc proteins were modified so that they could easily pass through the cell membrane. Both human and mouse iPS cells were established using these proteins (Hongyan et al., 2009; Dohoon et al., 2009). Another recent method used synthetic modified mRNA to generate iPS cells. RNA usually is unstable, and cells with foreign RNA are usually destroyed by the interferon response. The authors modified the medium and RNA to reduce the interferon response and improve RNA stability. The reprogramming factor from the introduced RNA was expressed stably and highly in the cells. Using this method, the possibility of genomic integration is very low, because of the nature of RNA. However, the possibility of cell damage in iPS cells generated using this method is slightly increased through the stressful induction method that requires consecutive introduction of RNA into cells for 2 weeks and artificial inhibition of the cell interferon response. A newer method to establish both human and mouse iPS cells used just miRNA, miR-200c, 302 and 369. These iPS cells were named mi-iPS cells (Miyoshi et al., 2011).
Regardless of the method used to generate iPS cells, the quality of the cells should be examined from various points of view and in depth before using the iPS cells for clinical applications.

8. Applications of iPS cells

The major benefit of iPS cells is that they make it possible to obtain differentiated cells in the required quantities. It is expected that iPS cells can be used for regenerative medicine and drug discovery (Fig. 6).

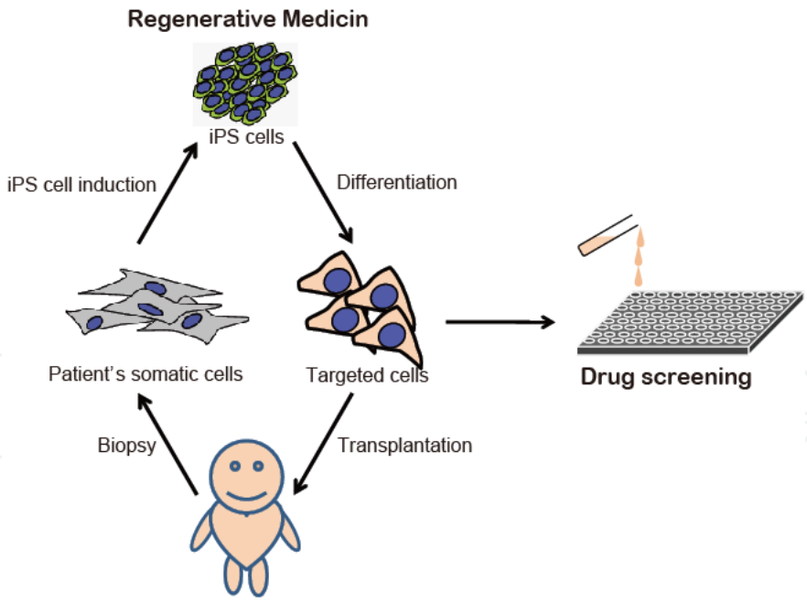


Fig. 6. A Schematic illustration for the application of iPS cells

iPS cells derived from patients are useful for regenerative medicine and drug discovery. Somatic cells are taken by biopsy from patients. Patient specific iPS cells are then established from the somatic cells and differentiated into targeted cells. If the targeted cells are transplanted into a disease site, then this would bethat is very promising for regenerative

medicine. Moreover, if disease phenotypes are reproduced using patient specific iPS cells in vitro, patient specific responses to drugs could be evaluated for individual therapies.

8.1 Using iPS cells for regenerative medicine

Differentiated cells from iPS cells derived from the recipients are not rejected by the immune system upon transplantation, because they have the same immune markers as the recipient. Hence, it is expected that it will be possible to use iPS cells for cell therapy and regenerative medicine. Under this scheme, iPS cells are differentiated into the targeted cells, and the differentiated cells are then implanted into the diseased area where they can improve the patient's symptoms. Experiment procedures utilizing lab animals have already proven the effectiveness of this scheme for cell therapy. For example, in a rat model of Parkinson's disease, the implantation of dopamine-producing neurons that were differentiated from iPS cells led to a clear improvement in the symptoms (Hargus et al., 2010).

It is also possible to use an approach which is a combination of cell therapy and gene therapy. Sickle cell anemia is a genetic blood disorder. The patient's red blood cells are abnormally sickle-shaped, thus decreasing the oxygen transport ability of these patients' red blood cells compared to unaffected individuals. This abnormality is caused by a mutation in one gene. The mutation was repaired using gene therapy technology in iPS cells derived from model mice. The repaired iPS cells were differentiated into hematopoietic stem cells. The hematopoietic stem cells transplanted into the model mice started to generate normal red blood cells and cure the disease (Hanna et al., 2007). The effectiveness of these procedures has not yet been examined in humans. However, Geron and Advanced Cell Technology announced that they plan to start clinical trials of transplantation of cells derived from ES cells for spinal cord injury and muscular degeneration, respectively. The current advances in the differentiation induction technology are likely to facilitate human studies. For example, the three dimensional structure of the neural retina differentiated from mouse ES cells was recently demonstrated (Eiraku et al., 2011). The combinations of the various differentiation technologies will likely provide new sources and methods for regenerative medicine.

8.2 Concerns about using iPS cells for regenerative medicine

Before using iPS cells for clinical applications, the safety of iPS cells should be sufficiently verified. In the paper introduced previously about curing Parkinson disease model mice, the authors suggested several problems that need to be overcome before this strategy can be clinically used. The major problem was that the model mice transplanted with the cells differentiated from iPS cells eventually developed teratomas (Hargus et al., 2010). The formation of teratomas in donor mice was caused by the undifferentiated cells that were present in the differentiated cells used for transplantation. It will therefore be necessary to develop an efficient differentiation system that allows for the invariably selection of targeted somatic cells and complete removal of all residual undifferentiated cells. In fact, attempts have already been made to select or generate iPS cells which can easily be differentiated into targeted cells. Recently, our group demonstrated that iPS cells have various differentiation potentials, and we found that several iPS cell clones were highly resistant to neural differentiation (Miura et al., 2009). Additional, studies to identify the genes responsible for the resistance are currently underway.

In addition, it was reported iPS cells carry epigenetic memory of the original somatic cells during early passages. This memory affects their differentiation potential. For example, iPS cells from B cells differentiated into blood progenitor cells more efficiently than iPS cells derived from fibroblasts (Kim et al., 2010; Polo et al., 2010). The origin of iPS cells was therefore reflected in the differentiation potential of the iPS cells. Further accumulation of this knowledge will help create smooth path toward the clinical application of iPS cells.

8.3 Using iPS cells to understand pathological conditions and for drug discovery

Utilizing the advantages provided by the iPS cell technology, differentiated cells which are difficult to harvest from patients and culture in vitro can now be obtained in sufficient quantities for researchers to study the pathogenesis of diseases and to perform drug screening.

The first disease-specific iPS cells were established from patients with familial amyotrophic lateral sclerosis (ALS). However, the authors could not reproduce disease phenotypes using differentiated cells from ALS-iPS cells in vitro (Dimos et al., 2008). The first in vitro reproduction of a disease phenotype was achieved with iPS cells derived from a spinal muscular atrophy (SMA) patient. The motor neurons differentiated from SMA iPS cells exhibited the specific phenotype, such as a decreased number and size of neurons (Ebert et al., 2009). Recently, many disease models have been generated in vitro with iPS cells from patients with Familial dysautonomia (FD), myeloproliferative disorders, Dyskeratosis congenital, Leopard syndrome, Rett syndrome (REFS) and further diseases (Lee et al., 2009; Ye et al., 2009; Suneet et al., 2010; Carvajal et al., 2010; Marchetto et al., 2010).

The effects and side effects of drugs are generally tested using laboratory animals, primarily rodents. However, the effects of drugs are different between humans and animals, and such studies were one of obstacle to developing new therapeutic agents. Moreover, using laboratory animals is cost- and time-intensive. The ability to test new agents on specific types of cells will greatly facilitate research on drug effects and toxicity.

It was previously very difficult to collect sufficient amounts of targeted cells from patients for analyses until the iPS cell breakthrough. Many disease models using iPS cells will likely be established in the near future, because the safety of iPS cells (with regard to teratoma formation) is not an issue affecting basic research involving these cells. Therefore, iPS cell technologies will greatly facilitate our understanding of the pathogenesis of various diseases and will help in the development of novel treatments.

9. Conclusion

About sixty years ago, humans started to deeply and systematically investigate living things from a molecular point of view. The major purposes were to achieve a better understanding of the basic function of living things and to try to regulate and use these findings to enhance human lives. The biological systems improved by nature for several billion years are much more efficient than the engineered systems developed by humans. For example, fireflies emit thermal free light while producing their fluorescence, while electric lights produce heat. This indicates that biological systems are very efficient. Understanding and using these biological systems can therefore have a major impact on the quality of human life.

The development of iPS cells is a prime example of using such biological systems for human benefit. The development of iPS cells has demonstrated that the characteristics of differentiated cells could be changed artificially by employing appropriate factors and

methods. Recently, new direct reprogramming technologies have been developed, which allowed somatic cells to be directly reprogrammed into targeted somatic cells without involving iPS cells.

However, precisely what occurs during iPS cell inductionstill remains unclear. Human fertilized eggs differentiate into somatic cells for several months in the mother's womb. During the induction of iPS cells, the somatic cells are artificially induced to regress into pluripotent stem cells within just a few weeks. There is a possibility that abnormalities are accumulated in iPS cells due to artificial reprogramming stresses. It will be necessary to uncover the full mechanism of iPS cell induction, and many questions remain to be answered, including: Exactly what is happening during iPS cell induction? Can abnormalities of cells be caused by what is happening during iPS induction? Moreover, it is also important to evaluate the established iPS cells in comparison to ES cells. Such research will help pave the way for iPS cells to move from a scientific finding to a medical revolution.

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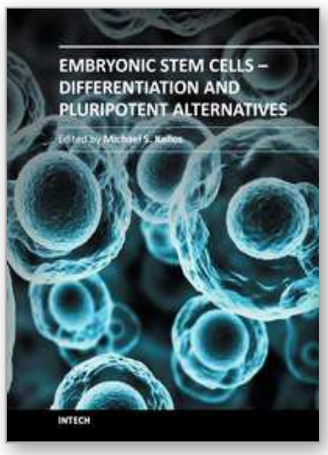

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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