Pluripotent Stem Cells

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The Winding Road to Pluripotency (Nobel Lecture)**

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Introduction

Dr. John Gurdon received recognition for his landmark achievement in 1962, which provided the first experimental evidence of reprogramming by the transplantation of amphibian somatic cell nuclei into enucleated oocytes.[1] This breakthrough in technology introduced a new paradigm: that each nucleus of a differentiated cell retains a complete set of blueprints for the whole body, while oocytes possess a certain potential for reprogramming.

Inspired by this paradigm shift and subsequent research achievements, we identified four transcription factors that could induce pluripotency in somatic cells by their forced expression, and successfully consolidated effective reprogramming methods in mouse cells in 2006[2] and in human cells in 2007.[3] The established reprogrammed cells were named "induced pluripotent stem (iPS) cells". I would like to provide an overview, focusing on the experimental background of the generation of iPS cells and the future perspectives regarding iPS cell research, which has been developing rapidly.

My Early Days As a Scientist

I graduated from Kobe University, School of Medicine, Japan, and obtained my medical license in 1987. I decided to become an orthopedic surgeon and started my training as a resident in Osaka, Japan. During my school days, I had practiced judo and played rugby, and injured myself many times, including more than 10 fractures throughout my body. It was thus natural for me to have become interested in orthopedic surgery. I especially wanted to treat patients suffering from sport injuries and overtraining.

In 1989, however, my life took a new turn from clinical medicine in orthopedic surgery to basic science research for two reasons. First, I found that I was not a very talented surgeon. Second, I saw many patients suffering from intractable diseases and injuries, which even highly talented surgeons and physicians were not able to cure. For example, I had encountered patients suffering from spinal cord injuries, amyotrophic lateral sclerosis, and osteosarcomas. Furthermore, I lost my father due to liver cirrhosis during my residency. Basic medical research is the only way to find cures for these patients. For these reasons, I decided to go back to school. I became a PhD student at Osaka City University Medical School in April of 1989.

Among the many departments at the school, I applied to the Department of Pharmacology, directed by Dr. Kenjiro Yamamoto. At the interview, I was not able to answer many questions about pharmacology, because I had not studied pharmacology well enough when I was a medical student. Instead, I tried to convince the interviewer, Dr. Fumio Ikemoto, that I really wanted to do basic medical research, despite my lack of knowledge. I am so grateful that Dr. Ikemoto accepted me into the department. Dr. Ikemoto repeatedly told me that we should not perform research that simply reproduced somebody else's results. Rather, we should do something unique and new. During my training as a scientist, I was very fortunate to have two types of teachers: namely, great mentors and unexpected results from my experiments.

My direct mentor at the graduate school was Dr. Katsuyuki Miura. In my first few months as a PhD student, Dr. Miura told me to read as many manuscripts as possible and propose new projects. I felt like I was given a blank canvas and told that I could draw whatever I wanted. This mentorship was very different from what I had experienced during my residency. At the hospital, I'd had little freedom, and had to follow instructions from senior physicians and textbooks. I thought "wow, I like this system!" Another thing that Dr. Miura often told me was that we were competing worldwide. Whatever project you choose, you will compete with other scientists throughout the world, mostly in the USA or Europe, on the same or similar projects. This was again very different from my experience at the hospital, where I was competing only with other residents at the same hospital. The idea of "worldwide" competition had never entered my mind when I was working at the hospital. For all of these reasons, I found that basic research was a more suitable career, based on my interests and temperament.

In the summer of 1989, I was still struggling to find my project. Dr. Miura proposed a simpler project to begin my research studies. He suggested that I examine the role of a vasoactive molecule, platelet activating factor (PAF), in dogs to study the regulation of blood pressure (Figure 1). Because it was known that the intravenous injection of PAF

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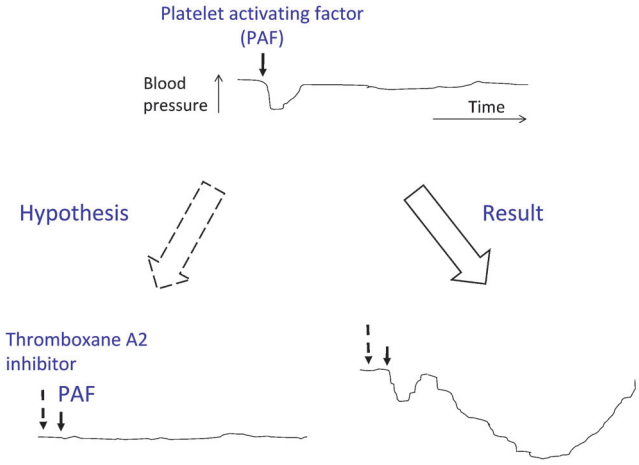

Figure 1. My first experiment as a graduate student. Intravenous injection of a vasoactive molecule platelet activating factor (PAF) caused a transient decrease in blood pressure in dogs (top). We hypothesized that this hypotension would be blocked by pretreatment with a thromboxane A2 inhibitor (bottom left). Unexpectedly, we observed a profound hypotension (bottom right).

into dogs caused a transient decrease in blood pressure (transient hypotension), Dr. Miura hypothesized that this decrease in blood pressure would be mediated by another vasoactive molecule, thromboxane A2. If that hypothesis was correct, then pretreatment with a thromboxane A2 inhibitor should block the PAF-induced transient decrease in blood pressure. My first experiment, where I treated dogs with an inhibitor of thromboxane A2, was performed based on his hypothesis, and I had expected no decrease in the blood pressure in the pretreated dogs. It should have been a simple experiment suitable for a beginner. However, the result was totally unexpected. In the beginning, the thromboxane A2 inhibitor did not seem to be effective, with subsequent PAF treatment inducing the normal transient decrease in the blood pressure. Surprisingly, however, a few minutes after the treatment, a profound and prolonged decrease in blood pressure was observed, which we had never observed following treatment with PAF alone (Figure 1). I got so excited! I ran into Dr. Miura's office to report this result excitedly. Although the result did not support his hypothesis, Dr. Miura responded with excitement too, and encouraged me to explore the finding further. I spent another two years uncovering the mechanism responsible for this unexpected result.[4,5] I was extremely lucky to obtain this kind of unexpected result in my very first experiment as a graduate student.

The fact that I got very excited with the result clearly told me that I had found the correct career. During my thesis work, however, I was often frustrated by my scientific approach, which relied on pharmacological tools, such as inhibitors and agonists. No drug can be 100% specific or effective, so there are always nonspecific activities or incomplete blockade of the targets. In contrast, I was fascinated by the emerging gene engineering technologies being demonstrated in mice, especially the knockout mouse technology, by which any gene of interest could be deleted with 100% specificity and efficacy. There were a few groups in Japan who brought the technology from the USA or Europe to their pharmacological studies. This technology seemed like a miracle to me. I really wanted to utilize the knockout mouse technology in my own research. Therefore, in order to learn about the genetic engineering of mice, I decided to become a postdoctoral fellow in the USA, where the technology was being widely used in many laboratories. I checked advertisements in journals such as Nature, Science, and Cell, and applied to as many laboratories as possible. The very first person who replied to my application was Dr. Thomas (Tom) Innerarity at the Gladstone Institute of Cardiovascular Disease, San Francisco (Figure 2). After a short interview by telephone, Tom offered me a position. In April 1993, I crossed the Pacific Ocean with my wife and two little daughters.

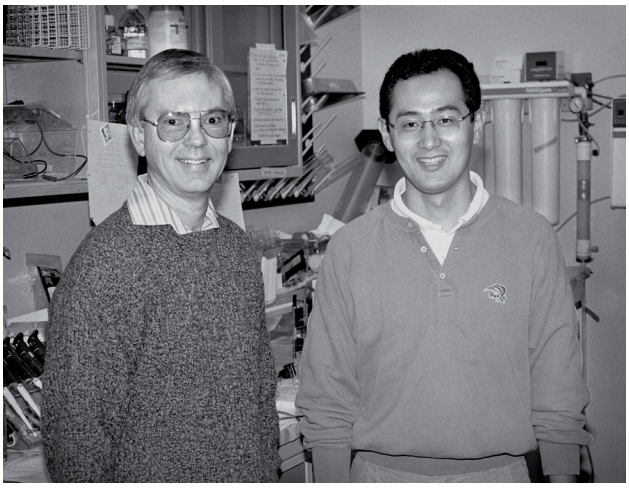

Figure 2. Days at the Gladstone Institute as a postdoctoral fellow. A picture taken with Dr. Thomas Innerarity in his laboratory (1995, Gladstone Institutes).

In Tom's laboratory, I stared working on the mechanisms underlying the gene expression of Apo B (apolipoprotein B), which is a constituent of LDL (low-density lipoprotein) and thought to be important in cholesterol regulation. In particular, we focused on an mRNA editing factor, APOBEC1 (Apo B mRNA editing catalytic subunit 1) to analyze its gene function. Our original objective was to explore the possibility of using gene therapy for familial hypercholesterolemia to prevent atherosclerosis. Tom hypothesized that the overexpression of APOBEC1 in the liver would lower the plasma cholesterol levels, and he planned to examine this possibility using transgenic mice that overexpressed APOBEC1 in a liver-specific manner. I worked very hard and was able to quickly generate transgenic mouse lines. However, we observed totally unexpected results. We found that there was abdominal expansion of transgenic mice as if they were pregnant, regardless of whether they were male or female. An exceptionally high incidence of liver tumors in these mice was confirmed by autopsy (Figure 3).[6] It turned out that APOBEC1 is a very potent oncogene. Therefore, we can never use this gene for gene therapy. Again, I got very excited

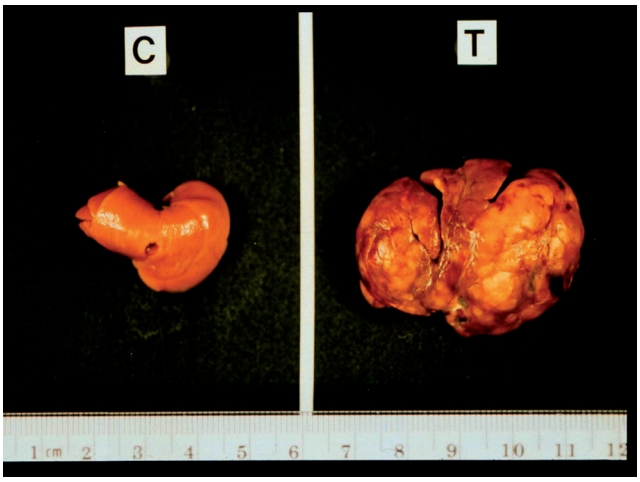

Figure 3. Hepatocellular carcinoma in APOBEC-1 transgenic mouse. Livers from nontransgenic control mouse (left) and transgenic mouse (right).[6] (Copyright 1995, the National Academy of Sciences.)

about this unexpected result, although it indicated that APOBEC1 could not be used to prevent atherosclerosis, thereby effectively halting my previous line of research. Although it contradicted his hypothesis, Dr. Innerarity kept supporting me while I decided to work on liver cancer, showing a similar excitement as Dr. Miura did in response to the unexpected results. He encouraged me to keep working on APOBEC1 to elucidate the mechanism by which it led to cancer formation. I became the only person who worked on liver cancer at the Gladstone Institute of Cardiovascular Diseases.

I met another person who ended up being very important in my life at Gladstone. It was the then president of the institute, Dr. Robert Mahley. He once told us (postdoctoral fellows) about how to become successful in science. He said the secret was "VW". He had and still has a Volkswagen car, but in this case, VW did not mean Volkswagen. Instead, he meant vision and hard work. Dr. Mahley told us to have a clear vision and then work hard toward that goal. I was working very hard day and night. However, I realized I did not have a clear vision. Why am I doing many of my experiments? Why did I quit working at hospitals to become a postdoctoral fellow in the USA? I then realized that my vision or motivation for doing science was to contribute to patient health and longevity. Of course, we cannot immediately help any patients by doing experiments in laboratories. However, basic medical research has the potential to help thousands of patients suffering from intractable diseases and injuries.

As noted above, I very was fortunate to have two types of great teachers in my early days as a scientist. First, my mentors, including Dr. Miura, Dr. Innerarity, and Dr. Mahley, encouraged me to continue my projects despite the fact that the results contradicted their hypotheses. They served as models of good mentors for me to follow. The other great teacher was nature itself, which gave me totally unexpected results that led me to completely new research themes. Without these two types of great "teachers", I could have never initiated my research that led to the generation of iPS cells.

The Research that Led to the Production of iPS Cells

What brought me to cell reprogramming biology was another set of totally unexpected experimental results and an encounter with ES (embryonic stem) cells through my research experience, along with related scientific streams. While trying to elucidate the molecular mechanisms underlying the carcinogenesis induced by APOBEC1, I discovered an interesting molecule which was a novel target of APOBEC1. I named this molecule NAT1 (Novel APOBEC1 Target #1).[7] We found that the overexpression of APOBEC1 resulted in aberrant editing at numerous sites of the NAT1 mRNA, including those that generated premature termination codons. Consequently, the NAT1 protein levels were markedly reduced. I also found that the NAT1 protein was similar to eukaryotic translation initiation factor (eIF) 4G, and likely functioned as a translational regulator. Based on these observations, I hypothesized that NAT1 could function as a tumor suppressor gene. In order to examine that hypothesis, I decided to generate NAT1 knockout mice. At that time, I had learned the skills necessary for generating knockout mice, including the preparation of targeting vectors and cultivation of mouse ES cells from Dr. Robert Farese, Jr., a friend of mine who established the ES cell and knockout core laboratory at Gladstone. This was how I first encountered ES cells.

Again I worked very hard. Three years had passed since I joined Gladstone, and my wife had to go back to Japan because we decided to send our elder daughter to an elementary school in Japan. After my family left San Francisco, I worked even harder. I really wanted to find out the function of NAT1, the gene I had identified myself. Without my family at home, I did not have anything else to do. I literally worked day and night. I generated a targeting vector quickly and was able to obtain targeted ES cell clones. I asked the knockout core laboratory to inject the targeted ES cells into mouse blastocysts to generate chimeric mice. I was happy about the scientific progress I was making.

At the same time, however, I felt lonelier and lonelier without my family. The only way to live with my family was to go back to Japan. However, I was unable to find a good position in Japan. Fortunately, I obtained a fellowship from the Japanese Government and decided to go back to Japan for a second postdoctoral fellowship. Owing to the invaluable help and generosity of Dr. Innerarity, I was able to continue the research on NAT1 after I returned to Japan at Osaka City University Medical School. The following year, I became an assistant professor and continued working on NAT1.

The generation of the NAT1 knockout mice went smoothly. We obtained good chimeric mice, and subsequently, F1 heterozygous mutant mice. Tom sent those F1 mice to me. However, I could not obtain homozygous mutant mice by intercrossing the heterozygous mutants, thus suggesting that NAT1 was indispensable for mouse development. I then

struggled with the analyses of mutant embryos, since neither I nor anyone around me had ever worked on mouse embryogenesis. I learned how to dissect embryos by reading textbooks. With great encouragement from my colleagues, including Dr. Katsuyuki Miura, I finally showed that NAT1 mutant embryos died around the time of implantation.

In order to further characterize the functions of NAT1, I generated homozygous deletion mutant ES cells. I found that NAT1-null ES cells proliferated normally when they were maintained on undifferentiated feeder cells. However, when they were cultured without feeder cells or leukemia inhibitory factor, I observed marked differences. Under these conditions, the wild-type ES cells rapidly differentiated in terms of their morphology and gene expression. In contrast, mutant ES cells showed resistance to differentiation. This meant that NAT1 is essential for maintaining the pluripotency of ES cells.[8] This was the pivotal moment when ES cells became my main research subject. My future career developed from merely a research tool (knockout mouse construction), thanks to the unexpected results of the NAT1 functional analysis. This unexpected result changed my project again—from cancer to ES cells.

Mammalian ES cells were first derived from mouse embryos in 1981 by Dr. Martin Evans, and also by Dr. Gail R. Martin. ES cells have two important properties.[9,10] The first is their rapid proliferation, which can be considered to provide them with immortality. The other important property is pluripotency, the ability to differentiate into virtually all types of somatic and germ cells that exist in the body. NAT1 is essential for pluripotency, but not for the rapid proliferation of mouse ES cells. Because of the important role of NAT1, I became very interested in the biology of ES cells.

Although I was obtaining important results about the molecular functions of NAT1, I started to become frustrated and wondered whether my basic research could eventually contribute to clinical medicine, which was my true goal. I was working at the medical school, where most of my colleagues participated in medical research projects, such as drug development or for understanding the pathophysiology of diseases. My vision was (and still is) to contribute to the lives of patients through basic research, but I was not sure whether working on NAT1 and mouse ES cells could realize my vision. At that time, my colleagues often told me that, "Shinya, those mouse cells may be interesting, but you should do something more closely related to human disease and human medicine." Very luckily, however, two events happened in the late 1990s, which encouraged me to continue working on ES cells.

The first event was the generation of human ES cells by Dr. James Thomson at the University of Wisconsin in 1998.[11] Immediately after this paper was published, the ES cell research field began to draw public attention because of their potential value in regenerative medicine. Human ES cells have the two same properties as mouse ES cells: rapid proliferation and pluripotency. Human ES cells can be expanded indefinitely, and they can be used to generate various types of human somatic cells, such as dopaminergic neurons, neural stem cells, cardiac cells, and so on. These human cells should then be able to be used to treat patients suffering from various diseases and injuries, such as Parkinson's disease, spinal cord injuries, etc. So again, it turned out that ES cells themselves could help patients, not mouse patients, but human patients. When I first read the landmark paper by Dr. Thomson, I got very excited—I still remember that moment. However, the generation and use of human ES cells is associated with an ethical obstacle regarding the use of human embryos. In Japan, we were not allowed to use human ES cells. Thus, human ES cell research was a distant and forbidden world to me then.

The other event that encouraged me was my promotion. I had a chance to organize my own laboratory. In 1999, I moved to the Nara Institute of Science and Technology (NAIST) as an associate professor. In 1998, I found an advertisement for that position in a Japanese scientific magazine. It said that the institute was seeking a scientist at the associate professor level who would run his or her own laboratory while organizing a core facility for mouse genetic engineering for the institute. It seemed a perfect position for me, and I decided to apply for the position. I did not expect to be chosen, since I had only published a few papers using the technology. Surprisingly, however, I was provided an opportunity to give a job seminar at the institute, and more surprisingly, I got the position!

In December of that year, I entered through the main gate of the NAIST with excitement and nervousness. The NAIST is one of only a few national universities that only have graduate schools in Japan (most also have medical and dental schools). It has a beautiful campus, good equipment, talented faculty members, and, most importantly, highly motivated and brilliant graduate students. My frustration disappeared unconsciously. Because of these two events, the generation of human ES cells and my promotion to associate professor at the NAIST, I was able to continue my research on ES cells.

Then the word "VW" came to my mind again. Now that I was starting my own laboratory, I decided that I needed to have a clear vision or long term goal that I was going to share with future lab members. Many laboratories working on ES cells, including those famous in the field, were working on in vitro directed differentiation of cells into various lineages, such as cardiac myocytes and neuronal cells. I did not think it would be wise to compete with these laboratories, since my lab was very small and new. I decided to do the opposite. I decided that the goal of my laboratory would be to establish ES cell-like pluripotent stem cells that were not derived from embryos, but from differentiated somatic cells. By achieving this goal, we would be able to overcome the obstacles facing the development of medicine using human ES cells, namely, the use of human embryos and immune rejection after transplantation. I thus started trying to reprogram somatic cells back into the embryonic state.

I knew reprogramming was possible, at least in theory. Somatic cell reprogramming techniques had been developed by several groups. For example, Dr. Ian Wilmut succeeded in generating a first cloned mammal, "Dolly" the sheep, by transplanting the nucleus of a fully developed cell into an enucleated egg.[12] However, the efficiency of the method was extremely low. In addition, it had been pointed out that this system was technically quite difficult to apply for primates, including humans. Another example was the cell fusion technique between ES cells and somatic cells to attain

pluripotency.[13] However, the resultant fused cells did not seem to be suitable for application in a clinical setting due to the generation of tetraploid cells. However, the fact that somatic cells were able to attain pluripotency following nuclear transplantation or fusion with ES cells provided a lot of scientific encouragement, because it led us to hypothesize that oocytes or ES cells contain intrinsic factors that can reprogram somatic cells into a pluripotent state.

In addition to this background, there were also other discoveries of master transcription factors involved in vertebrate development, such as antennapedia in the fly[14] or MyoD in the mouse.[15] From these findings, it was a simple logical step to deduce that a combination of factors should be able to induce pluripotency in somatic cells. I just did not know which or how many factors were required. When we first started our research, it could have been one, several, one hundred, or even more, and we thought at that time that the project would take 10, 20, 30 years, or even longer to complete.

I hypothesized that many of the reprograming factors are expressed predominantly in eggs and ES cells. In order to search for factors that are specifically expressed in ES cells, we planned to utilize an EST (expressed sequence tag) database, which is a kind of catalog of genes that are expressed in each tissue or organ, obtained by random sequencing of cDNA libraries made from tissues or organs in various species. In a timely fashion, large quantities of mouse EST data were disclosed by RIKEN.[16] Furthermore, a program that analyzed EST databases to predict the expression pattern of each gene became available from the National Center for Biotechnology Information (NCBI).[17] I utilized this program, designated an in silico differential display, to compare EST libraries from mouse ES cells and those from various somatic tissues. I was immediately able to identify multiple genes that were highly and specifically expressed in undifferentiated mouse ES cells and early embryos. Among them, we particularly focused on the genes with the highest enrichment. These included well-known genes, such as Oct3/4,[18,19] Utf1,[20] and Rex1,[21] which had been experimentally identified as ES cell specific markers. This confirmed the usefulness of this approach. We designated other ES cell enriched genes "ES cell associated transcripts" (ECATs). We confirmed the ES cell specific expression of ECATs by performing northern blot analyses.[22–24]

I characterized the functions of ECATs in ES cells and mice with the new members who had joined my lab, including three graduate students, Eiko Kaiho, Yoshimi Tokuzawa, and Kazutoshi Takahashi. I was lucky to have these talented and hard-working students with me from the beginning. Furthermore, I was very fortunate to have Tomoko Ichisaka in my lab, as a technical staff member of the core facility for mouse molecular engineering. I believe that Tomoko is one of the best technicians in terms of the manipulation of mouse embryos in Japan, and maybe in the world. Thanks to Tomoko and the core facility, we were able to generate knockout mice to examine many ECATs.

The first gene we knocked out in mice at the NAIST was ECAT3, also known as Fbox15. These mice were part of Yoshimi Tokuzawa's project. Yoshimi, Tomoko, and I were very happy when we obtained the first targeted ES line, the first chimeric mice, and then germ-line transmission. However, when we generated homozygous mutant mice lacking the functional ECAT3 gene, we did not observe any obvious phenotypes.[22] Because of its specific expression in mouse ES cells and embryos, we expected that its disruption would result in early lethality during embryogenesis. Furthermore, we showed that Fbox15 is a direct target of Oct3/4 and Sox2, another transcription factor essential for the maintenance of pluripotency.[25] On the contrary, we obtained homozygous mutant mice in accordance with the Mendelian law from heterozygous intercrosses. Yoshimi then generated homozygous mutant ES cell lines, hoping that she would observe drastic phenotypes. However, again, we did not see any significant changes. ECAT3-null ES cells proliferated normally and showed normal differentiation potentials. Thus, both the ECAT3 knockout mice and ES cells were apparently normal. This often happened with other ECATs. These experiences reminded us that science is often tough.

An exception was ECAT4, a transcription factor that was later renamed Nanog. We and others found that Nanog played important roles in the maintenance of pluripotency in mouse ES cells.[24,26] Nanog was also essential for mouse embryonic development before implantation. In addition to Nanog, Yoshimi Tokuzawa identified another transcription factor, Klf4, that played important roles in mouse ES cells. Another group reported the important role of the well-known oncogene, c-Myc, in pluripotency. By 2004, we had identified a total of 24 factors, including Oct3/4, Sox2, c-Myc, Nanog, other ECATs, and Klf4, as candidate reprogramming factors.

What we then needed was a sensitive and rapid assay system to screen these candidate factors. It turned out that the Fbox15-null knockout mice provided such an assay system. When we made knockout mice of Fbox-15 and other ECATs, we utilized a gene trap strategy, in which we knocked the neomycin resistance gene into the gene of interest. Thus, in ECAT3 knockout cells, the neomycin resistance gene is expressed from the enhancer and promoter of ECAT3, which was active only in ES cells and early embryos, but not in somatic cells. Somatic cells, such as mouse embryonic fibroblasts (MEFs) derived from the ECAT3 knockout mice are sensitive to G418, whereas ECAT3 knockout ES cells were resistant to high concentrations of G418. Based on these results, we expected that if any of the 24 candidates could actually induce pluripotency in ECAT3 knockout MEFs, the reprogrammed cells would become resistant to G418. We confirmed this strategy by using a fusion reprogramming system. The ECAT3 knockout mice that showed few phenotypes and thus disappointed Yoshimi and me turned out to provide a very useful assay system to evaluate candidate reprogramming factors.

My lab moved to Kyoto University in 2005, with the 24 gene candidates, the ECAT3-based assay system, and Tomoko Ichisaka and Kazutoshi Takahashi. In Kyoto, I asked Kazutoshi Takahashi to examine the 24 factors by using the assay system.[2] To tell the truth, we did not expect that we had the answer among these 24 factors. We thought we had to screen many more factors, and had already started to prepare cDNA libraries from mouse ES cells and testes.

Nevertheless, Kazutoshi introduced each of the 24 candidate genes, one by one, into ECAT3 knockout MEFs by retroviral transduction. As, in a sense, expected, we did not obtain any drug-resistant colonies using any single factor, thus indicating that no single candidate gene was sufficient to elicit reprograming and induce pluripotency. In addition to the single factor transduction, Kazutoshi proposed to transduce all 24 factors together into ECAT3 knockout MEFs as a practice for performing a cDNA library screening. It was like a mini library consisting of 24 cDNAs. To our surprise, four weeks after transduction, we obtained a several G418-resistant colonies. I thought this might be some kind of mistake, such as contamination with ES cells. I asked Kazuthoshi to repeat the experiment again and again. It always worked. Kazutoshi picked up the G418-resistant colonies for expansion. We found that these cells were expandable and showed a morphology similar to that of mouse ES cells. A reverse transcription PCR (RT-PCR) analysis revealed that the iPS-MEF24 clones expressed ES cell markers, including Oct3/4, Nanog, E-Ras, Cripto, Dax1, Zfp296,[24] and Fgf4.[27]

Next, to determine which of the 24 candidates were critical, Kazutoshi examined the effects of withdrawal of individual factors from the pool of transduced candidate genes. ES cell-like colonies did not form when either Oct3/4 or Klf4 was removed. The removal of Sox2 resulted in only a few ES-like colonies. When he removed c-Myc, the ES cell-like colonies did emerge, but these had a flatter, non-ES-cell-like morphology. Removal of the remaining factors did not significantly affect the colony numbers or characteristics. We finally showed that a combination of four genes, Oct3/4, Klf4, Sox2, and c-Myc was sufficient to produce ES cell-like colonies. These data demonstrated that pluripotency could be induced from MEF culture by the introduction of four transcription factors: Oct3/4, Sox2, c-Myc, and Klf4. I designated the new pluripotent stem cells "iPS cells", short for induced pluripotent stem cells.

We examined the pluripotency of iPS cells by the teratoma formation assay in animals. We obtained tumors from iPS cells after subcutaneous injection into nude mice. A histological examination revealed that the iPS cells differentiated into all three germ layers, including neural tissues, cartilage, and columnar epithelium. We also examined the ability of iPS cells to produce adult chimeras. We injected iPS cells into mouse-derived blastocysts, which we then transplanted into the uteri of pseudo-pregnant mice. We obtained adult chimeras from those injected iPS cells, as determined by the coat color of the resulting pups. From these chimeras, we were able to obtain F1 mice through germline transmission. Based on these results, we concluded that iPS cells are comparable to ES cells in terms of their pluripotency.[28]

The following year, we reported the generation of iPS cells from human fibroblasts using the same factors.[3] In the case of ES cells, it took 17 years to move from the mouse to human cells. This was in part because, although mouse ES cells and human ES cells share many similar features, they are very different in many aspects, including the culture conditions and morphology. In the case of iPS cells, it took much less time. This was because we already knew both how to culture human pluripotent stem cells and what they should look like. In other words, we could have never generated human iPS cells without the previous reports on human ES cells.

The generation of iPS cells was an exceptional experience in my scientific career, in that everything went smoothly. In all other cases, my career has been full of failures. This luck resulted from the dedicated work of young researchers, especially three people, Kazutoshi Takahashi, Yoshimi Tokuzawa, and Tomoko Ichisaka. It was these three who generated the iPS cells. Without these three young lab members, we could never have generated the iPS cells in my laboratory. Therefore, I am extremely grateful to these three and the other members of my lab for their tireless efforts.

When I initiated my basic research 25 years ago, I did not imagine at all that I was going to work on stem cells in the future. It was the unexpected results from PAF, APOBEC1 transgenic mice, and the NAT1 knockout mice that brought me to the new field. The encouragement from my mentors, including Drs. Yamamoto, Miura, Innerarity, and Mahley was essential for me to continue my work as a scientist. I am grateful to my two types of teachers: these mentors and nature itself.

The Potential Applications of iPS Cells

One of the advantages of the iPS cell technology is its simplicity and reproducibility. We can now generate human iPS cells not only from skin fibroblasts, but also from other somatic cells, including peripheral blood cells. Hundreds of laboratories all over the world are now working on iPS cells, trying to apply the technology in medicine and in the pharmaceutical industry (Figure 4). Without the iPS cell technology, it would be difficult to obtain sufficient numbers of somatic cells, such as heart cells or brain cells, from patients suffering from diseases affecting the heart or brain. With the iPS cell technology, all that is needed is a tiny amount of blood cells from the patients. We can then generate iPS cells, expand the cells as much as we want, and then make heart cells or brain cells to specifically study the affected tissues. These cells have the same genetic information as the patients and will

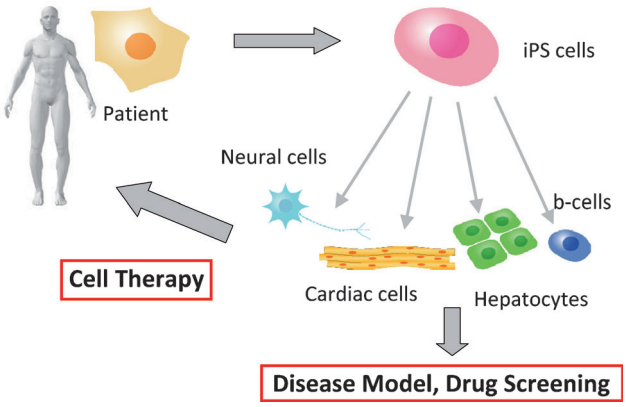

Figure 4. Potential applications of iPS cells.

provide unprecedented opportunities for predicting the toxicity of drugs, making disease models in petri dishes, performing drug screening, and for cell transplantation therapies.

Many effective drugs have been retracted from the market because of side effects such as cardiac arrhythmia and liver toxicity. The best known cardiac toxicity is long QT syndrome, as characterized by prolonged intervals between the Q and T waves in electrocardiograms, which often cause lethal arrhythmia. Since human heart cells are hard to obtain, pharmaceutical companies have been using noncardiac cancer cell lines, into which only one cardiac gene has been transfected, to predict the development of long QT syndrome and other types of cardiac toxicity of their drug candidates. However, this artificial system suffers from both false-positive and false-negative results. Now, human cardiac myocytes derived from iPS cells are commercially available from multiple companies. Pharmaceutical companies are now beginning to use these cells to predict cardiac toxicity. Similar approaches can be taken to predict the side effects in the liver and brain. These new types of medical application of the iPS cell technology are considered to be just around the corner.

I believe that the biggest potential of this technology resides in disease modeling and drug screening. Hundreds of diseases can be studied this way. Progress has been made in modeling intractable diseases while searching for new drugs with patient-derived iPS cells by many groups all over the world. To my surprise, it has been shown that iPS cells can be used to recapitulate the phenotypes not only of monogenic diseases, but also some late-onset polygenic diseases, such as ALS (amyotrophic lateral sclerosis) and Alzheimer's disease.[29,30]

ALS is a late-onset motor neuron disease. Most cases of ALS are sporadic, and are not caused by a mutation in a single gene. It has been more than 100 years since this disease was first recognized. However, there is still no effective treatment, despite numerous scientific efforts. In many diseases, animal models have been useful to understand the mechanisms and identify effective drugs. In the case of ALS, animal models do exist and many drugs have been developed that are effective on those animal models. However, the same drugs are not effective in human ALS patients. Therefore, drug screening for ALS needs to be conducted with human cells, but it has been difficult to obtain sufficient numbers of motor neurons and other affected cells from patients.

Now that iPS cells can be utilized to produce the cells of interest, many scientists have been generating iPS cells from ALS patients and producing large numbers of motor neurons having the same genetic information as the patients. Among them is Dr. Haruhisa Inoue at our institute (CiRA, the Center for iPS Cell Research and Application).[29] Dr. Inoue demonstrated that motor neurons from patients had significantly shorter projections, which are necessary for signal transduction from the brain to muscles, than did those from healthy control individuals (Figure 5). He also found that a histone acetyltransferase inhibitor, called anacardic acid, reverted the abnormal ALS motor neuron phenotype. In addition to ALS, scientists at the CiRA have been working on other intractable diseases, such as FOP (fibrodysplasia ossificans progressiva) and CINCA (chronic infantile neurological cutaneous and articular) syndrome.[31] The findings of their studies have shown that patient-derived iPS cells can provide a useful tool for elucidating the disease pathogenesis and for screening drug candidates.

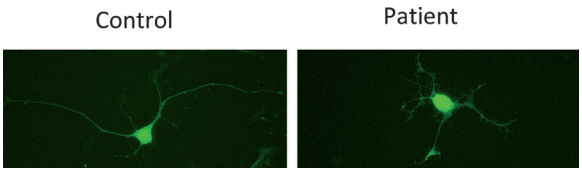

Figure 5. Disease modeling of motor neurons from ALS patient-derived iPS cells. Microscopy images of motor neurons that were differentiated from iPS cells derived from a control donor (left) and an ALS patient (right).[29] (Copyright 2012, American Association for the Advancement of Science.)

Stem cell therapy is another promising medical application of iPS cells. In Japan and other countries, researchers are conducting preclinical studies to prove the efficacy and safety of iPS cells for treating various diseases and injuries, such as Parkinson's disease, macular degeneration, cardiac failure, spinal cord injury, and platelet deficiency.[32] The iPS cell technology has progressed rapidly in the last five years. We and others have reported an integration-free method that can be used to generate human iPS cells using episomal vectors.[33] We also have shown that p53 shRNA,[34] L-Myc,[35] and Glis1,[36] when strongly expressed, can replace the oncogene c-Myc, and efficiently generate human iPS cells. We have also developed systems to evaluate iPS cells. We believe that the technology is getting closer to clinical trials. Dr. Masayo Takahashi at the Riken Center for Developmental Biology (CDB) has already applied for permission to conduct an iPS cell based clinical trial for macular degeneration from the Japanese Ministry of Health and Labor.

Some of these clinical trials will initially begin with autologous iPS cells derived from the patients' own somatic cells. However, for larger scale trials and more standard therapies, preparing autologous iPS cells from each patient may not be practical, since it will be time-, labor-, and cost-intensive. Generating, expanding, and differentiating iPS cells under a good manufacturing protocol are expensive. In addition, these processes take several months. In the case of spinal cord injuries, it has been shown that the best result can be expected when cells are transplanted within a month after the onset of injury. Therefore, if the generation, expansion, and differentiation of iPS cells is started after a patient is injured, the cells will never be ready in time. To overcome these practical problems, we are now trying to establish iPS cell stocks for regenerative medicine purposes. To minimize immune-mediated rejection, our plan is to generate iPS cells from donors with HLA homozygous alleles. In Japan, we estimate that iPS cell lines from 140 HLA homozygotes will cover up to 90% of the Japanese population.

Scientific Streams Toward the Future

I receive this prize on behalf of numerous researchers and scientists who have contributed to the generation and rapid progress of the iPS cell technology. As I described, iPS cells were established on the basis of three preexisting scientific streams (Figure 6).

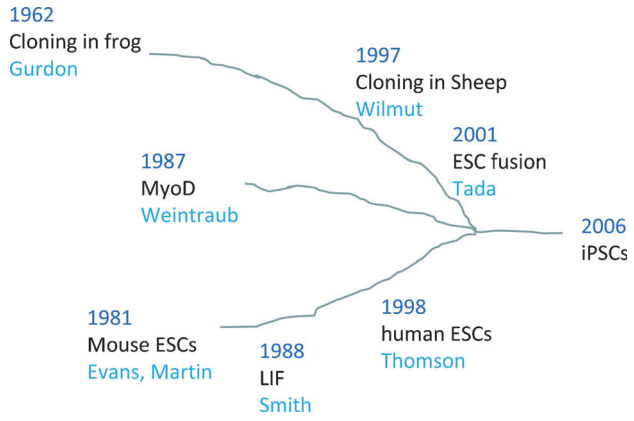

Figure 6. Three scientific streams that led to the development of iPS cells. Shown are landmark events with the name of senior authors and published years.

The first stream was nuclear reprogramming, initiated by Dr. John Gurdon more than 50 years ago. A nucleus from a fully differentiated intestinal cell of a tadpole was transplanted into an unfertilized egg in which the nucleus had been destroyed by ultraviolet light. The egg with the transplanted nucleus developed into an adult frog, thus achieving cloning.[1] A few decades later, Dr. Ian Wilmut succeeded in the somatic cloning of a mammal, a sheep, for the first time in 1997.[12] Although nuclear transfer in mammals is more technically demanding due to the smaller cell size and physiological adjustment of the egg's cell cycle, his team demonstrated the birth of a lamb by nuclear transfer from an adult mammary gland or differentiated fetal cells into an enucleated sheep egg. Their successes in somatic cloning demonstrated that even differentiated cells contain all of the genetic information required for the development of entire organisms, and that oocytes contain factors that can reprogram somatic cell nuclei. In 2001, Dr. Takashi Tada demonstrated that mouse ES cells also contain reprogramming factors by showing that the fusion of somatic cells and ES cells can induce reprogramming in somatic nuclei.[13] This scientific stream was essential for me to initiate our project that led to the development of iPS cells.

The second important stream was factor-mediated cell-fate conversion, first demonstrated by Dr. Weintraub.[15] They converted mouse fibroblasts into myoblasts by forced expression of one of the myoblast-specific transcription factors, "MyoD". These results led to the concept of a "master" transcription factor that determines the fate of the cell lineage.

The third essential stream was ES cell research, which was initiated by Dr. Martin Evans and Dr. Gail Martin in 1981.[9,10] Until then, pluripotent cell lines had been obtained only from teratocarcinoma cells. They established pluripotent cell lines with a normal karyotype, which had been isolated directly from mouse early embryos in vitro. Dr. Austin Smith and others identified (and are still identifying) many factors which are essential for pluripotency, including Oct3/4 and Sox2.[19,37,38] In 1998, Dr. James Thomson succeeded in generating human ES cells with optimal culture conditions, which are very different from those for mouse ES cells.[11] All of these findings were indispensable for providing ideas about the existence of reprogramming factors, the factor combinations, and culture conditions for pluripotent cells, eventually leading to the generation of iPS cells.

New scientific streams have already emerged from the iPS cell studies (Figure 7). In 2007, Dr. Rudolf Jaenisch provided the first proof of concept of iPS cell based cell therapy in mouse models.[39] They demonstrated that mouse models of sickle cell anemia can be treated by transplantation with hematopoietic progenitors, which were obtained in vitro from gene-corrected autologous iPS cells.

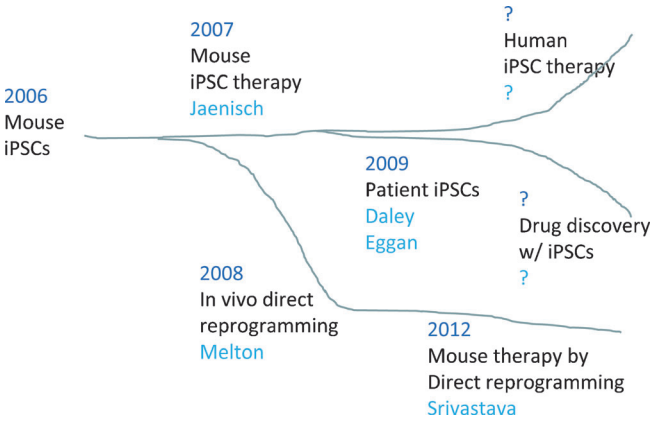

Figure 7. New scientific streams derived from iPS cells.

In 2008, Dr. George Daley[40] and Dr. Kevin Eggan[41] first generated iPS cells from patients. Dr. Daley established iPS cells from patients with a variety of intractable diseases, such as adenosine deaminase deficiency related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson's disease, Huntington's disease, juvenile-onset type 1 diabetes mellitus, Down syndrome, Lesch-Nyhan syndrome, X-linked adrenoleukodystrophy, dyskeratosis congenita, Hurler syndrome, fragile X syndrome, and NEMO deficiency.[40,42–45] Dr. Eggan produced patient-specific iPS cells directly from an elderly (82-year-old) familial ALS patient, and successfully differentiated the iPS cells into motor neurons.[41]

Another important scientific stream that emerged from the iPS cell technology is "direct reprogramming", which was first shown by Dr. Douglas Melton's group in 2008.[46] They reported that the cell fate can be directly transdifferentiated in vivo, in living mice, by introducing just a small number of transcription factors. They identified a combination of tran-

scription factors which converted differentiated pancreatic exocrine cells into endocrine cells that secreted insulin. Many studies have followed, and in 2012 Dr. Deepak Srivastava provided the first proof of concept of therapies based on direct reprogramming in mice.[47] They succeeded in directly converting cardiac fibroblasts into cardiac myocytes in situ after coronary ligation. Their results showed decreased infarct size and recovery of some cardiac functions.

The history of iPS cell research has only just begun, and this technology has a remarkable potential for use in cell therapy, drug screening, and personalized medicine. Unexpected results have opened up an entirely new research field. I hope that iPS cells will be utilized by many scientists in multiple research areas related to medicine or biology, and that some of those researchers will also receive a Nobel Prize for their excellent work in the near future.

I would like to express my sincere gratitude to all of the members of the Center for iPS Cell Research and Application at Kyoto University, and the Gladstone Institutes in San Francisco for their continuous support of my scientific research and life. I wish to thank all of the previous and current colleagues in my laboratory for their dedicated and tireless efforts. My deepest gratitude goes to my family for their unflagging support throughout my life. I could not be here today without them. Although my father, who talked me into pursuing a career in medicine, and my father-in-law, who showed me how to be a good doctor, are no longer with us, I believe they all share our happiness and joy in heaven. I sincerely wish to help make this iPS cell technology a clinical reality before I meet both of my fathers again in the future.

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