Since his fundamental discovery in 2006, Shinya Yamanaka and his group have shown that different types of cells – not only fibroblasts, but different types of cells including, for example, those of the digestive tract, liver cells etc. – can be reprogrammed. The work accomplished since then tends to reduce or even eliminate the risk of tumorigenicity in iPS cells, thus demonstrating that cellular reprogramming can also be obtained in some circumstances in the absence of retroviral vectors and the oncogene *c-Myc*.
Shinya Yamanaka's experimental results, which have the virtue of being easily reproduced, have made it possible to take a giant step in research on stem cells and regenerative medicine. They are also of great importance in the field of fundamental research because they will help in understanding the mechanisms of cellular differentiation and associated abnormalities which can lead to cancer and other diseases.
Shinya Yamanaka is Director of the Center for iPS Cell Research and Application (CiRA), Kyoto University, Professor at the Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University and Senior Investigator at the Gladstone Institute of Cardiovascular Disease, San Francisco, California, U.S.A. The CiRA laboratory in Kyoto indeed has been purpose built for him to develop his research.

Shinya Yamanaka:
Induced Pluripotent Stem (iPS) Cells

Thank you very much, Madame Le Douarin, for the kind introduction.
Chairman of the Balzan Foundation, ladies and gentlemen, this is a distinct honour to receive this year's Balzan Prize. I would like to thank the Balzan Foundation and the General Prize Committee for giving me this great opportunity.
In order to describe my work, let me explain briefly what embryonic stem cells are. Embryonic stem (ES) cells are derived directly from embryos. In 1981 researchers in the UK removed mouse embryos just before implantation from the uterus and succeeded in culturing the cells in vitro that were taken from the mouse embryos over an extended period of time. They designated these cells ES cells, embryonic stem cells.
ES cells exhibit two definitive properties: proliferation and pluripotency. These cells have abilities to grow robustly and to differentiate into any of the lineages that give rise to the cells of the adult body, a capacity referred to as pluripotency, which enables ES cells to be differentiated into various types of cells, such as neurons and cardiac muscle cells. The expression of many genes that are expressed specifically in ES cells is necessary to maintaining their pluripotency. When I was a postdoctoral fellow at the Gladstone Institute of Cardiovascular Disease in San Francisco, the U.S.A., in the mid 1990s, I discovered a gene called NAT1. Returning to Japan, I continued to study the functions

of this gene and found that NAT1 is essential to maintain pluripotency in ES cells. In 1998, Professor James Thomson of the University of Wisconsin in the U.S.A. announced that his research team had successfully generated human ES cells. This achievement, which came 17 years after the isolation of mouse ES cells was first reported, was heralded as opening up the possibility of cell transplantation therapies to treat otherwise intractable diseases.

Human ES cells can theoretically be differentiated into any somatic cell type, such as neurons, cardiomyocytes, pancreatic beta cells, and hepatic cells. If ES derived functional cells are produced in large quantities, they may one day find uses in clinical applications. For instance, ES cell derived dopaminergic neurons may in the future be used for cell transplantation in patients with Parkinson's disease, and neural stem cells made from ES cells may be transplanted into patients suffering spinal cord injuries to aid in the recovery of motor function. For these reasons, human ES cells are regarded as an attractive source for cell transplantation therapies.

However, the clinical application of ES cells also faces two major hurdles. One is that immune rejection is likely to occur after functional cells derived from ES cells are transplanted; the second is due to the ethical issues surrounding the use of human embryos to generate ES cells. When I had my laboratory at the Nara Institute of Science and Technology (NAIST) in Nara, Japan, in 1999, I decided that my group's research would be directed toward the generation of a new type of pluripotent stem cell capable of circumventing these two obstacles. My concept was to generate embryonic-like stem cells by reprogramming somatic cells from patients, in a sense turning back the clock on somatic cells to restore them to pluripotency.

This research theme was challenging as, at that time, many laboratories around the world were engaged in the differentiation of ES cells into functional cells. The concept of cellular reprogramming was in fact established many years ago. In 1962, the year I was born, Sir John Gurdon reported the generation of frog offspring by transferring tadpole intestinal cell nuclei into enucleated eggs from the African clawed frog, *Xenopus laevis*. Dolly the sheep, reported in 1997, was another example, as was a 2001 report showing that thymocytes acquire pluripotency upon electrofusion with mouse ES cells. These experiments clearly showed that eggs and ES cells contain factors that induce pluripotency in somatic cells.

I first hypothesized that those factors that maintain pluripotency in mouse ES cells might be used to induce pluripotency in somatic cells. So members of my laboratory started searching for factors that play important roles in the maintenance of ES cell identity – genes that are expressed specifically in mouse ES cells. By the time that I

moved to Kyoto University in Kyoto, Japan, in 2004, along with some of the members of my NAIST lab, our group had identified 24 such factors and tried to narrow down which among them was capable of reprogramming. We had observed that when all 24 factors were introduced together into somatic cells, typical ES-like cell colonies appeared. To determine which of the 24 candidates are critical, we repeated the experiment by removing each factor from the 24-factor combination in a process of elimination, and watching as more limited sets of factors were introduced into somatic cells to see if the factors removed were essential to the generation of ES-like cells. Finally, we found four transcription factors indispensable for inducing pluripotent cells.

In 2006, we reported that embryonic-like stem cells could be induced by introducing the four factors – Oct3/4, Sox2, Klf4, and c-Myc – into mouse fibroblasts or skin cells via retroviral vectors. These ES-like cells showed rapid proliferation and differentiated into various somatic cell lineages. The cells also expressed several ES cell marker genes, such as Oct3/4, ERas, and Esg1. When injected subcutaneously into nude mice, they differentiated into various cell types characteristic of all three germ layers, such as gut-like epithelium, cartilage, skeletal muscle, and neural tissue, demonstrating their pluripotency. We named the new cells "induced pluripotent stem cells," or iPS cells. As our final goal is to make the cells usable in the clinic, we worked on development of protocols for inducing pluripotency in human cells. In 2007, we reported the generation of human iPS cells, simultaneously with an independent report by Dr. Thomson's group. These first reports were followed in close succession by reports from other labs as well.

Soon after the announcement of the human iPS cell generation, Kyoto University established the Center for iPS Cell Research and Application (CiRA) to advance iPS cell research. I have since served as the director, and a new research building was opened in February this year. This facility has five aboveground stories and one basement floor, and a cell processing center and animal research facility onsite. At CiRA, 19 laboratories work on research projects ranging from basic research to preclinical and clinical studies, utilizing the center's state-of-the-art facilities. Our goal is to improve iPS cell technology and develop new drugs and therapies for patients with intractable diseases at the earliest possible stage.

Currently, we generate iPS cells from somatic cells provided by patients. Physicians conduct biopsies to obtain tiny amounts of skin cells, or fibroblasts. The fibroblasts are cultured in a petri dish for two to three weeks, and then the four genes are introduced into the fibroblasts. After culturing the cells for a few weeks, iPS cell colonies emerge. Each iPS cell colony comprises several hundred iPS cells. iPS cells grow

rapidly and, like ES cells, can be differentiated into any functional cell type in the body. iPS-cell derived cardiac muscle cells, for example, show the same synchronized pulsing as beating heart muscle in the body.

iPS cells have enormous potential. As iPS cells can in principle be steered to differentiate into any kind of cell, the prospect of patient-specific iPS cells has raised great hope for future medical applications, such as understanding pathogenesis, drug screening, and toxicology, as well as the development of regenerative medical approaches, such as cell transplantation. In iPS-based cell therapies, various types of somatic cells derived from pluripotent stem cells may one day be used to repair tissues damaged through disease or injury. Years of research and rigorously designed clinical studies will be required to determine whether these applications are safe and effective. Many scientists around the world are conducting research aimed at the development of clinical applications using iPS cells. The therapeutic effects of mouse iPS cells have so far been reported in animal models of sickle cell anemia, Parkinson's disease, hemophilia A, and spinal cord injury. The Center for Regenerative Medicine in Barcelona, Spain, reported in 2009 that human iPS cells were effective to treat animal models of Fanconi's anemia.

iPS cell technology can also be used for drug screening or toxicology testing *in vitro* and for creating disease models in culture, which are regarded as comparatively shorter-term goals than the development of applications in regenerative medicine. For example, liver cells generated from individuals with different cytochrome p450 enzymes would be of value for predicting the liver toxicity of new drugs. The disorder long QT syndrome (LQTS) is caused by mutations in genes involved in generating cardiac action potentials resulting in lethal arrhythmias. LQTS can also be induced by certain drugs in sensitive individuals. By generating beating cardiac myocytes from iPS cells derived from these sensitive individuals, drug candidates could be tested *in vitro*.

Generating *in vitro* disease models using iPS cell technology may also prove useful in elucidating mechanisms of disease pathogenesis. Many groups have already generated iPS cells from patients with various neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Parkinson's disease, a variety of genetic diseases with either Mendelian or complex inheritance, and spinal muscular atrophy (SMA).

An important challenge is how to recapitulate disease in cells derived from patient-specific iPS cells. In genetically inherited diseases, specific pathologies may be easier to model. Indeed, motor neurons generated from iPS cells derived from a SMA patient exhibit selective deficits compared to those generated from iPS cells derived from the patient's healthy mother. However, in many neurodegenerative diseases such as ALS, it takes years for symptoms to develop in patients. For instance, a professional baseball player who played in major league baseball in the U.S.A. in the 1930s, Lou Gehrig,

was one of leading batters with a batting average of .350 in 1936 and 1937. When his average dropped in 1938, people thought he had fallen into a slump, and he was forced to retire. Only later was he diagnosed with ALS, and he died in 1941 at the age of 37. ALS is a progressive, fatal neurodegenerative disease caused by the degeneration of motor neurons that control muscle movement. About 70 years after Lou Gehrig died, no major progress has been made in finding effective therapies for the disease, due in part to the lack of good disease models. If researchers use iPS cell technology, iPS cells generated from patients with ALS are derived into motor neurons that have the same DNA as the patient's. If the symptoms of the disease can be recapitulated in the cells, it may be possible to use them to elucidate the mechanism of ALS and screen drug compounds that may be effective to the disease.

However, the development of clinical applications using iPS cells faces many obstacles, some similar to those facing ES cells, and others that are unique. Common obstacles to realize cell therapies are that we have to develop methods to differentiate ES/iPS cells into any functional cells we need and to transplant them into patients. The most challenging common hurdle is teratoma formation. Even a small number of undifferentiated cells can result in the formation of teratomas, a form of tumor. Another key goal is to induce differentiation of human ES/iPS cells into required cell types while leaving few undifferentiated cells behind. One unique hurdle to be overcome before iPS cells can be used in the clinic is primarily related to the induced reprogramming of somatic cells. We need a reliable evaluation of whether nuclear reprogramming for each iPS cell is complete. Aberrant reprogramming may result in impaired ability to differentiate, and may increase the risk of teratoma formation after directed differentiation. One of the most important challenges is to develop simple, yet sensitive and reliable methods to evaluate the effectiveness and safety of the many iPS cell clones and subclones generated by many different technologies. To use iPS cells for disease modeling and drug and toxicology screening *in vitro*, finding methods for recapitulating pathology in these cells is of the utmost importance.

Despite all these obstacles and challenges, iPS cells offer enormous and unprecedented potential for disease research, drug screening, toxicology, and regenerative medicine. An increasing number of laboratories worldwide working with this technology have reported many new findings in the short time since the first report was published. I cannot emphasize strongly enough that it would not have been possible to generate iPS cells and advance the field at such rapid speed without the many studies using mouse and human ES cells that appeared since the isolation of mouse ES cells in 1981. I believe that the concerted efforts of researchers around the world will make the promise of iPS cells a reality in the not-too-distant future.

Finally, I would like to thank all members in my laboratory and CiRA for their valuable discussions, technical support, and administrative assistance. Without their hard work, iPS cells would never have been made. I was lucky enough to have several talented colleagues and students in my laboratory. In particular, Drs. Yoshimi Tokuzawa and Kazutoshi Takahashi, and the excellent technician Tomoko Ichisaka have greatly contributed to the development of iPS technology. Yoshimi identified the transcription factor Klf4, one of the four transcription factors used to generate iPS cells. Kazutoshi, who now works with me at CiRA, conducted the painstaking experiments needed to test 24 candidate factors, and identified the four indispensable factors. These two were the first students in my lab at the NAIST. Tomoko, who also works in my lab, has been an excellent technician, and without her skills, we could not have conducted the functional analysis of these factors.

As Madame Le Douarin has recounted, I manage two laboratories – one in Kyoto and the other in San Francisco. With the students and colleagues working in my labs and many other researchers around the world, I hope to continue to make progress in iPS cell research. It may take many years to reach the goal, but I sincerely hope that iPS cell technology will contribute to the development of new cures for people suffering from various diseases and injuries.

I just want to add one more thing. I would like to thank my wife and my family for their continuous support.

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Paolo Matthiae:
Thank you, Shinya Yamanaka, for this very interesting presentation. I have the pleasure to invite now Mario Stefanini, who is Professor of Histology at the University of Rome "La Sapienza" and a member of the Lincei, to make some comments.

Comments, Questions and Preliminary Discussion

Mario Stefanini:
Shinya Yamanaka has helped pioneer a whole new field in Biology[1-3]. There are more than 40 major research laboratories around the world working on the production of safe iPS cells for clinical application, investigation concerning etiology of diseases, and drug screening.
I would like to ask Dr. Yamanaka to elaborate on the recent data on iPS cell biology, produced by his own laboratories as well as by other research centres.
The first issue regards iPS cells and tumorigenesis. As we just heard, iPS cells were made by using viruses to insert at least four key genes, Oct3/4, Sox2, c-Myc, and Klf4, into their genome. This procedure, even in the absence of one gene c-Myc, carries the risk of turning the cell cancerous. A new method has just been published by Derrick Rossi and colleagues that claims not only to do away with genes, but also to be more efficient[4]. The authors chemically modified RNAs transcribed from the four genes, Klf4, c-Myc, Oct4 and Sox2, and introduced these into human fibroblast cells. The method proved more efficient at generating iPS cells than the virus method. Furthermore, treating the iPS cells with an additional RNA transcript turned them into muscle cells.

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