Progress in Stem-Cell Research and New Medical Care*1

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Abstract

Stem cells are the key to success in regenerative medicine, a field attracting considerable attention as a new form of medical care in the 21st century. Embryonic stem (ES) cells, which are representative pluripotent stem cells, have the highest potential in terms of multipotency and proliferative ability in a test tube. However, ES cells are produced employing embryos about 1 week after fertilization, and it is difficult to generate ES cells from patients. Furthermore, of course, there are important ethical concerns.

However, a technique for preparing induced pluripotent stem (iPS) cells has recently been established, allowing us to readily produce patient-derived pluripotent stem cells. We can use iPS cells not only as a new source of human cells for drug development and toxicology testing of new drugs but also as the supply source for blood products, providing an alternative to donated blood. These cells also raise the possibility of totally new gene and cell therapies such as gene correction therapy or novel immune treatments using rejuvenated antigen-specific T-cells.

In addition, we have endeavored to produce iPS cell-derived organs in model animals with the aim of reproducing parenchymal organs such as the heart and pancreas, and have already succeeded in producing rat iPS cell-derived pancreas in individual mice. Currently, we are exploring the possibility of the development of completely new regenerative medical technologies such as regeneration of human parenchymal organs in the bodies of individual large animals.

Key words ES cell, iPS cell, Cloned embryo, Cancer stem cell, Gene correction therapy

Definition of Stem Cells

Stem cells are defined as undifferentiated cells that have two distinct features, i.e., multi-lineage potential and self-renewal capacity, which means that stem cells can differentiate into a wide range of different cell types, and can self-renew to produce more stem cells with multi-lineage potential. These cells produce, repair, and maintain tissues and organs while also maintaining themselves. There are two types of stem cells. One, pluripotent stem cells, can differentiate into all cell types other than those of the placenta and amniotic membrane. Examples of this type are the well-known embryonic stem (ES) cells and the recently developed innovation known as induced pluripotent stem (iPS) cells. The other type is tissue stem cells, often referred to recently as adult stem cells, which can differentiate into a limited range of specific tissues and organs. Stem cells of this type include hematopoietic stem cells, which have been the most thoroughly studied, neural stem cells, and hepatic stem cells.

What Are Hematopoietic Stem Cells?

Hematopoietic stem cells are the best studied among the various types of stem cells. It has recently become apparent that these cells are present in the bone marrow and are hibernating

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Hematopoietic stem cells have both the multi-lineage potential and self-renewal capacity, and maintain the blood systems throughout a person's life by producing 20 billion blood cells every day.

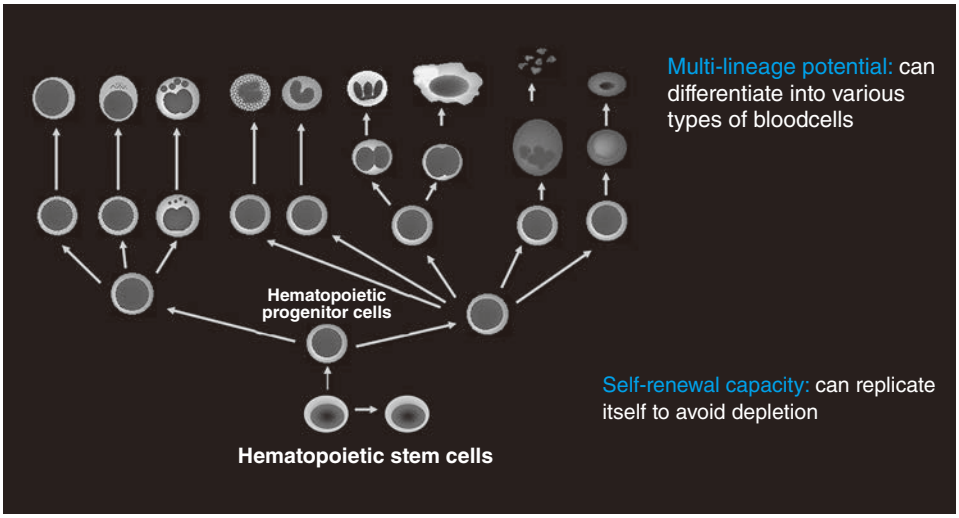


Fig. 1 Self-renewal and multi-lineage potential of stem cells

most of the time. They do not divide often, but when they do on occasion, they seem to undergo asymmetric division and produce one hematopoietic stem cell and one hematopoietic progenitor cell. Hematopoietic stem cells reproduce themselves and then go into hibernation. The hematopoietic progenitor cells produced thereafter continue dividing and differentiating into various functionally and morphologically different blood cell types. This is the aforementioned multi-lineage potential. Thus, self-renewal capacity and multi-lineage potential allow stem cells to provide a lifelong supply of various cell types while maintaining their own population.

To summarize, hematopoietic stem cells divide to reproduce themselves and hematopoietic progenitor cells. Because hematopoietic progenitor cells do not have self-renewing capacity, they will eventually disappear, but only after having actively differentiated and proliferated to form various types of blood cells. Because these processes are repeated in an ongoing cycle, our blood systems are maintained (**Fig. 1**).

What Are ES Cells?

ES cells are briefly described below because these cells are very important in terms of recent studies of stem cells and the possibilities of future clinical applications. When a sperm fertilizes an egg, the fertilized egg begins to undergo rapid cell division. In humans, a fertilized egg becomes a round mass of about 100 cells, which is an embryo, in about 1 week. To produce ES cells, a small group of cells (referred to as an inner cell mass) in the blastocyst stage, embryologically, destined to later develop into a human being, are extracted, and then cultured under specific conditions (**Fig. 2**). Once formed, ES cells can be propagated indefinitely by *in vitro* culture. In addition, ES cells basically can differentiate into various types of cells if culture conditions are altered accordingly. Therefore, these cells are considered to be an excellent material for regenerative medicine.

On the other hand, however, the embryo is unavoidably destroyed to produce ES cells. Such an embryo is capable of forming a human being, if implanted in a uterus. We prevent implantation, and destroy the embryo to produce ES cells. According to this principle, ES cells cannot be produced from either healthy individuals or patients because they already exist as the consequence of the growth of the embryo.

ES cells can be propagated by culture indefinitely, and have extensive differentiation capacity.

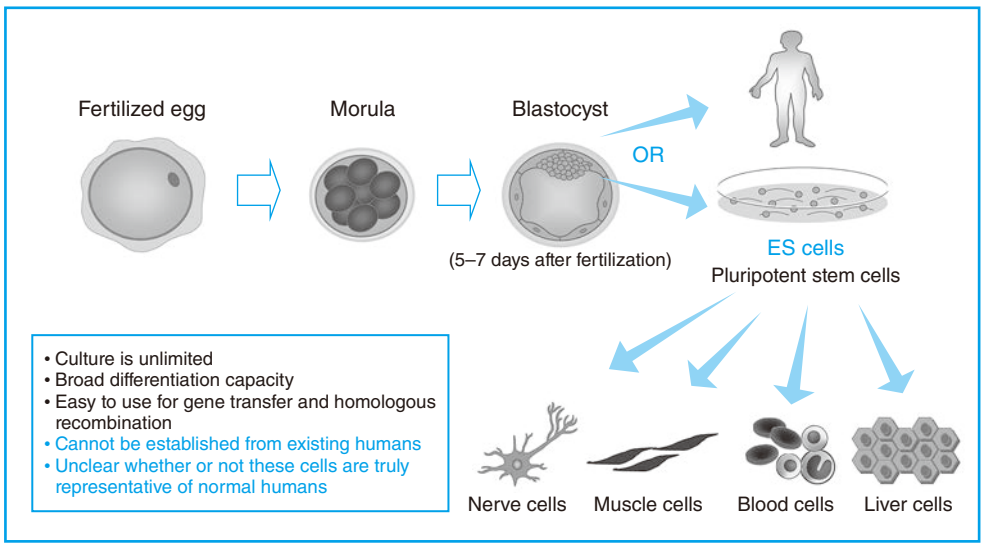


Fig. 2 Embryonic stem (ES) cells

Because they can be cultured, gene transfer and gene recombination can be carried out during the process of culture. However, the following issues must also be taken into account. ES cells cannot be produced from a human being, i.e. someone who has already been born. Because ES cells are produced from an embryo destroyed prior to implantation, whether such an embryo would eventually have formed a healthy individual, if implanted in a uterus and allowed to grow, remains unknown.

Process of the Establishment of ES Cells

This principle of ES cells was first established in mice, as reported by Dr. Martin Evans in 1981.[1] In 2007, he was awarded the Nobel Prize in Physiology or Medicine for this accomplishment.

The procedure for the production of ES cells is the same in murine models, as noted above. ES cells are produced from the inner cell mass obtained from the embryo at the blastocyst level, which is at several days after fertilization and would otherwise later develop into a mouse. If these ES cells are returned to the blastocyst and placed in a uterus, they will grow into a mouse. Thus, we can certainly say that this is a marvelous invention and discovery.

About 17 years after Dr. Evan's pioneering work, Dr. James Thomson at the University of Wisconsin in the US established human ES cells, and reported his study.[2] In the same manner as described previously, human ES cells were established by using the inner cell mass of the blastocyst.

Although ES cells constitute a very good material for regenerative medicine, the problem is that they are not cells from patients themselves. Therefore, basically, ES cells may be associated with infections or immune reactions, as in the case of the allogeneic transplants currently in use. In regenerative medicine, an ideal practice is to implement safe cell or organ transplantation using cells from patients themselves, which are free of such problems as immune rejection.

A procedure to this end is to establish pluripotent stem cells from patients themselves by means of therapeutic cloning. Therapeutic cloning is basically a technique to rejuvenate already differentiated cells. The nucleus containing the genome, which is a cell design diagram, is taken out of a differentiated mature cell. The nucleus is inserted into an unfertilized egg from another female animal of the same species after enucleation of the cell. This is called somatic cell nuclear transfer (**Fig. 3**). Employing this procedure, the genome reacts with certain proteins in the unfertilized egg, resulting in initialization of the nucleus. It was first demonstrated in

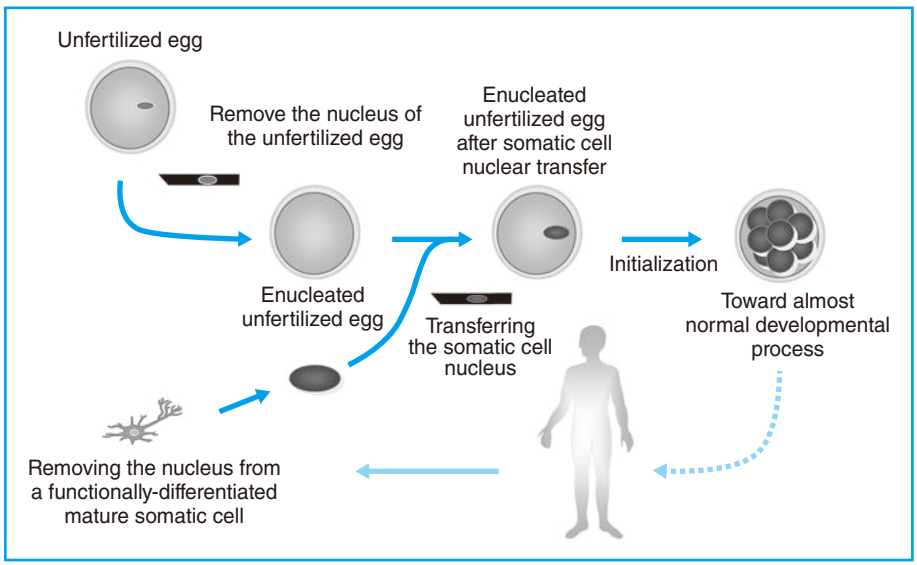


Fig. 3 Rejuvenation of the genome by nuclear transfer

an experiment using frogs that restoring the status of the nucleus to the same level as that of an fertilized egg, which has recently been called reprogramming, is feasible.[3] Last year, Dr. John Gurdon, who carried out this experiment, was awarded the Nobel Prize together with Dr. Shinya Yamanaka. This procedure was first proven to be feasible in frogs, and later in mammals. Using this technique, it is possible to produce patient-derived pluripotent stem cells from the patient's own somatic cells, and to apply them to regenerative medicine.

Rejuvenating the Genome by Nuclear Transfer

Suppose that there is a patient, and that the nucleus taken out of any somatic cell of this patient's body is then physically injected into an enucleated unfertilized egg of a different female individual, the injected nucleus then reacts with the cytoplasm, and behaves as though it were a fertilized egg. As mentioned previously, this egg differentiates to the blastocyst level after about 1 week of culture.

Although it is not possible to rejuvenate patients themselves, their cells can be rejuvenated to the level of fertilized eggs employing the procedure for rejuvenating genomes. Through this procedure, pluripotent stem cells can be produced from patients, i.e. from already living human beings.

Preparation of Clone Animals

The principle of cloning animals was first demonstrated with frogs. Thereafter, about 15 years ago, cloning was proven to be possible in a mammal, the sheep,[4] and became a sensational topic.

Let me explain again the principle of cloning in sheep. There was a mature sheep, and its mammary cells were harvested. When the nucleus of the mammary cell was transferred into an enucleated unfertilized egg of another female sheep, and appropriate stimulation was delivered, the egg began to divide like a fertilized egg. A blastocyst had formed a week later. When this blastocyst was placed in the uterus of a foster parent, surprisingly it grew normally into a sheep, famously named Dolly. Dolly and the sheep that donated the mammary cell had the same genes; their relationship was, in simple terms, that of monozygotic twins of different ages. These are called clone animals (**Fig. 4**).

High Hurdles for Application in Humans

Once this technique was proved possible in mammals, its application in medical care of humans was naturally attempted. If a somatic cell of basi-

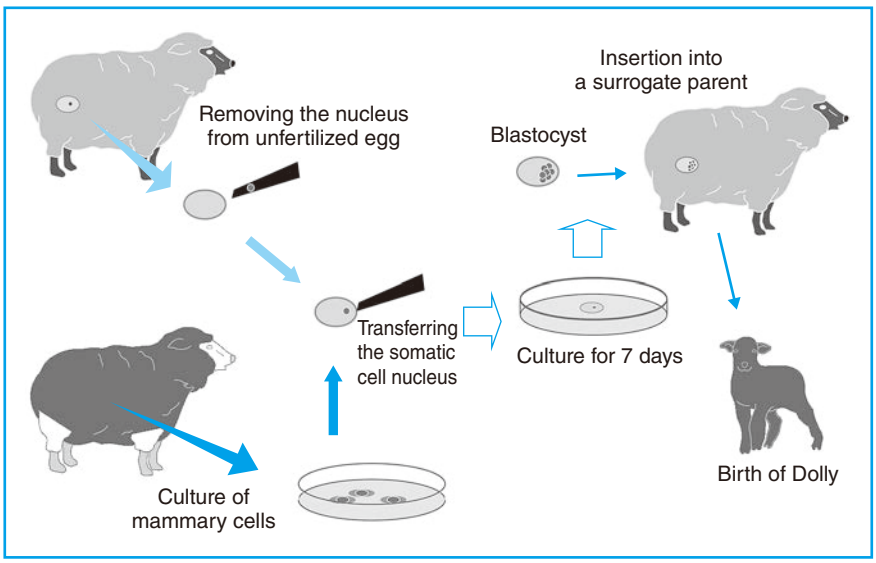


Fig. 4 Production of cloned animals by nuclear transfer

cally any type—fibroblast, cutaneous cell, etc.—is taken from a human donor, and then injected into an enucleated unfertilized egg, a blastocyst is obtained in one week if the process goes as well as in sheep. The inner cell mass from this blastocyst allows the production of ES cells. In this manner, patient-derived ES cells, or pluripotent stem cells, can be obtained. This technique enables regenerative medicine and cell therapy using cells from the patient him- or herself. This method is called therapeutic cloning. ES cells thus prepared are primarily defined as nuclear transfer ES (ntES) cells, which distinguishes them from ES cells produced by the conventional method.

By this method, it was expected that ES cells could be produced from already living individuals, i.e. fully formed human beings. However, various issues are associated with this procedure (**Fig. 5**). For example, first, many unfertilized human eggs must be collected to carry out nuclear transfer. Second, as a problem peculiar to the production of ES cells, these cells cannot be produced without destroying embryos, which at least theoretically have the potential to grow into individual human beings in the future. Thus, there is the bioethical problem that the destruction of embryos, i.e., the origin of life, is unavoidable.

In addition, public anxiety arises from the possibility of placing these embryos in uteruses of surrogate mothers, which might allow the production of many clone humans who have the same genes but different ages. This procedure faced major obstacles in terms of regenerative medicine, with both technical and social hurdles being very high.

(Note: Success in the establishment of human ntES cells by nuclear transfer was reported by an American research group in June 2013.[5])

Epoch-Making Discovery of iPS Cells and Its Significance

The aforementioned obstacles were overcome by iPS cells in a very simple and unexpected way. Dr. Yamanaka transferred only 4 genes chosen from among genes frequently expressed in ES cells into adult skin cells, i.e., skin cells from human donors. Employing this simple procedure, the skin cells underwent a change *in vitro*, resulting in the production of cells that closely resemble ES cells. He gave these cells the name iPS cells (**Fig. 6**).

In the beginning, because people believed that such cells could not be produced easily, his achievement was apparently not taken very seriously. However, this method was ultimately shown to be highly reproducible. We also tried his method, and obtained iPS cells just as he

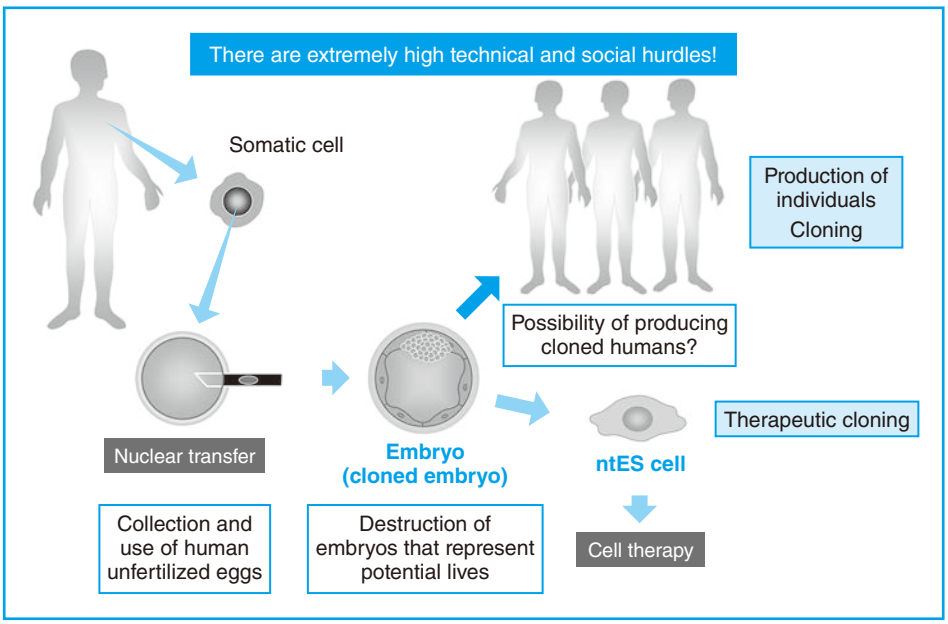


Fig. 5 Principle and problems of nuclear transfer ES cell production

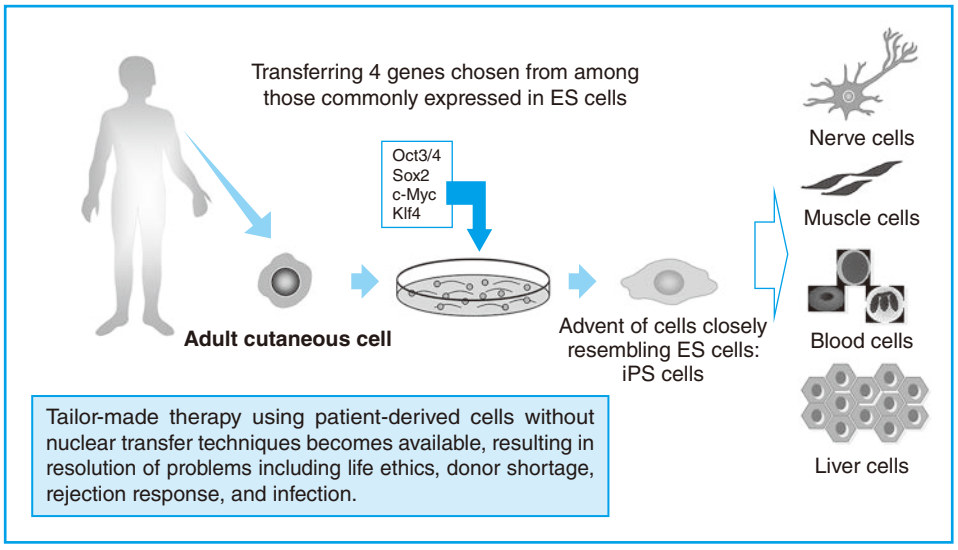


Fig. 6 What are induced pluripotent stem (iPS) cells?

reported. The iPS cells produced in this way have been demonstrated by various researchers to have properties the same as those of ES cells. Thus, this method is currently used in a variety of places.

This iPS cell technology has increased the possibility of implementing tailor-made treatments using patient-derived cells, without the need for the aforementioned technique of nuclear transfer. This iPS cell technology is attracting considerable attention because there is high expectation that various problems associated with ES cells may be

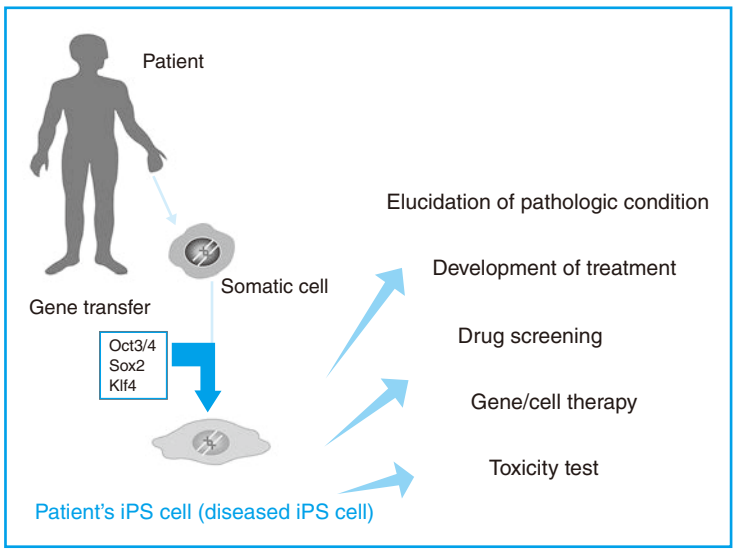


Fig. 7 Patient-derived iPS cells are useful as an inexhaustible source of human cells

resolved with a single blow. This work was first reported in mice in 2006.[6] One year later, it was shown that use of the same 4 genes made it possible for human somatic cells to change into pluripotent stem cells.[7] Based on this accomplishment, Dr. Yamanaka was awarded the Nobel Prize in Physiology or Medicine last year, only 6–7 years after his success with iPS cells.

Thus, transfer of only 4 genes can cause skin cells to change into cells with completely different appearances and functions. However, because genomes remain the same, producing such change means that this is an utterly epoch-making technique. Biologically, this is absolutely revolutionary work, and is also an extremely important contribution to medicine. For instance, if there was a patient here needing treatment, iPS cells could be produced using somatic cells derived from this patient. Once produced, iPS cells can be multiplied as much as we want in the same manner as ES cells, and they can also be stored. It is also possible to make iPS cells differentiate into various cell types (**Fig. 7**).

Therefore, the possibilities for iPS cells include elucidation of various pathological conditions, development of treatments, and drug screening. In addition, gene or cell therapy of a new type, virtually unthinkable before, can now be realized in the relatively near future. It may also become easier to carry out toxicity studies of drugs using human cells. Thus, the potential broad applications of iPS cells make this a very important technology.

Merits of iPS Cells

In summary, iPS cells, in a sense, lie between ES and tissue stem cells. Like ES cells, iPS cells have high capacities to proliferate *in vitro* and to differentiate, but they also have the problem that induction of differentiation is necessary to produce the desired target cells before clinical application.

Tissue stem cells are different in this respect. As shown in bone marrow transplantation, if hematopoietic stem cells are infused intravenously, they go to the bone marrow without aid, where they begin to produce blood cells by themselves. In this regard, tissue stem cells are both easy to use and safe. ES and iPS cells are associated with the risk of teratoma induction if transplanted while they are still undifferentiated. Tissue stem cells, however, are free of such risk.

However, a significant difference between iPS and ES cells is that the former can be produced from the same person, precluding the possibility of an immune rejection response. The absence of immune rejection seems to be the greatest medical merit of iPS cells. In addition, iPS cells do not raise the difficult ethical issues associated

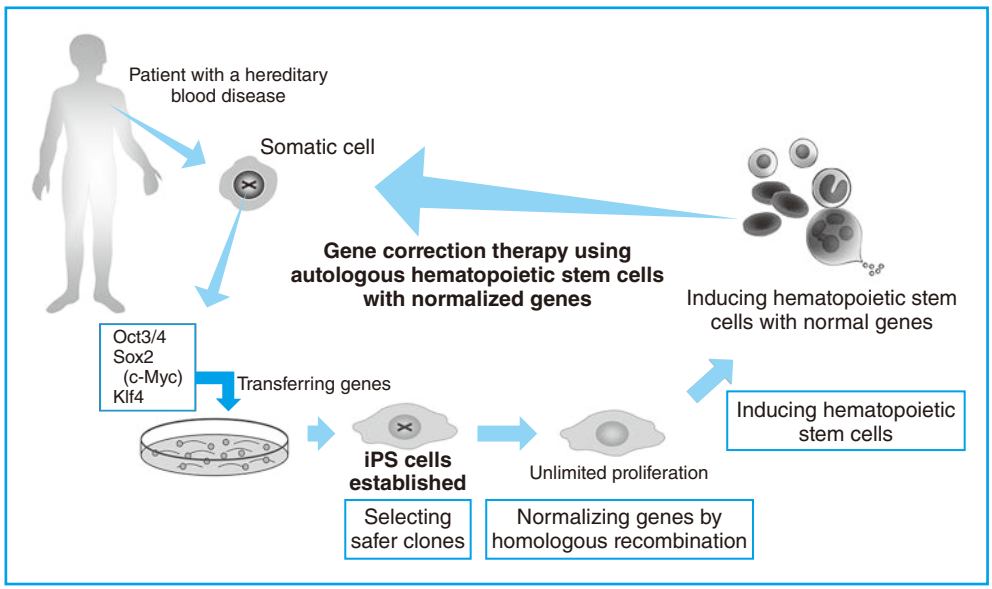


Fig. 8 Treating hereditary disease by using iPS cells: gene correction therapy

with ES cells. As a whole, the merits of iPS cells are that they can be cultured without limit, have high differentiation capacity, allow easy implementation of gene transfer and homologous recombination, and can be obtained from the patients themselves.

Application to Gene Correction Therapy

Next, I would like to explain the possibilities of or progress in medical care research that will be brought about with not only iPS but also stem cells as a whole. This is a pattern of gene therapy called gene correction therapy, which has not been available for long, despite our expectations (**Fig. 8**).

For instance, if there is a patient with a hereditary blood disease, somatic cells of this patient can be collected to establish iPS cells. Because the patient has a blood disease attributable to a genetic disorder, the somatic cell shown in **Fig. 8** is marked with an X, meaning that there is a problem somewhere in the genome. Then, when iPS cells are produced, they have the same genome, and the same X is thus also applied. However, once iPS cells are produced, they can be cultured in whatever volume desired and for however long they are needed, and can be used for gene homologous recombination or gene transfer. Therefore, we believe that it is possible to repair abnormal genes in the stage of iPS cell culture in most, if not in all, cases.

These iPS cells are identical to the patient's pluripotent stem cells except that the hereditary problem intrinsic to the patient has already been eliminated. If hematopoietic stem cells can be induced from these iPS cells, these hematopoietic stem cells that are identical to the patient's own hematopoietic stem cells but have normal genes can be transplanted into the patient. This means that the patient's hereditary blood disease is radically treated by hematopoietic stem cells having the same genome except for the corrected genes.

This type of treatment has not yet become possible despite the growing demand to date. The advent of iPS cell technology, however, has at least made this treatment theoretically feasible. It is now being demonstrated worldwide that this type of treatment is feasible, at least in animal models.[8]

Unexpected Activity of Hematopoietic Stem Cells

Hematopoietic stem cells, that are representative somatic stem cells, are present in the bone marrow. Research on and developments in hemato-

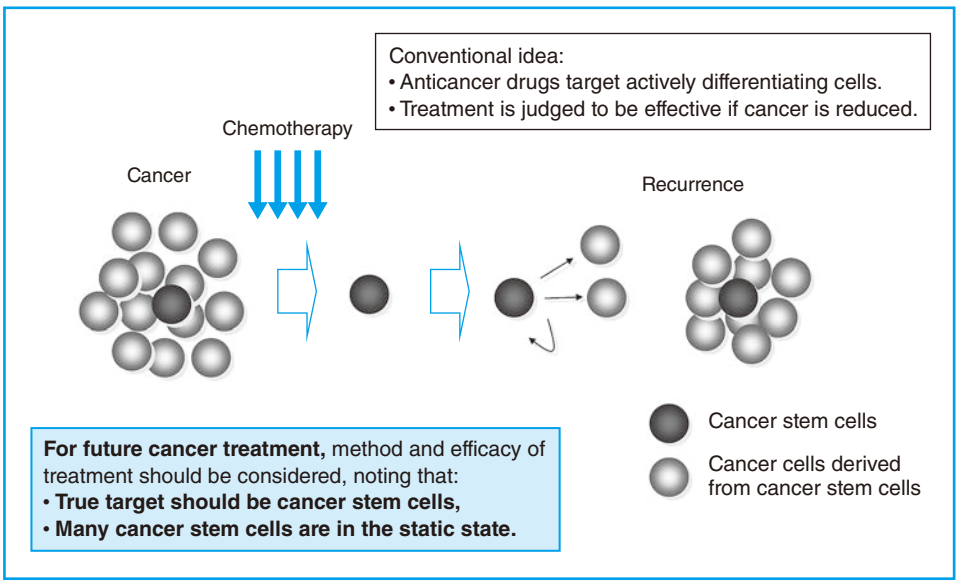


Fig. 9 Need for cancer treatment taking cancer stem cells into account

poietic stem cells have shown that, unexpectedly, most hematopoietic cells in the bone marrow are in the static phase, rather than energetically dividing to produce blood cell components. They only occasionally produce hematopoietic progenitor cells, and these progenitor cells then rapidly supply the blood with new cells.

As you may know, cancer occurs after the accumulation of mutations. Because mucosal cells of the stomach or intestine and blood cells have quite a rapid turnover, even if there is a mutation, the cell will die soon. Therefore, accumulation of mutations requires cells that have a rather long lifespan. Accordingly, stem cells that usually do not divide vigorously but can self-renew and have a long lifespan are potentially a very good target for malignant transformation, in view of the accumulation of mutations.

In fact, about 15 years ago, a Canadian hematologist found that leukemic cells, which had been thought to be a uniform cell population, were not actually so, and that cells responsible for leukemic proliferation accounted for only several percent or less of all leukemic cells.[9] It became apparent that this small minority of leukemic cells responsible for leukemic proliferation had characteristic cell surface marker features, i.e., positive for CD34 and negative for CD38. These markers were found to also be hematopoietic stem cell markers. These findings led to the view that hematopoietic stem cells may actually be accumulating mutations. These researchers then proposed the concept of leukemia stem cells.

Interestingly, the validity of this concept was demonstrated not only in leukemia, a neoplasm of the blood, but also in some solid tumors. As a result, after more than 10 years of research, it seems that the concept of cancer stem cells has become widely accepted in recent years.

Treatment Aimed at Cancer Stem Cells

I consider it unlikely that this concept applies to all cancers. However, the current cancer treatment strategies basically target cancer cells defined as those that are undergoing vigorous division. A treatment is judged to be effective when the tumor size is reduced. Cancer stem cells comprise only a very small proportion of the cancer cell population (**Fig. 9**).

For instance, if it is assumed that cancer stem cells are in the static phase like hematopoietic stem cells, it is highly likely that the most important among them, i.e., cancer stem cells that are responsible for malignant proliferation, remain alive even after all vigorously dividing cancer

precursor cells have been destroyed by chemotherapy. Then, the remaining cancer stem cells occasionally leave the static state and provide precursor cells, which in turn produce tumors. Apparently, this is the mechanism of cancer recurrence. However, in essence, this means that the most important cells have been left untreated. This concept is anticipated to be applicable to at least some types of future cancer treatments.

Unlike the conventional idea that all cancer cells are homogeneous and keep dividing, there is a new idea that some cells responsible for malignant proliferation, inducing cancer, are in a static phase and are resistant to chemotherapy and radiation. It is necessary to introduce this new idea.

Along with this new idea, it is necessary to devise a method of identifying such latent cancer stem cells and to consider treatments specific to cancer stem cells or a comprehensive treatment that attacks all cancer cells including cancer stem cells.

Treatments Using ES and iPS Cells

Next, various studies using ES or iPS cells, carried out in our laboratory, will be described.

I have shown you that hematopoietic stem cells have the capacity for self-renewal and also have multi-lineage potential. If hematopoietic stem cells can be allowed to proliferate freely *in vitro*, various cells can be induced using such hematopoietic stem cells. However, despite extensive efforts by a great number of researchers, there has as yet been no success in achieving proliferation of hematopoietic stem cells in the *in vitro* environment.

However, as mentioned previously, it is relatively easy to get ES and iPS cells to proliferate *in vitro*. The techniques for achieving such proliferation are already established. Therefore, if hematopoietic stem cells or various blood cells can be induced by using ES or iPS cells, which developmentally precede hematopoietic cells, they can be very useful. For instance, iPS cells may be produced from patients with an allergy to induce mast cells, and they can then be used for screening of anti-allergy drugs and for elucidation of the pathological condition. If erythrocytes or platelets can be produced, they may be used as a very stable and safe blood supply source as an alternative to donated blood. If lymphocytes, neutrophils, etc., can be induced from ES or iPS cells, fairly potent treatments for infections or malignancies may well become available.

A group led by Dr. Koichiro Tsuji at the Institute of Medical Science, the University of Tokyo, induced iPS cells from patients with allergic disease, as I mentioned just now. They developed a system to induce mast cells from such iPS cells. They are currently screening candidate substances for anti-allergic drugs.

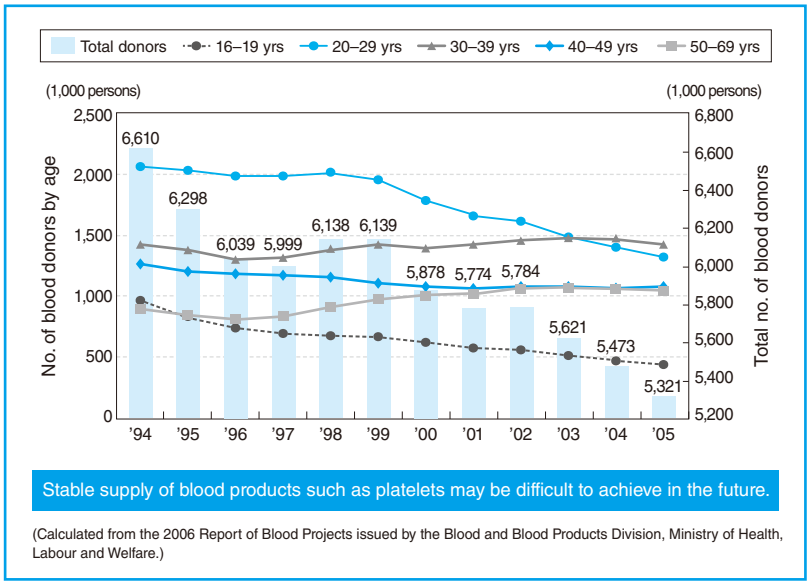
Thus, patient-derived diseased iPS cells can be used for these studies. It may also be possible in the future to provide tailor-made treatments for allergic diseases, if the cause of the disease becomes apparent with the use of such iPS cells.

Assuring a Stable Blood Supply

Although blood transfusion currently depends entirely on blood donation, there has been a steady annual decline in the number of blood donors, as shown in **Fig. 10**. The major reason for this decrease in the number of blood donors is the falling birthrate, as shown by this line graph. If this trend continues, it is anticipated that we will face a shortage of domestic blood product supplies for transfusion within about 15 years (**Fig. 10**).

Under these circumstances, if iPS cells or ES cells allow us to produce platelets or erythrocytes, they can potentially serve as a very stable blood supply source for transfusion not dependent on donated blood. We initially used ES cells, but later attempted to produce platelets from iPS cells. The process of such production will be described briefly. When iPS cells are cultured with feeder cells over a period of approximately 2 weeks, they proliferate to form a sac-like structure. We named this the iPS-Sac (or ES-Sac in the case of ES cells). Most cells contained in the sac are blood precursors. These blood precursor cells are taken out of the sac, and cultured again.

The production process up to this point is common to all types of blood cells. From this point forward, if megakaryocytes are the production target, the blood precursor cells obtained from the sac should be cultured in the presence of thrombopoietic factors such as thrombopoietin (TPO) and stem cell factor. Mature megakaryocytes can be obtained in about 7–10 days. During this course, platelets are also produced,



(Calculated from the 2006 Report of Blood Projects issued by the Blood and Blood Products Division, Ministry of Health, Labour and Welfare.)

Fig. 10 Blood donors are decreasing due to the declining birthrate

and these platelets will be used for various functional analyses. If erythropoietin is added instead of TPO, erythrocytes can be produced. Thus, we have developed a system to induce platelets and various other blood cells from ES or human iPS cells.[10,11]

We produced human iPS cell-derived platelets, and infused these platelets into immune-deficient mice to examine their functions. Most of the cells running through capillary vessels are erythrocytes. When the vascular endothelium is injured by laser application, platelets attach to the injured site to form a blood clot and thereby halt blood flow.[12]

These human-derived platelets and mouse-derived platelets adhered to each other and to the vascular wall to form a blood clot. Namely, it was demonstrated that human iPS cell-derived platelets were functioning normally to form blood clots in the bodies of individual mice. Thus, we have obtained human iPS cell-derived platelets that are functionally very close to actual platelets. Preparation for initiating clinical research and trials using these platelets is currently underway.

Immune Therapy Using iPS Cell Technology

The next topic is the currently available immune therapy. T cells are harvested from patients with cancer or an infection, allowed to proliferate *in vitro*, and then returned to these patients as treatments for their illnesses. Such attempts have been carried out for quite a long time, but their effects have so far been rather limited.

Various factors appear to be responsible for the unsatisfactory results of these attempts. First, although cell division is necessary to achieve amplification of T cells, continued cell division causes the telomere structure at the end of the chromosome to shorten, causing the cells to become aged and worn out. Therefore, the proliferating ability of the cells decreases and their killer activity against other cells also decreases. Namely, continued cell division in the *in vitro* environment wears the T cells out, and returning these worn-out T cells to the patient's body does not achieve particularly good results.

In this regard, we thought that iPS cell technology might be useful for producing young and vigorous T cells, capable of providing potent immune therapy.

New Immune Therapy With Antigen-Specific T Cells

Suppose there is a patient here. T cells that react with a virus or a cancer antigen are collected

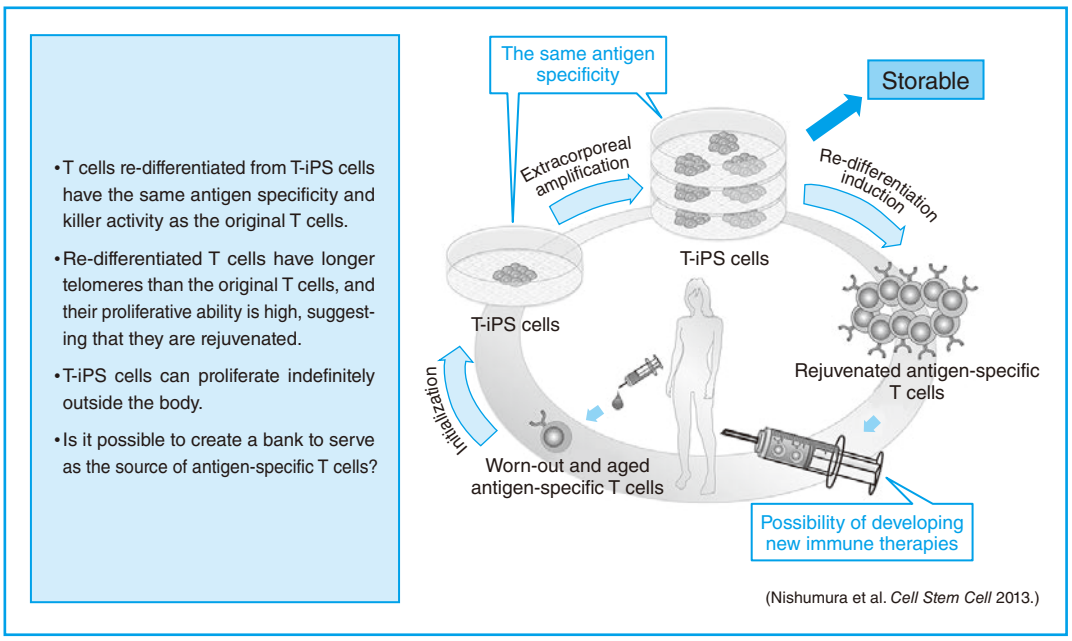


Fig. 11 Potent immune therapy using rejuvenated T cells obtained employing the iPS cell technology

from this patient. After collection, iPS cells are produced from these T cells. Technically speaking, these T cells have T-cell receptors that recognize the virus or the cancer antigen, but the specificity of recognition is stored in the genome rather than in memory at the protein level. The specificity is regulated by rearrangement of genes, which is the finding clarified by the Nobel-awarded work of Dr. Susumu Tonegawa in 1987. When an iPS cell is produced from the T cell, the T-cell receptor gene of the iPS cell has an accurate memory in the genome of what antigen the T cell was recognizing. Once this T-iPS cell is produced, it can be multiplied to any extent at this stage.

Another key feature is that if T cells are induced from these T-iPS cells, they are newly-formed, so-to-speak rejuvenated T cells which can all recognize the same antigen. Thus, young T cells that recognize the same antigen can be produced on a massive scale. As shown in **Fig. 11**, this is a very roundabout approach, because iPS cells are produced from T cells (T-iPS cells), and T cells are then produced from these iPS cells. However, during this process, aged and worn-out antigen-specific T cells are rejuvenated. Because T-iPS cells can be produced without limit and can be stored, the future role T-iPS cells as a ready and useful tool appears to be promising (**Fig. 11**).[13]

Thus, such a method has been developed. New immune therapies using these rejuvenated antigen-specific T cells are possible in the future. As evidence of the rejuvenation of these T cells, it has become apparent that the length of their telomeres is increased, and that their proliferative ability is higher than that of the original T cells. Therefore, we believe that potent treatments for cancer or infections, using rejuvenated T cells, will become available.

Regenerative Medicine for the Next Generation

Finally, I would like to introduce a project that represents a form of regenerative medicine for the next generation. I have so far described the production of target cells from iPS or ES cells, with the aim of providing cell therapy. Now, the next topic is the production of organs from iPS cells.

In Japan, there are currently more than 300,000 patients on dialysis treatment because of chronic renal failure. For these patients, about

5 million yen per patient and over 1,300 billion yen as a whole are required annually in medical care expenditures. Although renal transplantation is a radical treatment, it is extremely difficult to obtain donor kidneys for transplantation in 300,000 patients.

However, on the other hand, as the prevalence of diabetes mellitus increases, patients with renal failure are anticipated to increase by 10,000 individuals annually. Although there are many patients who hope to receive various donor organs such as the heart, liver, lung, and pancreas, as well as kidneys, many cannot undergo transplantation in Japan because there is an overwhelming shortage of donor organs or because the cost of transplantation is prohibitively high. Although a considerable number of patients go abroad to undergo transplantation, this situation is similar overseas. Because the World Health Organization (WHO) is giving serious thought to a ban on travel for transplantation in the future, it may soon become difficult to go abroad to undergo transplantation. At any rate, the extreme shortage of donor organs is an enormous problem and requires resolution.

Experiments Using Mice

Now, there is an idea from another viewpoint about how to tackle this difficult problem. We thought about producing organs in individual animals, aside from producing organs in the *in vitro* environment.

In principle, this would be very simple. First, animals that lack a certain organ should be prepared. For instance, knockout (KO) mice with specific deletion of the gene Pdx1 are known to have no pancreas. Because of this, they appear normal at birth, but soon die. After alteration of the gene, ES or iPS cells from another normal mouse are inserted into the embryo of an organ-depleted mouse. This mouse and the ES cells grow cooperatively to form a murine chimera.

Namely, this mouse has a mixture of host-derived cells and pluripotent stem cell-derived cells. However, because this original Pdx1-KO mouse cannot produce a pancreas, all cells are considered to be derived from ES cells (or iPS cells) where the pancreas is concerned.

In this connection, we attempted to transplant ES cells marked with green fluorescent protein (GFP) into the blastocyst of Pdx1 deficient mouse. Basically, as mentioned previously, we assumed that an ES cell-derived (or iPS cell-derived) pancreas might be created. In actuality, as we expected, a pancreas was present in the abdomen of the Pdx1-KO mouse that should have been lacking this organ, and the pancreas was homogeneously positive for GFP under fluorescent light. Namely, most of the cells constituting the pancreas were found to be iPS cell-derived. The tissue sections of this pancreas also clearly showed that the whole pancreas was GFP-positive. On the other hand, in control mice which were whole body chimeras, the pancreas consisted of coexisting host-derived cells and iPS cell-derived cells. This was also apparent histologically.

Namely, it was demonstrated that iPS cell-derived or ES cell-derived organs could be created according to this method, as we expected.[14]

Possibility of Application in Regeneration of Various Organs

Considering whether the same principle can be applied to other organs, we used KO mice with depletion of the gene Sall-1, which are kidney-deficient mice, as the host animals. Pluripotent stem cells were similarly inserted into the embryos of Sall-1-KO mice. We expected that these mice would develop into whole body chimeras, but the kidneys should consist entirely of ES cell-derived or iPS cell-derived cells.

In the actual experiment, kidneys that would inevitably have been absent otherwise were created as expected, and they were properly connected with urinary ducts. The bladder filled and distended properly, showing that the kidneys were functioning adequately and producing urine.

Under fluorescent light, the kidneys alone generated red fluorescence, while the urinary ducts and bladder, which were chimera organs, produced only mild fluorescence. Thus, it was apparent that the kidneys consisted almost entirely of ES cells.

When the same experiment was carried out using GFP-marked iPS cells, it was found that there were kidneys, that would inevitably have been absent otherwise, and that the bladder was filled with urine. Under fluorescent light, although the urinary ducts and bladder contained some iPS cell-derived cells, the kidneys

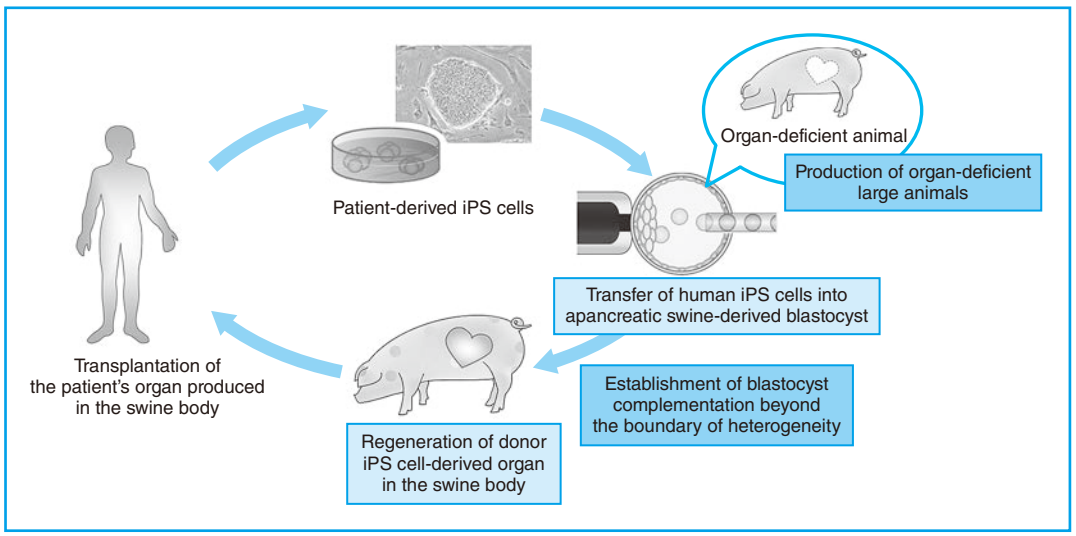


Fig. 12 Can a human organ be produced in an animal body?

were found to be totally composed of iPS cell-derived cells. Thus, based on the fundamental principle, this method proved to be applicable not only to the pancreas but also to regeneration of various other organs.[15]

Application Between Swine and Humans

I have so far described procedures between mice and mice. Naturally, without modification, this technique is not applicable to humans. Our vision related to this technique is as follows. Suppose there is a patient with heart failure who is barely kept alive with the aid of an artificial heart. We can produce iPS cells from this patient, and in the meantime a swine model lacking a heart is produced. Then, iPS cells are inserted into the embryo of the swine. If the principle for applying this technique between mice and mice works between swine and humans, piglets with human hearts should be born in due course. When the swine has grown to an adequate size, the heart could be harvested and transplanted into the patient.

Although produced in the body of a swine, this heart would consist of cells derived from the patient's iPS cells. Although there may well be various many problems to overcome, this is basically transplantation of a heart consisting of the patient's own cells.

Problems in Application Between Swine and Humans

Considering these potentials, we are aiming for the realization of our plans. Reasonably, a major question is whether the aforementioned blastocyst complementation works well when crossing the interspecific boundary between humans and swine. Major questions include whether it is possible to create several swine that lack a heart or any other organ and how to keep them alive (**Fig. 12**).

Experiments in Mice and Rats

First, we tried to go beyond the interspecific boundary, using pancreas-deficient mice and rat iPS cells. The experiment was relatively simple. Rat iPS cells were transplanted into the blastocyst of a pancreas-deficient mouse. The hypothesis, or the purpose of this experiment, was that the chimera consisting of rat and mouse cells would have an iPS cell-derived pancreas. In this connection, a biologically very interesting question was whether the pancreas, if successfully created, would be the size of a rat pancreas or of a mouse pancreas.

As we wished, we did obtain such chimera animals. It was amazing that laparotomy demonstrated the presence of a pancreas in these ani-

mals that otherwise would not have had one. The pancreas showed uniform expression of GFP. Namely, it became apparent that most of the cells constituting the pancreas were derived from rat iPS cells. Nevertheless, the pancreas was found to be the size of those in mice. Thus, it was shown that at least for the pancreas, the size would probably be determined by the environment, rather than by the cells constituting the pancreas.[15]

Thus, we successfully demonstrated that blastocyst complementation can be established beyond the interspecific boundary, using mice and rats, i.e., xenogeneic animals. Next, we attempted the creation of large animals lacking an organ.

Preparation of Swine Without a Pancreas

First, animals that lack an essential organ do not survive. Therefore, whether such animals can be mass-produced is a common-sense question. Then, the next question is whether our success with small animals like mice and rats could be translated into large animals like swine, assuming that such organ-deficient animals are available. In this regard, we were challenged to carry out organ regeneration in large animals.

In experiments using mice, we had already identified the genes that control the genesis of the pancreas. The mouse genes, i.e., the construct Pdx1-Hes1, were transferred into swine to produce transgenic swine. Employing this strategy, we were successful in creating swine without a pancreas.

Because these apancreatic swine die soon after birth, we cannot breed them by mating. However, in the case of swine, somatic cell nuclear transfer is easier than in mice. Therefore, collecting sufficient somatic cells, e.g., fibroblasts, of swine fetuses and using them as donor cells for nuclear transfer allowed us to produce an unlimited amount of apancreatic swine embryos without mating.

In fact, we have a collection of many fibroblasts from apancreatic swine fetuses. When we carry out somatic cell cloning using these fibroblasts as donor nuclei, the cloned embryos obtained have the same genes as those of apancreatic swine. Therefore, the newborn swine soon die because they have no pancreas.

Success in Creation of Swine Chimeras With an Exogenous Pancreas

If swine blastocysts can be complemented by normal iPS cells or ES cells as we did in mice or rats, an embryo-derived or iPS cell-derived pancreas may be produced, allowing the blastocysts to grow and the animals to survive after birth. This is what we thought. However, unfortunately, we have not yet produced qualified ES or iPS cells that can be inserted into blastocysts to yield chimeras in any animal species except for mice and rats.

At this point, instead of using ES cells or iPS cells, we collected germ cells from normal swine embryos having a red fluorescent gene, and inserted them into blastocysts. Employing this complementation procedure, the blastocyst is basically the same as that obtained using iPS cells. Consequently, as expected, apancreatic swine fetuses that had undergone this procedure were found to have a pancreas which showed uniform red fluorescence. Thus, it was demonstrated that normal embryo-derived pancreases were produced.[16] The swine born with a pancreas grew normally to maturity. We have been keeping them for more than a year. They are still alive and in good health.

Accordingly, these swine chimeras with an exogenous pancreas show normal growth and have normal blood glucose levels, and we thus think that their pancreases are functioning normally. Therefore, we believe that pancreatic islets obtained from these swine are sufficient to be applicable to pancreatic islet transplantation. In addition, these swine that underwent blastocyst complementation had a pancreas, grew to maturity, and actually were able to be mated. Now, we have store of sperm obtained from these swine. If we use these sperm for *in vitro* fertilization or normal fertilization without nuclear transfer, half of the resultant embryos will have no ability to produce a pancreas. Therefore, we are now capable of obtaining many embryos that will not produce a pancreas. We were able to go beyond the boundary of heterogeneity and create animals devoid of an organ, even swine without a pancreas, and have established a method for mass production of such animals.

Consequently, we have so far overcome various problems, and can now say that we are ready to produce a human pancreas in the body of

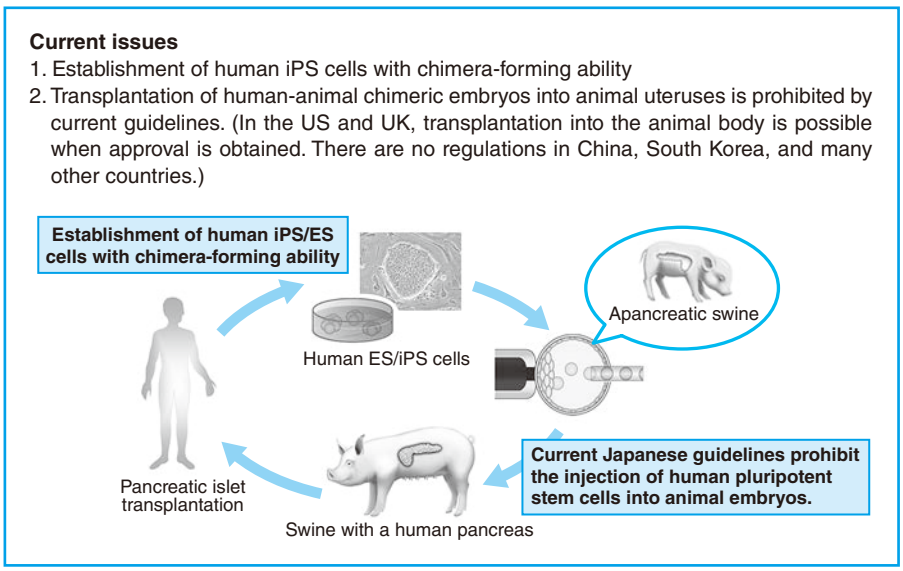


Fig. 13 The possibility of producing human organs in the bodies of heterogeneous animals has now come into sight

a swine fetus. We cannot know whether this attempt will work out as well as hoped until we conduct a trial. If this attempt turns out well, it will open the way for a future supply of pancreatic islets for severely ill patients with diabetes mellitus who are, for instance, waiting for donor organs. We expect that the supply will meet the needs of several tens of thousands of patients.

Issues Involved in Utilization of Organs From Heterogeneous Animals

We think that the possibility of producing human organs in the bodies of heterogeneous animals is coming into ever sharper focus. However, the current problem is that human iPS cells that have the capability of chimeric formation are not yet available (**Fig. 13**).

I mentioned previously that human ES and human iPS cells were produced. However, in fact, it has recently become apparent that human ES and iPS cells currently produced worldwide are slightly different from mouse ES or iPS cells; they are more differentiated pluripotent stem cells in the epiblastic stage. For example, it has been speculated that their transfer into swine blastocysts may not produce chimeras. At present, researchers worldwide are conducting studies to produce completely reprogrammed human iPS and ES cells, similar to those of mice or rats. Therefore, we think that this problem will soon be solved.

There is another problem, which is peculiar to Japan. In the current Japanese guidelines, transplantation of human pluripotent stem cells into animal embryos and culture them *in vitro* is permitted, but it is prohibited to return the embryos to the animal bodies to allow them to grow. Therefore, in actuality, this type of research cannot be carried out in Japan. In this regard, we have been requesting the Ministry of Education, Culture, Sports, Science and Technology for more than 2 years to revise the guidelines and allow us to proceed with this type of research. Currently, however, the guidelines remain unchanged, and our research is suspended for the time being.

We are faced with these two major problems. If these problems can be overcome, it may become possible in the near future for human organs to be produced using domestic animals.

For Medical Care in the 21st Century

In summary, what I have talked about so far is only a small part of the research being conducted on stem cells. We think that progress in stem cell research is astounding, and holds the promise

of major innovative changes in medical care in the 21st century. The important feature of iPS cells is that they can be produced from patients' cells. These cells have a very broad range of applications, serving as an inexhaustible source of tissues, cells, and organs as well as playing a significant role in regenerative medicine and gene and cell therapies.

In the initial stage of research, it was often said that iPS cells were dangerous because they were produced using viruses. However, the technologies for producing iPS cells have progressed markedly, and there are currently several ways to establish these cells, without using such viral vectors as those inserted into genomes. Therefore, there seems no longer to be any risk of simple insertion mutation, as was initially feared. However, cell therapy using ES or iPS cells requires very prolonged cell culture, during which various mutations may occur. The first application of these cells to actual treatment of diseases in humans requires higher safety, and we advocate the development of a method to assure this level of safety.

Finally, along with advances in embryologic engineering and progress in research on stem cells, the future success of germ cell production and the creation of human organs in individual animals, as already referred to in this communication, has certainly come into sight. We assume that a balance between medical care needs and societal consensus will be required henceforth.

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