# GMP Manufacturing of iPS Cells and Cell Bank for Regenerative Medicine

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Induced pluripotent stem cells (iPSC) can differentiate into a large variety of cells in the body. iPSC is expected to be applied in the field of regenerative medicine. Sumitomo Dainippon Pharma Co., Ltd. is now developing some iPSC derived cellular therapeutic products for treatment of nervous system and eye diseases. In this article, we report on the establishment of iPSC using Sendai virus vectors and on the construction of cell banks for clinical use under GMP compliance.

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## Introduction

Induced pluripotent stem cells (iPSC) can differentiate into various tissues and organs in the body, and are expected to be used not only for drug discovery and safety evaluation of agricultural chemicals and chemical substances, but also for regenerative medicine by administrating differentiated cells and tissues into patients (**Fig. 1**).

At Sumitomo Dainippon Pharma Co., Ltd., we have been researching regeneration of the central nervous system using semaphorin inhibitors, and in addition, we have accumulated experience with embryonic stem cells (ES cells) including differentiation technology as an aspect of safety evaluation research. Taking advantage of these backgrounds, we are now developing some iPSC derived cellular therapeutic products for treatment of nervous system and eye diseases.

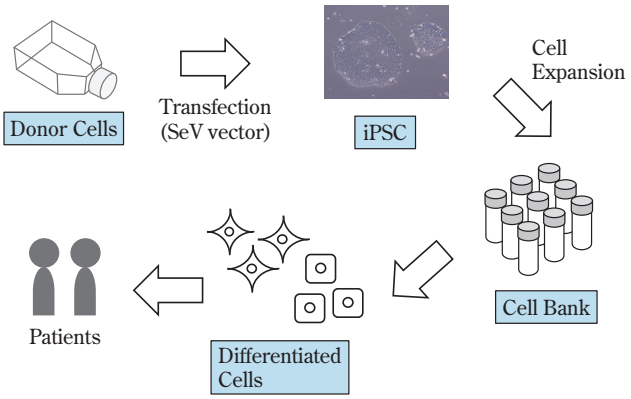


**Fig. 1** Clinical application of iPSC

In the clinical use of cells and tissues differentiated from iPSC, autologous products using iPSC derived from cells from the patient himself is an option. An alternative option is allogeneic products, using iPSC derived from cells from a healthy donor. As our initial approach to the regenerative medicine business, in consideration of long-term reproductively and manufacturing cost, we have selected allogeneic products.

In the manufacturing of cellular therapeutic products for clinical use, regulations related to good manufacturing practice (GMP), etc., must be applied, and the same is true of iPSC as the raw material of differentiated cells. From the standpoint of increasing the scale of business, we are aiming at developing cellular therapeutic products in the United States in addition to Japan, and therefore, iPSC that comply with both Japanese and U.S. regulations are necessary. In this article, we report on the establishment of allogeneic iPSC for clinical use in both Japan and the United States using the Sendai virus vector and the construction of cell banks. Thus, raw materials for stable manufacturing of high quality cellular therapeutic products have been secured.

## Donor Cells

### 1. Requirements for Donors

To establish iPS cells for use in regenerative medicine for allogeneic transplantation, starting cells must be collected from healthy donors.

The requirements for donors are specified in regulations in both Japan and the United States. In other words, items (healthy condition, medical history, etc.) that must be checked for donor candidates are stipulated in the "Standards for Biological Materials" (Notification of the Ministry of Health, Labor and Welfare No. 375, September 26, 2014) and "Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from Processing of Autologous Human Induced Pluripotent Stem (-like) Cells" (Notification No. 0907 No. 5 of the PFSB, September 7, 2012) in Japan and the Code Of Federal Regulations Title 21 Part 1271 Subpart C in the United States. In addition to these regulations, we have added some original items such as age, sex, medical history of our target diseases (including relatives) into the criteria for donors.

We used peripheral blood mononuclear cells (PBMC) separated from whole blood collected from donors as starting cells for iPSC. It was considered that the separation procedure for PBMC from the whole blood was preferably done under GMP control because PBMC will be used as starting cells for cellular therapeutic products, but we could not find an appropriate contract manufacturing organization (CMO) in Japan, and it was difficult to recruit donor candidates conforming to U.S. regulations in Japan. Therefore, we contracted the procedures from donor recruiting to PBMC separation to a major CMO in United States based on our plan.

Prior to recruiting donor candidates, we received approval of this project from ethical review boards at our company and the CMO. We were provided with voluntary written consent from the donor candidate. We received PBMC in an anonymous state from the CMO, and the iPSC were established at our company.

### 2. Viral Testing for Donors

Since cellular therapeutic products are administrated directly to the human body, they can be a cause of severe health hazards if those products are infected by viruses. When donors have a history of persistent viral infections in the past, there is a risk of viral infection in the cells even if the current health state of the donors is good; furthermore, it is difficult to perform operations for reducing viruses such as virus filtration and gamma irradiation on the cells. Therefore, confirming that donor candidates have not had persistent viral infections is important and is a requirement of both Japanese and U.S. regulations.

In terms of the viral tests that should be conducted for donor candidates, the types of viruses and test methods are stipulated in the regulations. Tests for the viruses which can be causes of severe diseases and which are distributed worldwide, such as human immunodeficiency virus (HIV) and hepatitis viruses, are required both in Japanese and U.S. regulations.

On the other hand, in terms of viruses which are not distributed worldwide, West Nile virus, for example, U.S. regulations require the test because this virus is widely distributed in the United States, but Japanese regulations suggest the necessity of the test can be determined by the history of overseas travel of donor candidates because there have been no reports of infections by this virus at present in Japan. Thus, the requirements of both regulations are not identical, but since we will use the iPSC in both Japan and the United States, even viruses stipulated by the regulations of only one of these countries were included in those to be tested.

Furthermore, in consideration of cases where test results are negative because the virus has not sufficiently proliferated in the body immediately after infection by the virus, we concluded that it was necessary to perform virus tests twice for the donor. The first test was conducted immediately before blood collection for PBMC separation, and then tests were conducted for the second time after a fixed period of time (window period). To determine the window period, the pharmacokinetics of the virus after infection and the immunological response were considered, and "Guidelines for Later Testing of Blood for Transfusion" (Japan Red Cross Society Blood Operations Division) were also referred to. PBMC derived from donors satisfying the criteria in both the tests before and after the window period were used for establishing iPS cells.

## Raw Materials

### 1. Sendai Virus Vector

To establish iPS cells from somatic cells acquired from donors, transfection of reprogramming genes are necessary to convert the cells to the early development

stage. There are various methods for gene transfection, but if transgenes and/or vectors are inserted into the host genome, there is a risk of increased carcinogenesis; therefore, it is important to avoid occurrences of this genome insertion from the standpoint of clinical uses. In addition, it is preferable that the reagents used in gene transfer and/or transgenes do not remain in the product from the standpoint of safety. As a method in accordance with these conditions, we used gene transfection using the Sendai virus vector.

In September 2014, we concluded a patent licensing agreement with ID Pharma Co., Ltd. regarding technology to produce clinical iPSC for use in human regenerative medicine through nucleus reprogramming by the Sendai virus vector and used CytoTune®-iPS 2.0LG (SeV vector)[1] to establish iPSC. This kit (SeV vector) includes three kinds of vectors as shown in **Fig. 2** (published with permission from ID Pharma Co., Ltd.). Regarding the SeV vector, when we established the iPSC, only the research grade kit was on the market; therefore, we received a supply of the clinical grade kit before it was on the market.

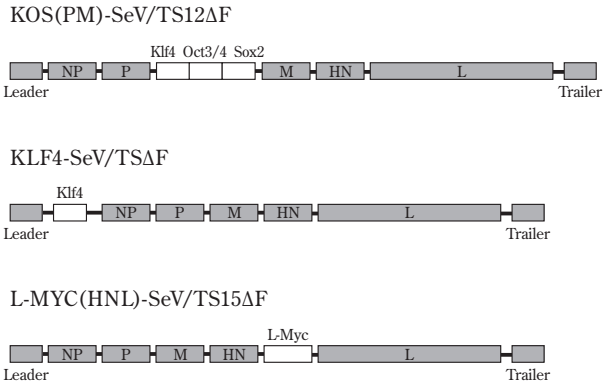


**Fig. 2** Structure of SeV vector

The SeV vector is an RNA virus vector and replicates, transcribes and translates in the cytoplasm. The transgene is not present as a DNA state in the cell, and in principle there is no integration into the genome DNA sequence of the host; therefore, it is a vector with which the risk of mutations to the host genome is small. The SeV vector is being investigated for use in gene therapy.

Furthermore, this SeV vector is a non-transmissible vector that does not produce infectious virus particles from infected cells because of the loss of the gene coding for the F protein (ΔF) necessary for infection.[2] In addition, a mutation is introduced into the polymerase related gene to inhibit genomic RNA replication at specific conditions (temperature); therefore, the residue of the transgene in the cells can be reduced by adjusting the culture conditions.[3]

## 2. Raw Material

Cellular therapeutic products are manufactured using a greater variety of raw materials (medium, factors added to medium, solution for cryopreservation, etc.) than those for conventional medical products. Since cellular therapeutic products are directly administered into the body, the product must be sterile. In the general manufacturing processes for pharmaceuticals, sterile filtration or autoclaves are used for sterilization, but it is difficult to apply these to cellular therapeutic products. Therefore, if the raw material is contaminated by microorganisms, it is practically impossible to eliminate the microorganisms during manufacturing processes. The use of raw materials that are sterile is extremely important from the standpoint of sterility assurance. Additionally, the risk of infection by mycoplasma and viruses is high for raw materials derived from animals; therefore, the absence of infection by these must be confirmed by testing. The testing for infections in these raw materials is required in the Japanese and U.S. regulations.

Thus, raw materials including starting cells (PBMC) and the SeV vector, that have been confirmed to be sterile in advance, were used in this manufacturing. Furthermore, in addition to performing tests for the absence of mycoplasma and viruses for raw materials from animals, these materials were confirmed to be compliant with Standards for Biological Materials that stipulate provisions such as a prohibition in using raw materials derived from the spinal cord, etc. which is a high risk location in ruminants, from the viewpoint of bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE).

In addition, in GMP manufacturing, it is important to confirm that there are no problems with the manufacturing environment, management systems, quality tests, etc. performed by raw material vendors, and we evaluated the vendors by audit and so on. The risk for each raw material was assessed from the evaluation results of vendors and quality tests performed by the vendors, and if necessary, additional tests were performed by us to confirm that risks were low.

### 3. Materials

The materials such as flasks and pipettes, used in manufacturing of cellular therapeutic products, directly contact the cells; therefore, appropriate quality control similar to that for raw materials is necessary. Sterility is also an important quality aspect for materials, and all of the materials used in this manufacturing were confirmed to be appropriately sterilized using gamma radiation and so on. In addition, low risk for bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) were confirmed in the same manner as for the raw materials.

All of these used in this manufacturing were disposable plastic materials. Since there is a possibility that plasticizers and other components in plastic may affect the cells, containers with eluted components that were confirmed to show no cell toxicity according to USP Class VI and Japanese Pharmacopoeia medical plastic container tests, etc., were used.

In addition, if foreign matter is mixed into the cells, it leads to a risk of the foreign matter migrating into the body; therefore, all major materials were visually checked so that no foreign matter was found and then used for manufacturing.

## Manufacturing

### 1. GMP Management

The iPSC and the cell bank were manufactured at the Cell Processing Center (CPC) at Regenerative & Cellular Medicine Kobe Center of Sumitomo Dainippon Pharma Co., Ltd. Since the cell bank is for clinical use, manufacturing was carried out under GMP control, which is regulations for manufacture and quality control of pharmaceuticals. Systems and facilities in compliance not only with Japanese GMP but also with cGMP (Code of Federal Regulations Title 21 Part 210-211), which is the GMP regulations in the United States, were constructed.

In terms of GMP for cellular therapeutic products, detailed regulations are insufficient since this is an advanced field and we also had not accumulated the experiences. Thus, we used consultations with the Pharmaceuticals and Medical Devices Agency (PMDA). Furthermore, since there is a difference in the regulatory requirements between Japan and the United States, we requested our subsidiary in the United States (Sunovion Pharmaceuticals Inc.) to participate in this activity, and also used overseas consultant opinions for reference.

To prevent microbiological contamination of cellular therapeutic products, maintaining cleanliness in the manufacturing environment is an important point in facility management. The degree of cleanliness in the CPC was managed according to Microbiological Evaluation of Processing Areas for Sterile Pharmaceutical Products, Japanese Pharmacopeia and U.S. regulations (cGMP, etc.). Safety cabinets for cellular manipulations were managed at Grade A (equivalent to ISO 14644-1 Class 5), and the environments in the cleanrooms where the safety cabinets were installed were managed at Grade B (equivalent to ISO 14644-1 Class 6).

### 2. Cell Bank

Since it is difficult to maintain homogeneity of the cells themselves in the manufacturing of cellular therapeutic products, controlling of manufacturing processes and raw materials are important for obtaining a product with constant quality. In particular, the quality of the raw material cells has a large impacts on the quality of the product, and it will be possible to repeatedly manufacture stable quality products over many years from the same raw material cells by having a large stock of them. This cell stock is called a “cell bank” and is a system that is commonly used even in conventional biopharmaceutical manufacturing. A master cell bank (MCB) is prepared from seed cells (seed stock), and furthermore, a working cell bank (WCB) is prepared from the MCB. This two-stage cell bank system where the WCB is used for manufacturing the final products is commonly used.

The cellular therapeutic products being developed by us are allogenic products, and we plan to manufacture multiple products by differentiation of iPSC. Thus, after establishing the iPSC using the SeV vector from PBMC, the MCB and WCB are prepared. Considering the manufacturing scale and the market and production scale for our cellular therapeutic products under development, it could be assumed that several hundred for each of the MCB and WCB would be necessary. Furthermore, considering storage on the level of 10 year units, cryopreserved cells are necessary. Therefore, we investigated and constructed a manufacturing process for the cell bank (**Fig. 3**) that conforms to these.

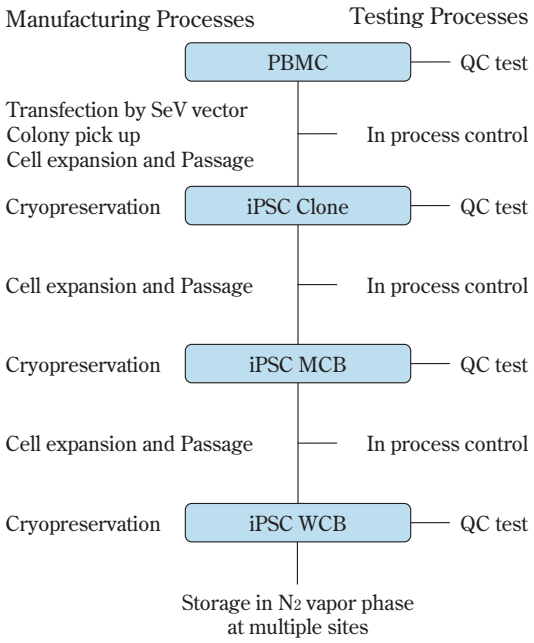


**Fig. 3** Manufacturing process of iPS cell bank

### 3. Investigation of Manufacturing Method

(1) Establishment of iPSC

Regarding the process for transfection of reprogramming genes into PBMC using the SeV vector, based on the original protocol of the SeV vector's developer, we established our original method for establishing them after going through the various investigations described in the following. CytoTune®-iPS 2.0LG, which is the SeV vector used to establish iPSC, includes three vectors as described above. There are optimal concentrations for each vector according to the type of cells and other conditions; therefore, iPSC were actually established from PBMC in multiple concentrations and the efficiency of establishing, the residual transgenes and other properties of established iPSC were compared. In addition, residual transgenes in the cells varied depending on the culture temperature; therefore, optimization investigations for culture temperature were carried out and we found sensitive changes in the residual level with extremely small differences in temperature. Thus, in GMP manufacturing of iPSC, calibrated temperature gauges were used, and the cells were cultured in a condition with even stricter control of temperature inside the incubator than in normal culturing.

In addition, even though petri dishes and plates with covers which are easily opened and closed are commonly used in research laboratories, we considered that closed cell culture containers would be preferable. Thus, we established a manufacturing process using flasks (with vent filters), which are closed system containers, for all manufacturing processes. When flasks are used, operations with normal culture equipment are difficult in some manufacturing processes; therefore, operability was assured by using equipment with suitable shapes for flasks.

From the results of process development, it was confirmed iPSC was able to be established from PBMC acquired from multiple donors. We concluded that a robust process for establishment of iPSC had been developed.

(2) Cell bank manufacturing

A manufacturing process for expanding and cryopreserving the established iPSC which met our requirements for a raw material for commercial manufacturing was investigated based on a protocol provided by the Center for iPS Cell Research and Application (CiRA), Kyoto University.

The manufacturing scale of the cell banks for iPSC is generally several tens, but since the needed manufacturing scale was 10 times that in our case, increasing scale was an important subject. In the cryopreservation process, the iPSC are frozen after being suspended in a cryopreservation solution, but during that time the cells are exposed to the cryopreservation solution; therefore, the operation needs to be conducted in as short a time as possible to reduce the effects of the components in the cryopreservation solution on the quality of the cells. When preparing a large number of cell banks, a long period of time is necessary for carrying out the freezing operation by one operator; therefore, we established a procedure with several operators working in parallel. The consistency of the quality of cells from each operator was confirmed through the verification process described in the following.

### 4. Validation and Verification of Manufacturing Process

Validation in the manufacturing of pharmaceuticals is an approach stipulated in regulations for confirming (prior confirming) that the product manufactured according to the manufacturing process has the expected quality.

Since sterility is an important quality aspect in cellular therapeutic products, validation to assure the sterile manufacturing was focused on. This validation was done by mimicking (simulating) the manufacturing

process using a dedicated medium for detecting microorganisms instead of cells and reagents to confirm that no microorganisms contaminations occurred in the operations. This validation is called process simulation testing (PST) and is a method that is commonly used for assurance of sterile manufacturing processes for pharmaceuticals. With typical sterile pharmaceuticals, only a part of the process is simulated during PST because sterile filtering is possible during the final filling process. In addition, if the final product can be sterilized, there is no need to carry out PST. However, in this manufacturing, sterile filtering and sterilization of the final product can not be carried out; therefore, all of the sterile operations over the several months from the culture of PBMC and to the cryopreservation of WCB had to be assured using PST.

PST was repeated three times, and GMP manufacturing was carried out after completing the PST.

In addition, verification is confirming (post-production confirming) that the product manufactured according to the manufacturing process has the expected quality using product that is actually manufactured and is a concept focusing specifically on the cellular therapeutic products. In this cell bank manufacturing, multiple operators carried out the cryopreserving process simultaneously in parallel; therefore, it was necessary to verify that there were no differences in the quality of cells among the operators. Thus, for verification, cells coming from each operator were sampled, and the absence of differences in quality among the operators was confirmed.

### 5. GMP Manufacturing

GMP manufacturing was carried out by the investigated and validated manufacturing process (a picture of operation in the manufacturing facility is shown in Fig. 4).

**Fig. 4** Manufacturing operation in CPC

In the establishment process of iPSC, PBMC was initially thawed and cultured, and the reprogramming genes were transferred using the SeV vector by the method described above. After gene transfection, culturing was carried out under the validated conditions, and multiple iPSC colonies were obtained. The iPSC were cloned by picking up the colonies, then expanded and cryopreserved. Quality tests and confirmation of differentiation properties, etc., were carried out on the cloned iPSC obtained, and a clone suitable as the raw material for our cellular therapeutic products under development was selected according to these results.

In the manufacturing of cell banks, culturing of the selected clone was carried out, and then MCB of iPSC was constructed by cryopreservation. Furthermore, WCB of iPSC was manufactured in the same manner from one tube of the MCB. In the production of the cell banks, the proliferation rate for the iPSC under our culture conditions was comparatively fast, and since cell density greatly affects the quality of cells, the timing for carrying out passage and cryopreservation operations was adjusted in one hour units to obtain cells with optimal quantity. During a total period of several months from the initial culture of PBMC to the cryopreservation of the WCB, the cells had to be maintained in good and sterile condition, but manufacturing was completed without occurrences of deviations that affect the quality.

The constructed MCB and WCB were stored under the vapor phase of liquid nitrogen. From the standpoint of distributing the risk of disasters, storage was distributed across multiple locations instead of storage all together in a single location.

## Quality Control

### 1. Quality Control Items

Quality control for pharmaceuticals is carried out to confirm that the pharmaceutical product has the intended efficacy and safety. The appropriate performing of quality control is a major prerequisite for pharmaceuticals.

In addition, in the quality control tests for cellular therapeutic products, not only the analytical methods used for conventional pharmaceutical, but also the methods that are not frequently used in pharmaceutical analysis, such as flow cytometry, need to be used. We worked on developing methods and control procedures for using these new technologies under GMP control.

The test items and specifications set for the cell banks are given in **Table 1**. For these settings, we referred to "Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products" (ICH Guideline Q5D, July 14, 2000, PAB/ELD Notification 873) which is an internationally harmonized guideline for the quality control of the cell bank and guidance from the International Stem Cell Banking Initiative (ISCBI).[4] We confirmed the justification of the strategy of quality control in consultation with PMDA.

**Table 1** Specification of iPS cell bank

| Test item | Specification |
|---|---|
| Cell morphology | Human iPSC |
| Identification (Cell surface marker) | Positive |
| Purity (Residual RNA from SeV vector) | Negative |
| Sterility | No growth of microorganism |
| Endotoxin | < 0.5 EU/mL |
| Mycoplasma | Negative |
| Karyotype | Normal |
| STR | Identical to the cells from donor |
| Viral tests | Negative |

We confirmed a cell surface marker unique to human iPSC by flow cytometry in addition to confirming cell morphology to identify that the cells were iPSC. In addition, we confirmed the absence or presence of the residual SeV vector and biological contamination as described in the following. Furthermore, karyotype analysis was performed to confirm the absence or presence of chromosomal abnormalities, and short tandem repeat (STR) analysis was performed to assure that other donor cells were not mixed in. Process control tests such as confirmation of sterility were performed at various points in the manufacturing process. An example of the test results is shown in **Fig. 5**. To confirm appropriateness of test methods from the standpoint of the specificity, precision, sensitivity, etc., analytical method validation was carried out as necessary. For the validation, we referred to "Validation of Analytical Procedures: Text (Items to Be Implemented)" ICH Guidelines Q2A, October 28, 1997, Notification No. 338) and "Validation Analytic Procedures: Methodology (Methods to Be Implemented)" (ICH Guideline Q2B, October 28, 1997, PAB/ELD Notification No. 338).

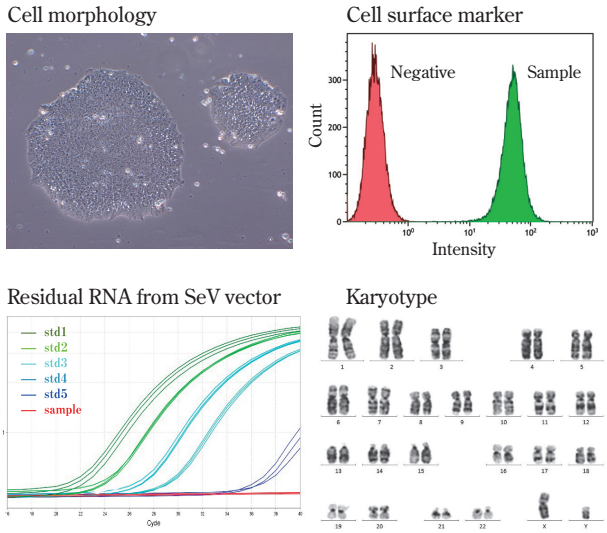


**Fig. 5** Testing results (example) of iPS cell bank

## 2. Residual Vector Evaluation

The SeV vector used in establishing the iPSC has an ability of transfecting the reprogramming gene into the cell; therefore, if the vector remains in the established iPSC, it could be a safety risk. From this standpoint, it is important to assure that the vector does not remain in the iPSC.

We considered the possibility of the remaining SeV vector itself, which was added in the gene transfection process, from the stability of the vector. In our evaluating of the stability of the SeV vector under the iPSC culturing conditions, inactivation to the level at which infectious ability was not detected in several days was confirmed. The total time for the actual manufacturing process reaches several months, and the SeV vector is only used in the initial step of manufacturing. Furthermore, in consideration of the dilution by culture medium exchange during the manufacturing process, the risk of the remaining SeV vector can be thought of as theoretically negligible.

Furthermore, in terms of the residual SeV vector inside the infected cells, the SeV vector is designed as a temperature sensitive vector that disappears at the cell culture temperature to reduce the residual risk. To show the absence of residual SeV vector inside the cells by actual data, we confirmed the absence of infectious factors in the iPS cells using a cell based assay with which the detectability of the SeV vector had been confirmed.

In addition, the gene (RNA) transferred into the cell with the SeV vector is one that is not originally

present in the human body; therefore, the residual level in the cells must be controlled from the standpoint of safety. A limiting test method by reverse transcription polymerase chain reaction (RT-PCR) was developed for evaluating the residual level of the gene (RNA) transferred into the cells with high sensitivity. We confirmed that the residual gene was not detected (below detection limits) in the cell bank by the validated method.

### 3. Microbiological Contamination Tests

The absence of microbiological contamination is an important quality aspect for cellular therapeutic products; therefore, sterility tests, endotoxin tests and mycoplasma tests were conducted for the cell banks. In addition to the virus tests for donors described above, comprehensive tests of the cell bank for virus negativity is stipulated in "Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" (ICH Guideline Q5A, February 22, 2000, PAB/ELD Notification 329). With reference to this guideline, we carried out nonspecific tests to confirm the absence of infection using animals and indicator cells in addition to evaluating more than ten kinds of specific viruses by PCR.

### 4. Differentiation Potential

To show that the established iPSC have pluripotent properties, we confirmed differentiation into three germ layers (endoderm, mesoderm and ectoderm) with *in vitro* evaluation systems. Furthermore, since the cell banks are planned for use as raw material cells for various products, we confirmed that actual differentiation to our target cells (**Fig. 6**).

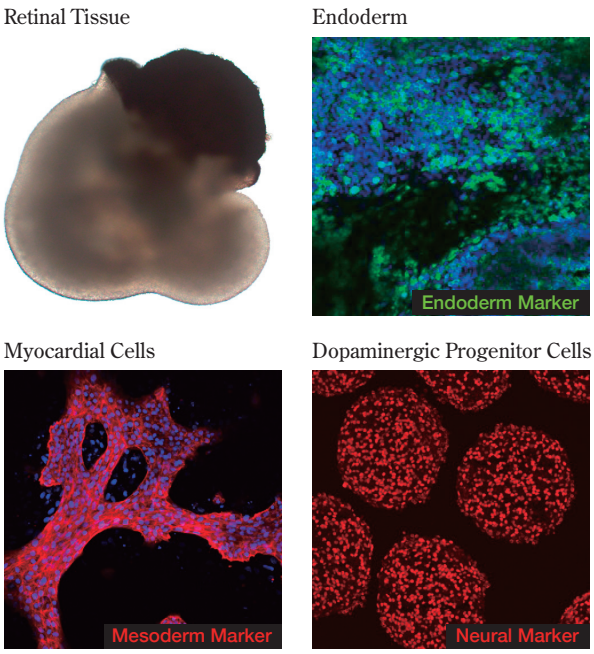


**Fig. 6** Pluripotency of iPSC

## Summary

We established iPSC and constructed cell banks which can be clinically used in both Japan and the United States, under GMP control. We believe that the iPSC and the cell banks will be our cornerstone for regenerative medicines and will lead to contributions to society through the providing of innovative regenerative medicines to meet unmet medical needs.

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