controlled conditions [6]. High process reproducibility can be achieved by automation, and several effective automatic cell culture systems have been reported [7-12]. These automated platforms have the potential to provide cost-effective, large-scale expansion of stem/progenitor cells with consistent phenotype for clinical use and improved operational safety [13]. Progress in robot platforms for cell culture has resulted in several prototypes to implement large-scale expansion and harvesting of stem/progenitor cells with yield and phenotypic reproducibility. An automated culture system by "The Automation Partnership Biosystems (TAP Biosystems)" has cultivated human embryonic stem cells and bone marrow-derived cells [14,15]. Kawasaki Heavy Industries (Tokyo, Japan) has created AutoCulture® (Additional file 1), which can automate many manual steps in cell culture, including media exchange, centrifugation of cells, splitting and passaging, and recording of cell morphology (Figure 1A). To the best of our knowledge, no cell products obtained from an automated culture apparatus have actually been transplanted into humans for regenerative therapy.

Our institute recently completed a phase I clinical trial using autologous cardiac stem cells (CSCs) isolated by manual cell culture techniques to treat ischemic cardiomyopathy [16]. The trial is registered in the Japanese government database for clinical trials using human stem cells and ClinicalTrials.gov, which is a world-wide registry and results database for clinical trials involving humans, as AutoLogous Human CArdiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy (ALCADIA; Identifier: NCT00981006). CSCs are manually cultivated by a single experienced investigator for approximately 1 month to minimize variability of the final cell products. To advance this trial from a single-center to a multi-center randomized trial, we evaluated AutoCulture® by comparing the growth rate, morphology, and phenotype of cells cultivated using this method with those of manually cultured CSCs.

Results

Cellular morphology and growth

Calculations based on the net cell number and doubling time obtained in the ALCADIA trial (Additional file 2)

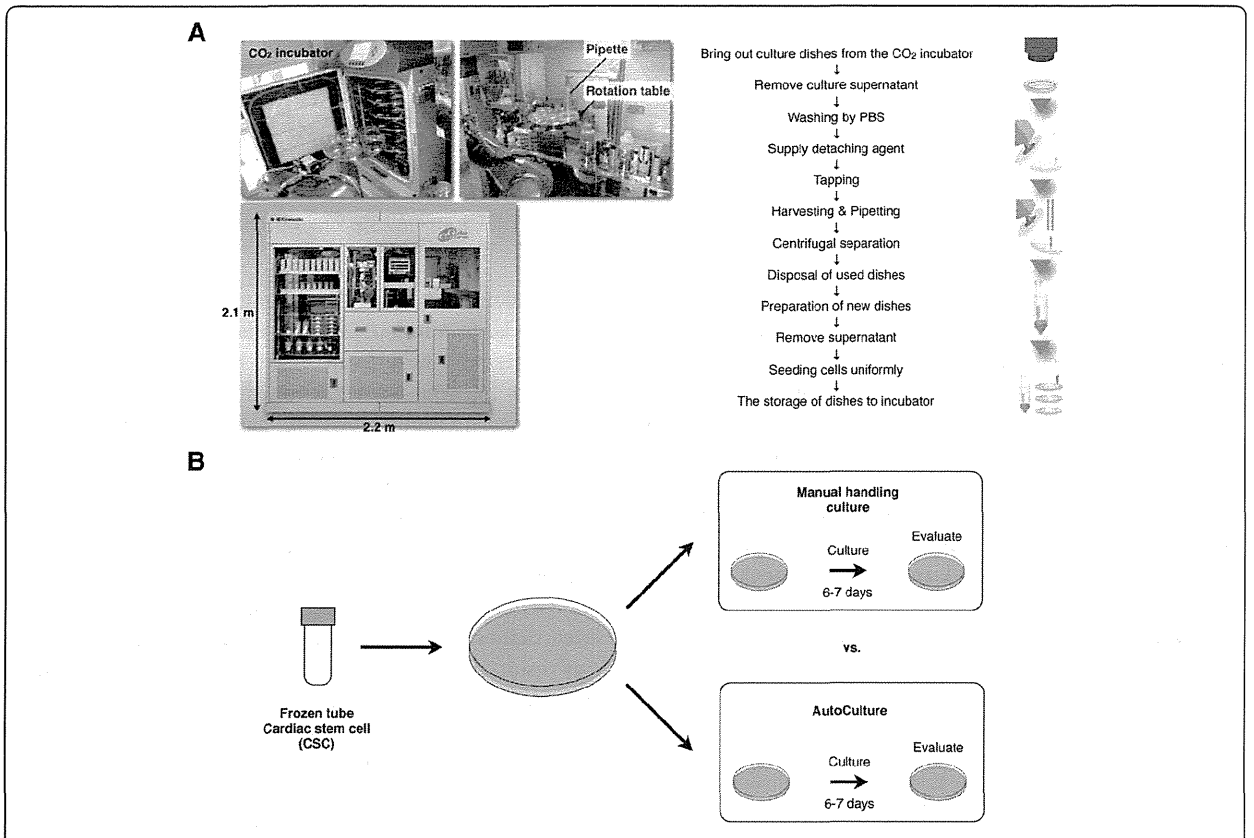

Figure 1 Process maps showing the key steps in human cardiac stem cell culture using manual methods and the AutoCulture® system. **(A)** The automated culture system AutoCulture® by Kawasaki Heavy Industries (left) can automate every step of manual cell culture under current good manufacturing practice (cGMP) grade. **(B)** Schematic representation of the experiment. Human cardiac stem cells (CSCs) were thawed and split into 2 dishes for either manual culture or automated culture using AutoCulture®.

indicated that a culture duration of 2 weeks was sufficient to obtain the appropriate cell number for clinical trial when cells after the second passage (P2) were used as the starting material. Identically seeded culture plates were maintained manually or by automation using AutoCulture® (Figure 1B). The morphology of CSCs cultured using the automated system was similar to that of manually cultured CSCs on day 7 and 14 after seeding (Figure 2A). Under both the conditions, the cells were of similar size, exhibited a low nucleus/cytoplasm ratio, and had a spindle-like shape. In addition, the growth rate was not significantly different, as indicated by cell counts at passage (Figure 2B). Trypan blue staining revealed no significant difference in cell viability between the culture methods. Moreover, both the methods effectively washed out the cells, as indicated by the paucity of adherent cells on discarded culture dishes (data not shown). These results suggest that manual passage was effectively replicated using AutoCulture®.

Gene expression

To investigate the gene expression profiles, RT-PCR analysis was performed according to the shipping criteria for cultivated cells in the current clinical trial (ALCADIA). We examined expression levels of the pluripotency related genes *NANOG*, *OCT4*, *SOX2*, and *REX1* and 2 transcription factor genes involved in cardiomyocyte development, *NKX2.5* and *GATA4* (Figure 2C). The stem cell markers *OCT4*, *REX1*, and *GATA4* were expressed by both cell populations; however, neither *NANOG* nor *NKX2.5* expression was detectable. Moreover, expression levels were not significantly different between the 2 groups on either day 7 or day 14.

Cell surface marker expression profiles

Cell surface markers indicative of CSCs and other phenotypes were detected by fluorescence-activated cell sorting (FACS) (Figure 3A). Under both the culture conditions, the cells were positive for the mesenchymal stem cell (MSC) markers CD29 and CD90 and the vascular endothelial marker CD105 and negative for the hematopoietic lineage marker CD45 and MHC class II. In addition, fluorescent intensities measured by FACS were similar for all positive markers, indicating that equal proportions of cells in both the populations expressed these proteins. Moreover, almost all the cells were CD29 positive, whereas at least 2 populations were distinguished on the basis of CD90 expression. Furthermore, STRO-1, which is expressed by mesenchymal stem cells in the bone marrow, was negative in both the populations. Although the surface expression profiles of CSCs and bone marrow-derived stem cells overlap, STRO-1 expression can discriminate cardiac MSCs from bone marrow-derived MSCs.

Surface glycan expression profile by lectin microarray analysis

Recently, glycan expression profiling has been reported to be an effective cell validation tool to complement phenotype analysis by epigenetic and gene expression analyses

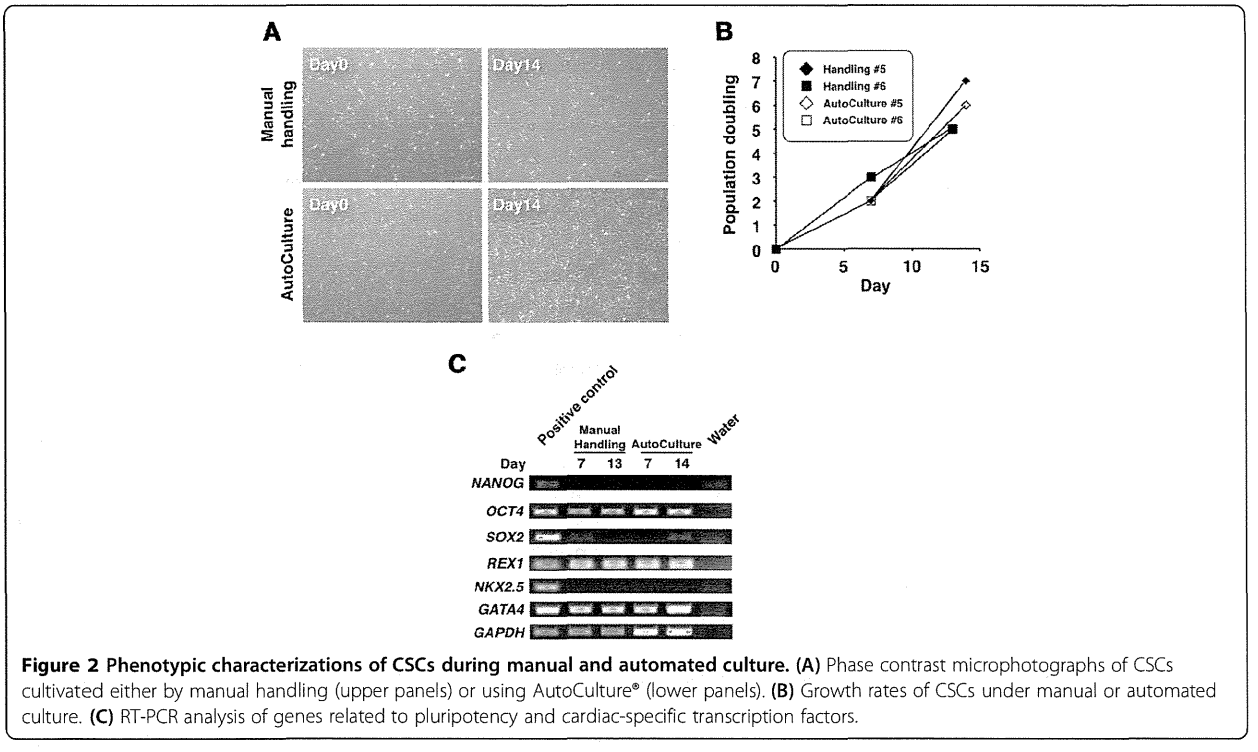

Figure 2 Phenotypic characterizations of CSCs during manual and automated culture. (A) Phase contrast microphotographs of CSCs cultivated either by manual handling (upper panels) or using AutoCulture® (lower panels). **(B)** Growth rates of CSCs under manual or automated culture. **(C)** RT-PCR analysis of genes related to pluripotency and cardiac-specific transcription factors.

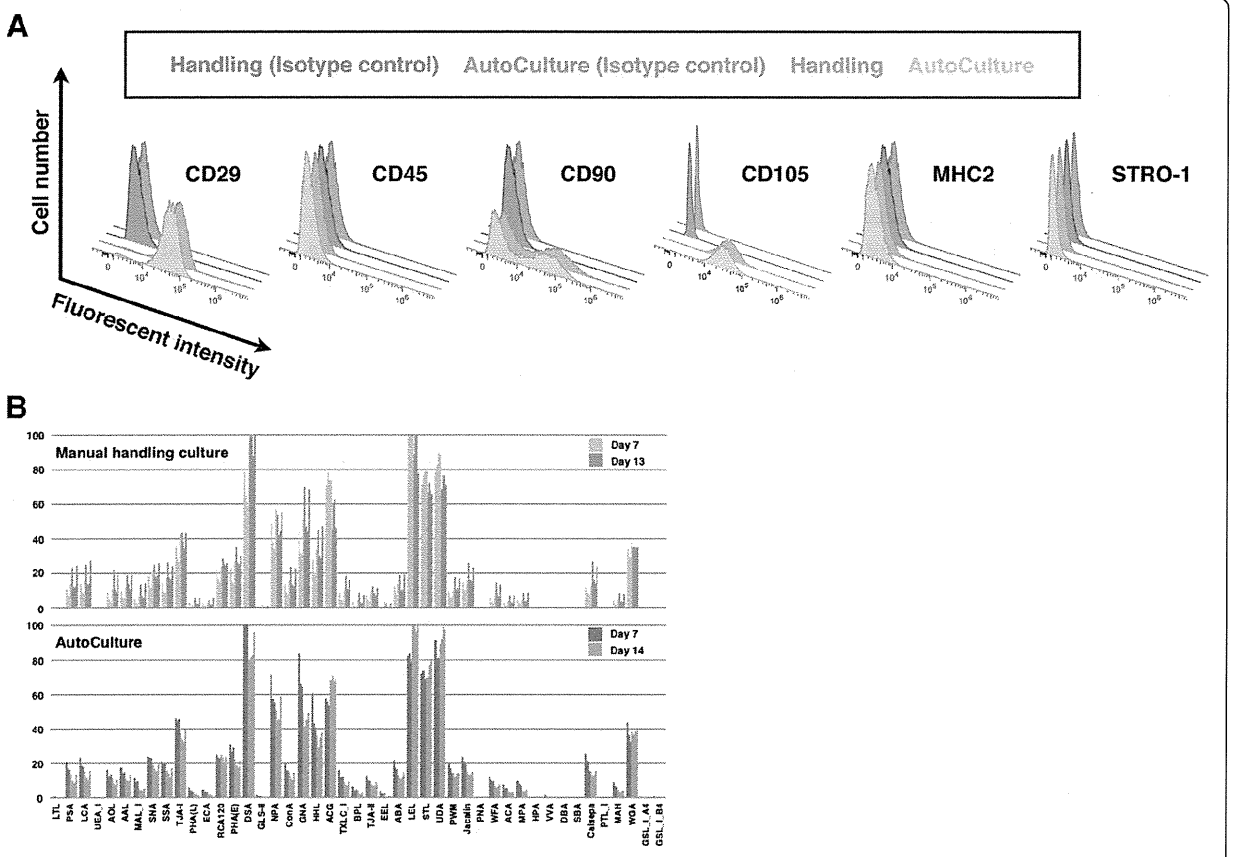

Figure 3 Analysis of cell surface protein and glycan expression in CSCs expanded by manual culture or AutoCulture®. (A) Cells were stained with fluorescence-conjugated primary antibodies and analyzed for surface expression by fluorescence-activated cell sorting. **(B)** Lectin microarray analysis of manual cultures and cells maintained using AutoCulture® on day 7 and day 13/14. The glycan profiles showed little difference between the two culture groups at any time.

[17]. These lectin profiles showed similar patterns, and no significant differences in expression intensities were observed between the 2 culture groups on either day 7 or day 13/14 after seeding (Figure 3B). The washing process used to harvest adherent cells may have profound effects on the cell surface structure and expression. CSCs harvested from the AutoCulture® system exhibited similar surface expression profiles and overall viability to those cultured manually.

Analysis of array comparative genomic hybridization (aCGH) and microarray

To detect genomic DNA mutations on AutoCulture®, we performed aCGH analysis (Agilent technologies) on day 7 and day 14 and compared them with the tissue derived from human right atrial appendage (RAA) (Figure 4A). There were no differences in genomic DNA mutation between CSCs in AutoCulture® and RAA. To investigate the global gene expression profile changes between CSCs in manual culture and CSCs in AutoCulture®, we performed a pairwise comparison of gene expression microarray data using NIA array analysis [18]. The results revealed a similar gene pattern between them (Figure 4B, Additional file 3). The "Symbol" of 162 gene probes was left blank in 258 overexpressed gene probes.

Discussion

Cell-based regenerative medicine is still in the early stages of development [19,20]. The quality of cells for transplant depends on the ability of skilled personnel to isolate, expand, and harvest cultured cells. For consistency of cell yield and phenotype, it is imperative that methodological consistency is strictly maintained. Automation greatly enhances the consistency of culture conditions and may thus reduce the variability in cell quality that is one of the great impediments to the widespread application of cell-based therapy till date. In this study, we used the automated cell culture system AutoCulture® to expand human CSCs isolated from the RAA for use in the ALCADIA clinical trial designed to assess the safety of cell-based therapy for patients with ischemic cardiomyopathy. RAA-derived cells containing CSCs exhibited similar growth rates and gene

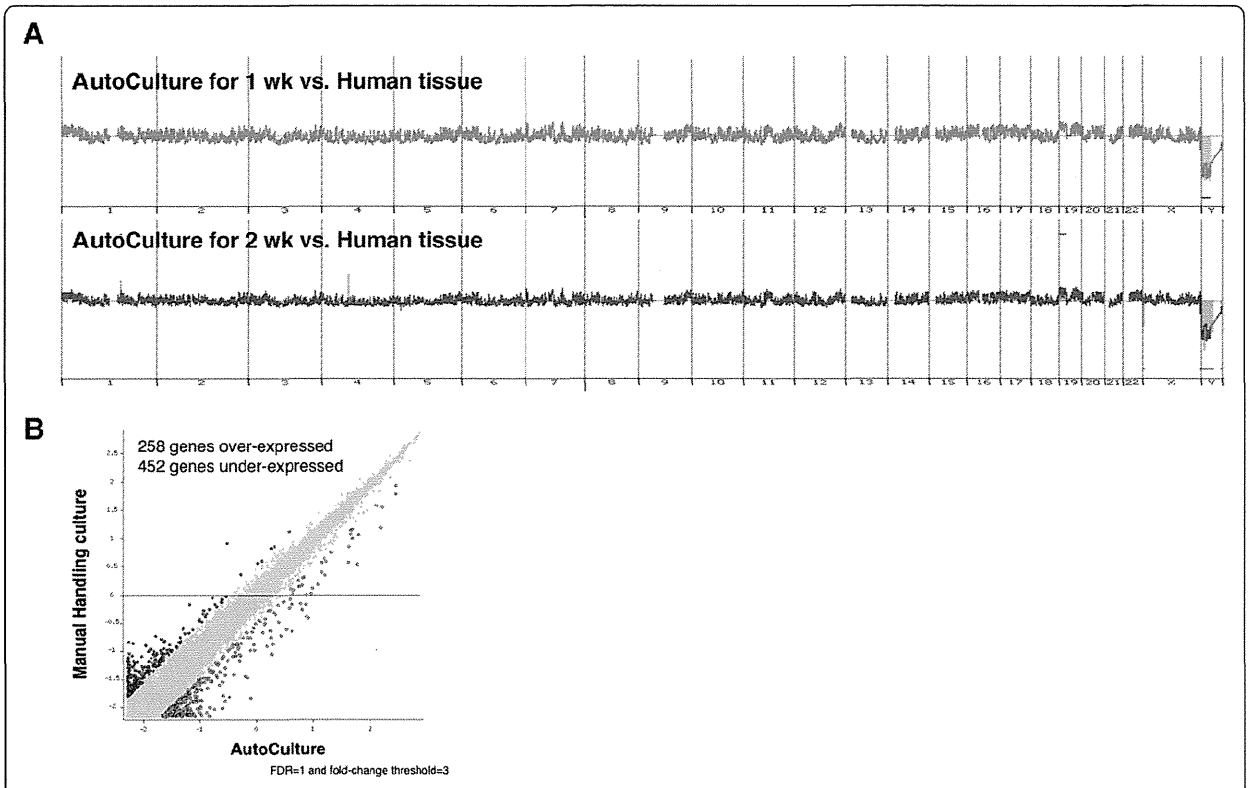

Figure 4 Global analysis of gene expression and genomic DNA mutations. (A) Comparison of array comparative genomic hybridization analysis. Human gDNA (from RAA) was compared to gDNA extracted from CSCs cultivated using AutoCulture® after 1 and 2 weeks using CGH arrays (arrays G4413A). **(B)** Microarray analysis of gene expression profiles. The pairwise scatter plot presents differences in gene expression on day 7 after seeding between manually cultured CSCs and those cultured using AutoCulture®. Grey dots represent transcripts with a subthreshold difference in expression. Red or green dots indicate those with at least a 3-fold difference in expression levels between the two groups. Gene expression levels are depicted on a log2 scale. The number of differentially expressed genes is indicated above.

expression profiles between manual and automated cultures. Thus, the AutoCulture® system effectively replicated manual culture and demonstrated scalability and stability in addition to safety and cost-effectiveness. Indeed, we found no significant differences in phenotype between the two culture methods. Cells in both the populations had similar morphologies, mean growth rates, and expression levels of genes associated with pluripotency and the mesenchymal lineage. In addition, the surface glycan profile was virtually identical, while aCGH analysis revealed no difference in genomic DNA mutation frequency. Finally, the approximately 41,000-probe Agilent Whole Human Genome Microarray chip G4112F showed that only approximately 1% of transcripts measured were significantly under- or overexpressed. The successful transfer of manual to automated cell culture may be attributable to the high flexibility of the machine, which can faithfully copy every step and condition, including media changes, splitting, and passaging in a controlled environment.

AutoCulture® is an all-in-one automated cell culture system consisting of robot arms, tube and flask decappers, flask holders, flask tappers, media pumps, a pipette head, a centrifugal separator, a rotating plate, and a CO_2 incubator. In addition to media change and passage, it permits routine observation. To automate these culture steps, it is necessary to program the humidity, temperature, volume and flow of liquid, and robot arm motion that transfers flasks from or into the CO_2 incubator or flask holder. Another automated cell culture platform, TAP CompacT, was also shown to be an effective system for culture of adherent cells by the Healthcare Engineering group [14]. However, the lack of a centrifugal separator in that system may result in differences between the manual and automated processes, possibly explaining why automation resulted in a smaller population of STRO-1+ cells and overall lower cell yield after the first passage [21]. STRO-1 expression is not a necessary or specific marker for stem or progenitor cells, and somatic stem cells may be more resistant to nutritional and chemical stress [22]. Residual trypsin in the culture media may have adversely affected the survival of differentiated cells, but it is not clear whether stem or progenitor cells can survive or not. On the other hand, the AutoCulture® system efficiently removes trypsin/EDTA by washing and centrifugation. There were no significant

differences in the surface marker expression profile or the mean rate of proliferation between these cells and those maintained manually, strongly suggesting that both populations of RAA-derived CSCs contain equal properties.

The AutoCulture® system can save labor and costs by expanding the scale of production and maintaining uniformity of results. In addition, this system can simultaneously cultivate different cells without cross-contamination because it can be equipped with a connecting hatch to multiple CO_2 incubators. Large-scale production and multisample cell culture capacity for cell transplantation may be a prerequisite for commercialization of cell products under current good manufacturing practice (cGMP) grade. Production methods for cell therapy should be designed to ensure that the end product is standardized and safe. cGMP is a quality assurance system that ensures that the cell product meets preset specifications with minimal lot-to-lot variability [23]. It requires traceability of raw materials used in cell culture and validated standard operating procedures (SOPs) throughout the process [24,25]. Current good tissue practice (cGTP) is intended to prevent human cells, tissues, and cellular and tissue-based products from contamination by infectious disease agents and to ensure that these cells and tissues maintain their integrity and function. The controlled environment of a carefully designed, constructed, validated, and maintained clean room will minimize the risks of environmental contamination and decrease the possibility of cross-contamination [26]. Based on cGMP, aseptic handling and filling of raw materials should be performed in a grade A environment (class 100) with a grade B background (class 1,000). Clean room disciplines, gowning procedures, cleaning programs, and maintenance of air handling units are included in SOPs. Environmental monitoring is essential in clean room quality control. Proper cleaning, maintenance, repair, and attire are major issues for cGMP [27].

Construction and maintenance of a cGMP facility is so expensive that it may be difficult to conform to these standards on a large scale without automation. Unlike manual culture, the robots enabled the environment in the cell culture cabinet to be completely separated from the external environment. Moreover, automated cell culture machines can be equipped with cleaning and monitoring systems to prevent contamination by microorganisms and cross-contamination by other cell types cultured in tandem. These properties may meet the stringent conditions for a human cell processing facility while reducing both construction and maintenance costs.

In Japan, the regulatory path of a regenerative cell therapy using this automated machine will be to obtain an approval for the end products, such as cells or tissues, based on the new guidelines and philosophy at an initial phase. An important requirement for obtaining approval is publication of the safety and reliability of the machine to produce the final biological products in a peer-reviewed journal. The similar properties of cell products between those obtained by machine and those obtained by manual culture, as demonstrated in this study, could support approval of a clinical trial using this machine, which is currently being planned.

Conclusion

AutoCulture® is one of the best candidates to solve the problems inherent in large-scale production and harvesting of human cells for clinical applications. The automated cell processing system can reproduce many complex operations performed by professional staff and can maintain multiple cell lines automatically. Thus, this automation system will be a powerful tool for both clinical trials exploring the potential of autologous or allogeneic cell-based regeneration therapies and for the commercialization.

Methods

Isolation of human CSCs containing atrial appendage

After this study was approved by the ethics committees of Tokyo Metropolitan Geriatric Hospital (ID: #220106), human cardiac tissue samples from RAA were surgically excised from 7 patients (60–75 years old) during cardiac surgery. All patients provided written informed consent. A cell population containing CSCs was acquired according to the current protocol for ALCADIA [28]. In brief, the tissue fragments were cut into 5 × 5-mm pieces and incubated with 0.2% collagenase type II and 0.1% DNase I (Worthington Biochemicals) at 37°C for 30 min. The cells were cultivated in a basic culture medium of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and 40 ng/ml basic fibroblast growth factor (bFGF). The cells were seeded in 60-mm dishes coated with collagen type I. The cultured cells were passaged twice, harvested, and frozen until used in this experiment. P2 cell population was utilized as the starting material for this comparison experiment.

Cell expansion and harvesting

After thawing, the cells derived from the human atrium were seeded at 1×10^5 cells per 100-mm culture dish and cultivated for 5–7 days. The cells were split at 1:10 at 80%–90% confluence. The basic culture medium was replaced every 3 or 4 days. For automated culture, we used the same lot of CSCs. After seeding, the culture dishes were placed in the AutoCulture® chamber and transferred into the internal CO_2 incubator by the robot arm (Figure 1A, Additional file 4). For media replacement, the robot arm retrieved the culture dishes from the incubator and set them on a rotating table. The dish covers were removed by the robot arm, a specified amount of medium was discarded, fresh medium was supplied by a new pipette, the covers were returned,

and the culture dishes was transferred back to the CO_2 incubator. For passage, the old medium was removed and DPBS was pipetted onto the dishes under gentle shaking. After washing in DPBS, AutoCulture® supplied trypsin, oscillated the culture dishes, and returned them to the CO_2 incubator for a 5-min incubation. Following this, the robot arm moved the culture dishes onto the rotation table, added a prespecified volume of the basic culture media, and transferred the cell suspension from each dish to a separate 50-ml centrifuge tube. The cell suspension was centrifuged at 200 × *g* for 5 min at room temperature, and the supernatant was discarded. Fresh basic culture medium was supplied to the cell pellet, which was then resuspended. The washed cell suspension was subcultured at approximately 1:10 onto new culture dishes and returned to the CO_2 incubator.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell populations containing CSCs, from human iPSCs, and raw human heart tissue samples using the RNeasy Plus Mini Kit (QIAGEN) as positive/negative control. Total RNA from human iPSCs and the human heart (Clontech Laboratories) was used as the positive control for each primer. Total RNA (500 ng per reaction) was converted to cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol. Primers for the cardiac-specific transcription factors *NKX2.5* and *GATA4*; the stem cell markers *NANOG*, *OCT3/4*, *SOX2*, and *REX1*; and the housekeeping gene *GAPDH* were obtained from PrimerBank (Additional file 5).

Flow cytometric analysis

The cells (1×10^6 per reaction) were stained in autoMACS Running Buffer (Miltenyi Biotec.) with fluorescence-conjugated primary antibodies for 30 min at 4°C. The cells were then analyzed on the Attune Acoustic Focusing Cytometer (Applied Biosystem), and the data were analyzed using FlowJo 8.8.7 software (TOMY Digital Biology). Antibodies used for phenotyping included anti-human CD29-PE, CD90-PE, CD105-FITC, STRO-1-FITC, CD45-PE, and MHC class II-PE. Isotype controls were FITC-conjugated mouse IgG_1, PE-conjugated mouse IgG_1, and FITC-conjugated mouse IgM.

Lectin microarray analysis

Proteins were extracted from each cell population in hydrophobic and hydrophilic fractions using the CelLytic MEM Protein Extraction Kit (Sigma-Aldrich), as described previously [29]. Lectin microarray analysis was performed as described previously, with only minor modifications [30]. The glycoprotein (200 ng) was labeled with Cy3 monoreactive dye (GE Healthcare) in DPBS containing 0.5% Triton X-100 (PBSTx) at room temperature for 1 h. The Cy3-labeled glycoprotein solution (60 μl) was applied to the LecChip (GP Bioscience), which has triplicate spots specific for 45 lectins on each glass slide. An evanescent-field fluorescence scanner (GlycoStationTM Reader) was used to analyze the LecChip. All data were analyzed with GlycoStationTM Tools Signal Capture 1.0 and GlycoStationTM Tools Pro 1.0 software (GP Bioscience). To expand the dynamic range, the data were subjected to a gain-merging procedure, and the merged data were then normalized with max-normalization, as described previously [29].

aCGH analysis

Genomic DNA from the heart tissue and cultured cells was isolated using the DNeasy Blood & Tissue Kit (QIAGEN). Labeled test and reference DNAs were combined, denatured, preannealed with Cot-1 DNA (Invitrogen) and blocking agent, and then hybridized to the arrays (SurePrint G3 Human CGH Microarray 2x400K, Agilent Technologies). After hybridization and washing, the arrays were scanned at 3-μm resolution using an Agilent G2505C scanner. Images were analyzed with Feature Extraction software 10.7.3.1 (Agilent Technologies) using the CGH 107 Sep09 protocol for background subtraction and normalization.

Gene expression analysis

Gene expression analysis was performed using the Agilent Whole Human Genome Microarray chip G4112F (Agilent Technologies), which contains >41,000 probes. Raw data were normalized and analyzed by GeneSpring GX11 software (Silicon Genetics). Pairwise scatter plot analysis of the global gene expression profiles of both manually cultured cells and autocultured cells was performed on day 7 after thawing. The number of differentially expressed genes is indicated over each scatter plot. The NIA Array [18] web tool was used for pairwise scatter plot analysis. Gene expression microarray data have been submitted under accession number GSE 44032. Analysis of microarray experiments was conducted using the Aberration Detection Method-2 statistical algorithm (Agilent Technologies) on the basis of the combined log2 ratios at a threshold of 6.0. The data were centralized, and calls with average log2 ratios <0.3219 were filtered to exclude false positives.

Additional files

Additional file 1: Document 1. Specialization of the automated cell processing machine (Auto Culture®).

Additional file 2: Document 2. Quantitative cellular aspects for ALCADIA clinical trial.

Additional file 3: Table S1. Results of microarray analysis of CSCs in manual culture and AutoCulture®. To investigate the differences in global gene expression profile between CSCs in manual culture and CSCs in AutoCulture®, we performed a pairwise comparison of gene expression

microarray data using NIA array analysis. The results revealed similar gene expression patterns between them.

Additional file 4: Movie 1. AutoCulture®.Movie of the culture robot in AutoCulture®.

Additional file 5: Table S2. RT-PCR primer sequences. RT-PCR primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Abbreviation

CSC: Cardiac stem cells; FACS: Fluorescence-activated cell sorting; MSCs: Mesenchymal stem cells; aCGH: Array comparative genomic hybridization; RAA: Right atrial appendage; cGMP: Current good manufacturing practice; SOPs: Standard operating procedures; cGTP: Current good tissue practice; bFGF: Basic fibroblast growth factor.

Competing interests

DK, MYI, KM, TK, YI, MT, AU and SG declare that they have no competing interests. KW and TS are employees of Kawasaki Heavy Industries, Ltd.

Authors' contributions

DK, MT, AU, and SG designed the research; DK, KW, YI, KM, MYI, and performed the experiments; DK, MT, and SG analyzed the data; and DK, TK, YI, and SG wrote the manuscript. All authors read and approved the final manuscript.

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Current Topics

Stem Cell Research for Regenerative Medicine/Personalized Medicine

Tumorigenicity Studies for Human Pluripotent Stem Cell-Derived Products

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Human pluripotent stem cells (hPSCs), *i.e.* human embryonic stem cells and human induced pluripotent stem cells, are able to self-renew and differentiate into multiple cell types. Because of these abilities, numerous attempts have been made to utilize hPSCs in regenerative medicine/cell therapy. hPSCs are, however, also tumorigenic, that is, they can give rise to the progressive growth of tumor nodules in immunologically unresponsive animals. Therefore, assessing and managing the tumorigenicity of all final products is essential in order to prevent ectopic tissue formation, tumor development, and/or malignant transformation elicited by residual pluripotent stem cells after implantation. No detailed guideline for the tumorigenicity testing of hPSC-derived products has yet been issued for regenerative medicine/cell therapy, despite the urgent necessity. Here, we describe the current situations and issues related to the tumorigenicity testing of hPSC-derived products and we review the advantages and disadvantages of several types of tumorigenicity-associated tests. We also refer to important considerations in the execution and design of specific studies to monitor the tumorigenicity of hPSC-derived products.

Key words pluripotent stem cell; embryonic stem cell; induced pluripotent stem cell; tumorigenicity; regenerative medicine; cell therapy

1. INTRODUCTION

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have two abilities: 1) pluripotency, which is the ability to differentiate into a variety of cells, and 2) self-renewal, which is the ability to undergo numerous cell division cycles while maintaining their cellular identity. Because of these two characteristics, they are expected to provide new sources for the robust and continuous production of a variety of cells and tissues for use in regenerative medicine/cell therapy. Additionally, human iPSCs (hiPSCs) offer a possible solution to the ethical problems and immune rejection of cell products derived from human ESCs (hESCs), thus creating novel avenues for individual patient-specific cell therapy. As a practical example of therapy using hESC-based products, the world's first clinical trial started in spinal cord patients in the United States in 2011. Other clinical trials have also been conducted with retinal pigment epithelial (RPE) cells derived from hESCs to treat patients with dry age-related macular degeneration (AMD) and Stargardt's macular dystrophy by Advanced Cell Technology.[1] Following Yamanaka's establishment of hiPSCs in 2007, new innovations in regenerative medicine/cell therapy have been made that relate to cell substrates, manufacturing materials, and application methods. As one of the promising seeds for practical use in regenerative medicine/cell therapy, the clinical application of hiPSC-derived RPE cells is expected to start in 2013 in Japan for wet AMD patients.

For the proper development of hPSC-derived products, it is essential to assess their risk and to study their quality and safety. In this review, we place a particular focus on the current situations and issues related to the evaluation of tumorigenicity, which is one of the potential concerns in an attempt to conduct clinical research on hPSC-derived products.

2. THE TUMORIGENICITY OF HUMAN PLURIPOTENT STEM CELLS

Tumorigenicity is defined as the capacity of a cell population inoculated into an animal model to produce a tumor by proliferation at the site of inoculation and/or at a distant site by metastasis.[2] Undifferentiated hPSCs have the ability to form teratoma in animals, and this is ascribed to their pluripotency.[3] In general, to demonstrate the pluripotency of established cells, they are injected into immunodeficient mice, *e.g.*, nude mice, to form spontaneous teratomas. Teratomas are defined as tumors of multiple lineages containing tissue derived from the three germ layers (*i.e.*, endoderm, mesoderm, and ectoderm). hPSCs are tumorigenic and differ greatly from somatic cells and somatic stem cells in terms of tumorigenic potential.[4] Residual pluripotent cells in hPSC-derived products have the ability to initiate ectopic tissue formation, tumor development, and/or malignant transformation.

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3. INTERNATIONAL GUIDELINES OF TUMORIGENICITY STUDIES

At present, the World Health Organization (WHO) TRS 878 guideline, titled, "Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks,"[2,5] is the only international guideline addressing tumorigenicity studies. The International Conference on Harmonization's "ICH guideline Q5D: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products" also cited the tumorigenicity tests of the WHO TRS 878 guideline. These guidelines provide documented study design advice and general principles for tumorigenicity studies. Several *in vitro* test systems, such as cell growth in soft agar and muscle organ culture, have been explored as alternatives to *in vivo* tests for tumorigenicity[6]; however, as a result of technical difficulties, the correlations with *in vivo* tests have not yet been clearly proved. Therefore, in the WHO TRS 878, *in vivo* tests remain the standard for assessing tumorigenicity. Simply put, the model protocol of the *in vivo* tumorigenicity test in the WHO TRS 878 is that 10^7 animal cells are administrated to 10 nude mice and observed for 3 to 16 weeks; HeLa cells are recommended as the positive control reference preparation. Applying this test to hPSC-based cell therapy products, we must learn about its coverage and purpose. The *in vivo* tumorigenicity test proposed in WHO TRS 878 covers cells used to manufacture biological products but not cells transplanted into patients. Its purpose is to examine the tumorigenic phenotype range, from non-tumorigenic to weakly- or highly tumorigenic, of the cell banks, but not to detect the slightly contaminated tumorigenic cells in hPSC-derived products. The WHO TRS 878 also requires the master or working cell bank to check the tumorigenicity whenever cultured for predetermined passage times. WHO TRS 878 has therefore not directly addressed the tumorigenicity of hPSC-derived products. Importantly, the tumorigenicity described in WHO TRS 878 is not a direct risk index for humans but that estimated by animal testing, which examines tumor formation at transplanted sites and metastasis at remote sites.

4. ALTERNATIVE APPLICATION OF WHO TRS 878 TO hPSC-DERIVED PRODUCTS

As mentioned above, one of the risks of hPSC-derived products is the possibility of tumor formation following transplantation. To ensure safety, the tumorigenicity of hPSC-derived products must be evaluated to identify undifferentiated and/or abnormal cells that might exist in minute quantities in final products. It should be noted that the tumorigenicity of final products is clearly different from the tumorigenicity of the cell bank of serially passaged cell lines defined as cell substrates in WHO TRS 878. However, we must consider how the WHO TRS 878 protocol of tumorigenicity testing should be applied to hPSC-derived products because the WHO TRS878 guideline is the only one that directly addresses tumorigenicity studies. First, we understand the grounds of the WHO TRS 878 tumorigenicity test protocol to require administratation of 10^7 cells to nude mice. Tumor-producing doses at the 50% endpoint (TPD_{50}) (the number of cells required for tumor development with 50% probability) are used as units of tumorigenic phenotypes. The strengths of TPD_{50} vary greatly according to cell strain. For example, the TPD_{50} values of Endo-CA (human endometrial carcinoma cells), A594 (human lung cancer cells), HeLa (human cervical carcinoma cells), and 293 (human kidney cells) are 10, 3×10^3, 3×10^4 and 3×10^6 cells/nude mouse, respectively.[7] Administrating 10^7 cells to 10 nude mice should result in the tumor formation of 293 weakly tumorigenic cells in several nude mice out of ten. On the other hand, 10^7 cells of highly tumorigenic HeLa cells should form tumors in all ten nude mice. Therefore, HeLa cells are recommended as the positive control.

In general, treatment with hPSCs-derived products is thought to require from several tens of thousands to hundreds of millions cells, depending on the disease. Several tens of thousands of prepared RPE cells may be required for retinal degeneration diseases, whereas in the case of treatment for heart failure, hundreds of millions of cardiac muscle cells may be necessary. Assuming that 1 in 10^4 of the final product cells (0.1%) is tumorigenic, the *in vivo* tumorigenicity test would require an inoculum of 3×10^8 or 3×10^{10} cells to detect tumor formation in nude mice when contaminated cells have tumorigenic activity equal to HeLa cells (TPD_{50}: 3×10^4) or 293 cells (TPD_{50}: 3×10^6), respectively. The alternative application of WHO TRS 878 "Administrate 10^7 cells to 10 nude mice" to hPSC-derived products may thus lead to false-negative test results.

5. AVAILABILITY OF HIGHLY IMMUNODEFICIENT MICE

To detect slightly contaminated tumorigenic cells in hPSC-derived products, several new generations of severely immunodeficient animal models are now available. Rag2-γC double-knockout (DKO) mice,[8] NOD/SCID/γC^{null} (NOG) mice,[9] and NOD/SCID/IL-2rgKO (NSG) mice[10] are reported to be T, B, and NK cell-defective and to show high engraftment rates of human cells and tissues compared with traditional nude (T cell-defective) mice.[11,12] Using these severe combined immunodeficient mouse lines, which are likely to be useful for sensitive *in vivo* tumorigenicity tests, the small amount of residual tumorigenic cells in the hPSC-derived products could be detected. Since scientific risk assessment needs to standardize the tumorigenicity evaluation of hPSC-derived products, the following points should be taken into consideration in the development of *in vivo* tumorigenicity tests: (a) validation of the limit of detection, sensitivity, and precision, (b) positive and negative control selection, (c) number of tested cells, (d) test duration, (e) route/method of administration, and (f) comparison with nude mouse.

6. *IN VITRO* TUMORIGENICITY-ASSOCIATED TESTS

Some tests are indicated to detect tumorigenic cells contaminating cell populations *in vitro*. Table 1 summarizes the advantages and disadvantages of the tests associated with product tumorigenicity. The soft agar colony formation assay is a conventional method to monitor anchorage-independent growth, and is considered the most appropriate *in vitro* assay to detect the malignant transformation of cells.[6] Previous

Table 1. Comparison of Tumorigenicity-Associated Assays

Assay	Soft agar colony formation assay	Flow cytometry	qRT-PCR	*In vivo* tumorigenicity test using SCID mice[15]
Measurement standard	Colony formation	Expression of marker protein for pluripotency	Expression of marker gene for pluripotency	Tumor formation
Purpose	Detection of anchorage-independent growth	Detection of tumorigenic and undifferentiated cell	Detection of tumorigenic and undifferentiated cells	Detection of tumorigenic of undifferentiated pluripotent cells
Time	30 d	1 d	6 h	12–16 weeks
Advantage	Inexpensive	Rapid	Rapid and simple	Direct
		Analyzes individual cells	Quantitative	Analyzes tumor formation in a specific microenvironment
			Highly sensitive	
Disadvantage	Indirect	Indirect	Indirect	Costly
	Not applicable to hiPSCs	Detects only the cells that express the known marker proteins	Detects only the cells that express the known marker genes	Time-consuming
		Gating techniques strongly influence the results		
Limit of detection	1% of PA-1 teratocarcinoma cells	0.1% of hiPSCs (TRA-1-60)	≦0.002% of hiPSCs (Lin28)	245 Undifferentiated hESCs with 10^6 feeder fibroblasts (0.025%)

reports have shown that hPSCs undergo apoptosis when dissociated into single cells.[13] This test requires the scattering of cells and their enclosure in agar so it may be difficult to utilize it for tests of hPSC-derived product tumorigenicity. Flow cytometry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) tests were found to be able to detect a trace amount of undifferentiated cells. The advantage of the flow cytometry test is that it can be used to identify undifferentiated cells. Unfortunately, the results are greatly affected by gating, and only the cells expressing the marker proteins are detectable. The advantages of qRT-PCR are its rapidity, quantitative nature, and high sensitivity. Its disadvantage is that only the cells expressing the marker gene are detectable. Our previous report demonstrated that the soft agar colony formation assay is unable to detect hiPSCs, even in the presence of a Rho-associated protein kinase (ROCK) inhibitor that permits survival of dissociated hiPSCs/hESCs. The flow cytometry test using anti-TRA-1-60 antibody has detected 0.1% undifferentiated hiPSCs spiked in primary RPE cells. The qRT-PCR method with a specific probe and primers has been found to detect a trace amount of Lin28 mRNA, which is equivalent to that present in a mixture of a single hiPSC and 5×10^4 RPE cells.[14] As tumorigenic cells are commonly highly proliferative and immortalized, observation of the cell growth rate by culturing for a limited period also seems to be useful to detect rapidly growing contaminated immortalized cells. If combinations of these *in vitro* tumorigenicity-associated tests do not demonstrate the existence of both undifferentiated and immortalized cells, the tumorigenic potential of final products can be considered extremely low. More importantly, the validity of advancing to clinical trials should be confirmed for each product and judged by the following points: (a) methods of cell inoculum, (b) sites of injection, (c) risk management plans, (d) results from *in vivo* tumorigenicity tests.

7. CONCLUSION AND FUTURE PROSPECTS

No guideline for the tumorigenicity testing of cell-/tissue-derived products, including hPSC-derived products, has been issued. Because the subject and purpose described in WHO TRS 878 are not suitable for hPSC-derived products, the direct application of the WHO TRS 878 tumorigenicity test to hPSC-derived products is unreasonable. Tumorigenicity studies for hPSC-based products should examine (a) the existence of residual undifferentiated pluripotent cells, (b) the existence of tumorigenic transformants, and (c) whether the transplant forms tumor in microenvironments at the site of transplantation. As a countermeasure, highly sensitive *in vivo* tumorigenicity tests using severely immunodeficient mice may be a viable option. We now address the current problems with the development and standardization of *in vivo* tumorigenicity tests for hPSC-derived products.

Safety assessments of hPSC-derived products must choose among various tumorigenicity tests, considering the limitations of each. The overall safety of each product should be estimated on the basis of the results of an appropriate set of tumorigenicity tests. The following points should also be taken into account in order to decide on the items to evaluate: (a) properties of the raw materials, (b) properties of the products, (c) target diseases, and (d) risk management. Of course, the results/assessments of even the most appropriate tumorigenicity tests cannot guarantee safety in humans. After understanding the limitation of each tumorigenicity test, we should develop a risk assessment and risk management plan and obtain informed patient consent.

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TECHNOLOGY REPORT

Optimization of Slow Cooling Cryopreservation for Human Pluripotent Stem Cells

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Summary: Human pluripotent stem cells (hPSCs) have the potential for unlimited expansion and differentiation into cell types of all three germ layers. Cryopreservation is a key process for successful application of hPSCs. However, the current conventional method leads to poor recovery of hPSCs after thawing. Here, we demonstrate a highly efficient recovery method for hPSC cryopreservation by slow freezing and single-cell dissociation. After confirming hPSC survivability after freeze-thawing, we found that hPSCs that were freeze-thawed as colonies showed markedly decreased survival, whereas freeze-thawed single hPSCs retained the majority of their viability. These observations indicated that hPSCs should be cryopreserved as single cells. Freeze-thawed single hPSCs efficiently adhered and survived in the absence of a ROCK inhibitor by optimization of the seeding density. The high recovery rate enabled conventional colony passaging for subculture within 3 days post-thawing. The improved method was also adapted to a xeno-free culture system. Moreover, the cell recovery postcryopreservation was highly supported by coating culture surfaces with human laminin-521 that promotes adhesion of dissociated single hPSCs. This simplified but highly efficient cryopreservation method allows easy handling of cells and bulk storage of high-quality hPSCs. genesis 52:49–55, 2014. © 2013 Wiley Periodicals, Inc.

Key words: ectoderm; tissue endoderm; tissue mesoderm; tissue other; tissue germ layer; process; early development; single cell dissociation; slow freezing

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have an infinite proliferative potential and capacity for differentiation into all cells of the three germ layers. For successful application of hPSCs in transplantation therapy or drug discovery, it is necessary to prepare large numbers of hPSCs with various genetic backgrounds (Serra *et al.*, 2012). There are several approaches to improve the yield of hPSCs, but cryopreservation is a key operation because it enables long-term preservation and easy transportation of cells. Currently, hPSCs are cryopreserved by vitrification or slow cooling (Heng *et al.*, 2005; Reubinoff *et al.*, 2001). Vitrification involves flash cooling directly in liquid nitrogen, and some studies have recovered 20–90% of undifferentiated hPSC colonies postcryopreservation (Li *et al.*, 2010; Suemori *et al.*, 2006). However, vitrification requires skilled manipulation and strict temperature control during storage and transportation, because of the small volumes of cryoprotectant used, making it impractical for large-scale storage of hPSCs. In contrast, conventional slow cooling controls the cooling rate at 1°C/min in freezing medium that

Additional Supporting Information may be found in the online version of this article.

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typically contains 10% dimethylsulfoxide (DMSO). The slow cooling method is widely used because it is applicable to various cell types including mouse embryonic stem cells. However, for cryopreservation of hPSCs, the slow cooling method results in less than 10% survivability (Wagh *et al.*, 2011). Nevertheless, for bulk storage and easy handling, slow cooling is desirable for cryopreservation of hPSCs. In this study, we aimed to optimize the slow cooling method for hPSC cryopreservation.

Cryopreservation involves a series of processes including cell detachment, freezing, storage, thawing, and reseeding. During these processes, hPSCs may suffer necrotic cell death from cell detachment to thawing because of physical damage, and apoptotic cell death at reseeding because of adhesion failure. Therefore, hPSC survivability should be verified at each step. However, previous studies have reported different timings for their analyses, various methods of measurement, and counting either colonies or individual cells (Li and Ma, 2012). In particular, counting of colonies postseeding, although it has been often performed, is inaccurate because it leads to overestimation because of an increase of colony formation caused by fragmentation of the original colonies during freeze-thaw processes. For correct evaluation of the survival efficiency, in this study, we counted individual hPSCs in a stepwise manner.

To evaluate the effect of different methods of cell detachment from culture vessels, we first compared cell survival after conventional colony dissociation or single-cell dissociation. While the viability of cells detached by conventional colony dissociation before freezing were approximately halved (42.3-59.4% survival), the majority of hPSCs cryopreserved under a colony state were already dead immediately after freeze-thawing (15.5-32.5% survival) (Fig. 1a, c, e-h). Although the conventional dissociation method maintains the colony state to prevent apoptosis caused by failure to adhere at reseeding, hPSCs had already died by necrosis before attachment. This observation suggests that it is necessary to avoid cell death mainly caused by physical damage during cell dissociation and freeze-thawing processes. Next, we performed freeze-thawing of dissociated hPSCs. The majority of dissociated hPSCs showed higher viability before cryopreservation (68.1-77.7% survival). More importantly, single hPSCs were still alive just after thawing (59.7-66.1% survival), corresponding to 80-90% viability just after dissociation (Fig. 1b, d, e-h).

Although we found that single-cell dissociation of hPSCs is advantageous for freeze-thawing, it is necessary to avoid apoptotic cell death caused by dissociation (Amit *et al.*, 2000). Because we previously showed that the seeding density significantly affects the survivability of single hPSCs (Miyazaki *et al.*, 2012), we next assessed the effect of seeding density on the viability of freeze-thawed single hPSCs. The number of hPSCs

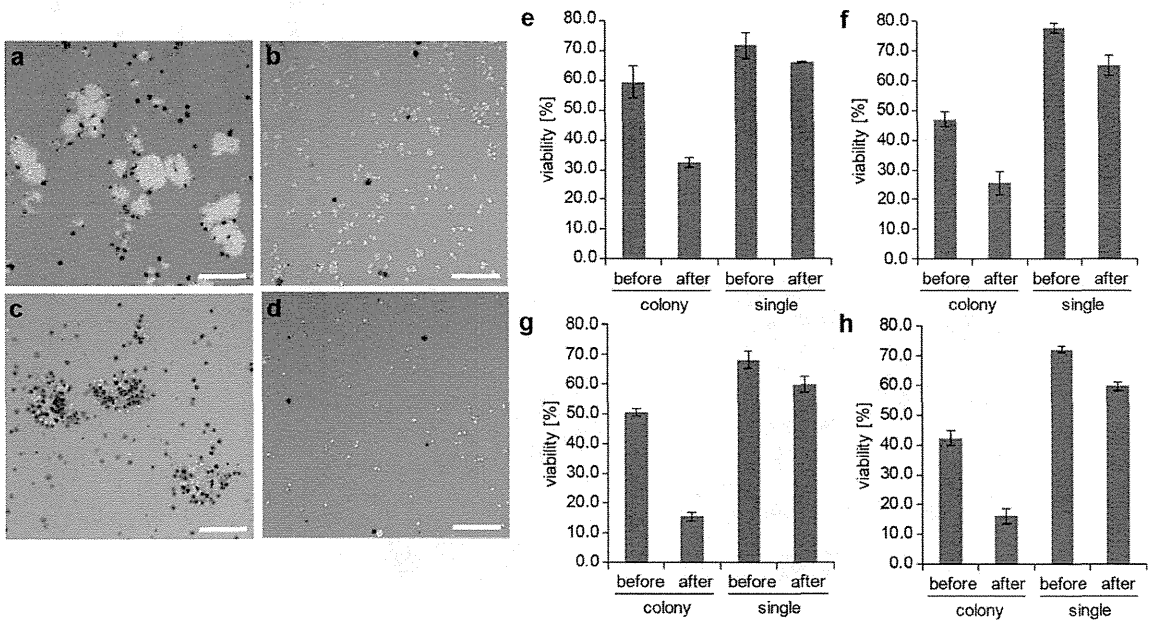

FIG. 1. hPSC survivability is influenced by the cellular state during cryopreservation. (**a–d**) Estimation of cellular state-dependent hPSC survivability by trypan blue exclusion. Representative bright field images of colony-dissociated H9 hESCs before (a) and after (b) cryopreservation and single dissociated H9 hESCs before (c) and after (d) cryopreservation. (**e–h**) Flow cytometric analysis of cellular state-dependent hPSC survivability during cryopreservation by fluorescent dye influx. e: H9 hESCs; f: 253G1 hiPSCs; g: KhES-1 hESCs; h: iPS(IMR90)-1 hiPSCs. Error bars indicate the standard deviation (SD) of three experiments. Scale bars: 200 μm.

attached to the substrate gradually decreases to the minimum at 12 h postseeding (Chen *et al.*, 2010). To evaluate survival correctly, we measured the viability of freeze-thawed single hPSCs at 12 h postseeding. On Matrigel in mTeSR1 medium, freeze-thawed single H9 hESCs seeded at 3×10^5 cells/cm^2 showed maximum survivability (36.2 ± 6.0% survival) (Fig. 2a, c), indicating the optimal condition for recovery of cryopreserved hPSCs. It is well known that administration of a Rho-associated protein kinase (ROCK) inhibitor dramatically improves the survival of single hPSCs (Watanabe *et al.*, 2007). However, ROCK inhibitor treatment did not significantly improve the survival of single hPSCs seeded at the optimal density (Fig. 2b). An effect of the ROCK inhibitor was only observed when single hPSCs were seeded at a lower cell density. These results indicate that a ROCK inhibitor is unnecessary for the optimized seeding density, whereas it should be administrated when single hPSCs are seeded at a lower seeding density. For stable survival of hPSCs, cell-cell contact is a key element (Chen *et al.*, 2010). Therefore, the seeding density of single hPSCs should be as high as possible for rapid formation of cell-cell contacts. However, seeding at very high densities leads to less efficient survival because of depletion of the adhesion surface. It is therefore important for freeze-thawed single hPSCs to be seeded at an appropriate density.

After adherence of single hPSCs, the majority of single hPSCs immediately formed cell-cell contacts as small clusters (Fig. 2c). By 3 days post-thawing, the cells had grown as colonies and reached confluency for passaging (Fig. 2d). Immediately after freeze-thawing, flow cytometric analysis showed that hPSCs remained in an undifferentiated state and maintained the undifferentiated status following subculture (Fig. 3a). After the first passage, hPSCs grew as colonies (Fig. 3b) and sustained an undifferentiated state (Fig. 3a, c) and potential for differentiation (Fig. 3c) without karyotypic abnormalities

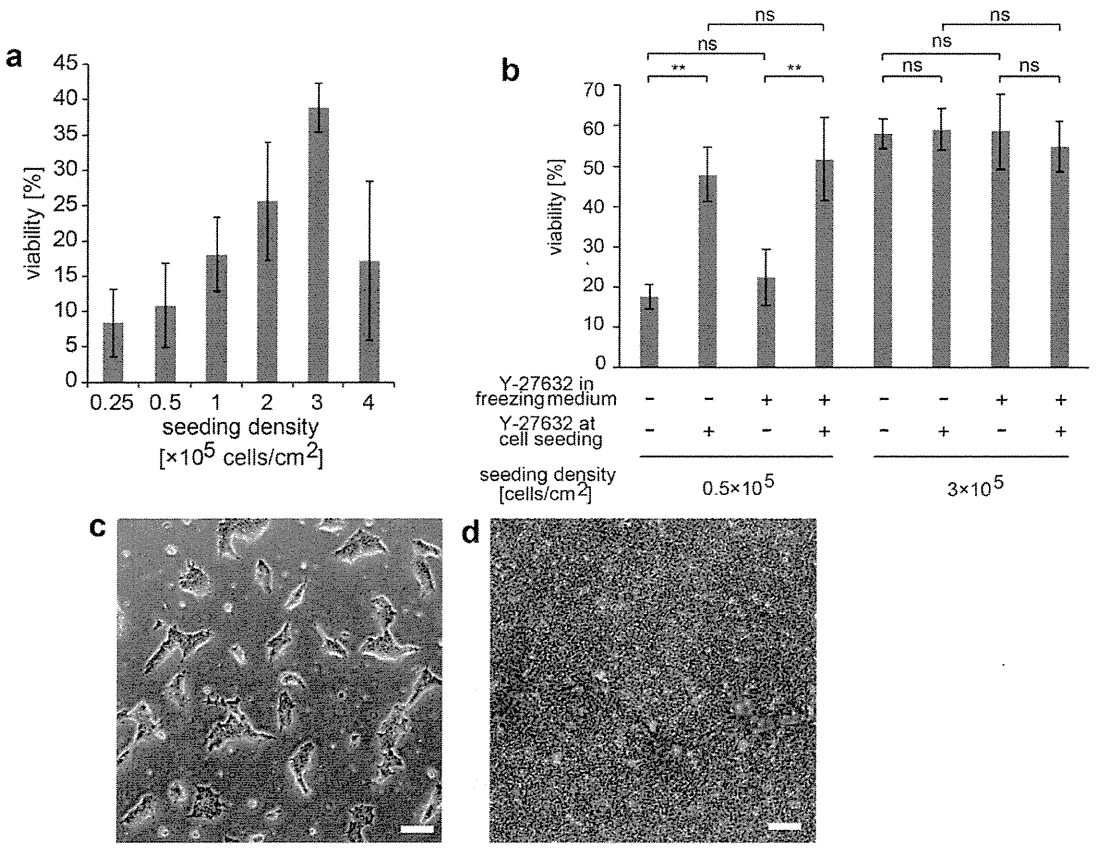

FIG. 2. Recovery of freeze-thawed single hPSCs. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**) Effect of the ROCK inhibitor Y-27632 on adhesion of freeze-thawed single H9 hESCs at 12 h postseeding. Error bars indicate the SD. of three experiments. $^{**}P < 0.05$; ns, not significant, two-tailed Student's *t*-test. (**c**, **d**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (c) and 3 days (d) postseeding at 3×10^5 cells/cm^2. Scale bars: 200 μm.

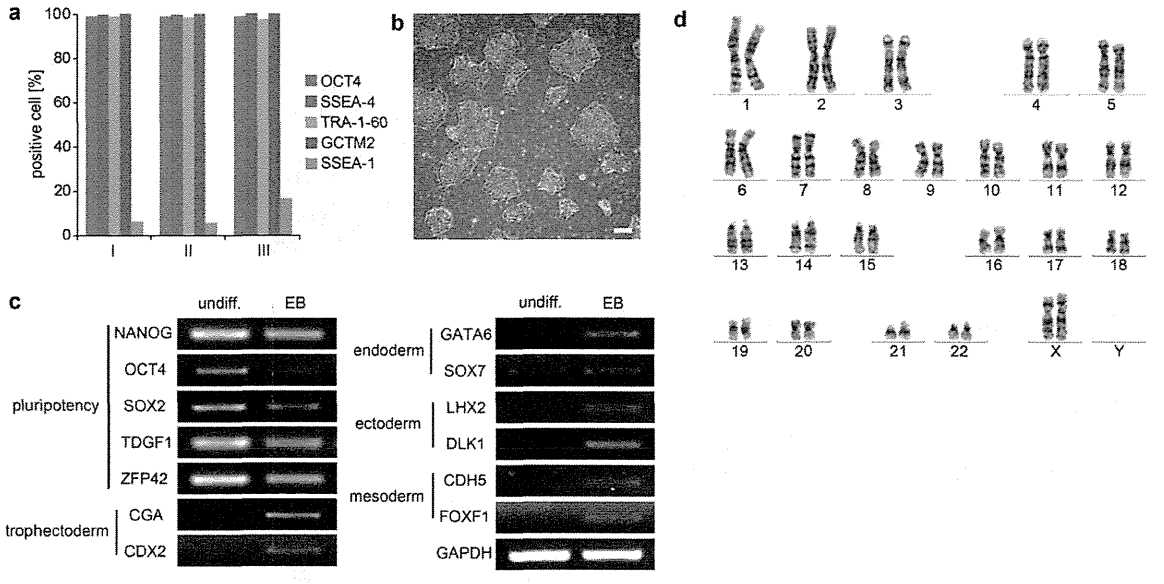

FIG. 3. Characterization of freeze-thawed hPSCs. (**a**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**b**) Phase contrast image of H9 hESCs at subculture. (**c**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. undiff. indicates cells at subculture. (**d**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

(Fig. 3d). Thus, hPSCs can be rapidly and efficiently cryopreserved by slow cooling while maintaining their pluripotent state.

Because hPSCs used for transplantation therapy would be expanded under xeno-free culture conditions, we evaluated xeno-free TeSR2 medium and recombinant human laminin-521 as the culture substrate. Laminin-521 is a laminin isoform used to improve the adhesion of dissociated hPSCs. Freeze-thawed single hPSCs in TeSR2 medium showed high survivability (86.8 ± 3.1% survival) immediately after freeze-thawing. Considering the promotion of adhesive by laminin-521, we re-evaluated the seeding density and found that freeze-thawed single hPSCs seeded at 1×10^5 cells/cm^2 showed maximal survival (59.7 ± 15.5% survival for H9 hESCs and 43.2 ± 11.2% survival for 253G1 iPSCs) (Fig. 4a and Supporting Information Fig. 1a). Moreover, on the laminin-521-coated culture surface, a higher number of single hPSCs were able to adhere and survive even at a lower seeding density (36.2 ± 6.1% survival for H9 hESCs and 32.5 ± 8.7% for 253G1 iPSCs at 0.25×10^5 cells/cm^2). After reseeding, the freeze-thawed hPSCs rapidly formed colonies on laminin-521 (Fig. 4b) and could be passaged within 3 days (Fig. 4c). The freeze-thawed hPSCs maintained their undifferentiated state (Fig. 4d, e, and Supporting Information Fig.4b), potential for differentiation (Fig. 4e), and a normal karyotype after passaging on laminin-521 in TeSR2 medium (Fig. 4f). Therefore, freeze-thawing of single hPSCs is applicable to xeno-free culture conditions, and human laminin-521 enhances the survival of single hPSCs after thawing.

In the present study, we obtained a high recovery rate following cryopreservation by optimizing the cellular state during freezing and the seeding density after thawing. These modifications achieved 80–90% survival of hPSCs post-thawing and around 60% survival following subculture on laminin-521, even in the absence of a ROCK inhibitor. hPSCs are delicate cells as compared with other types of cultured cells. Therefore, hPSCs require specific conditions not only for maintenance in culture but also for cryopreservation. Most previous approaches for effective hPSC cryopreservation have focused on development of a cryoprotectant specific for hPSCs (Ha *et al.*, 2005; T'Joen *et al.*, 2012). However, our study demonstrated that a fundamental solution for cryopreservation of hPSCs is their cellular state. It is therefore necessary to consider the cellular state for further investigation of hPSC cryopreservation. Moreover, our examination of the survivability of individual cells in a stepwise manner will contribute to estimation of the precise survival efficiency of hPSC cryopreservation.

It is still unclear why hPSC colonies are susceptible to injury after freeze-thawing. One risk is that hPSC colonies undergo unavoidable physical damage. Because cell scraping and pipetting for cell detachment is usually associated with cell rupture, freeze-thawed colonies of hPSCs may still be damaged following cell detachment. In addition, cell colonies have poor penetration of cryoprotectant. During cryopreservation, cells are

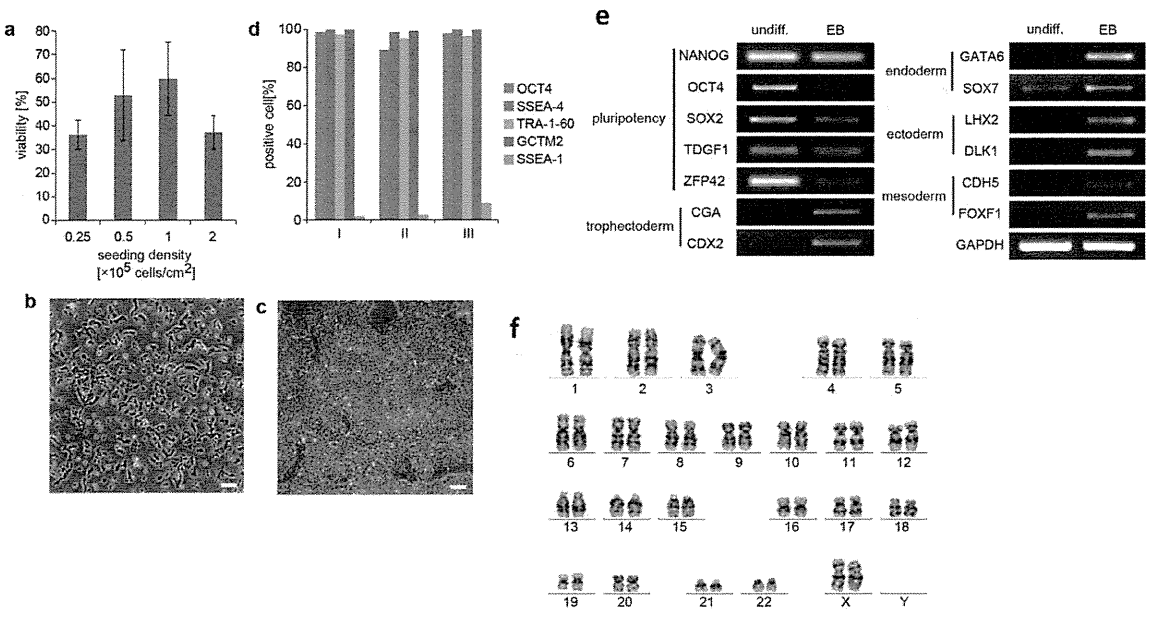

FIG. 4. Cryopreservation of single hPSCs under xeno-free culture conditions. (**a**) Seeding density-dependent adhesion of freeze-thawed single H9 hESCs on laminin-521 in TeSR2 medium at 12 h postseeding. Cell viability is expressed as the ratio of the attached cell number to live H9 hESCs at seeding. Data represent the means ± SD of three freeze-thawing experiments of one cryopreserved batch of cells. (**b**, **c**) Phase contrast images of freeze-thawed single H9 hESCs at 12 h (b) and 3 days (c) postseeding at 1×10^5 cells/cm^2. (**d**) Flow cytometric analysis of undifferentiated markers before cryopreservation (I), at 3 days postseeding (II), and at subculture (III). (**e**) Reverse transcription-PCR analysis of differentiation marker gene expression in embryoid bodies. Total RNA was extracted from day 14 embryoid bodies generated from cells after subculture. (**f**) G-banding analysis of H9 hESCs after subculture. Data for subcultured cells were obtained at two passages after freeze-thawing. Scale bars: 200 μm.

often ruptured by rapid osmotic changes caused by the cryoprotectant (Hunt, 2011). hPSC colonies are tightly connected by cell–cell interactions such as adherence junctions, and their formation generates a heterogeneous cellular state for cryoprotectant penetration. When compared with a complex cellular structure, single cells may be more suitable for cryopreservation in terms of cryoprotectant penetration. In addition, dissociation to single cells may minimize cellular damage at detachment because physical manipulation is unnecessary. Here, we used TrypLE Select as the dissociation solution, but other dissociation solutions used for single cell passaging, such as accutase and trypsin, would provide similar results.

In conclusion, our method enables highly efficient slow cooling of hPSCs. The cellular manipulation is simple and very similar to commonly used techniques for culturing most mammalian cell lines. Furthermore, our method is suitable for large-scale cryopreservation of pluripotent stem cell lines and machine-aided automation.

METHODS

Matrix Coating

Six-well flat-bottomed plates (BD Falcon, 353046) and 12-well flat-bottomed plates (BD Falcon, 353043) were coated with Matrigel (BD Biosciences, 354230) at 25 μg/cm^2 or laminin-521 (Bio Lamina, BLA-LN521-02) at 3 μg/cm^2 for 3 h at room temperature (RT) just prior to use. Matrigel and laminin-521 were diluted in DMEM/F12 or D-PBS, respectively.

Maintenance and Passaging of hPSCs

hESC lines, H9 and KhES-1, and hiPSC lines, iPS(IMR90) clone 1, and 253G1 were maintained on mitomycin C-treated mouse embryonic fibroblasts as described previously (Suemori *et al.*, 2006). hPSCs were transferred onto Matrigel-coated culture vessels in mTeSR1 medium (StemCell Technologies, ST-05850) or laminin-521-coated culture vessels in TeSR2 medium (StemCell Technologies, ST-05860) following the manufacturer's instructions.

Subculture was performed by colony dissociation as follows. Semi-confluent hPSCs were incubated with 2 mg/ml dispase in DMEM/F12 at 37°C for 3 min, and then rinsed twice with DMEM/F12. After addition of culture medium, weakly adherent colonies were detached using a cell scraper. The cells were collected and centrifuged at 200*g* for 3 min at 4°C. Small colonies were passaged onto fresh matrix-coated culture vessels at a ratio of 1:4. The cells were cultured at 37°C with 3% CO_2 in a humidified incubator. Medium changes were performed daily.

Cell Freezing and Thawing

For cryopreservation, hPSCs were dissociated as follows. Semiconfluent hPSCs were treated with 4.8 m*M* EDTA/PBS for 3 min at RT and then TrypLE Select (Invitrogen, 12563011) for 1 min at 37°C. The cells were collected and pipetted for complete dispersal and then centrifuged at 200*g* for 3 min at 4°C.

For freezing, 2.0–2.2 × 10^6 hPSCs were resuspended in 0.5 ml prechilled culture medium, gently mixed with the same volume of 2× prechilled freezing medium containing 20% DMSO (final concentration: 10% DMSO), and then transferred to cryovials (NUNC, 377224). The cryovials were placed in a Mr. Frosty Freezing Container (NUNC, 5100-0001) and stored at −80°C for 24 h. The cryovials were then transferred to liquid nitrogen and stored for at least 1 week before analysis.

For thawing, the cells were warmed at 37°C in a water bath and then diluted with cold culture medium. The cells were collected by centrifugation at 200*g* for 3 min and seeded onto matrix-coated culture vessels at the indicated seeding densities.

Cell Viability Assays

The viability of hPSCs cultured with mTeSR1 medium was estimated by trypan blue exclusion or flow cytometric detection of fluorescent dye influx. To obtain accurate data of cell viability before and after cryopreservation, cell preparation for viability assays was performed by centrifugation at 500*g* for 5 min to ensure maximum recovery of more than 90% of the frozen cells. For trypan blue staining, hPSCs were incubated with a 0.2% trypan blue solution (Wako, 207-17081) for 1 min at RT, and then observed under an optical microscope in a bright field. For flow cytometric detection, hPSCs were rinsed with D-PBS by centrifugation at 500*g* for 5 min at 4°C, and then completely dissociated by treatment with 4.8 m*M* EDTA/D-PBS for 2 min at RT. The cells were rinsed with culture medium and resuspended in D-PBS. The cell suspension was incubated with 105 n*M* thiazole orange for 5 min and then 11 μ*M* propidium iodide for 2 min just prior to analysis. Considering cell damage during preparation for FACS analysis, single hPSCs were additionally treated by a dissociation process similar to that for hPSC colonies.

Cell Adhesion Assays

Freeze-thawed single hPSCs were seeded at several cell densities in 24-well plates coated with Matrigel or laminin-521. After 12 h of incubation, the cells were rinsed with prewarmed D-PBS, and the remaining live cells were detached with 0.25% trypsin/EDTA and counted using a hemocytometer.

To evaluate the effect of Y-27632 treatment, freeze-thawed single hPSCs were seeded at two cell densities and then incubated with 10 μ*M* Y-27632 (Wako, 253-00513). After 12 h, adherent live cells were counted as described above.

Karyotype Analysis

hPSCs were treated with 100 ng/ml colcemid (Life Technologies, 15212-012) for 2–3 h. After dissociation in 0.25% trypsin/EDTA, the cells were treated with a hypotonic solution and then fixed in Carnoy's solution. Cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were then analyzed by randomly counting 50 cells using the Ikaros Karyotyping System (METASYSTEM).

Flow Cytometric Analysis

Cells were dissociated by treatment with 4.8 m*M* EDTA/PBS for 2 min and then TrypLE Select for 1 min. To detect surface markers, the cells were rinsed twice with 10% FBS/DMEM, and 1 × 10^5 cells were incubated with primary antibodies diluted in staining buffer (0.1% BSA/D-PBS) for 30 min at 4°C. The cells were rinsed twice with staining buffer and then incubated for 30 min at 4°C with the secondary antibody diluted in staining buffer. After rinsing twice with staining buffer, the cells were resuspended in D-PBS and stained with propidium iodide just prior to analysis. To detect intracellular markers, dissociated cells were fixed with 4% formaldehyde/PBS for 15 min at 4°C. After rinsing with D-PBS, cells were incubated with saponin permeabilization buffer (SPB) (1 mg/ml saponin and 1% BSA in D-PBS) for 15 min, and then the primary antibody for 30 min at 4°C. Cells were rinsed twice with SPB, and then incubated for 30 min at 4°C with the secondary antibody diluted in SPB. After three rinses with SPB, the cells were resuspended in staining buffer for analysis by a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Antibodies against the following markers used were SSEA-4 (Developmental Studies Hybridoma Bank (DSHB), MC-813-70, 1 μg/ml), Tra-1-60 (Millipore, MAB4360, 1 μg/ml), GCTM2 (Millipore, MABD90, 1 μg/ml), TRA-2-54 (DSHB, 1 μg/ml), and SSEA-1 (DSHB, MC-480, 2 μg/ml) as primary antibodies and a mouse anti-Ig/FITC (Becton Dickinson, 731735, 1 μg/ml) as the secondary antibody.

Differentiation Assay

Differentiation potential was estimated by semi-quantitative polymerase chain reaction (PCR) analysis of differentiation marker genes expressed in embryoid bodies. Freeze-thawed hPSCs subcultured for two passages were detached by treatment with CTK solution (ReproCell, RCHETP002) and using a cell scraper. The cell clumps were cultured as a suspension in petri dishes with hESC medium without FGF-2. Medium changes were performed every 2 days. After 14 days of

culture, total RNA was extracted using an RNeasy Mini Kit (Qiagen, 74104), and cDNA was synthesized from 1 to 2 μg RNA using an Omniscript™ RT Kit (Qiagen, 205111) according to the manufacturer's instructions. PCR conditions were optimized to facilitate semiquantitative comparison with the log phase of amplification. The gene-specific primers are described elsewhere (Kumagai *et al.*, 2013; Miyazaki *et al.*, 2012). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis

The statistical significance of differences was determined by the two-tailed Student's *t*-test. Differences with a value of $P < 0.05$ were considered significant.

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Identification of small molecules that promote human embryonic stem cell self-renewal

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ABSTRACT

Human embryonic stem cells (hESCs) and induced pluripotent cells have the potential to provide an unlimited source of tissues for regenerative medicine. For this purpose, development of defined/xeno-free culture systems under feeder-free conditions is essential for the expansion of hESCs. Most defined/xeno-free media for the culture of hESCs contain basic fibroblast growth factor (bFGF). Therefore, bFGF is thought to have an almost essential role for the expansion of hESCs in an undifferentiated state. Here, we report identification of small molecules, some of which were neurotransmitter antagonists (trimipramine and ethopropazine), which promote long-term hESC self-renewal without bFGF in the medium. The hESCs maintained high expression levels of pluripotency markers, had a normal karyotype after 20 passages, and could differentiate into all three germ layers.

1. Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have a self-renewal ability and pluripotency to differentiate into all three germ layers *in vitro* and *in vivo* [1–3]. Because of these notable properties, hESCs and hiPSCs are tools for basic biology, drug discovery research, and a cell source for regenerative medicine.

Undifferentiated hESCs and hiPSCs are usually maintained on mouse embryonic fibroblasts (MEFs) as feeders, or MEF-conditioned medium (CM) on Matrigel, which limits their clinical application owing to the potential risk of using animal components. In recent years, many commercial and non-commercial media have been reported to maintain hESCs and hiPSCs in culture under a feeder-free condition [4], but they have not been fully developed for the large-scale culture of cells because these media are expensive and often have batch-to-batch variations. One method to overcome such issues might be the addition of small molecules to the media as a replacement for growth factors and other components.

In this study, we aimed to identify small molecules to replace the role of basic fibroblast growth factor (bFGF). Most defined/xeno-free media for hESCs contain bFGF at a higher concentration. Therefore, it is thought that bFGF is one of the most important components for robust expansion of hESCs in an undifferentiated state. Moreover, small molecules are not fully defined as replacements of bFGF for the expansion of undifferentiated hESCs in culture.

Here, we adopted a high-content screening (HCS) system using green fluorescent protein (GFP) expression regulated by the OCT4 promoter to monitor changes of cell fate in media. Furthermore, we focused on not only the intensity of GFP expression under the OCT4 promoter induced by each small molecule, but also the similarities in structure and the pharmacological effects of hit compounds. We found that selected small molecules support long-term hESC self-renewal in the absence of bFGF as evidenced by various pluripotency markers, a normal karyotype and differentiation into all three germ layers.

2. Materials and methods

2.1. Construction of the hOCT4pro-EGFP reporter gene

We created an enhanced GFP (EGFP) reporter under the control of the human OCT4 promoter (hOCT4pro-EGFP) using a modified method from a previous report [5]. Briefly, the promoter region of human OCT4 was cloned from the genomic DNA of KhES-1 cells by PCR using the following primers: forward, 5′-TTCCCATGTCAAGTAAGTGGGGTGG-3′; and reverse, 5′-ACCGGTGGGGAAGGAAGGCGCCCCAAGCC-3′. The PCR product was cloned into a pBSSK(−) vector and the sequence was confirmed by DNA sequencing. The

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