and K^d- restricted cytotoxic T-lymphocyte (CTL) epitope peptide $GPC3_{298-306}$ (EYILSLEEL) in BALB/c mice. This peptide-specific CTL showed specific cytotoxicity against GPC3-expressing or peptide-pulsed cancer cell lines, suggesting that GPC3 was highly immunogenic and could elicit effective antitumor immunity in mice. Importantly, there was no evidence of autoimmune reactions in the treated mice.[64] Because of the similarities in the peptide binding motifs between H-$2K^d$ and HLA-A24, this peptide was applicable for immunotherapy in HLA-A24-positive patients.

HLA-A2 is also expressed in 40% of Japanese individuals, as well as other ethnic populations.[60,65] An HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide was also identified using *HLA-A2.1* transgenic mice.[58] A binding assay was performed, and it was reported that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could bind to HLA-A*02:06 and HLA-A*02:07. This suggests that HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) might be effective in HLA-A*02:06 and HLA-A*02:07 patients.

These GPC3-derived peptide-specific CTLs could be induced from the peripheral blood mononuclear cells of HCC patients by in vitro stimulation with peptide. The adoptive transfer of these GPC3-derived peptide-specific CTLs reduced the mass of human HCC tumors implanted into non-obese diabetic/severe combined immunodeficiency mice.[66]

GPC3-targeted vaccine therapy

The authors recently completed an investigator-initiated Phase I clinical trial of GPC3-derived peptide vaccines to evaluate their safety, tolerability, and efficacy in patients with advanced HCC.[67] Thirty-three advanced HCC patients were enrolled and received escalating doses of GPC3-derived peptide vaccine (0.3, 1.0, 3.0, 10, and 30 mg/patient). On days 1, 15, and 29, peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant by intradermal injection. $GPC3_{298-306}$ (EYILSLEEL) peptide was used in 17 HLA-A24-positive patients, and $GPC3_{144-152}$ (FVGEFFTDV) peptide was used in 16 HLA-A2-positive patients.

Dose-limiting toxicity and dose-specific adverse events were not seen, and GPC3-derived peptide vaccine treatment was well tolerated. One of the thirty-three patients was judged to have a partial response, whereas 19 patients exhibited stable disease after 2 months according to Response Evaluation Criteria In Solid Tumors (RECIST).[68] The disease control rate (partial response plus stable disease) was 60.6% after 2 months. The median time to tumor progression was 3.4 months (95% confidence interval [CI] 2.1–4.6), and the median overall survival was 9.0 months (95% CI 8.0–10.0).

Immunologically, the frequency of GPC3-peptide-specific CTL in the peripheral blood correlated with the overall survival of HCC patients. In the multivariate analysis, GPC3 peptide-specific CTL frequency was a predictive factor for overall survival. The median overall survival of all 33 patients was 12.2 months (95% CI 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs compared with 8.5 months (95% CI 3.7–13.1) in individuals with a low frequency (P=0.033). Moreover, the infiltration of cluster of differentiation (CD)8-positive T-cells into HCC cells was confirmed.

Based on this Phase I study, a Phase II study of the GPC3-derived peptide vaccine is ongoing in an adjuvant setting (UMIN-CTR: 000002614). Forty-four patients with HCC who had undergone surgery or radiofrequency ablation were enrolled. The primary end points of this study were the 1- and 2-year recurrence rates, and the secondary end point was the immunological response. Patient enrollment has been completed, and the study is ongoing. An additional sponsor-initiated Phase I clinical trial of a three-peptide cocktail vaccine, which includes a GPC3-derived peptide, is also underway.

Anti-GPC3 antibody therapy

GPC3 has been suggested as a potential target for antibody-based therapy in liver cancer because of its high-level expression in HCC. The murine monoclonal antibody GC33, which binds specifically to the C-terminal region of GPC3 with a high affinity, caused significant antibody-dependent cellular cytotoxicity against HCC cells, and exhibited potent antitumor activity in xenograft models.[69–72] For the clinical application of GC33, a humanized GC33 was generated using complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. To improve the stability of the humanized GC33, it was further optimized by replacing the amino acid residues that might affect the structure of the variable region of its heavy chain.[73]

Because of these preclinical data highlighting the relevance of GPC3 as a potential therapeutic target in HCC, a first-in-man Phase I clinical trial to assess the safety, tolerability, and pharmacokinetics of GC33 in patients with advanced HCC was performed.[74] A total of 20 patients were enrolled, and were assigned to receive GC33 at one of four sequentially increasing dose levels (2.5, 5, 10, and 20 mg/kg) weekly by intravenous infusion. The tumor expression of GPC3 was examined in biopsied specimens using immunohistochemical staining. A total of 56% of the patients had a high total GPC3-staining score. This study provided the

initial clinical data regarding the safety profile and pharmacokinetic features of GC33, and revealed potential antitumor activity that might be associated with the expression of GPC3 in tumors. Stable disease was seen in four patients, all of whom exhibited high GPC3 expression. The median time to progression was significantly longer in patients with tumors expressing high levels of GPC3 than in patients with low GPC3 expression.

GC33 is now being assessed in Phase II clinical trials in second-line HCC patients who have progressed after one line of systemic therapy and whose tumors exhibit positive GPC3 immunohistochemical staining (NCT01507168). Additional antibodies that target GPC3 for HCC treatment, human (MDX-1414 and HN3) and humanized mouse (YP7) antibodies, are at different stages of preclinical development.[75] These trials will define the potential of GPC3 as a novel antibody therapy.

Potential of GPC3 for other cancers

GPC3 is also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian clear-cell carcinoma (CCC), and lung squamous cell carcinoma.[37-41] However, Kim et al reported that GPC3 is downregulated in lung cancer. Thus, the overexpression of GPC3 in lung cancer is controversial.[76] GPC3 has been investigated in some of these tumors as a potential immunotherapeutic target or diagnostic marker.

Melanoma

GPC3 messenger RNA and protein was identified in >80% of melanoma and melanocytic nevus patients.[39] In the authors' previous study, GPC3 protein was detected in the sera of 39.6% melanoma patients, but not in healthy donors. The positive detection of serum GPC3 was significantly higher than that of 5-S-cysteinyldopa and melanoma-inhibitory activity, both of which are well-known tumor markers for melanoma. Surprisingly, GPC3 could be detected even in patients with stage 0 in situ melanoma.[37] The combination of secreted protein acidic and rich in cysteine (SPARC) and GPC3 was also a useful tumor marker for melanoma: 66.2% of melanoma patients at stages 0–II exhibited positive SPARC or GPC3 expression.[47] This suggests that GPC3 is a novel tumor marker that is useful for the diagnosis of melanoma, particularly during the early stages.

Ovarian carcinoma

Ovarian CCC is the second most common epithelial ovarian carcinoma subtype in Japan. Ovarian CCC is associated with a poor prognosis and increased chemoresistance compared with other epithelial ovarian carcinoma subtypes.[77,78] GPC3 was expressed in ~40% of CCC patients, and there was a tendency toward poor progression-free survival in GPC3-positive patients at stage I.[79] GPC3 expression was responsible for CTL recognition, and subtoxic dose chemotherapy made tumor cells more susceptible to the cytotoxic effects of CTL.[80] A Phase II trial of a GPC3-derived peptide vaccine in ovarian CCC patients is ongoing (UMIN-CTR: 000003696), and some chemotherapy-refractory ovarian CCC patients have achieved a significant clinical response.[81]

Pediatric tumors

A Phase I trial using a GPC3-derived peptide vaccine for pediatric patients with hepatoblastoma, nephroblastoma, or yolk sac tumors is ongoing (UMIN-CTR: 000006357). The safety and optimal dose of GPC3 peptide vaccines for pediatric cancer patients has not yet been reported.

Conclusion

Although immunotherapy is a potentially attractive treatment modality, its antitumor effects in advanced HCC are not dramatic. GPC3 is overexpressed in HCC but its expression in most adult normal tissues is low. GPC3 expression is correlated with poor prognosis in HCC, suggesting it to be an ideal tumor antigen. GPC3 is thought to play a role in regulating cancer cell growth, although our structural and biological knowledge of GPC3 remain limited. Recent studies have shown the utility of GPC3 as a serum and immunohistochemical marker for the diagnosis of HCC. In addition, although studies assessing GPC3-targeted immunotherapies against HCC (such as vaccine and antibody therapies) have shown good safety and tolerability, sufficient clinical effects have not yet been observed. Further analysis and knowledge of GPC3 biology and its potential as an immunotherapeutic target are needed to allow the development of more effective GPC3-targeted cancer therapies. Although current GPC3-targeted immunotherapies for HCC are in the preclinical and clinical trial phases of development, they are expected to yield clinical success in the near future.

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Disclosure

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Derivation of Transgene-Free Human Induced Pluripotent Stem Cells from Human Peripheral T Cells in Defined Culture Conditions

Yoshikazu Kishino[1], Tomohisa Seki[1,2], Jun Fujita[1], Shinsuke Yuasa[1], Shugo Tohyama[1,2], Akira Kunitomi[1], Ryota Tabei[1], Kazuaki Nakajima[1], Marina Okada[1], Akinori Hirano[3], Hideaki Kanazawa[1], Keiichi Fukuda[1]*

1 Department of Cardiology, Keio University School of Medicine, Shinjuku, Tokyo, Japan, 2 Japan Society for the Promotion of Science, Chiyodaku, Tokyo, Japan, 3 Department of Cardiovascular surgery, Keio University School of Medicine, Shinjuku, Tokyo, Japan

Abstract

Recently, induced pluripotent stem cells (iPSCs) were established as promising cell sources for revolutionary regenerative therapies. The initial culture system used for iPSC generation needed fetal calf serum in the culture medium and mouse embryonic fibroblast as a feeder layer, both of which could possibly transfer unknown exogenous antigens and pathogens into the iPSC population. Therefore, the development of culture systems designed to minimize such potential risks has become increasingly vital for future applications of iPSCs for clinical use. On another front, although donor cell types for generating iPSCs are wide-ranging, T cells have attracted attention as unique cell sources for iPSCs generation because T cell-derived iPSCs (TiPSCs) have a unique monoclonal T cell receptor genomic rearrangement that enables their differentiation into antigen-specific T cells, which can be applied to novel immunotherapies. In the present study, we generated transgene-free human TiPSCs using a combination of activated human T cells and Sendai virus under defined culture conditions. These TiPSCs expressed pluripotent markers by quantitative PCR and immunostaining, had a normal karyotype, and were capable of differentiating into cells from all three germ layers. This method of TiPSCs generation is more suitable for the therapeutic application of iPSC technology because it lowers the risks associated with the presence of undefined, animal-derived feeder cells and serum. Therefore this work will lead to establishment of safer iPSCs and extended clinical application.

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* E-mail: kfukuda@a2.keio.jp

Introduction

Induced pluripotent stem cells (iPSCs) are expected to provide new cell sources for revolutionary therapies[1]. In initial studies of generating human iPSCs, human fibroblasts were reprogrammed using forced expression of reprogramming factors[2]. Further studies subsequently showed successful reprogramming of several types of human somatic cells[3]. A range of donor cell types has been used to generate iPSCs; however, blood cells have attracted much of the attention because of the ease of sampling. Of these, T cells are a unique cell source for the generation of iPSCs because T cell-derived iPSCs (TiPSCs) have monoclonal T cell receptor (TCR) gene rearrangements in their genomes[4–7] and they can be differentiated into antigen-specific T cells for use in novel immunotherapy[8,9]. Furthermore, clones and descendants of TiPSCs can be identified via the unique rearrangement patterns in the TCR gene locus[5].

Human T cells have been successfully reprogrammed with the exogenous expression of four transcription factors, *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*[4–7,10]. Among these methods, Sendai virus (SeV) is an RNA virus of the Paramyxoviridae family that replicates in the cytoplasm and does not go through a DNA phase that can integrate into the host genome[11–13]. iPSCs generation using a combination of activated T cells and SeV has therefore showed benefit due to the high induction efficiency and lack of genome insertion of exogenous genes[5].

On another front, the application of culture systems to reduce potential risks of unpredictable agent becomes increasingly vital for applying iPSCs clinically[14]. The initial culture system for ESC generation used fetal calf serum in the culture medium and mouse embryonic fibroblast (MEF) as a feeder layer, introducing possibilities for transferring exogenous antigens, unknown viruses, or zoonotic pathogens to the generate cell populations[15]. An animal product-free medium that supports the derivation and long-term feeder-independent culture of human ESCs was recently developed[16], and in the field of iPSC generation, many efforts have been made to remove potentially harmful agents from the culture system[17–20].

Therefore, we investigated generating transgene-free human TiPSCs by applying a combination of SeV and activated T cells into defined culture medium and feeder-free conditions towards future clinical application of TiPSCs.

Materials and Methods

Cell Culture and SeV Infection

Blood samples were obtained from healthy donors. Samples were obtained only after the donors had provided written informed consent for blood sampling for the subsequent generation of iPSC. The handling these samples was approved by the Ethics Committee of Keio University (20-92-5). In addition, the study conformed to the principles outlined in the Declaration of Helsinki regarding the use of human tissue or subjects.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors (four men [31, 30, 30, and 31 years of age] and one 30-year-old woman) by the centrifugation of heparinized blood over a Ficoll-Paque PREMIUM (GE Healthcare) gradient, according to the manufacturer's instructions. PBMCs were cultured at 37°C in 5% CO_2 with plate-bound anti-CD3 monoclonal antibody (BD Pharmingen) in GT-T502 medium (KOHJIN BIO) that contained rIL-2 at 175 JRU/ml. After 5 days of culture, activated PBMCs were collected and transferred at 1.5×10^6 cells/well to a new 6-well plate coated with the anti-CD3 mAb, and incubated for an additional 24 hours. Then, cells were transduced with the CytoTune iPS Reprogramming Kit (DNAVEC) that contained SeV vectors individually encoding for each of *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* at an MOI of 10. After 24 hours of infection, the medium was changed to fresh GT-T502 medium. At 48 hours post-infection, the cells were collected and transferred to a 10 cm-dish coated with Matrigel (BD Biosciences) in mTeSR1 media (StemCell Technologies Inc.). After an additional 24 h incubation, the medium was changed to new mTeSR1 medium and was changed thereafter daily until the colonies were selected. The TiPSCs generated were also maintained on Matrigel in mTeSR1 medium. The mTeSR1 medium was changed every other day, and the cells were passaged using dissociation solution for human ES/iPS Cells (CTK solution, ReproCELL) every 6–7 days. M-TiPSCs1 and M-TiPSCs2 were derived from a 31 year old man. In this series of experiments, the human ESC line KhES-2 [21] was used as a positive control and was cultured using the same protocol as for the iPSC culture described above. The KhES-2 cells were obtained from the Department of Development and Differentiation, Institute for Frontier Medical Sciences (Kyoto University) and used in line with the Guidelines for Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

ALP and Immunofluorescence Staining

ALP staining was performed with the ALP substrate (1-step NBT/BCIP; Pierce) after fixation with 4% paraformaldehyde (PFA; MUTO Pure Chemicals, Japan). Immunofluorescence staining was performed using the following primary antibodies:

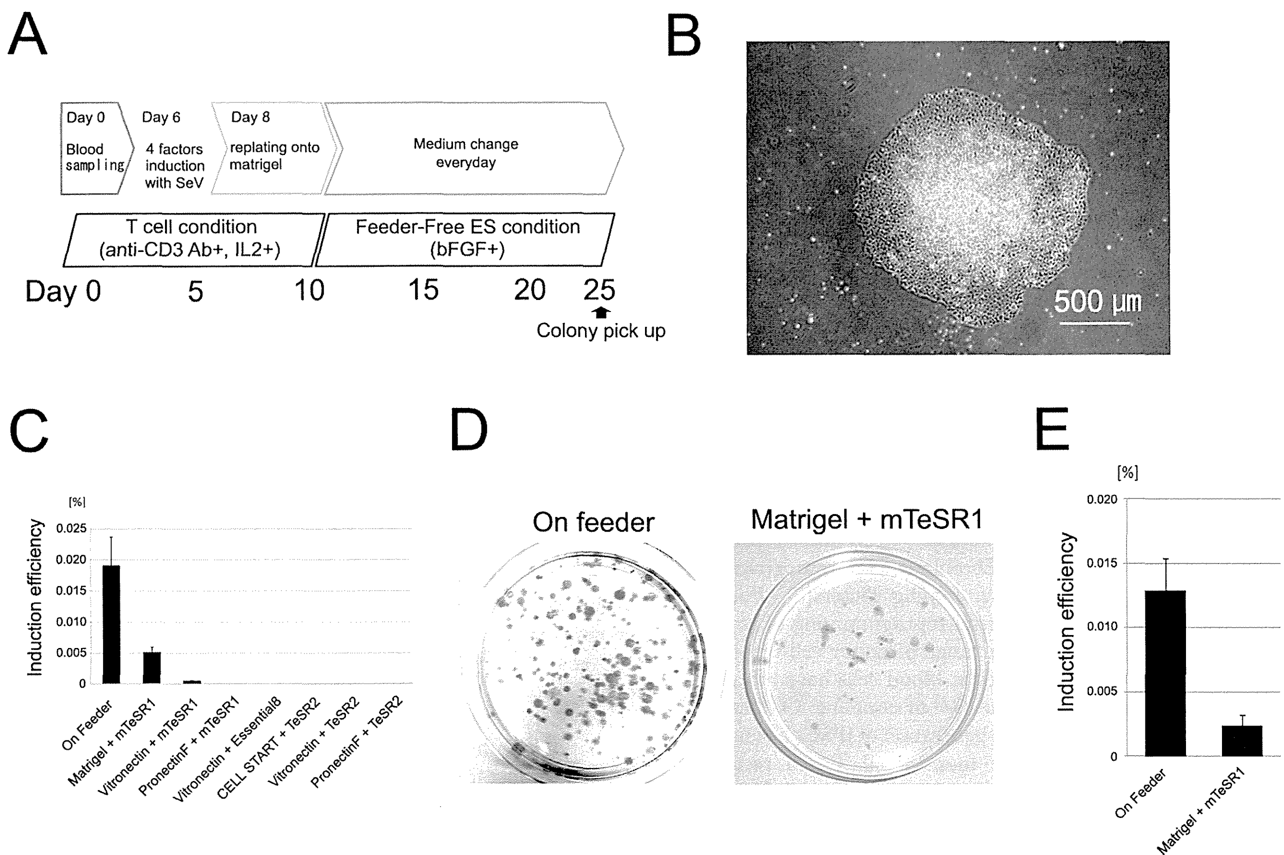

Figure 1. Generation of human TiPSCs under defined conditions. (A): Strategy used in the present study for reprogramming T cells. (B): Typical ESC-like TiPSC colony on day 25 after blood sampling under the defined culture condition. (C): Comparison of reprogramming efficiencies between the culture system using a feeder cell layer and that using defined culture conditions. Data show the mean ± s.d. (D): Comparison of representative 10-cm dishes stained for ALP (red spots) between feeder layer condition and defined culture condition (Matrigel) on day 25. (E): Comparison of reprogramming efficiencies between a culture system using a feeder cell layer and one using Matrigel and mTeSR1 medium for samples from five donors. Data show the mean ± s.d.
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anti-NANOG (RCAB0003P, ReproCELL), anti-OCT3/4 (sc-5279, Santa Cruz), anti-SSEA 3 (MAB4303, Millipore), anti-SSEA 4 (MAB4304, Millipore), anti-Tra-1–60 (MAB4360, Millipore) and anti-Tra-1–81 (MAB4381, Millipore), anti-human smooth muscle actin (IR61161, DAKO), anti-human Sox17 (AF1924, R&D Systems), or anti-Nestin (N5413, Sigma). The fluorescence signals were detect using a conventional fluorescence laser microscope (IX70; Olympus) equipped with a color charge-coupled device (CCD) camera (CS220; Olympus). DAPI (Molecular Probes) and Hoechst (33342; Lonza) were used for nuclear staining. The secondary antibodies used were: anti-rabbit IgG and anti-mouse IgG and IgM conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes).

Quantitative PCR Analysis

Total RNA samples were isolated using TRIZOL reagent (Invitrogen) and RNase-free DNase I (Qiagen), according to the manufacturer's instructions. The concentration and purity of the RNA were determined using an ND-1000 spectrophotometer (Nanodrop), and the cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen). Quantitative PCR (QT-PCR) was performed using a 7500 real-time PCR system (Applied Biosystems), with SYBR Premix ExTaq (Takara, Otsu, Japan). The amount of mRNA was normalized against that of *GAPDH* mRNA. Primer sequences and cycling conditions are listed in Table S1.

Bisulfite Sequencing

Genomic DNA was isolated from bulk cell culture samples or undifferentiated colonies. A total of 5 µg DNA was used as the input for bisulfite conversion with EZ DNA Methylation-Gold Kit (ZYMO RESEARCH) according to the manufacturer's protocol. The converted DNA was used as a template for conventional nested PCR to amplify the regions of the *OCT3/4* and *NANOG* promoters[22]. The primers were specific for conversion of the sense DNA strands (Table.S1). The purified PCR products were TA-cloned into the pGEM-T vector (Promega), and insert sequences of randomly picked clones were analyzed using the ABI 3700 DNA analyzer (Applied Biosystems).

Analysis of TCR Gene Rearrangement in Genomic DNA

Genomic DNA was extracted from approximately 5×10^6 cells using a QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. For *TCRB* gene rearrangement analysis, PCR was performed according to BIOMED-2 protocols[23]. The dominant band within the expected size range was purified using a Wizard SV Gel and PCR Clean-Up System (Promega) and the purified PCR products were TA-cloned into the pGEM-T vector (Promega). The insert sequences of randomly picked clones were analyzed using the ABI 3700 DNA analyzer (Applied Biosystems). V, D, and J segment usages were identified by comparison to the ImMunoGeneTics (IMGT) database (http://www.imgt.org/) and by using an online tool (IMGT/V-QUEST) [24]. Gene-segment nomenclature follows IMGT usage.

Global Gene Expression Analysis

For transcriptional profiling, total RNA was isolated from TiPSCs using the RNeasy Mini Kit (Qiagen). Cyanine-labeled antisense RNA was amplified using the Quick Amp Labeling Kit (Agilent), hybridized with the Gene Expression Hybridization Kit onto a Whole Human Genome Oligo Microarray (Agilent), and analyzed using the Agilent Microarray Scanner. The data were analyzed with the GeneSpring GX12.0 software (Agilent). Two normalization procedures were applied. Initially, the signal intensities with values less than 1 were assigned a value of 1. Then, each chip was normalized to the 50th percentile of the measurements taken from that chip. Finally, each gene was normalized to the median of that gene in the respective controls, to enable comparisons of relative changes in gene expression levels between different conditions. The microarray data in this experiment have been deposited in GEO and given the series accession number GSE56234.

Teratoma Formation

TiPSCs (at a concentration corresponding to 25% of the cells from a confluent 150-mm dish per mouse) were injected into the testis of SCID mice (CLEA, Japan). Prior to injection, mice were anesthetized using a mixture of ketamine (50 mg/kg), xylazine (10 mg/kg), and chlorpromazine (1.25 mg/kg). Adequate anesthesia was maintained by monitoring the heart rate, muscle relaxation, and loss of a sensory reflex response (i.e. no response to tail pinching) in mice. At around 8 weeks after injection, mice were killed by cervical dislocation and the teratomas were dissected, fixed in 10% PFA overnight, and embedded in paraffin. The sections were stained with hematoxylin and eosin. All experiments were performed in accordance with the Keio University Animal Care Guidelines and were approved by the Ethics Committee of Keio University (20-041-4), which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

In vitro Differentiation

Cells were harvested using CTK solution (ReproCELL), and transferred to ultralow attachment plates (Corning) in hiPSC medium without bFGF. After 8 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for an additional 8 days. The cells were incubated at 37°C in 5% CO_2 and the medium was replaced every other day.

Chromosome Karyotyping

TiPSCs treated with colcemid solution (60 ng/ml) were cultured for 5 hours at 37°C to stop cell growth in metaphase. These cells were then incubated in 0.075 M KCl solution for 10 min at room temperature, followed by fixation in Carnoy's solution. Fixed cells were sent to Nihon Gene Research Laboratories Inc. (Sendai, Japan) for analysis.

Results

Generation of Human TiPSCs under a Defined Culture Condition

First, we attempted to find a culture system involving alternatives to feeder cells in the generation of human TiPSCs. Previously, Matrigel[25], pronectin F[26], vitronectin[18], and CellStart[27] have been used as feeder cell substitutes for the successful culture of human pluripotent cells. Therefore we examined the feasibility of generating TiPSCs on these substitutes in a chemically defined culture medium, mTeSR1 or TeSR2. Activated human peripheral blood mononuclear cells (PBMCs) were infected with SeV vectors expressing *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*, and then replated onto feeder cells or one of the substitutes (Fig. 1A). Within 3 weeks of infection, we identified colonies that resembled human ESCs using mTeSR1 and Matrigel (Fig. 1B) or vitronectin. Although the efficiency was generally lower than culturing on feeder cell layers, the Matrigel and mTeSR1 combination resulted in higher efficiency than the other

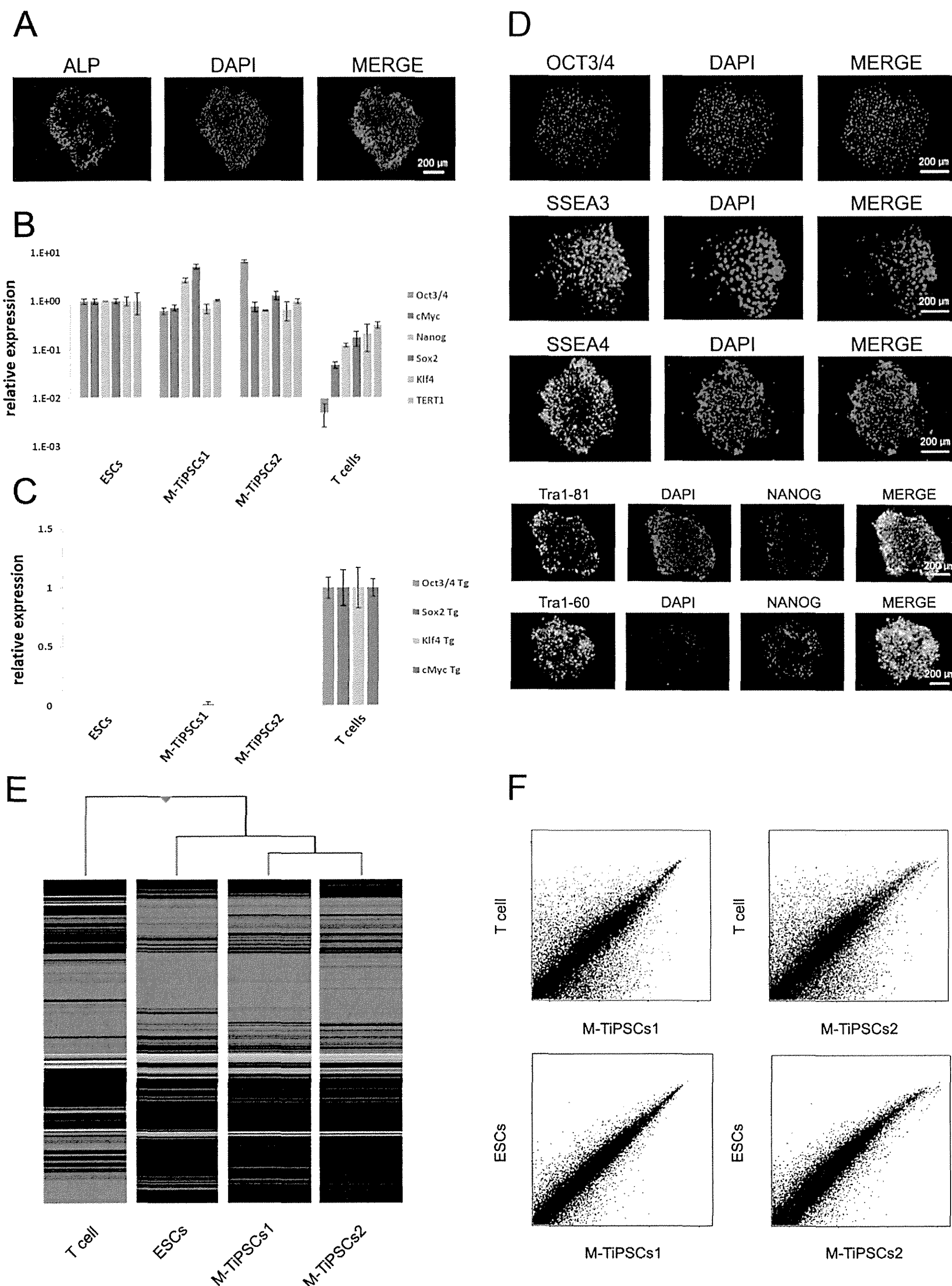

Figure 2. Characterization of M-TiPSCs generated under a defined culture condition. (A): ALP staining in M-TiPSCs. (B): QT-PCR analyses of M-TiPSCs for the ESC marker genes *OCT3/4, NANOG, SOX2, KLF4, c-MYC*, and *TERT1*. (C): QT-PCR analyses of M-TiPSCs for the transgenes, *OCT3/4, SOX2, KLF4*, and *c-MYC*. (D): Immunofluorescence staining for pluripotency and surface markers (NANOG, OCT3/4, SSEA3, SSEA4, TRA-1-60, and TRA-1-81) in M-TiPSCs1. (E): Heat map analyses of M-TiPSCs, ESCs, and the parental human T cells. (F): Scatter plots comparing the global gene expression profiles of M-TiPSCs with those of T cells and ESCs.
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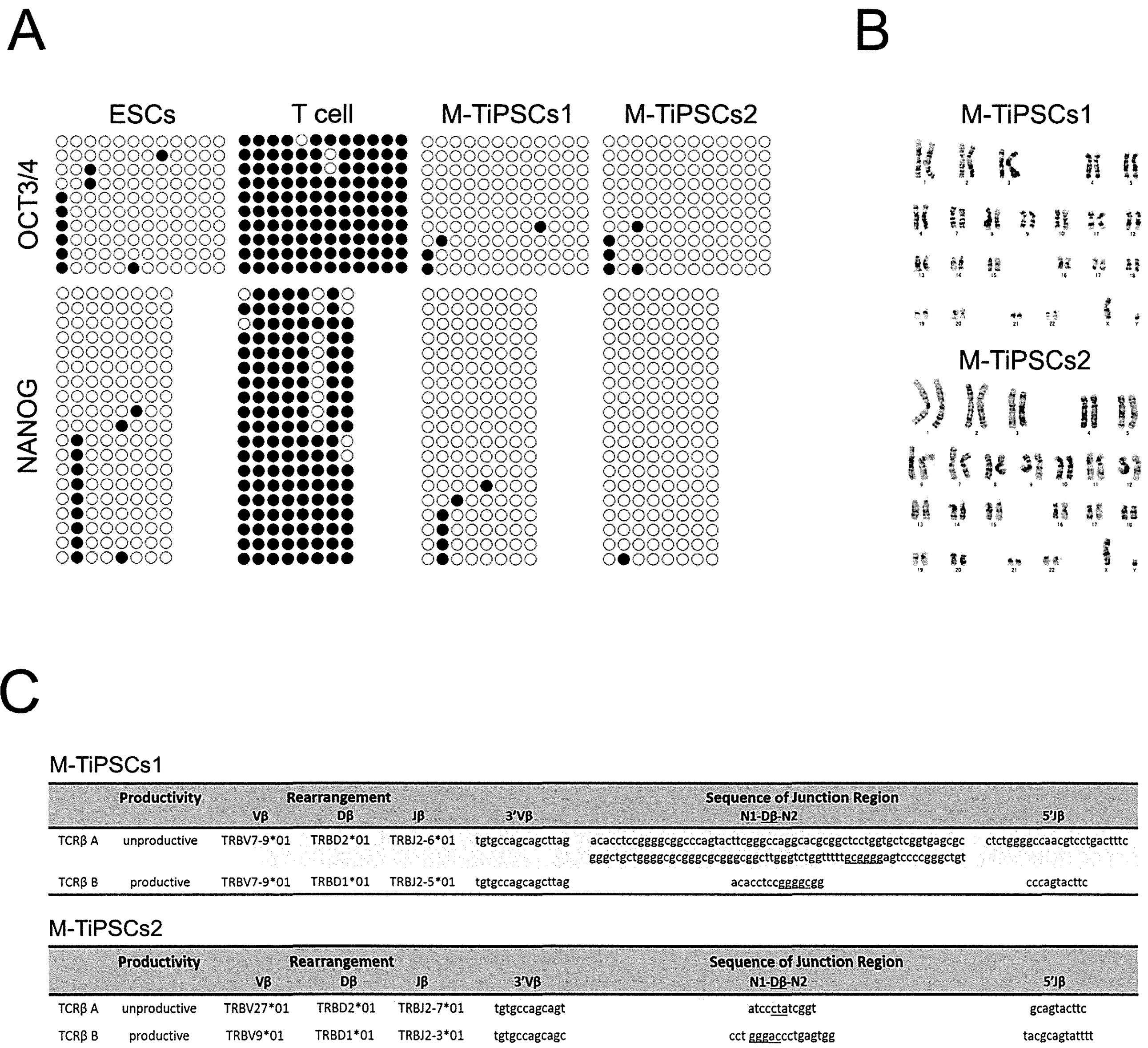
feeder-free conditions (Fig. 1C,1D), with successful iPSC generation from all donors (Fig. 1E). After picking these colonies on Day 25 from blood sampling, we established cell lines that maintained human ESC-like morphology.

Characterization of TiPSCs Generated under a Defined Culture Condition

To confirm that these TiPSCs generated on Matrigel (M-TiPSCs) had the characteristics of typical ESCs and iPSCs, we analyzed stem cell marker expression at the protein and mRNA levels, by DNA chips, and with bisulfite sequencing of the *NANOG* and *OCT3/4* promoters. Immunostaining and QT-PCR analyses revealed typical pluripotent marker expression in the M-TiPSCs



M-TiPSCs1

	Productivity	Rearrangement Vβ	Dβ	Jβ	Sequence of Junction Region 3'Vβ	N1-Dβ-N2	5'Jβ
TCRβ A	unproductive	TRBV7-9*01	TRBD2*01	TRBJ2-6*01	tgtgccagcagcttag	acacctccggggcggcccagtacttcgggccaggcacgcggctcctggtgctcggtgagcgc gggctgctggggcgcgggcgcgggcggcttgggtctggtttttgcggggagtccccgggctgt	ctctggggccaacgtcctgactttc
TCRβ B	productive	TRBV7-9*01	TRBD1*01	TRBJ2-5*01	tgtgccagcagcttag	acacctccggggcgg	cccagtacttc

M-TiPSCs2

	Productivity	Rearrangement Vβ	Dβ	Jβ	Sequence of Junction Region 3'Vβ	N1-Dβ-N2	5'Jβ
TCRβ A	unproductive	TRBV27*01	TRBD2*01	TRBJ2-7*01	tgtgccagcagt	atccctatcggt	gcagtacttc
TCRβ B	productive	TRBV9*01	TRBD1*01	TRBJ2-3*01	tgtgccagcagc	cct gggaccctgagtgg	tacgcagtatttt

Figure 3. Analysis of TiPSCs genome modification and karyotype. (A): Bisulfite sequencing analysis of the *NANOG* and *OCT3/4* promoter regions in peripheral T cells, ESCs, and M-TiPSCs. Each row of circles for a given amplicon represents the methylation status of the CpG dinucleotides in one bacterial clone for that region. Open circles represent unmethylated CpGs and closed circles represent methylated CpGs. (B): G-band analysis for karyotypes of M-TiPSCs generated under a defined culture condition. M-TiPSCs1 and M-TiPSCs2 at passages 6 and 15, respectively, were used for G-band analysis. (C): Analysis of TCR rearrangements. V, D, and J segment usages in the *TCRB* gene locus were sequenced and identified by comparison to the international ImMunoGeneTics information system database. M-TiPSCs showed rearrangements of Vβ/Dβ1,2 and Dβ1,2/Jβ2.
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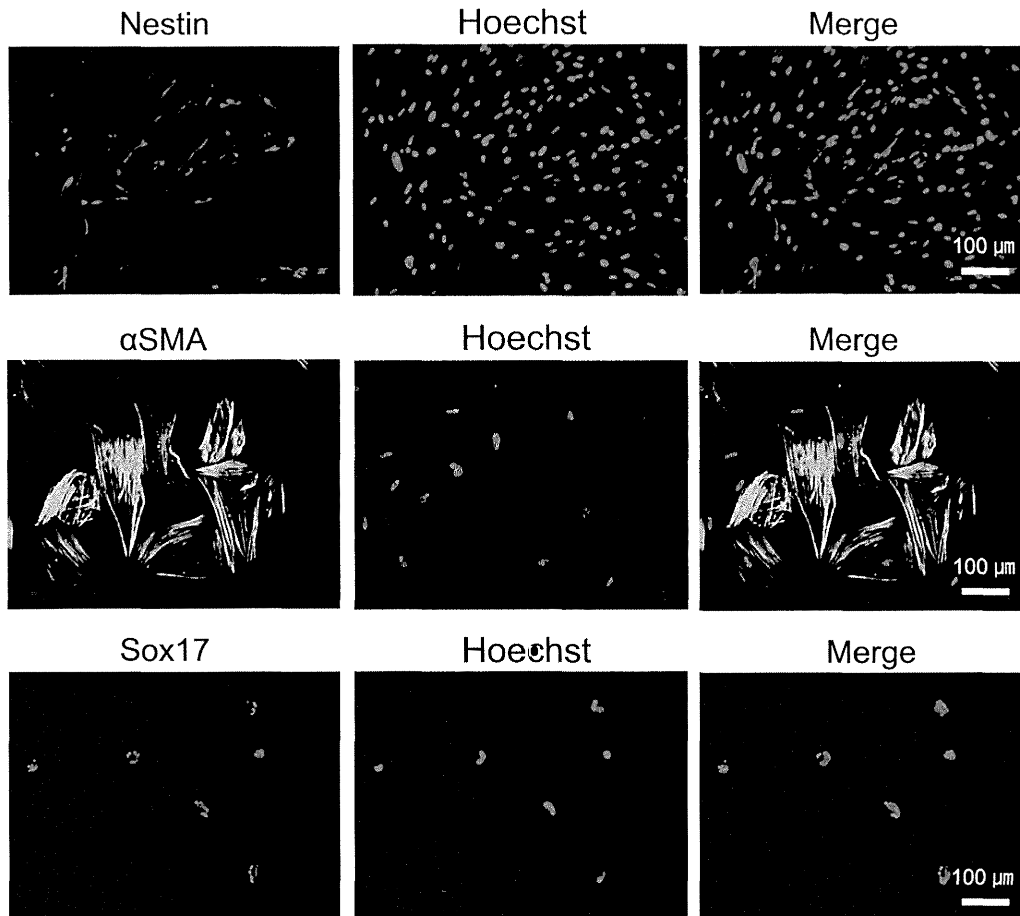
(Fig. 2A,2B,2D, Fig.S1A). On the other hand, QT-PCR analysis showed no SeV transgene expression in the M-TiPSCs (Fig. 2C). Heat map and scatter plot analyses of human peripheral circulating T cells, ESCs, and M-TiPSCs showed that the global gene expression profiles of M-TiPSCs were overall similar to ESCs, and different from the parental human T cells (Fig. 2E, 2F). Bisulfite sequencing of M-TiPSCs showed that CpGs in the promoter regions of *NANOG* and *OCT3/4* were predominantly unmethylated in the M-TiPSCs, as is the case in ESCs (Fig. 3A). Although previous reports have raised the possibility of leading chromosomal instabilities of human ESCs in feeder-free cultures [28,29], G-band analysis in this study revealed that the M-TiPSCs had normal karyotypes (Fig. 3B). These results suggested that SeV-mediated gene transfer on our defined culture conditions successfully reprogrammed human T cells into iPSCs that are similar to ESCs.

Analysis of TCR Rearrangements

To confirm that the M-TiPSCs were derived from mature T cells, we analyzed the TCR rearrangements by sequencing the V, D, and J segment usages in the T-cell receptor beta (*TCRB*) gene locus. The specific rearrangement pattern identified in the *TCRB* gene locus of the M-TiPSCs (Fig. 3C) confirmed their mature T cell origins.



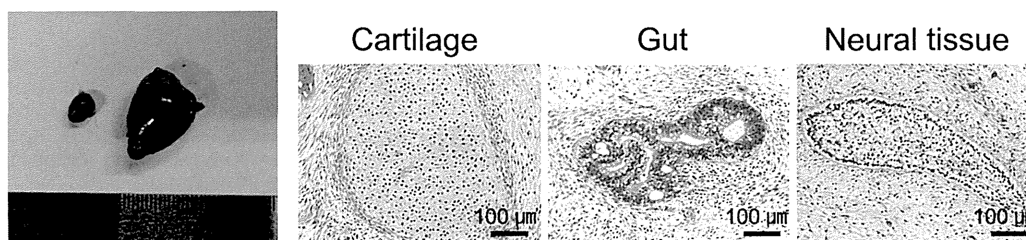


Figure 4. In vitro and in vivo differentiation of M-TiPSCs. (A): Immunofluorescence staining for Sox17 (endodermal marker), αSMA (mesodermal marker), and Nestin (ectodermal marker) in each TiPSCs1-derived differentiated cell in vitro. (B): Gross morphology of representative teratomas derived from TiPSCs1 in vivo (hematoxylin and eosin staining).
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In vitro and In vivo Differentiation

To then demonstrate the pluripotency of our M-TiPSCs, we checked their differentiation capability. In vitro differentiation assays revealed M-TiPSC-generated embryoid bodies that contained derivatives of all three germ layers (Fig. 4A, Fig.S1B). In addition, M-TiPSCs injected into the testes of SCID mice gave rise to teratomas that contained derivatives of all three germ layers (Fig. 4B). These results indicate that the M-TiPSCs generated herein are pluripotent stem cells.

Discussion

In this report, we successfully generated iPSCs from human peripheral T cells without fetal calf serum in the culture medium and MEF as a feeder layer. These substrates are associated with

the possibility of the transfer of exogenous antigens, unknown viruses, or zoonotic pathogens to the cell populations generated[15]. Although our protocol uses some substrates (e.g. Matrigel[30], anti-CD3 mAb, CTK solution[31], and SeV[32]) that are associated with a risk of transferring animal-derived pathogens, reducing the number of animal-derived substrates in the culture system will have a significant impact on the clinical application of iPSCs. However, the use of such products must be managed with strict quality control to avoid the risk of pathogen transfer and/or, in the future, these products should be removed from the protocol.

T cells are clinically suitable for iPSC generation because of their ease of sampling and culturing[5]. T cells have been successfully reprogrammed into a pluripotent state with retrovirus[7,10], lentivirus[4,6], or Sendai virus[5] using MEF feeder layers for the reprogramming culture conditions. Here, we report using a combination of SeV and activated T cells to successfully reprogram human T cells into a pluripotent state using defined culture conditions with verification of rearranged TCR genes. For clinical applications of iPSCs, establishment of such conditions are highly desirable for maximal safety and feasibility. Although reactivation of exogenous genes in the iPSCs still carries a risk of tumorigenesis[33], RNA-based vectors such as Sendai virus are much safer in this respect than DNA-based vectors, because they never integrate into a host genome[11–13]. The resultant feeder-independent TiPSCs thus have greatly enhanced safety for use in regenerative medicine.

Moreover TiPSCs have two unique features that are advantageous for clinical applications. First, because each TiPSC line contains a single rearranged TCR gene in its genome[4–7], they can be applied to novel immune therapy. Indeed, successful differentiation of human TiPSCs generated from antigen-specific T cells was reported[8,9]. Second, the rearrangement patterns of T-cell receptor loci are readily traceable to identify the clonality and the descendants of TiPSCs without insertion of exogenous marker genes[5]. Therefore, generating human TiPSCs under a defined culture condition in which the potential risk of unpredictable agents is reduced, is valuable to extend applications of TiPSCs and is significant for implementing regenerative therapies using iPSCs.

In conclusion, we developed a method for safer iPSCs generation using Sendai virus and activated T cells under a defined culture condition for clinical use. This reprogramming system also provides advantages for the development of novel immune therapy and genetic markers for use as a clinical research tool for future applications of regenerative medicine.

Supporting Information

Figure S1 Characterization of M-TiPSCs2 generated under defined culture conditions. (A): ALP and immunofluorescence staining for pluripotency and surface markers (NANOG, OCT3/4, SSEA3, SSEA4, TRA-1–60, and TRA-1–81) in M-TiPSCs2. (B): Immunofluorescence staining for Sox17 (endodermal marker), αSMA (mesodermal marker), and Nestin (ectodermal marker) in each TiPSCs2-derived differentiated cell in vitro.
(TIF)

Table S1 Oligonucleotide primers used for PCR.
(DOCX)

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Author Contributions

Conceived and designed the experiments: YK TS KF. Performed the experiments: YK TS. Analyzed the data: YK TS. Contributed reagents/materials/analysis tools: JF SY ST AK RT KN MO AH HK. Wrote the paper: YK TS JF.

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REVIEW

Methods of induced pluripotent stem cells for clinical application

Tomohisa Seki, Keiichi Fukuda

Tomohisa Seki, Keiichi Fukuda, Department of Cardiology, Keio University School of Medicine, Tokyo 160-8582, Japan
Tomohisa Seki, Department of Emergency and Critical Care Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
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Correspondence to: Keiichi Fukuda, MD, PhD, FACC, Professor and Chair, Department of Cardiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. kfukuda@a2.keio.jp
Telephone: +81-3-53633874
Fax: +81-3-53633875
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Abstract

Reprograming somatic cells using exogenetic gene expression represents a groundbreaking step in regenerative medicine. Induced pluripotent stem cells (iPSCs) are expected to yield novel therapies with the potential to solve many issues involving incurable diseases. In particular, applying iPSCs clinically holds the promise of addressing the problems of immune rejection and ethics that have hampered the clinical applications of embryonic stem cells. However, as iPSC research has progressed, new problems have emerged that need to be solved before the routine clinical application of iPSCs can become established. In this review, we discuss the current technologies and future problems of human iPSC generation methods for clinical use.

Key words: Induced pluripotent stem cells; Cell reprogramming

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Core tip: Each induced pluripotent stem cells methodology has advantages and disadvantages, as in the case of autologous *vs* allogenic transplantation, and the choice of appropriate strategy may vary depending on the intended use. Additionally, to avoid tumorigenesis and to establish effective differentiation into the intended cells, further investigation is needed to identify the most suitable iPSC line and how these lines should be selected.

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INTRODUCTION

In 2006, Takahashi *et al*[1] established a novel method of reprogramming mouse somatic cells using exogenetic expression of genes related to pluripotency. The cell lines established by this group were named induced pluripotent stem cells (iPSCs) and demonstrated the same pluripotency and self-renewal properties that are characteristic of embryonic stem cells (ESCs). The following year, the

same group also succeeded in generating iPSCs from human somatic cells[2]. These groundbreaking steps have been expected to lead to novel regenerative cell therapies with the potential to solve many problems surrounding incurable diseases. In particular, the clinical application of iPSCs is expected to solve the problems of immune rejection and ethics that are currently key obstacles in the clinical use of ESCs. However, as research into iPSCs has progressed, new problems to solve have emerged before iPSCs can be established as cell sources for patients. In this review, we discuss the current technology and future problems surrounding human iPSC generation methods for clinical applications.

BENEFITS AND PROBLEMS OF ESCS AND IPSCS IN CLINICAL APPLICATION

For treating diseases that lack self-repairing cells, the transplantation of artificially generated cells is one attractive means for curing the diseases. In fact, regenerative cell transplantation therapies have been expected to treat incurable diseases, such as spinal cord injury[3], neurodegenerative disease[4], heart failure[5,6], diabetes[7], and retinal disease[8].

ESCs have the capacity to self-renew and differentiate into cells of the three germ layers. The development of suitable cultivation systems for maintaining the pluripotency of ESCs marked their promise as a cell source for regenerative medicine since human ESCs were first generated in 1998[9]. Numerous efforts have been made since then to realize the promise of making specific differentiated cells from ESCs. However, the ethical problem of needing human zygotes to generate human ESCs has remained unsolved. Additionally, immune rejection remains an issue because of the limited number of ESC cell lines and the ability of the cell lines to match the huge number of human leukocyte antigen (HLA) type combinations found in patients.

These problems with ESCs and the ongoing need for regenerative therapies drove further research efforts, such as Dr. Yamanaka's 2006 success in generating iPSCs from somatic mouse cells[1] and reports in 2007 by Takahashi *et al*[2] and Yu *et al*[10] of the successful generation of human iPSCs. In the original mouse work, 24 transcription factors showing high expression in ESCs were chosen as candidate reprogramming triggers[1]. Finally, the forced expression of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* together with a retrovirus evoked mouse somatic cell reprogramming into the pluripotent state, and the same combination of four factors forced human fibroblasts into iPSCs[2]. At the same time, Yu *et al*[10] successfully reprogrammed human fibroblasts using the combination of *OCT4*, *SOX2*, *NANOG*, and *LIN28*. These methods attracted much attention because these iPSC lines could potentially overcome the immune rejection and ethical issues hampering the development of ESCs for clinical use. Therefore, iPSCs showed promise as the breakthrough technology in regenerative medicine.

COMBINATION OF REPROGRAMMING FACTORS FOR IPSC GENERATION

Since iPSC generation methods were first reported, numerous efforts have been made to adapt them to clinical applications[5,11]. The reported generation methods vary in the combinations of reprogramming factors, vehicles for exogenous genes, and cell types to generate the iPSCs. Therefore, current discussions in the literature focus on selecting the most appropriate iPSC generation method for clinical use.

In relation to the combination of reprogramming factors for iPSC generation, the first reported combination of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*, known as the Yamanaka factors, are generally used for iPSC generation. Combining only three of these factors and omitting *C-MYC* was also reported to achieve successful iPSC generation, although with reduced reprogramming efficiency[12,13]. These alternative methods arose following concerns about using *C-MYC*, which is a known oncogene in human cells. Another group also reported the generation of mouse iPSCs, which showed more efficient germline transmission in chimeric formation experiments using the combination of *OCT3/4*, *SOX2*, *KLF4*, and *TBX3* as reprogramming factors[14]. Subsequently, iPSC generation using *L-MYC* instead of *C-MYC* was reported with improved efficiency in both reprogramming somatic cells and germline transmission of generated iPSCs[15]. Furthermore, *GLIS1* was also reported as a candidate alternate factor for *C-MYC* that showed high reprogramming efficiency, less incomplete reprogramming, and reduced tumor formation in iPSC-derived mice[16]. Recently, improved iPSC quality was also achieved by introducing *Zscan4*, which is highly expressed at the zygotic genome activation stage. Forced expression of *Zscan4* in combination with the Yamanaka factors improved iPSC quality as demonstrated by tetraploid complementation[17].

Recently, lineage-specific genes were substituted for *OCT3/4* or *SOX2*[18]. In this report, *OCT3/4* was replaced with an early mesendodermal lineage marker, such as *GATA3*, and *SOX2* was replaced with an early ectodermal lineage marker, such as *ZNF521*, in reprogramming using the Yamanaka factors. This finding raised the possibility that *OCT3/4* and *SOX2* might act as lineage specifiers for cell reprogramming and showed that reprogramming factors are not limited to genes associated with pluripotency[18].

Although methods for checking the quality of iPSCs such as germline transmission experiments are not applicable to human iPSCs, such studies can be useful for seeking the best combination of reprogramming factors to generate human iPSCs that are of sufficient quality for clinical use.

GENE-DELIVERY VEHICLES FOR IPSC GENERATION

Gene-delivery vehicles are also an important for selecting a suitable method of iPSC generation for clinical use, and to date, many gene delivery vehicles have proven to be applicable to iPSC generation. Initially, retrovirus vectors, such as pMXs[2,19,20], pLib[21] or pMSCV[22,23], were used for the delivery of reprogramming factors into somatic cells. Importantly, transgenes introduced with retrovirus vectors have been known to be silenced in pluripotent states[22,24], and therefore, silencing of transgene expression in iPSCs has been thought to be an important result of successful reprogramming[25]. Lentiviral vectors have also been used for successful iPSC generation because lentiviral vectors achieve a higher efficiency of infection than retrovirus vectors[10,26]. Importantly, transgenes introduced by lentiviral vectors are more resistant to being silenced in pluripotent states than those transtenes introduced by retrovirus vectors[27].

The genomic integration of transgenes that occurs as a result of these virus vectors was thought to be a problem for the clinical use of iPSCs because the delivered transgenes have the potential to be reactivated after cell reprogramming and thus drive oncogenesis in the iPSC-derived cells[28]. Furthermore, these transgenes have the potential to disrupt functional genes, even if they are silenced and not expressed. Therefore, many efforts have been made to generate iPSCs without the genomic insertions. For example adenovirus vectors are routinely used to introduce transient gene expression in target cells. Furthermore, a replication-defective pHIHG-Ad2 vector, was used to successfully reprogram somatic cells into iPSCs[29]. Therefore, although adenoviral vectors still integrate into the genome of target cells at extremely low frequencies[30] and the reprogramming efficiency is significantly lower than that with retrovirus, this method generates iPSCs that do not transfer residual transgenes into the host genomes.

More recently, a Cre-deletable lentivirus system was used for the successful generation of iPSCs[31]. However, although these systems can avoid transgene reactivation, the risk of gene breaks being introduced near the insertion site is present because the LoxP sequence remains in the host genome after removing the insert sequence by Cre recombinase[32].

In addition to virus vectors, the Sendai virus was also successfully used to generate iPSCs[33,34]. The Sendai virus genome is negative-sense single-stranded RNA. Because replication occurs in the cytoplasm, this virus vector does not pose a danger of genome insertion. Therefore, this method solves both problems of gene disruption near the insertion site and reactivation of transgenes. Additionally, the residual Sendai virus RNA can be removed from the infected cells using siRNA[35], and temperature-sensitive mutations[36] were also reported, improving the potential clinical suitability of this iPSC generation method.

As another approach for safe iPSC generation, a virus-free reprogramming method has received attention. For example, an iPSC generation method using episomal vectors has also been developed[37-40]. The early attempts using this method yielded lower efficiencies of successful reprogramming than those achieved using retrovirus, and only a low percentage of iPSC lines generated using this method had no plasmid integration. However, later modifications of the episomal vector method yielded a higher reprogramming efficiency using a combination of plasmids encoding *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and shRNA for *TP53*[41,42].

Generating iPSCs using a piggyBac transposon was reported as another method to avoid the reactivation of residual transgenes and gene breaks in the host genome[43-45]. The piggyBac transposon is a moth-derived DNA transposon[46] that is highly active in mammalian cells and able to be completely eliminated from the host genome using the piggyBac transposase[47]. Despite generating integration-free iPSCs by this method, the reported reprogramming efficiency was lower than that with retrovirus, and thus further improvements are needed[43-45].

As another way to avoid introducing genetic material, introducing reprogramming factors such as RNAs or proteins has attracted much attention. Indeed, the direct delivery of synthetic mRNAs has been shown to successfully reprogram somatic cells to a pluripotent state[48]. In this study, *in vitro* transcribed RNAs were modified to avoid the endogenous antiviral cell defense. As a result, this method achieved a higher iPSC generation efficiency than the original retrovirus system[48]. Successful reprogramming of somatic cells has also been achieved using microRNAs[49], whereby expression of the *miR302/367* cluster containing five different miRNAs, *miR302a/b/c/d* and *miR367*, reprogrammed human fibroblasts more efficiently than previous retrovirus systems[49]. Such RNA-based reprogramming avoids both breaks in existing genes and the reactivation of transgenes. Therefore, these methods hold much promise as novel iPSC generation methods that could be applicable for clinical use.

Similarly, recombinant proteins were also reported as a successful means of gene introduction for generating iPSCs[50,51]. These protein-based methods are also attractive for clinical application because of the absence of breaks in existing host genes and the reactivation of transgenes; however, the generation efficiencies remain lower compared to those in the existing retrovirus systems[50,51].

Finally, small-molecule drugs have been investigated for establishing safe methods of iPSC generation for clinical application because they are nonimmunogenic, cost-effective, and easy to handle[52]. Recently, successful reprogramming of mouse somatic cells without transgene introduction was achieved with small-molecule drug combinations[53]. This strategy has many merits for applying the iPSC generation method for clinical use, and therefore, further research into applying this method to human somatic cells is expected in the near future.

TYPES OF DONOR CELLS FOR IPSC GENERATION

Generating iPSCs in clinical practice also requires the consideration of the most appropriate type of donor cells. At first, iPSCs were generated from mouse fibroblasts[1], followed by successful reprogramming of mouse hepatocytes and gastric epithelial cells[54]. Subsequently, terminally differentiated somatic cells have also been reprogrammed, including mouse B lymphocytes[55] and pancreatic beta cells[56]. With respect to human cells, iPSCs have been generated from human dermal fibroblasts[2,10] and many types of human somatic cells[5].

As a matter of course, clinical applications of cell therapies require that tissue collection from patients be as minimally invasive as possible, and harvesting human dermal fibroblasts by biopsy leaves a small scar on the patient's body. Recently, iPSCs were generated from human keratinocytes induced from plucked hair, a process that is much less invasive than biopsy[23,57]. However, several hairs are needed to obtain the successful cell outgrowth of keratinocytes in some cases, and therefore, a more stable protocol for primary culture is needed for routine clinical practice.

Dental tissue has also been proposed as a unique cell source for iPSC generation. Dental stem cells[58,59] and mesenchymal stromal cells derived from human third molars[60] were successfully reprogrammed and thought to be potentially useful material for clinical iPSC generation. Oral gingival[61] and oral mucosa fibroblasts[62], which can be obtained less invasively, were also investigated for iPSC generation. These methods are advantageous for clinical application because they involve a minimally invasive approach for the patients.

Cord blood was also reported as another cell source for generating iPSCs[63,64]. Early studies with cord blood yielded lower reprogramming efficiencies than those achieved with lentivirus or retrovirus systems. However, a modified method involving the knockout of p53, which was previously shown to increase the efficiency of reprogramming[65-70] for iPSCs, increased the efficiency of generating iPSCs from CD34-positive cells which were sorted from cord blood cells[71]. Additionally, gene introduction using Sendai virus vectors successfully reprogrammed CD34-positive cord blood cells more efficiently than lentiviral or retroviral vector used to reprogram cord blood cells[36]. Cord blood cells have attracted much attention because cord blood-derived cells do not require laborious mobilization or an invasive biopsy before introducing reprogramming factors. Using cord blood cells for iPSC generation also avoids the risk of transferring over accumulated genetic mutations into iPSC. Additionally, banked cord blood cells are relatively uncomplicated for use in iPSC generation because their immunological information is already available in cord blood banks[72].

Peripheral blood cells are also an attractive cell source because the method for cell sampling from patients is less invasive. Peripheral blood cells are more easily accessible as a cell source than the dermal fibroblasts obtained by skin biopsy. In the first study to generate iPSCs from human blood cells, the donor needed to be injected for 3 days with G-CSF to mobilize the CD34-positive cells, and the reprogramming efficiency was not higher than that of previous studies[73]; however, since then there have been many efforts to effectively generate iPSCs from peripheral blood cells. For example, less invasive methods have since been reported for generating iPSCs from peripheral mononuclear blood cells[34,74-77], whereby mononuclear blood cells from donors or frozen samples were induced using the Yamanaka factors with a retrovirus[74,77], lentivirus[75,76], or Sendai virus[34]. In these experiments, the majority of iPSCs generated from mononuclear cells had *TCR* gene rearrangements, indicating that these cell lines were derived from T lymphocytes, and the reprogramming efficiencies with the Sendai virus were similar to those with the previous retrovirus system[34]. Additionally, to avoid generating iPSCs containing genome rearrangements, methods were developed in which iPSCs were generated from peripheral mononuclear cells cultured under conditions that inhibit the proliferation of lymphocytes[78,79] and from CD34-positive cells that were mobilized without additional drug administration to the donors[80]. Sampling of peripheral blood is one of the least invasive procedures available, and therefore, generating iPSCs from peripheral blood could be one of the most appropriate methods for the clinical applications of iPSCs.

REMOVING ANIMAL PRODUCTS FROM CULTURE SYSTEMS

One of the most important issues to address in applying iPSCs to clinical therapy is that the culture systems for generating iPSCs contain animal-derived products with potential and unpredictable risks to patients[81]. The initial culture system for ESC generation contained fetal calf serum in the culture medium and mouse embryonic fibroblasts as a feeder layer. These animal-derived products conferred a risk of transferring exogenous antigens, unknown viruses, or zoonotic pathogens to the generated cell populations[9]. Thus, many investigations have be conducted to reduce such risks by establishing animal product-free culture systems for human iPSCs.

First, human-derived feeder cells have proven to be a useful alternative to mouse cells for human iPSC generation[82]. However, these feeder cell preparations need significant time and effort, and in the case of clinical therapies requiring the mass culture of human iPSCs in some situations, using human-derived feeder cells for culturing human iPSCs is not an optimal strategy. As an option for culturing human iPSCs without feeder cells, Matrigel has proven to be a useful alternative that enables the stable culture of human pluripotent stem cells[83-85]. Although Matrigel allows the generation of human iPSCs without animal-derived feeder cells[86,87], Matrigel itself was derived from Engelbreth-Holm-Swarm mouse

sarcoma cells[88]. Therefore, other types of matrices, such as CellStart[89,90], recombinant proteins[91-93], and synthetic polymers[94,95], which do not contain animal-derived agents, have been tested and used as feeder-cell substitutes for the successful maintenance and generation of human pluripotent cells.

Developing animal product-free medium for iPSC generation is also an important practice for achieving safe therapy using iPSCs. The culture media used in the early generation of human ESCs contained fetal bovine serum[9]. To remove unpredictable agents that might cause the differentiation of human ESCs, knockout serum replacement (KSR) has now been established as a defined material for maintaining human ESCs[96] and is also used for human iPSC generation[2]. Additionally, mTeSR1 medium was developed as a chemically defined medium for maintaining human pluripotent cells and is used for defined condition cultures of human pluripotent stem cells[97]. However, because KSR and mTeSR1 also contain animal-derived products, new media have now been commercially developed as xeno-free media for maintaining human pluripotent stem cells and have already been used successfully for iPSC generation; these media include TeSR2[98], NutriStem[99], Essential E8[91], and StemFit[100].

SELECTING THE MOST APPROPRIATE IPSC LINE FOR CLINICAL USE

One of the most intractable problems for applying iPSCs to clinical therapy is the variety of iPSC lines with respect to differentiation tendency and tumorigenic risk. Additionally, the laboratory in which the iPSCs are generated could influence global gene expression patterns of those iPSCs due to small possible differences in the culture conditions[101]. Therefore, how to select iPSC lines that are appropriate for a specific clinical use in terms of safety and differentiation ability remains a topic of intense discussion.

As described above, in contrast to generating iPSCs with Yamanaka factors, introducing *TBX3*[14], *L-MYC*[15], or *GLIS1*[16] instead of *C-MYC* has yielded high-quality iPSCs with respect to the efficiency of germ line transmission and prognosis of iPSC-derived mouse cells. These reports implied that selecting suitable combinations of reprogramming factors was an important consideration for clinical therapy using iPSCs. However, these reports were derived from mouse experiments, and how these findings translate to human iPSCs remains unknown. Of course, the quality index of iPSCs using chimeric formation is not applicable to human iPSCs. Therefore, another index of iPSC quality that is applicable to human iPSCs is required.

With respect to assessing the effects of the type of donor cell on the quality of generated iPSCs, the teratoma-forming propensity of neural stem and progenitor cells derived from mouse iPSCs was shown to differ depending on the donor cell type[102]. This finding suggested that the quality of the iPSCs should be considered when the type of donor cell is selected. Since that early report, an epigenetic memory of tissue of origin donor cell type that affected the differentiation tendency of iPSCs was reported[103,104]. Importantly, the effect of epigenetic memory was not demonstrated when pluripotent stem cells generated by nuclear transfer were used. Additionally, another recent study showed that human iPSCs contain more CpG sites that retain the DNA methylation pattern of the parental donor cells than human ESCs generated by nuclear transfer[105]. Although the fact that the differentiation tendency derived from epigenetic memory will not remain after long-term culture[103], these reports also suggested the importance of selecting the donor cell type when applying iPSCs clinically.

The effect of donor cell type on differentiation tendency was also reported in human iPSCs[106], but experiments that compared donor cell type, gene vehicle type, and volunteers on hepatic differentiation tendencies of iPSCs showed that the differentiation tendency of iPSCs depended on the volunteer from which the iPSCs were generated[107]. Interestingly, in this latter study, hepatic differentiation tendencies that were derived from donor cells were not observed, implying that such differences derive from the volunteer cell donor when epigenetic memory does not remain after long-term culture.

With respect to the tumorigenic tendencies of human iPSCs, a study of gene expression and DNA methylation in 21 human iPSC lines and 2 human ESC lines showed activated expression of genes containing specific LTR7 sequences in some human iPSC clones that showed a neural differentiation-defective phenotype and formation of teratomas when they were differentiated into dopaminergic neurons and transplanted in mouse brains[108]. Another report on 21 human iPSC lines and 6 human ESC lines indicated that certain human iPSC clones were in a pro-oncogenic state, as shown by the ectopic presence of secretory tumor tissue during *in vitro* cartilage differentiation[109]. These reports together implied that a marker foreseeing pro-oncogenic differences in iPSC lines would be required for establishing safe therapy using iPSCs.

AUTO-TRANSPLANTATION AND ALLO-TRANSPLANTATION OF IPSCS

One of the most important advantages expected of iPSCs for transplantation therapy is avoiding immune rejection and therefore avoiding combination immunosuppressive treatment. Indeed, autologous iPSCs were used in the first human clinical trial of iPSCs started by Dr. Masayo Takahashi at the RIKEN Center for Developmental Biology in Kobe in 2013 for treating age-related macular degeneration with iPSC-derived sheets of retinal pigment epithelium. With respect to the immunogenicity of autologous iPSCs, in the first report that aroused discussion on its credibility, immunogenicity showed in the case of iPSCs was not observed in the case of ESCs[110,111]. Subsequently, experiments with isogenic transplantation of mouse iPSCs revealed the successful engraftment of iPSC-derived tissue without immunosuppressive treatment and

verified the advantages of using auto-transplantation of iPSCs for avoiding immunosuppressive treatment[112,113]. However, a recent study demonstrated immune rejection upon transplanting autologous undifferentiated mouse iPSCs *in vivo*, and rejection was imperceptible upon transplanting autologous terminally differentiated mouse iPSCs[114]. Although this report supports the presence of immunogenicity in undifferentiated iPSCs[91] and conflicts with the successful autologous engraftment of undifferentiated iPSCs in other reports[93,94], it supports the advantages of using auto-transplantation of iPSC-derived terminally differentiated cells for avoiding immunosuppressive treatment. On another front, whether the immunogenicity in undifferentiated iPSCs contributes to removing contaminated undifferentiated cells from iPSC-derived cell populations and avoiding tumorigenesis after transplantation in the same manner as removing undifferentiated cells from cell populations before transplantation[115-117] remains to be clarified.

Allo-transplantation of iPSC-derived cells was also expected to provide a useful strategy for transplantation therapy using iPSCs due to saving cost and time in generating autologous iPSC lines for transplantation. Furthermore, as described above, selecting appropriate iPSC lines on a patient-by-patient basis will require significant numbers of studies for verification. Therefore, although the transplantation of iPSC-derived cells from an allogenic donor with foreign HLA requires lifelong immunosuppressive treatment of the recipient, the concept of homozygous HLA-typed iPSC banks may be feasible for achieving generalized therapy using iPSCs[41,118,119] and, indeed, this type of approach is already progressing[120].

CONCLUSION

The invention of iPSCs was groundbreaking for novel regenerative medicine and has been expected to lead to regenerative therapies with the potential to advance the treatment and management of incurable diseases. Importantly, iPSCs could overcome the problems of immune rejection and ethics that remain with ESCs. However, each strategy of using autologous or allogenic iPSCs has advantages and disadvantages, and the choice of appropriate strategy may vary depending on the intended use. Additionally, there remain many factors that affect establishing transplantation therapy using iPSCs. To avoid tumorigenesis and establish effective differentiation into the intended cells, further investigation is needed to clarify which iPSC line is the most suitable and how these lines can be best selected.

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