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Figure 4: Poor survival of patients with BORIS positive ESCC

Overall survival rate was calculated by Kaplan-Meier method. A) The 5-year overall survival rate was 70.0% in the BORIS-negative group (N=22) and 29.9% in the BORIS-positive group (N=28), and the difference between the two groups was significant ( $p=0.028$ ). B) The 5-year survival rate in the patients with pT2/3 (N=32) was 60% in BORIS-negative group (N=17) and 0% in BORIS-positive group (N=15), and the difference between the two groups was significant ( $p=0.015$ ).

Figure 5: BORIS is involved in the cell proliferation and invasive ability of ESCC cell lines

A) BORIS expression was inhibited by BORIS-specific siRNA #1 and #3 in ESCC cell lines, TE5 and TE10, shown by qPCR at 48 hours (left panel) and RT-PCR at each time point (right panel). B) Cell proliferation of TE5 and TE10 was significantly inhibited by transfection with BORIS-specific siRNA #1 and #3 when measured by WST-1 assay. The number of cells was determined by absorbance of O.D. 450nm. \*\*:  $p<0.01$  C) Matrigel invasion assay showed the invasive ability of TE5 and TE10 was significantly inhibited by siRNA #1 and #3. \*:  $p<0.05$ . We used Stealth RNAi Negative Control Kit with medium GC content (Invitrogen, Carlsbad, CA) as a negative control.

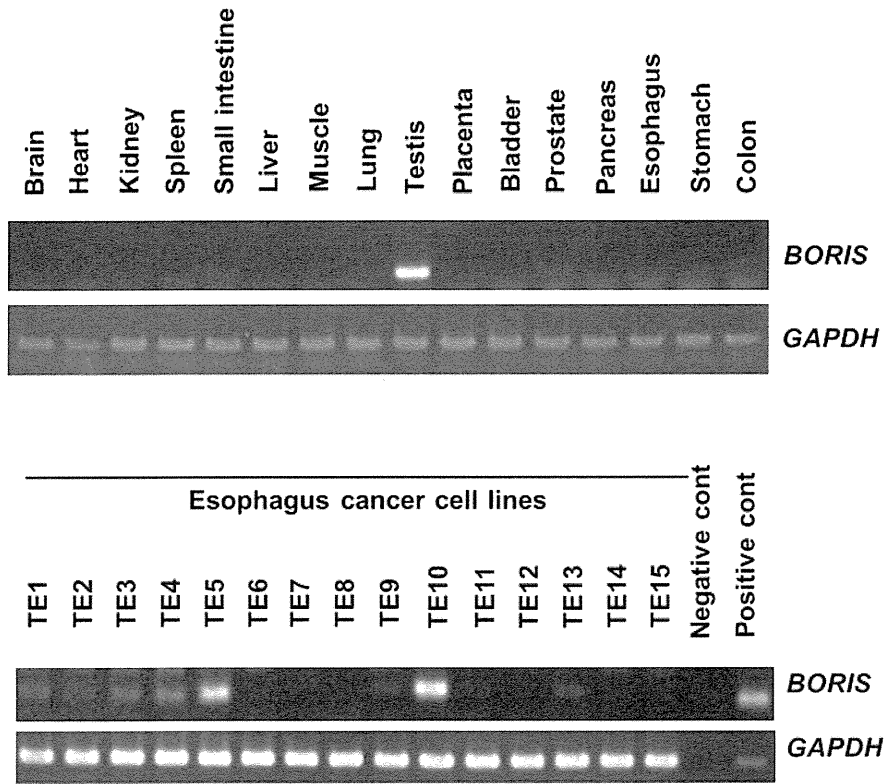


Figure 1. BORIS is expressed in ESCC cell lines and testis among normal tissues. BORIS was expressed in 7 of 15 esophageal cancer cell lines (RT-PCR).  
94x85mm (300 x 300 DPI)

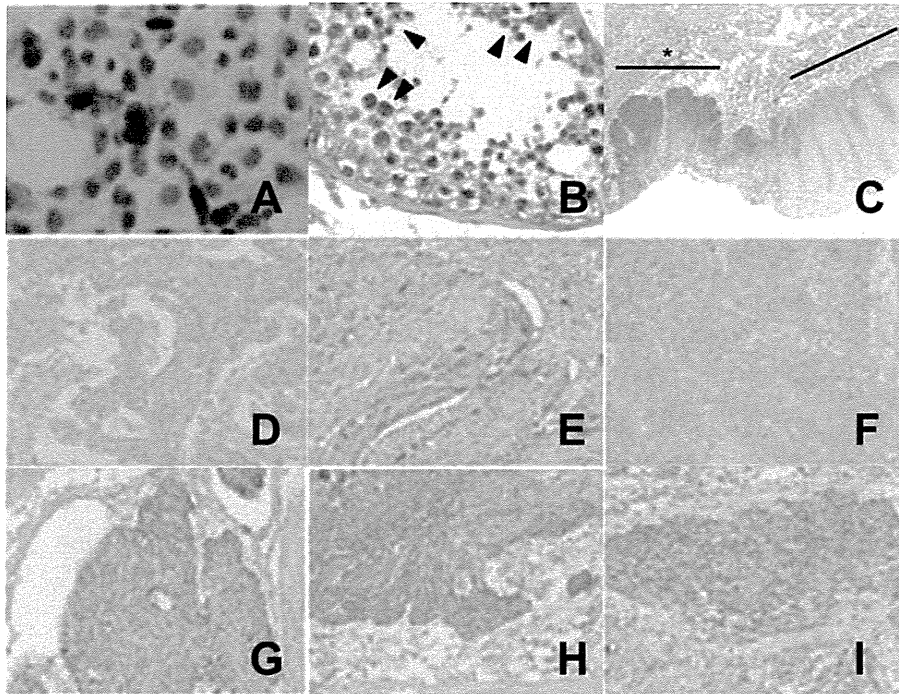


Figure 2: Differential expression of the BORIS protein in ESCC tissues and normal testis  
 A) Specific recognition of BORIS transfected COS-7 by the polyclonal Ab. Only BORIS transfected COS-7 cells were stained in IHC. B) Staining of spermatocytes in normal testis (x400). All the cells related with spermatogenesis (arrow head) were stained. C) The expression of BORIS in cancer cells (\*) is much higher than those normal cells. Heterogenous BORIS staining of ESCC tissues (D-I), D) negative (x200): focal staining or <5% of the cells stained, E, F) moderate staining (x200): 25% to 50% of the cells stained. G, H, I) strong staining (x200): >50% of the cells stained. BORIS expression was mainly localized to the cytoplasm.

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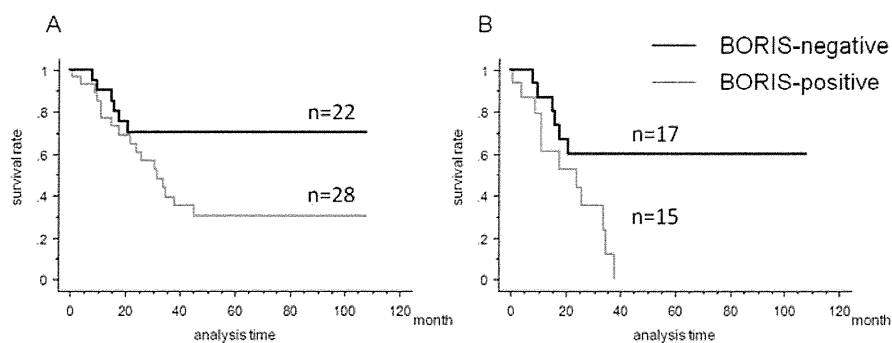


Figure 4: Poor survival of patients with BORIS positive ESCC  
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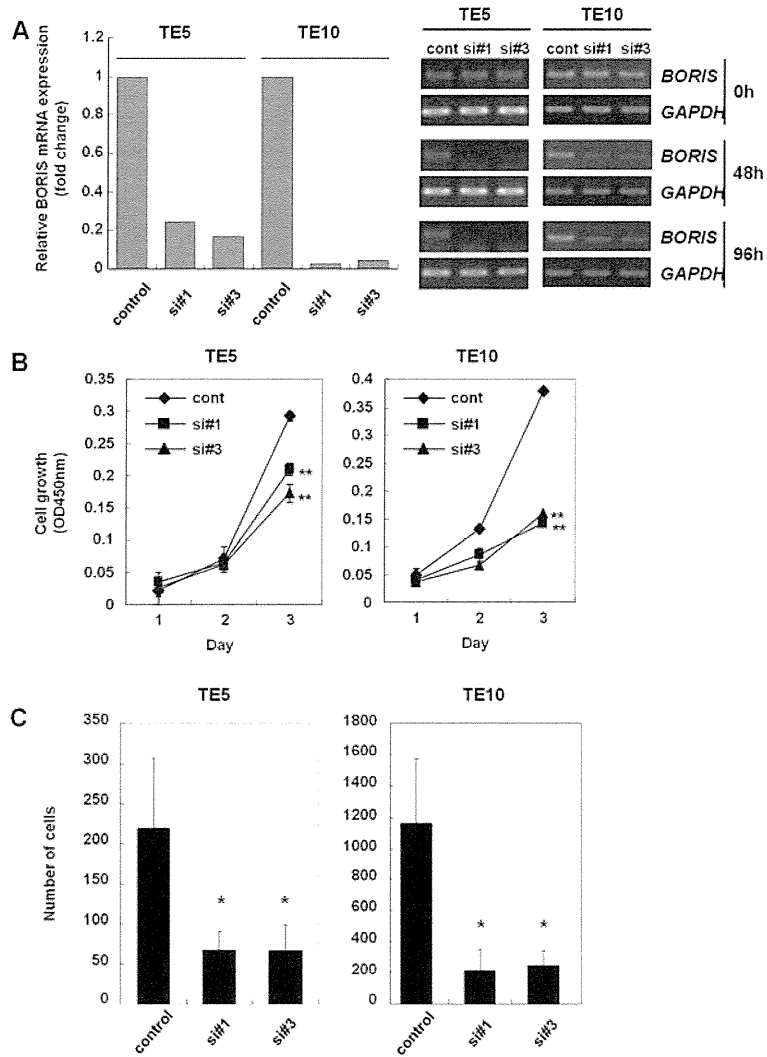


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107x148mm (300 x 300 DPI)

## An Efficient Non-viral Method to Generate Integration-Free Human iPS Cells from Cord Blood and Peripheral Blood Cells

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**Key words.** iPS cells • reprogramming • plasmid • peripheral blood • T cells • cord blood

### ABSTRACT

The generation of induced pluripotent stem cells (iPSCs) provides the opportunity to use patient-specific somatic cells, which are a valuable source for disease modeling and drug discovery. To promote research involving these cells, it is important to make iPSCs from easily accessible and less invasive tissues, like blood. We have recently reported the efficient generation of human iPSCs from adult fibroblasts using a combination of plasmids encoding OCT3/4, SOX2, KLF4, L-MYC, LIN28 and shRNA

for TP53. We herein report a modified protocol enabling efficient iPSC induction from CD34<sup>+</sup> cord blood cells and from peripheral blood isolated from healthy donors using these plasmid vectors. The original plasmid mixture could induce iPSCs, however, the efficiency was low. The addition of EBNA1, an essential factor for episomal amplification of the vectors, by an extra plasmid greatly increased the efficiency of iPSC induction, especially when the induction was performed from  $\alpha\beta$ T cells. This

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improvement enabled the establishment of blood-derived iPSCs from 7 healthy donors ranging in age from their 20s to their 60s. This induction method will be useful for the derivation of patient-specific

integration-free iPSCs, and would also be applicable to the generation of clinical grade iPSCs in the future.

## INTRODUCTION

Pluripotency can be induced with a combination of a few defined factors in somatic cells [1-3]. The generation of iPSCs provides an opportunity to develop and use patient-specific somatic cells like neurons, cardiomyocytes, and beta islet cells, which are otherwise difficult to obtain. These cells make it possible to examine the cause(s) and mechanism(s) underlying various diseases *in vitro*. Disease-specific iPSCs have already been established for Huntington's disease, Parkinson's disease, spinal muscular atrophy, and Rett syndrome [4, 5].

Human iPSCs can be generated from a wide spectrum of somatic cells, including fibroblasts, keratinocytes, and mesenchymal stem cells in adipose tissue [2, 6, 7]. Among them, cord blood cells and peripheral blood mononuclear cells (PMNCs) are attractive sources to use for the generation of iPSCs because of the low invasiveness of their collection. For instance, local anesthesia, suture, and suture removal are necessary for isolation of fibroblasts by skin biopsy but not for blood isolation. In addition, cord blood banks have already collected large repertoires of HLA haplotypes, which can become a source for histocompatible iPSC stocks. The induction of iPSCs from human peripheral blood cells was first reported by Loh et al. in 2009 [8]. They used retrovirus vectors for gene delivery, and infected them into

mobilized CD34<sup>+</sup> blood cells isolated from a donor after a 3-day injection of granulocyte colony stimulating factor. Although effective, this method is relatively labor-intensive, and places a major burden on the donor, thus making it difficult to use as a conventional tool. In addition, retrovirus integration disrupts the endogenous genomic organization and might influence the *in vitro* disease modeling. To overcome these issues, Seki et al. employed a non-integrating virus vector, using the Sendai virus, as a tool for iPSC induction [9]. They isolated mononuclear cells from peripheral blood and stimulated the expansion of T cells with an anti-CD3 antibody and IL-2. The T cells were then used for the Sendai virus infection to generate iPSCs. Their vectors had temperature-sensitive mutations in the RNA genome. These mutations allowed Sendai virus amplification in the T cells and maintenance of transgene expression when the cells were cultured at the permissive temperature (37°C). After the establishment of iPSCs, the Sendai virus vectors could be removed by culturing the cells at the non-permissive temperature (38 °C), although the elimination needs to be experimentally confirmed. However, this method uses an infectious virus, which requires special care, particularly when it encodes oncogenic genes like C-MYC, and it is difficult to modify reprogramming factors and vector sequences in conventional laboratories because they lack the equipment and facilities for handling such viruses.



Episomal plasmids were also used for the deviation of non-integrating iPSCs [10]. The widely used vectors have two components of the Epstein-Barr virus, OriP and EBNA1. The EBNA1 sequence encodes a protein and expresses it from its viral promoter after transduction into human somatic cells. The EBNA1 protein in turn recognizes the OriP sequence and induces plasmid amplification coincident with DNA amplification of the host cell. This system enables relatively high and long term expression of the reprogramming factors. Yu et al. generated a combination of three plasmids, pEP4EO2SEN2K, pEP4EO2SET2K, and pCEP4-M2L (here called the T1 mix), which encode OCT3/4, SOX2, KLF4, c-MYC, NANOG, LIN28, and SV40 Large T antigen, and showed iPSC induction from cord blood cells [11]. Chou et al. established iPSCs from PMNCs with their plasmids, pEB-C5 and pEB-Tg (termed C1), containing OCT3/4, SOX2, KLF4, c-MYC, LIN28, and SV40 Large T antigen, but not NANOG [12]. However, to the best of our knowledge, iPSC induction from peripheral blood with plasmid vectors is still inefficient.

We have recently reported an efficient combination of plasmids for human iPSC induction from fibroblasts, which encode OCT3/4, SOX2, KLF4, L-MYC, LIN28 and a shRNA for TP53 [13]. We herein report a highly efficient method of iPSC induction from PMNCs using these plasmid vectors, with slight modification. We were able to establish hundreds of iPSCs from  $1 \times 10^6$  PMNCs isolated from healthy donors ranging in age from their 20s to their 60s. The iPSCs had genomic rearrangement at the TRB and TRD loci, indicating their T cell origin. We also

established iPSCs which did not show any evidence of genomic rearrangement in the TRB, TRD, and IGH loci by changing the culture medium. Most of these iPSCs were integration-free, karyotypically normal, and effectively differentiated into various cell types *in vivo*. This represents a reliable method to generate patient-specific iPSCs, and would be applicable to the generation of clinical grade iPSCs in the future.

## MATERIALS AND METHODS

### Cell culture

Peripheral blood was obtained from healthy donors whose written informed consent was obtained in accordance with the institutional review board guidelines. CD34<sup>+</sup> cord blood cells were obtained from the Stem Cell Resource Network in CD34<sup>+</sup> Japan (Banks at Miyagi, Tokyo, Kanagawa, Aichi, and Hyogo) through the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan, <http://www.brc.riken.jp/inf/en/index.shtml>). Human fibroblasts, HDF1388, were purchased from Cell Applications, Inc. Human fibroblasts were cultured in DMEM (Nacalai Tesque, Kyoto, Kyoto, Japan, <http://www.nacalai.co.jp/english/index.html>) supplemented with 10% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA, <http://www.invitrogen.com/site/us/en/home.html>). Human ESC lines (KhES-1 and KhES-3) were obtained from Kyoto University. H1 and H9 were from WiCell Research Institute. Mouse embryonic fibroblasts (MEF) were isolated from day 13.5 embryos of C57BL/6 mice. MEF and SNL cells [14] were cultured in DMEM supplemented with 7% FBS, 2 mM

L-glutamine, and 50 units and 50 mg/ml of penicillin and streptomycin, respectively. Established iPSCs and ESCs were maintained on mitomycin C-treated SNL cells in Primate ESC medium (ReproCELL, Yokohama, Kanagawa, Japan, <http://www.reprocell.com/en/>) containing 4 ng/ml of bFGF (Wako, Osaka, Osaka, Japan, <http://www.wako-chem.co.jp/english/>) as described previously [15].

### Vector construction

Efficient gene expression was achieved by inserting the woodchuck hepatitis post-transcriptional regulatory element (WPRE) upstream of the polyadenylation signal of pCX-EGFP [16]. Its SV40-ori sequence was removed by BamHI digestion. The modified vector was then digested with EcoRI and was ligated with the EBNA1 coding region, which was amplified by PCR from pCEP4 (Invitrogen). This vector was designated “pCXWB-EBNA1”. The coding regions or genomic fragments of NANOG, TERT, SALL4, ESRRB, TBX3, GLIS1, mir-302s, Wnt3a, cyclinD1, Utl1, and SV40LT, were amplified by PCR. The fragments were then cloned into pENTR-D/TOPO (Invitrogen), and were recombined with pCXLE-gw by a LR reaction (Invitrogen), which has a Gateway cassette, rfc.1 (Invitrogen), into the EcoRI site of the pCXLE plasmid [13]. We also constructed a red fluorescent protein expression vector, pCXLE-DsRed, and a TP53 shRNA expression vector, pCXLE-DsRed-shp53, to use as controls. The episomal vectors described by Yu et al. [10] and Chou et al. [12] were obtained from Addgene (#20924, 20925, 20926, 20927, 28213, and 28220; Cambridge, MA, USA, <http://www.addgene.org/>).

### Generation of iPSCs from PMNCs with episomal vectors

PMNCs were purified by density gradient centrifugation with Ficoll-paque Plus (GE Healthcare, Waukesha, WI, USA, [http://www3.gehealthcare.com/en/Global\\_Gateway](http://www3.gehealthcare.com/en/Global_Gateway)) or BD Vacutainer CPT (BD Biosciences, Franklin Lakes, NJ, USA, <http://wwwbdbiosciences.com/home.jsp>) according to the manufacturer’s instructions. Three micrograms of expression plasmid mixture were electroporated into  $3-5 \times 10^6$  PMNCs with the Nucleofector 2b Device (Lonza) with an Amaxa Human T cell Nucleofector kit according to the manufacturer’s instructions. The program used was V-24. The cell viability and transfection efficiency of PMNCs in our lab were 70-80% and 40-60%, respectively. The plasmid mixtures used in the experiments are shown in Supporting Information Table S1. The cells equivalent to  $3 \times 10^4 - 1 \times 10^6$  of input cells were then seeded onto 6 well plates covered with a MEF feeder layer. For the induction from  $\alpha\beta$  T cells, the transfected cells were cultured in X-vivo10 (Lonza) supplemented with 30 U/ml IL-2 (PeproTech Inc., Rocky Hill, NJ, USA, <http://www.peprotech.com/>) and 5  $\mu$ l/well of Dynabeads Human T-activator CD3/CD28. For non-T cell induction,  $\alpha$ MEM medium containing 10% FCS, 10 ng/ml IL-3, 10 ng/ml IL-6, 10 ng/ml G-CSF, and 10 ng/ml GM-CSF was used. Two days after the transfection, an equal volume of Primate ESC medium containing bFGF and 10  $\mu$ M Y27632 was added into each well without aspiration of the previous medium. The culture medium was then replaced with Primate ESC medium containing bFGF and Y27632 4 days after the transfection. The colonies were counted 16 to 25 days after

plating, and the colonies similar to human ESCs were selected for further cultivation and evaluation. Generation of iPSC from CD34<sup>+</sup> rich population was performed as described previously with slight modification[17]. Briefly, purified CD34<sup>+</sup> cells from PMNC was expanded for 6 days in StemSpan SFEM medium (StemCell Technologies Inc, Vancouver, BC, Canada, <http://www.stemcell.com>) supplemented with 10 ng/ml IL-3, 100 ng/ml IL-6, and 300 ng/ml of Flt3 ligand, SCF, and TPO. Three micrograms of the plasmid mixture were then electroporated into  $1 \times 10^5$  cells. The cells equivalent to  $0.2 - 6 \times 10^4$  of input cells were then seeded onto 6 well plates coated with Retronectin (Takara Bio Inc., Otsu, Shiga, Japan, <http://www.takara-bio.com>) in the StemSpan medium. The culture medium was gradually changed to the Primate ESC medium as described above. The iPSC clones used in this study are summarized in Supporting Information Table S2. We calculated the iPSC induction efficiency based on the number of iPSC colonies per number of seeded cells which were estimated from the number of cells used for the electroporation.

### **Generation of iPSCs from cord blood CD34<sup>+</sup> cells with episomal vectors**

The frozen CD34<sup>+</sup> cells were thawed and cultured in  $\alpha$ MEM medium containing 10% FCS, 50 ng/ml IL-6, 50 ng/ml sIL-6R, 50 ng/ml SCF, 10 ng/ml TPO, 20 ng/ml Flt3 ligand, and 20 ng/ml IL-3 for a few days before transfection. Three micrograms of expression plasmid mixtures were then electroporated into  $5-40 \times 10^5$  CD34<sup>+</sup> cells with a Nucleofector 2b Device using an Amaxa Human CD34<sup>+</sup> cell Nucleofector kit according to the

manufacturer's instructions. The program used was U-08. The cells were then cultured for 2 or 5 days, and  $0.5-35 \times 10^4$  cells were re-plated onto 100 mm dishes covered with an SNL or MEF feeder layer. Equal volumes of  $\alpha$ MEM medium containing cytokines and Primate ESC medium containing bFGF and 10  $\mu$ M Y27632 were mixed and used for cultivation, followed by replacement with Primate ESC medium in 2 days. The colonies were counted 17 to 26 days after the transduction, and the colonies similar to human ESCs were selected for further cultivation and evaluation.

### **Characterization of iPSC clones**

The isolation of total RNA, RT-PCR of marker gene expression, DNA microarrays, episomal copy number detection and teratoma formation were performed as previously described [13]. A Southern blot analysis was carried out as previously described [18]. PCR-based detection of the TRB rearrangement was performed with TCRB Gene Clonality Assay (InVivoScribe, San Diego, CA, USA, <http://www.invivoscribe.com/>) according to the manufacturer's instructions. The chromosomal G-band analyses were performed at the Nihon Gene Research Laboratories (Sendai, Miyagi, Japan, <http://www.ngrl-japan.com/index.html>).

### **Exome Analysis**

One microgram of genomic DNA was sheared with the Covaris E210 system and was subjected to exome enrichment using SeqCap EZ Exome Library v3.0 (Roche Diagnostics GmbH, Mannheim, Germany, <http://www.roche-applied-science.com/>) according to the manufacturer's instructions. Illumina sequencing libraries were made by PCR with Illumina sequencing adaptors and

were sequenced with HiSeq2000 (Illumina, Inc., San Diego, CA, USA, <http://www.illumina.com/>). The sequenced reads were mapped to protein coding sequences of reference human genome (hg19) by BWA 0.6.2. Local realignment was performed around indels with the Genome Analysis Toolkit (GATK v1.6)[19]. Optical and PCR duplicates were marked in BAM files with Picard 1.68. Only the regions with over 30 × coverage observed in all samples were subjected to the following SNV call. Original HiSeq base quality scores were recalibrated with GATK TableRecalibration. SNVs were called with GATK UnifiedGenotyper and eliminate false-positive calls according to the Broad Institute's best-practice guidelines (Best Practice Variant Detection with the GATK v3 [http://www.broadinstitute.org/gsa/wiki/index.php/Best\\_Practice\\_Variant\\_Detection\\_with\\_the\\_GATK\\_v3](http://www.broadinstitute.org/gsa/wiki/index.php/Best_Practice_Variant_Detection_with_the_GATK_v3)).

### Statistical analyses

Data are shown as the means ± standard deviation. The statistical significance of differences between two groups was evaluated with the Wilcoxon signed-rank test.

## RESULTS

### iPSC generation from cord blood cells

We have previously reported the establishment of plasmid-based vectors which can efficiently generate iPSCs from adult fibroblasts and dental pulp cells [13]. The most effective combination of reprogramming factors consists of OCT3/4, SOX2, KLF4, L-MYC, LIN28, and shRNA for TP53. We called this combination of

plasmids Y4 (Supporting Information Table S1).

As the first step, we tried to generate iPSCs from CD34<sup>+</sup> cells isolated from cord blood with the Y4 combination (Fig. 1A). This population was enriched in hematopoietic stem cells, and has been shown to possess the potential for reprogramming [20]. The frozen CD34<sup>+</sup> cells purified from two donors were cultured for a few days with cytokines, IL-3, IL-6, soluble IL-6 receptor, SCF, TPO, and Flt3 ligand. The Y4 mixture was then transduced by electroporation (Fig. 1A). The cells were recovered in the medium for a few days and replated onto feeder cells in a 1:1 mixture of human ESC medium and the medium containing cytokines. The mixed culture medium was then changed to the medium for human ES cells after 2 days. We added an inhibitor for Rho kinase to support the reprogramming process by inhibiting cell death [21].

The ES cell-like colonies began to emerge 10 days after the plasmid transfection and were selected for expansion (Fig. 1B). The reprogramming efficiency was relatively high (up to 0.1%; Supporting Information Table S3). Both MEF and SNL feeder cells resulted in a similar colony number. Established clones formed flat and compacted colonies, and showed high nucleus-to-cytoplasm ratios, which were typical morphology of human ES cells (Fig. 1C). When injected into immunodeficient mice, the clones developed into teratomas, where they differentiated into various types of cells of all three germ layers, like neurons, gut-like epithelium, cartilage, and muscle (Figs. 1D-F). Therefore, the Y4 mixture

can induce iPSCs from CD34<sup>+</sup> cells derived from cord blood.

### iPSC generation from peripheral blood

We next attempted to carry out reprogramming from peripheral blood with the Y4 mixture. The experiments were performed with blood from a healthy Japanese donor (donor #1, male, 30s). After the isolation of peripheral blood, the fraction of PMNCs was separated by density gradient centrifugation, and three million cells were used for the plasmid transduction (Fig. 2A). The PMNCs were then directly plated into the culture dish covered with MEF feeder cells. PMNCs mainly consist of T lymphocytes, but also contain other cell types, such as B lymphocytes, monocytes, and macrophages. We employed two different types of media for their cultivation. One medium contained IL-2 and antibodies against CD3 and CD28 to stimulate the proliferation of T cells. The other medium, termed non-T medium, was supplemented with FCS, IL-3, IL-6, G-CSF, and GM-CSF. The PMNCs were cultured in each of these media for 2 days. The media were then diluted with the same volume of ESC medium supplemented with bFGF and a Rock inhibitor, followed by complete replacement on day 4.

There was extensive cell proliferation from day 4 to day 10 in the T cell stimulating medium. A few tiny cell colonies firmly attached to the culture dish were observed around two weeks after the plasmid transduction. At this early time point, these cells showed a round morphology and clear cell-to-cell boundaries. Thereafter, these cells gradually adhered to each other and formed tightly packed colonies by three weeks. On the other hand, obvious proliferation did not

occur in the non-T medium. However, we were able to find colony formation with a similar time course as was observed with the T cell stimulating conditions. Around three weeks after the transduction, we counted the number of ES cell-like colonies in the cultures under both conditions. In the medium for T cell stimulation,  $42.2 \pm 8.4$  colonies were obtained from  $1 \times 10^6$  cells with the Y4 plasmid mixture (Fig. 2B; Table 1). On the other hand, only  $2.5 \pm 1.0$  colonies emerged from  $1 \times 10^6$  cells in the non-T medium (Table 1). The established cells had morphology typical of human ES cells (Fig. 2C). A microarray analysis of gene expression revealed that they had a gene expression profile similar to that of ES cells (Supporting Information Table S4). Their linear correlation coefficient against ESCs (KhES3) was 0.993 on average, which is similar to the correlation coefficient of a different ESC line, H9, and fibroblast-derived iPSCs, 201B7 and 253G4, against KhES3 ( $R^2 = 0.991$  and  $0.988$ , respectively). The iPSCs showed the potential to differentiate into cells of all three germ layers by teratoma formation in mice (Figs. 2D-F). Most of the iPSCs were karyotypically normal (Supporting Information Table S5).

Terminally differentiated  $\alpha\beta$ T cells have genomic rearrangements in the TRB and TRA loci which encode the T cell receptor  $\beta$  and  $\alpha$  chains, respectively. B cells have rearrangement in the immunoglobulin heavy locus, IGH. Therefore, we examined the cell origin of established iPSCs by detecting their genomic rearrangement by a Southern blot analysis. The iPS clones established under T cell conditions (585A1, 585B1, 604A1, and 604B1) showed genomic rearrangement in TRB loci and did not show a specific band with the probe for the

TRD locus (Fig. 2G; Supporting Information Fig. S1). The TRD locus is located within the TRA locus and is excised coincident with TRA rearrangement during the maturation of  $\alpha\beta$ T cells. Hence, these data suggested that the 4 iPSC clones were derived from  $\alpha\beta$ T cells. In contrast, we could not detect any rearrangement of the three loci (TRB, TRD, and IGH) in iPSC clones that developed in the non-T medium (692D2, 692E1, 648A1, and 648B1), thus indicating that they originated from non-lymphoid lineages.

We also tested other combinations of plasmids for PMNC reprogramming (Supporting Information Table S1). One combination, Y3, contains the same reprogramming factors as Y4 except for the shRNA against TP53. With the Y3 combination, iPSCs were obtained under both T cell and non-T cell conditions, but Y3 seemed to be less effective than Y4 (Table 1). We used other combinations of episomal plasmids which have been reported to induce iPSCs from blood cells [11, 12]. However, at least under our culture conditions, no iPSCs were obtained from PMNCs with T1 and C1 combinations (Table 1). We also employed a combination, termed T2, containing the same reprogramming factors as T1, but contained them in two plasmids, pEP4EO2SCK2MEN2L and pEP4EO2SET2K [10]. Although the T2 combination could generate iPSCs from fibroblasts at better efficiency than T1 [13], we also failed to induce iPSCs from PMNCs using T2 (Table 1). Thus, Y4 had the highest induction efficiency among the various plasmid combinations we tested.

We then examined whether Y4 could efficiently generate iPSCs from other donors (Table 2). PMNCs were isolated from 6 healthy male and

female donors ranging in age from their 20s to their 60s, and induced reprogramming. However, the iPSC induction efficiency was different among the donors, and the efficiency of the 6 donors was relatively lower than that of donor #1. As described above, we could consistently obtain more than 25 iPSC colonies from  $1 \times 10^6$  input cells from donor #1 when they were cultured under the T cell condition. In contrast, only around 5 colonies emerged under the same conditions for donors #4 and #7. In addition, iPSC colonies were rarely obtained in the non-T cell medium from the other donors. Two out of the three experiments failed to induce iPSC colonies from  $1 \times 10^6$  input cells from donors #3, #4, and #7.

#### **The addition of an extra EBNA1 expression vector greatly enhanced iPSC formation from T cells**

In order to reproducibly generate iPSCs from T lymphocytes from multiple donors, it was necessary to improve our induction protocol. To this end, we took two approaches. First, we tried to improve the efficiency by means of pro-reprogramming factors. Several factors have recently been reported to increase the reprogramming efficiency of human and/or mouse somatic cells using viral transduction methods [3, 22-31]. We cloned these 11 candidate genes into an episomal plasmid, and transduced them one by one into human fetal dermal fibroblasts with the Y3 combination, and screened for their effects on the reprogramming efficiency. As expected, the addition of shRNA for TP53 increased the number of iPSC colonies compared to Y3 alone, however, SALL4, TERT, Wnt3a, and Utf1 did not show any apparent influence on the reprogramming (Supporting Information Fig.

S2A). Plasmids encoding ESRRB, TBX3, and SV40 large T antigen seemed to suppress iPSC induction. On the other hand, transduction of NANOG, GLIS1, mir-302s and cyclin D1 increased the number of iPSC colonies.

Notably, GLIS1 showed the highest enhancement of reprogramming, as it induced an approximately 4-fold increase in the number of colonies compared to the control Y3 combination (Supporting Information Fig. S2A). GLIS1 also showed additional effects on TP53 suppression, and could generate 2-fold more iPSC colonies when used with the Y4 combination in fibroblasts (Supporting Information Fig. S2B). Based on these results, we expected that GLIS1 would enhance the iPSC induction from PMNCs. However, when cells were transduced with Y3 or Y4, the addition of GLIS1 showed only a slight or no increase in the number of iPSCs induced under both the T cell and non-T cell conditions (Figs. 3A and B). As a result, the pro-reprogramming effect of GLIS1 varies depending on the origin of the somatic cells.

As an alternative way to enhance the reprogramming efficiency, we aimed to increase the protein expression from our episomal plasmids. The maintenance of EBV-based plasmids is largely dependent on the EBNA1 protein. Previous reports have shown that a high transfection efficiency could be achieved by cell lines which constitutively express EBNA1 from an integrated genomic construct [32]. Based on this knowledge, we employed an extra plasmid, pCXWB-EBNA1, encoding EBNA1 under the control of the CAG promoter (Supporting Information Fig. S3) [16]. To ensure early removal of the

reprogramming plasmids, the additional vector did not include an OriP sequence, an essential component for episomal plasmid replication. Hence, the EBNA1 vector itself is not amplified in human cells, and gives only a transient expression of EBNA1.

To examine the effect of this plasmid on the transgene expression, fibroblasts were transduced with episomal plasmids encoding EGFP in the presence and absence of the EBNA1 expression vector (Supporting Information Fig. S4). Six days after the transduction, EGFP fluorescence was analyzed by flow cytometry. The addition of EBNA1 greatly increased both the mean expression level and the population of EGFP<sup>+</sup> cells (from 8.5% to 17.2%). However, this high expression quickly diminished during cultivation. One month after the transduction, only 0.3% of cells remained positive for the fluorescence. Therefore, the transient expression of EBNA1 promoted amplification of the episomal vector at the early time points, but it had a minimal influence on the maintenance of the vectors in the later phase. Consistent with this result, the addition of the EBNA1 vector to the Y4 mix led to a 2-fold increase in the number of iPSC colonies generated from fibroblasts (Supporting Information Fig. S5). The established clone, 923B1, showed expression of pluripotent genes similar to ES cells (Supporting Information Table S4). In addition, no obvious genomic integration was observed in the clones (Fig. 3C; Supporting Information Fig. S6).

We then performed iPS induction from PMNCs using this extra plasmid. The addition of the EBNA1 vector to the Y4 mixture increased the number of iPS colonies in 7 different donors

(Figs. 3D and E; Supporting Information Fig. S7; Tables 1 and 2). The Wilcoxon signed-rank test indicated that this modification led to a moderate but significant increase of the reprogramming under the non-T cell conditions (median; 2.5 fold,  $p < 0.05$ ) and achieved a dramatic enhancement of reprogramming in the cells cultivated under the T cell culture conditions (median; 28-fold,  $p < 0.05$ , Fig. 3D). The reprogramming efficiency under the T cell culture conditions was up to 0.1% at the maximum (Table 2). However, this was likely overestimated because of the vigorous expansion of transduced T cells before the initiation of the reprogramming and attachment to the culture plates. To assess whether this was the case, 15 iPS clones were randomly chosen from 31 colonies in one dish, and their genomic rearrangement pattern at the TRB locus was examined (Supporting Information Fig. S8). A Southern blot analysis and PCR-based detection revealed that two and three clones among these had an identical rearrangement pattern. On the other hand, there were 12 different TRB rearrangements among 15 clones. These data suggested that some clones were derived from daughter cells of the same T cells, but that most of them were derived independently from different cells.

This induction method enabled the generation of more than one hundred iPS clones from all of the donors we examined. The iPS clones induced with Y4 and the extra EBNA1 vector were isolated for a further analysis. The established clones (929D2 and 929D3) showed the expression of pluripotent genes, and no obvious genomic integration was found (Fig. 3C; Supporting Information Figs. S6 and S9; Supporting Information Table S4). They were

karyotypically normal and had the potential to differentiate into various cell types of all three germ layers, indicating their pluripotency (Supporting Information Table S5; Fig. S10). When performed exome analysis, the numbers of non-silent variations in iPSCs induced with Y4 and EBNA1 were 7 and 6 under the T cell condition, and 1 and 6 under the non-T cell conditions (Supporting Information Table S6 and S7). These numbers were almost comparable with that observed in fibroblast-derived iPSCs [33]. The plasmid mixture could induce iPSCs from frozen PMNCs at almost the same efficiency as freshly isolated cells (Table 1). Moreover, when hematopoietic stem and progenitor cells were enriched from PMNCs by CD34 expression, the extra EBNA1 vector also enhanced iPSC generation (Supporting Information Table S8).

## DISCUSSION

In this study, we developed a method that can be used to generate iPSCs from cord blood and PMNCs. The efficiency from PMNCs was up to 0.1% at the maximum. This means that only a few milliliters of peripheral blood is sufficient for the induction of iPSCs. Frozen cells can also be applied for the induction. Thus, our report provides a practical and efficient way to generate patient-specific iPSCs. This will also be valuable for the generation of clinical-grade iPSCs for future therapeutic applications.

T cell-derived iPSCs have their own genomic rearrangements in TRB and TRA loci, which would prevent the generation of large repertoires of TCR in re-differentiation towered T cells. Mice having successful pre-rearranged



TCR were reported to show early TCR expression and aberrant T cell development [34]. Therefore T cell-derived iPSCs would have some limitation in their use, such as clinical reconstitution of T lymphocytes and *in vitro* recapitulation of normal T cell development.

The iPS induction was greatly enhanced when using the extra EBNA1 vector and the Y4 combination in the T cell stimulating medium. The proliferation of T cells can be activated by the combination of IL-2 and the stimulation of TCR complexes, along with co-stimulatory signaling via anti-CD3 and anti-CD28 antibodies [35]. The replication of EBV-based episomal vector occurs along with the host cell division. Therefore, cell proliferation is important for the maintenance of the episomal vectors in human cells. In addition, proliferation itself has been reported to increase the chance of fully reprogramming in a stochastic manner [36]. These features could be the cause of the dramatic enhancement of the reprogramming efficiency from PMNCs in the T cell stimulating medium.

EBNA1 was also reported to have a correlation with the TP53 signaling. TP53 is an important molecule that regulates the cell cycle. EBNA1 interacts with the N-terminal domain of a ubiquitin-specific protease, USP7 [37]. USP7 can also bind TP53 through its N-terminal domain, which results in the stabilization of TP53 [38]. Hence, both EBNA1 and TP53 interact with USP7, and EBNA1 is thought to interfere with the stabilization of TP53 by blocking their interaction. Therefore, transient high expression of EBNA1 and a shRNA for TP53 encoded by Y4 would synergistically

suppress the endogenous TP53 and enhance the reprogramming process.

When  $3 \times 10^6$  PMNCs were used for electroporation, one tenth of them generated 130.8 colonies in the T cell medium (0.04%) and one third of them gave 5.4 colonies in the non-T cell medium (0.0005%) on average. During the reprogramming process under the T cell condition, however, one transduced T cell would produce dozens of daughter cells, which would inherit episomal vectors. In support of this, identical TRB rearrangements were observed in different iPS clones established from the same dish, suggesting that the cells were derived from one parental T cell (Supporting Information Fig. S8). Only a portion of these amplified daughter cells would enter the reprogramming process, attach to the culture dish, and form iPS colonies. Hence, the actual efficiency was likely much lower than the estimated efficiency. On the other hand, we replaced the culture medium on day 4, in which most cells were floating. The supernatant still had the potential to induce iPS colonies when they were seeded onto new feeder cells (data not shown). Therefore, our culture protocol likely led to the loss of many cells that had acquired some initiation into the reprogramming process. The reported iPS induction efficiency by retro/lentivirus vectors is around 0.002% [18, 39] and 0.02% [8] from normal and mobilized peripheral blood, respectively. The non-integration method using episomal vector C1 would be less efficient (0.001%) than those [12]. Mack et al. purified and expanded CD34<sup>+</sup> cells from PMNCs and carried out transduction with episomal vectors, which were similar to T1, but used L-MYC instead of C-MYC [17]. They estimated that the reprogramming

efficiency was 0.03% on average, and obtained approximately 5 iPSC colonies per one milliliter of blood. With similar approach, our mixture Y4+EBNA1 showed the reprogramming efficiency around 0.06%, which was 2-fold higher than that of the previous report. To the best of our knowledge, the present study describes the most efficient simple method to generate iPSCs from peripheral blood by a non-viral vector.

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#### Competing Financial Interests

K.O. and S.Y. are filing a patent application based on the results reported in this paper. S.Y. is a member of the Scientific Advisory Board for iPSC Academia Japan Inc. and iPierian Inc., which manages the patents.

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