Successful Generation of Human Induced Pluripotent Stem Cell Lines from Blood Samples Held at Room Temperature for up to 48 hr

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SUMMARY

The collection sites of human primary tissue samples and the receiving laboratories, where the human induced pluripotent stem cells (hIPSCs) are derived, are often not on the same site. Thus, the stability of samples prior to derivation constrains the distance between the collection site and the receiving laboratory. To investigate sample stability, we collected blood and held it at room temperature for 5, 24, or 48 hr before isolating peripheral blood mononuclear cells (PBMCs) and reprogramming into IPSCs. Additionally, PBMC samples at 5- and 48-hr time points were frozen in liquid nitrogen for 4 months and reprogrammed into IPSCs. hIPSC lines derived from all time points were pluripotent, displayed no marked difference in chromosomal aberration rates, and differentiated into three germ layers. Reprogramming efficiency at 24- and 48-hr time points was 3- and 10-fold lower, respectively, than at 5 hr; the freeze-thaw process of PBMCs resulted in no obvious change in reprogramming efficiency.

INTRODUCTION

Adult somatic cells from patients can be now reprogrammed to a pluripotent state by the ectopic expression of transcription factors such as OCT3/4, SOX2, KLF4, and c-MYC (Takahashi et al., 2007).

Several large-scale initiatives have been launched to fully explore the pharmaceutical and clinical potentials of human induced pluripotent stem cells (hIPSCs) (Soares et al., 2014). However, there are major challenges associated with large-scale production of hIPSC lines, including the availability of somatic tissues and potential degradation of collected tissue during transportation and storage. Furthermore, primary tissue collection from a diversity of patients located in a variety of referral centers can be difficult since it may require transportation over long distances. Factors that affect the stability of biological samples include temperature and time elapse before samples can be processed in laboratories. Thus, any delays between collection and processing can affect the sample integrity, viability, and other factors necessary for successful reprogramming. Such information remains to be systematically gathered for the main cell types used for generating hIPSC lines. Traditionally, hIPSCs are derived from skin punch biopsies (Chen et al., 2011). These are often assumed to be more traumatic for patients as local anesthesia and a stich may be required, i.e., for 4-mm punch biopsies, and the procedure can

leave a scar. This may result in a decrease in the number of volunteers for tissue donation. The derived fibroblasts require a prolonged period of expansion in culture prior to reprogramming. Moreover, concerns have been raised over the potential risks of mutations associated with exposure of epidermis to UV light (van der Pols et al., 2006) and raises a clinical concern on the safety of the IPSCs cells derived from skin (Loh et al., 2010). The ideal somatic cell type for hIPSC derivation should be easily accessible and require a less traumatic sampling procedure.

Less traumatic, non-invasive methods of collecting cells for reprogramming from blood, hair follicle, and urine have been described (Raab et al., 2014). Each of these methods has its advantages and disadvantages.

Peripheral blood is an advantageous alternative to skin for hIPSC derivation (Loh et al., 2010; Zhang 2013) since it is widely used in clinical diagnostics, and moreover, the method of blood collection is standardized and relatively less traumatic than skin biopsy.

In this study, we set out to establish the permissible time during which blood samples can be stored at room temperature after collection without severely compromising their ability to be successfully reprogrammed into IPSCs. We also assessed the capacity for self-renewal, differentiation, and the genomic integrity and to ensure that the derived IPSCs are of good quality for disease modeling and clinical application.
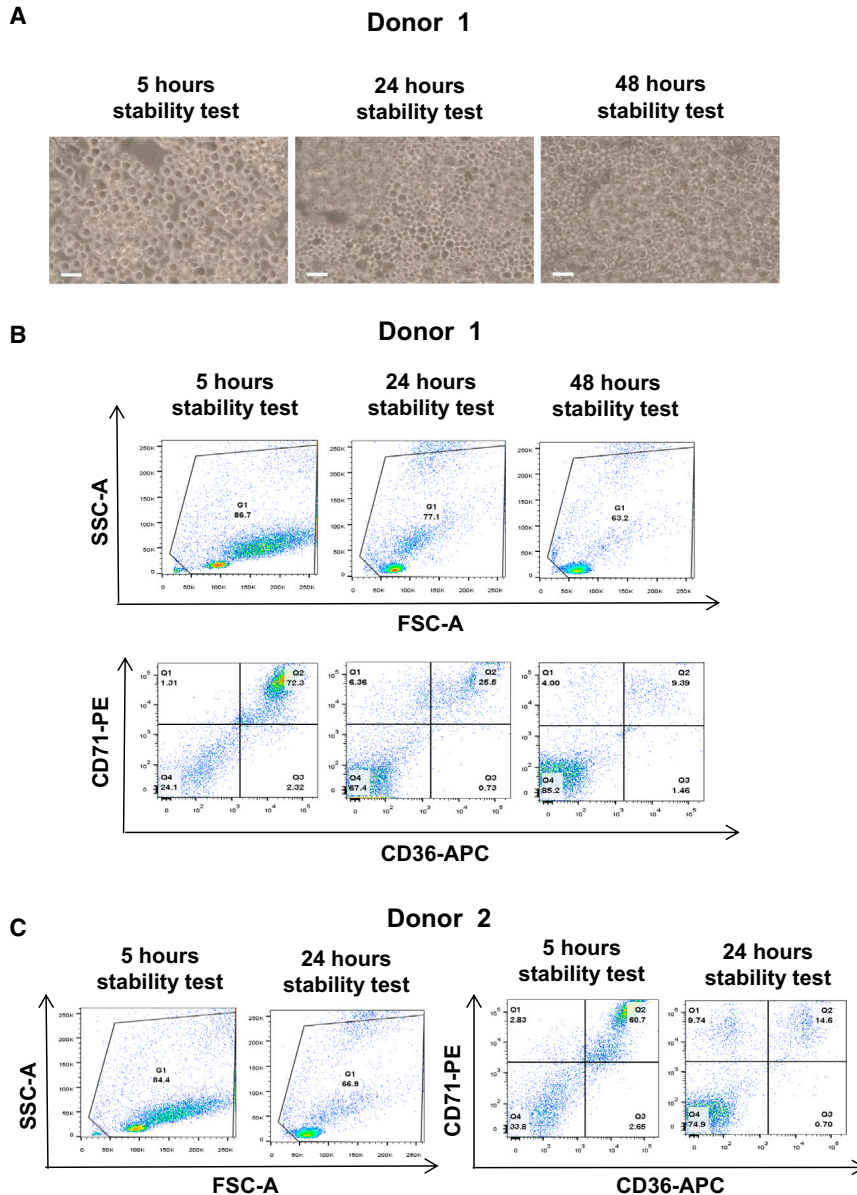
CrossMark

A

Donor 1

5 hours
stability test

24 hours
stability test

48 hours
stability test

B

Donor 1

C

Donor 2

PBMCs isolated from blood held at RT for 5-, 24-, and 48 hr postcollection were expanded into erythroblasts for 9 days in the presence of growth factors.

(A) Representative phase-contrast images of erythroblast-enriched population (donor 1: codr) with characteristic clustering behavior in all the three stability time points at day 9 postexpansion (scale bars represent 250 μm).

(B and C) Erythroblast populations expanded from blood samples held at RT for 5-, 24-, and 48 hr postcollection were detected by immunostaining with antibodies specific for erythroid markers (CD71 and CD36) analyzed by flow cytometry. Representative flow cytometry data of donor 1 and donor 2 are shown.

RESULTS

IPSCs Can Be Derived from Blood Samples Stored at Room Temperature for up to 48 hr Postcollection

hIPSCs are routinely derived from fresh blood, but whether hIPSCs with similar properties can be derived from long-term-stored human peripheral blood remains to be evaluated. We sought to examine the effects of blood stability on reprogramming (Figure S1). Briefly, blood samples were collected from two patients (3 × 10 ml for each donor) and kept for 5, 24, or 48 hr at room temperature. Then PBMCs were isolated from each sample and grown in media favoring the expansion of the erythroid lineage for 9 days.

Representative images of expanded erythroblast of donor 1 displaying characteristic clustering behavior in all the three stability time points (Figure 1A). On day 9, postexpansion cells were stained with CD71 and CD36 antibodies specific for erythroid markers and analyzed for expression by flow cytometry; 72.3% of cells expressed both markers, indicating enrichment of erythroblasts for donor 1 (Figure 1B). Blood samples kept at room temperature for 24 or 48 hr display a lower capacity with 3- or 7.5-fold, respectively, to produce CD71 and CD36 expressing erythroid cells than blood samples kept at room temperature for 5 hr, confirming that long-term storage of primary samples could be detrimental. A similar observation was made with
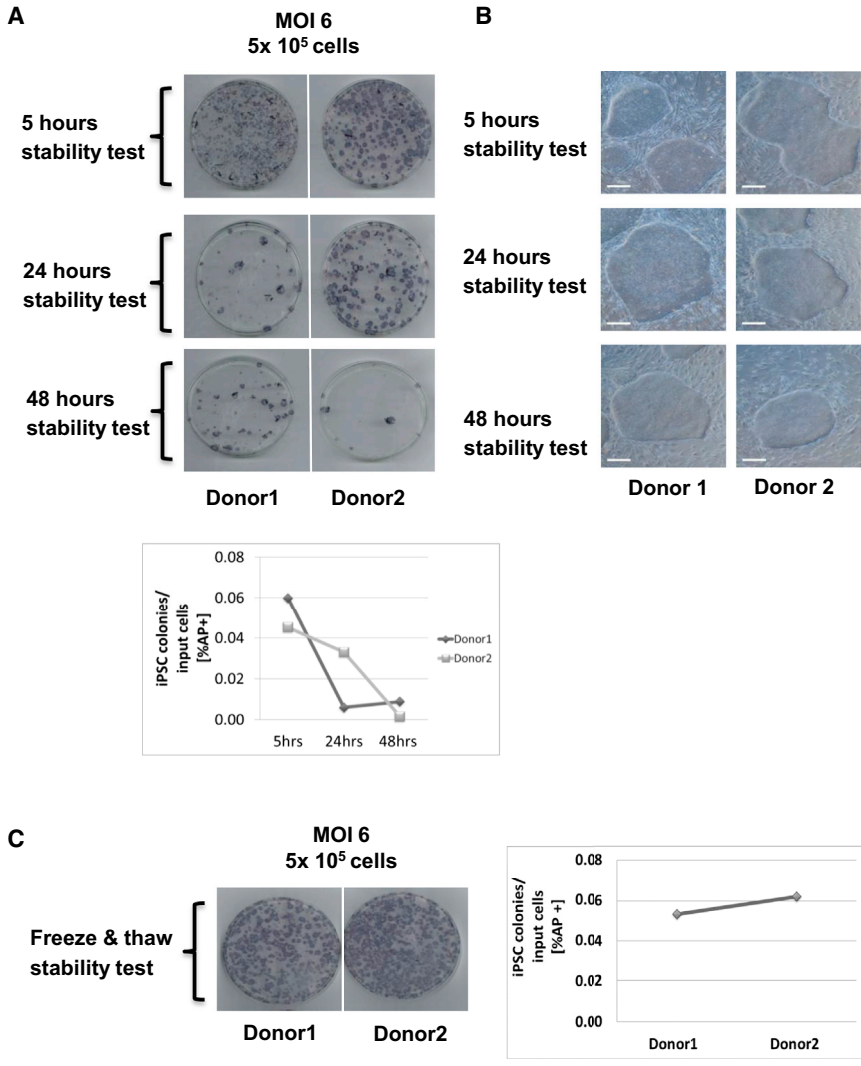
Figure 2. Reprogramming of Erythroblast-Enriched Populations

(A) Representative images in the top show AP-stained IPSC colonies derived from samples that had been held at room temperature for 5-, 24-, and 48 hr postdraw. Reprogramming efficiency was quantified by determining the percentage of AP-positive colonies (bottom).

(B) Representative phase-contrast images of IPSCs with characteristic typical human ES cell-like morphology in all the three stability time points are shown. Donor 1 (codr), donor 2 (gabq). Scale bars represent 250 μm.

(C) Representative images show AP stained IPSC colonies derived from two erythroblasts donors (donor 1 and donor 2) that had been held at room temperature for 5 hr postdraw followed by a freeze and thaw cycle (linked to Figure S2). Reprogramming efficiency was quantified by determining the percentage of AP-positive colonies.

donor 2 where the percentage of enriched erythroblast population was also dramatically reduced to 4-fold with respect to 24-hr incubation at room temperature compared with 5 hr (Figure 1C).

We next sought to determine whether the erythroblasts obtained from these samples could produce IPSCs. Cells were transduced with Sendai viruses expressing human OCT3/4, SOX2, KLF4, and c-MYC at an MOI of 6 and resulting IPSC colonies stained with alkaline phosphatase (AP) (Martí et al., 2013; Bar-Nur et al., 2015). Small IPSC colonies became visible 2-weeks posttransduction in all the transduced lines at each of the three stability time points. However, consistent with our findings on PBMCs stability by immunostaining with erythroblast marker, a decrease in the number of IPSC colonies was observed for cells at 24- or 48-hr stability time points compared with samples processed 5 hr postdraw (Figure 2A). Reprogramming efficiencies ranging from 0.06% to 0.009% (donor 1) and

0.05% to 0.0015% (donor 2) were observed for 5- to 48-hr storage time points. As the enriched erythroblast population of day 9 culture has not been purified, there is still a possibility of reprogramming other cell lineages such as lymphocytes or monocytes. IPSCs derived from the three storage conditions showed the typical compact morphology seen in undifferentiated human embryonic stem cells (hESCs) (Figure 2B). Taken together, these results indicate that up to 48 hr blood storage at room temperature is compatible with immediate reprogramming but can affect the efficiency of IPSC derivation.

Freezing and Thawing of PBMCs Has No Effects on Conversion to Erythroblasts and Reprogramming into hIPSCs

To exclude the possibility that freezing and thawing of PBMCs might have an effect on reprogramming, we next performed experiments whereby PBMCs isolated from

donor 1 and donor 2 stored at room temperature for 5 hr immediately cryopreserved in liquid nitrogen. One month later, PBMCs were thawed, expanded to erythroblasts, and reprogrammed into IPSCs (Figure 2C). Importantly, the number of IPSC colonies formed was comparable prior to PBMC freezing (i.e., for samples kept at room temperature for 5 hr) (Figure 2A). IPSCs were also derived from PBMC samples processed 48 hr postdraw that had been stored in liquid nitrogen for 1 and 4 months prior to expansion and reprogramming (Figure S2). Thus, our data suggest that adding a freezing and thawing cycle of PBMC samples does not affect the efficiency of subsequent reprogramming.

Increasing the Number of Input Cells and Virus Transduction Volume Rescue Reprogramming Potential of Blood Samples Kept at Room Temperature for 24 and 48 hr

The results above suggest that whole blood samples deteriorate over a period of time at room temperature and hence decrease the fraction of erythroblasts available for reprogramming (Figure 2A). Therefore, scaling up input target cells and/or virus transduction volume may improve reprogramming efficiencies of those samples. To test this hypothesis, we next performed experiments in which PBMCs derived from two donor samples held at room temperature for 5, 24, and 48hr were cultured in media favoring expansion into erythroblasts. Triple staining was performed following expansion with surface markers such as CD71 and CD36 to identify erythroblasts population and CD14 for monocyte population and analyzed expression by flow cytometry. Consistent with our findings in (Figures 1B and 1C), erythroblast populations were also enriched in donor 3 and donor 4 with 88.2% and 50.9%, respectively, when samples are held at room for 5 hr postdraw as judged by CD 71 and CD36 double positives. These populations dramatically decrease when blood samples were stored at room temperature for 24 and 48 hr (Figures 3A and 3B). The vast majority of expanded cells were negative for CD14 monocyte-specific marker. In marked contrast, CD14 was expressed in PBMC parental populations, and CD71 expression was not detected in vast majority of the cells (Figures S3 and S4). These results indicate that the majority of expanded cells were erythroblasts.

We doubled the quantity of enriched population of erythroblasts (1×10^6 cells) and virus volume (MOI 12) for reprogramming and observed a dramatic improvement in the IPSC colony formation with up to 3- and 5-fold (donor 3) and 15- and 6-fold (donor 4) at 24- and 48-hr stability points, respectively (Figures 3C and 3D). The colonies derived from the three storage conditions show the typical compact IPSC morphology, similar to undifferentiated hESCs (Figure 3E).

These results demonstrate that increasing the number of target cells and virus transduction volumes enhances the efficiency of IPSC derivation from blood samples stored at room temperature for up to 48 hr postcollection.

In Vitro Differentiation of hIPSCs

We next examined the pluripotency and differentiation properties of three IPSC donors derived from the three storage conditions (n = 2–3 lines per donor). Briefly, hIPSCs were individually picked and expanded for approximately 15–30 passages. The resulting hIPSCs were cultured under conditions favoring the formation of cells of the three germ layers, subsequently immunostained with markers specific for the pluripotency and the three germ layers, and then analyzed on a Cellomics Array scan. Representative images of one IPSC donor (donor 1) are shown in Figures 4 and S5. These analyses show that, prior to the initiation of cell differentiation, all of our derived IPSC lines expressed pluripotent markers such as OCT4, NANOG, and SOX2, thereby confirming their pluripotent status. After several days in respective differentiation media, cells were positive for endoderm (SOX17, CXCR4, GATA4), mesoderm (BRACHYURY, EOMES, MIX1L), and neuroectoderm (NESTIN, SOX1, SOX2), indicating their differentiation into all three germ layers. Quantification of the three differentiation lineages using Cellomics Array software showed that the markers for endoderm, mesoderm, and neuroectoderm were upregulated (Figure 5). On the other hand, OCT4, a pluripotency marker, was downregulated in the three germ layers. These data indicate that IPSC derived from blood that had been stored up to 48 hr are pluripotent and can effectively differentiate into the three germ layers.

Blood Samples Held at Room Temperature for up to 48 hr PostDraw Produce Multiple IPSC Clones with Stable Genomes

To evaluate whether storage of blood at room temperature up to 48 hr affects the genomic stability of IPSCs, karyotype analysis by multiplex fluorescent in situ hybridization (M-FISH) was performed on hIPSC lines grown up to 30 passages. We randomly karyotyped metaphases obtained from six donors (n = 14 IPSC lines) derived from 5-hr blood storage, four donors (n = 10 IPSC lines) derived from 24 hr blood storage, and four donors (n = 9 IPSC lines) derived from 48-hr blood storage. At least ten metaphase spreads were analyzed per line. M-FISH revealed two normal male karyotypes [46,XY] (Figures 6A and 6B) and one female karyotype [46,XX] (Figure 6C). Of the 14 lines derived from 5-hr blood storage, four lines displayed chromosomal aberrations (Table S1), i.e., 2.1% abnormal karyotypes of the total 190 metaphases analyzed and 29% of the lines karyotyped harbor
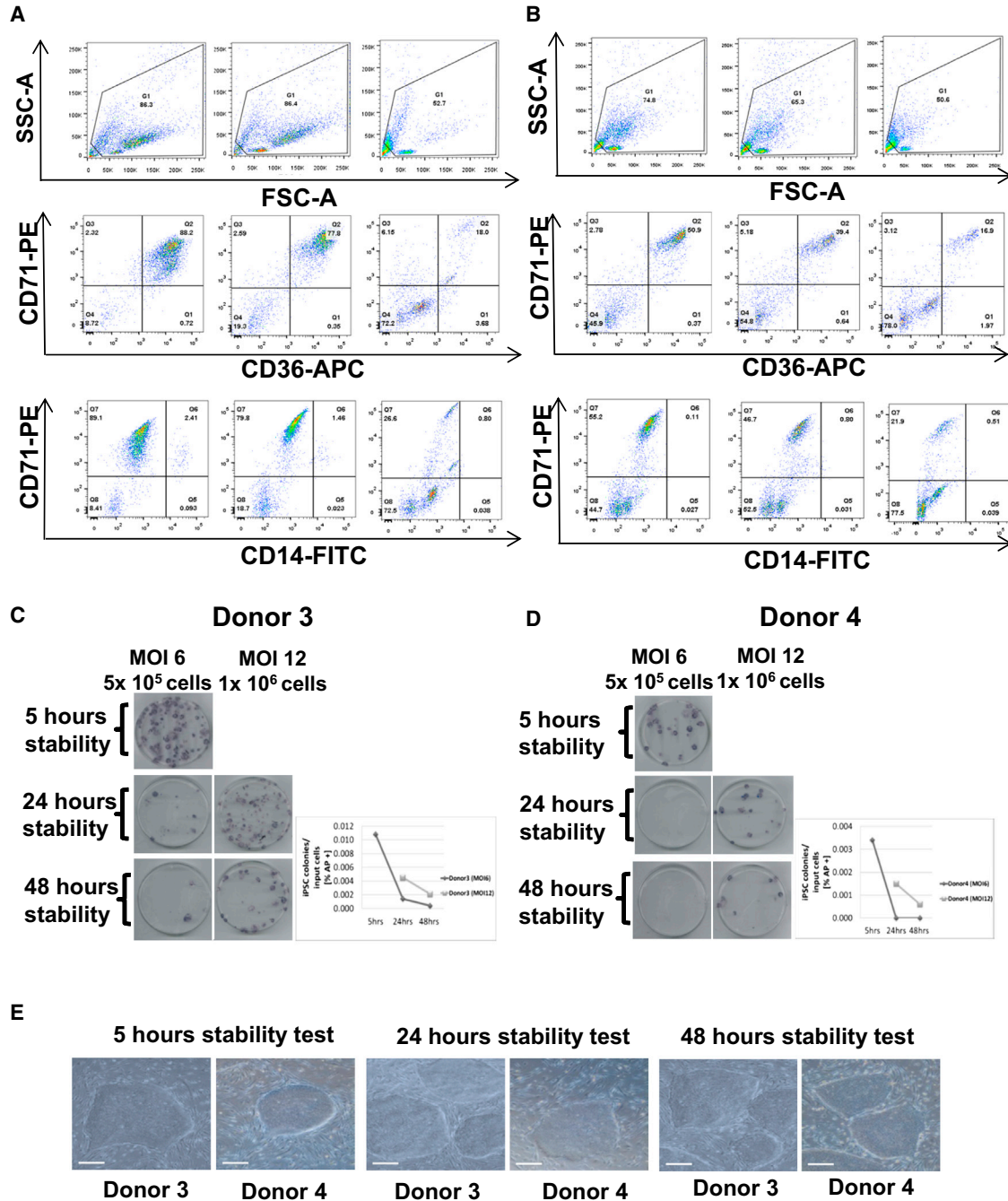
Figure 3. Reprogramming of Erythroblasts-Enriched Populations by Doubling the Input Cells and Viral Transduction Volumes

(A and B) Representative flow cytometry data of donor 3 and donor 4 (linked to Figures S3 and S4).

(C and D) Representative images show AP stained IPSC colonies derived from samples that had been held at room temperature for 5-, 24-, and 48 hr postdraw and reprogramming efficiencies quantified by determining the percentage of AP-positive colonies.

(E) Representative phase contrast images of IPSCs with characteristic typical human ES cell-like morphology in all the three stability time points are shown. Donor 3 (guuo), donor 4 (sefg). Scale bars represent 250 μm.

mutations (Figure 6D). Of the ten lines derived from 24-hr blood storage, one line had a translocation (Table S1); i.e., 0.62% abnormal karyotypes of the total 160 metaphases analyzed and 10% of the lines karyotyped harbor mutations (Figure 6D). Finally, we analyzed 130 metaphases from nine IPSC lines derived from 48-hr blood storage
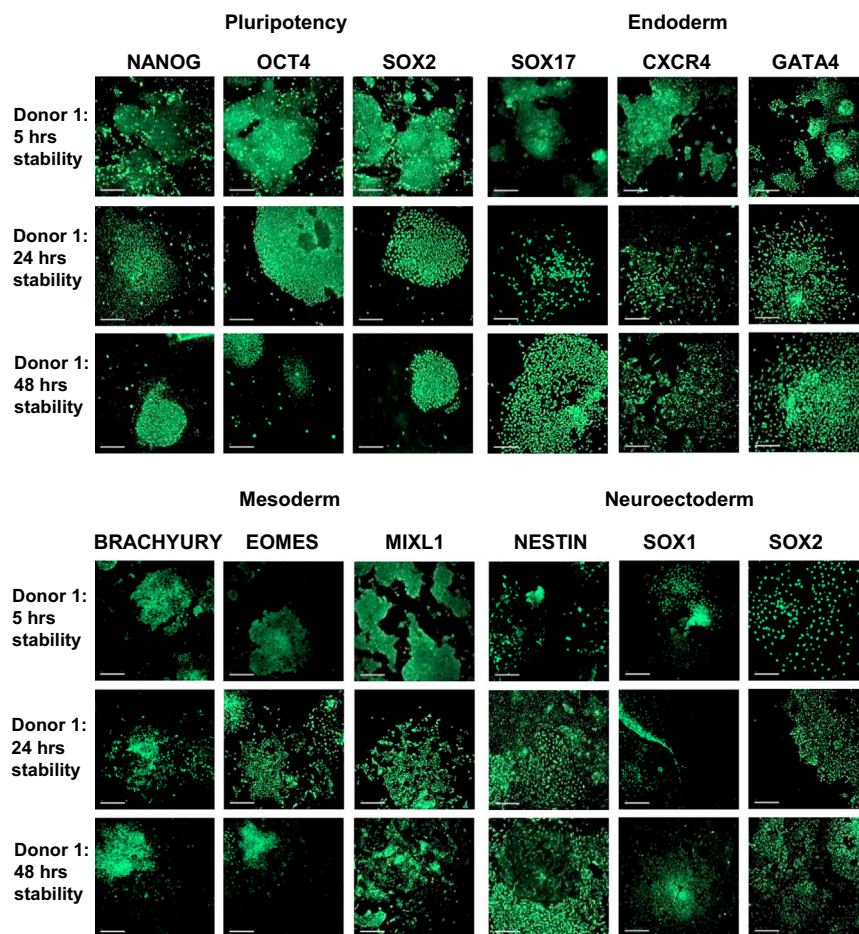
Figure 4. Characterization of hIPSC Lines Derived from Blood Samples Stored at Room Temperature for 5-, 24-, and 48 hr Postcollection

IPSCs derived from the three storage conditions were cultured in media favoring differentiation into the three germ layers and subsequently co-stained with DNA dye (DAPI) and antibodies against markers for endoderm, mesoderm, and neuroectoderm. Expression was analyzed on a Cellomics array scan. Representative images show immunofluorescence staining for markers for pluripotency (OCT4, SOX2, NANOG in green), endoderm (SOX17, CXCR4, GATA4 in green), mesoderm (BRACHYURY, EOMES, MIXL1 in green), neuroectoderm (SOX1, SOX2, NESTIN in green), and merged images are shown in Figure S5. The same microscopic fields are shown in Figures 4 and S5. Donor 1 (IPSC lines: codr_3, codr_5, codr_7). Scale bars represent 250 μm.

and identified two abnormal karyotypes (Table S1), 1.5% aberrant chromosomes of the total 130 metaphases analyzed and 22% of the lines karyotyped harbor mutations (Figure 6D). Taken together, our data suggest no dramatic incidence of chromosomal aberrations in IPSCs derived from blood samples stored at room temperature for 48 hr postcollection when compared with blood samples held at room temperature for 5 hr.

Increasing the Virus Transduction Doses Does Not Delay the Vector Clearance in Reprogrammed Cells

SeV reprogramming vectors tend to decrease over time in reprogrammed cells after several passages (Fusaki et al., 2009; Schlaeger et al., 2015). We next investigated how quickly the *SeV* RNA was lost during hIPSC expansion and whether reprogramming with increased virus transduction will delay viral clearance from the cells. Notably, residual Sendai transcripts were eliminated in 12 of 14 lines tested, including the samples that had been reprogrammed with viral MOI 12 (Figure 6E). Residual transcripts were observed in two lines reprogrammed with MOI 6 that had been derived from donor 1 (lane 1) and donor 2

(lane 5) even after up to 26 passages. This may be due to the variation of vector replication between cell lines (Fusaki et al., 2009). No RNA transcript was found in lines reprogrammed with virus at MOI 12 derived from donor 3 and donor 4. Taken these findings together, increasing the virus dose apparently does not delay vector clearance in reprogrammed cells.

DISCUSSION

Whole-blood and skin-punch biopsies are the most common tissues used for reprogramming to hIPSCs. One of the major challenges faced by clinical centers when collecting these biological samples for use in hIPSC production is how to minimize the potential tissue degradation associated with sample transportation and storage. Several groups have derived IPSCs from blood samples and recommend a 2- to 4-hr time elapse between biological sample collection and processing in order to preserve the integrity of primary cells prior to reprogramming (Daheron and D'Souza 2008; Yang et al., 2008; Freisleben et al.,
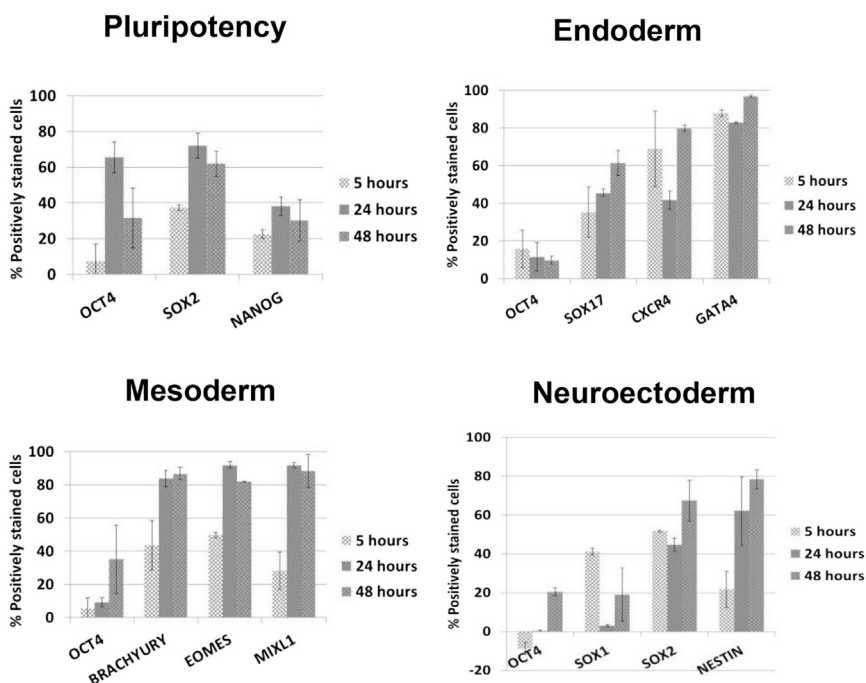
Figure 5. Characterization of hIPSC Lines Derived from Blood Samples Stored at Room Temperature for 5-, 24-, and 48 hr Postcollection

IPSCs derived from the three storage conditions were cultured in media favoring differentiation into the three germ layers and subsequently co-stained with DNA dye (DAPI) and antibodies against markers for endoderm, mesoderm, and neuroectoderm. Expression was analyzed on a Cellomics array scan. Representative data show the quantification of pluripotency and differentiation capacity of the IPSC lines obtained from Cellomics Array scan software (mean ± SD; n = 2 wells)

2011; Sommer et al., 2012). However, this will highly be dependent on the availability of relevant equipment, reagents, and trained personnel required for blood sample processing within the surgical suites.

For clinics with rural study sites with limited laboratory facilities that are not adequately equipped to process samples, long periods of transportation of samples to research laboratories, up to several hours, may be required.

To the best of our knowledge, nevertheless, no published data are available on the effects of the long-term stability of blood stored at room temperature on their ability to reprogram into IPSCs. Our study demonstrates that it is possible to store blood samples at room temperature for up to 48 hr before successfully reprogramming them into IPSCs.

This removes the need for sample processing within 2 to 4 hr of collections. Although the reprogramming efficiencies seen in IPSC derivation from blood at 24- and 48 hr storage time points was diminished when compared with fresh blood samples, we always obtained sufficient colonies required for further studies. Furthermore, the resulting hIPSC lines display a normal capacity of self-renewal and ability to differentiate into derivatives of the three primary germ layers. Importantly, it should be mentioned that similar results were obtained with samples collected from patients with rare disease (data not shown). Therefore, the results described here could be extended to a diversity of samples.

The genomic stability of hIPSCs has been extensively studied since this could be a major limitation for clinical applications (Aasen et al., 2008). Factors that affect the genomic integrity of stem cells include culture conditions, number of passages, and enzymatic and non-enzymatic dissociation (Mitalipova et al., 2005; Martins-Taylor et al., 2011; Taapken et al., 2011; Nguyen et al., 2013). However, it was unclear whether long-term incubation of tissue samples at room temperature over increasing period of time could impose a survival pressure on the somatic cells of the tissues and hence affect the quality of IPSCs derived from them. Notably, karyotype analysis of our blood derived IPSC lines clearly show that the rates of small chromosome abnormalities detected in the three storage conditions are similar to each other and no notable differences are seen. The karyotypic changes observed in our lines are deletions, translocation, and trisomies involving chromosomes 3, 7, 13, 20, 21, and X (Table S1). Interestingly, chromosome 20, 21 and chromosome X aberrations are known to be predominant for hESCs and hIPSCs (Martins-Taylor et al., 2011; Taapken et al., 2011). Therefore, 48 hr of storage of blood samples at room temperature does not affect the fundamental characteristic of subsequent hIPSC lines. Our findings suggest that storage up to 48 hr has no detrimental impact on the genome integrity.

In conclusion, the results from this study suggests that high-quality IPSCs can be derived from blood samples kept at room temperature for up to 48 hr, thus enabling
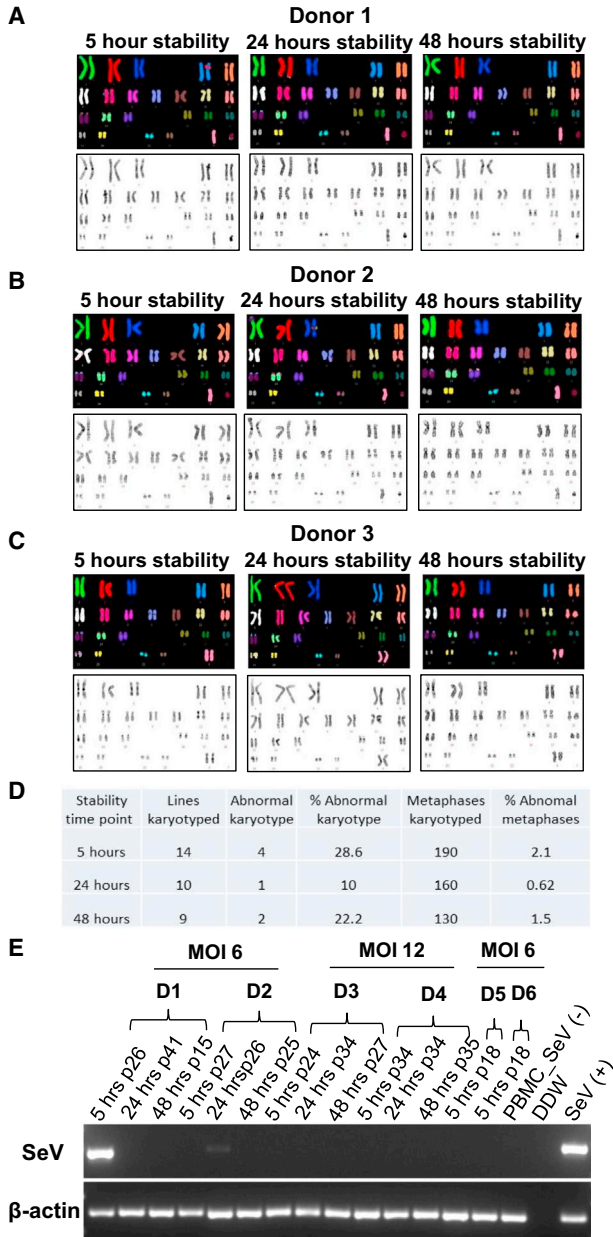
Figure 6. Screening for Genomic Integrity and SeV Clearance in IPSCs Derived from Blood Samples Stored at Room Temperature for 5-, 24-, and 48 hr Postcollection

M-FISH karyotypic analysis was performed on IPSCs derived from blood to evaluate the effect of storage duration (5-, 24-, 48 hr postdraw) on genomic stability. Randomly selected metaphases (10 or 20) were karyotyped on M-FISH and DAPI banding patterns.

(A and B) M-FISH shows normal diploid 46, XY male karyotypes at passage 20.

(C) Normal diploid 46, XX female karyotype at passage 20. Donor 1 (IPSC lines: codr_3, codr_5, codr_7), donor 2 (IPSC lines: gabq_2, gabq_4, gabq_6), donor 3 (IPSC lines: guuo_2, guuo_5, guuo_8).

(D) Table displaying the number of lines karyotyped, normal, and metaphases detected (related to Table S1). Abnormalities met the

sample collections from trial sites distance away from the research laboratories.

EXPERIMENTAL PROCEDURES

Ethics Statement

Human peripheral blood used in this study was approved by NRES Committee London-Fulham reference number 14/LO/0345. All subjects were informed of the purpose of the study, and a written consent was obtained.

Cell Culture

The hIPSCs were grown on irradiated mouse embryonic fibroblast (MEF) feeders (Globalstem) using stem cell media described below: Advanced DMEM (Life technologies) was supplemented as follows: 10% Knockout Serum Replacement (KoSR, Life Technologies), 2 mM L-glutamine (Life technologies), 0.007% 2-mercaptoethanol (Sigma-Aldrich), 4 ng/μl of recombinant zebrafish basic Fibroblast Growth Factor-2 (CSCR, University of Cambridge), and 1% Pen/Strep (Life Technologies). Media were changed daily, and the cells were passaged every 5 to 7 days depending on the confluence of the plates.

Processing Whole Blood Samples

Human blood samples were collected in sodium citrate collection tubes from consented individuals from Clinical Trials Laboratory Services London (CTLS). The age and gender of the donors are shown in Table S3. Blood samples were held at room temperature for 5-, 24-, or 48 hr postcollection, and subsequently peripheral blood mononuclear cells (PBMCs) were separated by using Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation (Vissers et al., 1988). Briefly, 10 ml from each blood donor sample (n = 7) was diluted with 10 ml of Dulbecco's PBS and layered onto a Ficoll-Paque density gradient according to the manufacturer's instructions. The samples were centrifuged at 500 × g for 30 min at room temperature with the brake off so that the centrifuge speed decelerated slowly after the elapsed time. The PBMCs were carefully collected from the interphase and transferred into a fresh 50 ml Falcon tube, and PBS was added up to total volume of 50 ml. Cells were centrifuged at 300 × g for 20 min with the brake on, and the PBMC cell pellets were either frozen down in freezing media (ice cold) consisting of 10% DMSO and 90% complete KOSR media (and transferred into liquid nitrogen) or used directly for expansion into erythroblasts.

International System for Human Cytogenetics Nomenclature (2013).

(E) Loss of SeV RNA was detected by RT-PCR. SeV RNA clearance was seen in majority of lines except in two cases (D1 and D2) where viral RNA persisted over several passages up to p26. PBMC_SeV (−), non-reprogrammed human PBMCs; DDW, double-distilled water; SeV (+), human erythroblasts reprogrammed with SeV at MOI 6 and RNA extracted at passage 2. D1–D6 indicate donor IPSC lines. *β-actin* was included as internal control (see also Table S2).

Expansion of PBMCs into Erythroblasts

Following the PBMC separation as described above, samples were cultured in media favoring expansion into erythroblasts as described by Yang et al. (2008) and Soares et al. (2015) with modifications. Briefly, 2×10^6 PBMCs (frozen or fresh) samples were cultured in a well of a 12-well culture plate in 2 ml of serum-free expansion media (EM) containing StemSpan H3000 (SS, StemCell Technologies), SCF (100 ng/ml, R&D Systems), IL-3 (10 ng/ml, Life Technologies), erythropoietin (3 U/ml; Miltenyi Biotech), IGF-1 (40 ng/ml; Miltenyi Biotech), and dexamethasone (1 μM; Sigma-Aldrich) for 9 days. Spent media were changed every 3 days. After that, cells were analyzed by flow cytometry to monitor erythroblast expansion using antibodies specific for erythroblast cell surface markers CD71 and CD36 and PBMC cell surface marker CD14 as described below.

Flow Cytometry Analysis

Flow cytometric experiments were performed using a BD LSR Fortessa cell analyzer. Briefly, at day 9 of PBMC expansion, 5% of cell population was either dual or triple stained for 20 min at room temperature with PE-labeled anti-CD71 (BD Biosciences, UK, #555537), APC-labeled anti-CD36 (BD Biosciences, UK, #561822), and FITC-labeled anti-CD14 (BD Biosciences, UK, #561712) resuspended in PBS containing 0.5% BSA, 2 mM EDTA 1% PFA. Isotype controls IgG2a,κ, IgM,κ, and IgG2a,κ were used as negative controls for FACS gating for PE-labeled anti-CD71, APC-labeled anti-CD36, FITC-labeled anti-CD14, respectively, and single-color stained cells for compensation. Following the flow cytometric assays, a postacquisition analysis was subsequently performed using FlowJo software V10 (Tree Star).

Reprogramming of Erythroblast-Enriched Cell Populations Using Sendai Virus

For IPSC derivation from blood, Sendai vectors (each) expressing human OCT3/4, SOX2, KLF4, and c-MYC (CytoTune, Life Technologies, catalog number A1377801) were used to infect erythroblast-enriched populations following expansion. Briefly, 5×10^5 (or 1×10^6) erythroblast-enriched populations were resuspended in 0.3 ml (or 1.2 ml) EM media in a 15-ml Falcon tube, respectively. After that, Sendai virus vectors were reconstituted at MOI 6 (or MOI 12) in 15-ml Falcon tube containing 0.3-ml (or 1.2-ml) EM media supplemented with 8-μg polybrene, respectively. Subsequently, virus suspension was added to the cells in 15-ml falcon tube and resuspended and transferred into a 12-well plate. Cells were incubated overnight at 37°C and 5% CO_2. Next day cells were collected in a 15-ml falcon tube and washed with SS media by centrifugation at $300 \times g$ for 5 min with brake on. The transduced cell pellets were resuspended in 2-ml EM media and transferred into a well of 12-well plate. Three days postinfection, cells in a well of a 12-well plate were transferred to a 10-cm culture dish containing mouse embryonic fibroblast (MEF) feeder layer and cultured further for 2 days in EM media. Starting from day 5 posttransduction, cells were maintained in IPSC media (KoSR + FGF2), and media were changed daily. At 10 to 21 days posttransduction, the transduced cells began to form colonies with IPSC morphology, and visible colonies were handpicked and transferred onto 12-well MEF feeder plates.

Passaging of hIPSCs

To passage hIPCS, cells were washed with PBS and incubated with Collagenase and Dispase (Collagenase IV 1 mg/ml, Invitrogen 17104-019; Dispase 1 mg/ml, Invitrogen 17105-041) for 45 min. Colonies were collected in a falcon tube containing IPSC growth medium and were allowed to sediment for 30 s. The supernatant, containing residual Collagenase/Dispase was removed, and the colonies washed once with IPSC medium. The colonies were allowed to sediment again and the supernatant removed. Finally, they were mechanically broken up and plated onto fresh MEF feeders. Cells were passaged every 5 to 7 days (depending on the confluence of the plates) at the desired ratio.

AP Staining

Following reprogramming, cells were fixed in 4% paraformaldehyde and subsequently stained for AP with the AP BCIP/NBT ALP staining kit as recommended by the manufacturer (Promega).

hIPSCs Differentiation

Differentiation was performed as described previously (Vallier et al., 2009). Briefly, hIPSCs grown on feeder conditions were harvested using Collagenase and Dispase. The colonies were collected, washed in media, and mechanically broken up before being replated onto 24-well MEF feeder plates or precoated gelatine/FBS plates. For pluripotency assays, colonies were seeded on MEF feeder plates and for the differentiation assay colonies were grown on gelatine/FBS plates. Prior to differentiation to mesoderm, endoderm, and neuroectoderm, cells were cultured overnight in predifferentiation media CDM-PVA supplemented with recombinant ACTIVIN-A (10 ng/ml; CSCR, University of Cambridge) and zebrafish FGF2 (12 ng/ml; CSCR, University of Cambridge). For differentiation into mesoderm following culture in predifferentiation media, spent media were removed and replaced with fresh CDM-PVA media containing bone morphogenic protein 4 (BMP4, 10 ng/ml, R&D Systems), FGF2 (20 ng/ml; CSCR, University of Cambridge), recombinant ACTIVIN-A (10 ng/ml; CSCR, University of Cambridge), LY29004 (10 μM, Promega), and CHIR99021 (5 μM, Selleckchem) and subsequently cultured for 3 days. Media were changed daily.

For differentiation into endoderm following culture in predifferentiation, cells were cultured further in differentiation media for 3 days. Briefly, day 1 spent media were removed and replaced with fresh CDM-PVA media supplemented with recombinant ACTIVIN-A (100 ng/ml; CSCR, University of Cambridge), zebrafish FGF2 (80 ng/ml; CSCR, University of Cambridge), BMP4 (10 ng/ml R&D Systems), LY29004 (10 μM), and CHIR99021 (3 μM). Day 2 media were removed and replaced with fresh CDM-PVA supplemented with recombinant ACTIVIN-A (100 ng/ml), zebrafish FGF2 (80 ng/ml), BMP4 (10 ng/ml), and LY29004 (10 μM). Day 3 media were removed and replaced with RPMI media supplemented with B27 (1×, Life Technologies), recombinant ACTIVIN-A (100 ng/ml), zebrafish FGF2 (80 ng/ml), and Non-Essential Amino Acids (1×, Life Technologies).

For differentiation to neuroectoderm, IPSCs were grown for 12 days in CDM-PVA supplemented with SB431542 (10 μM; Tocris Bioscience), FGF2 (12 ng/ml, CSCR University of Cambridge), and NOGGIN (150 ng/ml, R&D Systems). Media were changed daily.

Immunostaining

For the detection of pluripotency and differentiation markers, cells grown in 24-well plates as described above were fixed with 4% paraformaldehyde for 20 min. After that, cells were permeabilized and blocked with 10% donkey serum and 0.1% Triton X-100 in PBS. Subsequently, cells were stained with primary antibodies overnight at 4°C and finally incubated with Fluorochrome-labeled secondary antibodies ALEXA FLUOR (Invitrogen). The primary antibodies used for detecting pluripotency markers were anti-OCT4 (SC-5279, Santa Cruz Biotech), anti-SOX2 (AF2018, R&D), and anti-NANOG (AF1997, R&D). The primary antibodies used for detecting endoderm markers were anti-SOX17 (AF1924, R&D), anti-CXCR4 (MAB173-100, R&D), and anti-GATA4 (SC-25310, Santa Cruz Biotech). The primary antibodies used for detecting mesoderm markers were anti-Brachyury (AF2085, R&D), anti-EOMES (Ab23345, Abcam), and anti-MIXL1 (SC-98664, Santa Cruz Biotech). The primary antibodies used for detecting neuroectoderm markers were anti-NESTIN (AB22035, Abcam), and anti-SOX1 (AF3369, R&D) anti-SOX2 (AF2018, R&D). The secondary antibodies used were donkey anti-goat AF488 (Invitrogen), donkey anti-mouse AF488 (Invitrogen), and donkey anti-rabbit AF488 (Invitrogen). Images were captured and quantified using a Cellomics array scan, and images were processed using Adobe Photoshop CS5.

Chromosome Harvest and Multiplex-FISH Karyotyping of Human IPSCs

Metaphase chromosomes from hIPSCs were harvested as described previously (Moralli et al., 2011), with slight modifications. Briefly, after refreshing, the medium cells were treated with 10 μM ROCK inhibitor (Tocris) for 2–3 hr, followed by incubation with 25–100 ng/ml Nocodazole (Sigma-Aldrich) for a further 2–3 hr. Subsequently, cells were dissociated using Accutase (Sigma-Aldrich) and treated with a buffered hypotonic solution (0.4% KCl in 10 mM HEPES [pH 7.4]) for 8–12 min at 37°C. The cells were then fixed and washed twice in a 6:1 (v/v) methanol:glacial fixative and kept in fixative in −20°C freezer until use.

For multiplex-fluorescence in situ hybridization (M-FISH), chromosome-specific DNA libraries were generated from 5,000 copies of flow-sorted chromosomes, provided by Flow Cytometry Core Facility of the Wellcome Trust Sanger Institute, using GenomePlex Whole Genome Amplification (WGA2) kit (Sigma-Aldrich). Human 24-color painting probe was made following the pooling strategy (Geigl et al., 2006). Five human chromosome pools were labeled with ATTO 425-, ATTO 488-, CY3-, CY5-, and Texas Red-dUTPs (Jena Bioscience), respectively, using WGA 3 re-amplification kit (Sigma-Aldrich) as described before (Gribble et al., 2013). The labeled products were pooled and sonicated to achieve a size range of 200–1,000 bp, optimal for use in chromosome painting. Then the sonicated DNA sample was precipitated with ethanol together with human Cot-1 DNA (Invitrogen) and resuspended in a hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulfate, 0.5 M phosphate buffer, 1 × Denhardt's solution [pH 7.4]). Metaphase preparations were dropped onto precleaned microscopic slides followed by fixation in acetone (Sigma-Aldrich) for 10 min and dehydration through an ethanol series (70%, 90%, and 100%). Metaphase spreads on slides were denatured by immer-

sion in an alkaline denaturation solution (0.5 M NaOH, 1.0 M NaCl) for 50–60 s, followed by rinsing in 1M Tris-HCl (pH 7.4) solution for 3 min, 1 × PBS for 3 min, and dehydration through a 70%, 90%, and 100% ethanol series. The M-FISH probe was denatured at 65°C for 10 min before being applied onto the denatured slides. The hybridization area was sealed with a 22 × 22-mm coverslip and rubber cement. Hybridization was carried out in a 37°C incubator for 2 nights. The posthybridization washes included a 5-min stringent wash in 0.5 × SSC at 75°C, followed by a 5-min rinse in 2 × SSC containing 0.05% Tween20 (VWR) and a 2-min rinse in 1 × PBS, both at room temperature. Finally, slides were mounted with SlowFade Gold mounting solution containing 4′6-diamidino-2-phenylindole (Invitrogen). Images were visualized on a Zeiss AxioImager D1 fluorescent microscope equipped with narrow band-pass filters for DAPI, DEAC, FITC, CY3, TEXAS RED, and CY5 fluorescence and an ORCA-EA CCD camera (Hamamatsu). M-FISH digital images were captured using the SmartCapture software (Digital Scientific) and processed using the SmartType Karyotyper software (Digital Scientific). Approximately 10–20 metaphase chromosomes per IPSC line were fully karyotyped.

Screening for SeV Clearance by RT-PCR

Total RNA was extracted from cells using Gen Elute Mammalian Total RNA Miniprep Kit reagent (catalog number RTN70, Sigma-Aldrich) with On-Column DNase I Digestion Set (catalog number DNASE70, Sigma-Aldrich). RT-PCR reactions were performed using SuperScript III Reverse Transcriptase (catalog number 18080-044, Invitrogen), and Random Primers (catalog number C1181, Promega) with 0.5 μg of total RNA per reaction. Total RNA was quantified using a NanoDrop ND-1000. PCR was performed with 1 μl of cDNA in 50 μl of Platinum Taq DNA Polymerase mix (catalog number 10966-034, Invitrogen) according to manufacturer's recommendation. For SeV PCR the following condition was used: denaturation 95°C, 30 s, annealing: 55°C, 30 s, elongation: 72°C, 30 s, for 35 cycles. The expected size of the PCR product is 181 bp.

The primer pair used for the detection of presence of Sendai virus vector has been previously described (Fusaki et al., 2009), and the sequences are shown in Table S2. Beta-actin primer pair used in this study has been previously described (Fischer et al., 2014), and the sequences are shown in Table S2. The expected size of the PCR product is 100 bp.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.08.012.

AUTHOR CONTRIBUTIONS

C.A.A. conceived and designed experiments, performed experiments, analyzed data, oversaw research, and wrote the manuscript. F.A.C.S., A.A., and M.P. provided intellectual input into the manuscript. R.A., S.P., S.F., and J.L. performed experiments and analyzed data. F.Y. performed experiments, analyzed data, and helped edit the manuscript. L.V. provided intellectual input into

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