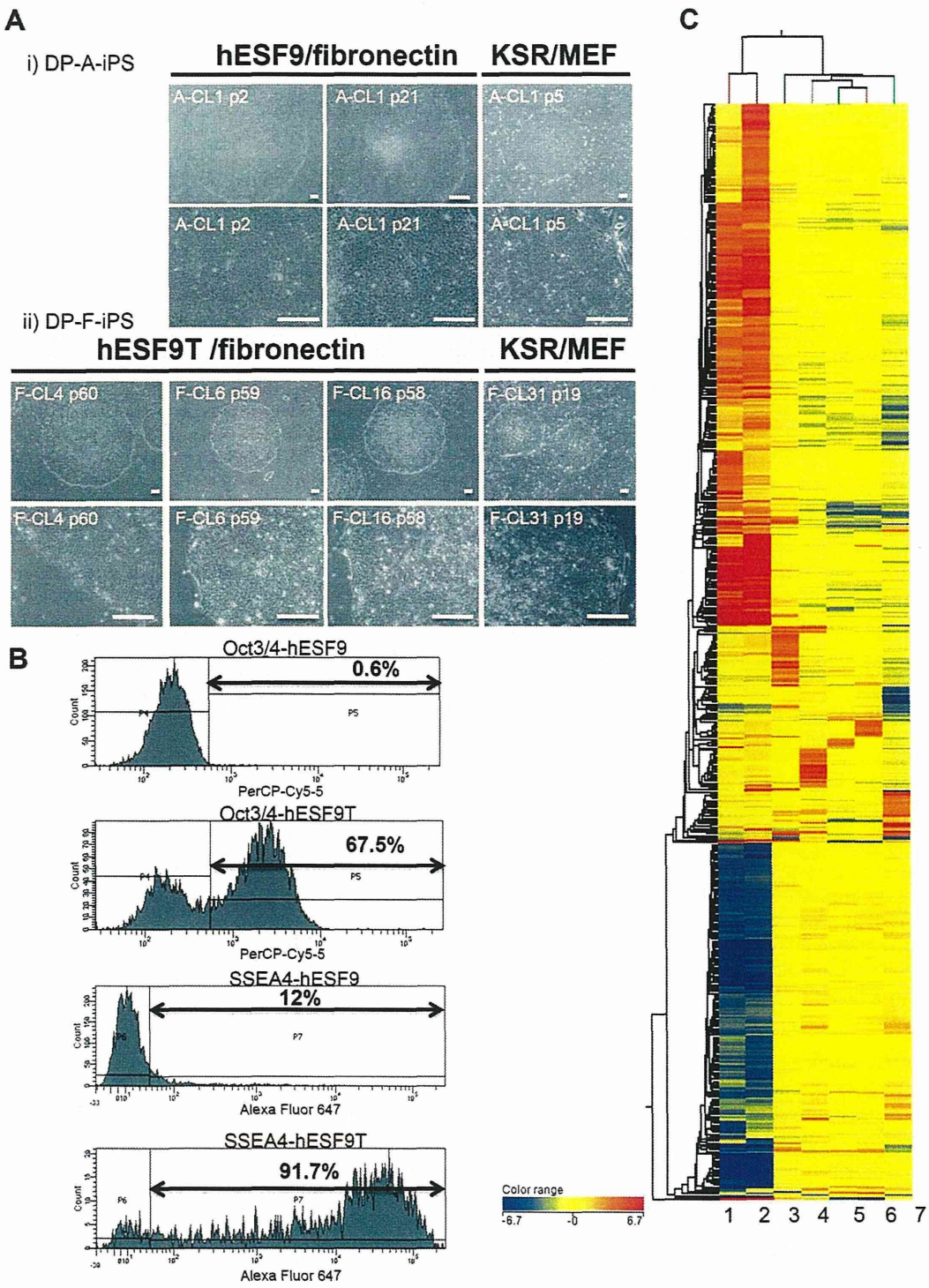

Figure 4. hiPSCs derived from DPCs in completely serum- and feeder-free culture conditions. A) Phase contrast images of iPSCs derived from DPCs (DP-A-iPS and DP-F-iPS). i) DP-A-iPS-CL1 at passage 2, or 21 on fibronectin-coated dish with hESF9 medium. Right panel showed the cells at passage 5 cultured on MEF with KSR-based conditions. ii) DP-F-iPS-CL4 at passage 60, CL6 at passage 59 and CL16 at passage 58 on fibronectin-coated dish with hESF9T medium. Right panel showed CL31 at passage 19 on MEF with KSR-based conditions. Bars indicate 200 μm. B) Flow cytometry analysis of Oct3/4 and SSEA-4 expression in hiPSCs generated and maintained in hESF9 medium supplemented with TGF-β1 (2 ng/ml) (hESF9T) or without TGF-β1 (hESF9) (DP-F-iPS-CL-8 at passage 33). The horizontal bar indicates the gating used to score the percentage of cells antigen positive. C) Comparison of the global gene expression analysis. Unsupervised clustering was performed using microarray data from parental cell (DPCs), DP-iPSCs cultured in hESF9 or hESF9T (DP-A-iPS, DP-F-iPS) and hiPSCs (Tic, DP-F-iPS). 1.DP cell (DP-A): passage 2 = before infection. 2.DP

cell (DP-F): passage 4 = before infection. 3.DP-A-iPS-CL1: passage 14 = serum-free condition (hESF9/on FN). 4.DP-F-iPS-CL12: passage 36 = KSR-based condition (KSR/on MEF). 5.DP-F-iPS-CL6: passage 37 = serum-free condition (hESF9T/on FN). 6.DP-F-iPS-CL8: passage 35 = serum-free condition (hESF9T/on FN). 7.Tic (hiPSC: JCRB1331): passage 58 = KSR-based condition (KSR/on MEF). A genome-wide gene expression profiling analysis demonstrated that hiPSCs cultured in hESF9 or hESF9T on fibronectin showed a similar gene expression pattern to those grown in a conventional feeder-dependent culture (KSR-based condition). Hierarchical combined tree on compare. (Fold change> = 20).
doi:10.1371/journal.pone.0087151.g004

hESF9 medium and DPCs tended to overgrow and inhibit the emergence of iPS colonies (data not shown).

TGF-β1 plays an important role in maintaining the pluripotency of hiPSCs

Newly generated hiPSCs were mechanically detached from culture dishes and transferred to fibronectin-coated dishes in hESF9 or hESF9T (containing TGF-β1) medium (Fig. 4-A). In hESF9 medium, hiPSCs attached to the dish, but they did not remain undifferentiated, and we occasionally observed spontaneous differentiation along the edges of colonies. Small differentiated cells were observed in hESF9 medium in the absence of TGF-β1. By contrast, hiPS colonies remained undifferentiated in hESF9T medium.

Increasing the dose of TGF-β1 up to 10 ng/ml promoted the growth of undifferentiated colonies as confirmed by QX100™ Droplet Digital™ PCR (Bio-RAD) analysis (Fig. 5, A-C). The greatest effect of TGF-β1 was seen at 2–10 ng/ml, whereas 0 ng/ml was markedly deleterious. The expression of pluripotency markers such as Nanog and Oct3/4 were up-regulated by TGF-β1. Moreover, the expression of differentiated marker genes was lower in hESF9T than in hESF9 medium (Fig.5-B). Plasminogen activator inhibitor-1 (PAI-1) is an indicator of mesoderm differentiation, and GATA binding protein 4 (GATA4) is a marker involved in the development of cardiac hypertrophy and remodeling, and it plays a critical role in regulating basal and agonist or stress-induced gene expression in cardiac and smooth muscle cell types. At passage 33, the percentage of SSEA4-positive-DP-F-iPS-CL8 cells cultured in hESF9T (91.7%) was higher than that in hESF9 medium (12%). Moreover, the percentage of Oct3/4-positive-DP-F-iPS-CL4 cells was 67.5% in hESF9T medium and 0.6% in hESF9 medium (Fig. 4-B). These results indicated that TGF-β1 supported to a large extent the undifferentiated growth of hiPSCs over a prolonged period. The hiPSCs generated and maintained in hESF9 did not survive beyond 30 passages. We continued to culture human iPSCs in hESF9T up to 60 passages.

Gene expression analysis confirmed the effect of TGF-β1 in maintaining pluripotency of hiPSCs

A genome-wide gene expression profiling analysis demonstrated that hiPSCs cultured in hESF9 or hESF9T on fibronectin showed broadly similar gene expression patterns to those grown in a conventional feeder-dependent culture (KSR-based condition) (Fig. 4-C, Fig. S5). In contrast, the cells cultured in hESF9 exhibited significantly different profiles in several signaling pathways from those cultured in hESF9T. In a pathway analysis the TGF-β signaling pathway (WP560), the WNT signaling and pluripotency pathway (WP399), the WNT signaling pathway (WP428), and Apoptosis modulation and signaling (WP1772) displayed significant differences between hESF9T and hESF9 (data not shown). Thus, DP-iPSCs cultured in hESF9T for a prolonged period remained undifferentiated and exhibited a similar gene expression pattern as cells grown in conventional feeder dependent cultures.

Characterization of DPC- derived iPS cells

An hiPS clone cultured in hESF9 or hESF9T showed a characteristic human ES cell-like morphology (Fig. 4-A), and reactivation of endogenous pluripotency marker genes such as *Oct3/4, Sox2, Nanog, Esg1*, and *Rex-1* was detected by RT-PCR (Fig. 5-D). These cells exhibited ALP activity and expressed SSEA-4, Tra-1-60, Tra-1-81, Nanog and Oct3/4 (Fig. 5-E). We confirmed the differentiation potential of the cells using an *in vitro* differentiation assay involving embryoid body generation. After 14 days of differentiation culture, the embryoid bodies contained a variety of differentiated cells characterized by germ-layer markers. These induced populations of cells were immunoreactive with antibodies to Nestin and βIII-tubulin (ectoderm markers), α-smooth muscle actin (SMA) (mesoderm marker), and α-fetoprotein (AFP) (primitive endoderm marker), but they did not react with anti-Oct3/4 (Fig. 6-A). The pluripotency of the iPS cell clone was also confirmed by the presence of cell derivatives of all three germ layers by teratoma formation after injection of undifferentiated iPS cells into severe combined immunodeficient (SCID) mice. Ten weeks after injection, histological analysis demonstrated that the formed tumors were derived from all three germ layers (n = 3). Neural tissues (ectoderm), epithelium (ectoderm), muscle (mesoderm), cartilage (mesoderm), adipose (mesoderm) and intestinal epithelial tissues (endoderm) were identified histologically in the hiPSCs-derived teratomas (Fig. 6-B).

Short Tandem Repeat Analysis

The genetic identity of DPCs and generated iPSCs was proven by a short tandem repeat analysis of genomic DNA (Table S3).

Cell growth and karyotype analysis of human iPS cells generated and maintained in define culture conditions

Growth curves were calculated from the split ratios at each passage. The population doubling time was 16.6±0.8 h (Fig. S6-A). The generated hiPSCs also had the property of self-renewal and pluripotency, and they possessed a normal karyotype. Karyotype analysis revealed that iPSCs at passage 20 were 46, XX (Fig. S6-B).

Discussion

We have established a fully defined serum-free culture system for the purposes of standardizing culture methods and protocols for deriving hiPSCs. Previously, we have demonstrated a defined serum- and feeder-free culture system based on use of hESF9 medium without TGF-β1 for human ES cell culture [2,5]. The hESF9 medium consists of a basal nutrient medium with known protein components, and it thus reduces the risk of contamination from adventitious pathogens. In this study, we showed that hiPSCs can be generated and maintained in a fully defined serum-free culture system from primary cell cultures of patient samples. The established hiPSCs are similar to hESCs in many respects, including morphology, proliferation, surface markers, gene expression, in vitro differentiation, and teratoma formation.

We first identified serum-free culture conditions that supported iPS cell generation. Several animal product-free culture media have been reported to support the derivation and/or maintenance

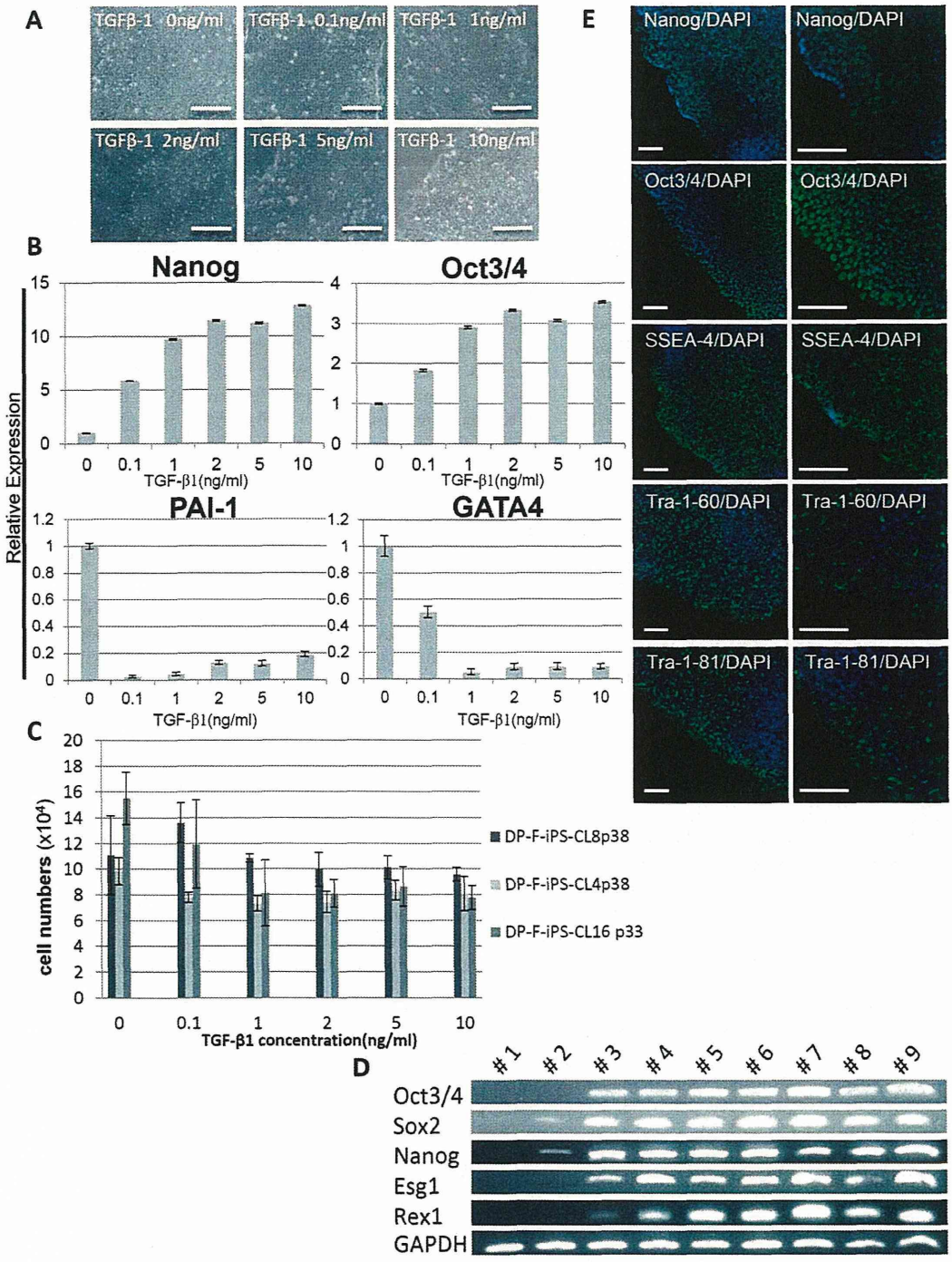

Figure 5. Culture of hiPSCs in hESF9T and Self-renewal marker expression of pluripotent stem cells in hiPSCs in defined culture conditions. A) Phase contrast photomicrograph of DP-F-iPS-CL16 (passage 28) supplemented with various concentration of TGF-β1 (0, 0.1, 1, 2, 5, 10 ng/ml). B) Digital-PCR analysis of gene expression of Nanog, Oct3/4, PAI-I and GATA4 in DP-F-iPS-CL6 in hESF9 medium supplemented with TGF-β1 (0, 0.1, 1, 2, 5, 10 ng/ml) on fibronectin. Expression levels were all normalized against GAPDH. C) Effects of TGF-β1 on hiPS cell proliferation. hiPSCs generated under hESF9 and cultured in hESF9T (CL-4 passage 38, CL-8 passage 38, CL-16 passage 33) were seeded in a 24 well plate coated with fibronectin at 1×10^4 cells/well and counted at every 24 hr. Each bar shows the number of cells in each concentration of TGF-β1 after 6 days of culture. Increasing the dose of TGF-β1 up to 10 ng/ml suppressed the growth of hiPSCs. Bars represent the mean±SEM. (n = 3). D) Expression of ES cell marker genes in iPSCs derived from DPCs. We used primers that only amplified the endogenous genes. #1: DP cell (DP-A): passage 2 = before infection. #2: DP cell (DP-F): passage 4 = before infection. #3: DP-A-iPS-CL1: passage 14 = serum-free condition (hESF9/on FN). #4: DP-F-iPS-CL4: passage 37 = serum-free condition (hESF9T/on FN). #5: DP-F-iPS-CL6: passage 35 = serum-free condition (hESF9T/on FN). #6: DP-F-iPS-CL8: passage 35 = serum-free condition (hESF9T/on FN). #7: DP-A-iPS-CL1: passage 8 = KSR-based condition (KSR/on MEF). #8: DP-F-iPS-CL12: passage 36 =

KSR-based condition (KSR/on MEF). #9: Tic (hiPSC: JCRB1331): passage 103 = KSR-based condition (KSR/on MEF). E) Immunocytochemistry of Nanog, Oct3/4, SSEA-4, Tra-1-60 and Tra-1-81. DP-F-iPS-CL16 grown under hESF9T-based culture conditions for 19 passages were fixed and reacted with antibodies (Nanog, Oct3/4, SSEA-4, Tra-1-61 and Tra-1-81). Binding of these antibodies was visualized with Alexa Fluor® 488-conjugated secondary antibodies (green). Nuclei were stained with DAPI (blue). Scale bars represent 100 μm.
doi:10.1371/journal.pone.0087151.g005

of hESCs, but their performance tends to be lower than that of KSR-based medium [14]. Our data showed that iPSCs can be generated in serum-free hESF9 medium by retroviral transduction of four transcription factors, *Oct3/4, Sox2, Klf4*, and *c-Myc* cultured on type I collagen or gelatin or fibronectin, and the absence of serum did not affect the efficiency of cell reprogramming. Subsequently, we demonstrated that serum-free medium also did not reduce retroviral transduction efficiency. In this study, we demonstrated that individual attachment factors could support generation of hiPSCs in place of MEF feeder cells. We showed that gelatin, collagen and fibronectin enabled rapid and steady generation of hiPSCs. However, once generated, iPS cells subcultured on gelatin or collagen did not retain their pluripotency, and these cells began to undergo differentiation.

It has been reported that fibronectin supports the maintenance of hESCs via α5β1 integrin [15], and Ras/MEK/MAPK signaling and kinases were stimulated via integrin ligation. Several groups have reported that vitronectin and one of its variants (VTN-NC) supports the maintenance of hESCs via αVβ5 integrin [4,16,17]. Another group has reported that the adhesion of hESCs and hiPSCs to laminin-511 is maintained via integrin α6β1, and Akt/ERK and kinases interacting with FAK are highly phosphorylated in human pluripotent stem cells [18–20]. These studies show that stem cell–ECM interactions are important in maintaining stem cell adhesion, survival and self-renewal both *in vivo* and *in vitro*.

In this study, we showed that individual ECM components differentially affected cell morphology and the efficiency of iPS induction through signals transmitted to the cell from the extracellular environment. The maintenance and proliferation of hiPSCs require a "niche" micro-environment [21]. Pluripotency was affected by many growth factors and cytokines interacting with cells. In the course of this process the acceleration of cell proliferation caused by cell cycle regulation is also important. The proliferation rate of ESCs is very rapid. The transduced cells acquired ESC-like characteristics, consequently the number of reprogrammed "iPSCs" increased. Moreover, the majority of transduced cells were incompletely reprogrammed and consequently did not generate ES cell-like colonies. Only a small proportion of transduced cells were able to form human ES cell-like colonies. It is unknown whether incompletely reprogrammed fibroblast-like cells supported the formation of ES cell-like colonies that emerged within fibroblast-like colonies through a feeder-like effect or through a non-autonomous effect. We believe that further studies using a fully defined medium will lead to a clarification of the reprogramming mechanisms and the advancement of stem cell research. This medium will greatly reduce variations in culture conditions arising from undefined medium constituents.

It is believed that the signaling requirements of human iPS cell generation and maintenance are different. In this report we have shown that hESF9 medium containing FGF-2 and heparin enhanced the derivation of iPSCs. However, the same conditions did not maintain the pluripotency of these cells. Moreover, TGF-β1 had an inhibitory effect when present at later stages of reprogramming. Therefore, we investigated whether TGF-β1 plays a role in maintaining hiPS cell pluripotency in fully defined culture conditions. TGF-β superfamily members participate in cell fate decisions in ESCs. However, the role of TGF-β in regulating the cell cycle of ESCs is poorly understood. TGF-β/Activin A are essential for the self-renewal of hESCs [22–25], and they function by activating Smad 2, 3 via binding to the Alk4/Activin receptor. Upon activation and dimerization, Smad 2, 3 maintains the pluripotent state through regulation of Nanog transcription [26,27]. Activation of Smad 2, 3 and its downstream targets, such as Nanog, by Activin A/Nodal, and the activation of PI3K/Akt signaling by factors such as IGF-1, heregulin, and FGF-2 are required to maintain pluripotency [26–28]. These two signaling requirements can be identified in all hES cell media formulations described to date. The activity of PI3K/Akt allows Activin A/Smad 2, 3 signaling to promote self-renewal. In the absence of PI3K signaling, Smad 2, 3 collaborates with Wnt pathway effectors to promote differentiation. Recently, TGF-β has been reported to repress activity of the telomerase reverse transcriptase [29]. The role of most members of the TGF-β superfamily in ESCs stemness or differentiation has not yet been investigated so it remains to be established what the specific role of each member of the family is and how it exerts its action under controlled experimental conditions.

In this study, we showed that TGF-β1 increased the expression levels of pluripotency markers such as Oct3/4 and Nanog in dose-dependent manner confirmed by Droplet digital PCR, microarray, and FACS analysis. On the other hand, increasing doses of TGF-β1 suppressed the growth rate of hiPSCs cultured under defined conditions. As with any pleiotropic factor, the effects of TGF-β superfamily members depend on their concentrations well as upon the presence of other factors. Furthermore, in the short term hiPSCs cultured in hESF9 or hESF9T exhibited similar morphologies, but the hiPSCs maintained in hESF9 did not survive beyond 30 passages. This result clearly confirmed that the iPSCs cultured in hESF9 medium absolutely required TGF-β1 to maintain pluripotency. At the same time, when TGF-β1 was present throughout the reprogramming procedure, DPCs tended to overgrow and inhibit or obscure the emergence of iPS colonies. Furthermore, activated p53 and TGF-β1 pathways act as roadblocks for iPSC formation from DPCs [4,24,29]. Decreasing the growth rate of DPCs led to the emergence of iPS cells without DPCs overgrowth. Regulation of TGF-β activity is important for hiPSCs generation and maintenance. Use of a feeder-free defined culture system to generate hiPSCs allowed us to clearly observe the reprogramming process and to begin to analyze the mechanisms involved.

In this study, we showed that iPSCs can be generated from adult DPCs by retroviral transduction of the four transcription factors Oct3/4, Sox2, c-Myc, and Klf-4. Human third molars are discarded as clinical waste and so could be obtained without any further surgical intervention. These teeth are aseptically obtained from the mandible and protected from UV and other damage by surrounding hard tissues. Therefore DPCs are a useful cell source for the generation of iPSCs [30–32]. Clonally expanded DPCs in serum-free medium could be reprogrammed with high iPS generation efficiency. Consequently, the cells are available for iPS generation by other methods using plasmids [33–35], chemicals and proteins [36,37], and microRNAs [38], aiming for the clinical use of the iPS cells in regenerative medicine.

The simplified defined medium described here consists of basal medium and known components that provide a much cleaner background for examining specific signaling pathways in

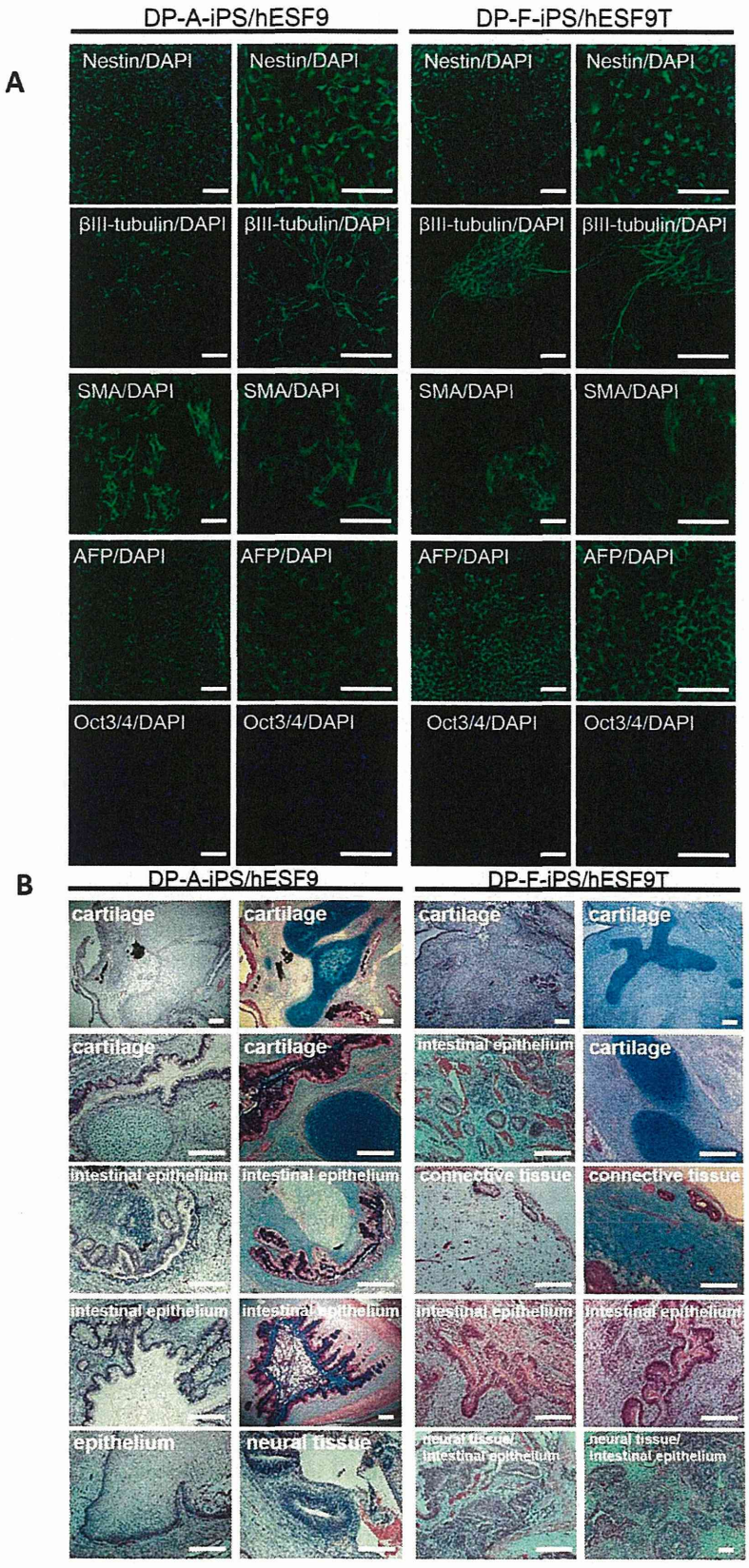
A
DP-A-iPS/hESF9
DP-F-iPS/hESF9T
Nestin/DAPI
βIII-tubulin/DAPI
SMA/DAPI
AFP/DAPI
Oct3/4/DAPI
B
DP-A-iPS/hESF9
DP-F-iPS/hESF9T
cartilage
intestinal epithelium
connective tissue
epithelium
neural tissue
neural tissue/
intestinal epithelium

Figure 6. Embryoid body-mediated differentiation of hiPSCs derived from DPCs in serum-free and feeder-free defined culture conditions and teratoma formation of hiPSCs in the defined culture conditions. A) Differentiation was performed using embryoid body formation, and the differentiated iPSCs (DP-A-iPS/hESF9 or DP-F-iPS/hESF9T) were fixed and reacted with antibodies. Shown were immunocytochemistry of Nestin, βIII-tubulin, α-smooth muscle actin (α-SMA), and α-fetoprotein (AFP). Binding of these antibodies was visualized with Alexa Fluor 488-conjugated secondary antibodies (green). Oct3/4 was also investigated. Binding of these antibodies was visualized with Alexa Fluor® 594-conjugated secondary antibodies (red). Nucleuses were stained with DAPI. (passage 25). Bar indicates 100 μm. B) Teratomas were generated in SCID mice (CB17/Icr-*Prkdc*^{scid}/CrlCrlj) from DP-A-iPS and DP-F-iPS grown under hESF9 or hESF9T-based conditions. Histological analysis with HE staining or Alcian Blue staining demonstrated that teratomas formed from iPS cells cultured in KSR-based (data not shown) or in hESF9T-based conditions contained derivatives of all three germ layers. Left panel shows teratomas from DP-A-iPS-CL1 at passage 22. Right panel shows teratomas from DP-F-iPS-CL14 at passage 6. Scale bars represent 200 μm.
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self-renewal, cell death, and cell differentiation, and it supports substantially improved reprogramming efficiencies. Although we have only demonstrated improved efficiencies for viral-based reprogramming, these conditions should be equally useful for other non-integrative reprogramming approaches [33–40]. Finally, since hESF9T medium is defined, it should also help facilitate the transfer of basic research on human pluripotent stem cells to the clinic and useful for understanding disease mechanisms, drug screening, and toxicology.

Conclusions

We have successfully generated hiPSCs from adult human dental pulp cells (DPCs) and maintained them in an undifferentiated state in serum-free defined medium. Furthermore these generated hiPSCs continued to proliferate and retained the properties of self-renewal and pluripotency for a prolonged period of time in the presence of appropriate amount of TGF-β1. As a result, we found TGF-β1 to be an important factor in maintaining pluripotency of hiPSCs. As this simple serum-free adherent monoculture system allows us to elucidate cellular responses to growth factors under defined conditions, these advantages will help to clarify the molecular mechanisms at play in early development.

Supporting Information

Figure S1 Transduction Efficiency of Retroviruses in TIG-3. TIG-3 was introduced with pMXs retroviruses containing the EGFP cDNA. After 4 days, cells were photographed under a fluorescence microscope and analyzed by flow cytometry. The upper panel shows the images of phase contrast and fluorescent microscope. The lower panel shows the result of flow cytometry. Shown are percentages of cells expressing EGFP.
(TIF)

Figure S2 Morphology of transduced TIG-3 on each ECMs in hESF9 medium. A) Upper figures: Twenty days after transduction TIG-3-derived human iPS colony were picked up and sub-cultured on each ECMs. Lower figures: Images of sub-cultured iPS colonies seeded on each ECMs with hESF9 medium for the indicated days at the left. B) Expression of ES cell marker genes in iPSCs derived from TIG-3 cultured on each ECMs with hESF9 medium at day 4. The expression of pluripotency marker genes; Nanog were weakened or disappeared when picked up and sub-cultured on collagen and gelatin. We used primers that only amplified the endogenous genes. #1: hiPSCs generated from TIG-3 on gelatin-coated dish and sub-cultured on gelatin-coated dishes with hESF9 medium at passage 2. #2: hiPSCs generated from TIG-3 on collagen-coated dish and sub-cultured on collagen-coated dishes with hESF9 medium at passage 2. #3: hiPSCs generated from TIG-3 on fibronectin-coated dish and sub-cultured on fibronectin-coated dishes with hESF9 medium at passage 2. Bars indicate 200 μm.
(TIF)

Figure S3 Transduction Efficiency of Retroviruses in Dental Pulp cells. DPCs were introduced with pMXs retroviruses containing the EGFP cDNA. After 4 days, cells were photographed under a fluorescence microscope and analyzed by flow cytometry. The upper panel shows the images of phase contrast and fluorescent microscope. The lower panel shows the result of flow cytometry. Shown are percentages of cells expressing GFP. Transfection efficiency of EGFP was 92.1% in serum-supplemented condition and 89.9% in serum-free culture condition of transfected cells. Bars indicate 200 μm.
(TIF)

Figure S4 hiPS cell generation from DPCs in serum- and feeder-free culture conditions. Images of DPCs (DP-F) plated on collagen-coated dish in RD6F medium. A) Images of DPCs (passage 2) on type I collagen-coated plate with RD6F medium. B) Transduced DPCs were cultured on fibronectin with hESF9 medium or on MEF with KSR-based conditions. After 20 days, iPS colony were picked up and sub-cultured on fibronectin. The reprogramming efficiency was 0.25% with a high success rate. C) ALP staining of iPSCs on fibronectin at 33 days after infection. Bars indicate 200 μm.
(TIF)

Figure S5 Global gene expression analysis of hiPSCs from DPCs. The gene expression of DP-hiPSCs generated in hESF9 and maintained in hESF9T is similar to that of the cells generated and maintained in conventional KSR-based condition or that of Tic (JCRB1331) maintained in conventional KSR-based condition.
(TIF)

Figure S6 karyotype of hiPSC generated in hESF9 and maintained in hESF9T defined culture. A) Growth curve of hiPSCs. Shown were averages. Growth curves for the hiPSC (DP-F-iPS-CL16) cultured under hESF9T at passage 21, 22, 23 and 24 were seeded in a 24-well plate coated with fibronectin and the cell numbers were counted every 24 h. The values are the mean± SEM (n = 4). Population doubling time: 16.6±0.843 h. B) Karyotype analysis of DP-F-iPS-CL14 cell at passage 20 maintained in hESF9T conditions. Normal diploid 46, XX karyotype.
(TIF)

Table S1 Composition of medium used for serum-free culture. The composition of the basal medium RD is described in Sato, JD et al., 1987[11]. hESF9 medium is described in Furue et al., 2008 [5].
(TIF)

Table S2 Primers used in this study listed.
(TIF)

Table S3 STR analyses of DP-derived iPSCs.
(TIF)

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Author Contributions

Conceived and designed the experiments: SY AS TO. Performed the experiments: SY YT HM. Analyzed the data: SY AS TO. Contributed reagents/materials/analysis tools: AS HT TO. Wrote the paper: SY TO.

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Reprogramming Suppresses Premature Senescence Phenotypes of Werner Syndrome Cells and Maintains Chromosomal Stability over Long-Term Culture

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Abstract

Werner syndrome (WS) is a premature aging disorder characterized by chromosomal instability and cancer predisposition. Mutations in *WRN* are responsible for the disease and cause telomere dysfunction, resulting in accelerated aging. Recent studies have revealed that cells from WS patients can be successfully reprogrammed into induced pluripotent stem cells (iPSCs). In the present study, we describe the effects of long-term culture on WS iPSCs, which acquired and maintained infinite proliferative potential for self-renewal over 2 years. After long-term cultures, WS iPSCs exhibited stable undifferentiated states and differentiation capacity, and premature upregulation of senescence-associated genes in WS cells was completely suppressed in WS iPSCs despite *WRN* deficiency. WS iPSCs also showed recapitulation of the phenotypes during differentiation. Furthermore, karyotype analysis indicated that WS iPSCs were stable, and half of the descendant clones had chromosomal profiles that were similar to those of parental cells. These unexpected properties might be achieved by induced expression of endogenous telomerase gene during reprogramming, which trigger telomerase reactivation leading to suppression of both replicative senescence and telomere dysfunction in WS cells. These findings demonstrated that reprogramming suppressed premature senescence phenotypes in WS cells and WS iPSCs could lead to chromosomal stability over the long term. WS iPSCs will provide opportunities to identify affected lineages in WS and to develop a new strategy for the treatment of WS.

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Introduction

Werner syndrome (WS) is a rare human autosomal recessive disorder characterized by early onset of aging-associated diseases, chromosomal instability, and cancer predisposition [1,2]. Fibroblasts from WS patients exhibit premature replicative senescence [3], and *WRN*, a gene responsible for the disease, encodes a RecQ-type DNA helicase [4–7], that is involved in maintenance of chromosome integrity during DNA replication, repair, and recombination [8,9]. WRN helicase is known to interact with a variety of proteins associated with DNA metabolism including proteins of replication fork progression, base excision repair, and telomere maintenance [8,9]. The dysfunction of WRN helicase causes defects in telomeric lagging-strand synthesis and telomere loss during DNA replication [10]. Further, it is also reported that telomere loss caused by a defect in WRN helicase involves chromosome end fusions that are suppressed by telomerase [11]. These observations suggest that premature senescence in WS cells reflects defects in telomeric lagging-strand synthesis followed by accelerated telomere loss during DNA replication.

Somatic cell reprogramming follows the introduction of several pluripotency genes including Oct3/4, Sox2, Klf4, c-myc, Nanog

and Lin-28 into differentiated cells such as dermal fibroblasts, blood cells, and other cell types [12–17]. During reprogramming, somatic cell-specific genes are suppressed, and embryonic stem cell (ESC)-specific pluripotency genes are induced, leading to the generation of iPSCs with undifferentiated states and pluripotency [18]. In addition, ESC-like infinite proliferative potential is directed by induction of the endogenous telomere reverse-transcriptase catalytic subunit (hTERT) gene and the reactivation of telomerase activity during reprogramming [13,18].

Recently, Cheung et al. demonstrated that cells from WS patients were successfully reprogrammed into iPSCs with restored telomere function, suggesting that the induction of hTERT during reprogramming suppresses telomere dysfunction in WS cells lacking *WRN* [19]. However, the effects of long-term culture on the undifferentiated states, self-renewal abilities, and differentiation potentials of WS iPSCs remain unknown. In a previous study, progressive telomere shortening and loss of self-renewal ability were observed in iPSCs from dyskeratosis congenital patient cells in a long-term culture [20], warranting the evaluation of the properties of patient cell-derived iPSCs with telomere dysfunctions over the long term.

In this study, we cultured WS iPSCs with self-renewal capacity and infinite proliferative potential for over 2 years and reported similar properties to those of normal iPSCs including undifferentiated states and differentiation ability. Notably, WS iPSCs maintained stable karyotypes and their potential to recapitulate premature senescence phenotypes during differentiation over the long term. The present data demonstrate that reprogramming suppresses premature senescence phenotypes in WS cells by reversing the aging process and restoring telomere maintenance over the long term.

Materials and Methods

Cell lines

WS patients were diagnosed on the basis of clinical symptoms and *WRN* gene mutations. A0031 WS patient fibroblasts from a 37-year-old male were obtained from Goto Collection of RIKEN Bioresource Center (https://www.brc.riken.jp/lab/cell/english/index_gmc.shtml) [21], and WSCU01 patient fibroblasts were isolated from a 63-year-old Japanese male who was diagnosed at Chiba University. Both fibroblast isolates had type 4/6 heterozygous mutations. TIG-3 human fetal lung-derived fibroblast cells and WS patient-derived fibroblasts were used to generate iPSC lines. PLAT-A cells (kindly provided from Dr. Toshio Kitamura) were used to produce retroviruses [22]. SNL 76/7 (SNL) cells (DS pharma biomedical) were used as feeder layers for reprogramming of fibroblasts and maintenance of iPSCs. The human fibroblast-derived iPSC line iPS-TIG114-4f1 was obtained from the National Institute of Biomedical Innovation [23].

PLAT-A cells, TIG-3 fibroblasts, TIG-114 fibroblasts from the 36-year-old male, and SNL cells were grown in the Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Hyclone) and antibiotics (Invitrogen). WS fibroblasts were maintained on collagen-coated dishes (Nitta Gelatin), SNL cells were maintained on gelatin-coated dishes (Nitta Gelatin), and iPSCs were maintained in the ES medium comprising Knockout DMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (Invitrogen), glutamine, non-essential amino acids, β-mercaptoethanol and 4-ng/ml basic FGF. All cells were maintained at 37°C under 5% CO_2 atmosphere.

Generation of iPSCs

The generation of iPSCs was performed as described previously [13]. Briefly, 2×10^6 PLAT-A cells were plated in T25 flasks (Biocoat, BD Falcon), and were transfected with 4 μg pMXs-OCT3/4, SOX2, KLF-4, and c-myc (Addgene) 1 day later. Twenty-four hours after transfection, the culture medium was replaced with a fresh medium and cells were incubated for 24 h prior to harvest of viral supernatants. Viral supernatants containing Yamanaka factors were combined in even ratios.

For reprogramming experiments, 3×10^5 fibroblasts were seeded on 60-mm dishes and were infected with viral supernatants containing Yamanaka factors in the presence of 8 μg/ml polybrene 1 day later. Four days after infection, fibroblasts were harvested, and 1×10^5 cells were reseeded onto mitomycin C-inactivated SNL feeder layers on 100-mm dishes. Twenty-four hours after reseeding, the medium was replaced with the ES medium, and cultures were maintained by replacing the medium every other day. Approximately 30 days after retroviral transduction, emerging iPSC colonies with ESC colony-like flat and round shapes were picked up by mechanical dissection and were plated onto fresh feeder layers on 4-well plates (Thermo Scientific Nunc). Subsequently, iPSC lines were established by successive passages onto fresh feeder layers with split ratios between 1:3 and 1:5 using dispase (Roche Applied Science).

Alkaline phosphatase activity

Undifferentiated states of emerging colonies were examined using alkaline phosphatase staining. After formalin fixation, colonies were stained with reaction buffer containing 100 mM Tris-Cl (pH 8.5), 0.25 mg/ml Naphthol AS-BI phosphate (Sigma) and 0.25 mg/ml fast red violet LB salt (Sigma).

Embryoid body formation and in vitro differentiation

Clumps of iPSCs were transferred to non-adherent polystyrene dishes containing the ES medium without basic FGF to form embryoid bodies (EBs). The medium was replaced every other day. After 8 days of floating culture, EBs were transferred onto gelatin-coated plates and were maintained in DMEM supplemented with 10% FBS, β-mercaptoethanol, and antibiotics for another 8 days. For detection of senescence phenotypes during differentiation, Y-27632-treated iPSCs were dissociated into single cell suspensions with Accutase (Innovative Cell Technologies) and 1×10^4 cells were transferred into 96-well V-shaped bottom plates (Greiner Bio-One) to form evenly sized EBs. After 12 days of EB formation in the ES medium without basic FGF, EBs were cultured in DMEM supplemented with 10% FBS, β-mercaptoethanol, and antibiotics.

Teratoma formation

After harvest, 1×10^6 iPSCs were injected into the testes of a severe combined immunodeficient (SCID) mice (CREA, Japan). Three months after injection, tumors were dissected and were fixed using 4% paraformaldehyde. Subsequently, dissected tumor tissues were embedded in paraffin and were sliced and stained with hematoxylin and eosin.

Western blot

Whole cell lysates were prepared in SDS sample buffer and subjected to electrophoresis on 8% SDS-polyacrylamide gels, and separated proteins were transferred onto PVDF membranes (FluoroTrans W, Pall Corporation). Membranes were blocked with TBS-T containing 5% skim milk and were then incubated with anti-WRN (1:500, 4H12, Abcom) or anti-β-actin (1:30000,

Ac-15, Sigma) monoclonal antibodies for 3 h at room temperature. Membranes were then washed with TBS-T and were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5000, NA931V, GE) for 1 h at room temperature. Chemiluminescence reactions were performed using Western Lightning Plus-ECL (PerkinElmer) and were detected using exposure of x-ray films.

Mutation analysis

The DNA fragments mut.4 (c.3139-1G>C) and mut.6 (c.1105C>T) were amplified with the primer pairs WS_mut4_U, GGTAAACGGTGTAGGAGTCTGC and WS_mut4_L, CTTGTGAGAGGCCTATAAACTGG, and WS_mut6_U, TGAAGATTCAACTACTGGGGGAGTAC and WS_ mut6_L, ACGGGAATAAAGTCTGCCAGAACC, respectively, using genomic DNA as a template. Mutations were analyzed by direct sequencing using these PCR primers.

Short tandem repeat (STR) analysis

Genomic DNAs were purified from WS fibroblasts and their derivative iPSC clones using phenol/chloroform extraction and were then used for analysis using a Cell ID System (Promega). PCR products were analyzed using an Applied Biosystems 3130xl Genetic Analyzer and GeneMapper software.

Gene expression profiling

Cy3-labeled total RNAs were hybridized onto Human Genome U133 Plus 2.0 Arrays (GeneChip, Affymetrix). Arrays were then scanned using the GeneChip Scanner 3000 7G (Affymetrix), and the obtained data were analyzed by Affymetrix Expression Console Software. The microarray dataset has been deposited in the NCBI Gene Expression Omnibus database under Series Accession GSE62114.

Measurement of telomere length

Genomic DNAs were digested using *Hinf*I restriction enzyme (TakaraBio), and were subjected to electrophoresis on 1% agarose gels. Size-fractionated DNAs were transferred onto Hybond-N+ membranes (GE). Membranes were hybridized with a digoxigenin-labeled $(CCCTAA)_4$ probe, and TRFs were detected using TeloTAGGG Telomere Length Assays (Roche Applied Science) according to the manufacturer's instructions.

RT-PCR and real-time qRT-PCR analysis of mRNA expression

Total RNA was prepared using RNeasy spin columns (Qiagen) according to the manufacturer's instructions. RT-PCR was performed with 0.1 μg of total RNA using SuperScript One-Step RT-PCR (Invitrogen). Semi-quantitative analysis was performed after converting total RNA into cDNA using a High Capacity RNA-to-cDNA kit (Life Technologies), and real-time PCR was performed using a Rotor-Gene SYBR Green PCR kit (Qiagen). Relative gene expression levels were analyzed according to the ΔΔCt method using Ct values of GAPDH mRNA as an internal control. Primer sequences are listed in Tables S1 and S2.

Immunofluorescence cytochemistry

Following fixation of iPSCs and differentiated cells with 4% paraformaldehyde for 15 min at 4°C, cells were permeabilized with 0.1% Triton X-100, washed with PBS containing 2% BSA, and incubated with primary antibodies diluted in PBS containing 2% BSA.

Primary antibodies against Nanog (1:200, Cell Signaling, D73G4), SSEA-4 (1:200, Cell Signaling, MC813), Tra-1-60 (1:200, Cell Signaling, #4746), Tra-1-81 (1:200, Cell Signaling, #4745), βIII-tubulin (1:200, Millipore, TU-20), desmin (1:200, Neomarkers, RB-9014-P0), vimentin (1:200, Santa Cruz, V9), and α-fetoprotein (1:500, Sigma, HPA010607) were detected using the secondary antibodies Alexa 488-conjugated anti-goat IgG (1:500, Invitrogen, A11055), Alexa 488-conjugated anti-mouse IgG (1:500, Invitrogen, A11001), Alexa 488-conjugated anti-mouse IgM (1:500, Invitrogen, A21042), and Alexa 488-conjugated anti-rabbit IgG (1:500, Invitrogen, A11013). Cell nuclei were stained with 1- μg/ml 4',6-diamidino-2-phenylindole (DAPI).

Karyotype analysis

After culturing iPSCs in the ES medium containing 100-ng/ml colcemid for 5 h at 37°C, cells were harvested using trypsin and were treated with 0.075 M KCl for 15 min at 37°C. Cells were then fixed in Carnoy's fluid, and chromosome slides were prepared. G-banding analysis was conducted using a previously described method [24].

M-FISH was performed with the Multi-color probe kit "24XCyte" (MetaSystems, Altlussheim, Germany) according to the manufacturer's protocol with slight modifications. Briefly, probes were denatured at 75°C for 5 min and were hybridized to metaphase spreads, which were denatured in 0.07 N NaOH at room temperature for 1 min. Slides were then incubated at 37°C for 2 nights and were then washed in 0.4× SSC at 72°C for 2 min, in 2× SSC containing 0.05% Tween 20 at room temperature for 30 s, and in 2× SSC at room temperature for 1 min, and the mounting medium (DAPI, 125 ng/ml) and a cover slip were applied. Acquisition and analysis of M-FISH images were performed using a CytoVision ChromoFluor System (Applied Imaging, Newcastle upon Tyne, UK).

Transduction of hTERT gene

PT67 retrovirus packaging cells (Takara Bio USA, Madison, WI, USA) were transfected with pMSCV-hTERT-puro using GenePorter II according to the manufacturer's protocol. After 24 h, the culture medium was replaced, cells were incubated for a further 24 h period, and viral supernatants were harvested, A0031 and WSCU01 WS fibroblasts were infected with viral supernatant in the presence of 8 μg/ml polybrene. Confluent infected cells were then split into 2 new dishes, and puromycin selection of infected cells was initiated at the following passage. Confluent infected cells were then passaged in 4-fold dilutions, leading to an increase in 2 population doubling levels for each passage.

SA-β-gal assay

SA-β-gal staining was performed as described by Debacq-Chainiaux et al. [25].

Ethical statement

This study was approved by the Ethics Review Board of the Graduate School of Medicine, Chiba University and was conducted in accordance with the Declaration of Helsinki. Written informed consents were obtained from patients prior to tissue harvesting and iPSC generation, and patients were entitled to the protection of confidential information. Genome/gene analyses performed in this study were approved by the Ethics Committee for Human Genome/Gene Analysis Research at Hiroshima University. All animal experiments were performed in strict compliance with the protocol approved by the Institutional Animal Care and Use Committee of Tottori University (13-Y-