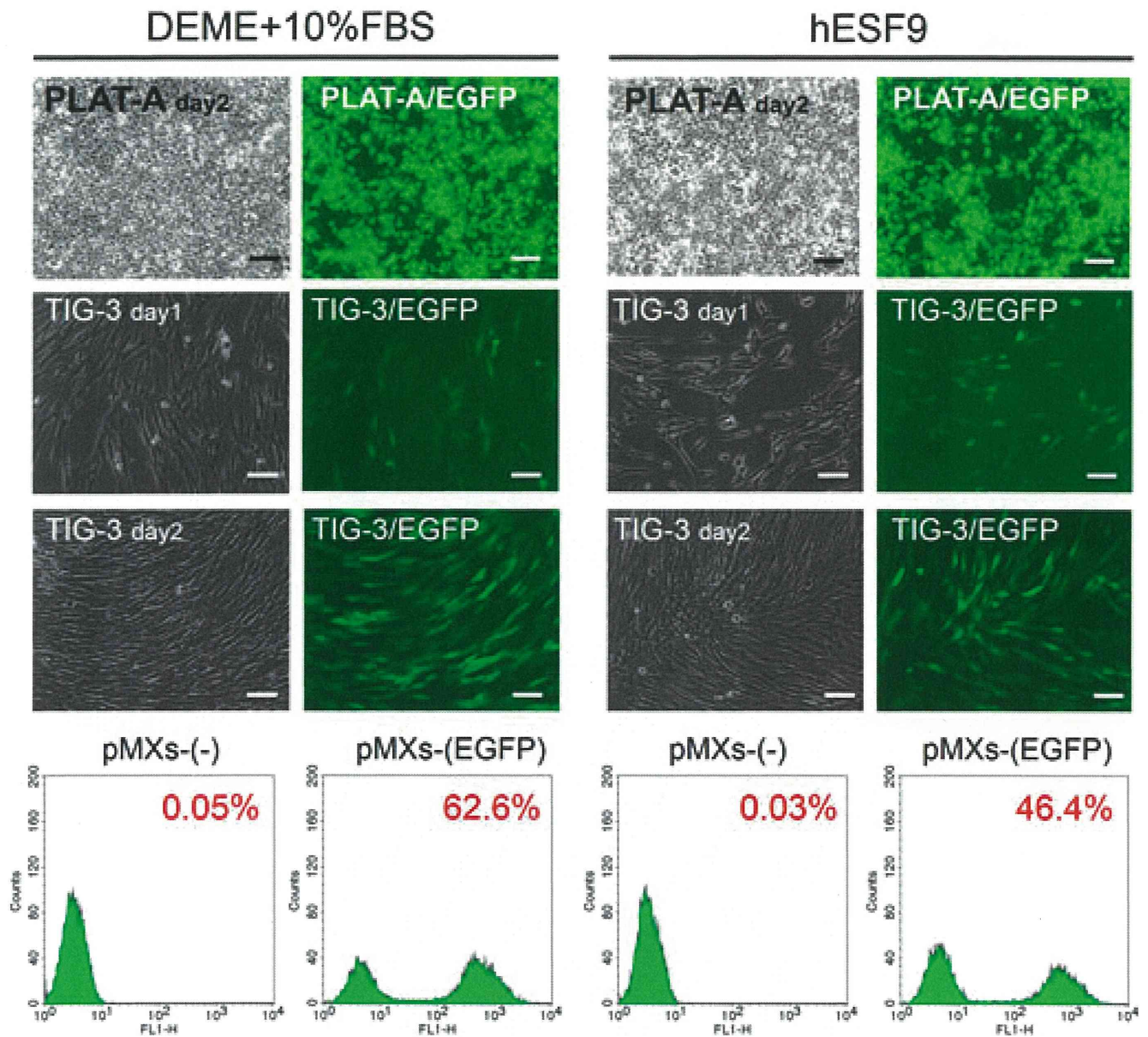


**Figure 1. Morphology of transduced TIG-3 on each ECMs in hESF9 medium.** A) Transduced TIG-3 cells were cultured on each ECMs with hESF9 medium or on MEF with KSR-based conditions. After 20 days, iPSC colony were picked up and sub-cultured on each ECMs. B) Images of sub-cultured iPSC colonies seeded after 2 days on each ECMs with hESF9 medium. C) ALP staining of iPSCs on gelatin, collagen, and fibronectin (infect after 36 days). Bars indicate 200  $\mu\text{m}$ . doi:10.1371/journal.pone.0087151.g001

and *c-Myc* in DMEM supplemented with 10% FBS. We produced retroviruses using PLAT-A cell line in serum-supplemented conditions as described in the manufacturer's protocol. Then we transduced four factors (*Oct3/4*, *Sox2*, *Klf4* and *c-Myc*) into TIG-3 (normal fibroblast cell line derived from fetal lung) [7] in DMEM supplemented with 10% FBS. At the same time, we transfected the EGFP-expressing retroviral vector and the empty control vector into TIG-3 with a constant amount of total DNA. After 4 days, cells were photographed under a fluorescence microscope and analyzed by flow cytometry. The transduction efficiency of the four factors was 26.6% as measured by EGFP expression (Fig. S1). After 4 days four factor-transduced cells were trypsinized and

plated on 0.1% gelatin, or type I collagen (0.3 mg/ml) or fibronectin ( $2 \mu\text{g}/\text{cm}^2$ ) in hESF9 medium. For comparison, we used KSR-based medium and mitomycin C-treated MEF [8-10] feeder cells (KSR-based conditions) [1,2]. After 13 days of culture, we observed human ES-like colonies, characterized by large nuclei and little cytoplasm (Fig. 1-A). After 20 days colonies were passaged and maintained in hESF9 medium on substrates coated with individual ECMs (Fig. S2-A). After 36 days of culture, alkaline phosphatase (ALP)-positive colonies were counted, and relatively high iPSC induction efficiency was observed. However, although type I collagen supported hiPSC cell generation more effectively than the other two ECMs, collagen and gelatin did not



**Figure 2. Transduction efficiency of retroviruses in serum-free hESF9 medium and serum-supplemented medium in TIG-3.** TIG-3 was introduced with pMXs retroviruses containing the EGFP cDNA. After 3 days, cells were photographed under a fluorescence microscope and analyzed by flow cytometry. The left panel shows the images of phase contrast, fluorescent microscope and the results of flow cytometry of the cells cultured in serum-supplemented condition (DMEM+10%FBS). The right panel shows the cells in serum-free culture conditions (hESF9). Transfection efficiency of EGFP was 62.6% in serum-supplemented condition and 46.4% in serum-free culture condition. Bars indicate 200  $\mu$ m. doi:10.1371/journal.pone.0087151.g002

support pluripotency of hiPSCs after further culture of these colonies (Fig.1-B). This inability to maintain pluripotency of hiPSCs was confirmed by ALP activity (Fig.1-C) and RT-PCR analysis ( Fig. S2-B).

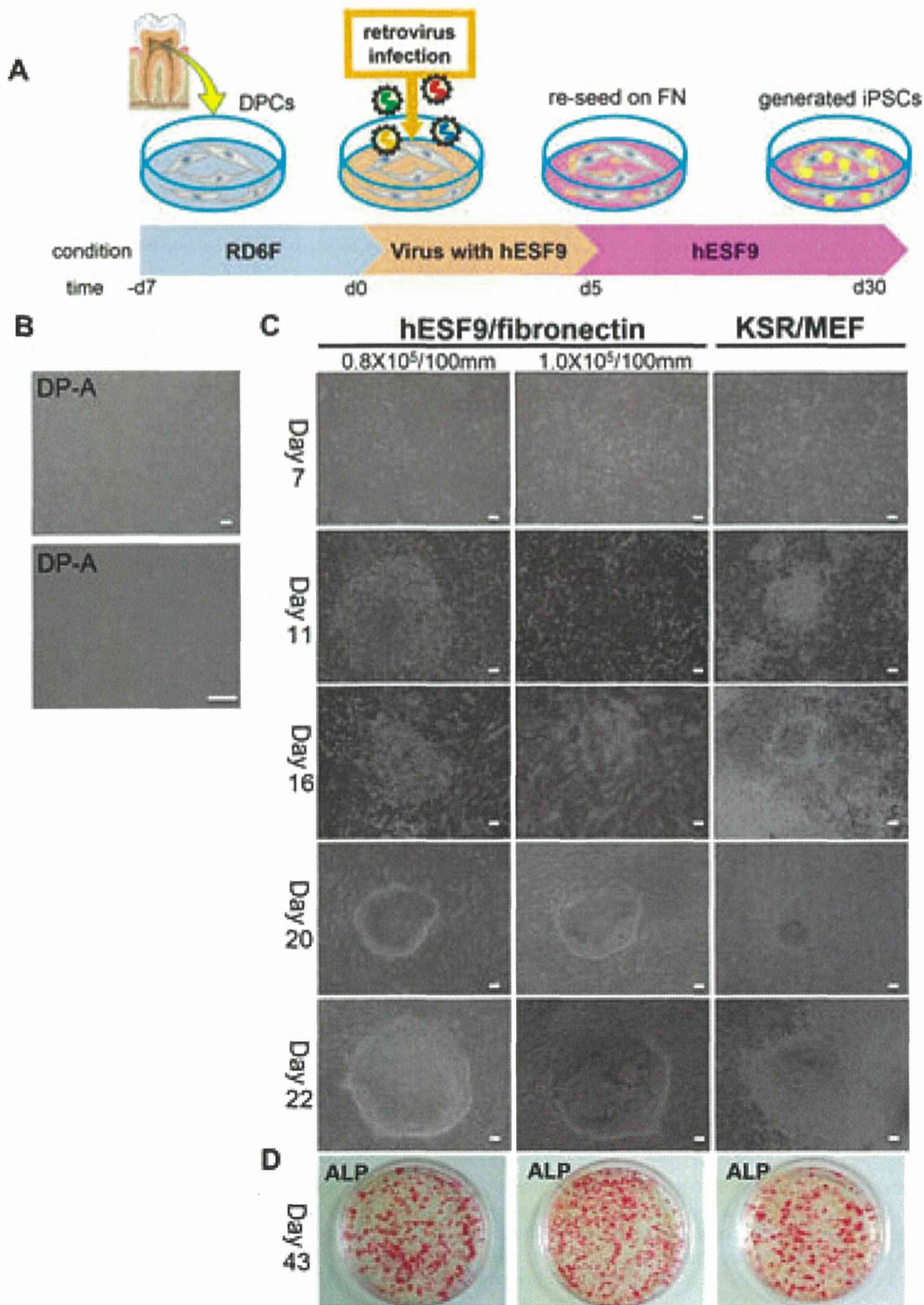
#### Retrovirus production using PLAT-A cell in serum-free conditions and analysis of transduction efficiency

We examined whether the serum-free culture condition could produce relatively high titer viral supernatants. Retroviral supernatants of pMXs(-empty) and pMXs(-EGFP) were produced in PLAT-A packaging cells in serum-free hESF9 medium or serum supplemented medium. The collected virus supernatants were used for infection. After 3 days, transduction efficiency was

measured by FACS analysis of EGFP expression. Transduction efficiency in serum-free hESF9 medium was 46.4%, whereas it was 62.6% in serum-supplemented medium (Fig. 2, Fig. S3). While the data showed a slightly lower titer in hESF9, the viral titer was sufficient to reprogram dental pulp cells (see below).

#### hiPS cell generation from adult DPCs in serum- and feeder-free culture condition

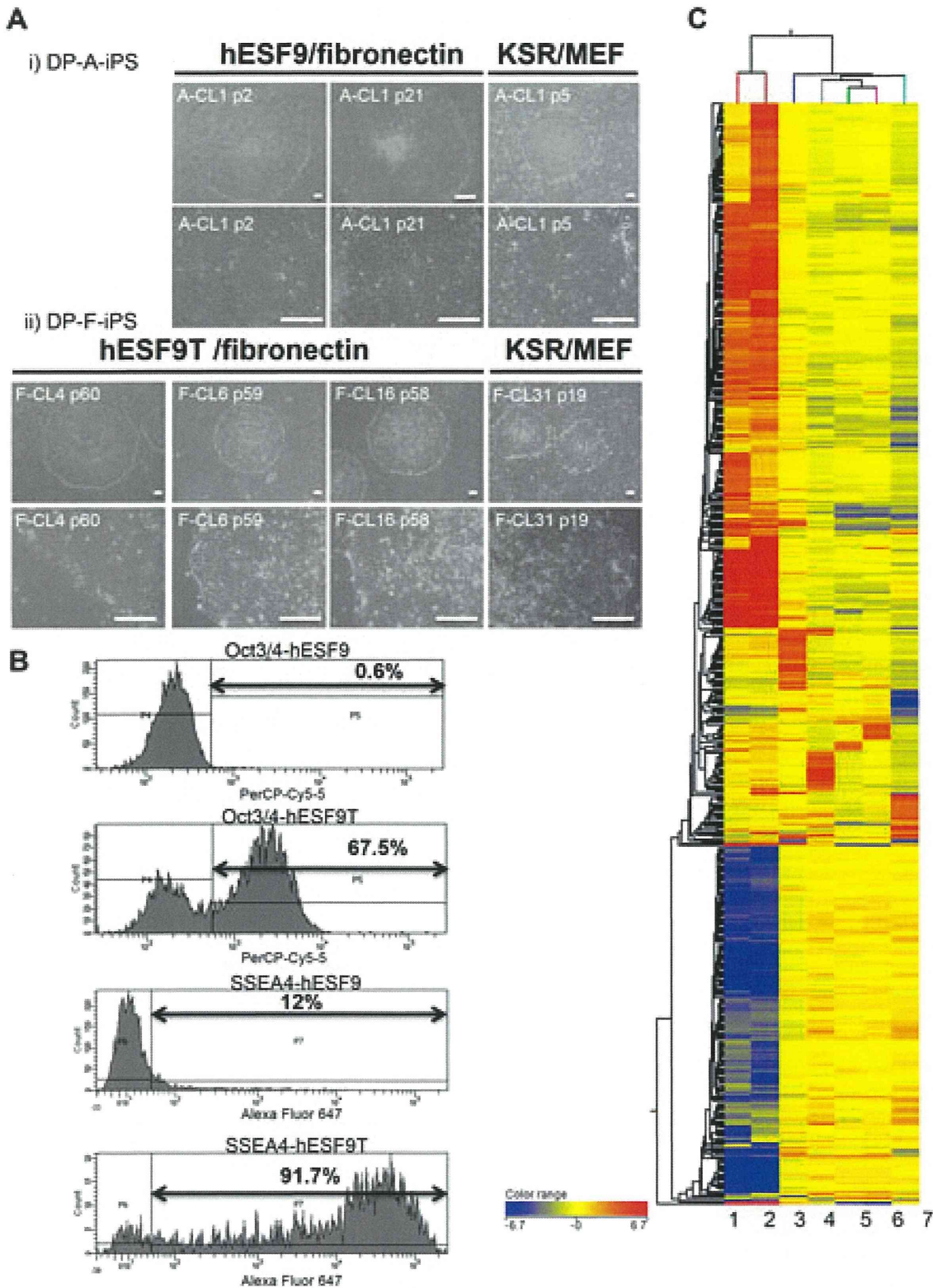
We obtained dental pulp cells (DPCs) from healthy patients (Fig. 3, Fig. S4). These DPCs were reprogrammed to hiPSCs after transduction with PLAT-A virus in completely serum-free culture conditions (Fig. 3). The transduced DPCs were trypsinized and plated on fibronectin-coated dish in hESF9 medium. Human iPSC



**Figure 3. hiPSC generation from DPCs in serum- and feeder-free culture conditions.** A) Time schedule of hiPSC generation. Day 7~0: DPCs were cultured in RD6F serum-free medium on type I collagen-coated dish. Day 0~4: Retroviral transduction (*Oct3/4*, *Sox2*, *KLF-4*, *c-Myc*) with hESF9 medium. Day 5: re-seeding on fibronectin-coated plate with hESF9 medium. Day 6~30: replace medium every other day. B) Images of DPCs (DP-A: passage 4) on type I collagen-coated plate with RD6F medium. C) 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 0.23–0.38% with a high success rate. D) ALP staining of iPSCs on fibronectin, 43 days after infection. Bars indicate 200  $\mu$ m. doi:10.1371/journal.pone.0087151.g003

cell colonies intermingled with partially reprogrammed colonies appeared at 15 days after transfection. After 20 days, individual iPS cell colonies were selected and subsequently passaged and maintained in hESF9 medium with TGF- $\beta$ 1 (2 ng/ml) (hESF9T) in dishes coated with fibronectin (Fig. 3-C). After 33 days in culture, ALP-positive colonies were counted; the efficiency of

reprogramming was 0.23–0.39%. By contrast, iPS cell colonies did not emerge at all when cells were not re-plated on fibronectin after viral infection (data not shown). When TGF- $\beta$ 1 was present throughout the reprogramming procedure using hESF9T medium, the number of iPS colonies were 10-fold lower than that in



**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-iPSC and DP-F-iPSC). i) DP-A-iPSC-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-iPSC-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  $\mu$ m. B) Flow cytometry analysis of Oct3/4 and SSEA-4 expression in hiPSCs generated and maintained in hESF9 medium supplemented with TGF- $\beta$ 1 (2 ng/ml) (hESF9T) or without TGF- $\beta$ 1 (hESF9) (DP-F-iPSC-CL8 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-iPSC, DP-F-iPSC) and hiPSCs (Tic, DP-F-iPSC). 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- $\beta$ 1 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- $\beta$ 1) 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- $\beta$ 1. By contrast, hiPS colonies remained undifferentiated in hESF9T medium.

Increasing the dose of TGF- $\beta$ 1 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- $\beta$ 1 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- $\beta$ 1. 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- $\beta$ 1 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- $\beta$ 1 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- $\beta$  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  $\beta$ III-tubulin (ectoderm markers),  $\alpha$ -smooth muscle actin (SMA) (mesoderm marker), and  $\alpha$ -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).

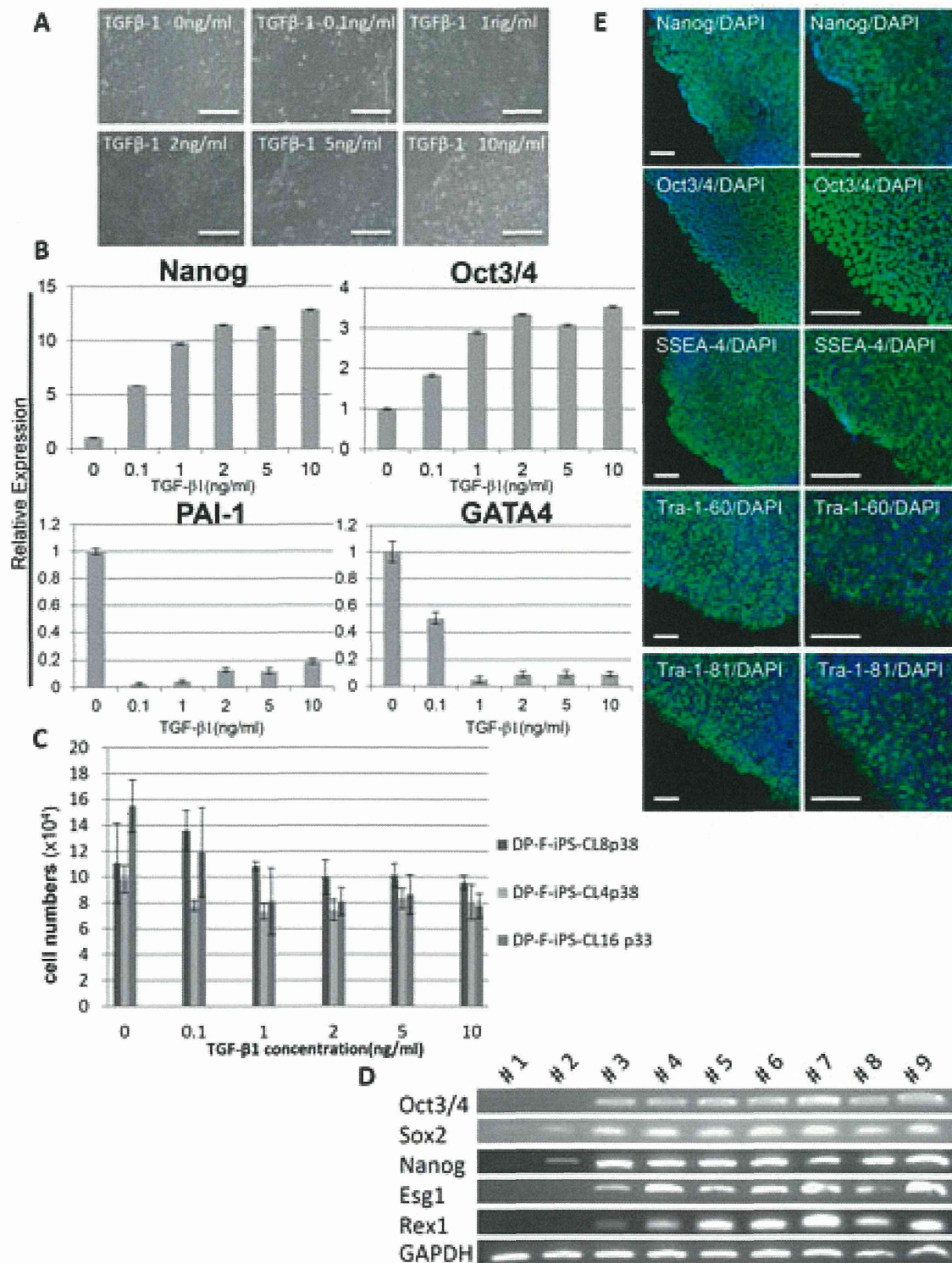
### Cell growth and karyotype analysis of human iPS cells generated and maintained in define culture conditions

Growth curves were calculated from the split ratios at each passage. The population doubling time was  $16.6 \pm 0.8$  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- $\beta$ 1 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-1 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  $1 \times 10^4$  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  $\pm$  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  $\mu$ m.  
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, iPSC 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  $\alpha 5 \beta 1$  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  $\alpha V \beta 5$  integrin [4,16,17]. Another group has reported that the adhesion of hESCs and hiPSCs to laminin-511 is maintained via integrin  $\alpha 6 \beta 1$ , 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 iPSC 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.

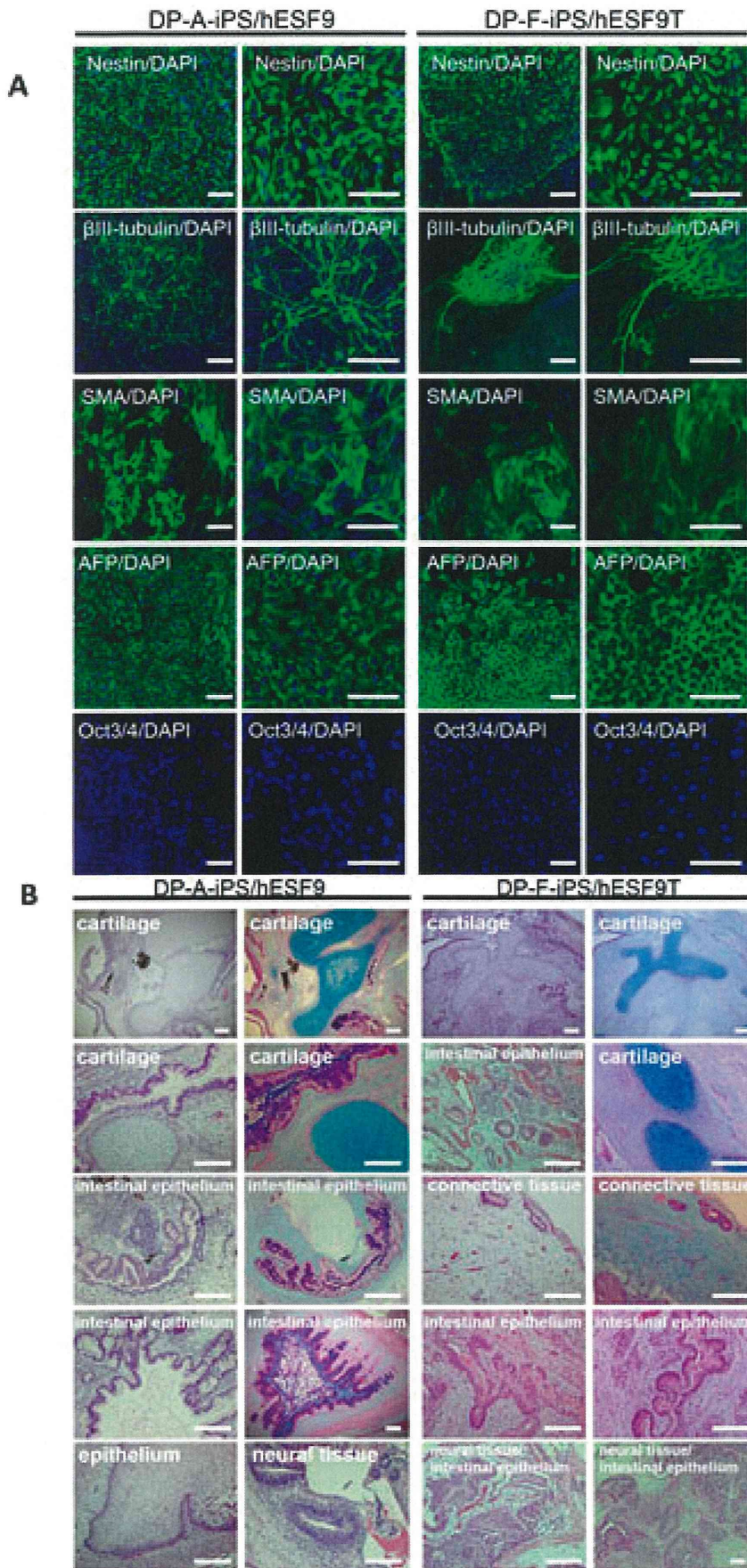
It is believed that the signaling requirements of human iPSC cell generation and maintenance are different. In this report we have shown that hESF9 medium containing FGF-2 and heparin enhanced the derivation of iPSCs. However, the same conditions did not maintain the pluripotency of these cells. Moreover, TGF- $\beta 1$  had an inhibitory effect when present at later stages of reprogramming. Therefore, we investigated whether TGF- $\beta 1$  plays a role in maintaining hiPSC cell pluripotency in fully defined culture conditions. TGF- $\beta$  superfamily members participate in cell fate decisions in ESCs. However, the role of TGF- $\beta$  in regulating the cell cycle of ESCs is poorly understood. TGF- $\beta$ /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- $\beta$  has been reported to repress activity of the telomerase reverse transcriptase [29]. The role of most members of the TGF- $\beta$  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- $\beta 1$  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- $\beta 1$  suppressed the growth rate of hiPSCs cultured under defined conditions. As with any pleiotropic factor, the effects of TGF- $\beta$  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- $\beta 1$  to maintain pluripotency. At the same time, when TGF- $\beta 1$  was present throughout the reprogramming procedure, DPCs tended to overgrow and inhibit or obscure the emergence of iPSC colonies. Furthermore, activated p53 and TGF- $\beta 1$  pathways act as roadblocks for iPSC formation from DPCs [4,24,29]. Decreasing the growth rate of DPCs led to the emergence of iPSC cells without DPCs overgrowth. Regulation of TGF- $\beta$  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 iPSC generation efficiency. Consequently, the cells are available for iPSC generation by other methods using plasmids [33–35], chemicals and proteins [36,37], and microRNAs [38], aiming for the clinical use of the iPSC 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





**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,  $\beta$ III-tubulin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and  $\alpha$ -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<sup>®</sup> 594-conjugated secondary antibodies (red). Nucleuses were stained with DAPI. (passage 25). Bar indicates 100  $\mu$ m. B) Teratomas were generated in SCID mice (CB17/1cr-Prkdc<sup>scid</sup>/Crj) 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 iPSC 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  $\mu$ m. doi:10.1371/journal.pone.0087151.g006

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- $\beta$ 1. As a result, we found TGF- $\beta$ 1 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 iPSC colony were picked up and sub-cultured on each ECMs. Lower figures: Images of sub-cultured iPSC 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  $\mu$ m. (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  $\mu$ m. (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, iPSC 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  $\mu$ m. (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  $\pm$  SEM (n=4). Population doubling time: 16.6 $\pm$ 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<sup>[11]</sup>. hESF9 medium is described in Furue et al., 2008<sup>[5]</sup>. (TIF)

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

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

## Acknowledgments

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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.