Table (Continued)

Small molecules	*C*[a]	*Host animal*	*Function*	*TFs to be replaced or function*	*Reference*
DZNep	0.05–0.1 μM	Mouse	Epigenetic modulators	Promotion of reprogramming	17
DNP	1 μM	Human	Oxidative phosphorylation uncoupler	Promotion of reprogramming	43
TTNPB	1 μM	Mouse	Retinoic acid receptor ligand	Promotion of reprogramming	17
Oxysterol	0.5–1 μM	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74
Purmorphamine	0.5–1 μM	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74
Shh	500 ng/ml	Mouse	Sonic hedgehog signaling	Sox2, Klf4, and C-Myc	74

Abbreviations: BayK, Bay K8644; BIX, BIX-01294; CHIR, CHIR99021; CK1, Casein kinase 1; DNMT, DNA methyltransferase; DNP, 2,4-dinitrophenol; DZNep, 3-deazaneplanocin; FSK, forkolin; HDAC, histon deacetylase; G9a HMTase, G9a histone methyltransferase; IP3K, inositol triphosphate 3-kinase; PDK1, 3′-phosphoinositide-dependent kinase 1; SAHA, suberoylanilide hydroxamic acid; TF, transcription factor; TSA, trichostain A; VPA, valproic acid; 2-Me-5HT, 2-methyl-5-hydroxytryptamine; 5-aza-CR, AZA, 5-azacytidine; 8-Br-cAMP, 8-Bromoadenosine 3′′5′′-cyclic monophosphate.
[a]C; Concentration used typical reprogramming experiments.

cell generation over a range of concentrations, such as 0.5–1 mM.[79,80] The effect of sodium butyrate on reprogramming seems to be mediated by *c-Myc* and works during an early stage of reprogramming. In addition, sodium butyrate enhances the percentage of fully reprogrammed iPSCs by reducing the number of incomplete or partially reprogrammed cells.[79] Genome-wide gene expression analysis suggests that the upregulation of several pluripotent genes in sodium butyrate-treated MEFs during reprogramming is not through the suppression of the p53-p21 pathway, even though reprogramming efficiency is also known to be increased by the suppression of the p53-p21 pathway.[80]

The anti-psychotic drug lithium chloride (LiCl) can accelerate the generation of mouse and human iPSCs.[70] The effect of LiCl on promoting reprogramming is partially dependent on its role as a GSK-3β inhibitor. However, LiCl also upregulates *Nanog*, which has not been observed after the treatment of somatic cells with other GSK-3β inhibitors. Furthermore, LiCl exerts its effects by promoting epigenetic modifications via the downregulation of LSD1, an H3K4-specific HDAC gene.[70]

PS48, an activator of 3-phosphoinositide-dependent protein kinase 1, can also accelerate the reprogramming efficiency into hiPSCs.[40] PS48 activates the phosphatidylinositol 3-kinase/Akt pathway and significantly induces the expression of glycolytic genes, which results in the facilitation of metabolic reprogramming from the mitochondrial oxidation process used mainly by adult somatic cells into the glycolysis process used in PSCs.[40,43] Owing to its lower oxidative stress, glycolytic metabolism is favorable over mitochondrial respiration in PSCs during cell proliferation and cell-cycle transition. The hypoxic condition and its effector gene, hypoxia-inducible factor 1α, which is extensively linked to promoting glycolytic metabolism, can also enhance the reprogramming efficiencies of both mouse and human cells.[40,81]

Several other small molecules, such as A83-01 (TGF-β inhibitor),[43,70] 3-deazaneplanocin (epigenetic modulator),[17] 2,4-dinitrophenol (DNP, oxidative phosphorylation uncoupler),[43] RSC133 (DNMT inhibitor),[82] rapamycin (mTOR inhibitor),[83] compound B6 (AKT-mediated inhibitor of GSK3-β),[84] compound B8 (inositol triphosphate 3-kinase inhibitor),[84] compound B10 (P38 kinase inhibitor),[84] and vitamin C,[70] can facilitate the reprogramming efficiency of somatic cells into mouse and/or human iPSCs (Table 2).

SMALL MOLECULES CAN REPLACE SEVERAL TFS DURING MOUSE iPSC REPROGRAMMING

From the available knowledge of small molecules that can substitute for the essential TFs *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, and promote reprogramming efficiency, several researchers tried to generate iPSCs by reducing the numbers of the TFs transduced into mammalian somatic cells. Figure 4 shows the schematic representation of some of the processes developed for miPSC generation from MEFs by reducing the numbers of TFs with and without small molecules. Table 3 summarizes the research for mouse somatic cell reprogramming into miPSCs by transduction with and without TFs and with the aid of small molecules.

NSCs and keratinocytes are known to endogenously express *Sox2*, *Klf4*, and/or *c-Myc* to some extent. Some examples of the gene expression levels of *Oct4*, *Sox2*, and *Nanog* in human fibroblasts, keratinocytes, CD133$^+$ cells from cord blood, and NSCs are described in Figure 5.[13,85,86] Several researchers tried to use endogenously expressing *Sox2*, *Klf4*, and/or *c-Myc* and to generate iPSCs with fewer numbers of pluripotent TFs. Kim *et al.* succeeded in preparing miPSCs from mouse NSCs using two exogenous TFs (*Oct4* and *Klf4* or *Oct4* and *c-Myc*).[87] They did not use small molecules to enhance reprogramming efficiency. Shi *et al.* also generated iPSCs from mouse NSCs using the G9a HMTase inhibitor BIX-01294 (BIX) in conjunction with the transduction of *Oct4* and *Klf4*, but without the use of oncogenic *c-Myc*.[67] BIX induced *Oct4* overexpression and facilitated the depression of *Oct4*, which promoted the iPSC reprogramming efficiency.[20,67]

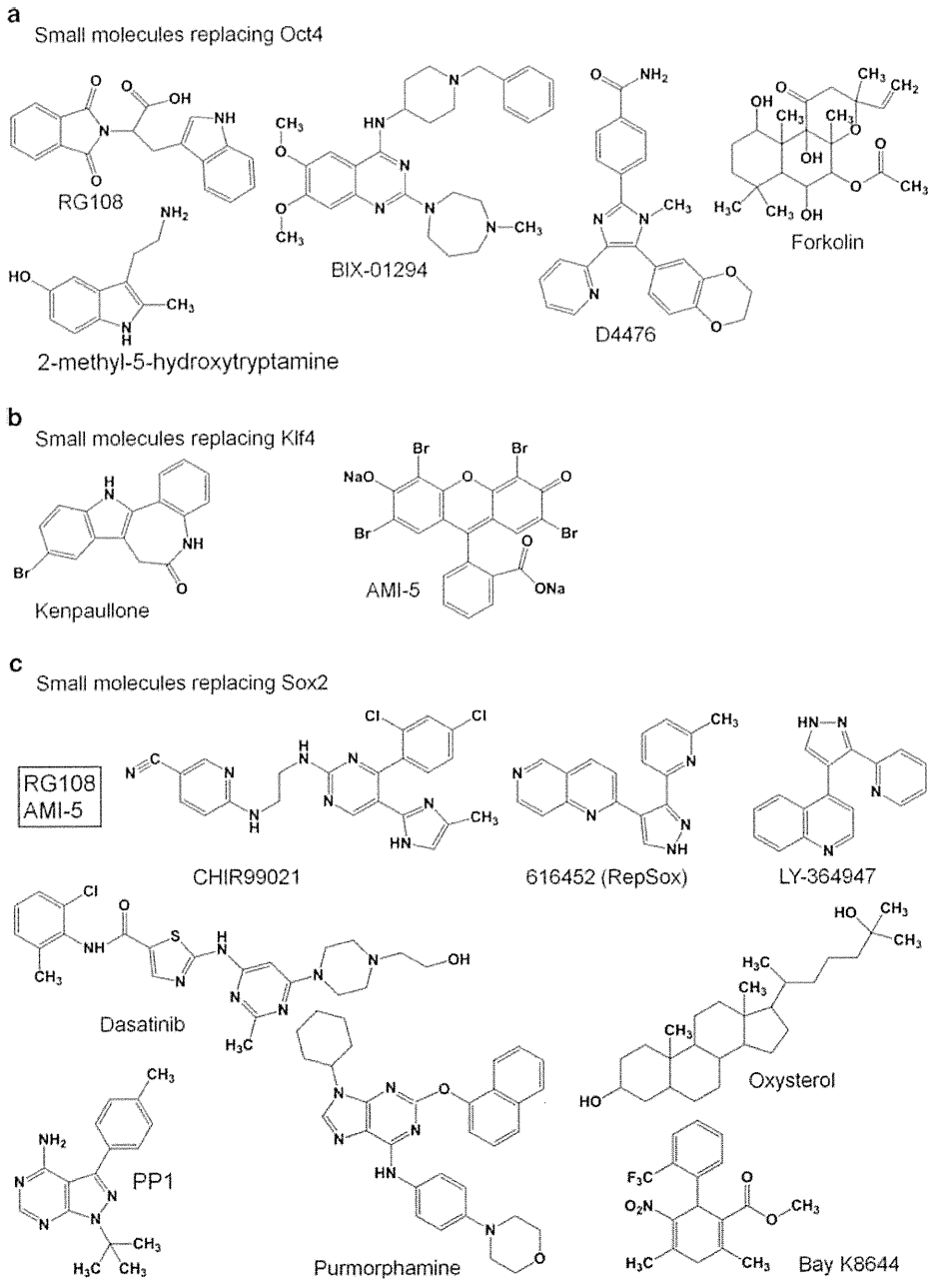

Figure 2 Representative schematic structures of small molecules that can replace *Oct4*, *Klf4*, and *Sox2* transduction in reprogramming to generate iPSCs. (**a**) Small molecules replacing *Oct4*; (**b**) small molecules replacing *Klf4*; (**c**) small molecules replacing *Sox2*.

Silva *et al.* also prepared miPSCs from mouse brain-derived NSCs using *Oct4* and *Klf4* transduction.[88] The cells rapidly adopted an undifferentiated morphology after a single round of transduction. However, these cells were partially reprogrammed and did not stably express endogenous *Oct4* or *Nanog*, and they were unable to colonize chimeras.[88] Therefore, the authors cultured the partially reprogrammed cells in medium containing a dual inhibitor of mitogen-activated protein kinase signaling and GSK-3, 2i medium, along with leukemia inhibitory factor, which is a self-renewal cytokine for mouse PSCs.[88] The cells cultured in 2i medium with leukemia inhibitory factor induced the stable upregulation of Oct4 and Nanog, transgene silencing, and competence for somatic and germline chimerism, which demonstrated that the cells were completely reprogrammed into miPSCs.[88] 2i medium is a powerful tool for generating miPSCs and

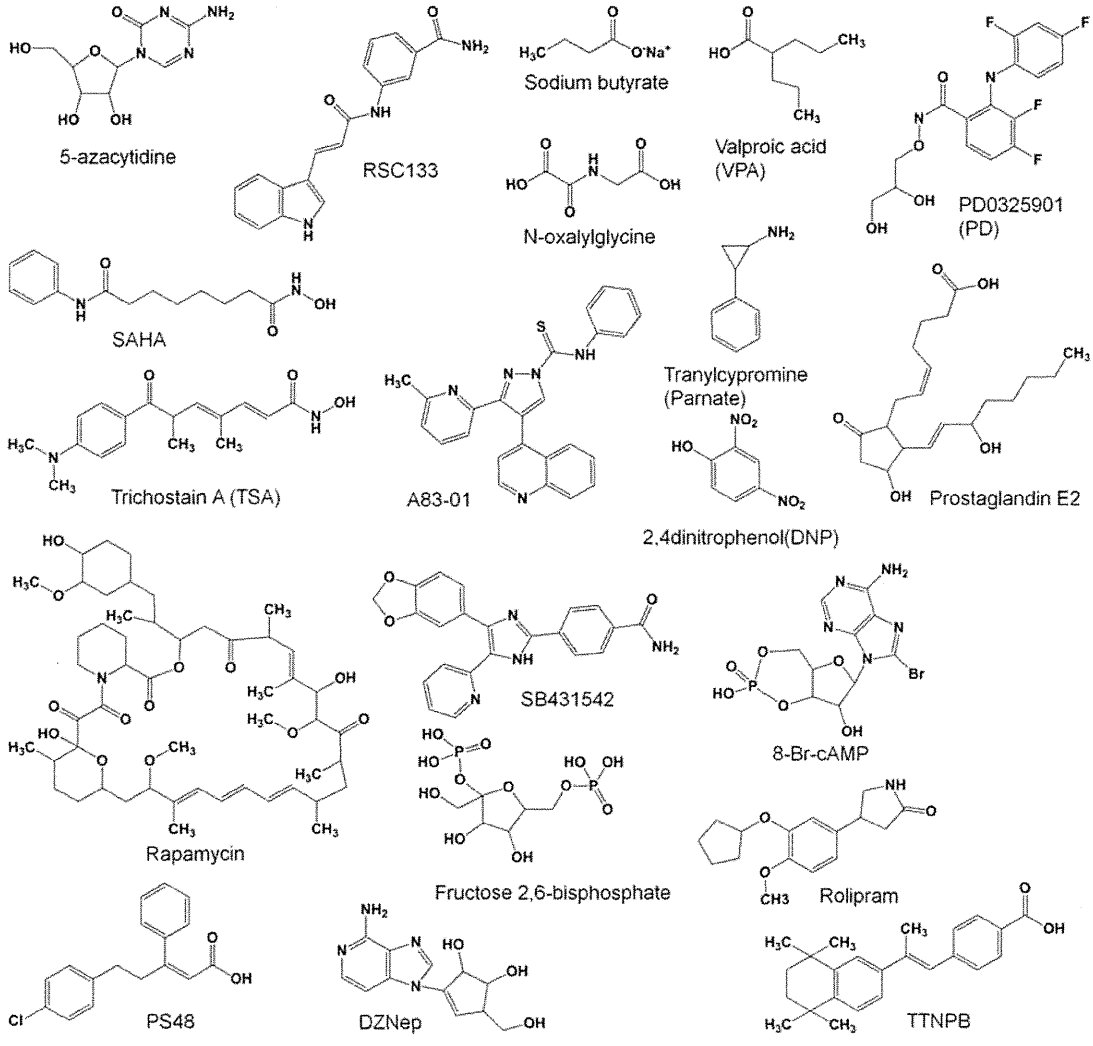

Figure 3 Representative schematic structures of small molecules that promote reprogramming to generate iPSCs.

seems to aid in the completion of mouse somatic cell reprogramming into miPSCs, although the effect of 2i medium on the generation of hiPSCs is currently unclear.

Other groups have also examined whether they could generate miPSCs from other somatic cells that do not endogenously express pluripotent TFs, such as MEFs, using small molecules and transducing fewer TFs. Huangfu *et al.* reprogrammed MEFs into miPSCs using VPA and transducing three factors without introducing the oncogene *c-Myc*. VPA, an HDAC inhibitor, improved the reprogramming efficiency by more than 100-fold under their conditions.[42]

Shi *et al.* screened several drugs to identify small molecules that can generate iPSCs from MEFs transduced with *Oct4* and *Klf4* and thus compensate for the lack of *Sox2* overexpression.[63] They found that a combination of BIX and Bayk8644 (BayK), an L-channel calcium agonist, was effective at compensating for the lack of *Sox2* expression.[63] This combination of small molecules (BIX and BayK) enabled the reprogramming of *Oct4*/*Klf4*-transduced MEFs, which do not endogenously express the TFs (ie, *Oct4*, *Sox2*, and *Klf4*) essential for reprogramming into iPSCs.[7,8] RG108, a DNMT inhibitor, can act as a direct epigenetic modifier to shift epigenetic landscapes for cell reprogramming. In combination with BIX, RG108 can reprogram cells transduced with *Oct4* and *Klf4* into miPSCs with high efficiency.[20,63]

VPA and CHIR99021 can improve the efficiency of miPSC colony generation after the transduction of three factors into MEFs, and Sox2 can be replaced by the TGF-β inhibitor 616452.[15] Therefore, Li *et al.* also generated miPSCs using only two TFs, *Oct4* and *Klf4*, in combination with VPA, CHIR99021, and 616452.[15] The authors further discovered that miPSC colonies were generated using only one TF, *Oct4*, in combination with VC6 (VPA, CHIR99021, and 616452)

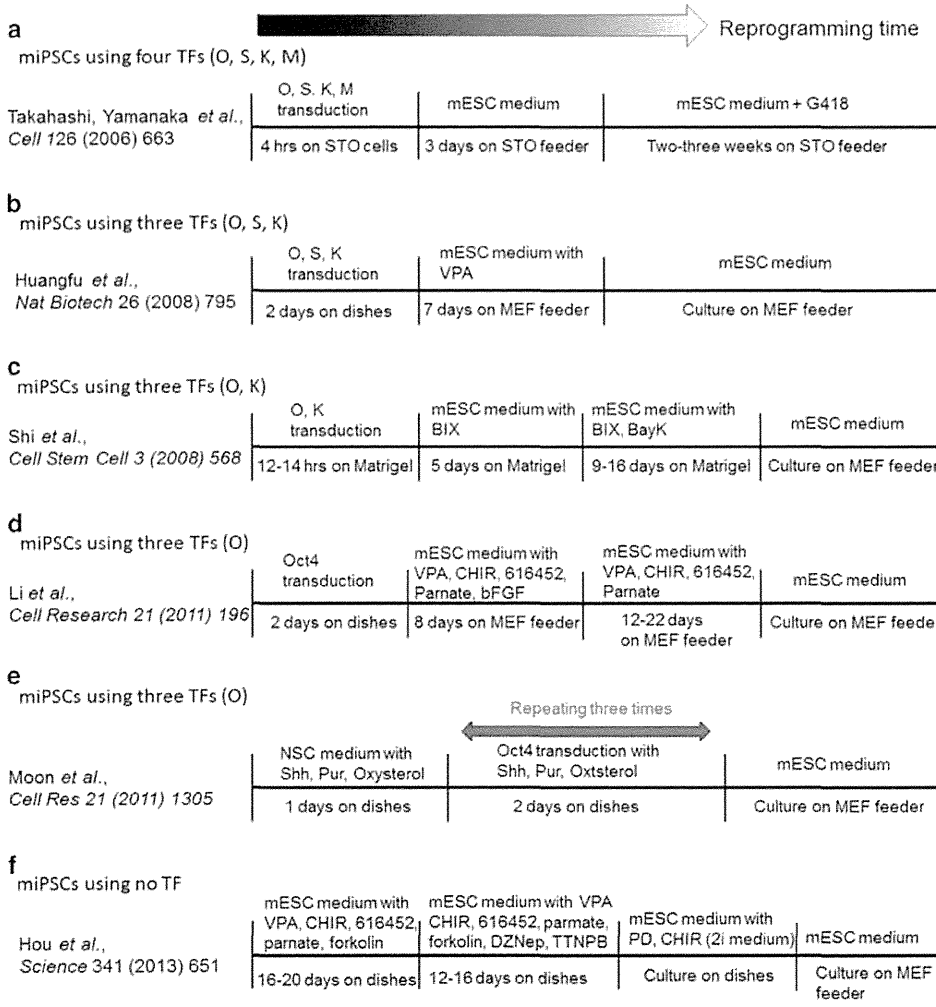

Figure 4 Schematic representation of the process of miPSC generation from MEFs by reducing the number of TFs with and without small molecules. (**a**) miPSCs using four TFs (Oct4 [O], Sox2 [S], Klf4 [K], and c-Myc [M]);[7] (**b**) miPSCs using three TFs (O, S, K);[42] (**c**) miPSCs using two TFs (O, K);[63] (**d**, **e**) miPSCs using one TF (O);[15,74] and (**f**) miPSCs using no TFs.[17]

treatment when MEFs and adult mouse fibroblasts were cultured for 30 days. However, the efficiency was only 0.0005% (one colony in 2×10^5 cells).[15] They confirmed that miPSCs could not be generated in the absence of VPA, CHIR 99021, or 616452 in their experiments.[15] Because tranylcypromine significantly promoted miPSC generation by approximately 20-fold, Li *et al.* succeeded in generating miPSCs by transduction of *Oct4* alone with the addition of VPA, CHIR99021, 616452, and tranylcypromine (VC6T) in the culture medium.[15] It is thought that the small-molecule combination including VC6T facilitated miPSC generation by lowering several major barriers to the reprogramming process. VPA (an HDAC inhibitor) and tranylcypromine (an H3K4 demethylation inhibitor) are epigenetic modulators that have been reported to facilitate iPSC generation.[42,89] From the effects of VPA and tranylcypromine, H3K4 demethylation and HDAC may be two major epigenetic barriers in the generation of miPSCs, suppressing the pluripotency transcriptional network. GSK3-β inhibition by CHIR99021 or TGF-β signaling inhibition by 616452 could efficiently replace *Sox2* for reprogramming.[15] GSK3-β and TGF-β signaling should also be another two critical barriers that suppress the reprogramming process. Therefore, overcoming these four major reprogramming barriers, ie, two epigenetic barriers and two signaling barriers, may allow researchers to generate miPSCs by *Oct4* induction alone.[15]

miPSC GENERATION USING ONE TF

Yuan *et al.* generated miPSCs from MEFs using only one factor, *Oct4*. They treated MEFs with a combination of small molecules, including AMI-5, a protein arginine methyltransferase inhibitor, and A83-01, a TGF-β inhibitor, to generate miPSCs.[73] miPSCs were able to generate live-born pups through tetraploid complementation assays, indicating

Table 3 Reprogramming of mouse somatic cells into miPSCs by transduction with and without transcription factors (TFs) with the aid of small molecules

TFs required[a]	*Somatic cells*	*PSCs*	*Small molecules*	*Efficiency (%)*	*Reference*
Four factors					
OSKM	MEFs	miPSCs	None	0.02%	7
OSKM	Mouse hepatocyte and gastric epithelial cells	miPSCs	None	0.01%	10
OSKM	Mouse liver and stomach cells	miPSCs	None	0.01%	10
OSKM	MEFs	miPSCs	Compound B6, B8, and/or B10	0.14–0.35%	84
Three factors					
KSM	Mouse NPCs	miPSCs	None	0%	67
KSM	Mouse NPCs	miPSCs	BIX	0.004%	67
OSK	MEFs	miPSCs	None	0.001–0.0026%	12
OSK	MEFs	miPSCs	VPA	0.089%	42
OSK	MEFs	miPSCs	CHIR	0.18%	71
Two factors					
OK	Mouse NPCs	miPSCs	None	0.004–0.006%	87
OK	Mouse NPCs	miPSCs	BIX	0.034%	67
OK	Mouse NPCs	miPSCs	PD + CHIR (2i)	0.0125%	88
OK	MEFs	miPSCs	None	0.003%	69
OK	MEFs	miPSCs	BIX/BayK	0.022%	63
OK	MEFs	miPSCs	CHIR	0.006%	71
OK	MEFs	miPSCs	616452	0.05%	69
OK	MEFs	miPSCs	VPA, CHIR, 616452	0.01–0.04%	15
One factor					
O	Mouse NPCs	miPSCs	None	0.004%	16
O	MEFs	miPSCs	None	0%	15
O	MEFs	miPSCs	VPA, CHIR, 616452, Parnate	0.05–0.08%	15
O	MEFs	miPSCs	AMI-5, A-83-01	0.02%	73
O	MEFs	miPSCs	Shh, purmorphamine, or oxysterol	0.11%	74
No factor					
No factor	Mouse skeletal myoblasts	miPSCs	RG108	0.11%	68
No factor	MEFs	miPSCs	VPA, CHIR, 616452, Parnate, Forkolin, DZNep	0.04%	17
No factor	MEFs	miPSCs	VPA, CHIR, 616452, Parnate, Forkolin, DZNep, TTNPB	0.2%	17

[a]O, S, K, and M indicate Oct4, Sox2, Klf-4, and c-Myc, respectively.

that the miPSCs achieved full reprogramming. Their study suggests that the regulation of protein arginine methylation by inhibition with A83-01 is also involved in the reprogramming process into miPSCs.[73] The efficiency of miPSC generation was reported to be 0.02% in their study.[73]

Moon *et al.* also generated miPSCs from mouse fibroblasts using only one factor, *Oct4*, with the aid of the small molecules of Shh, purmorphamine, and oxysterol.[74] These small molecules can activate sonic hedgehog signaling, which compensates for Bmi1 gene expression. Bmi1 expression in

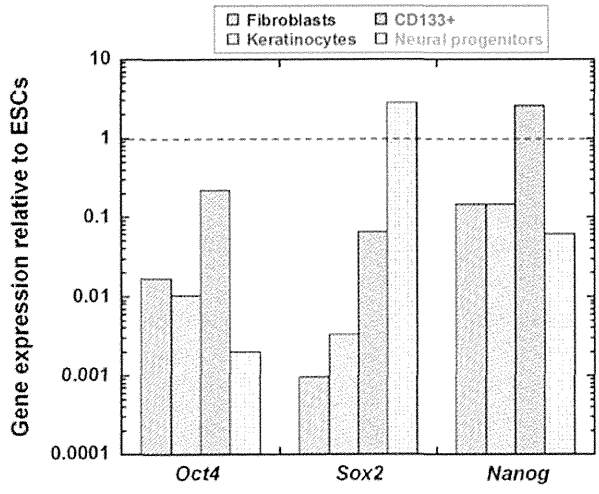

Figure 5 Relative gene expression levels of Oct4, Sox2, and Nanog in human fibroblasts, keratinocytes, CD133$^+$ cells from cord blood, and NSCs. The relative gene expression levels in fibroblasts, keratinocytes, and CD133$^+$ cells compared with hESCs were calculated from the data reported by Giorgetti *et al.*[85] and Page *et al.*[86] The relative gene expression levels in human NSCs compared with hESCs were used from the data reported by Kim *et al.*[13]

mouse fibroblasts leads to transdifferentiation of mouse fibroblasts into NSC-like cells, which can be subsequently reprogrammed into miPSCs by the transduction of *Oct4*.[74] The efficiency of miPSC generation was found to be 0.11% in their study.[74]

MOUSE CiPSC GENERATION USING SMALL-MOLECULE COCKTAILS

Hou *et al.* prepared miPSCs solely using a combination of seven small-molecule compounds that are chemically defined without using transduction or transfection of TFs.[17] It is difficult to select adequate small molecules that can replace the master regulatory gene *Oct4*. Therefore, Hou *et al.* first searched for small molecules that enabled MEF reprogramming in the absence of *Oct4* using MEFs expressing *Oct4* promoter-driven GFP to identify small molecules that facilitate cell reprogramming.[17] They identified FSK, 2-methyl-5-hydroxytryptamine, and D4476 as chemical substitutes for *Oct4* after screening up to 10 000 small molecules, and they chose to use FSK as the chemical substitute of *Oct4* in their research.[17] Because they had already developed the protocol to generate miPSCs using a small-molecule cocktail, 'VC6T' (VPA, CHIR99021, 616452, and tranylcypromine), with a single-gene transduction of *Oct4*,[15] they developed mouse CiPSCs using small-molecule cocktails in the absence of the transduction or transfection of small molecules. They used a four-step protocol to generate mouse CiPSCs (Figure 4f). (a) MEFs were first cultured with mESC medium containing VPA, CHIR99021, 616452, tranylcypromine, and FSK (VC6FT) for 16–20 days. (b) The cells were then cultured with mESC medium containing VC6FT, 3-deazaneplanocin (DZNep, epigenetic modulator), and TTNPB (a synthetic retinoic acid receptor ligand). In these cells, the expression levels of most of the pluripotency marker genes were elevated but appeared to still be lower than those found in mESCs, which indicated a partially reprogrammed cell state. (c) In the third stage, the cells were cultured in mESC medium containing PD0325901 and CHIR99021 (2i medium), which promotes complete cellular reprogramming. (d) The colonies exhibiting mESC-like morphologies were shifted onto MEFs and cultured in mESC medium.[17] The reprogramming efficiency was as high as 0.2%.[17] CiPSCs prepared in this research resembled mESCs in terms of their gene expression profiles, epigenetic status, and potential for differentiation and germline transmission. This research suggests that exogenous 'master genes' are dispensable for generating miPSCs using small molecules. This chemical reprogramming strategy opens the door to generate functional, desirable cell types for clinical applications, provided we can succeed in generating human CiPSCs in the future.

SMALL MOLECULES CAN INDUCE HUMAN iPSCs IN COMBINATION WITH A FEW TFs

Small molecules successively promote the generation of miPSCs by reducing the numbers of TFs required, as shown in the previous section. Researchers started to investigate the generation of hiPSCs by reducing the numbers of TFs with the aid of small molecules. Figure 6 illustrates some schematic representations of the process of hiPSC generation from human somatic cells. Table 4 summarizes reported studies for human somatic cell reprogramming into hiPSCs by transduction with and without TFs in combination with small molecules. Only a few small molecules have been reported to promote reprogramming and/or to replace TFs during hiPSC generation compared with miPSC generation. These molecules for hiPSC generation include sodium butyrate (HDAC inhibitor), VPA (HDAC inhibitor), CHIR99021 (GSK3-β inhibitor), LiCl (GSK3-β inhibitor), 616452 (TGF-β inhibitor), SB431542 (TGF-β inhibitor), PD325901 (MEK inhibitor), N-oxaloylglycine (prolyl-4-hydroxylase inhibitor), PS48 (3-phosphoinositide-dependent protein kinase 1 activator), 8-Br-cAMP (cAMP-dependent protein kinase activator), fructose 2,6-bisphosphate (phosphofructokinase 1 activator), quercetin (hypoxia-inducible factor pathway activator), and 2,4-dinitrophenol (oxidative phosphorylation uncoupler; Table 2). It will be important to evaluate whether other small molecules that have biological activities in miPSC reprogramming are valid for use in hiPSC reprogramming.

Currently, the minimum transduction of *Oct4* in combination with chemically defined small molecules is necessary to generate hiPSCs. This requirement is because the efficiency of hiPSC generation is much less than that of miPSC generation (eg, 0.0005–0.01%).

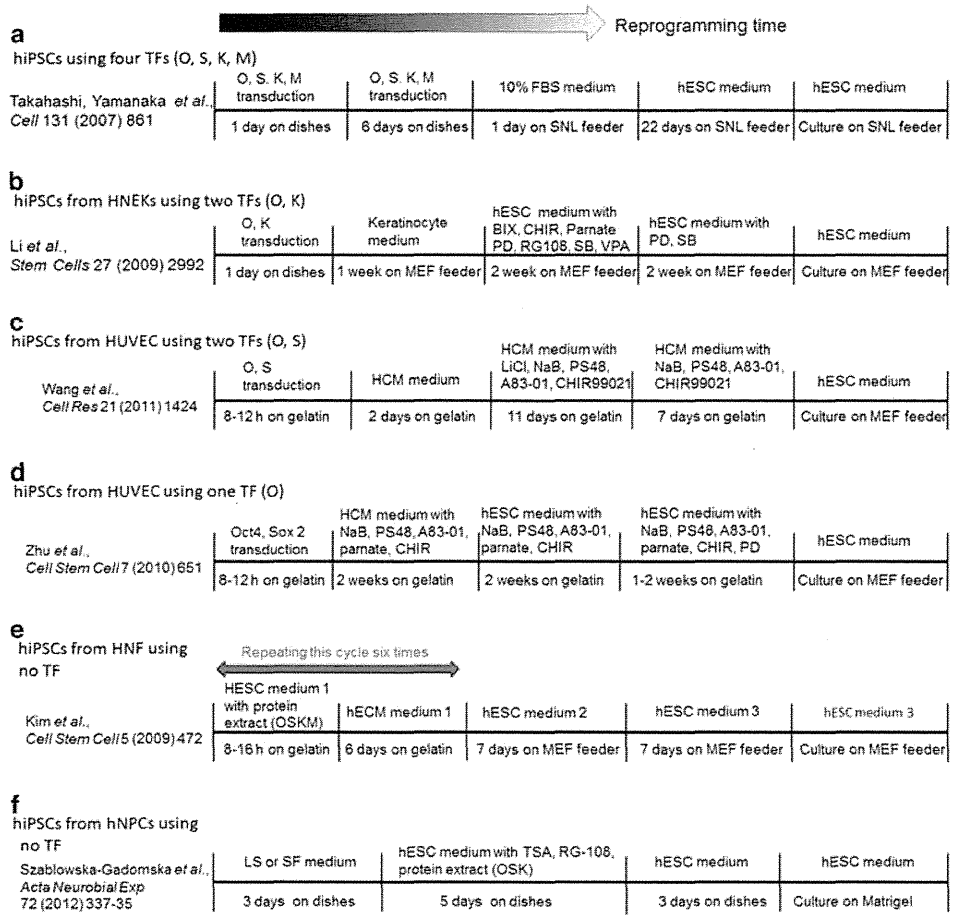

Figure 6 Schematic representation of hiPSC generation from human somatic cells by reducing the number of TFs with and without small molecules. (**a**) hiPSCs using four TFs (Oct4 [O], Sox2 [S], Klf4 [K], and c-Myc [M]);[8] (**b**) hiPSCs from human neonatal epidermal keratinocytes (HNEKs) using two TFs (O, K);[71] (**c**) hiPSCs from human umbilical vein endothelial cells (HUVECs) using two TFs (O, S);[70] (**d**) hiPSCs from HUVECs using one TF (O);[43] (**e**) hiPSCs from human newborn fibroblasts (HNFs) using no TFs;[18] and (**f**) hiPSCs from human NSCs using no TFs.[90] HCM medium indicates EndoGRO-VEGF complete medium (CHEMICON). LS and SF medium indicates low-serum and serum-free media, respectively. O, S, K, and M indicate the TFs Oct4, Sox2, Klf4, and c-Myc, respectively.

NSCs endogenously express *Sox2*, *Klf4*, and/or *c-Myc* to some extent, as was discussed in the previous section (Figure 5). Therefore, Kim *et al.* reprogrammed human fetal NSCs into hiPSCs by the transduction of only *Oct4*.[13] Their work suggests that only one TF, *OCT4*, should be sufficient to reprogram human fetal NSCs into hiPSCs. However, it is very difficult to obtain human fetal NSCs to generate patient-specific hiPSCs, and the efficiency of hiPSC generation is reported to be only 0.006% in their study.[13]

Zhu *et al.* developed their four-step protocol to generate hiPSCs from neonatal human epidermal keratinocytes, human umbilical vein endothelial cells (HUVECs), and amniotic-derived cells.[43] In the case of HUVEC-derived hiPSCs, the following protocol was used. (a) In the first stage, *Oct4* was transduced into cells cultured on gelatin-coated dishes for 8–12 h. (b) In the second stage, the cells were cultured in HUVEC medium containing sodium butyrate, PS48, A83-01, tranylcypromine, and CHIR99021 for 2 weeks on the gelatin. (c) In the third stage, the cells were cultured in hESC medium containing sodium butyrate, PS48, A83-01, tranylcypromine, and CHIR99021 for another 2 weeks on the gelatin. (d) In the fourth stage, the cells were cultured in hESC medium containing sodium butyrate, PS48, A83-01, tranylcypromine, CHIR99021, and PD0325901 for an additional 1–2 weeks on the gelatin to complete the full cellular reprogramming (Figure 6d). It was suggested that the single TF, *Oct4*, combined with a defined small-molecule cocktail is capable of reprogramming several human somatic cell types into hiPSCs that are functionally similar to pluripotent hESCs in terms of morphology, gene and protein expression, and differentiation capacity.[43] However, the reprogramming efficiency was extremely low (0.004%) because hiPSCs rather than miPSCs were generated.

Table 4 Reprogramming of human somatic cells into hiPSCs by transduction with and without TFs with the aid of small molecules

TFs required[a]	*Somatic cells*	*PSCs*	*Small molecules*	*Efficiency (%)*	*Reference*
Four factors					
OSKM	Human dermal fibroblasts	hiPSCs	None	0.02%	8
OSKM	hFFs	hiPSCs	RSC133	3 Times higher than control	82
Two factors					
OK	Human fetal NPCs	hiPSCs	None	0.006%	13
OS	Human cord blood-derived stem cells	hiPSCs	None	0.006%	64
OK	Human neonatal keratinocyte	hiPSCs	CHIR99021, Parnate (and PD0325901, SB431542)	0.005–0.01%	71
OK	Human neonetal epidermal keratinocytes	hiPSCs	A-83-01, PD032590, NaB, PS48 (+Parnate, CHIR99021)	0.025%	43
OS	HUVEC	hiPSCs	A83, NaB, PS48, CHIR88021, LiCl	0.0015%	70
One factor					
O	Human fetal NPCs	hiPSCs	None	0.004%	13
O	Human neonetal epidermal keratinocytes	hiPSCs	A-83-01, PD032590, sodium butyrate, PS48 (+Parnate, CHIR99021)	0.004%	43
O	HUVECs and amniotic fluid-derived cells	hiPSCs	A83-01, PD032590, NaB, PS48 (+Parnate, CHIR99021)	0.0025–0.01%	43
O	HUVEC	hiPSCs	A83-01, NaB, PS48, LiCl, CHIR99021 (or PD0325901)	0.0005%	70

Abbreviations: hFF, human foreskin fibroblast; TF, transcription factor.
[a]O, S, K, and M indicate Oct4, Sox2, Klf-4, and c-Myc, respectively.

Wang *et al.* also generated hiPSCs using either one transduced factor (*Oct4*) or two transduced factors (*Oct4* and *Sox2*) with the aid of small molecules.[70] They used small-molecule cocktails similar to those used by Zhu *et al.*[43] but also treated the cells with LiCl (5 mM) from days 3 to 18, which facilitated miPSC and hiPSC generation (Figure 6c).[70] hiPSC colonies were picked for expansion on feeder cells after approximately 1 month. The effect of LiCl on promoting reprogramming was only partially dependent on its major target, GSK-3β.[70] However, LiCl is unlike other GSK-3β inhibitors, as it enhances *Nanog* expression and also promotes its transcriptional activity. LiCl also works by promoting epigenetic modifications via the downregulation of an H3K4-specific histone demethylase, LSD1. However, the efficiency of hiPSC generation was still extremely low (0.0015 and 0.0005% for two-factor and one-factor transduction, respectively).

If hiPSCs can be generated from patient-specific cells and/or different human leukocyte antigen (HLA) types of human somatic cells using only chemically defined small molecules with high efficiency (eg, preferentially >0.1%), the door would be opened for the potential use of hiPSCs in clinical applications. Currently, to our knowledge, hiPSC generation solely based on chemically defined small molecules has not yet been reported, although hiPSCs have been generated using cell extracts and/or proteins without the use of TFs.[18,58,90]

CONCLUSIONS AND PERSPECTIVES

Introduction of small and large molecules can target signaling transduction pathways and can affect DNA replication, cell differentiation, apoptosis, and the reprogramming of the cells into iPSCs. Several specific small molecules can successfully enhance the reprogramming efficiencies of mouse and human somatic cells into iPSCs. Since 2013, miPSCs have been generated via treatment with small molecules without the use of genetic material.[17,68] However, it is still challenging to generate hiPSCs by treating cells with only small molecules. The generation of human and mouse piPSCs using pluripotent proteins or cell extracts without the

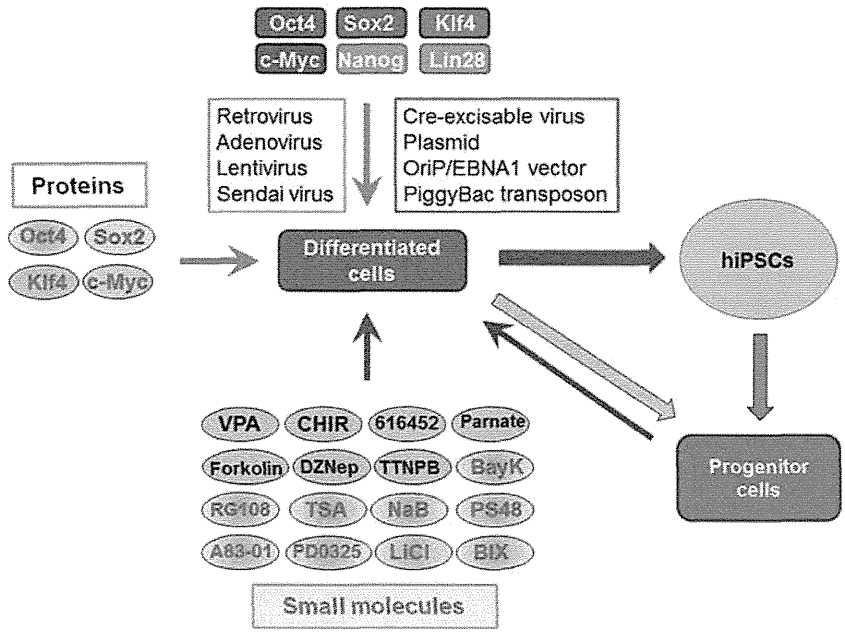

Figure 7 Schematic illustration of somatic cell reprogramming to iPSCs and progenitor or adult stem cells. Somatic cells can be reprogrammed to iPSCs using viral or non-viral introduction of exogenous TFs or protein transfection. Small molecules can either substitute for certain TFs and/or improve reprogramming efficiency by epigenetic modifications or signaling pathway regulation. The abbreviations of the small molecules are shown in Table 2.

use of genetic material has been reported.[18,21] However, currently, piPSCs are not a popular method for generating iPSCs, although more than 5 years has passed since the first reports of their generation. The hesitancy to adopt the method is likely due to the extremely low generation efficiency and the difficulty of preparing pluripotent proteins tagged with CPP. Figure 7 summarizes the reprogramming methods that have been developed and that may be promising in the future. Currently, target cells, such as dopamine-secreting cells, insulin-secreting β cells, and cardiomyocytes, may be prepared from PSCs as follows: (a) somatic cells from blood, fat tissue, or other sources of tissues are isolated; (b) the somatic cells are reprogrammed into iPSCs; and (c) iPSCs are differentiated into the targeted cells. In this case, which includes the use of hESCs and hiPSCs, it is extremely difficult to obtain highly pure populations of the target cells. Furthermore, it is impossible to avoid the potential for tumor generation when these targeted cells are transplanted into humans.[91] Therefore, we suggest that the direct reprogramming of somatic cells into progenitor (stem) cells, including adult stem cells, such as NSCs, cardiomyocyte progenitor cells, hepatocyte stem cells, hematopoietic stem cells, and mesenchymal stem cells, as an attractive alternative for clinical applications. It will be interesting to develop small molecules to guide human somatic cells into the transdifferentiation of tissue-specific progenitor cells or stem cells.[76,92–95] In this case, xeno-free cultures are much easier for culturing the progenitor cells compared with PSCs because MEFs are not required for the culturing of progenitor cells and adult stem cells. Although NSCs are reported to be able to proliferate for more than 20–130 passages,[96–98] mesenchymal stem cells, such as adipose-derived stem cells and amniotic fluid stem cells, have limited proliferation capacities (eg, 8–12 passages). Therefore, the establishment of small molecules to extend the proliferation of adult stem cells and progenitor cells should be a priority in regenerative medicine. Because bone marrow stem cells and hematopoietic stem cells are ready to be used in clinical applications, the only obstacle to the use of these adult stem cells in clinical applications is their limited proliferation and their overall shortage, which leads to low numbers of clinical trials. The shortages of progenitor cells and adult stem cells can be addressed by reprogramming somatic cells into progenitor cells and adult stem cells using small molecules, which do not disrupt or interrupt host cell genomes. The development of small molecules to guide human somatic cells into progenitor cells, adult stem cells, and iPSCs will open avenues for the clinical application of these types of progenitor cells and stem cells.[65,99,100]

ACKNOWLEDGMENTS

This research was partially supported by the Ministry of Science and Technology of Taiwan under Grant numbers 102-2120-M-008-002, and 102-2221-E-008-112-MY2. This work was also supported by the LandSeed Hospital project (NCU-LSH-101-A-001 and NCU-LSH-102-A-003), the National Defense Medical Center Project (102NCU-NDMC-01), and the Cathay General Hospital Project (101CGH-NCU-A2, 102NCU-CGH-02, CGH-MR-10115 and CGH-MR-A10204). A Grant-in-Aid for Scientific Research (number 24560968) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan is also acknowledged. We acknowledge the

International High Cited Research Group (IHCRG #14-104), Deanship of Scientific Research, King Saud University, Riyadh, Kingdom of Saudi Arabia. Akon Higuchi thanks King Saud University, Riyadh, Kingdom of Saudi Arabia, for the Visiting Professorship.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A Novel *In Vitro* Method for Detecting Undifferentiated Human Pluripotent Stem Cells as Impurities in Cell Therapy Products Using a Highly Efficient Culture System

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Abstract

Innovative applications of cell therapy products (CTPs) derived from human pluripotent stem cells (hPSCs) in regenerative medicine are currently being developed. The presence of residual undifferentiated hPSCs in CTPs is a quality concern associated with tumorigencity. However, no simple *in vitro* method for direct detection of undifferentiated hPSCs that contaminate CTPs has been developed. Here, we show a novel approach for direct and sensitive detection of a trace amount of undifferentiated human induced pluripotent stem cells (hiPSCs) using a highly efficient amplification method in combination with laminin-521 and Essential 8 medium. Essential 8 medium better facilitated the growth of hiPSCs dissociated into single cells on laminin-521 than in mTeSR1 medium. hiPSCs cultured on laminin-521 in Essential 8 medium were maintained in an undifferentiated state and they maintained the ability to differentiate into various cell types. Essential 8 medium allowed robust hiPSC proliferation plated on laminin-521 at low cell density, whereas mTeSR1 did not enhance the cell growth. The highly efficient culture system using laminin-521 and Essential 8 medium detected hiPSCs spiked into primary human mesenchymal stem cells (hMSCs) or human neurons at the ratio of 0.001%–0.01% as formed colonies. Moreover, this assay method was demonstrated to detect residual undifferentiated hiPSCs in cell preparations during the process of hMSC differentiation from hiPSCs. These results indicate that our highly efficient amplification system using a combination of laminin-521 and Essential 8 medium is able to detect a trace amount of undifferentiated hPSCs contained as impurities in CTPs and would contribute to quality assessment of hPSC-derived CTPs during the manufacturing process.

Citation: Tano K, Yasuda S, Kuroda T, Saito H, Umezawa A, et al. (2014) A Novel *In Vitro* Method for Detecting Undifferentiated Human Pluripotent Stem Cells as Impurities in Cell Therapy Products Using a Highly Efficient Culture System. PLoS ONE 9(10): e110496. doi:10.1371/journal.pone.0110496

Editor: Graca Almeida-Porada, Wake Forest Institute for Regenerative Medicine, United States of America

Received March 28, 2014; **Accepted** September 16, 2014; **Published** October 27, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Research Grants from the Japanese Ministry of Health, Labour and Welfare (H23-SAISEI-IPPAN-004, H23-SAISEI-IPPAN-005, H24-IYAKU-SHITEI-027, H25-JITSUYOKA(SAISEI)-IPPAN-008, and Marketing Authorization Facilitation Program for Innovative Therapeutic Products), http://www.mhlw.go.jp/english/policy/other/research-projects/index.html. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Cell therapy products (CTPs) are expected to offer promising treatments for serious and life-threating diseases for which no adequate therapy is currently available. An increasing number of CTPs derived from human pluripotent stem cells (hPSCs), i.e. induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs), are being developed for regenerative medicine/cell therapy because of their infinite self-renewal capacity and their ability to differentiate into various types of cells. Quality assessment of CTPs is critical to ensure their safety and efficacy for clinical application [1]. CTPs derived from hPSCs possibly include the cells of interest and also other cells such as undifferentiated cells, precursor cells and other differentiated cells. The presence of residual undifferentiated cells in CTPs derived from hPSCs is one of the most serious concerns for tumorigenicity because the undifferentiated hPSCs have a capacity to form teratoma in animals [1–4]. Hentze et al. previously reported that hundreds of undifferentiated hESCs were enough to produce a teratoma in immunodeficient SCID mice [5]. We cannot exclude the possibility that a trace amount of residual undifferentiated hPSCs in CTPs cause ectopic tissue formation, tumor development and/or malignant transformation after transplantation. Therefore, establishment of a detection method for residual undifferentiated cells is necessary for the safety and quality assessment of CTPs derived from hPSCs.

An *in vivo* teratoma formation assay is the only method to directly assess tumorigenicity of undifferentiated cells, but this assay is costly and time-consuming [2,3]. Several *in vitro* methods, such as flow cytometry and quantitative real-time PCR (qRT-PCR) analysis, can also detect residual undifferentiated hPSCs in CTPs [2,3]. Our previous report has shown that flow cytometry

using anti-TRA-1-60 antibody and qRT-PCR using a specific probe and primers for *LIN28* mRNA can detect as low as 0.1% and 0.002% undifferentiated hiPSCs spiked into retinal pigment epithelial (RPE) cells, respectively [3]. However, both of these methods have the disadvantage of detecting undifferentiated cell marker expression but not functionally undifferentiated cells *per se*. The soft agar colony formation assay is commonly used to detect tumorigenic cells with a property of anchorage-independent growth. However, this assay is not appropriate for the detection of hPSCs because they undergo apoptosis associated with dissociation into single cells [3,6]. At present, there is no simple method to directly detect a trace amount of hPSCs *in vitro*.

Recently, some cell culture matrices have been reported to sustain self-renewal of dissociated hPSCs without apoptosis [7,8]. We focused on a culture system enabling hPSC cell growth without apoptosis and developed a direct *in vitro* method for detecting a trace amount of undifferentiated hPSCs in CTPs. Laminin-521, a laminin isoform that is normally expressed in hESCs, is known to stimulate robust hPSC proliferation in an undifferentiated state in combination with mTeSR1 medium [7]. In the present study, we present a novel approach to detect undifferentiated hiPSCs contaminating CTPs through efficient amplification using a laminin-521-based cell culture system with Essential 8 medium [9] instead of mTeSR1 medium.

Materials and Methods

Cell culture

The hiPSC lines, 201B7, 253G1 and 409B2, were provided by the RIKEN BRC through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan [17–19]. hiPSCs were first cultured on mitomycin C-treated SNL cells (a mouse fibroblast STO cell line expressing a neomycin-resistance gene cassette and LIF) in primate ES cell medium (ReproCell, Kanagawa, Japan) supplemented with 4 ng/ml human basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, USA). hiPSC colonies were passaged as small clumps once every 5–6 days using CTK solution (ReproCell) and STEMPRO EZPassage (Invitrogen, Carlsbad, CA, USA). hiPSCs were then passaged onto Matrigel-coated dishes with mTeSR1 (Stem Cell Technologies, Vancouver, CAN) for at least 2 passages before plating on laminin-521 or directly subcultured onto laminin-521-coated dishes. Subculture on laminin-521-coated dishes was performed as follows: near-confluent cells were treated with 0.5 mM EDTA/D-PBS for 6–7 minutes at 37°C. Cells were pipetted to achieve single-cell suspension and centrifuged at 30× *g* for 4 minutes. After centrifugation, the cell pellet was suspended in Essential 8 medium (Life Technologies, USA) and seeded at $2-3\times10^4$ cells/cm^2 on laminin-521-coated dishes. Cells were grown in Essential 8 medium at 37°C in a 5% CO_2 atmosphere and passaged once in 3–4 days. Primary human mesenchymal stem cells (hMSCs) were purchased from Lonza and cultured in MSCGM medium (Lonza, Walkersville, MO, USA). hMSCs at passage 7 were used in this study. Primary human neurons were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA).

Cell proliferation assay

hiPSCs were dissociated into single cells and seeded on matrix-coated plates at a density of 3×10^4 cells/cm^2 or at the indicated density as described below. Tissue culture plates (BD Falcon, NJ, USA) were coated with laminin-521 (BioLamina, Sundbyberg, Sweden) dissolved in D-PBS at 4 μg/cm^2 at 37°C for 2 h. Control plates were coated with Matrigel (BD Biosciences, MA, USA) at 16 μg/cm^2. Viable cells were quantified every 24 h using CyQUANT Cell Proliferation Assay Kit (Life Technologies) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was treated with DNase I and isolated using RNeasy Mini Kit (Qiagen Hilden, Germany) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the QuantiTect Probe one-step RT-PCR Kit (Qiagen) on StepOnePlus Real Time PCR system (Life Technologies). Gene expression levels were normalized to *GAPDH* expression levels, which were quantified using TaqMan human GAPDH control reagents (Life Technologies). Primers and probes were obtained from Sigma-Aldrich. The sequences of primers and probes are listed in Table S1.

Teratoma assay

Teratoma formation experiments were performed by injecting 253G1 cells (1×10^6 cells/testis) that were cultured with laminin-521 and Essential 8 medium (passage 36), into the testes of severe combined immunodeficiency (SCID) mice at the age of 8 weeks under pentobarbital anesthesia. The mice were sacrificed with an overdose of pentobarbital 10 weeks after the transplant, and the isolated teratoma was fixed in 10% formalin. The paraffin-embedded section was stained with hematoxylin and eosin (HE). Animal experiments were performed at UNITECH Co., Ltd. (Chiba, Japan) in accordance with the animal ethical committee's approval (Permit Number: KIS-130712i-20 at UNITECH Co., Ltd. and 444 at NIHS).

Differentiation assay

Differentiation of hiPSCs into three germ layers was performed as follows: 253G1 cells were plated on laminin-521 at a density of 3×10^4 cells/cm^2 in Essential 8 medium and expanded until they were nearly confluent. A) Ectoderm lineage differentiation: Neural hiPSCs differentiation was performed according to the previously reported protocol with some modifications [10]. Briefly, culture medium was changed from Essential 8 to DMEM/F12 medium containing 20% knockout serum replacement (KSR, Life Technologies), 10 μM SB431542 (Sigma-Aldrich) and 500 ng/ml Noggin (R&D systems). After 4 days of differentiation, SB431542 was withdrawn and increasing amounts of N2 medium (25%, 50%, or 75%) was added to the KSR medium every 2 days. From day 10 of differentiation, the medium was changed to N2B27 medium without bFGF containing 500 ng/ml Noggin, and cells were cultured for 15 days. B) Mesoderm lineage differentiation: hiPSCs were cultured for 15 days in DMEM/F12 containing 10% FBS, 2 mM $_L$-glutamine 1% nonessential amino acids (Life Technologies) and 0.1 mM β-mercaptoethanol [8]. C) Endoderm lineage differentiation: hepatic differentiation of hiPSCs was performed according to the previously reported protocol with some modifications [11]. On the first day of differentiation, the medium was replaced with RPMI1640 (Sigma-Aldrich) containing B27 supplement (Life Technologies), 100 ng/ml activin A (R&D systems), 50 ng/ml Wnt3a (R&D systems) and 1 mM sodium butyrate (NaB) (Sigma-Aldrich). On the following 2 days, NaB was omitted from the medium. After 3 days of differentiation, the medium was replaced with knockout-DMEM containing 20% KSR, 1 mM $_L$-glutamine, 1% nonessential amino acids, and 1% DMSO for 5 days.

Differentiation of 253G1 cells into MSCs was performed according to the previously reported protocol with some modifications [14]. On the first day of differentiation, 253G1 cells

subcultured on laminin-521 in Essential 8 medium were dissociated into single cells and suspended in EB formation medium (AggreWell Medium, Stem Cell Technologies) with 10 μM Y-27632 (Wako, Japan), a ROCK inhibitor for generation of embryoid bodies (EBs). Cells (1×10^6) were then added to a well of the AggreWell Plate and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. After 24 h, EBs were plated on 35-mm dishes (BD) in Stemline II (Sigma-Aldrich) supplemented with 50 ng/ml BMP4 (R&D systems) and 50 ng/ml VEGF (R&D systems) for 2 days. Medium was changed to Stemline II containing BMP4 (50 ng/ml), VEGF (50 ng/ml) and bFGF (22.5 ng/ml), and the EBs were cultured for 2 days. EBs were dissociated into single cells and replated in Methocult H4536 (Stem Cell Technologies) containing Growth Enhancement Media Supplement (EX-CYTE, Millipore), 50 ng/ml VEGF, 50 ng/ml Flt3-ligand (R&D systems), 50 ng/ml thrombopoietin (R&D systems) and 30 ng/ml bFGF for hemangioblast formation. After 8 days, cultures were harvested and plated as defined passage 0 in MSC growth medium (αMEM+ 20% FBS) on Matrigel.

Immunofluorescence staining

Immunofluorescence staining was performed as follows: cells were fixed with 4% paraformaldehyde in PBS for 15 minutes. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and then blocked with Blocking One (nacalai tesque, Kyoto, Japan) at 4°C over night. Cells were incubated with primary antibody against α-fetoprotein (AFP, 1:400; Dako) for 30 minutes at room temperature, with antibody against smooth muscle actin (SMA, 1:400; Sigma-Aldrich), βIII tubulin (0.5 μg/ml; abcam) or TRA-1-60 (1:200; Millipore) for 1 hour at room temperature, or with antibody against CD105 (1:200; abcam) at 4°C over night. After washing with PBS three times, cells were incubated with secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) for 30 minutes at RT. VECTASHIELD mounting medium with DAPI (VECTOR) was used for nuclear staining. The samples were examined using an Olympus IX71 microscope equipped with cellSens Standard software (Olympus).

Embryoid body formation

Embryoid bodies were generated from hiPSCs using AggreWell 800 plates (Stem Cell Technologies) according to the manufacturer's instructions, with some modifications. hiPSCs dissociated with 0.5 mM EDTA were collected and suspended in EB formation medium (AggreWell Medium, Stem Cell Technologies) supplemented with 10 μM Y-27632 (Wako). The cells were added to each well (5×10^5 cells/well) in the AggreWell plate and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. EBs were harvested from AggreWell plate and cultured in 35-mm dishes (BD) with primate ES cell culture medium (ReproCell) without bFGF. The medium was changed every 3 days. After 10 days of incubation, total RNA was isolated from EBs. The expression levels of each differentiation marker were determined using quantitative RT-PCR, as described above.

Statistics

Statistical analysis was performed using SigmaPlot 12.5 Software (Systat Software Inc., CA). The data were analyzed using two-way ANOVA or two-way repeated-measures ANOVA followed by a Bonferroni t-test as a post hoc test. A probability below 0.05 was considered significant.

Results

Essential 8 medium promotes hiPSCs cell growth on laminin-521

hPSCs are known to easily undergo apoptosis induced by dissociation [6]. To achieve an efficient hPSC cell growth, we determined the optimal culture conditions that allow robust proliferation of hiPSCs dissociated into single cells. Here, we focused on laminin-521 as a cell culture matrix, which permits survival of dissociated hPSCs without a Rho-associated protein kinase (ROCK) inhibitor [7]. mTeSR1 medium is conventionally used to culture dissociated hiPSCs on dishes coated with laminin-521 [7], and other hPSC media besides mTeSR1 have not been fully characterized with laminin-521. To examine effects of medium on hPSC cell growth on laminin-521, we compared the hiPSC growth rate using conventional mTeSR1 medium with Essential 8 medium, with optimized components [9]. After subculture on Matrigel, 253G1 hiPSCs were dissociated into single cells and seeded on laminin-521-coated plates at a density of 3×10^4 cells/cm^2. No difference was observed between mTeSR1 and Essential 8 medium in the cell number of hiPSCs cultured on laminin-521 at 24 h after plating (Figure 1A and 1B). However, the cells cultured on laminin-521 in Essential 8 medium showed rapid expansion compared to those in the mTeSR1 medium, and they reached nearly confluent at 72 h after plating (Figure 1A). Cell growth quantification revealed that the number of cells in Essential 8 medium was 3-fold higher than those in mTeSR1 medium at 72 h after plating (Figure 1B). Similar results were also obtained using another hiPSC line, 201B7 (Figure S1A). These results suggest that Essential 8 medium promotes hiPSC proliferation more rapidly than mTeSR1 medium when grown on laminin-521. When dissociated hiPSCs were cultured on Matrigel, Essential 8 medium did not significantly promote cell proliferation compared with mTeSR1 medium (Figure 1B and Figure S1A). We found that Essential 8 medium enhanced the hiPSC growth rate even on LM511-E8, which is a fragment of laminin 511 [8], but this effect was weaker than that on laminin-521 (Figure S1B). Taken together, these results suggest that a combination of laminin-521 and Essential 8 medium is a potent cell culture system for efficient amplification of dissociated hiPSCs *in vitro* compared to other culture systems. Therefore, we decided to develop a novel method for detecting undifferentiated hPSCs using a combination of laminin-521 and Essential 8 medium.

Culture system using laminin-521 and Essential 8 medium maintains the undifferentiated state and pluripotency of hiPSCs

We next examined whether a culture system using laminin-521 and Essential 8 medium maintains undifferentiated states through serial passages. 253G1 cells were dissociated into single cells and sequentially subcultured on laminin-521 in Essential 8 medium. 253G1 cells exhibited vigorous proliferation for more than 30 passages under these conditions (data not shown). Quantitative RT-PCR analysis revealed that the expression levels of undifferentiated hPSC markers (*OCT3/4*, *NANOG*, *SOX2* and *LIN28*) in 253G1 cells cultured with laminin-521 and Essential 8 medium were similar to those cultured with Matrigel and mTeSR1 medium (Figure 2A). Moreover, the serial passages with laminin-521 and Essential 8 medium did not have any effect on expression of the undifferentiated markers. We also examined the effects of subculture on laminin-521 in Essential 8 medium using other hiPSC lines, 201B7 and 409B2, and showed that these cells expressed undifferentiated markers through serial passages (Figure S2A-B). We next tested the pluripotency of hiPSCs cultured on laminin-521 in Essential 8 medium using lineage-specific

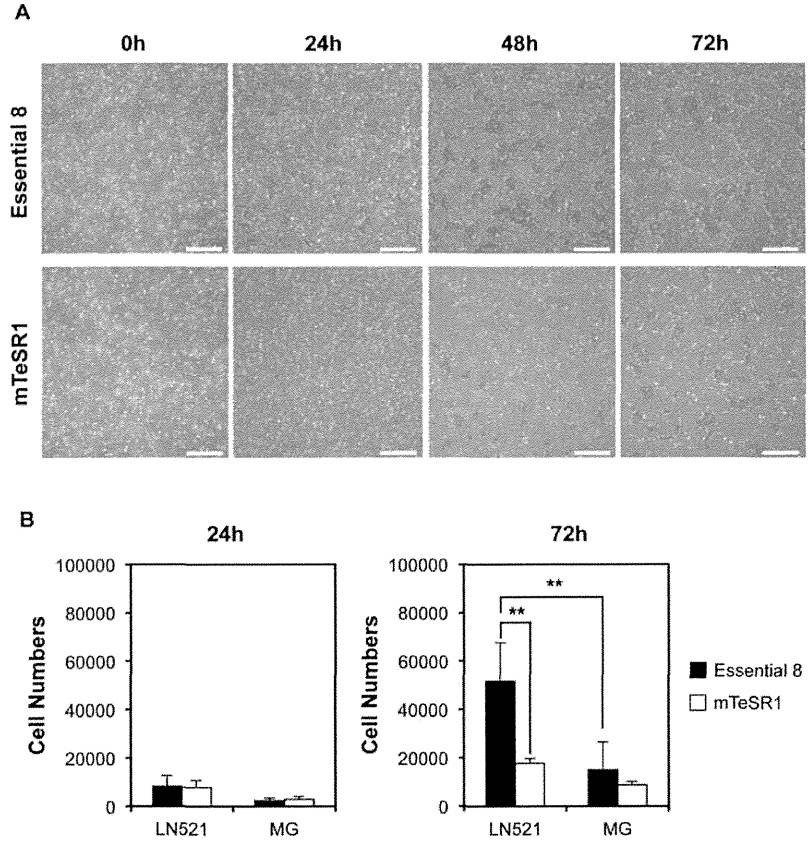

Figure 1. Robust proliferation of 253G1 cells cultured on laminin-521 in Essential 8 medium. (A) Morphology of the 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium after dissociation into single cells. Scale bars, 500 μm. (B) Quantification of the number of dissociated 253G1 cells expanded on laminin-521 or Matrigel in Essential 8 or mTeSR1 medium. Data are presented as the mean ± standard deviation (SD) of three independent experiments (**$P<0.01$, two-way ANOVA followed by Bonferroni t-test as post-hoc test). LN521, laminin-521; MG, Matrigel.
doi:10.1371/journal.pone.0110496.g001

differentiation protocols. Immunofluorescence analysis using antibodies specific for markers of three germ layers clearly demonstrated that 253G1 cells subcultured with laminin-521 and Essential 8 medium could selectively differentiate into endoderm, mesoderm and ectoderm expressing AFP, α-SMA and βIII tubulin, respectively (Figure 2B). To further examine pluripotency of hiPSCs subcultured with laminin-521 and Essential 8 medium, we facilitated spontaneous differentiation of cells grown as aggregates (embryoid bodies). Embryoid bodies derived from 253G1 cells increased gene expression of differentiated markers for all three germ layer lineages (Figure 2C), consistent with the observation obtained using 201B7 and 409B2 cells (Figure S2C-D). We also confirmed that 253G1 cells cultured with laminin-521 and Essential 8 medium were engrafted in testes of SCID mice and formed teratomas that involved all three germ layers (Figure 2D). These results strongly suggest that a culture system using laminin-521 and Essential 8 medium supports the undifferentiated state and pluripotency of hiPSCs through serial passages.

Essential 8 medium enables hiPSCs to proliferate rapidly from low cell density on laminin-521

Higher sensitivity is expected to ensure the accuracy and reliability of the detection of trace amounts of undifferentiated cells. To achieve higher sensitivity, culture system using laminin-521 and Essential 8 should have a capacity to rapidly expand hPSCs even at a low cell density. Therefore, we next tested whether Essential 8 medium promotes expansion of the hiPSCs on laminin-521 plated at a low cell density. 253G1 cells were seeded into laminin-521-coated plates at a density of 3.2×10^4 cells/cm^2, 1.6×10^4 cells/cm^2 and 8.0×10^3 cells/cm^2, and grown until nearly confluent. Cells grown in mTeSR1 reduced proliferative capacity as seeding density became lower. Conversely, cells cultured in Essential 8 medium showed robust propagation over a prolonged period of time even when they were seeded at low cell density (Figure 3A-C). Similarly, 201B7 and 409B2 cells seeded at lower density in Essential 8 medium also showed robust proliferation compared to the cells in mTeSR1 (Figure 3D-I). Essential 8 medium also promoted cell growth when hiPSCs were plated at lower density of 800 cells/cm^2 (Figure S3). These results indicate that Essential 8 medium promotes expansion of hiPSCs plated on laminin-521 at a low cell density. Thus, a culture system using laminin-521 and Essential 8 medium is considered to be well suited for direct detection of trace amounts of undifferentiated cells.

Culture system using laminin-521 and Essential 8 medium is useful for direct detection of undifferentiated hiPSCs contained in somatic cells

Residual undifferentiated cells contaminating hPSC-based CTPs are a quality concern associated with tumorigencity [1–4].

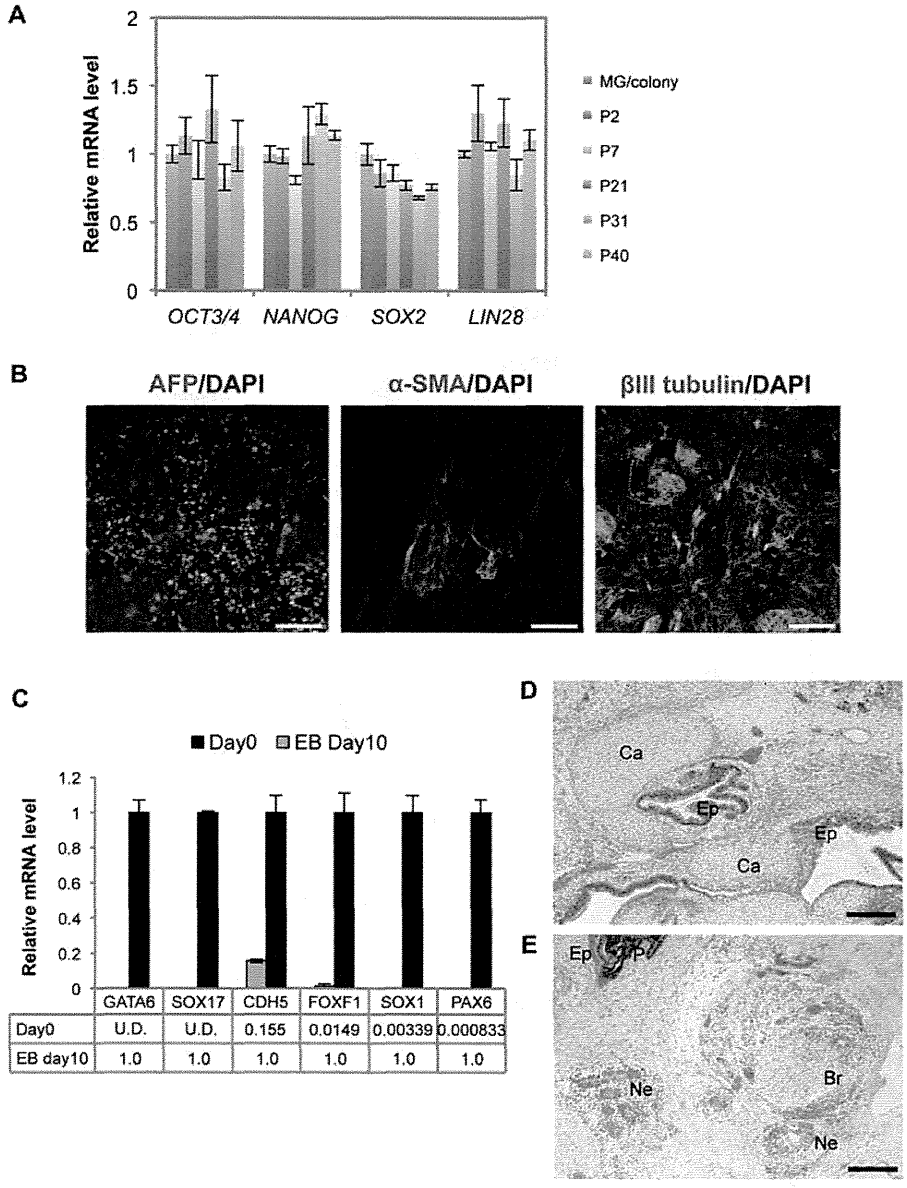

Figure 2. Characterization of 253G1 cells subcultured on laminin-521 in Essential 8 medium. (A) Expression levels of undifferentiated cell markers (*OCT3/4, NANOG, SOX2 and LIN28*) in 253G1 cells subcultured on laminin-521 in Essential 8 were determined using qRT-PCR. Relative mRNA expression levels are presented as ratios to the level of that in control cells on Matrigel. Results are the mean ± SD (n = 3). (B) *In vitro* differentiation analysis of 253G1 cells subcultured on laminin-521 in Essential 8 medium. Immunostaining of the markers for three germ layers are shown: endoderm (alpha-fetoprotein (AFP)), mesoderm (α-smooth muscle actin (SMA)) and ectoderm (βIII tubulin). Scale bars, 200 μm. (C) Expression levels of differentiated cell markers in embryoid bodies (EBs) derived from 253G1 cells: endoderm (*GATA6, SOX17*), mesoderm (*CDH5, FOXF1*), ectoderm (*SOX1, PAX6*). Relative mRNA expression levels are presented as ratios to the level of that in control cells (EBs at Day 10). Results are the mean ± SD (n = 3). (D-E) Teratomas derived from 253G1 cells cultured on laminin-521 in Essential 8 medium are shown. Hematoxylin and eosin staining showed the features of three germ layers: Ep, epithelium-like tissue (endoderm); Ca, cartilage (mesoderm); Ne, neural rosette-like tissue (ectoderm); P, pigmented neuroectodermal resembling meranocyte (ectoderm); Br, brain-like tissue (ectoderm). Scale bars, 200 μm.
doi:10.1371/journal.pone.0110496.g002

To determine whether a culture system using laminin-521 and Essential 8 medium can detect a trace amount of undifferentiated hiPSCs in CTPs, we spiked dissociated hiPSCs into primary human somatic cells and cultured these cells on laminin-521 in Essential 8 medium. As a model of the somatic cells, we employed human mesenchymal stem cells (hMSCs), because "off-the-shelf" hMSCs derived from hPSCs are a promising CTP [12–14]. We spiked 409B2 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells) into 1×10^5 hMSCs and plated these cells onto laminin-521-coated wells. hiPSCs were co-cultured with hMSCs on laminin-521-coated dishes in Essential 8 medium and formed distinctive colonies (Figure 4A). At day 7 after plating, we detected 362, 42.5 and 6 colonies (the mean of duplicate measurements) in 1%, 0.1% and 0.01% spiked samples, respectively (Figure 4B). We did not

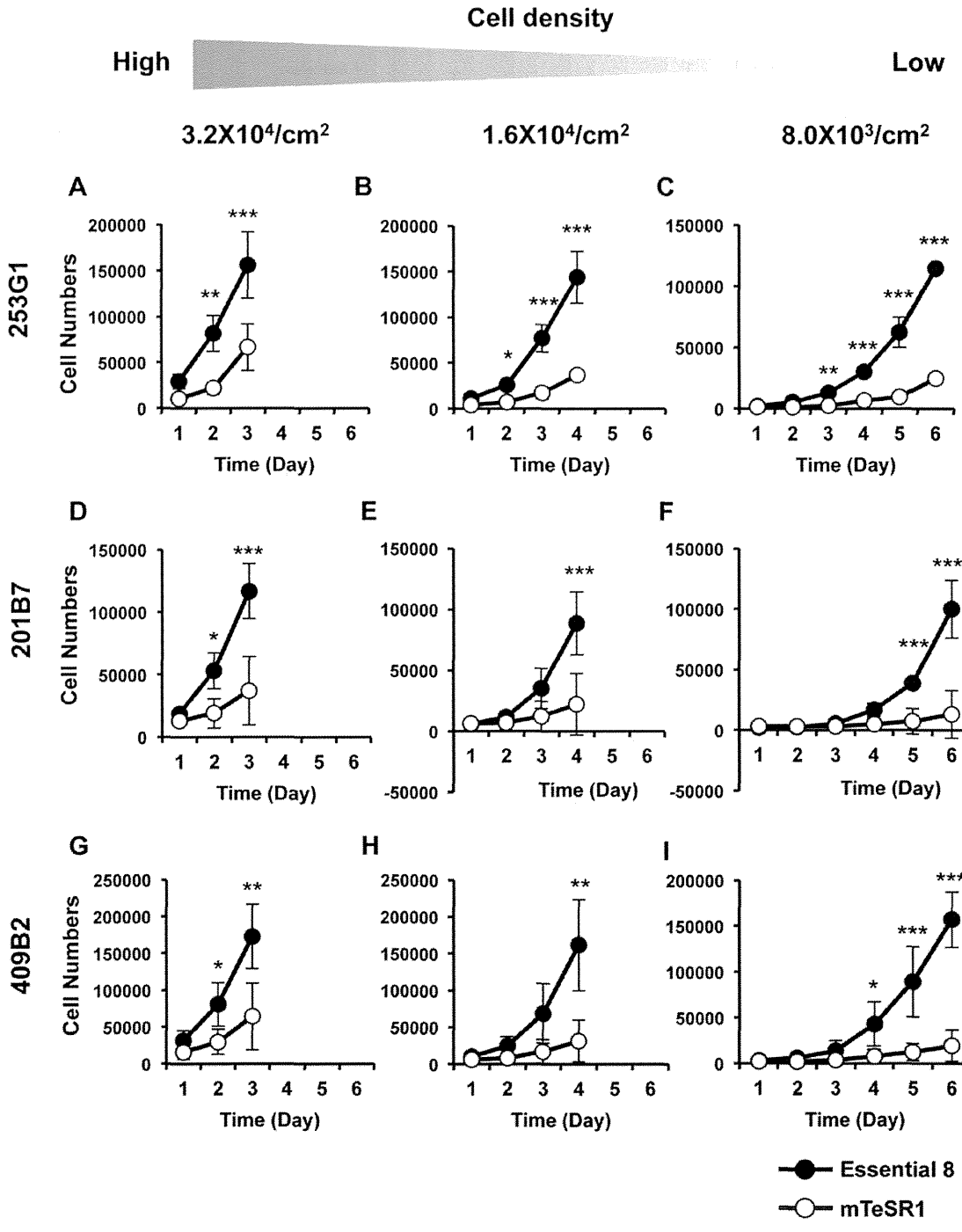

Figure 3. Rapid cell proliferation of hiPSCs plated at low cell density on laminin-521 in Essential 8 medium. (A-I) Quantification of the number of 253G1, 201B7 and 409B2 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted every 24 h after plating at 3.2×10^4 cells/cm^2 (A, D, G), 1.6×10^4 cells/cm^2 (B, E, H) and 8.0×10^3 cells/cm^2 (C, F, I), respectively. Data are presented as the mean ± standard deviation (SD) of three independent experiments (*$P<0.05$, **$P<0.01$, *** $P<0.001$, two-way repeated-measures ANOVA followed by a Bonferroni post-hoc test).
doi:10.1371/journal.pone.0110496.g003

find any colonies when only hMSCs were cultured on laminin-521 in Essential 8 medium. In addition, immunofluorescence staining with anti-TRA-1-60 antibody showed that these colonies formed in an undifferentiated state (Figure 4A), suggesting that colonies derived from hiPSCs were formed in an hMSC monolayer under conditions with laminin-521 and Essential 8 medium. We also

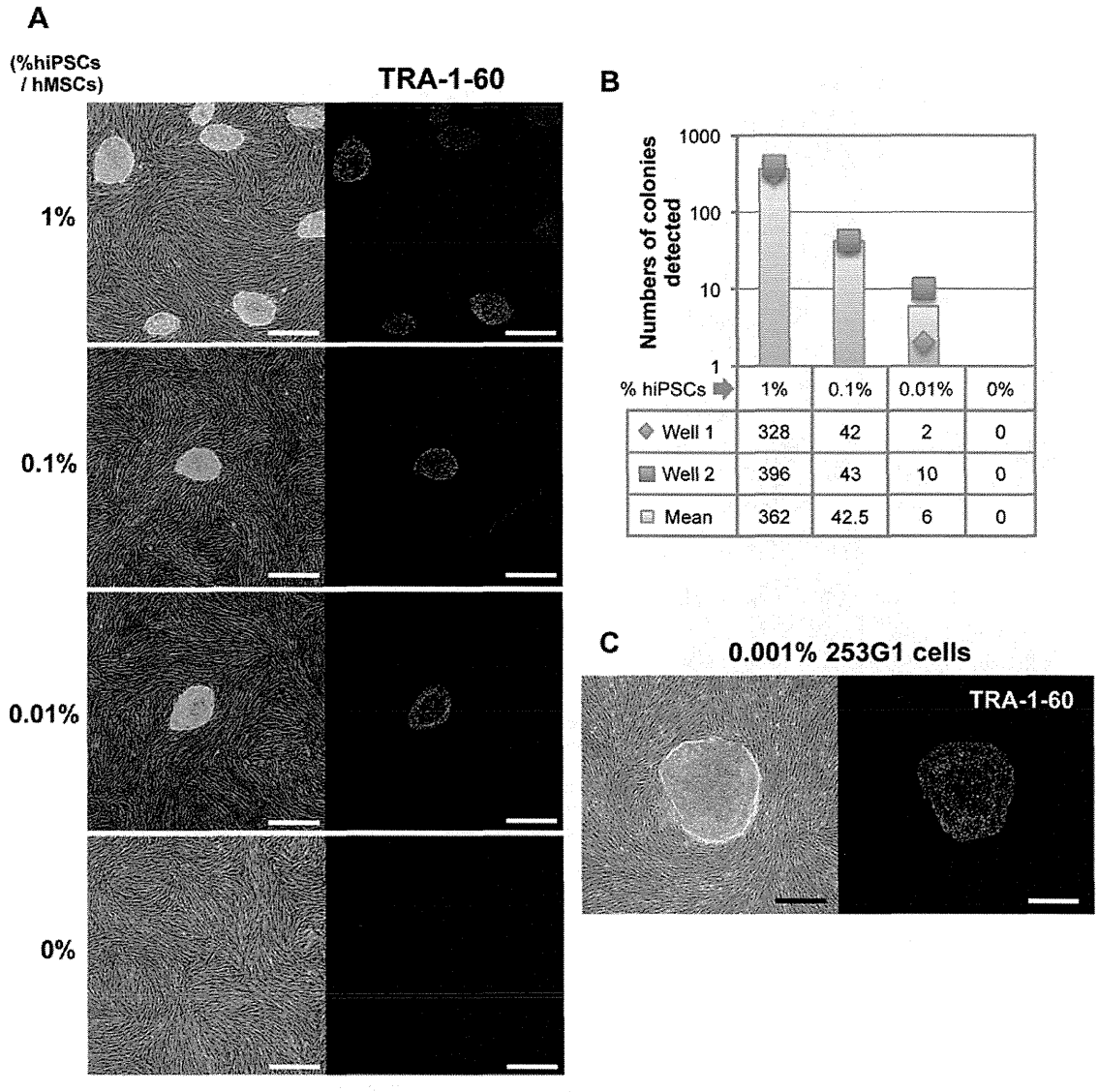

Figure 4. Detection of hiPSCs spiked into hMSCs on the culture system using laminin-521 and Essential 8 medium. (A) Morphologies of forming colonies derived from 409B2 cells spiked into hMSCs are shown (images in the left). 409B2 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 0 cells) were spiked into hMSCs (100,000 cells) and co-cultured on laminin-521-coated wells in 6-well plates in Essential 8 medium for 7 days. Expression of the undifferentiated marker, TRA-1-60, in these colonies was assessed using immunofluorescence staining (images in the right). Each experiment was carried out in duplicate. Scale bars, 500 μm. (B) Numbers of the colonies detected in each spiked sample in (A) are shown. Data are present as raw data in each well (shown by plots) or the mean of well 1 and well 2 (shown by bar graphs). (C) Morphology of a forming colony derived from 253G1 cells spiked into hMSCs at the ratio of 0.001% (6 hiPSCs to 600,000 hMSCs) is shown (images in the left). Mixture of those cells was co-cultured on a 100-mm cell culture dish coated with laminin-521 in Essential 8 medium for 9 days. Forming colony was stained with anti TRA-1-60 antibody (images in the right). Experiment was carried out in duplicate. Scale bars, 500 μm.
doi:10.1371/journal.pone.0110496.g004

tested another hiPSC line, 253G1, for undifferentiated cells spiked into hMSCs. We found that 253G1 cells spiked into hMSCs at the ratio of 1% and 0.1% formed approximately 100 and 20 colonies, respectively, on laminin-521 in Essential 8 medium (Figure S4). We detected one colony when 253G1 cells were spiked into hMSCs at a ratio of 0.01% or 0.001% and co-cultured on a laminin-521-coated dish in Essential 8 medium (Figure S4 and Figure 4C). Taken together, our culture system using laminin-521 and Essential 8 medium allows the direct detection of 0.001%–0.01% hiPSCs in hMSCs as a result of efficient cell amplification.

We also confirmed that no colonies were detected when a mixture of hiPSCs and hMSCs were cultured on laminin-521 in MSCGM medium instead of Essential 8 medium. In the absence of laminin-521, several colonies were detected in Essential 8 when hMSCs contained 1% hiPSCs but not when hMSCs contained 0.1% and 0.01% hiPSCs (data not shown). These results suggest that laminin-521 is required to detect trace amounts of hiPSCs in hMSCs (less than 0.1%).

To know whether this culture system also works in detecting trace amounts of hiPSCs contaminating other types of cells besides

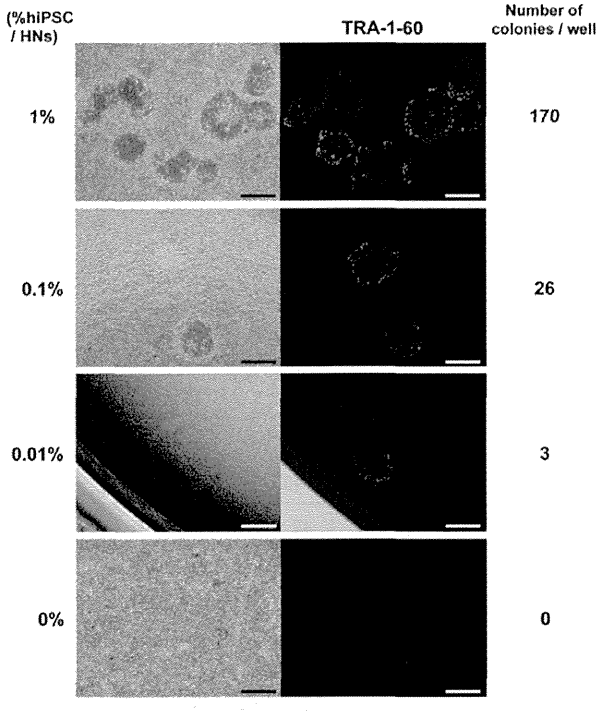

Figure 5. Detection of hiPSCs spiked into human neurons on the culture system using laminin-521 and Essential 8 medium. Morphologies of forming colonies derived from 253G1 cells spiked into human neurons are shown (images in the left). 253G1 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 0 cells) were spiked into human neurons (100,000 cells) and co-cultured on laminin-521-coated wells in 12-well plates in Essential 8 medium for 6 days. Forming colonies were stained with anti TRA-1-60 antibody (images in the right). HNs, human neurons. Scale bars, 500 μm.
doi:10.1371/journal.pone.0110496.g005

hMSCs, we next tested colony formation of hiPSCs spiked into primary human neurons. Spiked 253G1 cells were co-cultured with human neurons on laminin-521 in Essential 8 medium and clearly formed colonies (Figure 5), which is consistent with the observation using hiPSCs spiked into hMSCs. We detected 170, 26 and 3 colonies that were positive for TRA-1-60 when 253G1 cells were spiked into 1×10^5 human neurons at the ratio of 1, 0.1 and 0.01%, respectively. There was no colony when only human neurons were cultured on our system. These results suggest that this culture system is also useful for detection of trace amounts of hiPSCs not only in hMSCs but also in other types of cells such as human neurons. We also confirmed that no colonies were formed on the well that was not coated with laminin-521 even when human neurons containing 10% hiPSCs were plated (data not shown), indicating that formation of the colonies derived from hiPSCs in human neurons is dependent on laminin-521.

Culture system using laminin-521 and Essential 8 medium has a capacity for direct detection of residual undifferentiated cells contained in differentiating hiPSC cultures

Finally, we examined whether this culture system using laminin-521 and Essential 8 medium is applicable in direct detection of residual hiPSCs contained in differentiated cells derived from hiPSCs. We attempted to differentiate 253G1 cells into MSCs as described in Materials and Methods (Figure 6A). Using this protocol, we observed attached cells with fibroblast-like morphology at the stage of passage 0 MSCs. We confirmed that approximately 20% of these attached cells were positive for staining with anti-CD105 antibody, a MSC marker antibody (Figure S5). During the differentiation process of 253G1 cells into MSCs, we examined the expression levels of residual pluripotency markers in the cell cultures. qRT-PCR analysis revealed that expression of *OCT3/4*, *NANOG* and *LIN28* mRNA were clearly decreased in a time-dependent manner, however, expression levels at the same time point varied markedly among those genes (Figure 6B). In the cells at day 6 of differentiation, mRNA levels of *OCT3/4*, *NANOG* and *LIN28* were 7.3%, 4.8% and 86.4% of the control at day 1, respectively (Figure 6B). At day 14 of differentiation, although *OCT3/4* and *LIN28* were still at detectable levels of 2.6% and 17.2% of control cells, respectively, *NANOG* expression was not detected. These results indicate that the population of residual hiPSCs in differentiating cells, when estimated by the qRT-PCR data, greatly varies and depends on the pluripotency marker gene employed for the estimation. In addition, it is also possible that all the qRT-PCR signals were derived from partially differentiated cells, not from fully undifferentiated cells. To examine colony formation of residual undifferentiated cells in differentiating cell culture, cells at day 6 were dissociated into single cells and replated on laminin-521 in Essential 8 medium. Small cell clusters began to emerge 4 days after plating, rapidly expanded and formed colonies on laminin-521 in Essential 8, while other types of cells gradually decreased their numbers (Figure 6D). After 8 days of culture, 9.5 colonies (the mean of duplicate measurements) were formed from differentiating cells (5×10^4) (Figure 6C) and they were all positive for TRA-1-60 (Figure 6D), indicating that the colonies were derived from residual undifferentiated cells in the differentiating cell cultures. These results suggest that the culture method using a combination of laminin-521 and Essential 8 directly detects residual undifferentiated cells by highly efficient cell amplification. Based on our finding that approximately 0.3 and 6.7 colonies were formed from 1×10^4 MSCs containing 0.01% and 0.1% of 253G1 cells, respectively, in this culture system (Figure S4), and assuming that the sensitivity of the system for hPSCs in EBs are comparable to that in MSCs, the population of the undifferentiated cells in the differentiating cell cultures on day 6 (1.9 colonies/10^4 cells) was estimated to be in between 0.01% and 0.1%. When we tested colony formation using cell cultures on day 14 of differentiation, no colonies were detected on laminin-521 in Essential 8 medium (Figure 6C and data not shown), suggesting that the population of the residual hiPSCs was less than 0.01%.

Discussion

A method to detect residual undifferentiated hPSCs contained in CTPs is required to evaluate product quality during manufacturing processes. In the present study, we propose a novel method to detect a trace amount of undifferentiated hPSCs by highly efficient amplification of those cells *in vitro*. We showed that Essential 8 medium significantly promotes cell growth of hiPSCs dissociated into single cells on laminin-521 compared with the conventional medium, mTeSR1. In addition, Essential 8 medium allowed robust proliferation of hiPSCs even at low cell density on laminin-521. We also demonstrated that 0.001%–0.01% hiPSCs spiked into primary hMSCs were clearly detected and formed colonies on laminin-521 in Essential 8. Similarly, we confirmed that 0.01% hiPSCs spiked into primary human neurons were also detectable on this system. Moreover, we showed that residual undifferentiated hiPSCs contained in differentiating cells were

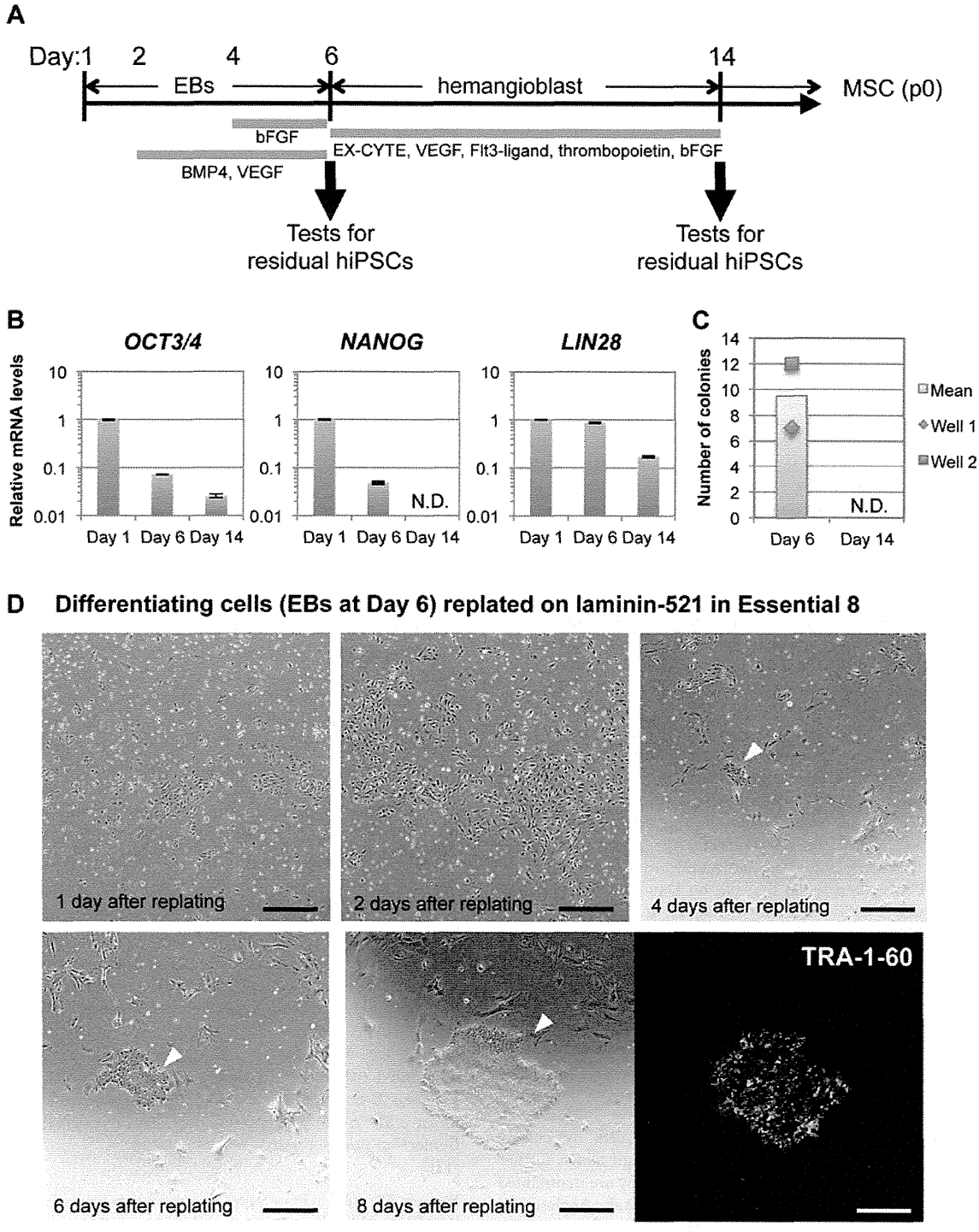

Figure 6. Detection of residual undifferentiated cells contained in differentiating cell cultures. (A) Differentiation scheme of 253G1 cells into MSCs is shown. (B) Expression levels of undifferentiated cell markers (*OCT3/4, NANOG* and *LIN28*) in each cell culture were determined using qRT-PCR. Relative mRNA expression levels are presented as ratios to the level of that in 253G1 cells at Day 1. Results are the mean ± SD (n = 3). (C) Numbers of the forming colonies derived from residual undifferentiated cells in differentiating cell culture at Day 6 or Day 14 are shown. Experiments were carried out in duplicate. Data are present as raw data in each well (shown by plots) or the mean of well 1 and well 2 (shown by bar graphs). (D) Phase contrast images of forming colonies derived from residual undifferentiated cells are shown. Cells at Day 6 of differentiation (EBs) were dissociated into single cells by Accutase and cultured on laminin-521-coated wells in Essential 8 medium (5×10^4/well). After 4 days of culture, small clusters emerged and then started to grow rapidly. Finally, they formed colonies that were positive for TRA-1-60 (shown by immunofluorescence staining, green). Arrowheads indicate a colony derived from same origin. Scale bars, 500 μm.
doi:10.1371/journal.pone.0110496.g006