transferred onto a well of 96-well plates, and then incubated at 4 °C for 30 min to allow the bottom agar layer to solidify. HeLa cells and hMSCs were dissociated into a single cell suspension by treatment with 0.25% trypsin-EDTA solution (Life Technologies) and passed through 40 μm nylon cell strainers (BD Falcon). Next, 25 μl of cell suspensions containing serially diluted HeLa cells (0, 10, 20, 30, 50, and 100 cells) and hMSCs (1.0×10^4 cells) in DMEM/10% FBS, were mixed with 25 μl of 2 × DMEM/20% FBS and 25 μl of 1.2% agar. After being placed on the bottom agar layer, the top agar layers were immediately solidified at 4 °C for 10 min to avoid false-positive signals derived from sedimentation-induced contact between the cells. The plates were incubated with 100 μl of DMEM/10% FBS per well for 10 and 20 days at 37 °C and 5% CO_2. The medium was changed every 2–3 days. Colonies were lysed and quantified with CyQuant GR dye using a fluorometer equipped with a 485/520 nm filter set (Wallac 1420 ARVOsx multilabel counter, PerkinElmer, Boston, MA). Results were evaluated as a relative fold change of the value of negative control (hMSCs only). The lower limit of detection (LLOD) of the assay signal was calculated as the mean plus 3.3 fold the standard deviation of the measurement of the three lots of hMSCs [14].

3. Results

3.1. The tumorigenic potential of HeLa cells in nude and NOG mice

We first tried to evaluate the tumorigenic potential of human tumorigenic cells in NOG mice in the absence or presence of Matrigel, compared with that in nude mice. Namely, we examined the tumor formation of HeLa cells transplanted into the subcutaneous spaces of mice, and tumorigenic incidence was compared between nude and NOG mice for 16 weeks (Fig. 1a–c and Table 1). The development of spontaneous tumors was not observed in non-transplant mice of either strain during the period of monitoring. Nude mice, which are traditional standards for tumorigenicity testing, showed no tumor formation when HeLa cells were transplanted at a dose of up to 1.0×10^5 cells. On the other hand, NOG mice developed tumors with a lower cell transplantation dosage (1.0×10^4 cells). Transplanting HeLa cells with Matrigel considerably increased the tumor formation potential of HeLa cells in NOG mice. Notably, subcutaneous transplantation of 1.0×10^3 HeLa cells gave rise to tumors in NOG mice when embedded with Matrigel (10/10 animals) but not without Matrigel (0/10 animals). Furthermore, 60% (6/10 animals) of NOG mice formed tumors within 16 weeks when 1.0×10^2 HeLa cells were transplanted with Matrigel subcutaneously. Next, to compare tumor forming potential of nude mice and NOG mice more quantitatively, we calculated a 50% tumor producing dose, TPD_{50}, of HeLa cells in NOG and nude mice. At the end of monitoring for 16 weeks, NOG mice exhibited $TPD_{50} = 1.3 \times 10^4$ when injected with HeLa cells in the absence of Matrigel (Table 1). As expected, tumorigenic potential of HeLa cells was enhanced 30-fold when transplanted in NOG mice compared with that in nude mice ($TPD_{50} = 4.0 \times 10^5$ at week 16). Furthermore, tumorigenic potential of HeLa cell was enhanced 5000-fold when HeLa cells are embedded with Matrigel and transplanted in NOG mice ($TPD_{50} = 7.9 \times 10$ at week 16) compared with that in nude mice without Matrigel. Thus, NOG mice showed superior tumor forming potential when the tumor cells are embedded with Matrigel.

Transplanted cells progressively formed a large spheroid tumor at the inoculation site without invading host subcutaneous tissue (Fig. 2d). Tumor mass increases in a dose- and time-dependent manner in both mouse strains (Fig. 2a–c). To confirm the origin of tumors engrafted in the NOG mice, embedded tissue sections were stained with anti-human HLA antibody. The immunohistochemical analysis demonstrated that the engrafted tumors originated from human cells (Fig. 2e). No histological difference was observed between tumor in nude mice and that in NOG mice (data not shown).

3.2. Detection of tumors in NOG mice inoculated with HeLa cells spiked into hMSCs

Next we attempted to determine the characteristics of the test using NOG mice and Matrigel for detection of tumorigenic cellular impurities in human somatic cells. For this end, HeLa cells (1.0×10, 1.0×10^2, 1.0×10^3, and 1.0×10^4) were spiked into 1.0×10^6 hMSCs, and then subcutaneously transplanted into NOG mice with Matrigel. Tumor formation at the transplanted site was continuously monitored for 16 weeks. Subcutaneous transplantation of hMSCs alone in NOG mice did not generate tumor in any mice during the monitoring period (Table 2). Within 16 weeks after transplanation, 50% of the NOG mice (3/6 animals) generated subcutaneous tumors derived from 1.0×10^2 HeLa cells spiked in 1.0×10^6 hMSCs (Fig. 3a). The TPD_{50} of HeLa cells transplanted in NOG mice with 1.0×10^6 hMSCs and Matrigel was 1.0×10^2 at week 16, which was almost the same as the TPD_{50} transplanted with HeLa cells alone (Tables 1 and 2). These results indicated that *in vivo* tumorigenicity tests using NOG mice and Matrigel are able to detect over 0.01% HeLa cell contamination in hMSCs, which is equivalent to a single tumor cell contamination in 10,000 somatic cells. To determine sensitivity of tumorigenicity tests using NOG mice, we spiked 1.0×10 (0.0001%) and 1.0×10^2 (0.001%) HeLa cells into hMSCs (1.0×10^7 cells), which was tenfold the dose used in the previous experiments, and subcutaneously transplanted them into NOG mice with Matrigel. Two and out of 6 NOG mice inoculated with 1×10^2 HeLa cells generated subcutaneous tumors within 16 weeks when a higher dose of hMSCs was co-transplanted (Fig. 3b). It is of note that one out of 6 mice transplanted with 1×10 HeLa cells and 1×10^7 hMSCs also showed tumor formation. The TPD_{50} of HeLa cells transplanted with 1.0×10^7 hMSCs and Matrigel in NOG mice was 1.8×10^2 at week 16 (Table 2). These results are quite

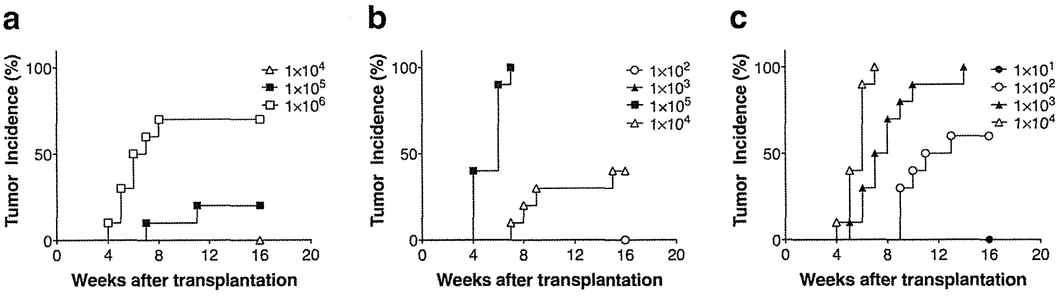


Fig. 1. Tumor incidence of HeLa cells in nude and NOG mice. The tumor formation of HeLa cells transplanted into the subcutaneous spaces of mice was examined for 16 weeks. The relationships between the dose and the tumorigenic incidence of HeLa cells in nude mice (a) and NOG without (b) or with (c) Matrigel are presented (n = 10 in each group).

Table 1
Tumor-forming capacity of HeLa cells in nude and NOG mice.

Strain	Group	Tumor incidence at indicated HeLa cell dose at 16w								TPD_{50} at week 16	Fold-change in TPD_{50} (vs. nude mice)
		0	1 × 10	1 × 10^2	1 × 10^3	1 × 10^4	1 × 10^5	1 × 10^6	1 × 10^7		
Nude	HeLa	0/10[a]	–	–	–	0/10	2/10	7/10	(10/10)[b]	4.0 × 10^5	1.0
NOG	HeLa	0/10	–	0/10	0/10	4/10	10/10	–	–	1.3 × 10^4	3.0 × 10
NOG	HeLa w/MG	0/10	0/10	6/10	10/10	10/10	–	–	–	7.9 × 10	5.0 × 10^3

–: Not tested.
MG: Matrigel.
[a] No. of mice in which tumors formed/total no. of mice inoculated.
[b] Since not all animals inoculated with the highest dose (10^6) formed tumors, it was assumed that the tumor incidence of animals at an even higher dose step (a dummy set of data) would have been 100% for the Spearman-Kärber method to be applicable.

consistent with those without a mixture of hMSCs (Fig. 1c), demonstrating that HeLa cells can grow under subcutaneous environments of NOG mice without significant effect from cotansplanted hMSCs. Taken together, our results indicated that *in vivo* tumorigenicity tests with NOG mice in the combination with Matrigel has the ability to detect 100 HeLa cells spiked into hMSCs in almost half of mice.

Morphological observation of tumors originated from HeLa cells spiked in hMSCs and HeLa cells alone was identical (Figs. 2d and 3c). The immunohistochemical analysis of the tissue sections using anti-human HLA monoclonal antibody clearly demonstrated that the engrafted tumors originated from human cells (Fig. 3d). Vimentin, an intermediated filament protein, is known to express in the process of epithelial to mesenchymal transition and is commonly used as one of the markers of mesenchymal stem cells [15,16]. Negative staining with anti-vimentin antibody suggested that formed tumors were attributed to the exceeding growth of HeLa cells (Fig. 3e).

3.3. Changes of TPD_{50} values depending on the time course

The tumor development of HeLa cells in nude and NOG mice under various conditions are shown as the transition of TPD_{50} at weekly intervals. Nude and NOG mice inoculated with HeLa cells without Matrigel demonstrated only a slight decrease in TPD_{50} values from 8 weeks following injection (Fig. 4). On the other hand, TPD_{50} values rapidly decreased until 12 weeks and then almost reached a plateau until 16 weeks when NOG mice were inoculated with cells in combination with Matrigel. These results suggest that Matrigel is able to support the growth of transplanted small number of tumor cells that cannot survive without Matrigel, and a tumor originated from small number of tumorigenic cells takes time to form visible mass.

3.4. Soft agar colony formation assay for detection of HeLa cell contamination

The soft agar colony formation assay is a suitable method to monitor anchorage-independent cell growth and a well-known *in vitro* assay for the detection of malignant transformed cells [17,18]. HeLa cells enclosed by soft agar showed progressive formation of colonies (Fig. 5a), whereas hMSCs did not form any colonies in a soft agar media with 1 × 10^4 cells/well by day 20 (Fig. 5b and c). We next spiked several concentrations of HeLa cells into 1 × 10^4 hMSCs to determine the minimum number of HeLa cells required for growth in a soft agar media. More than 0.2% spiked

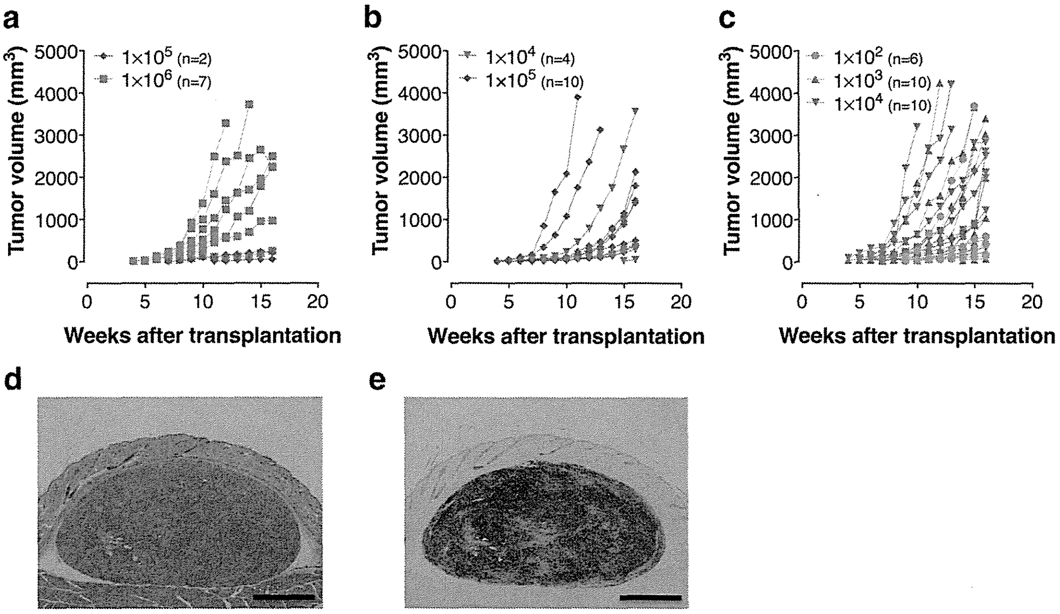


Fig. 2. Characterization of subcutaneous tumors formed by transplantation with HeLa cells in nude and NOG mice. Growth curves of subcutaneous tumors formed by inoculation with various dosages of HeLa cells were presented in respective mice (a: nude; b: NOG w/o Matrigel; c: NOG w/Matrigel). The tumor volume (TV) was calculated using the formula volume = 1/2 × length (mm) × (width [mm])^2. The successive engraftment was determined according to progressive nodule growth at the injection site. Mice were euthanized and necropsied when tumors reached approximately 20 mm in any dimension or when a sign of deconditioning was noted. Representative images from histology and immunohistochemistry analyses of subcutaneous tumors in NOG mice formed by transplantation with 1.0 × 10^2 HeLa cells suspended in Matrigel (d and e). Serial sections were stained with H&E (d) and HLA antibody (e) (magnification, 40×; scale bars, 1 mm).

Table 2
Tumor-forming capacity of HeLa cells mixed in hMSCs in NOG mice.

Strain	Group	Tumor incidence at indicated HeLa cell dose at 16w					TPD_{50} at week 16
		0	1 × 10	1 × 10^2	1 × 10^3	1 × 10^4	
NOG	HeLa[a]	–	–	–	–	6/6[b]	ND
NOG	HeLa/hMSC (1 × 10^6)	0/6	0/6	3/6	6/6	6/6	1.0 × 10^2
NOG	HeLa/hMSC (1 × 10^7)	0/6	1/6	2/6	–	(6/6)[c]	1.8 × 10^2

–: Not tested.

ND: Not determined.

[a] Single dose group as a positive control.

[b] No. of mice in which tumors formed/total no. of mice inoculated.

[c] Since not all animals inoculated with the highest dose (10^2) have formed tumors, it was assumed that the tumor incidence of animals at an even higher dose step (a dummy set of data) would have been 100% for the Spearman-Kärber method to be applicable.

HeLa cells gave rise to detectable colonies within 10 days (Fig. 5c). Fewer HeLa cells formed detectable colonies within 20 days (0.1%: 10 cells/well). Since the LLOD is calculated as means + 3.3 × standard deviation (SD) [14], the LLOD of the soft agar transformation assay was 2.06 based on signals from three lots of hMSCs as a negative control (1.0 ± 0.3 [fold over the background signal]) (Fig. 5d). Referring to the LLOD, even the minimum number of HeLa cells (0.1%) gave rise to distinct detectable colonies at day 20. These results indicated that the soft agar colony formation assay was able to detect colonies generated from at least 0.1% HeLa cells spiked into hMSCs within 20 days.

4. Discussion

The contamination of hCTPs with tumorigenic cells is an issue of concern for product manufacture. Although immunodeficient nude mice are commonly used to assess the tumorigenicity of cell substrates used for production of biological products, more sensitive methods are required to detect a trace amount of tumorigenic cellular impurities in hCTPs. Superior xeno-engraftment capacity of NOG mice were previously reported using HeLa S3 cells [6]. Here, in combination with Matrigel, we have further developed and quantitatively characterized highly sensitive *in vivo* tumorigenicity tests using NOG mice to detect trace amounts of tumorigenic cellular impurities in hCTPs. Subcutaneous transplantation into NOG mice with Matrigel allowed inoculation with 10^7 cells and actually achieved detection of 0.002% and 0.0001% HeLa cells spiked into hMSCs at probabilities of 50% and 17%, respectively. Moreover, the TPD_{50} of our methods was 5000-fold higher than that of tumorigenicity tests using traditional nude mice at 16 weeks following injection. We also showed that soft agar colony formation assay could detect at least 0.1% HeLa cells spiked into hMSCs within 3 weeks as a representative *in vitro* tumorigenicity tests.

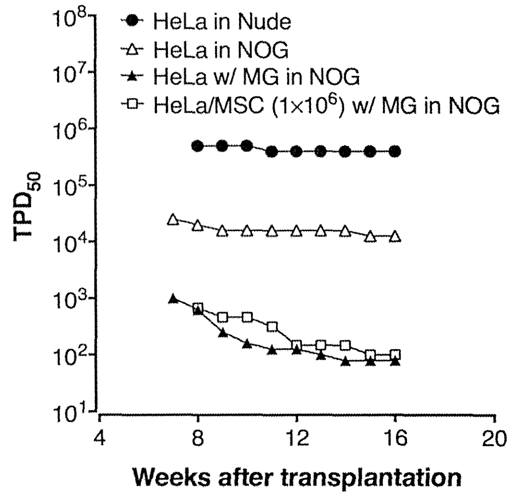


Fig. 4. Comparative development of HeLa cell tumors in nude and NOG mice under various conditions. HeLa cell tumor development in nude and NOG mice under various conditions are expressed as transition of TPD_{50} at weekly intervals. MG, Matrigel.

WHO TRS 878 recommends T-cell deficient nude mice for tumorigenicity tests. However, tumorigenicity tests using nude mice are not sensitive enough for detecting trace amounts of

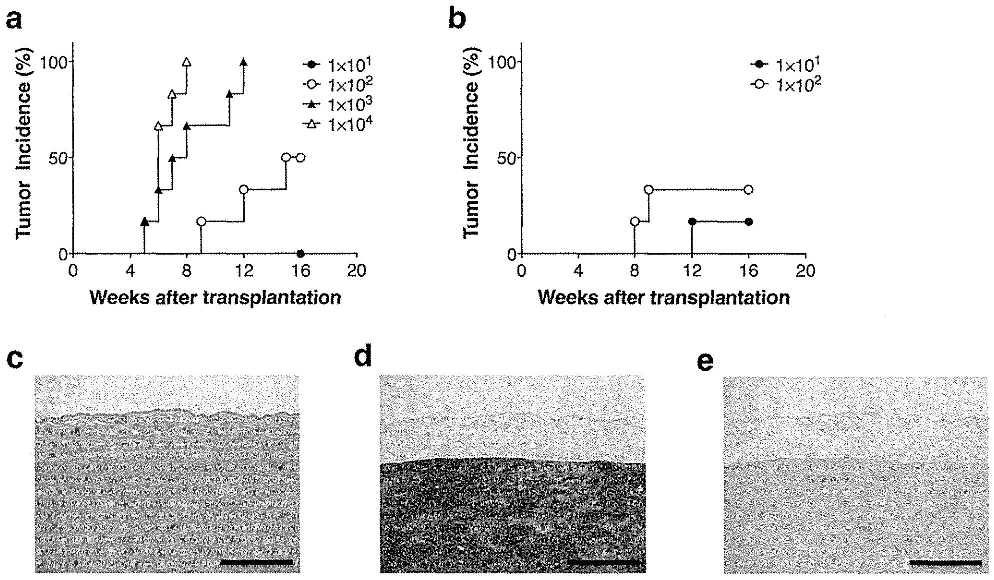


Fig. 3. Detection of tumors in NOG mice transplanted with HeLa cells spiked into hMSCs. The cohorts inoculated HeLa cells spiked in 10^6 and 10^7 hMSCs cells were observed for 16 weeks, respectively. The relationships between the cell dose and the tumorigenic incidence of HeLa spiked in 1.0 × 10^6 (a) and 1.0 × 10^7 (b) hMSCs cells in NOG mice are presented (n = 6 in each group). Representative images from histology and immunohistochemistry analyses of subcutaneous tumors in NOG mice formed by transplantation with 1.0 × 10^4 HeLa cells mixed in 1.0 × 10^6 hMSCs suspended in Matrigel (c, d, and e). Serial sections were stained with H&E (c), HLA antibody (d), and Vimentin antibody (e) (magnification, 100×; scale bars, 500 μm).

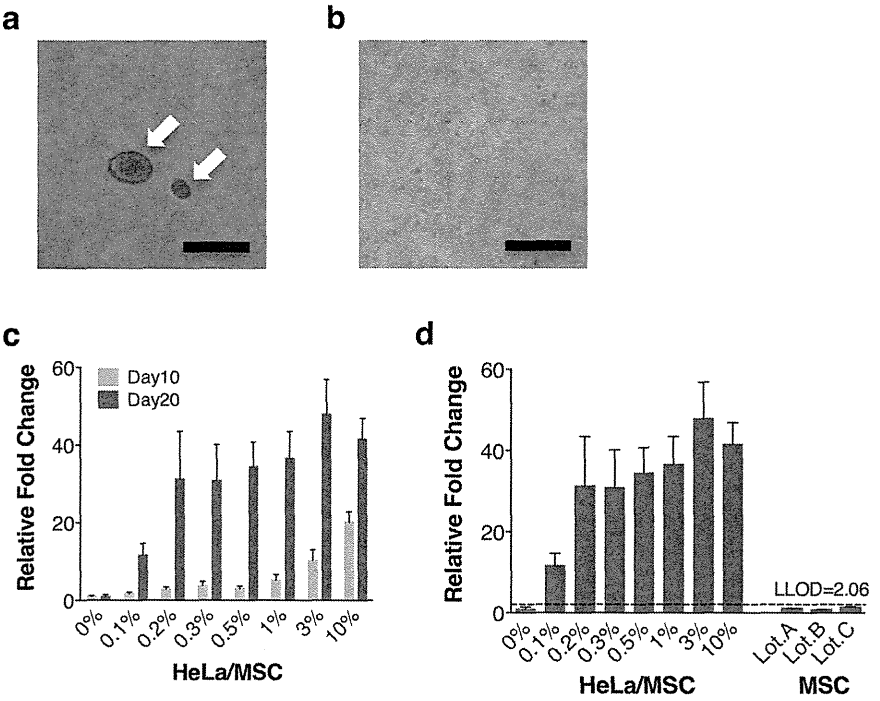


Fig. 5. Soft agar colony formation assay of HeLa cells. HeLa cells enclosed in soft agar showed progressive formation of colonies (a), whereas hMSCs did not form any colonies in a soft agar media with 1.0×10^4 cells/well by day 20 (b). Arrows indicate the colonies of HeLa cells (magnification, 100×; scale bars, 250 μm). HeLa cells (0%, 0 cells; 0.1%, 10 cells; 0.2%, 20 cells; 0.3%, 30 cells; 0.5%, 50 cells; 1%, 100 cells; 3%, 300 cells; 10%, 1000 cells) were spiked into 1.0×10^4 hMSCs and grown in soft agar for 10 and 20 days (c). HeLa cells spiked into hMSCs and three lots of hMSCs were grown in soft agar for 20 days (d). The lower limit of detection (LLOD) was calculated as the mean plus 3.3 fold the standard deviation of the measurement of the three lots of hMSCs. Quantification of the results is described in (d). Cell growth was quantified using a CytoSelect™ kit. Results were expressed as a relative fold change of the value of negative control (hMSC lot A). Error bars represent the standard deviation of the measurements ($n = 3$).

tumorigenic cells. In fact, the TPD$_{50}$ was previously reported to be 6.3×10^4 and 3.2×10^6 of human tumorigenic HeLa cells and HEK293 cells, respectively [19]. In the present study, tumorigenicity tests using nude mice transplanted subcutaneously with HeLa cells indicated TPD$_{50}$ = 4.0×10^5 (Table 1). Our method using NOG mice and Matrigel tremendously improved the TPD$_{50}$, and its value was 7.9×10 when transplanted with HeLa cells (Table 1). In addition to nude mice, NOD/SCID mice are frequently used for tumor biology and xeno-graft research. To establish the NOD/SCID mouse strain, SCID mutation impairing T and B cell lymphocyte development was transferred onto a non-obese diabetic background deficient in NK cell function [20,21]. NOD/SCID mice are known to demonstrate a high incidence of thymic lymphomas with age [22,23]. The NOG mice used in this study were generated by mating C57BL/6J-γC^{null} and NOD/Shi-scid mice [4]. The IL2 receptor common γ-chain is indispensable for IL2, IL4, IL7, IL9, IL15, and IL21 high affinity binding and signaling, and is also thought to play a key role in mediating susceptibility to thymic lymphomas in mice [5]. NOG mice never show the high incidence of thymic lymphomas characterized in NOD/SCID mice [24], which was consistent with our results. The incidence of thymic lymphomas often shortens the lifespans of mice and would lead to confusing tumorigenicity test results. Tumorigenicity tests using NOG mice are also able to resolve the issues arising from the pathological properties of NOD/SCID mice.

Matrigel is a tissue basement membrane matrix rich in extracellular matrix (ECM) proteins that was originally isolated from the mouse tumor the murine Engelbreth-Holm-Swarm sarcoma [25]. Matrigel is known to facilitate human tumor xenograft growth in rodents [26], which was confirmed in the present study using HeLa cells and NOG mice, presumably by providing the extracellular environment for tumor growth. However, Matrigel is derived from mouse cells, not of human origin. Since the human tissue-mimetic microenvironment is preferable for the estimation of tumorigenicity of hCTPs in clinical settings, development of a new human-derived ECM proteins as an alternative to Matrigel may be necessary for more precise estimation of the tumorigenic properties of hCTPs inoculated into human tissues.

Soft agar colony formation assay is an anchorage-independent growth assay in soft agar, which is considered the most stringent *in vitro* assay to detect transformed cells. In this study, the soft agar colony formation assay was able to detect colonies generated from at least 0.1% HeLa cells spiked into hMSCs within 20 days (Fig. 5c). Based on a standard curve by plotting the assay signals, the LLOD, which was calculated as 2.06 (Fig. 5d), corresponded to approximately 0.02% HeLa cell contamination. This method is easy, inexpensive, and time-saving, but its sensitivity to detect transformed cells is lower compared with *in vivo* tumorigenicity tests using NOG mice and Matrigel (Fig. 3a–b and Table 2). Understanding the abilities and limitations of individual tumorigenicity tests, we need to select appropriate tests to evaluate hCTPs.

Products derived from somatic cells, e.g. hMSCs, are extensively developed in industry all over the world. To our knowledge, human adult somatic cells have not yet been reported to form tumor after clinical application until now. Although at least four research papers have previously reported the *in vitro* spontaneous transformation of hMSCs [27–30], two of them were retracted later because their observations turned out to have come from cross-contamination with tumorigenic cells [31,32]. In the other two of the four papers, the immortal growth was easily detected *in vitro* [29,30], indicating that the process control to avoid cross-contamination and the monitoring of cell growth at/after the limit of *in vitro* cell age used for production would be more critical for the quality of products derived from human somatic cells, rather than the detection of malignant transformation during cell processing.

HeLa cells, the oldest immortal and tumorigenic human cell line in the history of the world, are well-characterized, currently available almost anywhere in the world, and extensively used for a wide variety of biomedical research. There have been many reports of HeLa cell cross-contamination in mammalian cell lines, and the use of HeLa-contaminated cell lines has been a big problem over several decades [33,34]. Therefore, we employed HeLa cells as a model of tumorigenic cellular impurities in the present study.

Alternatively, we also expect the application of our method for detecting the tumorigenic contamination of products derived from human pluripotent stem cells (hPSCs) as well as human somatic cells. It is actually being attempted for hPSCs such as embryonic stem cells to differentiate into hMSCs and they are expected to be used as or for hCTPs [35–37]. Although a lot of allogenic applications of primary hMSCs are currently being developed, hMSCs derived from clonal hPSCs are presumed to have an advantage of quality control in respect to robust manufacturing and restricted virus infection. However, since undifferentiated hPSCs are tumorigenic, the contamination of the final products with residual PSCs is one of the biggest concerns [2]. Recently, we showed that the NOG mouse was the most sensitive animal in terms of tumor formation from human induced pluripotent stem cells (hiPSCs) among several immunosuppressed animals, with the TPD_{50} value in between 10^2 and 10^3, when injected subcutaneously with Matrigel [38]. In the same paper, we also showed that, when hiPSCs were co-administered with Matrigel and retinal pigment epithelial cells derived from hiPSCs, the TPD_{50} value was increased to approximately 10^3 to 10^4, presumably attributable to paracrine action of pigment epithelium-derived factor with a pro-apoptotic effect on hiPSCs [38]. These results, combined with those in the present study, suggest that the *in vivo* tumorigenicity test using NOG mice is commonly applicable to the detection of tumorigenic or pluripotent cellular impurities in a variety of hCTPs.

To our knowledge, the *in vivo* tumorigenicity test using NOG mice in the present study is one of the most sensitive methods to detect a trace amount of tumorigenic cells in normal cells. However, hCTPs may contain quite a large number of cells in clinical setting. In some cases of cellular therapy, one hundred million or more cells are necessary [39]. For example, 1×10^8 or more cells are required for the treatment of liver disease [40]. For one patient with heart failure, preferably more than one billion cardiomyocytes need to be transplanted [41]. The sensitivity of the assay methods in the present study would be insufficient to evaluate tumorigenicity of hCTPs like above. The problem is that no method is currently available to detect one or a few tumorigenic cells in more than 10^7 normal cells. Further studies are necessary to develop more sensitive tests for hCTPs. Improvements of the tests (e.g. development of new animal models or matrix better than Matrigel), *in vivo/in vitro* tumorigenicity tests for cells cultured beyond the limit of *in vitro* cell age used for production, or combinations of the tests (e.g. *in vivo* test for cell colonies in soft agar) may be options.

Another problem is that scientific interpretations of the results from *in vivo* tumorigenicity tests have not been established, because the tests have not been well-characterized from a viewpoint of the evaluation of hCTPs. In the present study, we quantitatively characterized the *in vivo* tumorigenicity test, and determined its ability of detection of a trace amount of tumorigenic cells. According to the results in Table 2, the *in vivo* tumorigenicity test using NOG mice and Matrigel detects as low as 0.0001% cellular impurities at a probability of 17%, in case that the tumorigenicity of the impurities is comparable to that of HeLa cells in hMSCs. Conversely, the false negative rate (x) of an NOG mouse inoculated with the hCTP containing the 0.0001% cellular impurities is (100−17=) 83%. Thus, the false negative rate (y) of n mice can be expressed as follows:

$$y = x^n$$

Hence, we obtain:

$$n = \log y / \log x$$

For example, when permitting 1% false negative rate in the whole test, 25 (=log (0.01)/log (0.83)) mice are necessary to prove the absence of the 0.0001% cellular impurities. In other words, if 25 or more mice are used for a tumoriginicity test, and if no tumor formation is observed in all the mice, the test result indicates, at a false negative rate of 1%, that the hCTPs is not contaminated with 0.0001% or more HeLa-like tumorigenic cells.

This well-characterized test can be applicable at least to quality assessment/control of hCTPs. Namely, although "Negative in the NOG mouse test" may not directly guarantee the "safety" of the final product, the negative result can be one of critical "quality" attributes to demonstrate the absence a certain number of tumorigenic cells. To date, no international/domestic regulatory authority has issued guidelines for the tumorigenicity tests of hCTPs. Further studies are necessary to establish and share principles, paradigms, and standardized methods for measurements and interpretations of tumorigenicity of hCTPs, which will greatly contribute to the development of safe hCTPs of high quality.

5. Conclusions

In this study, we have demonstrated that NOG mice in combination with Matrigel demonstrated superior efficiency in engraftment of HeLa cells, compared with nude mice that are recommended in WHO guideline. They also showed an ability to detect as little as 100 HeLa cells present in hMSCs in almost half of mice. These results suggest that the *in vivo* tumorigenicity test using NOG mice with Matrigel is a highly sensitive and quantitative method to detect a trace amount of tumorigenic cellular impurities in human somatic cells, which can be useful in quality assessment of hCTPs.

Acknowledgments

This work was supported by a Strategic Fund for the Promotion of Science and Technology from the Japan Science and Technology Agency (AIRO-KAISHO Project), and Research Grants from the Japanese Ministry of Health, Labour and Welfare (H23-SAISEI-IPPAN-005, H24-IYAKU-SHITEI-027, H25-JITSUYOKA(SAISEI)-IPPAN-008, and Marketing Authorization Facilitation Program for Innovative Therapeutic Products).

Conflict of Interest

All authors declare no conflict of interest.

References

[1] World Health Organization. Requirements for the use of animal cells as in vitro substrates for the production of biologicals. WHO technical report series. 1998. No 878 Annex 1.
[2] Kuroda T, Yasuda S, Sato Y. Tumorigenicity studies for human pluripotent stem cell-derived products. Biological Pharm Bull 2013;36(2):189–92.
[3] Goldman JP, Blundell MP, Lopes L, Kinnon C, Di Santo JP, Thrasher AJ. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. Br J Haematol 1998;103(2):335–42.
[4] Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100(9):3175–82.

[5] Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 2005;174(10):6477–89.
[6] Machida K, Suemizu H, Kawai K, Ishikawa T, Sawada R, Ohnishi Y, et al. Higher susceptibility of NOG mice to xenotransplanted tumors. J Toxicol Sci 2009;34(1):123–7.
[7] Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ. Efficient tumour formation by single human melanoma cells. Nature 2008;456(7222):593–8.
[8] Lewis Jr AM, Alling DW, Banks SM, Soddu S, Cook JL. Evaluating virus-transformed cell tumorigenicity. J virological Methods 1999;79(1):41–50.
[9] Omeir RL, Teferedegne B, Foseh GS, Beren JJ, Snoy PJ, Brinster LR, et al. Heterogeneity of the tumorigenic phenotype expressed by Madin-Darby canine kidney cells. Comp Med 2011;61(3):243–50.
[10] U.S. Food and Drug Administration. Proposed use of a 50% limit of detection value in defining uncertainty limits in the validation of presence-absence microbial detection methods. 2006. Final Report and Executive Summaries from the AOAC International Presidential Task Force on Best Practices in Microbiological Methodology Appendix K.
[11] Hamilton MA, Russo RC, Thurston RV. Trimmed Spearman-Karber method for estimating Median Lethal concentrations in toxicity bioassays. Environ Sci Tech 1977;11(7):714–9.
[12] Lorenz RG, Bogel K. Methods of calculation. In: Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. 3rd ed. 1973. p. 321–35.
[13] Kuroda T, Yasuda S, Kusakawa S, Hirata N, Kanda Y, Suzuki K, et al. Highly sensitive in vitro methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPS cells. PloS one 2012;7(5):e37342.
[14] Miller JN, Miller JC. Statistics and Chemometrics for Analytical Chemistry. 5th ed. 2005. Published by Pearson Education Canada.
[15] Kato N, Shimmura S, Kawakita T, Miyashita H, Ogawa Y, Yoshida S, et al. Beta-catenin activation and epithelial-mesenchymal transition in the pathogenesis of pterygium. Invest Ophthalmol Vis Sci 2007;48(4):1511–7.
[16] Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. Vimentin and epithelial-mesenchymal transition in human breast cancer-observations in vitro and in vivo. Cells Tissues Organs 2007;185(1–3):191–203.
[17] Cifone MA, Fidler IJ. Correlation of patterns of anchorage-independent growth with in vivo behavior of cells from a murine fibrosarcoma. Proc Natl Acad Sci U. S. A. 1980;77(2):1039–43.
[18] Boyd DD, Levine AE, Brattain DE, McKnight MK, Brattain MG. Comparison of growth requirements of two human intratumoral colon carcinoma cell lines in monolayer and soft agarose. Cancer Res 1988;48(9):2469–74.
[19] **Lewis AM. Regulatory Implications of Neoplastic cell substrate tumorigenicity. 2005.** http://www.fda.gov/ohrms/dockets/ac/05/slides/5-4188s1_2.ppt **[Last accessed 3.12.2014].**
[20] Koyanagi Y, Tanaka Y, Tanaka R, Misawa N, Kawano Y, Tanaka T, et al. High levels of viremia in hu-PBL-NOD-scid mice with HIV-1 infection. Leukemia 1997;11(Suppl 3):109–12.
[21] Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB, Tennent B, et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 1995;154(1):180–91.
[22] Prochazka M, Gaskins HR, Shultz LD, Leiter EH. The nonobese diabetic scid mouse: model for spontaneous thymomagenesis associated with immunodeficiency. Proc Natl Acad Sci U. S. A. 1992;89(8):3290–4.
[23] Christianson SW, Greiner DL, Hesselton RA, Leif JH, Wagar EJ, Schweitzer IB, et al. Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice. J Immunol 1997;158(8):3578–86.
[24] Kato C, Fujii E, Chen YJ, Endaya BB, Matsubara K, Suzuki M, et al. Spontaneous thymic lymphomas in the non-obese diabetic/Shi-scid, IL-2R gamma (null) mouse. Lab Anim 2009;43(4):402–4.
[25] Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, et al. Basement membrane complexes with biological activity. Biochemistry 1986;25(2):312–8.
[26] Fridman R, Giaccone G, Kanemoto T, Martin GR, Gazdar AF, Mulshine JL. Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. Proc Natl Acad Sci U. S. A. 1990;87(17):6698–702.
[27] Rosland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, et al. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res 2009;69(13):5331–9.
[28] Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res 2005;65(8):3035–9.
[29] Tang DQ, Wang Q, Burkhardt BR, Litherland SA, Atkinson MA, Yang LJ. In vitro generation of functional insulin-producing cells from human bone marrow-derived stem cells, but long-term culture running risk of malignant transformation. Am J Stem Cells 2012;1(2):114–27.
[30] Wang Y, Huso DL, Harrington J, Kellner J, Jeong DK, Turney J, et al. Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy 2005;7(6):509–19.
[31] Garcia S, Bernad A, Martin MC, Cigudosa JC, Garcia-Castro J, de la Fuente R. Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. Exp Cell Res 2010;316(9):1648–50.
[32] Torsvik A, Rosland GV, Svendsen A, Molven A, Immervoll H, McCormack E, et al. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track – letter. Cancer Res 2010;70(15):6393–6.
[33] Chatterjee R. Cell biology. When 60 lines don't add up. Science 2007;315(5814):929.
[34] Buehring GC, Eby EA, Eby MJ. Cell line cross-contamination: how aware are mammalian cell culturists of the problem and how to monitor it? Vitro Cell Dev Biol Animal 2004;40(7):211–5.
[35] Jung Y, Bauer G, Nolta JA. Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. Stem Cells 2012;30(1):42–7.
[36] Chen YS, Pelekanos RA, Ellis RL, Horne R, Wolvetang EJ, Fisk NM. Small molecule mesengenic induction of human induced pluripotent stem cells to generate mesenchymal stem/stromal cells. Stem cells Transl Med 2012;1(2):83–95.
[37] Kimbrel EA, Kouris NA, Yavanian GJ, Chu J, Qin Y, Chan A, et al. Mesenchymal stem cell population derived from human pluripotent stem cells displays potent immunomodulatory and therapeutic properties. Stem Cells Dev 2014;23(14):1611–24.
[38] Kanemura H, Go MJ, Nishishita N, Sakai N, Kamao H, Sato Y, et al. Pigment epithelium-derived factor secreted from retinal pigment epithelium facilitates apoptotic cell death of iPSC. Sci Reports 2013;3:2334.
[39] Hayashi E, Hosoda T. Therapeutic application of cardiac stem cells and other cell types. BioMed Res Int 2013;2013:736815.
[40] Ohashi K, Park F, Kay MA. Hepatocyte transplantation: clinical and experimental application. J Mol Med 2001;79(11):617–30.
[41] Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature 2014;510(7504):273–7.

OPEN ACCESS Freely available online PLOS | ONE

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

Keiko Tano[1,2], Satoshi Yasuda[2], Takuya Kuroda[2], Hirohisa Saito[3], Akihiro Umezawa[1], Yoji Sato[2,4,5,6]*

1 Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo, Japan, **2** Division of Cellular & Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan, **3** National Research Institute for Child Health and Development, Tokyo, Japan, **4** Department of Quality Assurance Science for Pharmaceuticals, Graduate School of Pharmaceutical Sciences, Nagoya City University, Aichi, Japan, **5** Department of Cellular & Gene Therapy Products, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan, **6** Department of Translational Pharmaceutical Sciences, Graduated School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

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.

* Email: yoji@nihs.go.jp

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

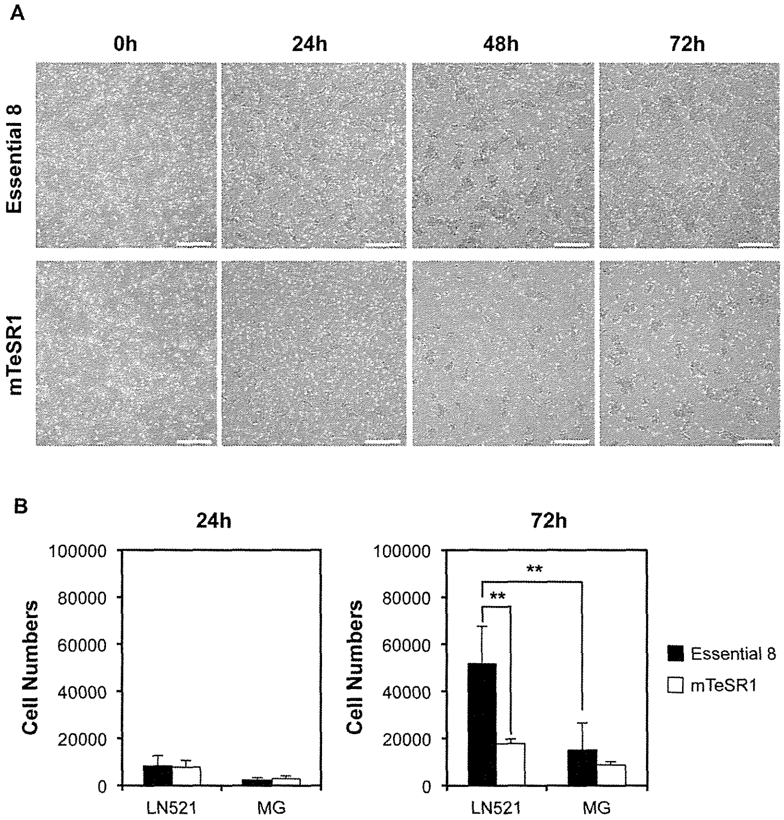


Figure 1. Robust proliferation of 253G1 cells cultured on laminin-521 in Essential 8 medium. (A) Morphology of the 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium after dissociation into single cells. Scale bars, 500 µm. (B) Quantification of the number of dissociated 253G1 cells 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

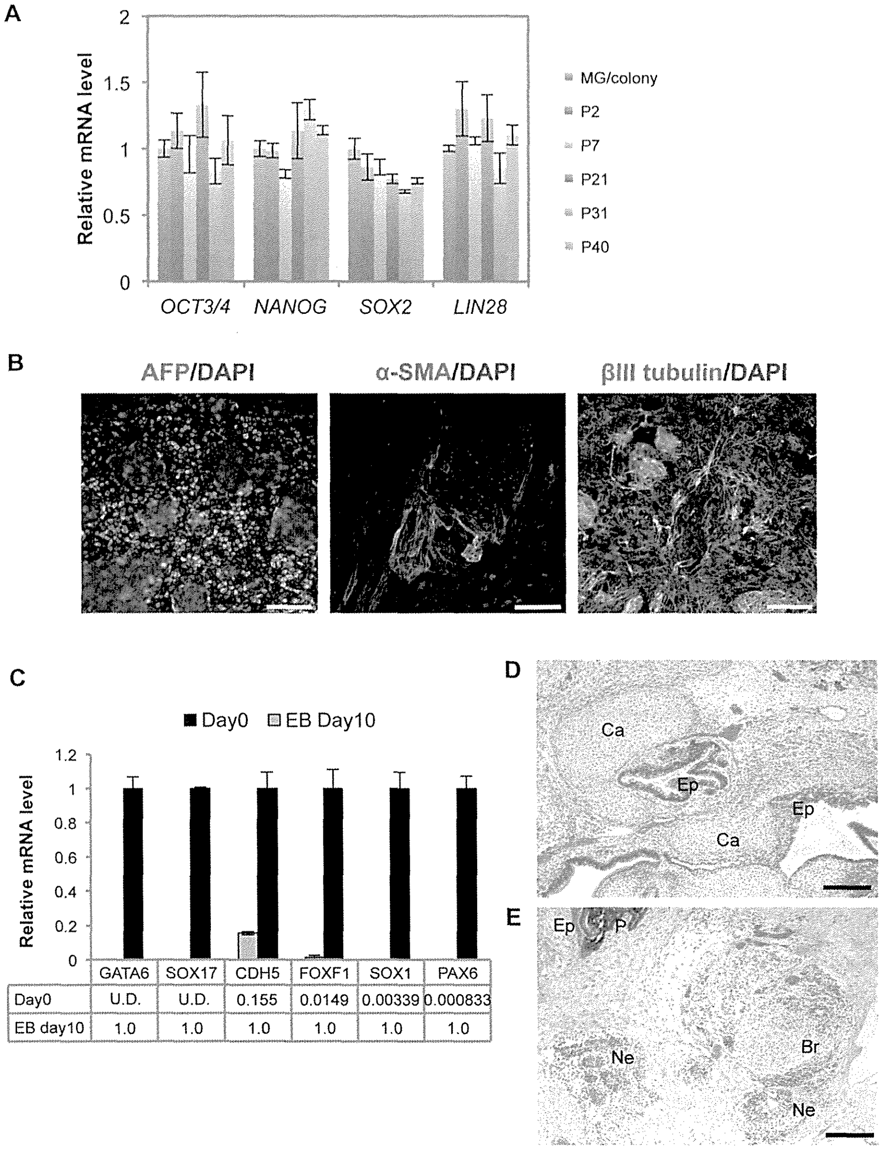
differentiation protocols. Immunofluorescence analysis using antibodies specific for markers of three germ layers clearly demonstrated that 253G1 cells subcultured with laminin-521 and Essential 8 medium could selectively differentiate into endoderm, mesoderm and ectoderm expressing AFP, α-SMA and βIII 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].



	GATA6	SOX17	CDH5	FOXF1	SOX1	PAX6
Day0	U.D.	U.D.	0.155	0.0149	0.00339	0.000833
EB day10	1.0	1.0	1.0	1.0	1.0	1.0

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

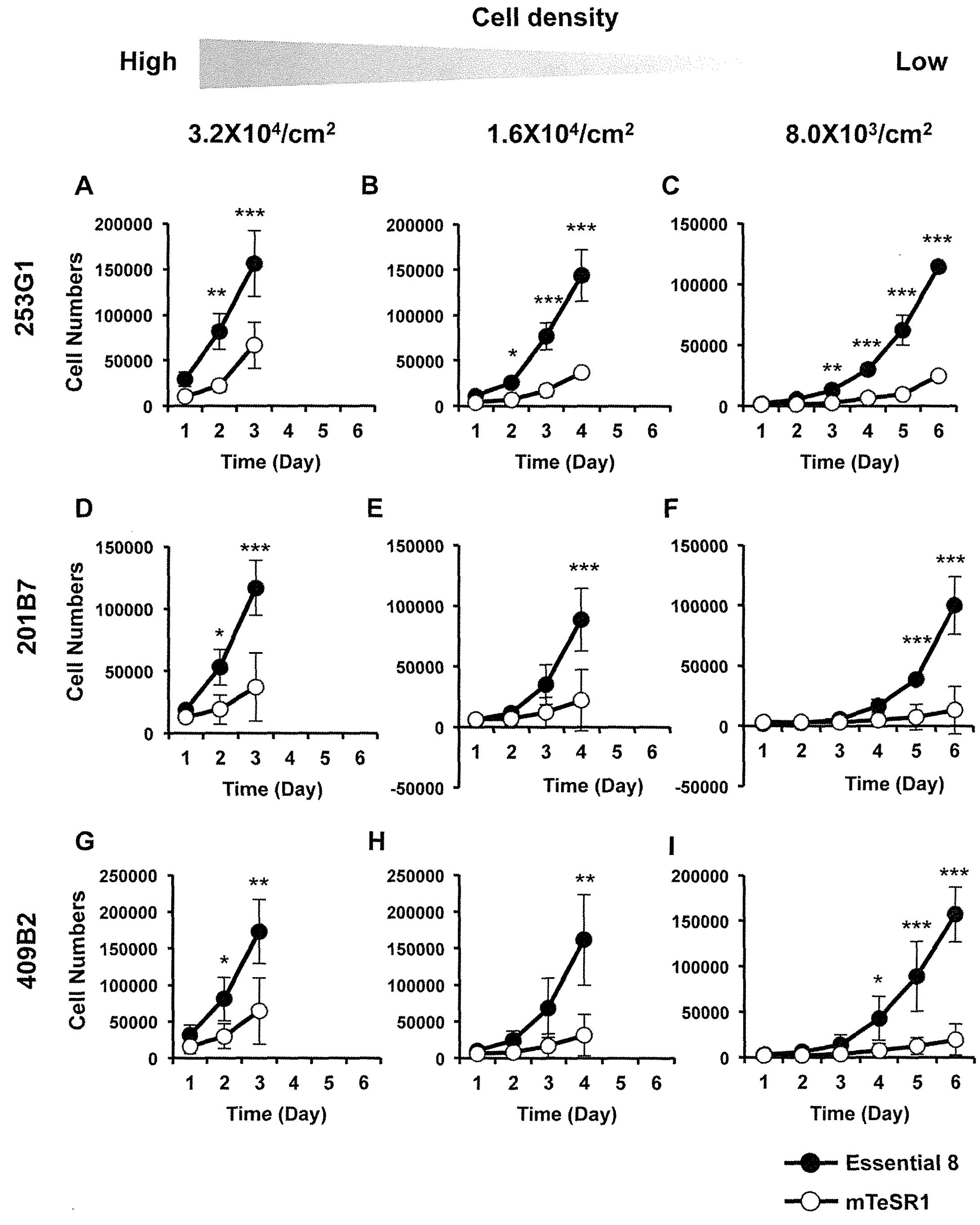


Figure 3. Rapid cell proliferation of hiPSCs plated at low cell density on laminin-521 in Essential 8 medium. (A-I) Quantification of the number of 253G1, 201B7 and 409B2 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted every 24 h after plating at 3.2×10^4 cells/cm^2 (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

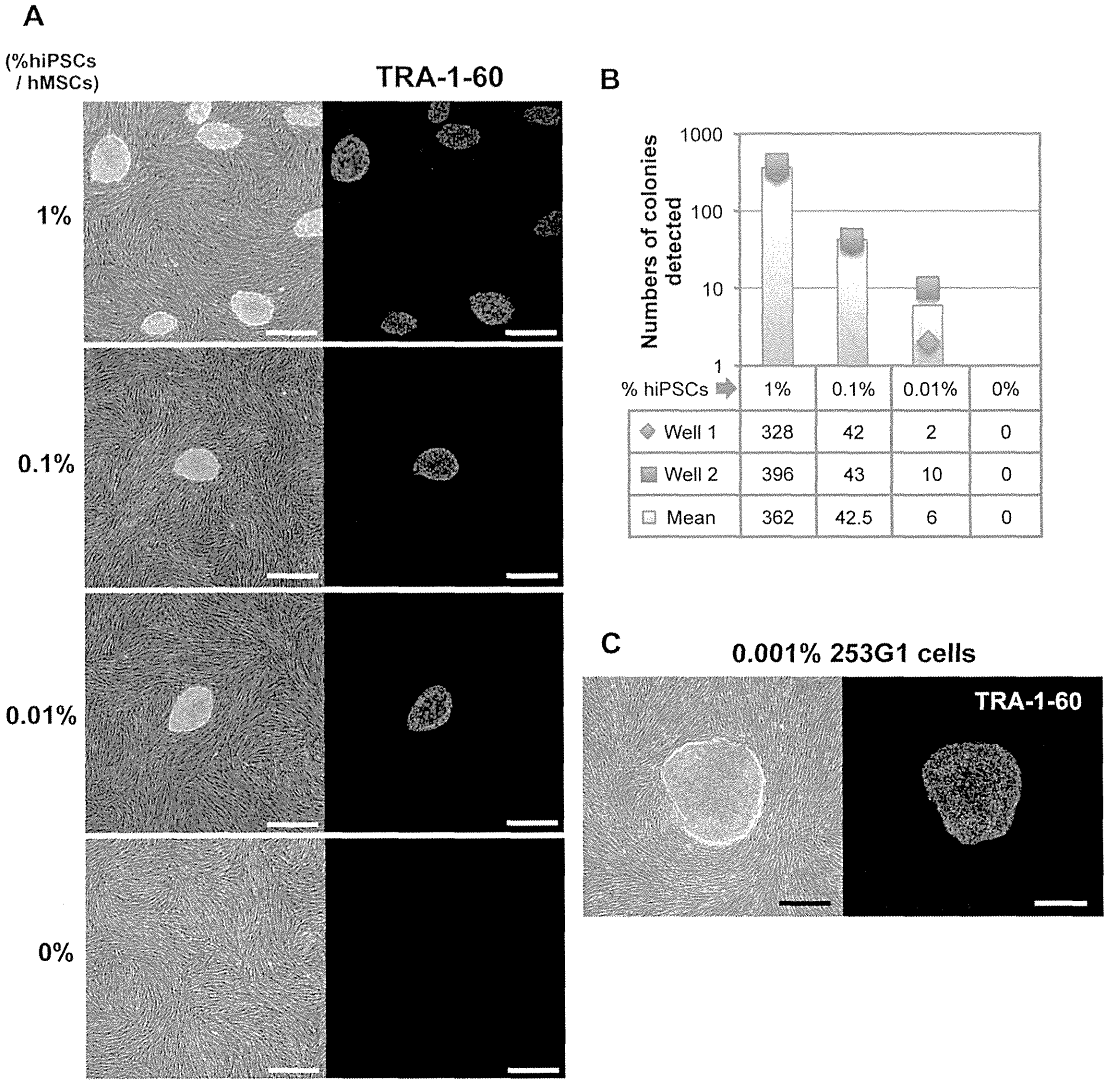


Figure 4. Detection of hiPSCs spiked into hMSCs on the culture system using laminin-521 and Essential 8 medium. (A) Morphologies of forming colonies derived from 409B2 cells spiked into hMSCs are shown (images in the left). 409B2 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 0 cells) were 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

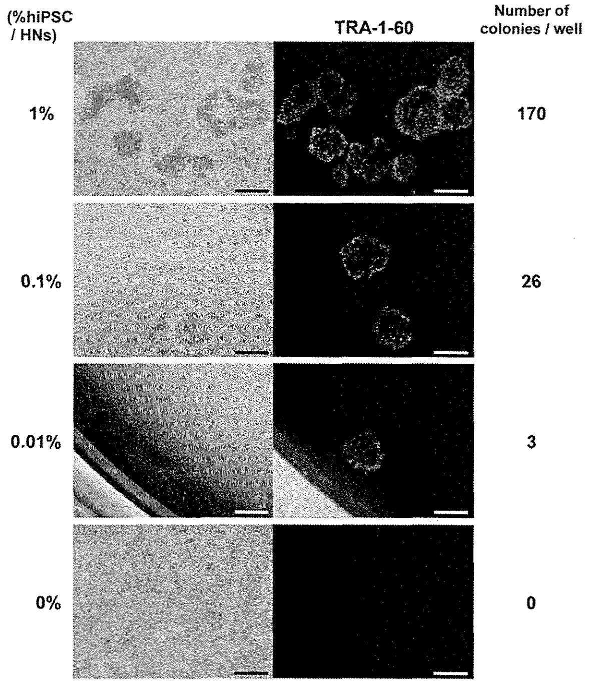


Figure 5. Detection of hiPSCs spiked into human neurons on the culture system using laminin-521 and Essential 8 medium. Morphologies of forming colonies derived from 253G1 cells spiked into human neurons are shown (images in the left). 253G1 cells (1%, 1000 cells; 0.1%, 100 cells; 0.01%, 10 cells; 0%, 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

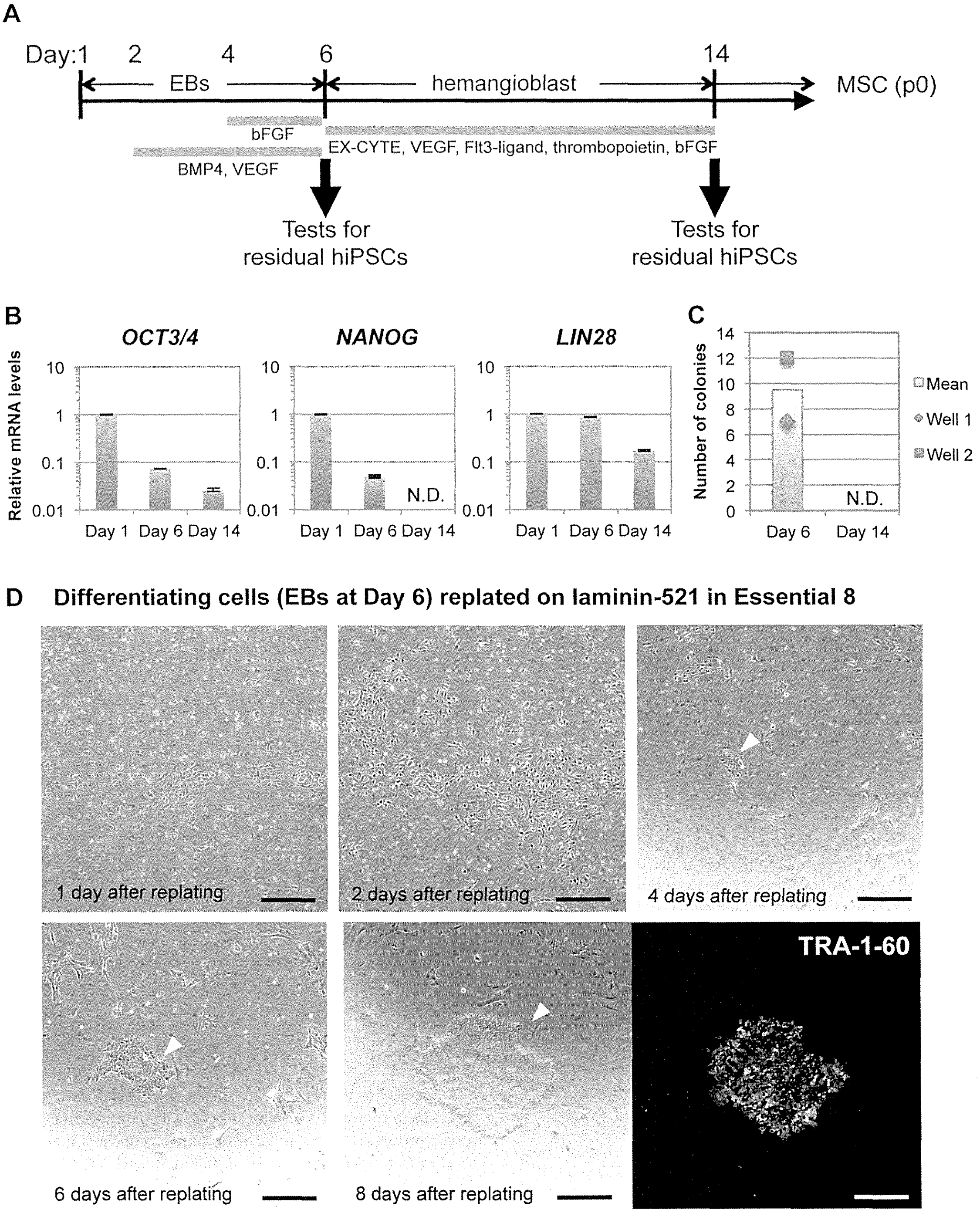


Figure 6. Detection of residual undifferentiated cells contained in differentiating cell cultures. (A) Differentiation scheme of 253G1 cells into MSCs is shown. (B) Expression levels of undifferentiated cell markers (*OCT3/4, NANOG* and *LIN28*) in each cell culture were determined using qRT-PCR. Relative 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

detectable by forming colonies on laminin-521 in Essential 8 in the process of hMSC differentiation. These results indicate that a culture system utilizing a combination of laminin-521 and Essential 8 medium provides a direct and highly sensitive method for detecting undifferentiated hPSCs. To our knowledge, this is the first report to show a direct and highly-sensitive *in vitro* method for detecting undifferentiated hPSCs as impurities in CTPs.

In this study, highly efficient amplification of undifferentiated hPSCs has been uniquely applied to quality control of CTPs. Amplified hPSC colonies were visible using phase-contrast microscopy and also immunofluorescence staining using pluripotency antibodies, which enabled direct detection of hPSCs contaminating CTPs. Our method distinguished between undifferentiated cells and other cells *in vitro*, and overcame the disadvantage of other *in vitro* methods such as flow cytometry and qRT-PCR. The flow cytometry analysis detects known marker molecules expressed in undifferentiated hPSCs using antibodies and proteins. Signals originating from non-specific detection commonly affect sensitivity of the assay as background. Our *in vitro* method can lower the background arising from non-specific detection and is expected to specifically detect residual undifferentiated hPSCs in CTPs. The qRT-PCR method is highly sensitive and can rapidly quantify undifferentiated cell contamination in CTPs. However, in the present study, gene expression levels of pluripotency markers during the differentiation process of hiPSCs into MSCs varied markedly among those marker genes (Figure 6B). Moreover, there remains a possibility that expression signals of marker genes were not derived from totally undifferentiated hPSCs, but from partially differentiated cells. Indeed, the expression level of *LIN28* did not decrease so much during the differentiation as those of the other genes, which was not obviously associated with the differentiation status of the cells in EBs on Day 6 (Figure 6B), although we have previously reported that *LIN28* was a useful marker for monitoring the level of residual hiPSCs in RPE cells derived from hiPSCs [3]. Thus, it is difficult to determine the presence of residual hiPSCs simply by qRT-PCRs. In contrast, direct detection method using the highly efficient amplification system can clearly detect the presence of intact undifferentiated cells. Based on the result from direct detection of residual hiPSCs when tested the cells on Day 6 of differentiation (approximately 0.01%–0.1%) (Figure 6C-D), it is conceivable that the qRT-PCR signals for the pluripotency marker genes (Figure 6B) are partly derived from residual hiPSCs but mainly derived from partially differentiated cells. Similarly, in the case of the cells at Day 14 of differentiation, the majority of the qRT-PCR signals of *OCT3/4* and *LIN28* (Figure 6B) are considered to be attributable to partially differentiating cells but not to intact hiPSCs. Combination of the *in vitro* methods including our cell culture method would mutually support useful quality assessment of CTPs to detect undifferentiated hPSCs.

In addition to the detection of undifferentiated cells, this culture system using laminin-521 and Essential 8 medium allows further characterization of the undifferentiated cells if they are maintained *in vitro* or inoculated into immunodeficient animals. Analyses for the properties of the residual undifferentiated cells would be necessary not only for the quality assessment of CTPs, but also for improvement of quality specifications of hPSCs as a raw/intermediate material for production of CTPs.

Here, we showed that our culture system is able to detect 0.01% of 409B2 hiPSCs and 0.001% of 253G1 hiPSCs, both of which were spiked into hMSCs (Figure 4). The detection sensitivity for hiPSCs spiked into hMSCs was different between the two hiPSC lines, although such a difference in cell growth on laminin-521 was not found between these two cell lines (Figure 3). This difference may be attributable to the difference in the growth potential of hPSCs in the specific environment provided by CTPs. Kanemura *et al.* have recently demonstrated that hiPSCs co-cultured with iPSC-derived RPE undergo apoptosis by pigment epithelium-derived factor (PEDF) secreted from hiPSC-derived RPE [15], showing that CTPs themselves have the potential to affect cell growth of hPSCs. In the present study, the influence of the co-culture system with hMSCs to the proliferation of hiPSCs might have been different between the two cell lines.

The mechanism by which laminin-521 and Essential 8 medium enhance hiPSCs cell proliferation remains unclear. Rodin *et al.* have recently shown that addition of E-cadherin to laminin-521 permitted the efficient clonal expansion of hESCs [7]. E-cadherin is known to be the primary cell-cell adhesion molecule and essential for hESC survival [16]. We observed that anti-E-cadherin antibody decreased growth potential of hiPSCs under our experimental conditions (data not shown). Therefore, E-cadherin signaling may play some important roles in the rapid cell growth on laminin-521 in Essential 8 medium.

Tumorigenicity is one of the major safety concerns for CTPs derived from hPSCs that are transplanted into patients. However, testing strategies for the tumorigenicity of hPSC-derived CTPs have not yet been established. Here, we introduced a novel testing method for directly detecting a trace amount of undifferentiated hPSCs *in vitro*. The ability of each tumorigenicity-associated test should be taken into consideration to evaluate tumorigenicity of residual undifferentiated hPSCs as impurities in products. *In vivo* tumorigenicity tests using immunodeficient animals can detect tumorigenic cells including undifferentiated hPSCs, but this method is costly and time-consuming. The flow cytometry analysis and qRT-PCR are rapid, but these methods indirectly detect tumorigenic cells depending on marker molecules. Risk of tumorigenicity in hPSCs-derived CTPs should be assessed, based on the results from an appropriate combination of these tumorigenicity-associated tests. Our novel method will contribute to establishment of the testing strategies for tumorigenicity in products, following evaluation of the quality of CTPs derived from hPSCs for the future regenerative medicine/cell therapy.

Supporting Information

Figure S1 (A) Quantification of the number of dissociated 201B7 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 a Bonferroni post-hoc test). LN521, laminin-521. MG, Matrigel. (B) Quantification of the number of dissociated 201B7 cells expanded on laminin-521 or LM511-E8 in Essential 8 or mTeSR1 medium. Results are presented as the mean ±SD (n = 3) (***$P<0.001$, two-way ANOVA followed by a Bonferroni post-hoc test).
(TIF)

Figure S2 (A-B) Expression levels of undifferentiated markers (*OCT3/4, NANOG, SOX2* and *LIN28*) in 201B7 cells (A) and 409B2 cells (B) 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 subcultured on Matrigel in mTeSR1 medium by colony passage. Results are presented as the mean ± SD (n = 3). (C-D) Expression levels of markers for the differentiation of embryoid bodies (EBs) derived from 201B7 cells (C) and 409B2 cells (D): endoderm (*GATA6, SOX17*), mesoderm (*CDH5, FOXF1*), and 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 presented as the mean ± SD ($n = 3$).
(TIF)

Figure S3 Quantification of the number of 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted at day 6, 9, and 12 after plating at 8.0×10^3 cells/cm^2 or 8.0×10^2 cells/cm^2.
(TIF)

Figure S4 Morphologies of forming colonies derived from 253G1 cells spiked into hMSCs are shown (images in the left). 253G1 cells (1%, 300 cells; 0.1%, 30 cells; 0.01%, 3 cells; 0%, 0 cells) were spiked into hMSCs (30,000 cells) and co-cultured on 12-well plates coated with laminin-521 in Essential 8 medium for 9 days. Expression of the undifferentiated cell marker, TRA-1-60, in these colonies was assessed using immunofluorescence staining (images to the right). Each experiment was carried out in duplicate.
(TIF)

Figure S5 Phase contrast images of the cells at day 18 of differentiation (at the stage of passage 0 MSCs) are shown. Expression of MSC marker, CD105, in these cells was examined using immunofluorescence staining (images to the right). Arrowheads indicate the cells that were positive for CD105.
(TIF)

Table S1 Sequences of the primers and probes for qRT-PCR.
(DOCX)

Acknowledgments

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).

Author Contributions

Conceived and designed the experiments: KT SY YS. Performed the experiments: KT. Analyzed the data: KT SY TK HS AU YS. Contributed reagents/materials/analysis tools: KT SY TK HS AU YS. Wrote the paper: KT SY YS. Acquired the funding: HS AU YS.

References

1. Bailey AM (2012) Balancing tissue and tumor formation in regenerative medicine. Sci Transl Med 4: 147fs28. Rev.
2. Kuroda T, Yasuda S, Sato Y (2013) Tumorigenicity studies for human pluripotent stem cell-derived products. Biol. Pharm. Bull 36: 189–192.
3. Kuroda T, Yasuda S, Kusakawa S, Hirata N, Kanda Y, et al. (2012) Highly sensitive in vitro methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPS cells. PLoS One 7: e37342.
4. Food and Drug Administration (2008) Cellular Therapies derived from Human Embryonic Stem Cells-Considerations for Pre-Clinical Safety Testing and Patient Monitoring-April 2008. Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC) Meeting #45. Available: <http://www.fda.gov/ohrms/dockets/ac/08/briefing/2008-0471B1_1.pdf>.
5. Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, et al. (2009) Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. Stem Cell Res 2: 198–210.
6. Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, et al. (2007) A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol 25: 681–686.
7. Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, et al. (2014) Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat. Commun 5: 3195.
8. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, et al. (2012) Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat. Commun 3: 1236.
9. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, et al. (2011) Chemically defined conditions for human iPSC derivation and culture. Nat. Methods 8: 424–429.
10. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, et al. (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol 27: 275–280.
11. Kajiwara M, Aoi T, Okita K, Takahashi R, Inoue H, et al. (2012) Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. Proc Natl Acad Sci U S A 109: 12538–12543.
12. Jung Y, Bauer G, Nolta JA (2012) Concise review: Induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. Stem Cells 30: 42–47.
13. Chen YS, Pelekanos RA, Ellis RL, Horne R, Wolvetang EJ, et al. (2012) Small molecule mesengenic induction of human induced pluripotent stem cells to generate mesenchymal stem/stromal cells. Stem Cells Transl Med 1: 83–95.
14. Kimbrel EA, Kouris NA, Yavanian G, Chu J, Qin Y, et al. (2014) Mesenchymal stem cell population derived from human pluripotent stem cells displays potent immunomodulatory and therapeutic properties. Stem Cells Dev 23: 1611–1624.
15. Kanemura H, Go MJ, Nishishita N, Sakai N, Kamao H, et al. (2013) Pigment epithelium-derived factor secreted from retinal pigment epithelium facilitates apoptotic cell death of iPSC. Sci Rep 3: 2334.
16. Xu Y, Zhu X, Hahm HS, Wei W, Hao E, et al. (2010) Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. Proc Natl Acad Sci U S A 107: 8129–8134.
17. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131: 861–872.
18. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, et al. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol 26: 101–106.
19. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, et al. (2011) A more efficient method to generate integration-free human iPS cells. Nat. Methods 8: 409–412.

Chapter 14

In Vitro Detection of Residual Undifferentiated Cells in Retinal Pigment Epithelial Cells Derived from Human Induced Pluripotent Stem Cells

Takuya Kuroda, Satoshi Yasuda, and Yoji Sato

Abstract

Human pluripotent stem cells (hPSCs) such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are a leading candidate for regenerative medicine/cell therapies because of their capacity for pluripotency and unlimited self-renewal. However, there are significant obstacles preventing the clinical use of hPSCs. A significant safety issues is the presence of residual undifferentiated cells that have the potential to form tumors in vivo. Here, we describe the highly sensitive qRT-PCR methods for detection of residual undifferentiated cells in retinal pigment epithelial (RPE) cells derived from hiPSCs. qRT-PCR using probes and primers targeting *LIN28A (LIN28)* transcripts can detect residual undifferentiated cell levels as low as 0.002 % in hiPSC-derived RPE cells. We expect this method to contribute to process validation and quality control of hiPSC-derived cell therapy product.

Key words Human induced pluripotent stem cells, Retinal pigment epithelial cells, Tumorigenicity, *LIN28*, qRT-PCR

1 Introduction

Pluripotent stem cells such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are pluripotent and have the ability to self-renew. Pluripotency is the ability to differentiate into a variety of cells and self-renewal is the ability to undergo numerous cell division cycles while maintaining cellular identity. Because of these two characteristics, it has been expected that these cell types will provide new sources for robust and continuous production of a variety of cells and tissues for regenerative medicine/cell therapy. Additionally, hiPSCs provide a possible solution to the ethical problems and the immune rejection of hESC-derived cells, thus raising novel avenues for patient-specific cell therapy. As previously reported [1, 2], many attempts are currently under way to differentiate hESCs and hiPSCs into various tissues. Cell therapy using hESCs or iPSCs has already entered

Chrissa Kioussi (ed.), *Stem Cells and Tissue Repair: Methods and Protocols*, Methods in Molecular Biology, vol. 1210,
DOI 10.1007/978-1-4939-1435-7_14, © Springer Science+Business Media New York 2014

the scope of clinical application. Indeed, a clinical trial using hESC-derived-RPE cells for Stargardt's disease and the dry type of age-related macular degeneration (dry AMD) have been initiated [3]. Clinical research using autologous hiPSC-derived RPE for the wet type of age-related macular degeneration (wet AMD) is also being planned in Japan.

One of the most important issues in the development of a safe pharmaceutical or medical device derived from hPSCs is ensuring that the final product does not form tumors after implantation [4]. There are two primary concerns. First, the pluripotent stem cell-based product may be unstable and transform to produce a tumor under some specific environment though the genomic stability of differentiated cells derived from hESCs/hiPSCs is currently unclear. Second, the product derived from human pluripotent stem cells might contain residual undifferentiated stem cells that would eventually proliferate and form a teratoma [5]. To address this second concern, it is critical to develop highly sensitive assays for detection of residual undifferentiated stem cells in the final products, and to determine their lower limit of detection (LLOD). An evaluation study of the in vivo tumorigenicity assay using severe combined immunodeficiency (SCID) mice has shown that 245 undifferentiated hESCs spiked into 10^6 feeder fibroblasts produce a teratoma [6]. However, some in vitro assays, such as quantitative real-time polymerase chain reaction (qRT-PCR), flow cytometry, and immunohistochemistry, have been used to indicate the undifferentiated state of stem cells with various markers (such as *POU5F1* (*OCT3/4*), *NANOG*, *SOX2*, *LIN28*, *MYC* (*C-MYC*), *REX1*, *KLF4*, *TRA-1-60*, *TRA-1-81*, *SSEA-3*, and *SSEA-4*) [7, 8]. We have demonstrated that the qRT-PCR assay can successfully detect as low as 0.002 % residual undifferentiated hiPSCs in hiPSC-induced RPE cells using *LIN28* as a target gene (designated as the *LIN28*/qRT-PCR method) [9]. To the best of our knowledge, the *LIN28*/qRT-PCR method is the most sensitive of the previously reported other methods in detecting undifferentiated PSCs. In this chapter, the *LIN28*/qRT-PCR protocol used in our laboratories is described in detail.

2 Materials

1. Pipetman (2, 10, 20, 200, and 1,000 μL).
2. Incubator, humidified, 37 °C, 5 % CO_2.
3. Sterile biosafety cabinet.
4. Liquid disposal system for aspiration.
5. Centrifuge.
6. Sampling tubes (1.5 mL).

7. Sterile serological pipets (5, 10, and 25 mL).
8. Sterile conical tubes (15 and 50 mL).
9. Cell culture plates (60 and 100 mm).
10. Tips (10, 20, 200, and 1,000 μL).

2.1 Maintenance and Culture of hiPS, RPE Cell, and SNL Cell

1. The hiPSC line 201B7 induced by transducing *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* (RIKEN Cell Bank).
2. Normal human retinal pigment epithelial cells (Lonza Biologics, 00194987).
3. SNL cells (a mouse fibroblast STO cell line expressing the neomycin-resistance gene cassette and LIF, CELL BIOLABS, CBA-316).

2.2 Cell Culture

2.2.1 SNL Culture

1. Dulbecco's modified Eagle medium (DMEM).
2. Phosphate-buffered saline (PBS), calcium free, magnesium free.
3. Fetal bovine serum (FBS).
4. L-Glutamine.
5. Penicillin/streptomycin.
6. Gelatin: To make 0.1 % gelatin solution, dissolve 1.0 g of gelatin powder in 1,000 mL of distilled water, autoclave, and store at 4 °C.
7. SNL medium, 500 mL: Mix 500 mL DMEM, 38.5 mL FBS, 5.5 mL L-glutamine, and 3 mL penicillin/streptomycin.
8. 0.25 % Trypsin/EDTA solution.
9. Mitomycin C.

2.2.2 hiPSC Culture

1. Primate ES Cell medium (ReproCELL, RCHEMD001).
2. Penicillin/streptomycin.
3. Recombinant human FGF basic 146 aa (bFGF).
4. hiPSC medium, 500 mL: Mix 500 mL primate ES cell medium and 2.5 mL penicillin/streptomycin. Add 4 ng/mL bFGF into the medium before use. Store at 4 °C for up to 2 weeks.
5. PBS (*see* Subheading. 2.2.1).
6. CTK solution (ReproCELL, RCHETP002).
7. StemPro EZPassage.
8. Cell scraper.
9. 100 μm cell strainer.

2.2.3 Primary PRE Cell Culture

1. RtEBM Bullet Kit (RtEBM medium; Lonza).
2. PBS (*see* Subheading 2.2.1).
3. 0.25 % Trypsin/EDTA solution.
4. FBS (contained in RtEBM Bullet Kit).