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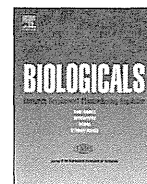

Short paper

Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells

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ABSTRACT

The analysis of *in vitro* cell senescence/growth after serial passaging can be one of ways to show the absence of immortalized cells, which are frequently tumorigenic, in human cell-processed therapeutic products (hCTPs). However, the performance of the cell growth analysis for detection of the immortalized cellular impurities has never been evaluated. In the present study, we examined the growth rates of human mesenchymal stem cells (hMSCs, passage 5 ($P = 5$)) contaminated with various doses of HeLa cells, and compared with that of hMSCs alone. The growth rates of the contaminated hMSCs were comparable to that of hMSCs alone at $P = 5$, but significantly increased at $P = 6$ (0.1% and 0.01% HeLa) or $P = 7$ (0.001% HeLa) within 30 days. These findings suggest that the cell growth analysis is a simple and sensitive method to detect immortalized cellular impurities in hCTPs derived from human somatic cells.

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1. Introduction

Human cell-processed therapeutic products (hCTPs) are expected to provide novel breakthrough therapies for currently life-threatening or incurable diseases. In the clinical applications of hCTPs to patients, however, one of the major concerns is the tumorigenic cellular impurities in the products. Since pluripotent stem cells (PSCs), such as embryonic stem cells and induced pluripotent stem cells, are tumorigenic [1–3], there is a risk of tumor formation if the products contain the residual undifferentiated PSCs [4]. On the other hand, somatic cells are considered to have little tumorigenic potential even after substantial manipulations like *in vitro* expansion, because they consistently pass into senescence [5]. Malignant transformation of the cells is believed to occur through multiple processes involving the accumulation of mutations in key regulatory genes that promote cell survival and proliferation [6,7]. Although a few individual groups reported the spontaneous transformation of human mesenchymal stem cells (hMSCs) during *in vitro* culture [8–11], two of them retracted their papers because the results appeared to be attributable to contamination with tumorigenic cells (fibrosarcoma, osteosarcoma, or glioma cell lines) [12,13]. The rest of the groups found the immortalization of the cells, which is closely associated with tumorigenicity, during *in vitro* culture, indicating that the good practices to avoid contamination with tumorigenic cells and the monitoring of cell growth are critical for the quality control of hCTPs derived from human somatic cells.

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Abbreviations

hCTP	human cell-processed therapeutic product
PSC	pluripotent stem cell
hMSC	human mesenchymal stem cell
STR	short tandem repeat
$P = n$	passage n
PBS	phosphate buffered saline
RT	room temperature
HPV	human papillomavirus

Cross contamination of cells with unidentified cells is usually evaluated by the short tandem repeat (STR) analysis [14]. However, the cell growth analysis which simply monitors the cell proliferation for a limited period may be adequately sensitive for the detection of the contamination of somatic cells with immortalized/tumorigenic cells, because somatic cells usually show slower growth, compared with that of immortalized/tumorigenic cells, as well as the attenuation of the growth after serial passaging [15–17]. In fact, the European Medicines Agency has considered that the evaluation of *in vitro* cell senescence after serial passaging is sufficient to prove the absence of immortalized/tumorigenic cells in a somatic cell-based product [18]. However, the performance of the cell growth analysis for detection of the immortalized/tumorigenic cellular impurities in somatic cells has never been studied. In the present study, we examined the growth of hMSCs contaminated with various doses of HeLa cells, a well-known cancer cell line, to determine the sensitivity of the cell growth analysis for the detection of the immortalized/tumorigenic cells in human somatic cells.

2. Materials and methods

2.1. Cells

hMSCs (Lonza, Walkersville, MD) at passage 2 ($P = 2$) were cultured in MSCGM BulletKit, a mesenchymal stem cell basal medium with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B (Lonza). HeLa cells (the Health Science Research Resources Bank, Osaka, Japan) were maintained in Eagle's minimum essential medium (Sigma), supplemented with 10% fetal bovine serum (FBS; Sigma), 0.1 mM non-essential amino acids (Life Technologies), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and were passaged upon reaching 90% confluence.

2.2. Cell growth analysis

At $P = 5$ of hMSCs, 1×10^6 of hMSCs were mixed with 1000, 100, or 10 of HeLa cells and seeded into T175 flasks (Corning). The cells were maintained in 40 ml of Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Upon reaching approximately 90% confluence, the cells were washed with phosphate buffered saline (PBS) and treated with 0.05% trypsin-EDTA solution (Gibco) for detachment from the flasks. The cells were centrifuged at 450 × g for 5 min and suspended with the fresh culture medium. Aliquots of the suspended cells were stained with Trypan Blue solution and counted by Countess Automated Cell Counter (Invitrogen) according to the manufacture's protocol. One million cells in the suspension were re-seeded into T175 flasks and cultured until the next passage. This process was repeated by $P = 10$. The growth rate (R_n) at $P = n$ was calculated by the following equation:

$$R_n = [\log_2(N_{n+1} - N_n)]/(D_{n+1} - D_n)$$

where N_k and D_k are the number of accumulated cells and the date at $P = k$, respectively.

2.3. Immunofluorescence microscopy

hMSCs contaminated with HeLa cells were fixed with 4% paraformaldehyde in PBS (Nacalai Tesque) for 10 min at room temperature (RT) and blocked in Blocking One (Nacalai Tesque) for 30 min at RT. The cells, then, were incubated with anti-HPV18 E7 antibody (8E2) (abcam) diluted at 1:500 in the blocking solution (PBS containing 5% Blocking One) for 1 h at RT for primary staining, and secondarily stained with goat anti-mouse IgG Alexa Fluor 488 (1:1000; Invitrogen) in the blocking solution for 45 min at RT. The cells were mounted with VECTASGIELD mounting medium with DAPI (VECTOR) and observed with a fluorescence microscope (IX71, Olympus).

3. Results and discussion

In the present study, we added 1000, 100, or 10 of HeLa cells to 1×10^6 of hMSCs of passage 5 ($P = 5$) and compared their growth with that of hMSCs alone (HeLa 0) until $P = 10$. The growth curves of three lots of hMSCs and the contaminated hMSCs are shown in Fig. 1. The cell numbers of HeLa 0 and the contaminated hMSCs were comparable at $P = 5$. The growth of HeLa 0 was constant during the early culture and getting slower with time (Fig. 1), while the growth of the contaminated cells was accelerated.

To confirm that the increases in the growth were attributable to the contamination with HeLa cells, we observed the cells with phase contrast microscopy. In the images of the contaminated hMSCs, we found small cells clearly different from hMSCs (Fig. 2A), and their relative abundance increased every passage. Because HeLa cells are infected with human papillomaviruses (HPV), we performed immunofluorescence analysis using HPV18 E7 antibody and confirmed that the cells were HeLa cells not transformed hMSCs (Fig. 2B). At $P = 10$, hMSCs were hardly identified in images of HeLa 1000 (Fig. 2C), because almost all of hMSCs were exchanged for HeLa cells at the five passages.

Next, we examined the growth rates of the contaminated cells (Fig. 3A). They were comparable to that of HeLa 0 at $P = 5$, and got significantly increased at $P = 6$ (HeLa 1000 and HeLa 100) or $P = 7$ (HeLa 10). These results indicated that the gross proliferation rate was not influenced by the spiked cells at $P = 5$ and then the population of HeLa cells in hMSCs increased in dose- and time-dependent manner. Eventually, the growth rate (doubling/day) of the contaminated hMSCs increased, and then reached plateau. The average growth rate of the contaminated cells at $P = 9$ and 10 was 0.73, suggesting that the growth rate of HeLa cells was approximately 0.7 in this culture condition. The average growth rates of the three lots are plotted along the passage number in Fig. 3B. The growth rates of HeLa 1000 and HeLa 100 at $P = 7$ and HeLa 10 at $P = 8$ were significantly increased compared with the growth rates at $P = 5$ ($^*P < 0.05$, two-way repeated measures ANOVA and Student-Newman–Keulis test). These results indicate that the cell cultures longer than $P = 7$ (about 20 days) and $P = 8$ (about 30 days) detect cross-contaminations of 100 (0.01%) and 10 (0.001%) HeLa cells, respectively, assuming that 10^6 hMSCs were contaminated at $P = 5$.

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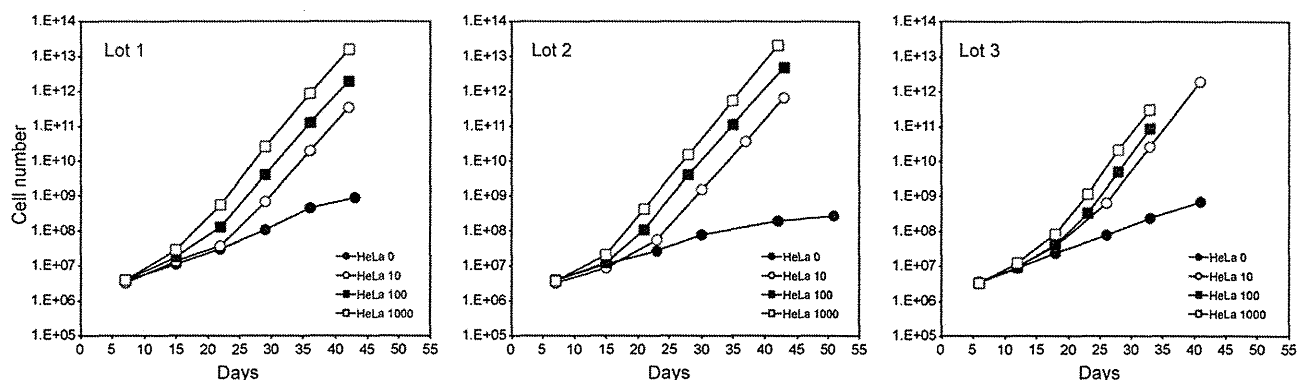

Fig. 1. Cell growth analysis of hMSCs contaminated with HeLa cells. At the passage 5 ($P = 5$) of hMSCs, 1×10^6 of hMSCs were mixed with 0, 10, 100, or 1000 of HeLa cells (HeLa 0, HeLa 10, HeLa 100, or HeLa 1000). Cells were passaged and counted at the indicated day from $P = 5$. The results of three lots of hMSCs are presented (Lot 1, 2, and 3).

The tumorigenicity has been evaluated by *in vitro* assays (e.g., soft agar formation assay, karyotype analysis) and/or *in vivo* assays (transplantation into immunodeficient animals) [8,9,19,20]. When these assays are performed to detect a trace amount of tumorigenic cells in hCTPs, they need to be of high sensitivity. Recently, we developed a highly sensitive *in vivo* tumorigenicity test using severely immunocompromised mice, NOG mice, in combination with Matrigel. Subcutaneous transplantation into NOG mice with Matrigel allowed inoculation with 1×10^7 cells and actually achieved detection of 0.002% HeLa cells spiked into hMSCs in a half of the mice (unpublished data). However, *in vivo* tumorigenicity test using immunodeficient animals requires a specific facility, and takes 3–4 months. In contrast, the cell growth analysis is not only simple and economical, but also detects as few as 0.001% immortal cellular impurities in hMSCs within 30 days, in case that the growth properties of the immortal cells are comparable to those of HeLa cells. Although immortality does not necessarily indicate tumorigenicity, it is known to be closely associated with cell transformation in many cases of tumorigenesis. Therefore, the present study can be said to be the first scientific basis for the usefulness of the cell growth analysis as one of tumorigenicity tests for hCTPs derived from human somatic cells.

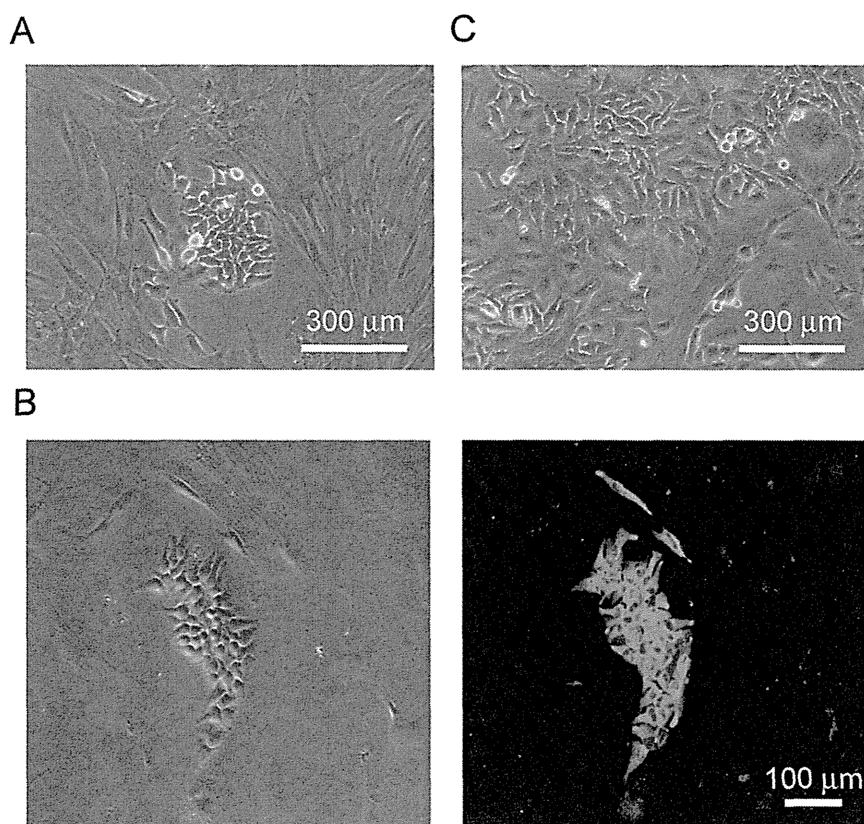

Fig. 2. Phase contrast and immunofluorescence microscopy of hMSCs contaminated with HeLa cells. (A) Representative image of the contaminated cells (HeLa 100 at $P = 8$) is presented. Red arrow pointed at the small size cells which were clearly different from hMSCs. (B) Phase contrast and immunofluorescence microscopy of HeLa 1000 at day 5 was conducted by using anti-HPV E7 antibody. Green: Alexa Fluor 488 goat anti-mouse IgG; blue: DAPI. (C) Representative image of HeLa 1000 at P10 is presented.

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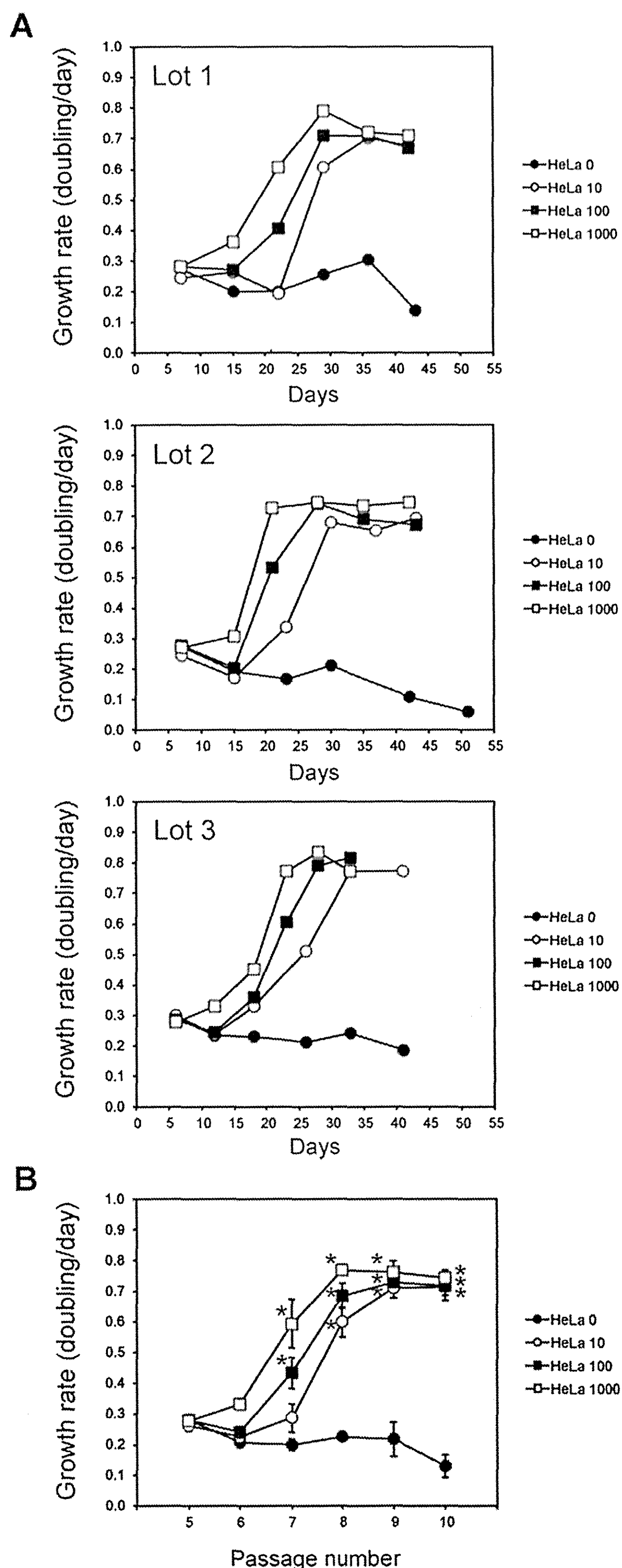

Fig. 3. Cell growth rate analysis of hMSCs contaminated with HeLa cells. (A) The Y-axis indicates the growth rate (doubling per day) of hMSCs. The results of three lots of hMSCs are presented (Lot 1, 2, and 3). (B) The growth rates of three lots of hMSCs are plotted along the passage numbers. Data are represented as mean ± S.E.M. of the three lots. Statistical significance was determined using two-way repeated measures ANOVA and Student-Newman–Keulis's post-hoc test (*$P < 0.05$ compared with the rate at $P = 5$).

Author contributions

Conceived and designed the experiments: KK NT SY RS YS. Performed the experiments: KK NT. Analyzed and Interpreted the data: KK NT SY RS SN AM YS. Contributed regents/materials/analysis tools: SN AM YS. Wrote the paper: KK NT SY YS. Acquired the funding: AM YS.

Competing interests

The authors have declared that no competing interests exist.

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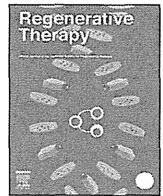

Original article

Characterization of *in vivo* tumorigenicity tests using severe immunodeficient NOD/Shi-scid IL2Rγ^{null} mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products

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ABSTRACT

The contamination of human cell-processed therapeutic products (hCTPs) with tumorigenic cells is one of the major concerns in the manufacturing and quality control of hCTPs. However, no quantitative method for detecting the tumorigenic cellular impurities is currently standardized. NOD/Shi-scid IL2Rγ^{null} (NOG) mice have shown high xeno-engraftment potential compared with other well-known immunodeficient strains, e.g. nude mice. Hypothesizing that tumorigenicity test using NOG mice could be a sensitive and quantitative method to detect a small amount of tumorigenic cells in hCTPs, we examined tumor formation after subcutaneous transplantation of HeLa cells, as a model of tumorigenic cells, in NOG mice and nude mice. Sixteen weeks after inoculation, the 50% tumor-producing dose (TPD_{50}) values of HeLa cells were stable at 1.3×10^4 and 4.0×10^5 cells in NOG and nude mice, respectively, indicating a 30-fold higher sensitivity of NOG mice compared to that of nude mice. Transplanting HeLa cells embedded with Matrigel in NOG mice further decreased the TPD_{50} value to 7.9×10 cells, leading to a 5000-fold higher sensitivity, compared with that of nude mice. Additionally, when HeLa cells were mixed with 10^6 or 10^7 human mesenchymal stem cells as well as Matrigel, the TPD_{50} values in NOG mice were comparable to those of HeLa cells alone with Matrigel. 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 the quality assessment of hCTPs.

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1. Introduction

Cell-processed therapeutic products (CTPs) derived from human somatic/stem cells are eagerly expected to treat patients with severe diseases involving functional damage of organs and tissues. To transplant hCTPs into patients, however, tumorigenicity is raised as one of the issues of these products. Tumorigenicity is defined as the capacity of a cell population transplanted into an animal model to

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produce a tumor by proliferation at the site of transplantation and/or at a distant site by metastasis [1]. Assessment of tumorigenicity is quite important to manufacture products with consistent quality. Currently, the World Health Organization (WHO) Technical Report Series (TRS) No. 878 Annex 1 is the only international guideline that addresses tumorigenicity tests of animal cells for the production of biologicals. However, the tumorigenicity test described in WHO TRS 878, which involves the administration of 10^7 cells to ten nude mice, would not be sensitive enough to detect a trace amount of tumorigenic cellular impurities in hCTPs [2]. In addition, the *in vivo* tumorigenicity test proposed in WHO TRS 878 covers only viable animal cells used as cell substrates for manufacturing biological products but not cells used directly for therapy by transplantation into patients. Thus, to date, no suitable tumorigenicity test has been established for hCTPs.

To establish methods to detect a trace amount of tumorigenic cellular impurities in hCTPs, the usage of several new generations of highly immunodeficient animal models are proposed. Rag2-γC double-knockout mice [3], NOD/Shi-scid IL2Rγ^{null} (NOG) mice [4], and NOD/SCID/IL-2rγKO (NSG) mice [5] indicate multiple immunodeficiencies, including defects in T, B, and natural killer (NK) cells, and a reduction in the function of macrophages and dendritic cells. NOG mice exhibit extremely high engraftment rates of human HeLa S3 cells compared with T-cell-deficient nude mice and T and B-cell-deficient SCID mice [6]. NSG mice are reported to show efficient tumor formation by single human melanoma cells in combination with Matrigel, a basement membrane-like extracellular matrix extract [7]. However, for the use of these highly immunodeficient mouse strains to detect tumorigenic cellular impurities in hCTPs as a part of the quality assessment/control, the performance of the tumorigenicity tests using these strains shall be validated using well known tumor cell lines.

In the present study, we examined the tumor formation potential of HeLa cells transplanted in NOG mice with Matrigel and compared their tumorigenicity with that in nude mice. To determine the sensitivity for the detection of tumor cells contamination in non-tumorigenic human somatic cells, we mixed various dose of HeLa cells in human mesenchymal stem cells and conducted tumorigenicity tests using NOG mice and Matrigel. We also performed soft agar colony formation assay, which is commonly used to detect anchorage-independent cell growth *in vitro*, and compared tumor cell detection level by soft agar with the *in vivo* tumorigenicity test.

2. Materials and methods

2.1. Cells

Human cervical cancer HeLa cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The cells were maintained in Eagle's minimum essential medium (Sigma), supplemented with 10% fetal bovine serum (FBS; Sigma), 0.1 mM non-essential amino acids (Life Technologies), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Human mesenchymal stem cells (hMSCs) were purchased from Lonza and cultured in MSCGM™ medium (Lonza). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and were passaged upon reaching 80% confluence. hMSCs were used at passage 6 and passages 6–8 for *in vivo* tumorigenicity tests and soft agar colony formation assay, respectively.

2.2. Preparation of cell suspensions for transplantation

Upon reaching approximately 80% confluence, cells were washed twice with phosphate buffered saline (PBS) and treated with 0.25% trypsin-EDTA solution (Life Technologies) for detachment from culture dishes. HeLa cells and/or hMSCs were counted and prepared in 100 μl of ice-cold HeLa cell culture medium or a 1:1 (v/v) mixture of HeLa cell culture medium and Matrigel (product #354234, BD Biosciences, San Jose, CA) for transplantation.

2.3. Tumorigenicity test with immunodeficient mice

Male BALB/cA nu/nu mice (nude; CLEA Japan, Inc., Tokyo) and male NOG mice maintained in the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan) were used for *in vivo* tumorigenicity studies. Prepared cell suspensions were injected using 1 ml syringes with a 25 G needle (Terumo) into 8-week-old mice ($n = 6$ or 10). The mice were palpated weekly for 16 weeks to observe nodule formation at the injection site. Tumor size was assessed by external measurement of the length and width of the tumors in two dimensions using a caliper as soon as tumors reached measurable size. The tumor volume (TV) was calculated using the formula volume $= 1/2 \times$ length (mm) $\times$ (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. The tumorigenicity of HeLa cells was evaluated by measuring tumor-forming capacity, which indicates the tumorigenic phenotype [8,9]. Tumor-forming capacity is defined as 50% tumor-producing dose (TPD_{50}), which represents the threshold dose of cells forming tumors in 50% of the animals. TPD_{50} values were calculated using the Spearman-Kärber method [10–12] at each time point. Not all animals transplanted with the highest dose formed tumors, in which case it was assumed that the tumor incidence of animals at 10 or 100 times the uppermost dose step (a dummy set of data) would have been 100% for the Spearman-Kärber method to be applicable [12].

The protocol of the present study was reviewed beforehand and approved by the Animal Ethics Committees of CIEA (Permit Number: 13041A) and the National Institute of Health Sciences (NIHS, Tokyo) (Permit Number: 359, 359-1, 359-2, 359-3). All animal experiments were performed according to the Ethical Guidelines for Animal Experimentation from the CIEA and the NIHS. All animals were sacrificed under isoflurane inhalation anaesthesia, and all efforts were made to minimize suffering.

2.4. Histology and immunohistochemistry

The engrafted tumors were isolated and fixed with 10% neutral buffered formalin (Wako). The paraffin-embedded sections were investigated by hematoxylin and eosin (H&E) stain and immunohistochemical studies using Bond-max stainers (Leica Biosystems). Some sections were incubated at 100 °C for 10 min in a target retrieval solution consisting of 10 mM citrate buffer (ER1; Leica Microsystems), and then placed at room temperature for 20 min. Mouse anti-human HLA class I-A, B, C monoclonal antibody (EMR8-5; Hokudo, Sapporo, Japan), and rabbit anti-vimentin monoclonal antibody (SP20; Nichirei Bioscience, Tokyo) were used as the primary antibodies. The antibodies for mouse immunoglobulin were visualized using Bond polymer refine detection kits (Leica Microsystems). Sections were counterstained with hematoxylin.

2.5. Soft agar colony formation assay

A soft agar colony formation assay was performed using a CytoSelect™ 96-well Cell Transformation Assay kit (CellBio labs, San Diego, CA) as previously described [13]. Prewarmed 25 μl of 2 × DMEM/20% FBS and 25 μl of 1.2% agar solution were mixed and

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

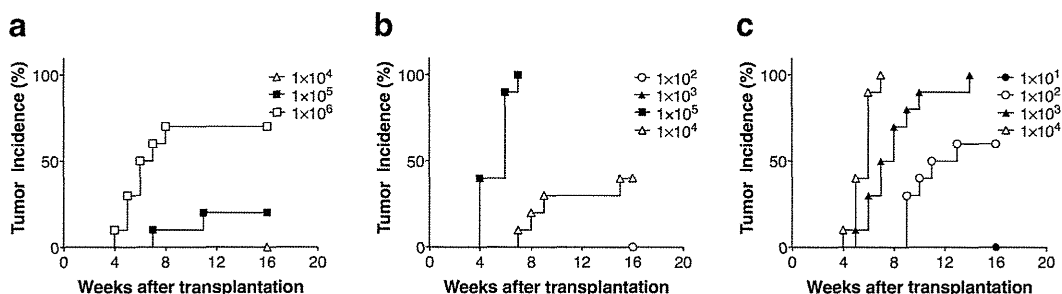

Fig. 1. Tumor incidence of HeLa cells in nude and NOG mice. The tumor formation of HeLa cells transplanted into the subcutaneous spaces of mice was examined for 16 weeks. The relationships between the dose and the tumorigenic incidence of HeLa cells in nude mice (a) and NOG without (b) or with (c) Matrigel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

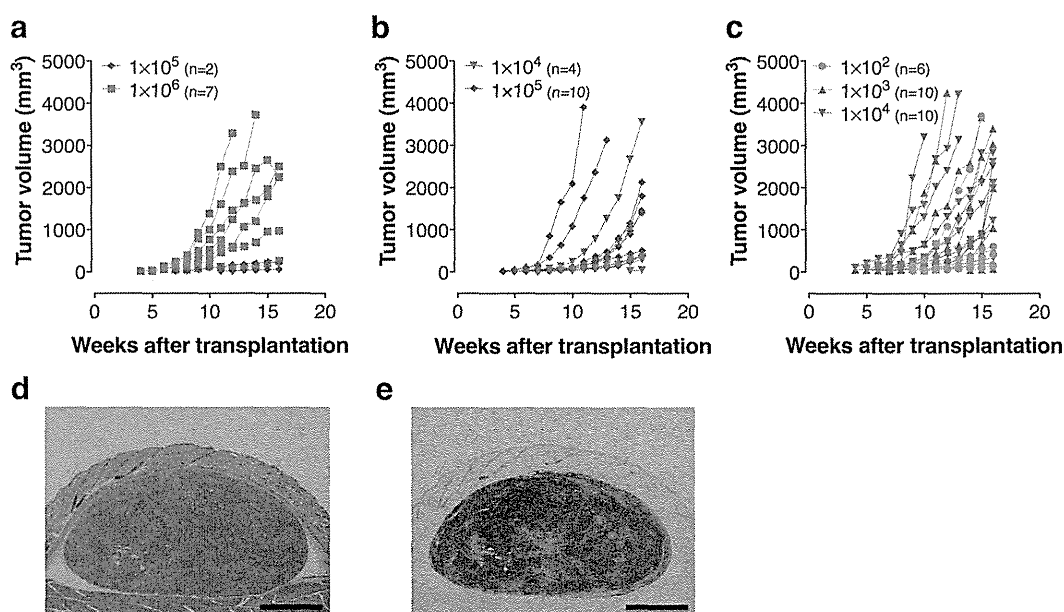

Fig. 2. Characterization of subcutaneous tumors formed by transplantation with HeLa cells in nude and NOG mice. Growth curves of subcutaneous tumors formed by inoculation with various dosages of HeLa cells were presented in respective mice (a: nude; b: NOG w/o Matrigel; c: NOG w/Matrigel). The tumor 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.

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

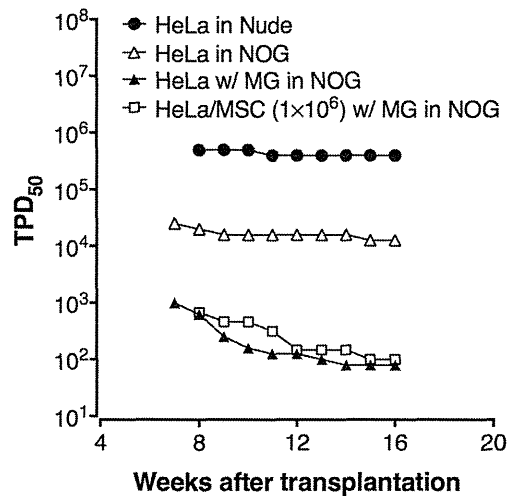

Fig. 4. Comparative development of HeLa cell tumors in nude and NOG mice under various conditions. HeLa cell tumor development in nude and NOG mice under various conditions are expressed as transition of TPD$_{50}$ at weekly intervals. MG, Matrigel.

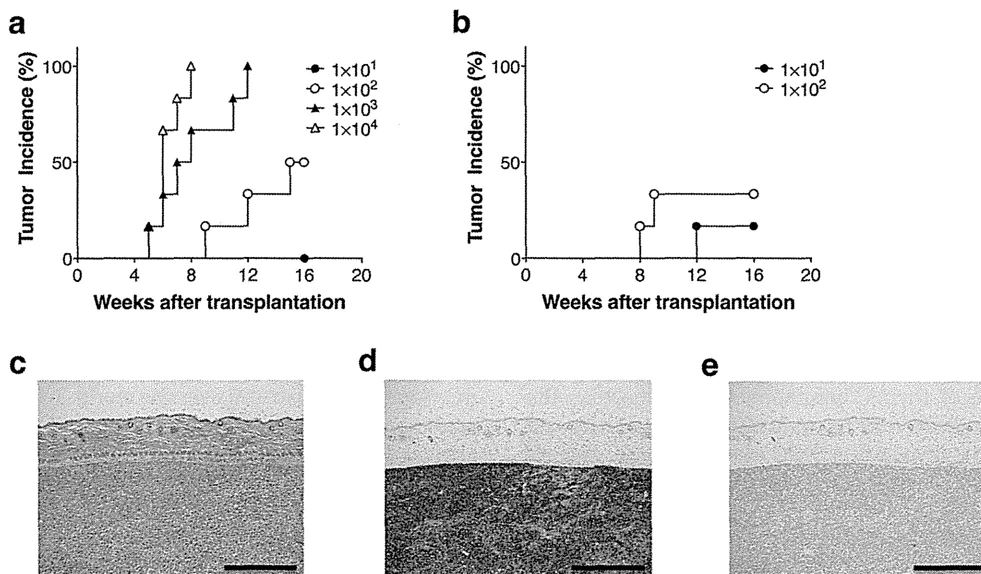

Fig. 3. Detection of tumors in NOG mice transplanted with HeLa cells spiked into hMSCs. The cohorts inoculated HeLa cells spiked in 10^6 and 10^7 hMSCs cells were observed for 16 weeks, respectively. The relationships between the cell dose and the tumorigenic incidence of HeLa spiked in 1.0 × 10^6 (a) 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).

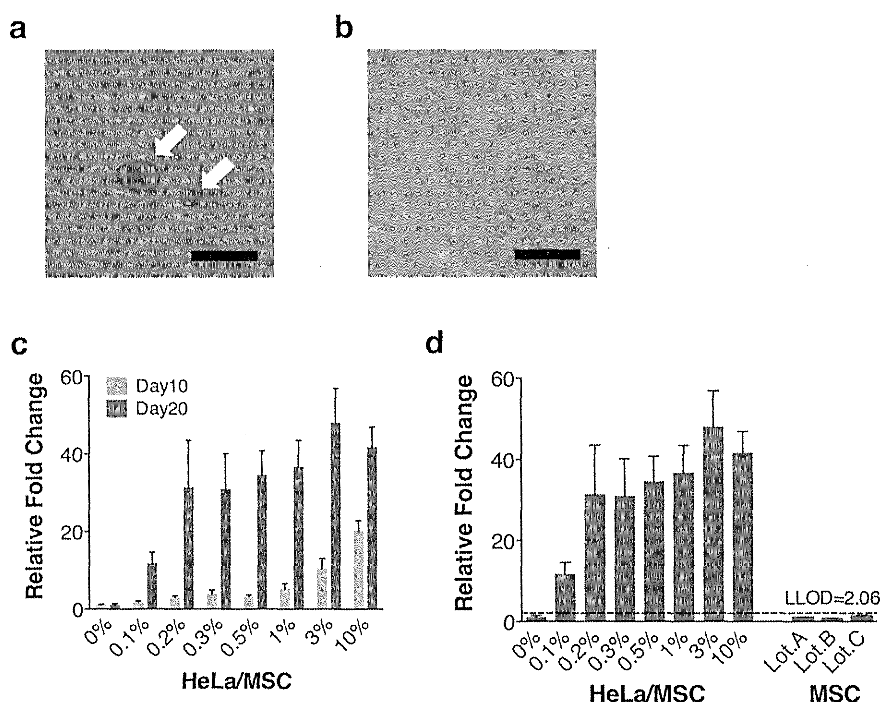

Fig. 5. Soft agar colony formation assay of HeLa cells. HeLa cells enclosed in soft agar showed progressive formation of colonies (a), whereas hMSCs did not form any colonies in a soft agar media with 1.0×10^4 cells/well by day 20 (b). Arrows indicate the colonies of HeLa cells (magnification, 100×; 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 $TPD_{50} = 4.0 \times 10^5$ (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

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Conflict of Interest

All authors declare no conflict of interest.

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

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Abstract

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

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Introduction

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

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

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

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

Materials and Methods

Cell culture

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

Cell proliferation assay

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

Quantitative RT-PCR

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

Teratoma assay

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

Differentiation assay

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

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

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

Immunofluorescence staining

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

Embryoid body formation

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

Statistics

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

Results

Essential 8 medium promotes hiPSCs cell growth on laminin-521

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

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

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

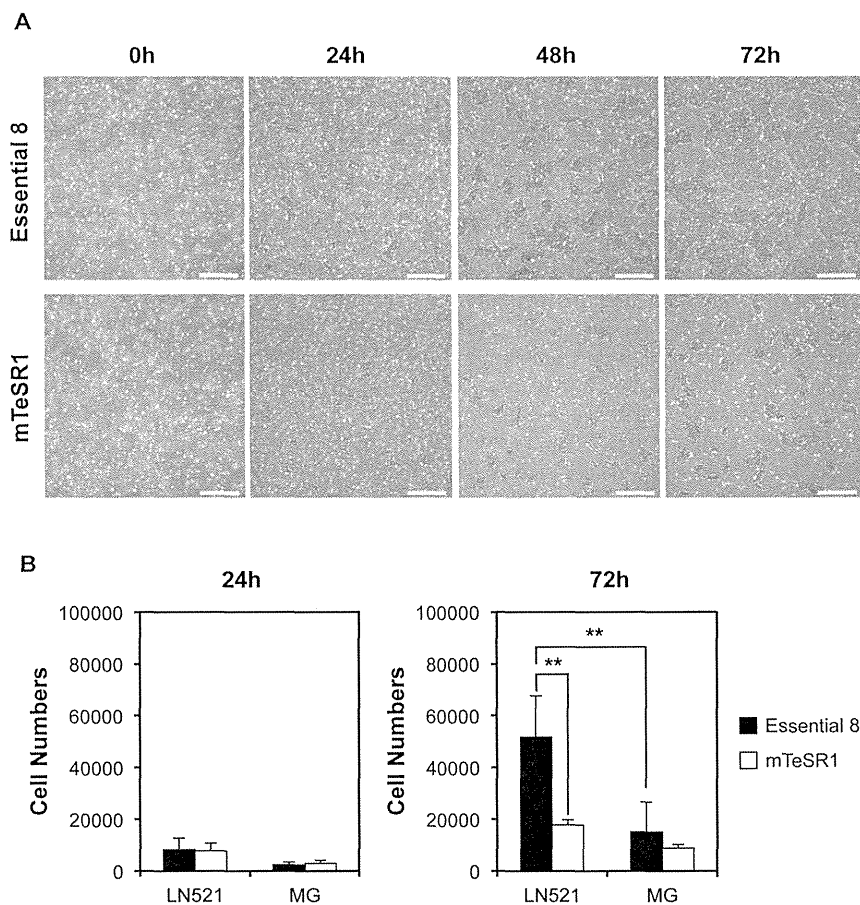

Figure 1. Robust proliferation of 253G1 cells cultured on laminin-521 in Essential 8 medium. (A) Morphology of the 253G1 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium after dissociation into single cells. Scale bars, 500 µm. (B) Quantification of the number of dissociated 253G1 cells expanded on laminin-521 or Matrigel in Essential 8 or mTeSR1 medium. Data are presented as the mean ± standard deviation (SD) of three independent experiments (**$P<0.01$, two-way ANOVA followed by Bonferroni t-test as post-hoc test). LN521, laminin-521; MG, Matrigel.
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differentiation protocols. Immunofluorescence analysis using antibodies specific for markers of three germ layers clearly demonstrated that 253G1 cells subcultured with laminin-521 and Essential 8 medium could selectively differentiate into endoderm, mesoderm and ectoderm expressing AFP, α-SMA and βIII tubulin, respectively (Figure 2B). To further examine pluripotency of hiPSCs subcultured with laminin-521 and Essential 8 medium, we facilitated spontaneous differentiation of cells grown as aggregates (embryoid bodies). Embryoid bodies derived from 253G1 cells increased gene expression of differentiated markers for all three germ layer lineages (Figure 2C), consistent with the observation obtained using 201B7 and 409B2 cells (Figure S2C-D). We also confirmed that 253G1 cells cultured with laminin-521 and Essential 8 medium were engrafted in testes of SCID mice and formed teratomas that involved all three germ layers (Figure 2D). These results strongly suggest that a culture system using laminin-521 and Essential 8 medium supports the undifferentiated state and pluripotency of hiPSCs through serial passages.

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

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

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

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

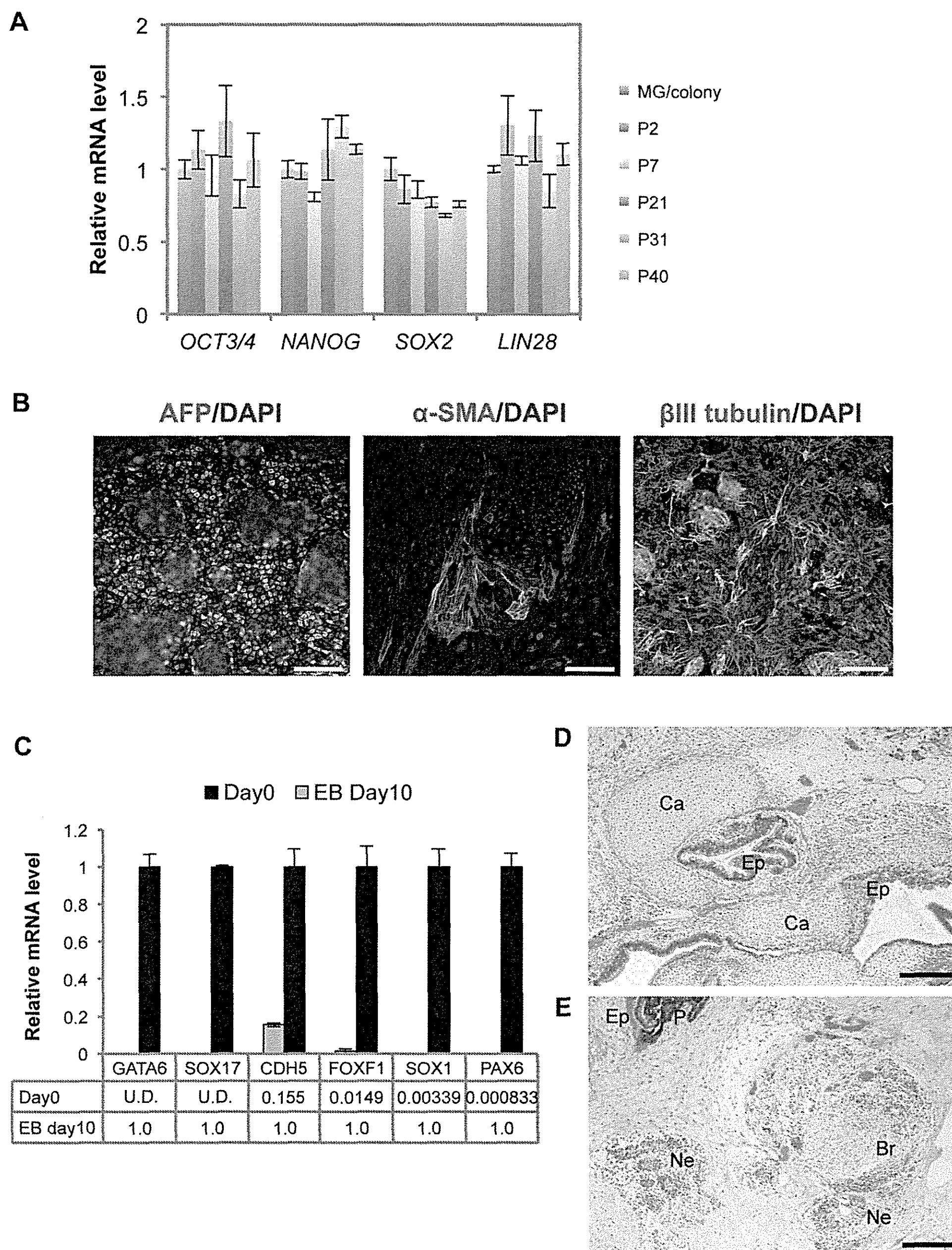

Figure 2. Characterization of 253G1 cells subcultured on laminin-521 in Essential 8 medium. (A) Expression levels of undifferentiated cell markers (*OCT3/4, NANOG, SOX2 and LIN28*) in 253G1 cells subcultured on laminin-521 in Essential 8 were determined using qRT-PCR. Relative mRNA expression levels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

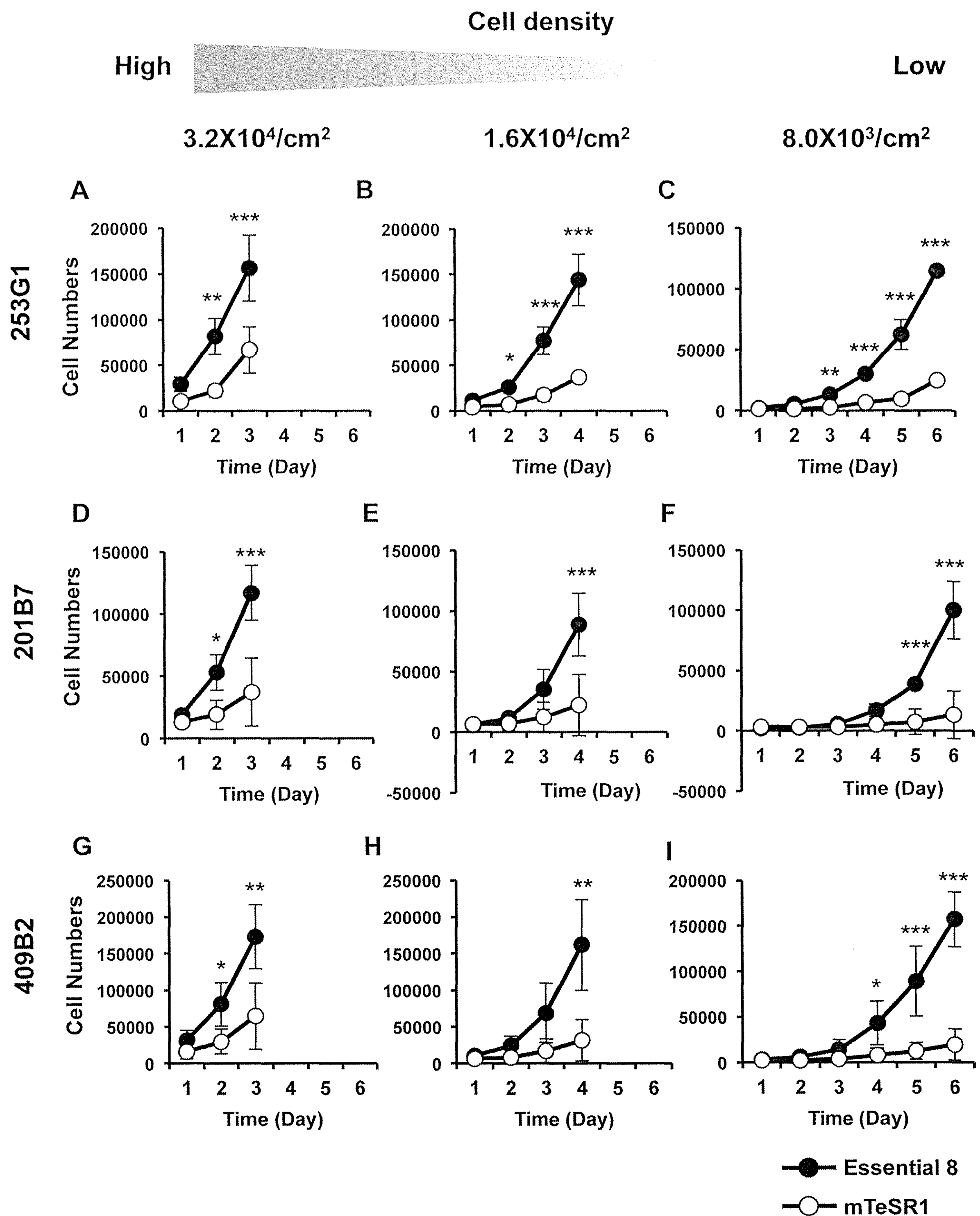

Figure 3. Rapid cell proliferation of hiPSCs plated at low cell density on laminin-521 in Essential 8 medium. (A-I) Quantification of the number of 253G1, 201B7 and 409B2 cells expanded on laminin-521 in Essential 8 or mTeSR1 medium. Cell numbers were counted every 24 h after plating at 3.2×10^4 cells/cm^2 (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).
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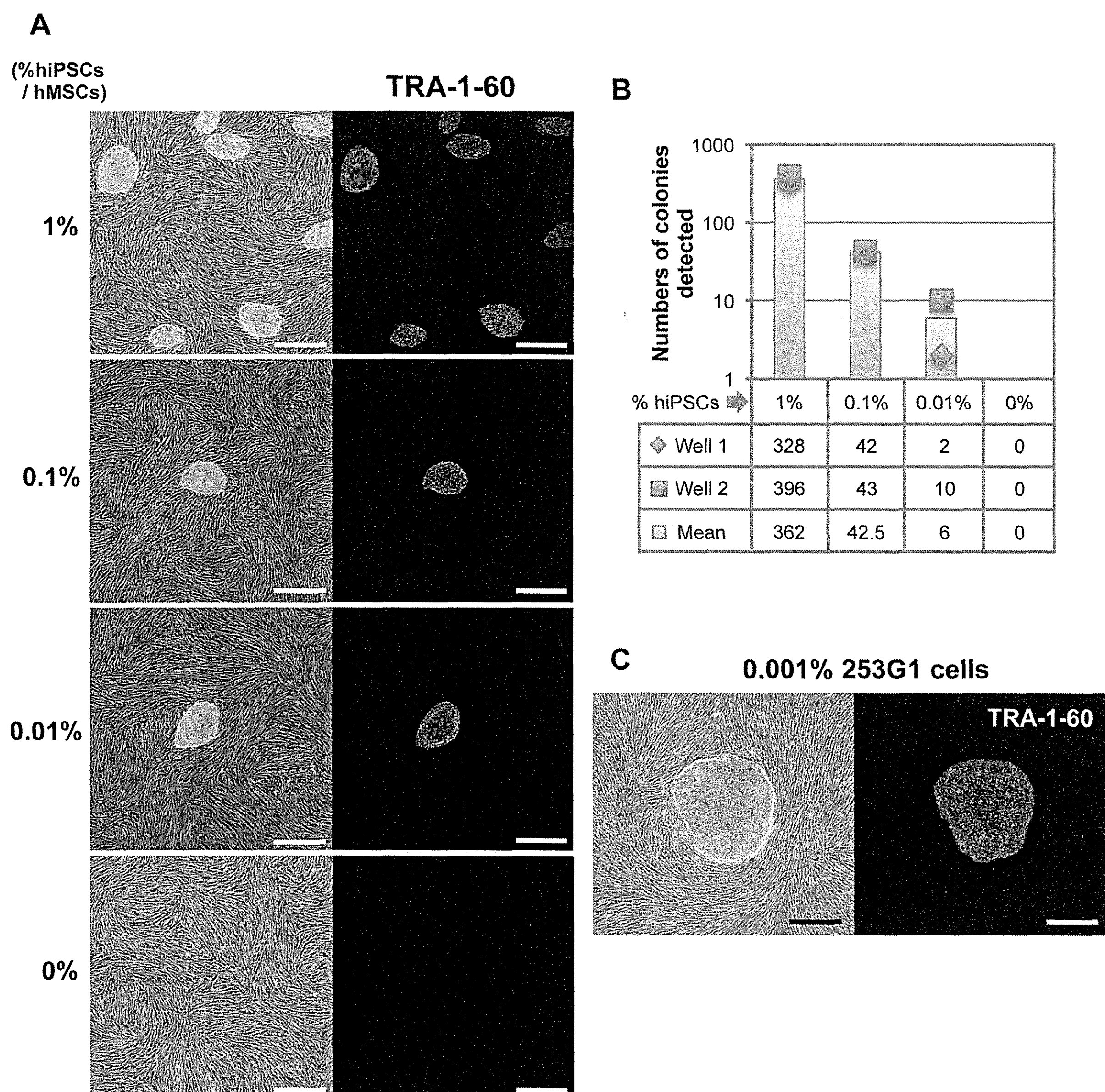
find any colonies when only hMSCs were cultured on laminin-521 in Essential 8 medium. In addition, immunofluorescence staining with anti-TRA-1-60 antibody showed that these colonies formed in an undifferentiated state (Figure 4A), suggesting that colonies derived from hiPSCs were formed in an hMSC monolayer under conditions with laminin-521 and Essential 8 medium. We also



% hiPSCs	1%	0.1%	0.01%	0%
◆ Well 1	328	42	2	0
■ Well 2	396	43	10	0
□ Mean	362	42.5	6	0

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.
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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