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Original Article

Application of cell growth analysis to the quality assessment of human cell-processed therapeutic products as a testing method for immortalized cellular impurities

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ABSTRACT

In human cell-processed therapeutic products (hCTPs) for clinical application, tumorigenic cellular impurities in the manufacturing process are a major concern. Because cellular immortalization is one of the prerequisite steps in tumorigenesis, we tested whether cell growth analysis can be employed to check for immortalized (and potentially tumorigenic) cellular impurities in hCTPs. We monitored the growth of human bone marrow-derived mesenchymal stem cells (BMSCs) mixed with HeLa cells at a ratio of $1/10^6$ or more and compared their growth rates with that of BMSCs alone. The cell growth analysis detected a significant increase in the growth rate of the BMSCs spiked with 0.0001% HeLa within 30 days at a probability of 47%. When human adipose-derived stem cells (ADSCs) were spiked with ASC52telo cells, a human telomerase reverse transcriptase (hTERT)-immortalized adipose-derived mesenchymal stem cell line, at a ratio of 0.001% or more, their growth rates were significantly increased within 15 passages, compared with that of ADSCs alone. These results indicate that cell growth analysis for the detection of immortalized cellular impurities in human somatic stem cells is simple and can be useful for the quality assessment of hCTPs in the manufacturing process.

1. Introduction

It is strongly expected that human cell-processed therapeutic products (hCTPs) will be developed to treat diseases that are currently life-threatening or incurable. The tumorigenic cellular impurities of hCTPs are a major concern for the clinical application of hCTPs. Unlike human pluripotent stem cells (i.e., embryonic stem cells and induced pluripotent stem cells [1,2]), human somatic cells are thought to have little tumorigenicity, regardless of the *in vitro* cell processing [3,4]. To the best of our knowledge, only three studies (therapies of ataxia telangiectasia with human fetal neural stem cells, spinal cord injury with olfactory mucosal cells, and full-thickness burn with cultured epidermal autograft) have reported tumor formation following the transplantation of human somatic cells into patients [5–7]. Four individual groups have reported the spontaneous transformation of human mesenchymal stem cells (hMSCs) after long-term *in vitro* culture [8–11]. However, two of these research papers were later retracted due to the cross-contamination of hMSCs with tumorigenic cells [12,13]. In the other two papers, the immortalization of hMSCs was initially found in the *in vitro* culture, which is closely associated with tumorigenicity [10,11]. These observations suggest that avoiding

Abbreviations: hCTP, human cell-processed therapeutic product; BMSC, bone marrow-derived mesenchymal stem cell; ADSC, adipose-derived stem cell; hTERT, human telomerase reverse transcriptase; hMSC, human mesenchymal stem cell; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; $P = n$, passage n; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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cross-contamination with tumorigenic cells and monitoring the growth of immortalized cells without senescence is critical for the quality control of hCTPs derived from human somatic stem cells. In fact, the European Medicines Agency has stated that the evaluation of *in vitro* cell senescence after serial passaging is sufficient to prove the absence of immortalized/tumorigenic cells in human somatic cell-based products [14].

In a previous study, we examined the growth rates of human bone marrow-derived mesenchymal stem cells (BMSCs) spiked with various doses of HeLa cells to determine the sensitivity of cell growth analysis for the detection of immortalized (and potentially tumorigenic) cells contained in somatic stem cells as impurities. The results indicated that as little as 0.001% of HeLa cells as impurities were detectable by cell growth analysis [15]. Here we attempted to detect 0.0001% of HeLa cells spiked into BMSCs to further confirm the sensitivity of cell growth analysis. We also characterized the performance of the cell growth analysis as a testing method for immortalized cellular impurities that show more modest growth, compared with HeLa cells, using human adipose-derived mesenchymal stem cells (ADSCs) and immortalized human telomerase reverse transcriptase (hTERT)-transduced ADSCs. Our data suggest the usefulness of cell growth analysis for the quality assessment for hCTPs.

2. Materials and methods

2.1. Cells

All of the cell cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. BMSCs at passage 2 ($P = 2$) and ADSCs at $P = 1$ were purchased from Lonza. ASC52telo cells, hTERT-immortalized adipose-derived mesenchymal stem cells, were obtained from ATCC. HeLa cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). BMSCs were cultured in an MSCGM BulletKit™, a mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B (Lonza). ADSCs and ASC52telo cells were cultured in an ADSC-BulletKit™, an ADSC basal medium supplemented with the necessary supplements (Lonza). HeLa cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS; Sigma), 0.1 mM non-essential amino acids (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Until they were used for cell growth analysis, cells were maintained in the medium as described above and passaged upon reaching 90% confluence.

2.2. Cell growth analysis

At $P = 5$ of BMSCs and ADSCs, 1×10^6 cells of BMSCs and ADSCs were spiked with HeLa cells (10 or 1 cells) and ASC52telo cells (1000, 100, or 10 cells), respectively. The spiked cells were prepared by the serial dilution of counted cells. The cell suspensions were seeded into T175 flasks (Corning) and maintained in 40 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Upon reaching approximately 90% confluence, the cells were rinsed with phosphate buffered saline (PBS; Nacalai Tesque, Kyoto, Japan) 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 0.4% trypan blue solution and counted using a Countess automated cell counter (Invitrogen) according to the manufacturer's protocol. One million cells in the suspension were re-seeded into T175 flasks and cultured until the next passage. These procedures were repeated by $P = 11$ and $P = 20$ in the experiments using the BMSCs spiked with HeLa cells and ADSCs spiked with ASC52telo cells, respectively. 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. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from ASC52telo cells spiked into ADSCs using an RNeasy Mini Kit with DNase I treatment (QIAGEN) according to the manufacturer's instructions. RNA concentration was measured using the NanoDrop ND-1000 (Thermo Fisher Scientific). qRT-PCR was performed with a QuantiTect Probe RT-PCR Kit (QIAGEN) on a 7300 Real-Time PCR System (Applied Biosystems). The PCR condition was as follows. After initial incubations at 50 °C for 30 min and 95 °C for 15 min, 40 cycles of amplification were carried out at 95 °C for 15 s and 60 °C for 1 min. Total RNA (5 ng per sample) was used for analysis. The levels of hTERT expression were normalized by those of GAPDH expression, which were quantified using TaqMan human GAPDH control reagents (Applied Biosystems). Primers and probes incorporating 5′-FAM reporter dye and 3′-TAMRA quencher dye for qRT-PCR were obtained from Sigma–Aldrich. The primer and probe sequences targeting hTERT gene were as follows: 5′-CCTGTTTCTGGATTTGCAGGTG-3′ (forward primer), 5′-GCACACATGCGTGAAACCTG-3′ (reverse primer), and 5′-CAGCCTCCAGACGGTGTGCACCAAC-3′ (probe).

3. Results and discussion

In our previous cell growth analysis, 10 HeLa cells spiked into 1×10^6 BMSCs (0.001%) showed a significant increase in the growth rate within 30 days [15]. Here we added a single HeLa cell (HeLa 1) or ten HeLa cells (HeLa 10) to 1×10^6 of BMSCs at $P = 5$ and monitored the number of cultured cells until $P = 11$. Although equal proportion assay of spiking 10 cells into 1×10^7 cells (0.0001%) appears to be more reproducible than that of spiking a single cell into 1×10^6 cells, we had technical difficulties in handling 1×10^7 cells of BMSCs with one culture dish. Considering the varied growth efficacy of a single HeLa cell, we performed experiments with ten samples of HeLa 1 for each lot of BMSCs. In contrast, we have previously reported that the results of HeLa 10 and BMSCs alone (HeLa 0) were convinced [15]. Here we determined to use BMSCs at $P = 5$ for the spike experiments because over 70% of clinical trials have used hMSCs from 1 to 5 passages [16]. BMSCs alone (HeLa 0) proliferated at almost constant rates during our observation (Fig. 1). As expected, the cell growth of HeLa 10 was distinct from that of HeLa 0 within 30 days. Several samples of HeLa 1 also indicated that their cell growth curves were rapidly accelerated within 30 days compared with those of HeLa 0. In contrast, cell growth in some samples of HeLa 1 did not differ from HeLa 0. The incidence rates of accelerated cell growth were 47 ± 9% when one HeLa cell was spiked into three lots of 1×10^6 BMSCs at the ratio of 0.0001% (Table 1). Next, we calculated the growth rates (R_n) according to the formula described in the Materials and methods section, and performed a statistical analysis to determine the passage number that showed a significant difference in growth rates compared with HeLa 0 at $P = 5$. The growth rates in HeLa 1 with accelerated cell growth gradually increased at $P = 8$ and were significantly higher than that in HeLa 0 at $P = 5$ (Fig. 2). These results indicated that cell growth analysis detects HeLa cells as 0.0001% impurities of

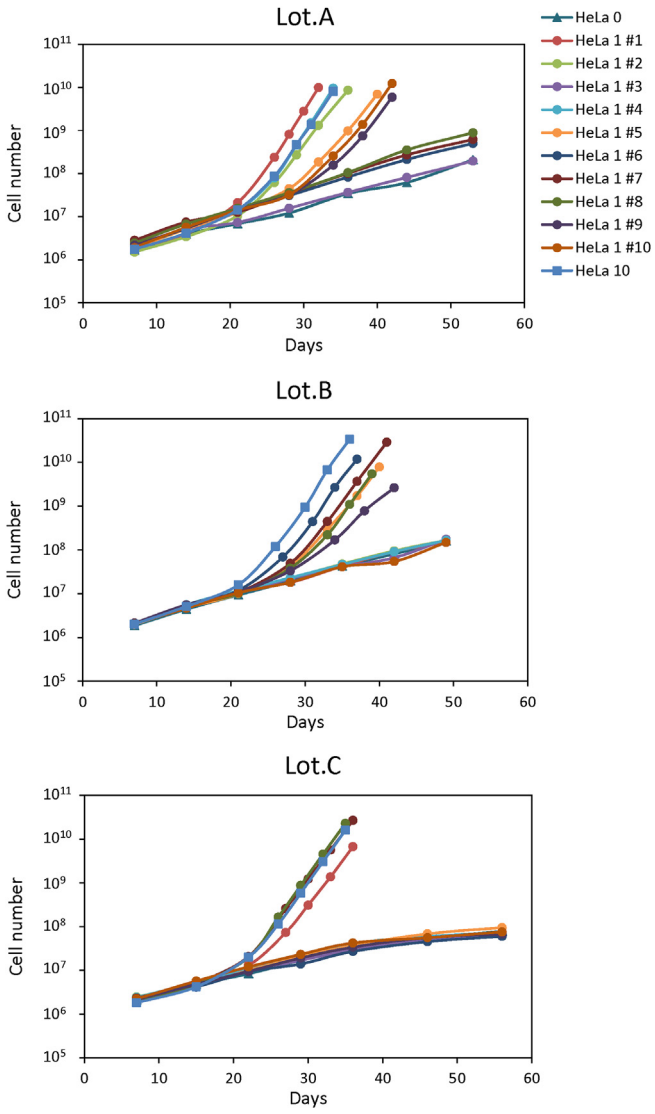

Fig. 1. Time course of the cell growth of BMSCs spiked with HeLa cells. One million BMSCs at passage 5 were mixed with none, 1, or 10 of HeLa cells (HeLa 0, HeLa 1, or HeLa 10, respectively) and cultured by passage 11 as described in the Materials and methods section. Ten samples spiked with one HeLa cell were used for each experiment with one lot of BMSCs. The results of three lots of BMSCs are presented.

immortalized/tumorigenic cells in 1×10^6 BMSCs within 3 passages with a 47% probability, which is the incidence rate of HeLa cells showing accelerated cell growth. To assess hCTP quality associated with safety, we need to avoid false negative results resulting from the failure to detect immortalized/transformed cells [14,15]. The false negative rate of one sample (x) was calculated as follows:

Table 1
The incidence rates of accelerated cell growth when one HeLa cell was spiked into BMSCs.

Lot.	HeLa 1		Incidence rate (%)
	+	−	
A	6	4	60
B	5	5	50
C	3	7	30
Average			47 ± 9[a]

[a] Values are means ± SEM of three lots.

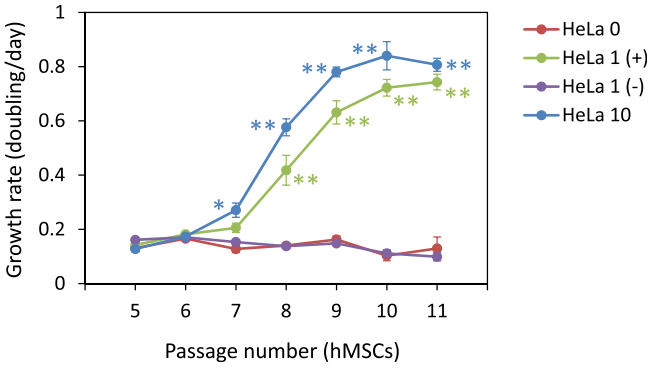

Fig. 2. Cell growth rate analysis of BMSCs spiked with HeLa cells. The growth rates of BMSCs spiked with one HeLa cell are plotted along the passage numbers after mixing with HeLa cells. HeLa 1 (+) represents the group of BMSCs spiked with one HeLa cell showing accelerated cell growth. HeLa 1 (−) represents the group of BMSCs spiked with one HeLa cell showing no accelerated cell growth. Data are represented as the mean ± standard error of the mean (SEM) of the three lots. Statistical significance was determined using two-way repeated measures analysis of variance (ANOVA) and the Student-Newman-Keulis's post-hoc test ($^{**}P < 0.001$ or $^{*}P < 0.05$ compared with the rate of HeLa 0 at $P = 5$).

$x = 1–0.47 = 0.53$. The false negative rate of n samples (y) can be expressed as follows: $y = x^n$. Hence we obtain: $n = \log y/\log x$. When the tests permit a 1% false negative rate, the n of samples can be calculated as follows: $n = \log(0.01)/\log(0.53) = 7.25$. Thus, 8 samples are necessary to prove the absence of 0.0001% immortalized cells comparable to HeLa cells in hCTPs with a growth property similar to BMSCs'.

HeLa cells are well-known immortalized/tumorigenic cells that are used as the control in common tumorigenicity tests with cell substrates for biological products [17]. We also attempted to detect another immortalized cell line, ASC52telo cells, which are hTERT-immortalized adipose-derived mesenchymal stem cells, contained in ADSCs as impurities, by using cell growth analysis. We spiked 1×10^3 (ASC52telo 0.1%), 1×10^2 (ASC52telo 0.01%), and 1×10 (ASC52telo 0.001%) of ASC52telo cells into 1×10^6 three lots of ADSCs at $P = 5$ and cultured them until $P = 20$ (Fig. 3). The ASC52telo cells alone proliferated almost constantly during their culture. On the other hand, all three lots of ADSCs containing ASC52telo cells quickly increased their cell growth at some time points, depending on the number of the spikedASC52telo cells. The cell growth rates of ADSCs alone (ASC52telo 0%) gradually decreased over the course of time and finally reached doubling rates/day of less than 0.1, likely due to the process of normal cell aging (Fig. 4). The cell growth rates in ASC52telo 0.1% were significantly higher than those in ADSCs alone after $P = 15$. ASC52telo 0.01% and ASC52telo 0.001% began to show significant difference in the cell growth rates at $P = 16$ and $P = 20$, respectively. The cell growth rate of ASC52telo cells slowly increased in the culture but not quickly, suggesting that whole cell adaptation to their culture condition occurred. As ASC52telo cells are similar to ADSCs in respect to morphology, we had difficulty distinguishing between ASC52telo cells and ADSCs under the microscope (Fig. S1). To confirm the impurities of ASC52telo cells in ADSCs, we consecutively measured the levels of hTERT transcript, which were exclusively expressed in ASC52telo cells, using qRT-PCR. hTERT mRNA was not detected in ADSCs alone during 15 passages after the spiking of ASC52telo cells under our experimental conditions. At the beginning of cell growth analysis, the levels of hTERT mRNA were dependent on the number of ASC52telo cells spiked in ADSCs. The levels of hTERT mRNA in all of the groups of ADSCs mixed with ASC52telo cells were gradually increased in successive passages and reached to the level comparable to that in ASC52telo cells

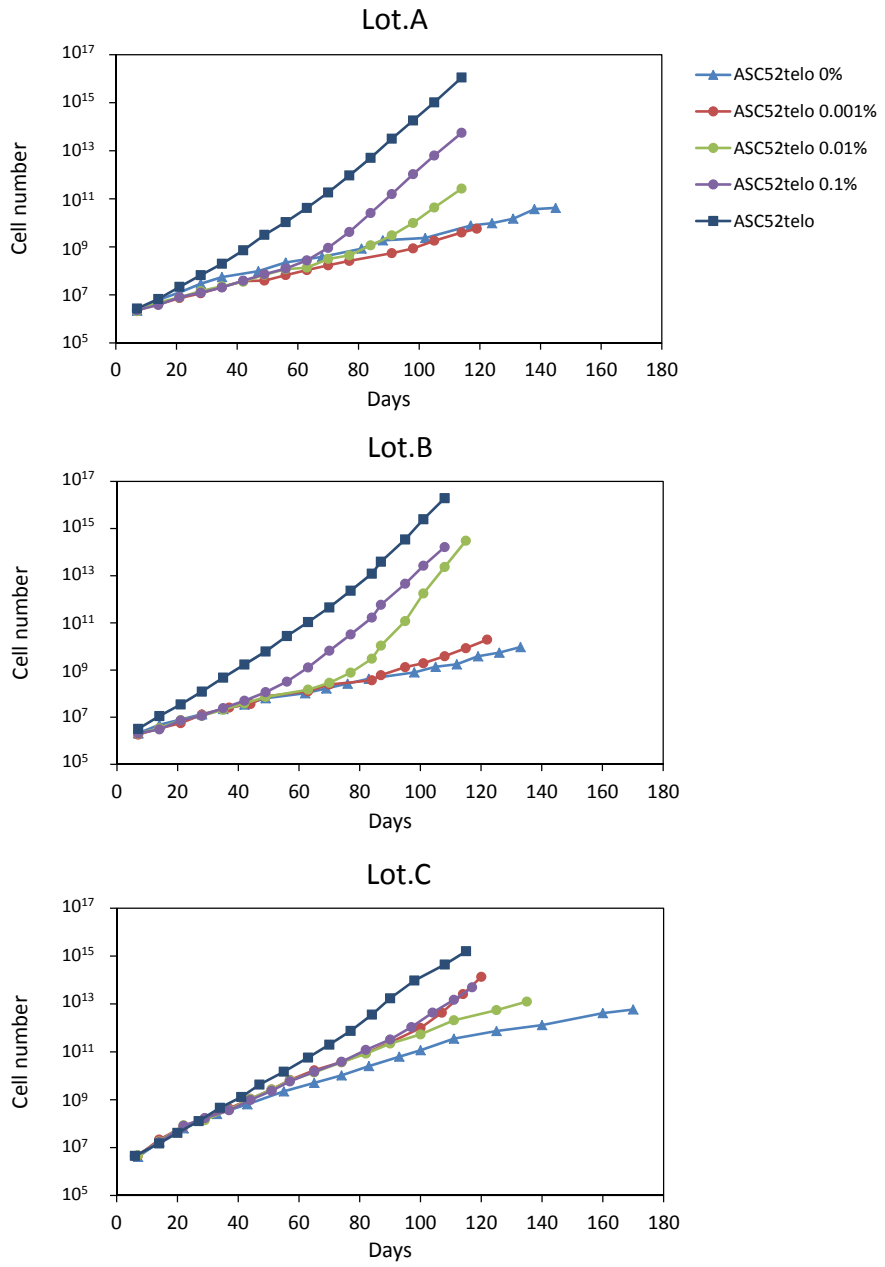

Fig. 3. Time course of the cell growth of ADSCs spiked with ASC52telo cells. One million ADSCs at passage 5 were mixed with 0, 10, 100, or 1000 of ASC52telo cells (ASC52telo 0%, ASC52telo 0.001%, ASC52telo 0.01%, or ASC52telo 0.1%, respectively) and cultured as described in the Materials and Methods section. ASC52telo cells alone were cultured as the control. The cells were passaged and counted at the indicated day from passage 5 until passage 20. The results of three lots of ADSCs are presented.

alone, within 15 passages after the spiking of ASC52telo cells (Fig. 5). These results indicated that most ADSCs were replaced with ASC52telo cells within 15 passages after the spiking in the cell growth analysis, even when as few as 0.001% ASC52telo cells were contained in ADSCs.

Cell growth analysis has great potential to detect a small amount of immortalized cellular impurities in hCTPs, even when the growth of the immortalized cells is relatively slow compared with HeLa cells. In the present study, the difference in the growth rate between ADSCs and ASC52telo cells was not so obvious as between BMSCs and HeLa cells, which makes difficult the detection of the immortalized cellular impurities by comparing the growth rate at an early passage with that at a later passage. These results suggest that negative time control data may be necessary to detect immortalized cellular impurities, when their growth rate is assumed to be modest.

We have recently reported several other methods for the detection of immortalized or tumorigenic cellular impurities in hCTPs besides cell growth analysis [15]. Digital analysis of soft agar colony formation, using a high-content image analyzer, can detect as little as 0.00001% HeLa cells in BMSCs [18]. In terms of sensitivity, this method is far superior to cell growth analysis. However, in principle, soft agar colony formation assay cannot be applied to immortalized cells not growing in agar. In fact, induction for a tumorigenic conversion upon hTERT transduction has not been shown with soft agar assays [19,20]. *In vivo* tumorigenicity testing using severely immunodeficient NOG mice is available to detect tumorigenic cells. They show tumor formation in one out of six

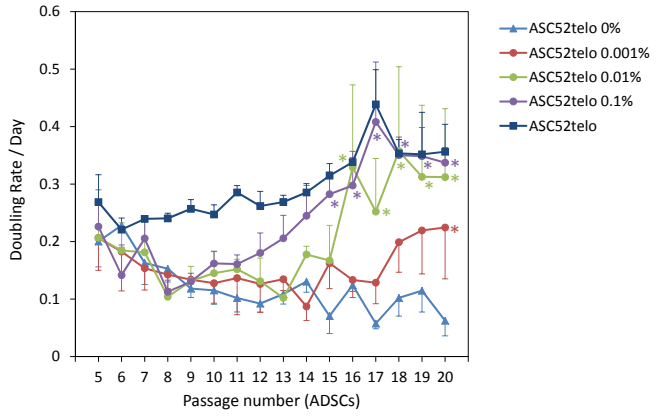

Fig. 4. Cell growth rate analysis of ADSCs spiked with ASC52telo cells. The growth rates of three lots of ADSCs spiked with ASC52telo cells are plotted. Data are represented as the mean ± SEM of the three lots. Statistical significance was determined using two-way repeated measures ANOVA and the Student-Newman-Keulis's post-hoc test (*P < 0.05 compared with the rate of ASC52telo 0%).

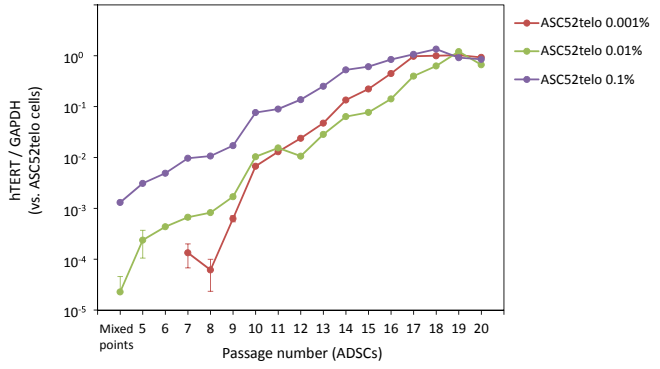

Fig. 5. Time-dependent changes in hTERT expression of ADSCs spiked with ASC52telo cells. One million ADSCs (Lot.C) mixed with 0, 10, 100, or 1000 of ASC52telo cells (ASC52telo 0%, ASC52telo 0.001%, ASC52telo 0.01%, or ASC52telo 0.1%, respectively) were cultured, and their total RNA was extracted. The mRNA levels of hTERT in the cells were determined with qRT-PCR analysis. hTERT mRNA was not detected in ADSCs alone (ASC52telo 0%) during their culture period. ASC52telo 0.001% did not show any expression of hTERT mRNA until 6 passages of ADSCs. Data are represented as the mean ± SEM of the triplicate experiments.

mice when transplanted with BMSCs containing 0.0001% HeLa cells subcutaneously [21]. *In vivo* tumorigenicity testing has an advantage of reflecting the *in vivo* microenvironment where hCTPs are transplanted. However, *in vivo* tests are costly and laborious. Appropriate methods should be chosen among the various tumorigenicity and related testings to evaluate immortalized or tumorigenic cellular impurities in hCTPs, taking the purpose and performance of the testings into consideration for decision making during the development of hCTPs [22]. We believe that the cell growth analysis characterized herein can contribute to the quality assessment of hCTPs and will suitably expedite cell therapy and regenerative medicine.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.06.005.

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