



Original Article

Evaluation of the reproducibility and positive controls of cellular immortality test for the detection of immortalized cellular impurities in human cell-processed therapeutic products

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ARTICLE INFO

Article history:

Received 24 August 2022

Received in revised form

14 October 2022

Accepted 20 October 2022

Keywords:

Cellular immortality test

Cell growth analysis

Cellular therapy

Tumorigenicity

Quality and safety

ABSTRACT

Introduction: Contamination of human cell-processed therapeutic products (hCTPs) with tumorigenic/immortalized cellular impurities is a major concern in the manufacturing and quality control of hCTPs. The cellular immortality test based on cell growth analysis is a method for detecting tumorigenic/immortalized cellular impurities in hCTPs. However, the performance of the cellular immortality test has not yet been well characterized. In this study, we examined the reproducibility of the cellular immortality test in detecting HeLa cells as a model of tumorigenic cellular impurities, as well as the applicability of other models of cellular impurities with different tumorigenicity to the cellular immortality test.

Methods: Using HeLa cells as a model for cellular impurities, we measured the growth rate of human mesenchymal stem cells (hMSCs) supplemented with HeLa cells at concentrations ranging from 0.01 to 0.0001% at each passage in three laboratories and evaluated the reproducibility of the detection of immortalized cellular impurities. In addition, HEK293 cells (another immortalized cell line) and MRC-5 cells (a non-immortalized cell line) were employed as cellular impurity models that exhibit different growth characteristics from HeLa cells, and the ability of the cellular immortality test to detect these different impurities when mixed with hMSCs was examined.

Results: In the multisite study, the growth rate of hMSCs supplemented with 1 and 10 HeLa cells (0.0001% and 0.001%) significantly increased and reached a plateau in all three laboratories, whereas those of hMSCs alone eventually decreased. Moreover, when hMSCs were supplemented with 10 and 100 HEK293 and MRC-5 cells (0.001% and 0.01%), the growth rate significantly increased. The growth rate of hMSCs supplemented with HEK293 cells increased with passage and remained high, whereas that of hMSCs supplemented with MRC-5 cells eventually decreased, as in the case of hMSCs alone.

Conclusions: These results indicate that the cellular immortality test is reproducible and can detect immortalized (i.e., potentially tumorigenic) cells such as HEK293 cells with a lower growth rate than HeLa cells by discriminating against normal cells, which could contribute to ensuring the safety and quality of hCTPs.

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Abbreviations: hCTPs, human cell-processed therapeutic products; hMSCs, human mesenchymal stem cells; PDL, population doubling level; PSCs, pluripotent stem cells; SACF, soft agar colony formation.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

<https://doi.org/10.1016/j.reth.2022.10.009>

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1. Introduction

Human cell-processed therapeutic products (hCTPs) derived from somatic cells such as human mesenchymal stem cells (hMSCs) and pluripotent stem cells (PSCs) such as embryonic stem cells and induced pluripotent stem cells are expected to provide promising treatments for life-threatening diseases for which no adequate therapy is currently available. However, tumorigenic/immortalized cellular impurities are a major concern for the manufacturing and quality control of hCTPs transplanted into patients. Immortalized cells (*i.e.*, potentially tumorigenic cells) found in hCTPs as impurities are attributable to residual undifferentiated PSCs [1,2], generation from the original component cells (*e.g.*, spontaneous transformation) [3–5], and/or cross-contamination with other tumorigenic/immortalized cells during the manufacturing processes [6–8]. The assessment of such cellular impurities is important for manufacturing products that are safe and of consistent quality.

Tumorigenic cells can proliferate rapidly and infinitely, whereas somatic cells such as hMSCs, usually exhibit a slower growth rate and attenuation of growth after serial passaging [9–11]. Therefore, the tumorigenicity of cells can be assessed using the growth characteristics of hCTPs through *in vitro* and *in vivo* assays [12,13]. The soft agar colony formation (SACF) assay [14,15] and digital SACF assay [16] are well-known *in vitro* assays for the detection of malignant transformed cells but not benign transformed cells. In contrast, the cellular immortality test based on cell growth analysis assesses the alteration of the growth rate on serial passaging, which makes it possible to detect immortalized cells, regardless of their tumorigenicity and malignancy. Our group has reported that 0.0001% HeLa cells, a well-known cancer cell line, and 0.001% mesenchymal stromal cells, which were derived from human adipose tissue and immortalized by transducing the telomerase reverse transcriptase gene, could be detected as contamination in hMSCs by the cellular immortality test [17]. In addition, the advantage of the cellular immortality test is that it is simple and inexpensive because it does not require any special equipment other than cell culture equipment. Therefore, the cellular immortality test has potential to be widely used to detect immortalized cellular impurities in hCTPs. However, the performance of this cellular immortality test remains to be characterized and fully understood. It is important to understand the performance of the assays to assess the safety and quality of the manufactured hCTPs. Multisite Evaluation Study on Analytical Methods for Non-Clinical Safety Assessment of Human-derived Regenerative Medical Products (MEASURE) is a Japanese experimental public–private partnership initiative launched by the National Institute of Health Sciences (NIHS) and FIRM-CoNCEPT (the Committee for Non-Clinical Safety Evaluation of Pluripotent Stem Cell-derived Therapeutic Products, the Forum for Innovative Regenerative Medicine). The objective of MEASURE is to validate experimental methods for the assessment of the tumorigenicity or biodistribution of hCTPs [18]. As a part of its activities, in the present study, we carried out a multisite validation of the cellular immortality test. Specifically, between-laboratory precision (*i.e.*, “reproducibility” as defined in the ICH Q2 (R1) guideline [19]) of detectability of the test for immortalized cellular impurities among three laboratories was investigated. HeLa cells were used as a positive control and a model of immortalized cellular impurities because they are highly proliferative and tumorigenic, widely accessible, and usually well characterized under a certain level of quality control as cell banks or stocks [15–17,20].

Furthermore, we investigated the ability of the cellular immortality test to detect other cell types with different rates of

proliferations, since other cellular impurities are not necessarily as proliferative as HeLa cells. In this study, we employed HEK293 and MRC-5 cells as other models of cellular impurities. HEK293 is a cell line derived from the kidney of a human female embryo and was originally immortalized in 1973 by the integration of a 4 kbp adenovirus 5 genome fragment, including the *E1A* and *E1B* genes at chromosome 19 [21,22]. HEK293 cells are used to produce bio-pharmaceuticals (*e.g.*, recombinant human coagulation factor VIII-Fc fusion protein, Efralocog Alfa; recombinant human coagulation factor IX-Fc fusion protein, Eftrenonacog Alfa) and gene transfer vectors (*e.g.*, adenovirus vector). It has been reported that HEK293 cells with high passage number (>65) possess tumorigenic potential in nude mice, but not those with low passage number (<52) [23]. Therefore, in this study, HEK293 cells with low passage numbers ($P < 52$) were used as another model of cellular impurities. MRC-5 is a normal diploid cell line derived from the normal lung tissue of a 14-week-old male fetus and is used to produce live attenuated varicella vaccine (*e.g.*, BIKEN, Takeda Pharmaceutical Company Limited; ZOSTAVAX, Merck & Co., Inc.). It has been reported that the senescence of MRC-5 cells begins at 42–46 population doubling levels (PDL) [24], indicating that the proliferative capability is attenuated by serial passaging, as in other somatic cells. We also investigated whether MRC-5 cells spiked into hMSCs could be detected by the cellular immortality test, along with HeLa cells.

2. Materials and methods

2.1. Cells

hMSCs at passage two ($P = 2$) were purchased from Lonza and cultured in MSCGM BulletKit (Lonza, Walkersville, MD, USA), a mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B. HeLa cells (#JCRB9004), HEK293 cells at $P = 42$ (#JCRB9068), and MRC-5 cells at $P = 23$ (#JCRB9008) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Merck, Darmstadt, Germany), 0.1 mM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), 50 U/mL penicillin, and 50 µg/mL streptomycin (Thermo Fisher Scientific). Cells were maintained in the medium described above and passaged upon reaching 90% confluence until they were used for cell growth analysis.

2.2. Cell growth analysis

The experiments for a multisite study were performed at Laboratories I, II, and III using HeLa cells spiked into hMSCs as a model of cellular impurities, and the experimental materials with the same model number were used across the three laboratories, according to a standardized protocol, as previously reported [17,20]. In addition, we performed the experiments that growth rate of hMSCs supplemented with HEK293 and MRC-5 cells were determined at every passage at Laboratories IV (NIHS) and I, respectively.

At $P = 5$, 1×10^6 hMSCs were supplemented with HeLa cells (1 or 10 cells), HEK293 cells (10 or 100 cells), or MRC-5 cells (10 or 100 cells). Ten or more cells were prepared by serial dilution of counted cells. Single HeLa cells were picked from Terasaki plates seeded at 1 cell/well. The cell suspensions were seeded into T175 flasks which are processed by hydrophilic coating (Nunclon Delta) to promote cell attachment (#178883, Thermo Fisher Scientific) and maintained in 40 mL Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine

serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Upon reaching approximately 90% confluence, the cells were rinsed with phosphate-buffered saline (Nacalai Tesque, Kyoto, Japan) and treated with 0.05% trypsin-EDTA solution (Thermo Fisher Scientific) for detachment from the flasks. The cells were centrifuged at 450×g for 5 min and resuspended in fresh culture medium. Aliquots of the cell suspension were stained with trypan blue or an acridine orange/propidium iodide viability kit (Logos Biosystems, Annandale, VA, USA), and living cells were quantified using a Countess Automated Cell Counter (Thermo Fisher Scientific) and a LUNA-FL dual-fluorescence cell counter (Logos Biosystems), respectively, according to the manufacturer's protocols to eliminate unconscious bias and human error. One million cells were reseeded into T175 flasks and cultured until the next passage. These procedures were repeated for P = 10 or 11. The experiments were conducted in triplicates. The growth rate (R_n) at P = n was calculated using 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 date at P = k, respectively.

2.3. Statistical analysis

Statistical significance was analyzed using SigmaPlot Version 14.5 software (Systat Software, San Jose, CA, USA) by two-way repeated-measures analysis of variance (ANOVA) with Dunnett's post-hoc test. P values < 0.05 were considered significant. The growth rate data of hMSCs spiked with HeLa and HEK293 cells at ratios of 10:1 × 10⁶ and 100:1 × 10⁶, respectively, were fitted to the Boltzmann function using GraphPad Prism (GraphPad Software, San Diego, CA, USA), to estimate the final growth rates after prolonged culture, which represent those of the dominant cells, *i.e.*, HeLa and HEK293 cells.

3. Results

3.1. Multisite study for the assessment of the reproducibility of the cellular immortality test

We first conducted a multisite study to evaluate the reproducibility of the cellular immortality test for the detection of immortalized cellular impurities in hCTPs. HeLa cells (1 or 10 cells) were added to 1 × 10⁶ hMSCs at P = 5, followed by the calculation of their growth rate from the number of living cells at every passage until P = 11. During cultivation of the cells, dead cells were hardly observed in all the three laboratories. The average growth rate per laboratory plotted against the passage number is shown in Fig. 1. The average growth rates of hMSC alone in laboratories I and III were significantly higher at P = 6 to 7 and P = 7 to 10, respectively, than at P = 5, whereas the average growth rates in all the three laboratories were lower at P = 11 than at P = 5 [two-way repeated measures ANOVA and Dunnett's post-hoc test]. In contrast, the average growth rates of hMSCs spiked with 10 and 1 HeLa cells were significantly higher in all the three laboratories at P = 7 to 11 and P = 9 to 11, compared with those at P = 5, respectively [two-way repeated measures ANOVA and Dunnett's post-hoc test]. Although one of the three tests for hMSCs spiked with 1 HeLa cell at Laboratories II and III showed little increase or a slight decrease in the growth rate (Supplementary Fig. S1), these results indicate that the cellular immortality test can detect HeLa cells intermingled at a concentration of 0.0001% or more in hMSCs, and was reproducible among the three laboratories, consistent with a previous report [17]. Non-linear regressions of the raw data for Fig. 1C, *i.e.*, the growth rates of the hMSCs spiked with HeLa cells at a ratio of

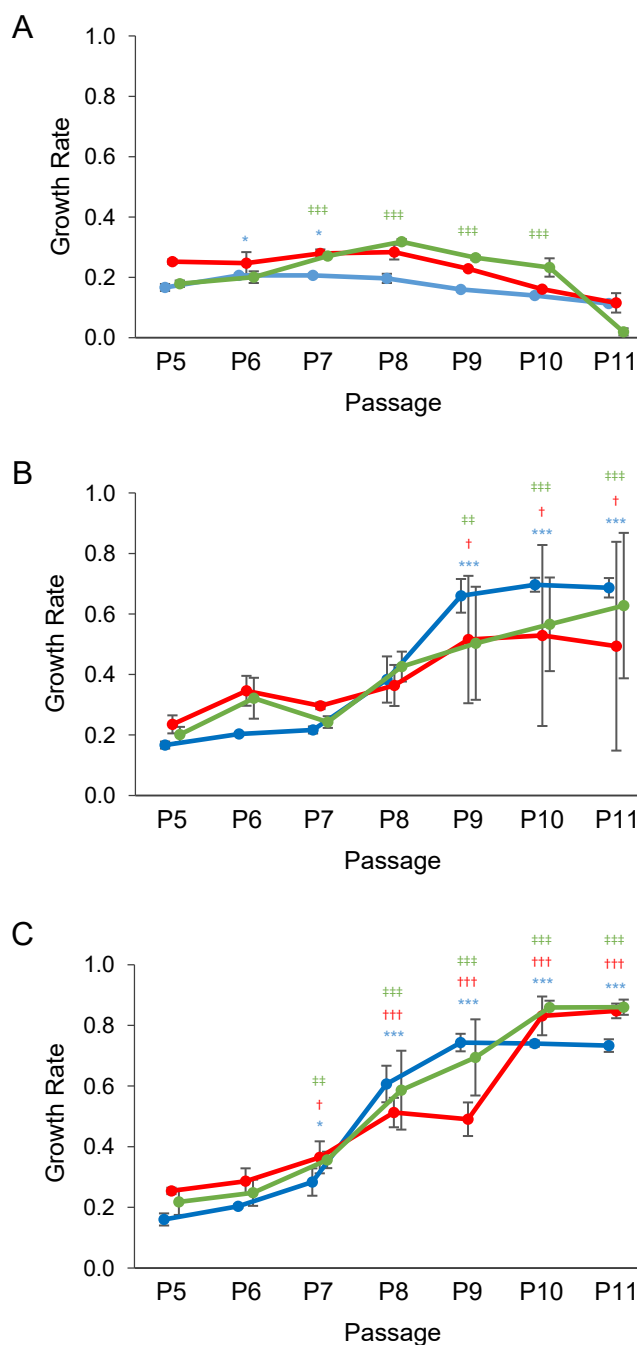


Fig. 1. Multisite study on cell growth analysis of hMSCs supplemented with HeLa cells. The y-axis indicates the growth rate (doubling per day) of hMSCs supplemented with HeLa cells (A: no cells, B: 1 cell, and C: 10 cells). The growth rates at the three laboratories were plotted against passage numbers (blue line, Laboratory I; red line, Laboratory II; green line, Laboratory III). Data are presented as mean ± standard deviation (SD) (n = 3, biological replicates). Statistical significance was determined using two-way repeated measures ANOVA and Dunnett's post-hoc test (*, Laboratory I; †, Laboratory II; †, Laboratory III, * P and † P < 0.05; †† P and ††† P < 0.01; †††† P , and ††††† P < 0.005 compared with the growth rate of each laboratory at P = 5).

10:1 × 10⁶, to the Boltzmann function (a sigmoidal curve with time as x-axis) indicated that the growth rate of the cells dominant after serial passaging, *i.e.*, HeLa cells, was estimated to be 0.88 ± 0.04 doubling per day (mean ± standard deviation with degrees-of-freedom (ν) of 6, Supplementary Table S1), which was comparable to previously reported results [17,20].

3.2. Contamination with HEK293 cells could be detected by the cellular immortality test

To evaluate the applicability of cell lines other than HeLa cells to the cellular immortality test as positive controls, we added HEK293 and MRC-5 cells (10 and 100 cells) to 1×10^6 hMSCs at P = 5 and calculated their growth rate from the number of living cells at every passage. During cultivation of the cells, dead cells were hardly observed. The average growth rates of hMSCs supplemented with HEK293 and MRC-5 cells plotted against passage numbers are shown in Figs. 2 and 3, respectively. As shown in Fig. 2, the average growth rates of hMSCs supplemented with 10 and 100 HEK293 cells were significantly higher at P = 9 than at P = 5 and then reached a plateau, whereas that of hMSC alone decreased until P = 10 [two-way repeated measures ANOVA and Dunnett’s post-hoc test], showing that not only HeLa cells but also HEK293 cells intermingled in hMSCs could be detected by the cellular immortality test. Non-linear regressions of the growth rates of hMSCs spiked with HEK293 cells at a ratio of 100:1 $\times 10^6$ (Fig. 2B) to the Boltzmann function indicated that the growth rate of the dominant cells after serial passaging, i.e., HEK293 cells, was estimated to be 0.64 ± 0.05 doubling per day (mean \pm standard deviation with degrees-of-freedom (ν) of 2 ($n = 3$), Supplementary Table S1).

In the case of hMSCs supplemented with 10 and 100 MRC-5 cells, the average growth rates were significantly higher at P = 8

and 6, respectively, compared to those at P = 5 [two-way repeated measures ANOVA and Dunnett’s post-hoc test] (Fig. 3). The increase in the growth rate of hMSCs supplemented with 100 MRC-5 cells was greater than that of hMSCs supplemented with 10 MRC-5 cells. However, unlike hMSCs supplemented with HeLa and HEK293 cells, the growth rate of hMSCs supplemented with MRC-5 cells decreased at P = 10 and was lower at P = 11 than at P = 5, as was the case for hMSCs alone (Figs. 1A and 3).

4. Discussion

In the quality control of hCTPs, such as hMSCs, one of the major concerns is contamination with immortalized cells and spontaneous transformation to immortalized cells during the manufacturing process since they increase the risk of tumor formation in patients. Therefore, the assessment of immortalized cellular impurities is critical for ensuring the quality and safety of hCTPs. The cellular immortality test based on cell growth analysis is a simple and low-cost method that can detect immortalized/tumorigenic cells based on differences in the growth rate between cells [17,20]. In this study, hMSCs supplemented with three different cell lines were used as models of contaminated hCTPs to demonstrate the performance of the cell lines as positive controls in the cellular immortality test.

The cellular immortality test can be easily performed in many laboratories because they do not need any special equipment other

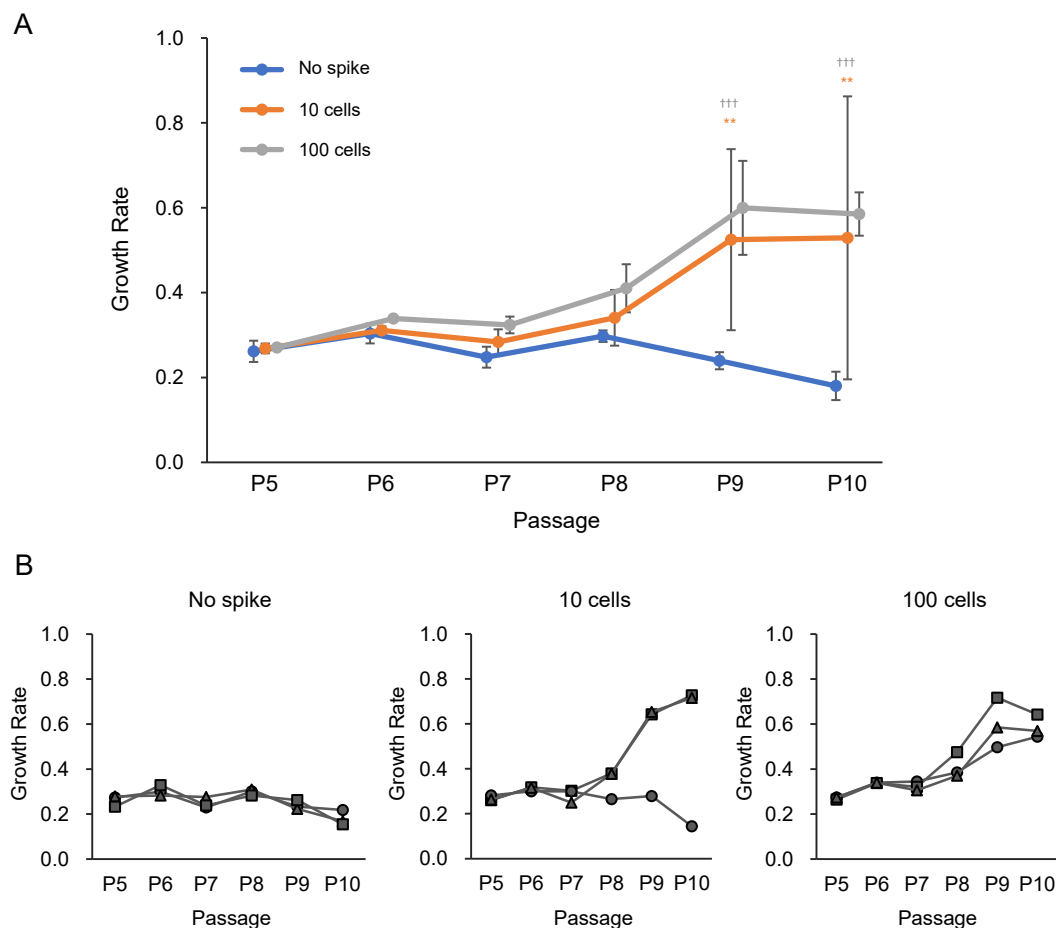


Fig. 2. Cell growth rate of hMSCs supplemented with HEK293 cells. The y-axis indicates the growth rate (doubling per day) of hMSCs supplemented with HEK293 cells (0, 10, and 100 cells). The growth rates are plotted against passage numbers (A, blue line, no cells; red line, 10 cells; gray line, 100 cells). Data are presented as the mean \pm standard deviation (SD) ($n = 3$, biological replicates) (A) and the value of each sample (B). Data were obtained from Laboratory IV. Statistical significance was determined using two-way repeated measures ANOVA and Dunnett’s post-hoc test (*, 10 cells; †, 100 cells, ** $P < 0.01$; ††† $P < 0.005$ compared to the rate at P = 5).

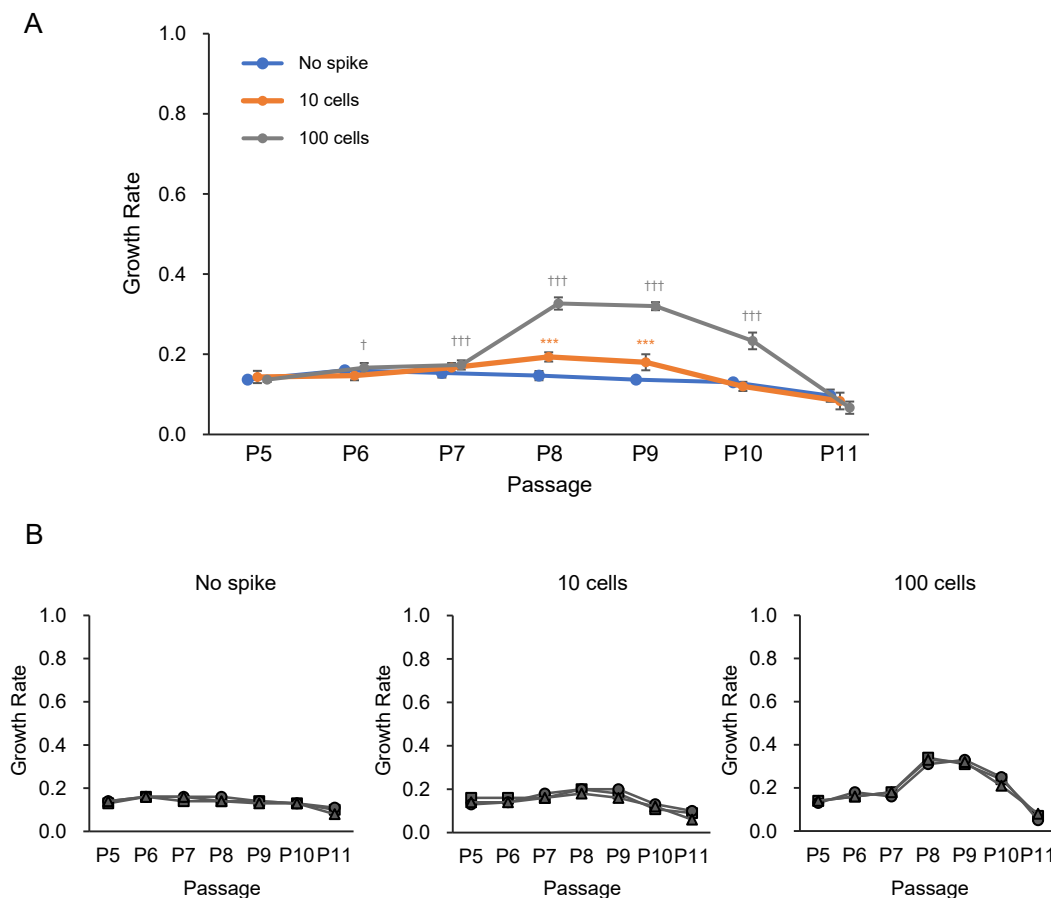


Fig. 3. Cell growth rate of hMSCs supplemented with MRC-5 cells. The y-axis indicates the growth rate (doubling per day) of hMSCs supplemented with MRC-5 cells (0, 10, and 100 cells). The growth rates are plotted along the passage numbers (A, blue line, no cells; red line, 10 cells; gray line, 100 cells). Data are represented as the mean \pm standard deviation (SD) (n = 3, biological replicates) (A) and the value of each sample (B). Data were obtained from Laboratory I. Statistical significance was determined using two-way repeated measures ANOVA and Dunnett's post-hoc test (*, 10 cells; †, 100 cells, †P < 0.05; ***P and †††P < 0.005 compared to the rate at P = 5).

than cell culture equipment. However, the detection limit of this test could be affected by basic cell culture skills as well as the experimental environment, especially when detecting trace amounts of cellular impurities intermingled with hCTPs. In the present study, all three laboratories confirmed significant increases in the average of growth rate of hMSCs spiked with 1 and 10 HeLa cells, suggesting that cellular immortality tests can detect cellular impurities with high reproducibility between laboratories, even when hCTPs are contaminated with trace amounts of immortalized/tumorigenic cells. However, at P = 9 to 11, the standard deviations of hMSCs supplemented with 1 HeLa cell were high in Laboratories II and III, because one out of the three tests showed little increase or a slight decrease in the growth rate (Supplementary Fig. S1). In these hMSC samples, a single HeLa cell was collected under a microscope and added to each hMSC sample; however, it is speculated that the HeLa cells may have been killed or lost during seeding into the assay plates.

The tumorigenicity of HEK293 cells has been reported to be weak and impossible to detect in immunodeficient animals without long-term culture and repeated passages [23], which is consistent with the fact that the estimated growth rate was lower than that of HeLa cells in the present study (Supplementary Table S1). However, we found that hMSCs supplemented with 10 HEK293 cells significantly increased the average growth rate, although one of the three tests showed no increase in the growth rate. This suggested that the *in vitro* cellular immortality test could

be a more sensitive method to detect cellular impurities with tumorigenicity comparable to HEK293 cells, compared with the *in vivo* tumorigenicity test using immunodeficient animals.

To assess the quality and safety of hCTPs reliably, it is necessary to understand the probability of false-negative results, in which the cellular immortality test misses immortalized/transformed cellular impurities. If the false-negative rate of a single sample is x, the false-negative rate y of n samples can be expressed as $y = x^n$. Hence, we obtain $n = \log y / \log x$. Using this equation, we can calculate the number of samples required to detect the cells of interest with a specified sensitivity after allowing for an arbitrary false-negative rate. For example, when the tests permit a 1% false-negative rate, the number of samples (n) can be calculated as follows: $n = \log (0.01) / \log x$. From the results of 10 HEK293 cells, $n = \log (0.01) / \log (1/3) = 4.15$, suggesting that at least five samples are necessary to prove the absence of 0.001% immortalized cells comparable to HEK293 cells in hCTPs with a growth property similar to that of hMSCs. In contrast, HeLa cells could be detected in all assays when 10 HeLa cells were added to hMSCs, suggesting that more samples are needed to prove the absence of HEK293-like cells compared with HeLa-like cells. Moreover, the false-negative rate (y) was altered by the number of spiked cells, similar to the results of 1 and 10 HeLa cells and 10 and 100 HEK293 cells intermingled in hMSCs, suggesting that a larger sample size is necessary to detect smaller amounts of cellular impurities. Therefore, precision and sample size should be considered to design optimal assays for the detection of

tumorigenic/immortalized cellular impurities. In addition, positive controls are important for confirming the reliability of tests and allowing estimation of the number of cellular impurities. In other words, the cellular immortality test based on cell growth analysis is susceptible to the quality of the positive control cells. Therefore, cell lines obtained from quality-controlled cell stocks, e.g., ATCC, ECACC, JCRB, Riken BRC, should be employed as positive controls.

The malignant transformation of cells is believed to occur through multiple processes involving the accumulation of mutations in key regulatory genes that promote cell survival and proliferation [25–27]. Not all cellular impurities of hCTPs are always tumorigenic or immortal. The growth rate of hMSCs supplemented with MRC-5 cells increased temporarily after $P = 5$ (Fig. 3). In our preliminary study, the growth rate of MRC-5 cells with low PDL (below 42–46) was approximately 0.7 (data not shown), which is higher than that of hMSCs, suggesting that the increased growth rate could have been attributable to the growth advantage of the spiked MRC-5 cells. Alternatively, MRC-5 cells could have facilitated the growth of hMSCs, via autocrine/paracrine signaling [28,29]. Also, if a non-immortalized subpopulation in the hMSCs had a high proliferative capability only under the cell culture condition for the test, such a subpopulation could have led to the transient increase in the overall growth rate.

The proliferative capability of MRC-5 cells with high PDL (over 42–46) is known to be attenuated due to senescence [24]. In the present study, the estimated PDL of MRC-5 cells intermingled in hMSCs was over 42–46 at $P = 8$, suggesting that the decrease in the growth rate of hMSCs supplemented with MRC-5 cells after $P = 8$ was due to the senescence of both MSCs and MRC-5 cells. As shown in Fig. 1A, slight but significant increases in the growth rate of hMSCs alone were also found at Laboratories I and III, which could be attributed to non-immortalized hMSC subpopulations with a high proliferative capability and/or those secreted humoral growth-promoting factors. The impact of such subpopulations with a growth advantage might be augmented by a change in the culture condition from that for the maintenance of an hCTP to that for the test. When the normal cells in the hCTP are composed of multiple subpopulations, a multi-phase (multi-step) proliferation curve may be observed, but if the overall proliferation rate of the hCTP is finally confirmed to be attenuated by this method with a relevant positive control, it suggests the absence of immortalized cell impurities. Therefore, in the cellular immortality test, if an increase in the growth rate is observed, it is important to determine whether the rate is attenuated after prolonged culture. In general, estimation and identification of characteristics of potential cellular impurities such as cell appearance and marker molecules are difficult. Although the methods such as flow cytometry and qRT-PCR are potent tools to detect cell types and marker molecules, these methods are inadequate to detect unknown cellular impurities. Similarly, the detection method using microscopy needs to clarify the differences in cell appearance between hCTPs and cellular impurities. As long as their characteristics are unknown, prolonged culture should be performed to demonstrate that no immortalized cells reside in hCTPs.

The cellular immortality test based on cell growth analysis aims to show the absence of immortalized cellular impurities with a proliferation rate comparable to or higher than that of the positive control cells after long-term culture. In general, cells in a continuous cell line that potentially serves as a positive control tend to have a high growth rate because cells with a growth advantage are selected during the strain generation process, and positive control cells with a low growth rate are usually not readily available. Since false negative results are hardly distinguished from the true negatives without a valid positive control, it could be difficult to detect

the cellular impurity with this method, when the growth rate of the immortalized cellular impurity is equal to (or lower than) the hCTP's. Cellular impurities in non-adherent normal blood cells can also be detected by the same principle with appropriate positive control cells. However, many continuous cell lines derived from blood cancers secrete humoral factors that affect the growth of normal blood cells, and if they have to be used as positive controls, the interpretation of test results can be very difficult.

5. Conclusion

In the present study, the cellular immortality test based on cell growth analysis was used to detect trace amounts (0.0001%) of HeLa cells used as a model for immortalized cellular impurities, which were intermingled with hMSCs, with good reproducibility between laboratories. Furthermore, hMSCs supplemented with HEK293 cells exhibited growth characteristics clearly different from hMSCs alone, suggesting that HEK293 cells can be used as a positive control with a lower growth rate than HeLa cells in the cellular immortality test. Somatic cells in cell therapy products are often heterogeneous, and subpopulations of normal cells that are well adapted to culture conditions may gain a growth advantage and temporarily increase the overall growth rate, like the results of the growth rate of hMSCs supplemented with MRC-5 cells. Therefore, when evaluating contamination of hCTPs with immortalized cellular impurities by the cellular immortality test, even if the growth rate is found to change, it is important to confirm with further long-term culture that it remains constant and does not decrease. Our findings provide important points to consider when conducting cellular immortality tests and will contribute to ensuring the quality and safety of hCTPs by controlling immortalized cellular impurities, a potential hazard related to the risk of tumor formation in patients.

Author contributions

K.K., S.K., S.Y., R.S., N.S., and Y.S. conceived and designed the experiments. T.H., K.K., A.M., S.H., A.W., T.K., R.T., and S.W. performed the experiments. T.H., K.K., S.K., S.Y., and Y.S. wrote the manuscript. Y.S. acquired the funding. All authors analyzed the data and reviewed the manuscript.

Data availability statement

Not applicable.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgements and Funding

The authors gratefully acknowledge the continuous support of all members of the MEASURE Project and FIRM-CoNCEPT, which has been led by Drs. Keiji Yamamoto, Hiroto Bando, and Takeshi Watanabe. This work was supported by research grants from the Japan Agency for Medical Research and Development to YS (grant numbers: JP19mk0104101, JP19mk0104080, JP20mk0104118, JP22mk0104177, JP22mk0104176, JP22mk0101195). The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2022.10.009>.

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