Current Topics

Stem Cell Research for Regenerative Medicine/Personalized Medicine

Tumorigenicity Studies for Human Pluripotent Stem Cell-Derived Products

Takuya Kuroda,[a,b] Satoshi Yasuda,[a,b] and Yoji Sato*,[a,b,c]

[a] *Division of Cellular and Gene Therapy Products, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [b] *Foundation for Biomedical Research and Innovation; Kobe 650–0047, Japan: and* [c] *Department of Quality Assurance Science for Pharmaceuticals, Graduate School of Pharmaceutical Sciences, Nagoya City University; Nagoya 467–8603, Japan.*
Received November 4, 2012

Human pluripotent stem cells (hPSCs), *i.e.* human embryonic stem cells and human induced pluripotent stem cells, are able to self-renew and differentiate into multiple cell types. Because of these abilities, numerous attempts have been made to utilize hPSCs in regenerative medicine/cell therapy. hPSCs are, however, also tumorigenic, that is, they can give rise to the progressive growth of tumor nodules in immunologically unresponsive animals. Therefore, assessing and managing the tumorigenicity of all final products is essential in order to prevent ectopic tissue formation, tumor development, and/or malignant transformation elicited by residual pluripotent stem cells after implantation. No detailed guideline for the tumorigenicity testing of hPSC-derived products has yet been issued for regenerative medicine/cell therapy, despite the urgent necessity. Here, we describe the current situations and issues related to the tumorigenicity testing of hPSC-derived products and we review the advantages and disadvantages of several types of tumorigenicity-associated tests. We also refer to important considerations in the execution and design of specific studies to monitor the tumorigenicity of hPSC-derived products.

Key words pluripotent stem cell; embryonic stem cell; induced pluripotent stem cell; tumorigenicity; regenerative medicine; cell therapy

1. INTRODUCTION

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have two abilities: 1) pluripotency, which is the ability to differentiate into a variety of cells, and 2) self-renewal, which is the ability to undergo numerous cell division cycles while maintaining their cellular identity. Because of these two characteristics, they are expected to provide new sources for the robust and continuous production of a variety of cells and tissues for use in regenerative medicine/cell therapy. Additionally, human iPSCs (hiPSCs) offer a possible solution to the ethical problems and immune rejection of cell products derived from human ESCs (hESCs), thus creating novel avenues for individual patient-specific cell therapy. As a practical example of therapy using hESC-based products, the world's first clinical trial started in spinal cord patients in the United States in 2011. Other clinical trials have also been conducted with retinal pigment epithelial (RPE) cells derived from hESCs to treat patients with dry age-related macular degeneration (AMD) and Stargardt's macular dystrophy by Advanced Cell Technology.[1)] Following Yamanaka's establishment of hiPSCs in 2007, new innovations in regenerative medicine/cell therapy have been made that relate to cell substrates, manufacturing materials, and application methods. As one of the promising seeds for practical use in regenerative medicine/cell therapy, the clinical application of hiPSC-derived RPE cells is expected to start in 2013 in Japan for wet AMD patients.

For the proper development of hPSC-derived products, it is essential to assess their risk and to study their quality and safety. In this review, we place a particular focus on the current situations and issues related to the evaluation of tumorigenicity, which is one of the potential concerns in an attempt to conduct clinical research on hPSC-derived products.

2. THE TUMORIGENICITY OF HUMAN PLURIPOTENT STEM CELLS

Tumorigenicity is defined as the capacity of a cell population inoculated into an animal model to produce a tumor by proliferation at the site of inoculation and/or at a distant site by metastasis.[2)] Undifferentiated hPSCs have the ability to form teratoma in animals, and this is ascribed to their pluripotency.[3)] In general, to demonstrate the pluripotency of established cells, they are injected into immunodeficient mice, *e.g.*, nude mice, to form spontaneous teratomas. Teratomas are defined as tumors of multiple lineages containing tissue derived from the three germ layers (*i.e.*, endoderm, mesoderm, and ectoderm). hPSCs are tumorigenic and differ greatly from somatic cells and somatic stem cells in terms of tumorigenic potential.[4)] Residual pluripotent cells in hPSC-derived products have the ability to initiate ectopic tissue formation, tumor development, and/or malignant transformation.

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: yoji@nihs.go.jp

3. INTERNATIONAL GUIDELINES OF TUMORIGENICITY STUDIES

At present, the World Health Organization (WHO) TRS 878 guideline, titled, "Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks,"[2,5] is the only international guideline addressing tumorigenicity studies. The International Conference on Harmonization's "ICH guideline Q5D: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products" also cited the tumorigenicity tests of the WHO TRS 878 guideline. These guidelines provide documented study design advice and general principles for tumorigenicity studies. Several *in vitro* test systems, such as cell growth in soft agar and muscle organ culture, have been explored as alternatives to *in vivo* tests for tumorigenicity[6]; however, as a result of technical difficulties, the correlations with *in vivo* tests have not yet been clearly proved. Therefore, in the WHO TRS 878, *in vivo* tests remain the standard for assessing tumorigenicity. Simply put, the model protocol of the *in vivo* tumorigenicity test in the WHO TRS 878 is that 10^7 animal cells are administrated to 10 nude mice and observed for 3 to 16 weeks; HeLa cells are recommended as the positive control reference preparation. Applying this test to hPSC-based cell therapy products, we must learn about its coverage and purpose. The *in vivo* tumorigenicity test proposed in WHO TRS 878 covers cells used to manufacture biological products but not cells transplanted into patients. Its purpose is to examine the tumorigenic phenotype range, from non-tumorigenic to weakly- or highly tumorigenic, of the cell banks, but not to detect the slightly contaminated tumorigenic cells in hPSC-derived products. The WHO TRS 878 also requires the master or working cell bank to check the tumorigenicity whenever cultured for predetermined passage times. WHO TRS 878 has therefore not directly addressed the tumorigenicity of hPSC-derived products. Importantly, the tumorigenicity described in WHO TRS 878 is not a direct risk index for humans but that estimated by animal testing, which examines tumor formation at transplanted sites and metastasis at remote sites.

4. ALTERNATIVE APPLICATION OF WHO TRS 878 TO hPSC-DERIVED PRODUCTS

As mentioned above, one of the risks of hPSC-derived products is the possibility of tumor formation following transplantation. To ensure safety, the tumorigenicity of hPSC-derived products must be evaluated to identify undifferentiated and/or abnormal cells that might exist in minute quantities in final products. It should be noted that the tumorigenicity of final products is clearly different from the tumorigenicity of the cell bank of serially passaged cell lines defined as cell substrates in WHO TRS 878. However, we must consider how the WHO TRS 878 protocol of tumorigenicity testing should be applied to hPSC-derived products because the WHO TRS878 guideline is the only one that directly addresses tumorigenicity studies. First, we understand the grounds of the WHO TRS 878 tumorigenicity test protocol to require administratation of 10^7 cells to nude mice. Tumor-producing doses at the 50% endpoint (TPD_{50}) (the number of cells required for tumor development with 50% probability) are used as units of tumorigenic phenotypes. The strengths of TPD_{50} vary greatly according to cell strain. For example, the TPD_{50} values of Endo-CA (human endometrial carcinoma cells), A594 (human lung cancer cells), HeLa (human cervical carcinoma cells), and 293 (human kidney cells) are 10, 3×10^3, 3×10^4 and 3×10^6 cells/nude mouse, respectively.[7] Administrating 10^7 cells to 10 nude mice should result in the tumor formation of 293 weakly tumorigenic cells in several nude mice out of ten. On the other hand, 10^7 cells of highly tumorigenic HeLa cells should form tumors in all ten nude mice. Therefore, HeLa cells are recommended as the positive control.

In general, treatment with hPSCs-derived products is thought to require from several tens of thousands to hundreds of millions cells, depending on the disease. Several tens of thousands of prepared RPE cells may be required for retinal degeneration diseases, whereas in the case of treatment for heart failure, hundreds of millions of cardiac muscle cells may be necessary. Assuming that 1 in 10^4 of the final product cells (0.1%) is tumorigenic, the *in vivo* tumorigenicity test would require an inoculum of 3×10^8 or 3×10^{10} cells to detect tumor formation in nude mice when contaminated cells have tumorigenic activity equal to HeLa cells (TPD_{50}: 3×10^4) or 293 cells (TPD_{50}: 3×10^6), respectively. The alternative application of WHO TRS 878 "Administrate 10^7 cells to 10 nude mice" to hPSC-derived products may thus lead to false-negative test results.

5. AVAILABILITY OF HIGHLY IMMUNODEFICIENT MICE

To detect slightly contaminated tumorigenic cells in hPSC-derived products, several new generations of severely immunodeficient animal models are now available. Rag2-γC double-knockout (DKO) mice,[8] NOD/SCID/γC^{null} (NOG) mice,[9] and NOD/SCID/IL-2rgKO (NSG) mice[10] are reported to be T, B, and NK cell-defective and to show high engraftment rates of human cells and tissues compared with traditional nude (T cell-defective) mice.[11,12] Using these severe combined immunodeficient mouse lines, which are likely to be useful for sensitive *in vivo* tumorigenicity tests, the small amount of residual tumorigenic cells in the hPSC-derived products could be detected. Since scientific risk assessment needs to standardize the tumorigenicity evaluation of hPSC-derived products, the following points should be taken into consideration in the development of *in vivo* tumorigenicity tests: (a) validation of the limit of detection, sensitivity, and precision, (b) positive and negative control selection, (c) number of tested cells, (d) test duration, (e) route/method of administration, and (f) comparison with nude mouse.

6. *IN VITRO* TUMORIGENICITY-ASSOCIATED TESTS

Some tests are indicated to detect tumorigenic cells contaminating cell populations *in vitro*. Table 1 summarizes the advantages and disadvantages of the tests associated with product tumorigenicity. The soft agar colony formation assay is a conventional method to monitor anchorage-independent growth, and is considered the most appropriate *in vitro* assay to detect the malignant transformation of cells.[6] Previous

Table 1. Comparison of Tumorigenicity-Associated Assays

Assay	Soft agar colony formation assay	Flow cytometry	qRT-PCR	*In vivo* tumorigenicity test using SCID mice[15]
Measurement standard	Colony formation	Expression of marker protein for pluripotency	Expression of marker gene for pluripotency	Tumor formation
Purpose	Detection of anchorage-independent growth	Detection of tumorigenic and undifferentiated cell	Detection of tumorigenic and undifferentiated cells	Detection of tumorigenic of undifferentiated pluripotent cells
Time	30 d	1 d	6 h	12–16 weeks
Advantage	Inexpensive	Rapid Analyzes individual cells	Rapid and simple Quantitative Highly sensitive	Direct Analyzes tumor formation in a specific microenvironment
Disadvantage	Indirect Not applicable to hiPSCs	Indirect Detects only the cells that express the known marker proteins Gating techniques strongly influence the results	Indirect Detects only the cells that express the known marker genes	Costly Time-consuming
Limit of detection	1% of PA-1 teratocarcinoma cells	0.1% of hiPSCs (TRA-1-60)	≦0.002% of hiPSCs (Lin28)	245 Undifferentiated hESCs with 10^6 feeder fibroblasts (0.025%)

reports have shown that hPSCs undergo apoptosis when dissociated into single cells.[13] This test requires the scattering of cells and their enclosure in agar so it may be difficult to utilize it for tests of hPSC-derived product tumorigenicity. Flow cytometry and quantitative reverse transcription polymerase chain reaction (qRT-PCR) tests were found to be able to detect a trace amount of undifferentiated cells. The advantage of the flow cytometry test is that it can be used to identify undifferentiated cells. Unfortunately, the results are greatly affected by gating, and only the cells expressing the marker proteins are detectable. The advantages of qRT-PCR are its rapidity, quantitative nature, and high sensitivity. Its disadvantage is that only the cells expressing the marker gene are detectable. Our previous report demonstrated that the soft agar colony formation assay is unable to detect hiPSCs, even in the presence of a Rho-associated protein kinase (ROCK) inhibitor that permits survival of dissociated hiPSCs/hESCs. The flow cytometry test using anti-TRA-1-60 antibody has detected 0.1% undifferentiated hiPSCs spiked in primary RPE cells. The qRT-PCR method with a specific probe and primers has been found to detect a trace amount of Lin28 mRNA, which is equivalent to that present in a mixture of a single hiPSC and 5×10^4 RPE cells.[14] As tumorigenic cells are commonly highly proliferative and immortalized, observation of the cell growth rate by culturing for a limited period also seems to be useful to detect rapidly growing contaminated immortalized cells. If combinations of these *in vitro* tumorigenicity-associated tests do not demonstrate the existence of both undifferentiated and immortalized cells, the tumorigenic potential of final products can be considered extremely low. More importantly, the validity of advancing to clinical trials should be confirmed for each product and judged by the following points: (a) methods of cell inoculum, (b) sites of injection, (c) risk management plans, (d) results from *in vivo* tumorigenicity tests.

7. CONCLUSION AND FUTURE PROSPECTS

No guideline for the tumorigenicity testing of cell-/tissue-derived products, including hPSC-derived products, has been issued. Because the subject and purpose described in WHO TRS 878 are not suitable for hPSC-derived products, the direct application of the WHO TRS 878 tumorigenicity test to hPSC-derived products is unreasonable. Tumorigenicity studies for hPSC-based products should examine (a) the existence of residual undifferentiated pluripotent cells, (b) the existence of tumorigenic transformants, and (c) whether the transplant forms tumor in microenvironments at the site of transplantation. As a countermeasure, highly sensitive *in vivo* tumorigenicity tests using severely immunodeficient mice may be a viable option. We now address the current problems with the development and standardization of *in vivo* tumorigenicity tests for hPSC-derived products.

Safety assessments of hPSC-derived products must choose among various tumorigenicity tests, considering the limitations of each. The overall safety of each product should be estimated on the basis of the results of an appropriate set of tumorigenicity tests. The following points should also be taken into account in order to decide on the items to evaluate: (a) properties of the raw materials, (b) properties of the products, (c) target diseases, and (d) risk management. Of course, the results/assessments of even the most appropriate tumorigenicity tests cannot guarantee safety in humans. After understanding the limitation of each tumorigenicity test, we should develop a risk assessment and risk management plan and obtain informed patient consent.

REFERENCES

1) Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet*, **379**, 713–720 (2012).
2) World Health Organization. “Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. Proposed replacement of TRS 878, Annex 1.”: ‹http://www.who.int/

biologicals/BS2132-CS_Recommendations_CLEAN_19_July_2010.pdf›, cited 19 July, 2010.
3) Müller FJ, Goldmann J, Löser P, Loring JF. A call to standardize teratoma assays used to define human pluripotent cell lines. *Cell Stem Cell*, **6**, 412–414 (2010).
4) Prockop DJ. Defining the probability that a cell therapy will produce a malignancy. *Mol. Ther.*, **18**, 1249–1250 (2010).
5) World Health Organization. "Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals. WHO Technical Report Series No.878, Annex 1.": ‹http://whglibdoc.who.int/trs/WHO_TRS_878.pdf›, cited 1998.
6) Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science*, **197**, 461–463 (1977).
7) Lewis AM. "Regulatory Implications of Neoplastic Cell Substrate Tumorigenicity.": ‹http://www.fda.gov/ohrms/dockets/ac/05/slides/5-4188S1_2.ppt›, cited 2005.
8) Garcia S, DiSanto J, Stockinger B. Following the development of a CD4 T cell response *in vivo*: from activation to memory formation. *Immunity*, **11**, 163–171 (1999).
9) Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, Heike T, Nakahata T. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood*, **100**, 3175–3182 (2002).
10) Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, Watanabe T, Akashi K, Shultz LD, Harada M. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor gamma chain(null) mice. *Blood*, **106**, 1565–1573 (2005).
11) Machida K, Suemizu H, Kawai K, Ishikawa T, Sawada R, Ohnishi Y, Tsuchiya T. Higher susceptibility of NOG mice to xenotransplanted tumors. *J. Toxicol. Sci.*, **34**, 123–127 (2009).
12) Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ. Efficient tumour formation by single human melanoma cells. *Nature*, **456**, 593–598 (2008).
13) Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.*, **25**, 681–686 (2007).
14) Kuroda T, Yasuda S, Kusakawa S, Hirata N, Kanda Y, Suzuki K, Takahashi M, Nishikawa S, Kawamata S, Sato Y. Highly sensitive *in vitro* methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPS cells. *PLoS ONE*, **7**, e37342 (2012).
15) Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, Dunn NR. Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. *Stem Cell Res.* (Amst.), **2**, 198–210 (2009).