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Current Perspective on Evaluation of Tumorigenicity of Cellular and Tissue-based Products Derived from induced Pluripotent Stem Cells (iPSCs)* and iPSCs as Their Starting Materials

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1. Introduction

The Cellular and Tissue-based Products Subcommittee (hereinafter, the subcommittee) of the Science Board to Pharmaceuticals and Medical Devices Agency (PMDA) has held multiple discussions from the scientific point of view on “tumorigenicity” that is the major safety concern of induced pluripotent stem cells (iPSCs)* for cellular and tissue-based products, and come to conclusion at present of the issues.

In order to appropriately promote the development of cellular and tissue-based products, measures should be taken to address scientifically appreciable matters of concern by clarifying the issues that are at present recognizable while taking feasibility into consideration. The available information is limited, hence various research projects are currently underway for development of cellular and tissue-based products. While the subcommittee presents the analysis of the current situation and measures available, it also recommends that subsequent review be performed when relevant data is accumulated in the near future.

* iPSCs only includes iPS cells and does **not** include embryonic stem cells (ESCs) nor somatic stem cells.

†This English translation of the document submitted to PMDA by the Science Board is intended to be a reference material to provide convenience for users. In the event of inconsistency between the Japanese original and this English translation, the former shall prevail. The PMDA will not be responsible for any consequence resulting from the use of this English version.

”Tumorigenicity” is defined as the ability of a cell population transplanted into an animal to give rise to malignant or benign tumors by proliferation. Human iPSCs and human embryonic stem cells (ESCs) are naturally tumorigenic, giving rise to teratoma upon transplantation, and these cells significantly differ from human somatic cells/somatic stem cells in this respect. Cellular and tissue-based products derived from these pluripotent stem cells may lead to formation of ectopic tissue or tumors due to residual or contaminant undifferentiated pluripotent stem cells. Therefore, assessment and appropriate management of tumorigenicity of the final product are important issues.

In order to ensure the safety of cellular and tissue-based products, the subcommittee summarized relevant issues focusing on “tumorigenicity.” Specifically, we reviewed the scientific methods for examining tumorigenicity available at present regarding the capabilities and limits of each test method, and presented the points for consideration. This report presents a summary on the development of cellular and tissue-based products from a scientific point of view, and not the requirements for regulatory approval of cellular and tissue-based products.

2. Tumorigenicity of Cellular and Tissue-based Products

Cellular and tissue-based products are derived from various types of cells (somatic cells, somatic stem cells, ESCs and iPSCs). The number of cells which constitute the final product varies by products: for example, 10^4 cells for retinal pigment epithelial cell product and 10^8 to 10^9 cells for cardiomyocyte product. Other variations include sources of the cells that are used, such as autologous, allogeneic, and HLA-homozygous allogeneic cells. In addition, considerable variety is expected in clinical application of cellular and tissue-based products including its form (e.g., cell suspension and cell sheet), route of application, site of application, use of immunosuppressant, urgency of patients’ condition. Therefore, comprehensive discussion is required for assessment of tumorigenicity with respect to such diversity. In fact, such a point of view is perceptible in the related guidelines by regulatory authorities in the US and the EU, and those by Japan’s Ministry of Health, Labour and Welfare (MHLW). However, there are no official guidelines for assessment of tumorigenicity of cellular and tissue-based products and cells of which these products are derived at present. (Note 1)

For the assessment of tumorigenicity of cellular and tissue-based products, it is important to note that the correlation or causal relationship between tumorigenicity of the (stem) cells used for manufacturing and that of the final product has not been

elucidated. For cellular and tissue-based products manufactured from pluripotent stem cells, particularly iPSCs, tumorigenicity will be examined separately for the iPSCs-stock, etc. from which the products are derived, and for the final products derived from iPSCs.

3. Assessment on the Undifferentiated Cells/Tumorigenic Cell Contaminants and Tumorigenicity in Cellular and Tissue-based Products Derived from iPSCs

If the final products manufactured by induced differentiation to iPSCs are tumorigenic, the tumorigenicity may be attributed to contamination by undifferentiated iPSCs or emergence of tumorigenic cells in the induced differentiation. There are several test systems for the assessment of these issues.

Test systems for assessment of contamination with potentially tumorigenic undifferentiated iPSCs include flow cytometry that utilize undifferentiated pluripotent cell-specific markers and quantitative RT-PCR (qRT-PCR). However, neither of them can detect contamination with undifferentiated pluripotent stem cells below a significant level of contamination, and thus it is important to establish a new method of testing. For example, it may be conceivable to subject the final product to the conditions for undifferentiated pluripotent stem cell culture, and assess the potential formation of colonies of iPSCs.

These test methods cannot detect tumorigenic cells that do not express undifferentiated pluripotent cell markers (i.e., unexpected tumorigenic cells). Therefore, detection of cells with tumorigenicity that arise due to unintended transformation during the manufacturing process is of significant importance.

Test systems for detection of malignant transformations include soft agar colony formation assay, focus formation assay, growth factor-independent growth assay, and tumorigenicity test by subcutaneous transplantation to nude mice (refer to Note 1, WHO TRS878). However, these methods were originally intended to be used for a characterization of a relatively homogeneous cell population, such as cell line or cell bank. Therefore, it should be taken into consideration whether these methods are sufficiently sensitive for detection of small number of tumorigenic cells within a population, as well as whether the results from these assay can be extrapolated to human use.

The tumorigenicity test by subcutaneous transplantation to severely immunodeficient mice, such as NOG mice or NSG mice, may be considered to be a test method that allows comprehensive and highly sensitive detection of tumorigenic cells. However,

measures for quantitation/standardization have not been established for these systems.

All the above test systems for cellular and tissue-based products test the amount or presence/absence of contamination of cells with tumorigenicity in the final products. As described above, final products are diverse, and thus, for each final product, it is necessary to discuss a practically safe level of tumorigenic cell contamination, establish test method that can detect it, and then determine a cut-off value. (Note 2)

One of the concerns regarding tumorigenicity of iPSCs-derived products is whether or not the microenvironment at engraftment affects tumorigenesis. As a system for nonclinical validation for this matter, there is a method that tests tumorigenicity by applying the relevant product to the site of test animals such as severely immunodeficient mice that corresponds to the application site for humans. However, extrapolation of this assay to clinical application has not been verified and will remain an issue in the near future.

4. Assessment and Management of Tumorigenicity of Human (Allogeneic) iPSC Cells Used for Manufacturing iPSCs-derived Products

iPSCs-stock is not intended to be developed for manufacturing of a specific final product but is a collection of human (allogeneic) iPSCs for manufacturing various final iPSCs-derived products. The iPSCs-collection is being established overseas as well, including at NIH in the United States. Accordingly, the subcommittee discussed how tumorigenicity of these human iPSC cell collections for clinical applications should be assessed and managed as a general consideration.

Although assessment of tumorigenicity of the final product is important as described above, tumorigenicity of the final product could possibly depend on that of each iPSC line in the iPSCs-collection from which the product is derived and/or on the process of induced differentiation for the final product (manufacturing process). Various final products with different intrinsic properties are manufactured from an iPSC line in the collection, while the same final product may be applied to a number of patients. Some patients may develop tumors depending on the properties of cells in the final product including level of differentiation and proliferativity, or the increase in the population size. Therefore, in this case, tumorigenicity of iPSCs used for manufacturing will be assessed in a thorough manner. If human iPSCs are to be involved in carcinogenesis in human application, “genetic abnormality that induces persistent cell proliferation” and “genomic instability” are considered to be the main concerns. Thus, the subcommittee discussed these points.

”Genetic abnormality that induces persistent cell proliferation” is considered to be an

abnormality caused by accumulation of several gene mutations related to carcinogenesis. Therefore, there is a concern over the increased risk of developing cancer if cells with genetic mutations related to carcinogenesis are introduced into patients via cellular and tissue-based products. In terms of induced genetic mutations in iPSCs, the risk of the insertion of exogenous gene fragments into the host genome is lower by the current gene transfer technique using plasmid (episomal vector) compared to gene transfer using retrovirus vector in which genes are always inserted into the host genome. However, the possibility of gene insertion to the host genome cannot be excluded and it is important to validate that the pluripotency inducing transgenes used for generation of iPSCs are not inserted into the host genome regardless of the approach used for transgene delivery. If exogenous pluripotency-inducing transgenes are detected by PCR, the risk of tumorigenicity of the cells may be increased. The test should be performed with sufficient knowledge on the detection sensitivity of PCR, which should be as high as feasibly possible. It is further ideal if test methods that detect plasmid fragment insertions are developed (array, whole genome sequencing, concentration and trap method, etc.). In addition, there is a concern that the promoter and enhancer sequence in the episomal vector could activate the endogenous genes adjacent to it, when inserted in the genome. Therefore, PCR should be conducted not only on the exogenous pluripotency genes, but also for the promoter and enhancer sequences.

Following the establishment of iPSCs, karyotype and abnormality in DNA sequences of all exons should be confirmed as the basic characterization. The genes that have been revealed to be causally implicated to carcinogenesis at present are listed in [Table 1]. It is important to confirm that mutations of these genes and consequent amino acid substitutions have not occurred (no new additional mutations) in the iPSCs. However, there are genes that have been reported to be related to carcinogenesis besides those listed in [Table 1] and knowledge/information on cancer-related genes is being renewed daily. Therefore, we note that the content of [Table 1] should be updated as necessary. Comprehensive detection of mutations in oncogenes and cancer-suppressing genes is difficult in practice. Also, depending on the set of the target gene examined, it may not be reasonable to exclude all the iPSCs with gene mutations whose actual contribution to carcinogenesis is extremely small as to being inappropriate for use for manufacturing of final products. When setting up such tests, target genes to be examined should be determined in a reasonable way based on the type of cells, manufacturing process, target disease and purpose of use, etc. with reference to existing cancer genome mutation database, such as COSMIC.

Another major issue is “genomic instability”. Genome instability is considered to

facilitate the selection of subclones that are advantageous to carcinogenesis *in vivo*. Genome instability is a driving force for evolution of cancer cells and all cancers are considered in general to have acquired some forms of genomic instability. Genome instability significantly contributes to oncogenesis in the long run, and it should be noted that it is a different issue from whether exogenous oncogenic genes remain in iPSCs or not. Also, it may be said that epigenetic instability of iPSCs can be high since they are derived by artificial reprogramming of differentiated cells, compared to ESCs that are derived from early embryos.

Majority of cancers in humans occur by accumulation of genetic hits that strongly contribute to proliferation (probably several to approximately 20 genes); therefore, it should be confirmed that the genomic mutation rate is not increased in iPSCs compared to normal cells in order to prevent promotion of delayed carcinogenesis. For example, it is considered useful to confirm the level of the genomic mutation rate compared to that of normal cells as the basic data when iPSCs are subcultured. An example of a method for confirmation is exome sequencing over time. For example, all exons of the genome in the original cells and 10th passage cells may be sequenced, and then the respective mutation rate in iPSCs may be compared to that of the normal cells. In addition, mutation rate data of the genome (exome) following induction of differentiation may also be useful in cases where differential induction is performed.

Moreover, those that result in chromosomal structural abnormalities do not necessarily overlap with those that result in nucleotide sequence abnormalities. Therefore, sequence information of the genome (exon) following subculture cannot be a substitute for information on karyotype analyses.

Also, there are two types of potential issues in the establishment of human iPSCs-stock for clinical applications: 1. the events associated with the introduction of several exogenous genes, and 2. genome instability associated with cell culture process. Under the present scientific knowledge, it is practically appropriate to analyze the genome instability at the time of gene introduction and after certain passage number without regard to the two possible causes. Analysis of all exons may be applied for this purpose since it also allows analysis of subpopulations in the event of genomic mutations.

For confirmation of tumorigenicity of human iPSCs-stock for clinical applications at the genetic level, it should be noted that genomic mutations and diversity of gene sequences in autologous somatic cells are also observed in healthy individuals. An investigation should also be performed from the point of view of identification of new mutations that occur through the process of generation of iPSCs and confirmation of whether these mutations induce amino acid substitution related to carcinogenesis. While

deliberate measures are required for evaluation of donor-originated mutations, appropriate measures should be taken to ensure the safety of cellular and tissue-based products with consideration for the scope of the consent obtained at cell donation.

5. Closing remarks

The subcommittee focused on “tumorigenicity” as a risk concerned with the development of cellular and tissue-based products and discussed the matter specifically with iPSCs. As a result, the knowledge available on the risk of tumorigenicity and the methods for assessment of tumorigenicity were summarized to a certain extent.

In the subcommittee, a clear consensus has been achieved on the fact that the present scientific technology cannot completely eliminate the risk of tumorigenicity of cellular and tissue-based products derived from iPSCs while practical application of these products is anticipated for patients with diseases at risk that may increase with time. Upon acknowledging this fact, efforts should be made to make use of the means available at present within a reasonable extent and to reduce the risk as much as possible. This report summarizes opinions at present based on such view.

In addition, cellular and tissue-based products derived from iPSCs are also associated with issues that are not discussed here, including presence/absence of increased tumorigenicity during the process of induced differentiation and tumorigenicity due to epigenetic factors, etc., and therefore, further discussion is necessary regarding such issues. Furthermore, final products for clinical applications are expected to take various forms. Therefore, it is considered necessary to appropriately select suitable tests based on the properties of each final product and to perform follow-up studies as long as possible for the clinical application.

The development of cellular and tissue-based products is rapidly advancing. The more advanced the field is, the more difficult it is to ensure quality and safety because nobody has sufficient experience. Efforts for continuously promoting development of novel assessment methods as well as discussion on the use of existing assessment techniques are required to overcome these difficulties. The subcommittee will continue its efforts to develop scientific consensus on the possibilities and limits of scientific technologies currently available, collecting the latest information and holding full discussions with accumulated knowledge on how the safety and efficacy of cellular and tissue-based products should be assessed.

Table 1.

Examples of Cancer Related Genes in Gene Symbol

ABL1	CBFA2T3	ERCC4	GATA1	MEN1	NUP214	SH3GL1
ABL2	CBLB	ERCC5	GATA3	MET	NUP98	SMAD4
ACVR1B	CBLC	ERCC6	GNA11	MITF	PALB2	SMARCA4
AFF3	CCND1	ETV4	GNAQ	MLH1	PAX8	SMARCB1
AKAP9	CCND2	ETV6	GNAS	MLH3	PBRM1	SMO
AKT1	CCND3	EVI1	GOLGA5	MLL	PDE4DIP	SOCS1
AKT2	CDC73	EWSR1	GOPC	MLL2	PDGFB	SRGAP3
ALK	CDH1	EXT1	GPC3	MLL3	PDGFRA	SRSF2
APC	CDH11	EXT2	H3F3A	MLLT3	PDGFRB	SS18
ARHGEF12	CDK6	EZH2	HMGA1	MPL	PIK3CA	STAT3
ARID1A	CDKN2A	FAM123B	HMGA2	MSH2	PIK3R1	STK11
ARID2	CDKN2C	FANCA	HNF1A	MSH6	PIM1	SUFU
ASXL1	CDX2	FANCB	HRAS	MUTYH	PLAG1	SUZ12
ATF1	CEBPA	FANCC	IDH1	MYB	PML	SYK
ATM	CHEK1	FANCD2	IDH2	MYC	PMS2	TCF3
ATR	CHEK2	FANCE	IKZF1	MYCL1	POLE	TCL1A
ATRX	CIC	FANCF	IL2	MYCN	POLH	TET2
AXIN1	COL1A1	FANCG	IL7R	MYD88	PPARG	TFG
AXIN2	CREB1	FANCI	IRF4	MYST3	PPP2R1A	TLX1
BAP1	CREBBP	FANCI	JAK2	NCOA2	PRKAR1A	TNFAIP3
BCL11A	CTNNB1	FANCL	JUN	NCOA4	PTCH1	TP53
BCL11B	CYLD	FANCM	KDM5C	NF1	PTEN	TPR
BCL2	DAXX	FANCP	KDM6A	NF2	PTPN11	TSC1
BCL3	DDB2	FBXW7	KDR	NFE2L2	RAD51C	TSC2
BCL6	DDIT3	FEV	KIT	NFKB2	RAF1	TSHR
BCOR	DDX5	FGFR1	KRAS	NIN	RB1	USP6
BCR	DDX6	FGFR1OP	LCK	NONO	REL	VHL
BHD	DEK	FGFR2	LMO2	NOTCH1	RET	WRN
BLM	DICER	FGFR3	MAF	NOTCH2	RNF213	WT1
BMPR1A	DNMT3A	FH	MAFB	NPM1	ROS1	XPA
BRAF	EGFR	FLCN	MAML2	NR4A3	RUNX1	XPC
BRCA1	ELK4	FLT3	MAP2K4	NRAS	SDHB	ZNF521
BRCA2	EP300	FOXL2	MDM2	NSD1	SDHD	
CARD11	ERBB2	FOXP1	MDM4	NTRK1	SETD2	
CARS	ERCC3	FUS	MED12	NTRK3	SF3B1	

This table is based on an article (*Cancer Research* 72:636-644, 2012) and a material* provided by an external expert (Dr. Tatsuhiro Shibata) on cancer related genes.

* A list including:

- (1) familial cancer genes reported in the Online Mendelian Inheritance in Man (OMIM) (<http://www.ncbi.nlm.nih.gov/omim>) of NCBI, US;
- (2) genes presumably responsible for primary somatic cell mutation in cancer, found from literature search;

- (3) frequently found genes (within the top 20th inclusive of the universal set of all cancers) the mutation database (UK Sangar Center COSMIC <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/etc.>).

Note 1: In order to ensure the quality and safety of cellular and tissue-based products, guidelines have already been issued by the MHLW including “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing of Human (Autologous) Cells and Tissue” (PFSB Notification No. 0208003 of the Pharmaceutical and Food Safety Bureau, dated February 8, 2008), “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing of Human (Allogenic) Cells and Tissue” (PFSB Notification No. 0912006 dated September 12, 2008), “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing of Human (Autologous) Somatic Stem Cells” (PFSB Notification No. 0907-2 dated September 7, 2012), “Guideline on Quality and Safety Assurance of Drug Product, etc. Derived from Processing of Human (Allogenic) Somatic Stem Cells” (PFSB Notification No. 0907-3 dated September 7, 2012), “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing of Human (Autologous) iPS(-like) Cells” (PFSB Notification No. 0907-4 dated September 7, 2012), “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing of Human (Allogenic) iPS(-like) Cells” (PFSB Notification No. 0907-5 dated September 7, 2012), and “Guideline on Ensuring the Quality and Safety of Drug Product, etc. Derived from Processing Human ES Cells (PFSB Notification No. 0907-6 dated September 7, 2012). At present, the only international guideline addressing tumorigenicity tests of cells is “Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals” in the Annex I of the 47th Report by Expert Committee on Biological Standardization (1998) (Technical Report Series No. 878, TRS 878) by World Health Organization (WHO). The tumorigenicity test described in WHO TRS 878 is briefly summarized as follows; “ 10^7 animal cells are administered to 10 animals, such as nude mice, and observed for 16 weeks. HeLa cells, etc. are recommended as the positive control.” The purpose of this test is to accurately grasp the level or presence/absence of tumorigenicity of a homogeneous bank of cell strain (cell bank) that is used as the cell substrate for biologics. A major change in the level of tumorigenicity or change in its presence/absence indicates an occurrence of certain abnormalities in cell characteristics. Therefore, WHO TRS 878 specifies that it is necessary to assess

tumorigenicity of the cell bank as one of the indicators of cell characteristics and use it for quality control as a measure for detection of occurrence of abnormalities in stability of the cell bank, regardless of the cause being infection with known or unknown viruses or genetic mutation or activation of oncogenes due to mutagenic substance or stress, etc. However, attention should be paid to its scope of application. WHO TRS 878 is only applied to human- or animal-derived cells that are used as *in vitro* substrates for manufacturing biotechnological /biological products for clinical applications including vaccines and protein drugs, and “cells for transplantation to patients” and “cells as the materials of cell lines that are transplanted into patients for treatment purposes” are not included in the scope.

Note 2: The ultimate objective of assessment of contamination by undifferentiated iPSCs or tumorigenic cells in iPSCs-derived products is to detect abnormal proliferation of cells in the product. Therefore, for iPSCs-derived products, it is important “to confirm on the absence of unintended transformation and/or abnormal proliferation of cells other than the target cells in cells that are cultured beyond the culture period” among matters to be confirmed in nonclinical safety studies that are exemplified in the guidelines by MHLW, “Guideline on Ensuring Quality and Safety of Drug Product, etc. Derived from Processing of Human (Autologous) iPS(-like) Cells” (PFSB Notification No. 0907-4 dated September 7, 2012) and “Guideline on Quality and Safety Assurance of Drug Product, etc. Derived from Human (Allogenic) iPS(-like) Cells” (PFSB Notification No. 0907-5 dated September 7, 2012).

Reference (members and meetings)

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2. Meetings

- 3rd Cellular and Tissue-based Products Subcommittee
Date: December 26, 2012
Topic: Discussion of given topics
Contribution: Kazutoshi Takahashi, Quality assessment of iPS cells

- 4th Cellular and Tissue-based Products Subcommittee
Date: February 6, 2013
Topic: Discussion of given topics
Contribution: Hiroyuki Mano, Mechanism of carcinogenesis and its verification methods

- 5th Cellular and Tissue-based Products Subcommittee
Date: April 25, 2013
Topic: Discussion of given topics
Contribution: Yoji Sato, Tumorigenicity assessment of regenerative medicine (cellular and tissue-based products)

- 6th Cellular and Tissue-based Products Subcommittee
Date: May 15, 2013
Topic: Discussion on given topics
Contribution by external experts: Akifumi Matsuyama (Foundation for Biomedical Research and Innovation), Regenerative medicine and regulatory science

- 7th Cellular and Tissue-based Products Subcommittee
Date: July 16, 2013
Topic: Discussion on given topics and development of report on tumorigenicity
Contribution by external experts:
Tatsuhiko Shibata (National Cancer Center), Risk assessment on tumorigenicity of iPS cells
Takashi Shimada (Nippon Medical School), Current situation and future of gene therapy