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"Challenges Toward Sound Scientific Regulation of Cell Therapy Products"

Tumorigenicity Tests for Cell-Processed Therapeutic Products

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“Tumorigenicity”

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.

Reference
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” (2010)

International Guidelines for Tumorigenicity Tests

- **WHO** "Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals" in WHO Expert Committee on Biological Standarization, 47th Report (1998) technical report series number 878, **TRS 878**
  w/ Proposed replacement of TRS 878, Annex 1"(2010)

World Health Organization

1. **Administrate 10^7 cells to 10 nude mice,**
2. **Observe for 16 weeks, and**
3. **Compare with a suitable positive control reference (e.g., HeLa cells)**

Purpose of tumorigenicity tests in WHO-TRS878

World Health Organization

- **Examining the tumorigenic phenotype range of cell banks used as cell substrates for biological products**

The extent of cell tumorigenicity has significantly changed.

Something affecting the characteristics of cell banks has occurred.

- **Virus infection, mutation and oncogenic activation by mutagen or stress, etc., could change the tumorigenic phenotype range of cell banks.**
- **No matter what the reason is, to detect abnormal stability of cell banks, WHO-TRS878 is used for quality control of cell substrates for biological products.**
- **WHO-TRS878 excludes viable animal cells when they are used directly for therapy by transplantation into patients or when they are developed into cell lines for the purpose of using them as therapeutic agents by transplantation**

Classification of CTPs based on the tumorigenicity of starting cells

- Human somatic/somatic stem cell-derived products

Cells used as raw materials are **little tumorigenic**

- Human ES/iPS cell-derived products

Cells used as raw materials are **tumorigenic**

Tumorigenicity: One of the Major Concerns of Human ES/iPS Cell-Derived Products

Tumorigenicity of Raw materials (ES/iPS cells)

Risk of tumor formation by residual undifferentiated ES/iPS cells

The undifferentiated/tumorigenic cells have to be eliminated as much as possible.

We need METHODS to check if the undifferentiated ES/iPS cells are really eliminated.

Purposes of Tumorigenicity(-Associated) Tests For Human ES/iPS Cell-Derived Products

1) **Quality control of cell substrates**

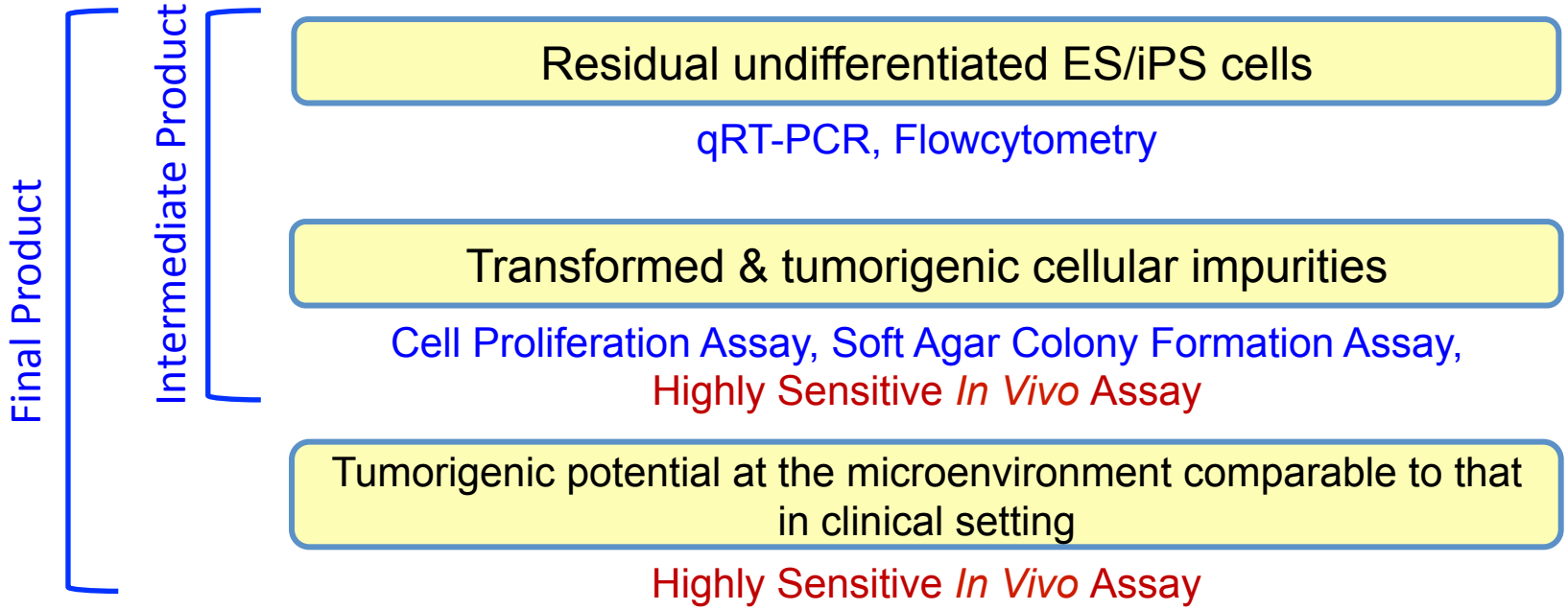
Tumorigenicity is one of critical quality attributes of homogeneous cell substrates **as in WHO-TRS878**.

2) **Quality control of intermediate/final products during manufacturing processes**

The amount of tumorigenic cellular impurities is an index for process control.

3) **Safety assessment of final products**

The results are used for nonclinical safety assessment of the final product



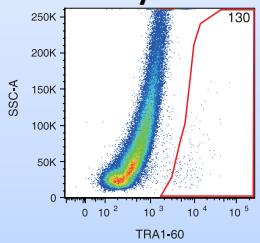
In Vitro Tumorigenicity-Associated Tests

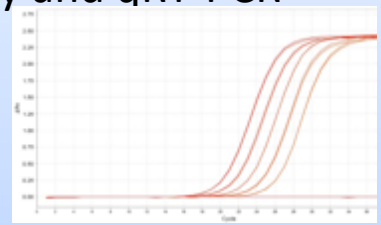
Assay	Soft agar colony formation assay	Flow cytometry	qRT-PCR
Measurement standard	Colony formation	Expression of marker protein for pluripotency	Expression of marker gene for pluripotency
Purpose	Detection of anchorage-independent growth	Detection of tumorigenic and undifferentiated cell	Detection of tumorigenic and undifferentiated cells
Time	30 days	1 day	6 hours
Advantage	Inexpensive	Rapid Analyzes individual cells	Rapid and simple Quantitative Highly sensitive
Disadvantage	Indirect Not applicable to hES/hiPS cells because of “dissociation-induced apoptosis”	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
Limit of detection	1% of PA-1 (teratocarcinoma cells)	0.1% of hiPSCs (Marker: TRA-1-60)	= <0.002% of hiPSCs (Marker: *LIN28*)

Kuroda *et al., PLoS ONE*. 2012;**7**:e50009

Common detection methods

Detection of markers for
undifferentiated cells
by Flowcytometry and qRT-PCR

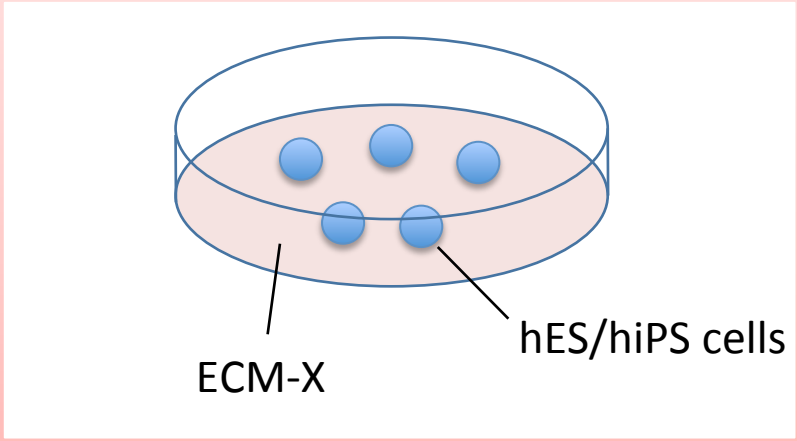




Advantages: simple and highly sensitive
Disadvantage: **indirect**

Novel detection method

Direct detection by amplification



Development of a highly efficient culture method using ECM-X

Direct detection of hiPSCs spiked into hMSCs in the culture system using ECM-X

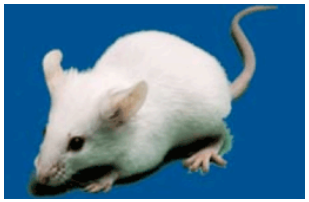
Unpublished Research Data

In Vivo Test: What's critical?

Sensitivity!

In vivo tumorigenicity tests need to be more sensitive for detection of **a trace amount** of transformed/tumorigenic cells in CTPs, compared with **WHO-TRS878** tests using nude mice, which are established for **homogeneous cell substrates**

Tumorigenicity Tests Using Highly Immunodeficient Mice



- **SCID or NOD-SCID mice**
  - Thymic lymphomas occurs spontaneously
- **NOD/SCID/γC^{null} (NOG) mice**
  - NOG mice are defective in T, B and NK cells and complement hemolytic activity, and show dysfunction of macrophages and dendritic cells.
  - Established in Central Institute for Experimental Animals in 2002 (available through Taconic or CLEA-Japan)
- **NOD/SCID/IL2rgKO (NSG) mice**
  - NSG mice show phenotypes similar to those of NOG mice.
  - Established in Jackson Lab. in 2005. (available through Charles River)

NOG and NSG mice show highly efficient engraftment of human cells and tissues, compared with common T cell-defective nude mice.

In vivo tumorigenicity tests with NOG mice and Matrigel

Unpublished Research Data

Tumor-forming capacity of HeLa cells mixed in hMSCs in NOG mice

Unpublished Research Data

For scientific risk assessment of CTPs, we are currently trying further evaluation and standardization of tumorigenicity tests using NOG mice.

Points-to-Consider for *In vivo* Tumorigenicity Tests Using Severely Immunodeficient Animals

- For quality assessment of intermediate/final products
  - Inoculation site

    Needs to be technically easy & to give reliable results (e.g., subcutaneous)
  - The number of cells to be administered

    Depends on the cell number for a clinical application and the detection limit of the test
- For preclinical safety assessment of final products
  - Inoculation site

    Should be the same as in clinical application---to evaluate tumorigenicity of the products in the microenvironment similar to that in clinical setting
  - The number of cells to be administered

    Preferable to 10-100 fold higher compared to that patients will receive

    (safety factor for species and individual differences)

    In case when physical hindrances make it difficult to administer so many cells, the cell number, not the inoculation site, should be adjusted, because the behavior of transplanted cells under specific conditions, such as immune privilege, inflammation, and ischemia, can be assessed only by *in vivo* tumorigenicity tests.

Are Tumorigenicity Tests Really Necessary for Human Somatic/Somatic Stem Cell-Derived Products?

"Cells and Tissues" are transplanted without tumorigenicity test

…because they are commonly regarded non-tumorigenic

Cell/tissue transplantation (Medical Practice)

Marketing authorization mandatory for commercial distribution

Classification of cell/tissue-based products

JAPAN	Cells OR Tissues	Cells OR Tissues	"Products for RM etc." (Cell-processed Therapeutic Products)	"Products for RM etc." (Cell-processed Therapeutic Products)
USA	Cells OR Tissues (OR 361HCT/P)	351HCT/P (Biologics OR Devices)	351HCT/P (Biologics OR Devices)	351HCT/P (Biologics OR Devices)
EU	Cells OR Tissues	ATMP (Medicinal Products)	ATMP (Medicinal Products)	ATMP (Medicinal Products)
More than minimal manipulation	NO	NO	YES	YES
Application	Homologous Use	Non-Homologous Use	Homologous Use	Non-Homologous Use

Cell proliferation assay to detect immortalized cellular impurities may be enough for the assessment of the product tumorigenicity.

The problem is "transformation of cells during manufacturing process"

Marketing authorization mandatory for interstate distribution

Tumorigenicity-associated assays of human cell/tissue-based products approved in the US and EU

Not Sensitive Enough?

	Products	Cells／Scaffolds	Treatment area	Tumorigenicity tests			Karyotype analysis	Other tests using immunodeficient animals	Notes
				In vivo	Soft agar colony formation assay	Cell growth analysis			
USA	**Carticel**	Autologous chondrocytes	Cartilage defects						
	Provenge	Autologous dendritic cell (expressing PAP antigen)	Metastatic prostate cancer						No preclinical safety studies were conducted because of autologous products.
	IaViv (azficel-T)	Autologous fibroblast	Nasolabial folds						No preclinical studies were conducted because of abundant experience in human. Tumor formation in one subject
	HemaCord (HPC-C)	Allogenic hematopoietic progenitor cells, cord blood	Hematopoietic progenitor cell transplantation			○			Measuring colony forming units
	Epicel	Autologous keratinocytes ／a layer of mouse cells	Burn	○ (Nude mice)	○		○	○ (Nude mice)	Nude mice (-), soft agar colony formation assay (-)
	Apligraf (Grafskin)	Allogenic keratinocytes ＋allogenic fibroblast ／bovine collagen	Skin ulcers	○ (Nude mice)			○	○ (hu-SCID mice)	MCB, nude mice (-)
	Gintuit (Apligraf (Oral))	Allogenic keratinocytes ＋allogenic fibroblast ／bovine collagen	Generation of new gum tissue	○ (Nude mice)					MCB, nude mice (-)
	TransCyte (Dermagraft-TC)	Allogenic fibroblast ／knitted nylon	Burn		○			○ (Nude mice)	Soft agar colony formation assay (-)
	Dermagraft	Allogenic fibroblast ／polyglactin mesh	Skin ulcers	○ (Nude mice)			○	○ (Nude mice)	Nude mice (-)
	OrCel	Allogenic keratinocytes ＋allogenic fibroblast ／bovine collagen	Burn Epidermolysis bullosa					○ (SCID mice, Nude mice)	
EU	**ChondroCelect**	Autologous chondrocytes	Cartilage defects			○		○ (Nude mice)	Evaluating senescence of cells after serial passaging

Spontaneous Transformation of hMSC in Culture: Facts or Fiction?

Cancer Res. 2005 Apr 15;65(8):3035-9.
Spontaneous human adult stem cell transformation.
Rubio D, Garcia-Castro J, Martín MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A.

Erratum in
Cancer Res. 2005 Jun 1;65(11):4969.
Retraction in
de la Fuente R, Bernad A, Garcia-Castro J, Martin MC, Cigudosa JC. Cancer Res. 2010 Aug 5;70(16): 6682.

Exp Cell Res. 2010 May 15;316(9):1648-50. Epub 2010 Feb 18.
Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells.
Garcia S, Bernad A, Martín MC, Cigudosa JC, Garcia-Castro J, de la Fuente R.

Cancer Res. 2009 Jul 1;69(13):5331-9. Epub 2009 Jun 9.
Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation.
Røsland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lønning PE, Bjerkvig R, Schichor C.

Cancer Res. 2010 Aug 1;70(15):6393-6. Epub 2010 Jul 14.
Spontaneous malignant transformation of human mesenchymal stem cells reflects **cross-contamination**: putting the research field on track - letter.
Torsvik A, Røsland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lønning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R.

→ GMP is critical, rather than spontaneous transformation

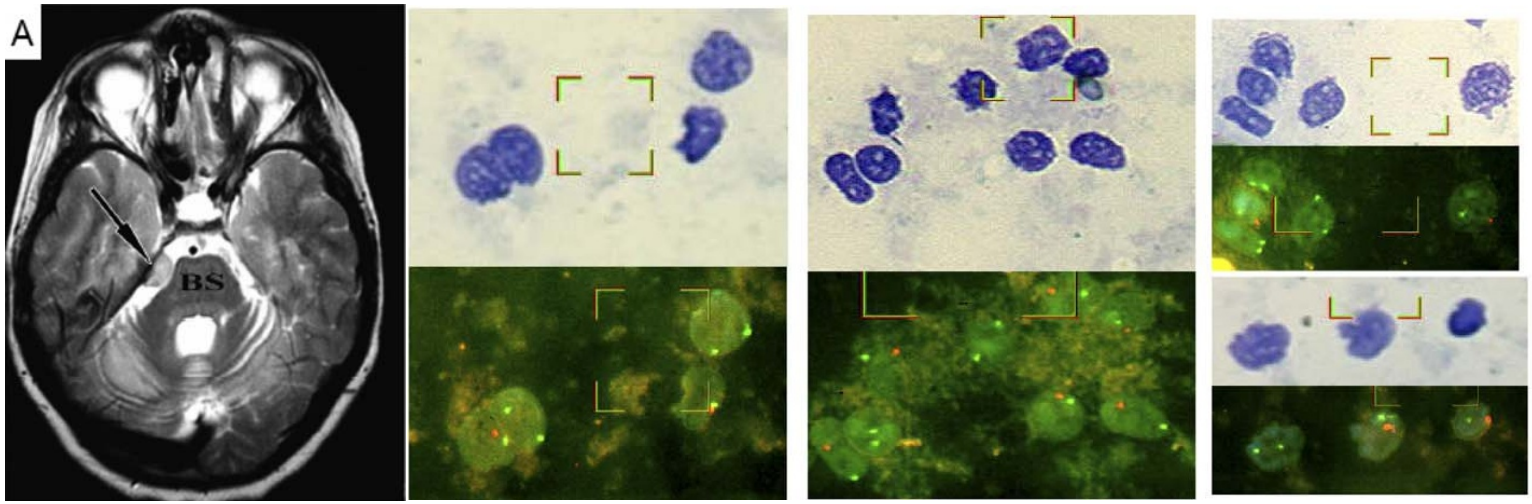
An Exceptional Case: Donor-Derived Brain Tumor Following Neural Stem Cell Transplantation

(Amariglio N *et al. PLoS Med.* 2009;**6(2)**:e1000029.)

A boy with ataxia telangiectasia:

→ treated with intracerebellar and intrathecal injection of human **fetal** neural stem cells

→ Four years after the first treatment he was diagnosed with a multifocal brain tumor



There has been **NO** scientific paper that reported tumor formation after administration of a product **derived from processing of human adult somatic /somatic stem cells.**

Conclusions

- Tumorigenicity is one of the major concerns for developing CTPs, particularly human ES/iPS cell-based products.
- However, no detailed guideline has been issued for tumorigenicity tests for CTPs.
  - Quality and safety assessments of CTPs are beyond the scope of tumorigenicity tests in WHO-TRS878. So, application of this guideline to CTPs would be unreasonable.
- Severely immunodeficient mice may be an option for tumorigenicity tests of CTPs. Standardization of such tumorigenicity tests needs to be achieved.
- Furthermore, *in vitro* tumorigenicity-associated tests should also be taken into consideration.
- By understanding the abilities and limitations of each tumorigenicity (or tumorigenicity -associated) test, appropriate tests should be selected to meet the criteria needed to evaluate each CTP.

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