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 Standardization, 47th Report (1998) technical report series number 878, **TRS 878**
  
  w/ Proposed replacement of TRS 878, Annex 1”(2010)

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

- 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

**Diagram:**
- **Final Product**
  - Residual undifferentiated ES/iPS cells
    - qRT-PCR, Flowcytometry
  - Transformed & tumorigenic cellular impurities
    - Cell Proliferation Assay, Soft Agar Colony Formation Assay,
      - Highly Sensitive *In Vivo* Assay
    - Tumorigenic potential at the microenvironment comparable to that in clinical setting
      - Highly Sensitive *In Vivo* Assay

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Transformed & tumorigenic cellular impurities

- Cell Proliferation Assay, Soft Agar Colony Formation Assay,
- Highly Sensitive *In Vivo* Assay

Tumorigenic potential at the microenvironment comparable to that in clinical setting

- Highly Sensitive *In Vivo* Assay
### In Vitro Tumorigenicity-Associated Tests

<table>
<thead>
<tr>
<th>Assay</th>
<th>Soft agar colony formation assay</th>
<th>Flow cytometry</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measurement standard</strong></td>
<td>Colony formation</td>
<td>Expression of marker protein for pluripotency</td>
<td>Expression of marker gene for pluripotency</td>
</tr>
<tr>
<td><strong>Purpose</strong></td>
<td>Detection of anchorage-independent growth</td>
<td>Detection of tumorigenic and undifferentiated cell</td>
<td>Detection of tumorigenic and undifferentiated cells</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>30 days</td>
<td>1 day</td>
<td>6 hours</td>
</tr>
<tr>
<td><strong>Advantage</strong></td>
<td>Inexpensive</td>
<td>Rapid</td>
<td>Rapid and simple</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Analyzes individual cells</td>
<td>Quantitative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Highly sensitive</td>
</tr>
<tr>
<td><strong>Disadvantage</strong></td>
<td>Indirect</td>
<td>Indirect</td>
<td>Indirect</td>
</tr>
<tr>
<td></td>
<td>Not applicable to hES/hiPS cells because of “dissociation-induced apoptosis”</td>
<td>Detects only the cells that express the known marker proteins</td>
<td>Detects only the cells that express the known marker genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gating techniques strongly influence the results</td>
<td></td>
</tr>
<tr>
<td><strong>Limit of detection</strong></td>
<td>1% of PA-1 (teratocarcinoma cells)</td>
<td>0.1% of hiPSCs (Marker: TRA-1-60)</td>
<td>= &lt;0.002% of hiPSCs (Marker: LIN28)</td>
</tr>
</tbody>
</table>

Common detection methods

Detection of markers for undifferentiated cells by Flow cytometry 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/γCnull (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

For scientific risk assessment of CTPs, we are currently trying further evaluation and standardization of tumorigenicity tests using NOG mice.
Points-to-Consider for \textit{In vivo} Tumorigenicity Tests Using Severely Immunodeficient Animals

\begin{itemize}
  \item For quality assessment of intermediate/final products
    \begin{itemize}
      \item Inoculation site
        Needs to be technically easy & to give reliable results (e.g., subcutaneous)
      \item The number of cells to be administered
        Depends on the cell number for a clinical application and the detection limit of the test
    \end{itemize}
  \item For preclinical safety assessment of final products
    \begin{itemize}
      \item 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
      \item 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 \textit{in vivo} tumorigenicity tests.
    \end{itemize}
\end{itemize}
Are Tumorigenicity Tests Really Necessary for Human Somatic/Somatic Stem Cell-Derived Products?
### Classification of cell/tissue-based products

<table>
<thead>
<tr>
<th>Region</th>
<th>Cells OR Tissues</th>
<th>Cells OR Tissues</th>
<th>“Products for RM etc.” (Cell-processed Therapeutic Products)</th>
<th>“Products for RM etc.” (Cell-processed Therapeutic Products)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAPAN</td>
<td>Cells OR Tissues</td>
<td>Cells OR Tissues</td>
<td>“Products for RM etc.” (Cell-processed Therapeutic Products)</td>
<td>“Products for RM etc.” (Cell-processed Therapeutic Products)</td>
</tr>
<tr>
<td>USA</td>
<td>Cells OR Tissues (OR 361HCT/P)</td>
<td>351HCT/P (Biologics OR Devices)</td>
<td>351HCT/P (Biologics OR Devices)</td>
<td>351HCT/P (Biologics OR Devices)</td>
</tr>
<tr>
<td>EU</td>
<td>Cells OR Tissues</td>
<td>ATMP (Medicinal Products)</td>
<td>ATMP (Medicinal Products)</td>
<td>ATMP (Medicinal Products)</td>
</tr>
<tr>
<td>More than minimal manipulation</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Application</td>
<td>Homologous Use</td>
<td>Non-Homologous Use</td>
<td>Homologous Use</td>
<td>Non-Homologous Use</td>
</tr>
</tbody>
</table>

- **Cell/tissue transplantation (Medical Practice)**
- **Marketing authorization mandatory for commercial distribution**
- **“Cells and Tissues” are transplanted without tumorigenicity test**
- **…because they are commonly regarded non-tumorigenic**
- **Marketing authorization mandatory for interstate distribution**

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


**Erratum in**

**Retraction in**


→ GMP is critical, rather than spontaneous transformation
An Exceptional Case: Donor-Derived Brain Tumor Following Neural Stem Cell Transplantation


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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