February 19, 2015

Workshop of IABS, PMDA, JST, NIBIO with support from WHO
"International Regulatory Endeavor towards Sound Development of Human Cell Therapy Products"

Tumorigenicity Tests for the Quality & Safety Assessment of Cell-Based Therapeutic Products

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Agenda

- WHO TRS 878 is NOT for CTPs.
- Tumorigenicity (-Associated) Tests for Quality Assessment of CTPs
  - *In vivo* tumorigenicity tests for CTPs
  - *In vitro* tests for detection of transformed cells
  - *In vitro* tests for detection of ES/iPS cells
- *In Vivo* Tumorigenicity Testing for Nonclinical Safety Assessment of CTPs
- Karyotyping & Omics/NGS Analysis

“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)

- **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**

- **There is no international guideline document for tumorigenicity testing of CTPs.**

Purposes of Tumorigenicity(-Associated) Testing for CTPs

1) Quality control of cell substrates (i.g., ESCs, iPSCs)

Tumorigenicity is a critical quality attribute of **homogeneous cell substrates**.

・・・WHO TRS 878 is applicable

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

The amount of tumorigenic cellular impurities is one of critical quality attributes.

・・・LOD is the Key

3) Non-clinical safety assessment of final products

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

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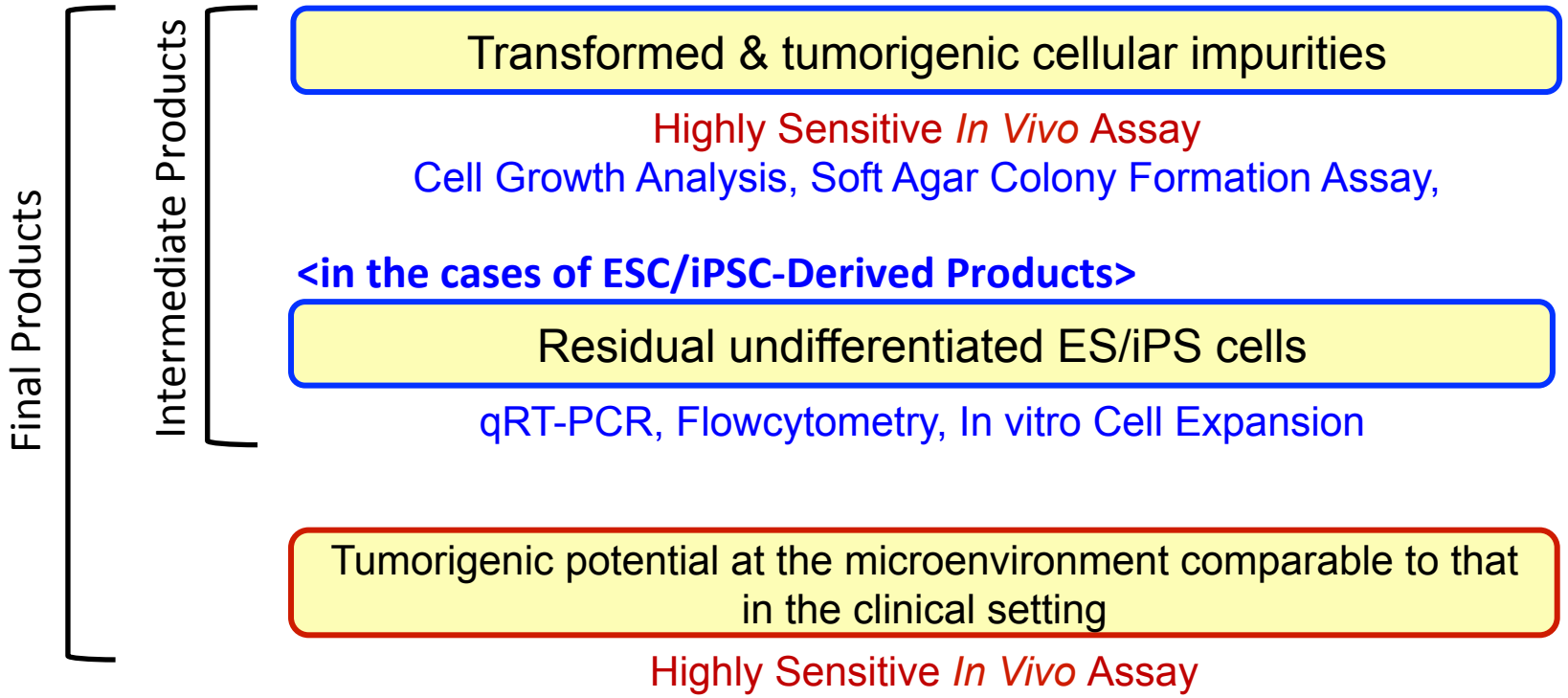

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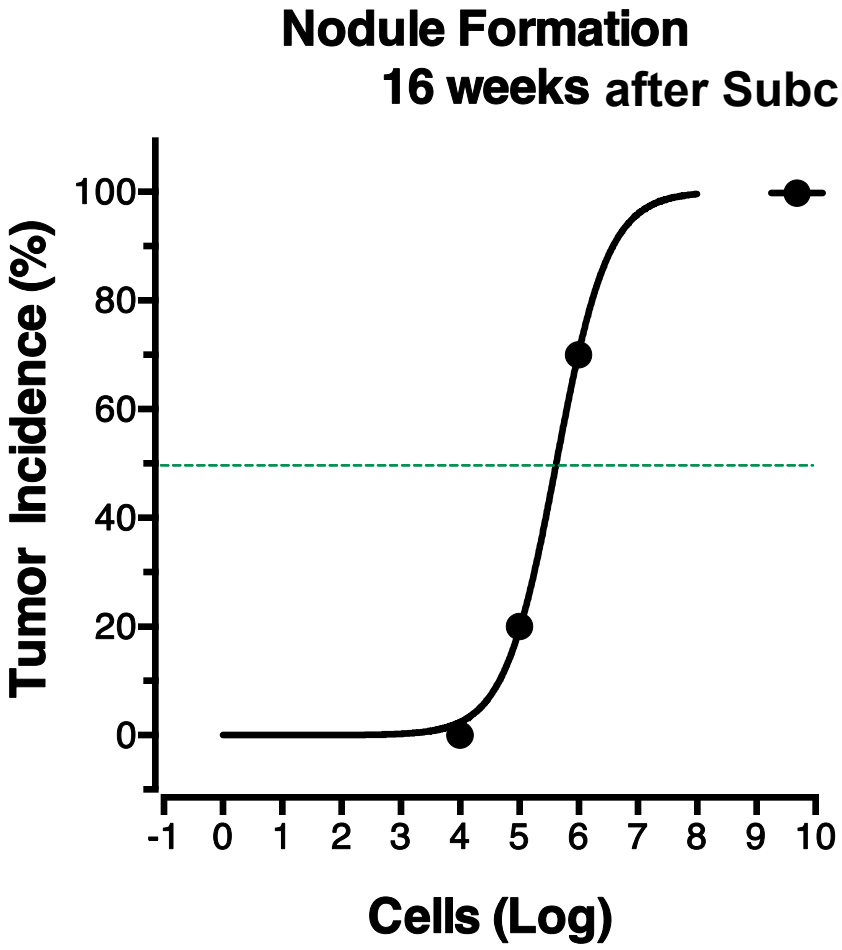
Sensitivity of Tumorigenicity Testing with Nude Mice (The Method in WHO TRS 878)



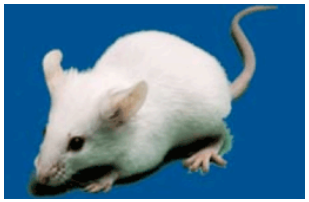
Dose to form a tumor in 50% of the animals

	TPD50
Nude	4.0×10^5

The sensitivity is not sufficient

Kusakawa *et al., Regen Therapy* 2015;1:30-7.

In Vivo Tumorigenicity Testing Using Highly Immunodeficient Mice



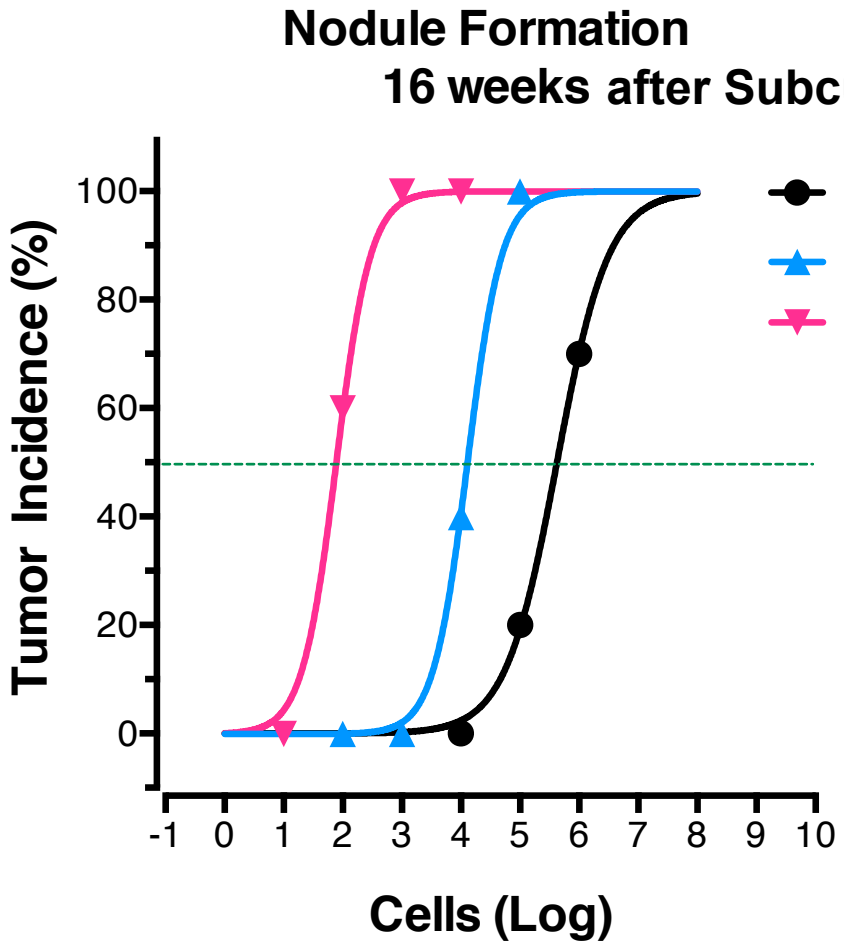
- **SCID or NOD-SCID mice**
  - Thymic lymphomas occurs spontaneously

- **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 for HeLa Cells with NOG Mice and Matrigel



	TPD50	Fold
Nude	4.0×10^5	1
NOG	1.3×10^4	25
NOG+ Matrigel	7.9 x 10	5,000

Kusakawa *et al., Regen Therapy* 2015;1:30-7.

Detection of Tumorignic Cellular Impurities (HeLa) in Normal Cells (hMSCs) by NOG mice and Matrigel

Kusakawa *et al., Regen Therapy* 2015;1:30-7.

Strain	Group	Tumor incidence at indicated HeLa cell dose at week 16					TPD_{50} at week16
		0	1×10	1×10^2	1×10^3	1×10^4	
NOG	HeLa/hMSC (1×10^6)	0/6	0/6	3/6	6/6	6/6	1.0×10^2
NOG	HeLa/hMSC (1×10^7)	0/6	1/6	2/6	-	(6/6)[a]	1.8×10^2

a: Since not all animals inoculated with the highest dose (10^2) have formed tumors, it was assumed that the tumor incidence of animals at an even higher dose step (a dummy set of data) would have been 100%.

-: Not tested; ND: Not determined

This method detects HeLa cells in hMSCs
at ratios of approx. $1/10^4$ and $1/10^6$, at probabilities of 50% and 17%, respectively.

If the acceptable false negative rate is 1%, sponsors need to confirm no tumor formation in [log0.01/log(1-0.17)=] 25 mice inoculated with 10^7 hMSCs, to show that the ratio of HeLa-like cellular impurities to hMSCs are less than $1/10^6$.

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Cell Growth Analysis

European Medicines Agency
Evaluation of Medicines for Human Use

EMEA/724428/2009

ASSESSMENT REPORT
FOR
ChondroCelect
Common name: characterised viable autologous cartilage cells expanded ex vivo expressing specific marker proteins
Procedure No. EMEA/H/C/000878

http://www.gezondheid.be/index.cfm?fuseaction=art&art_id=9251

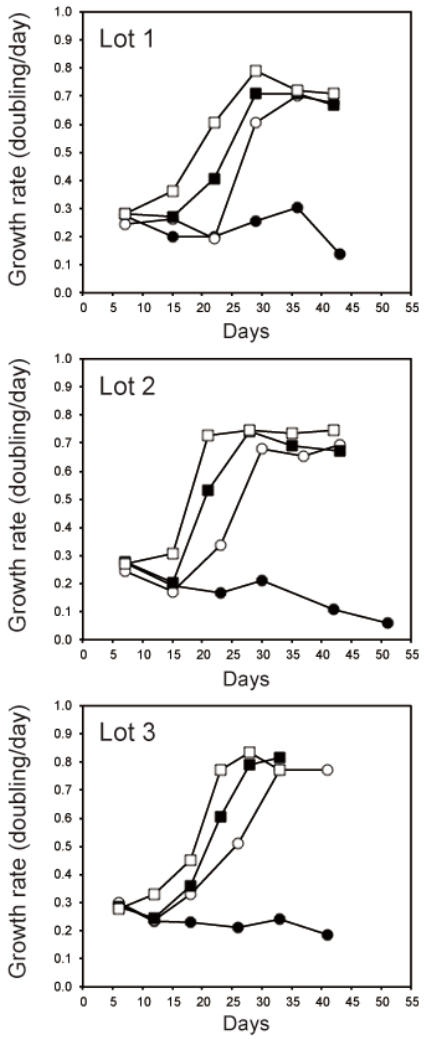
"In order to address the carcinogenic potential of ChondroCelect, the Applicant performed an *in vitro* study to evaluate senescence of human articular chondrocytes after serial passaging, using ChondroCelect culture conditions. Cells were kept beyond the routine cell culturing as suggested in EMEA/CHMP/410869/2006.

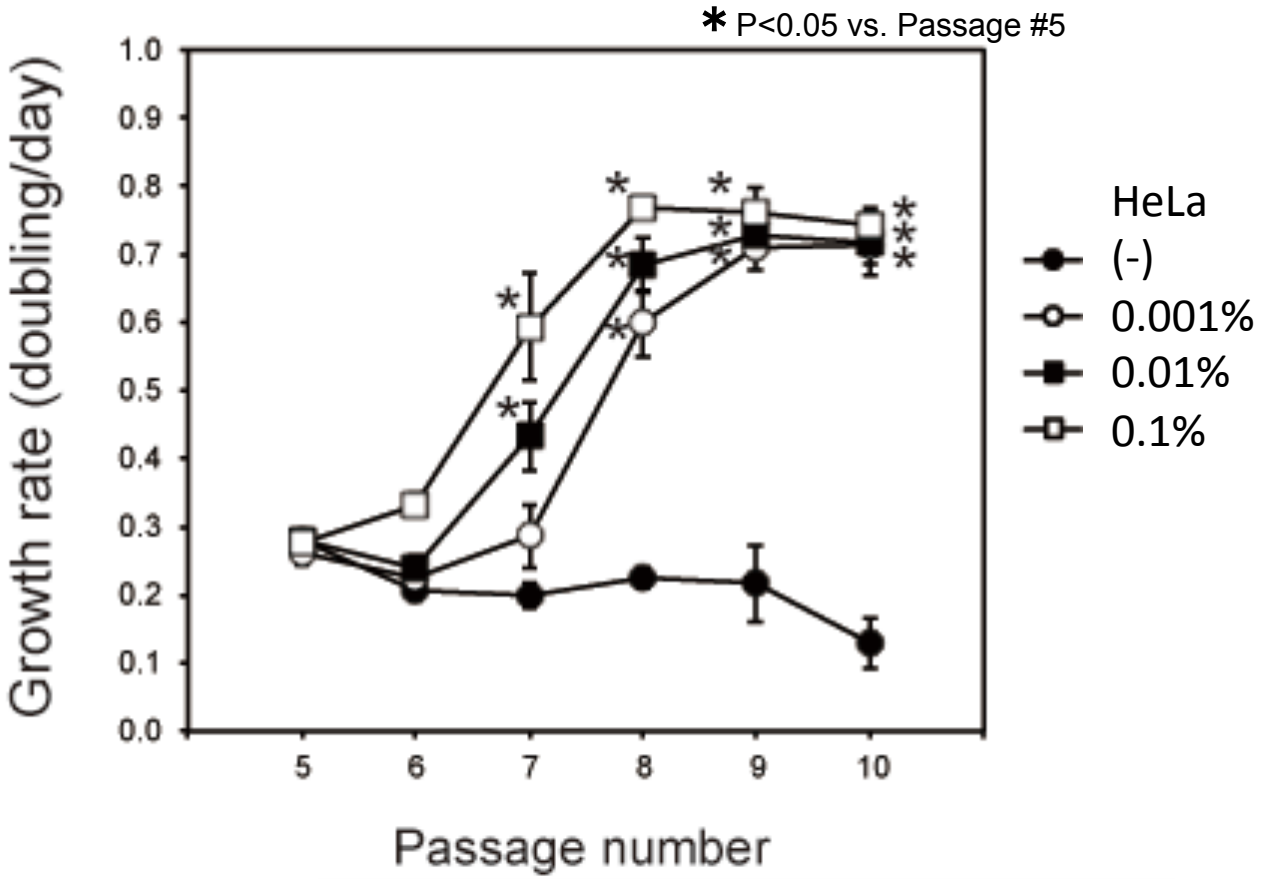
The results provide sufficient evidence that immortalisation of human chondrocytes during limited time in *in vitro* culture conditions would not occur, and that the risk of tumorigenic growth is negligible.

QUESTIONS: How sensitive is this kind of tests? How much is its LOD??

In view of
acceptable."

Detection of Immortal Cellular Impurities (HeLa) in Normal Cells (hMSCs) by Cell Growth Analysis

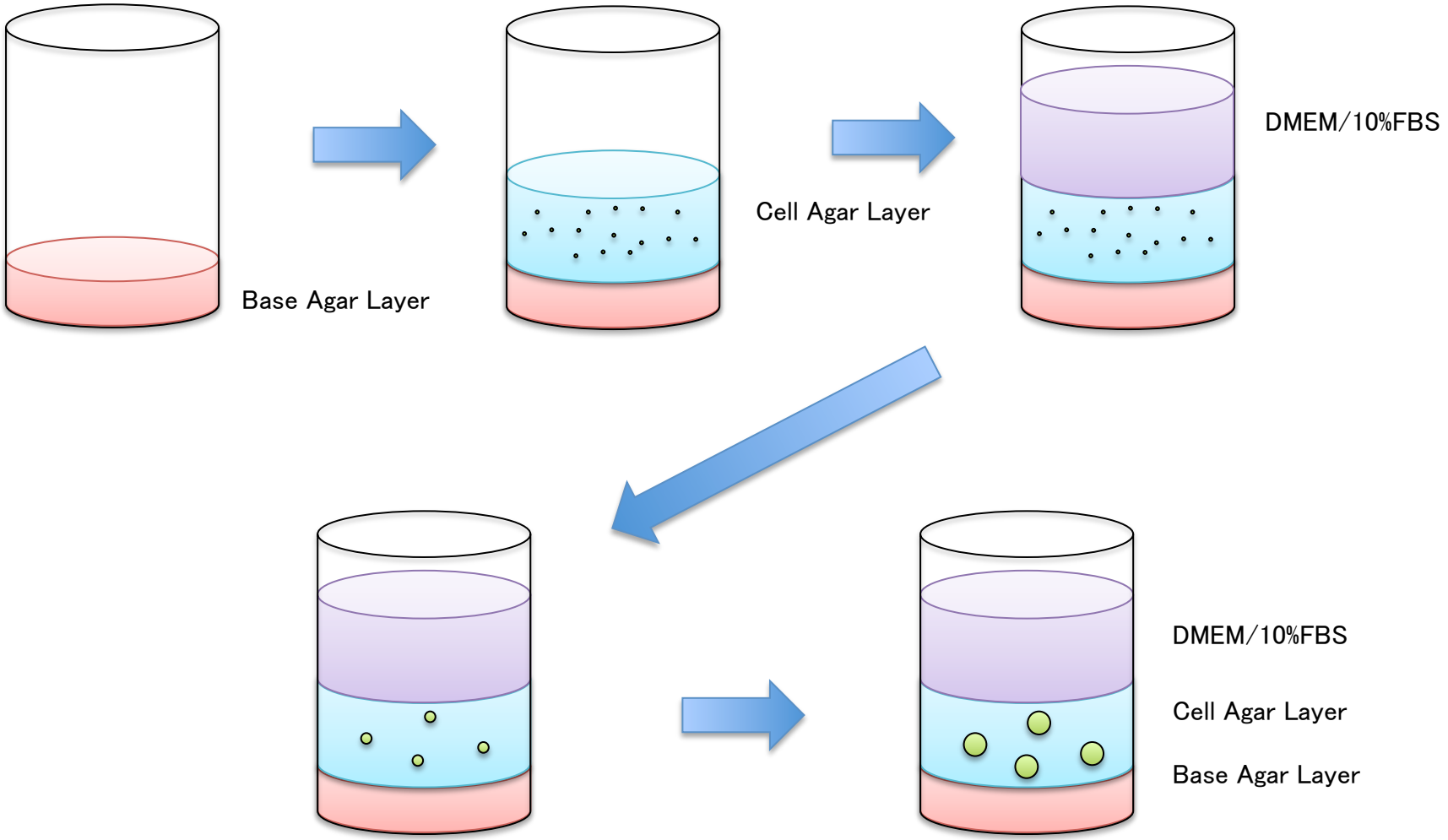




The sensitivity is pretty good.

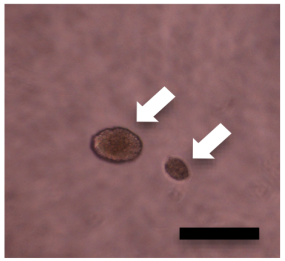
Kono *et al., Biologicals* 2015;43:146–9.

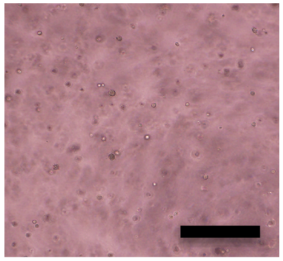
Soft Agar Colony Formation Assay



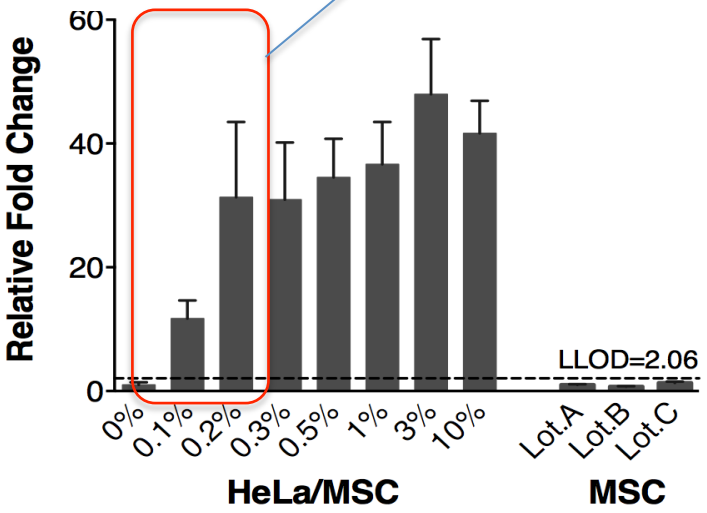
Detection of Tumorigenic Cellular Impurities (HeLa) in Normal Cells (hMSCs) by Soft Agar Colony Formation Assay

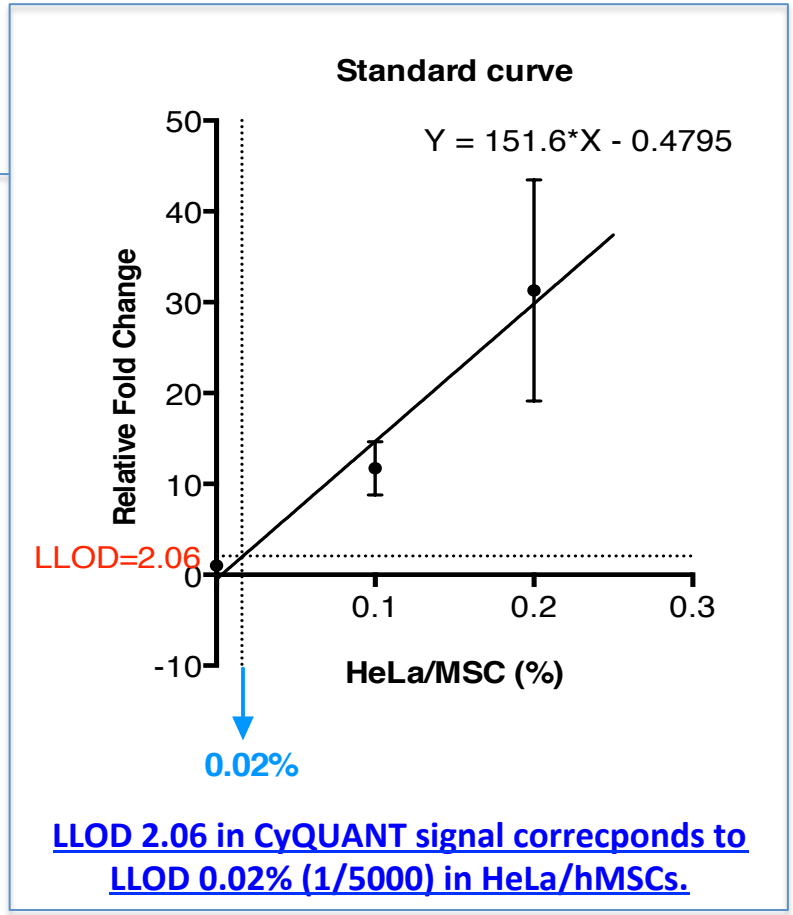
Soft-Agar Colony Formation Assay (20 days) → detected 0.1% (1/1000) HeLa/hMSCs※





* Quantitation by DNA-Binding Dye (CyQUANT)

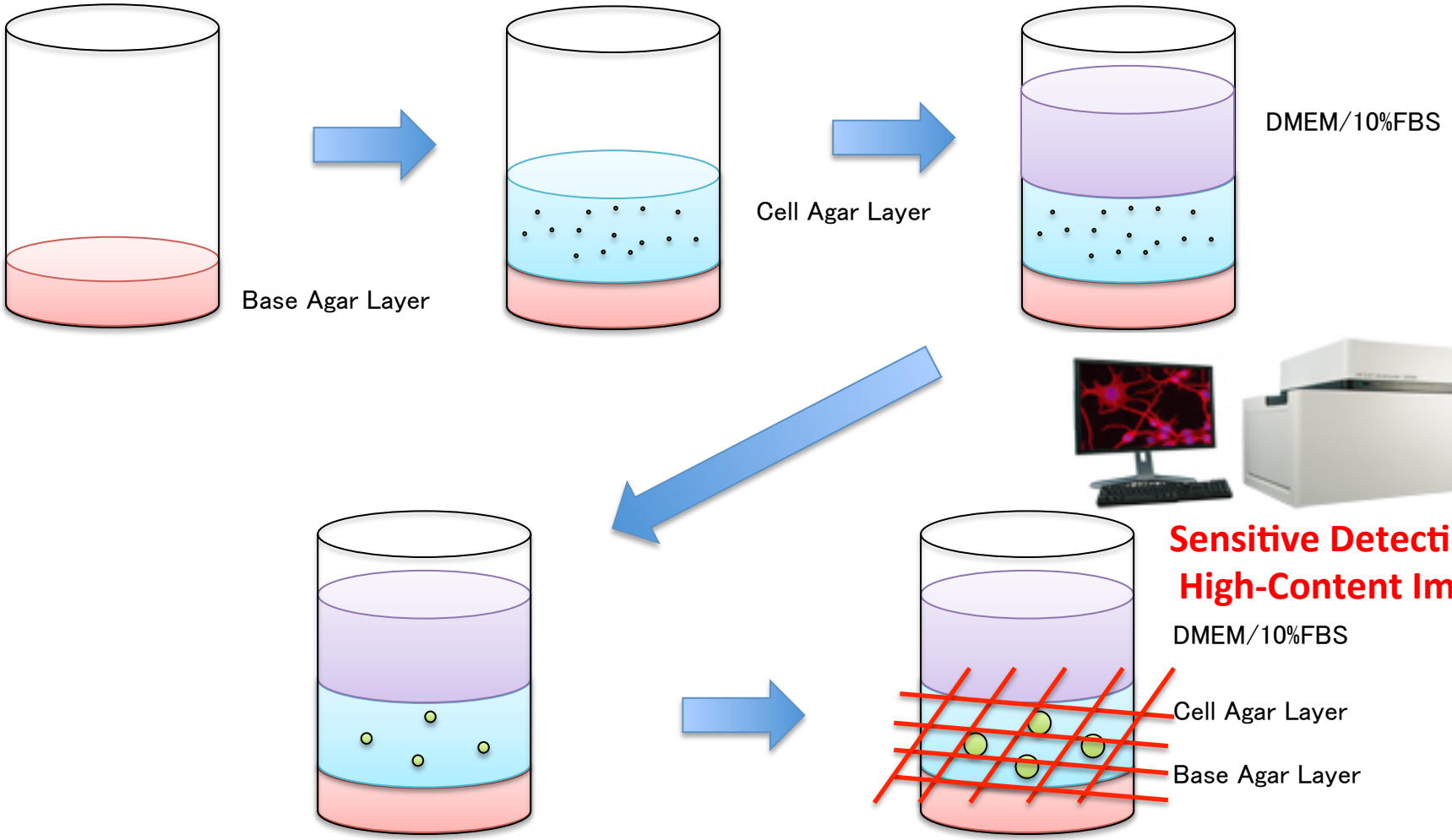




LLOD 2.06 in CyQUANT signal correcponds to LLOD 0.02% (1/5000) in HeLa/hMSCs.

Kusakawa *et al., Regen Therapy* 2015;1:30-7.

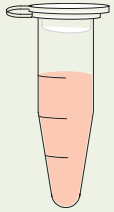
Soft Agar Colony Formation Assay



“Digital” Soft Agar Colony Formation Assay

Highly Sensitive Method for Quantitation of Tumorigenic Cellular Impurities in CTPs By Digital Counting of Single Tumorigenic Cells

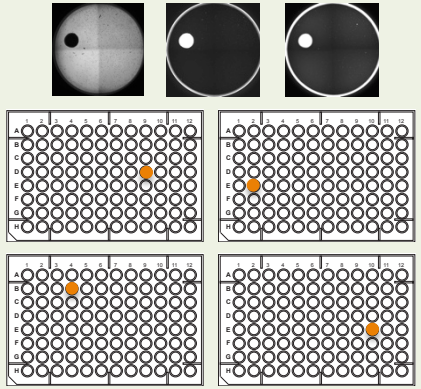
Cell Preparation



Sample Partitioning

※ A Sample Image

Colony Counting by High-Content Imaging



positive well

High-content imaging by *IN Cell Analyzer 2000*

Cell preparation : HeLa 1 / MSC 1,000,000 → 80wells (HeLa 0.0125 / MSC 12,500 / well)

Unpublished Research Data

When a cell suspension containing a single HeLa cell and 10^6 hMSCs was aliquated into 80 wells and cultured in the soft agar media, one "positive" well was detected in 3 out of 5 experiments, indicating its ability to detect as low as 0.0001% HeLa cells in hMSCs.

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Comparison of Detection Methods for Residual hPSCs in Normal Cells

Assay	*In vivo* tumorigenicity test using NOG mice	Soft agar colony formation assay	Flow cytometry
Purpose	Detection of tumorigenic cells	Detection of anchorage-independent growth (malignant cells)	Detection of undifferentiated/pluripotent cells
Time	12-16weeks		1 day
Advantage	● Direct ● Analyzes tumor formation in a specific microenvironment		● Rapid ● Analyzes individual cells
Disadvantage	● Costly & Time-consuming ● Specific Animal Facility	● Not applicable to hPSCs (Dissociation-induced apoptosis)	● Indirect ● Detects only the cells that express the known marker proteins ● Gating techniques strongly influence the results
LLOD	1000 iPSCs in 2.5E+5 hRPEs (0.4%)		0.1% of hiPSCs in hRPEs (TRA-1-60)

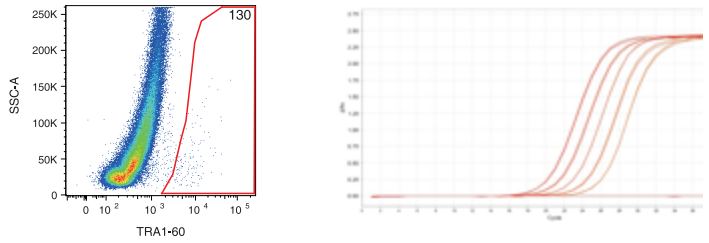
Assay	qRT-PCR	Droplet Digital PCR	Direct Expansion using Essential-8/LN521
Purpose	Detection of undifferentiated/pluripotent cells	Detection of undifferentiated/pluripotent cells	Detection of hPSCs
Time	6 hours	A few hours	About a week
Advantage	● Rapid ● Simple ● Quantitative ● Highly sensitive	● Rapid ● Simple ● Quantitative ● Highly sensitive	● Direct ● Easy ● Analyzes residual hPSCs
Disadvantage	● Indirect ● Detects only the cells that express the known marker genes	● Indirect ● Detects only the cells that express the known marker genes	● Time-consuming
LLOD	Approx. 0.002% of hiPSCs in hRPEs (*LIN28*)	0.001% of hiPSCs in human cardiomyocytes (*LIN28*)	0.01-0.001% of hiPSCs in hMSCs

Direct Detection of hiPSCs in CTPs

Sensitive detection methods

Detection of pluripotency markers

Flow cytometry **qRT-PCR**



TRA-1-60 *LIN28*

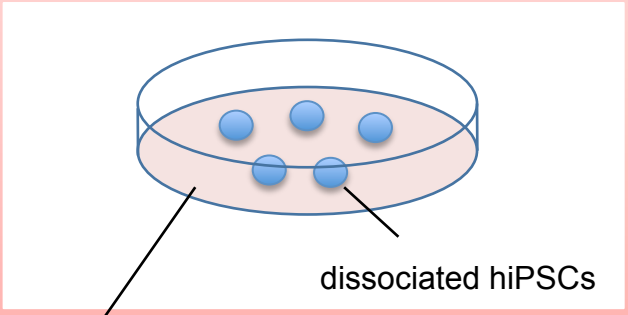
Advantage: simple and highly sensitive
Disadvantage: **indirect**

Novel detection method

Direct detection by efficient amplification

Problem:
cell dissociation is necessary for quantitation

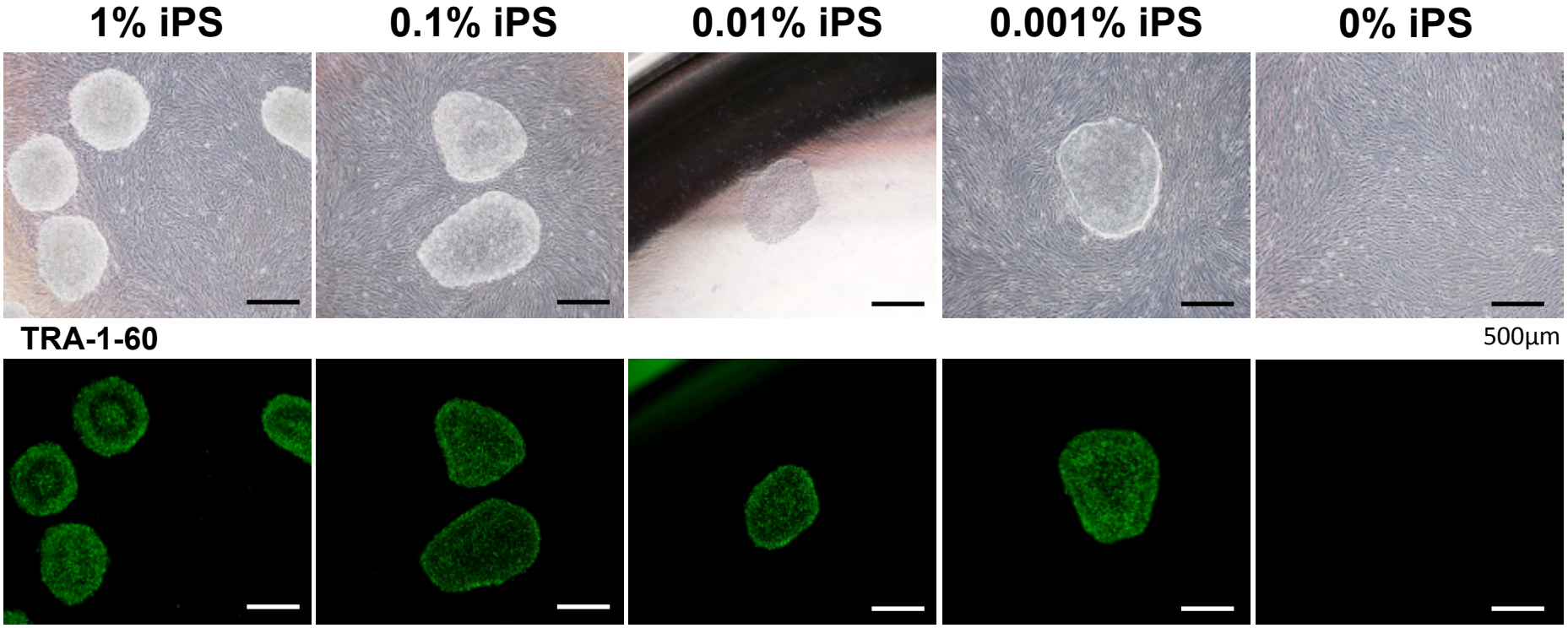
dissociation-induced apoptosis of iPSCs



laminin-521 coating amplification

Rodin S *et al*, *Nat Commn* (2014)

Direct detection of hiPSCs spiked into hMSCs in the culture system using laminin-521



	1% iPS	0.1% iPS	0.01% iPS	0.001% iPS	0% iPS
No. of colonies	100 (approximately)	20 (approximately)	1	1	0
No. of hMSCs plated	$3X10^4$	$3X10^4$	$3X10^4$	$6X10^5$	$3X10^4$

The culture method using laminin-521 can be applicable to direct detection of pluripotent cellular impurities in CTPs

Tano *et al., PLoS ONE* 2014;9:e110496

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Points to Consider for Nonclinical Safety Assessment by *in vivo* Tumorigenicity Testing

<Experimental Design>

- **Characteristics of the Animal Model**
  - Immunocompromisation
  - Limit of detection
  - Precision of the result
  - Positive (& negative) control cells
- **Test Protocol**
  - Observation period
  - Product dose
  - Site/route of administration (Influence of the microenvironment)
- **Biodistribution of the Cells**
  - Duration of engraftment
  - Cell migration

<Product>

- **Characteristics of the Product**
  - Identity
  - Purity
  - Viability
  - Formulation
  - Non-cellular components

<Patients>

- **Target Patient Population**
  - Autologous, allogeneic, xenogeneic
  - Immunological state
  - Pathological state
  - Size of target organ/tissue
  - Site/route of administration
  - Expected duration of engraftment

–Key Issue in Nonclinical Safety Assessment with in vivo Tumorigenicity Testing –

Site/Route of Product Administration

Bailey AM (CBER/FDA) *Sci Transl Med* 2012: **4:**147fs28

"…, an animal study that evaluates a route of product administration that is different from what is proposed clinically may not adequately account for the influence of the local host microenvironment, which could affect the product's ability to form tumors. For instance, **results generated from the subcutaneous implantation of a cell-based RM product may not accurately reflect the bioactivity of a product that is intended for intracranial implantation in humans**"

That's true…but is not always the case.

Tumorigenicity of iPSCs and HeLa Cells in Different Environments

Cell Line	TPD_{50}	TPD_{50}
iPSCs (201B7)	132	$5x10^4$
HeLa Cells	12.6	21
Route of Administration	Subcutaneous	Subretinal
Animal	NOG mouse	Nude rat

Secretion of PEDF, which has a pro-apoptotic effect on hiPSCs, from RPE cells

Kawamata *et al., J Clin Med.* 2015;4:159-71

iPS cells have very low tumorigenicity in the subretinal space of nude rats, compared with that in the subcutaneous space of NOG mice.

In the case of iPSC-derived retinal cell products, tumorigenicity testing by subcutaneous administration into NOG mice seems to be more sensitive to residual iPSCs in iPSC-derived retinal cell products.

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Karyotyping/Omics/NGS

- To discuss tumorigenicity of CTPs, based on the data from karyotyping/omics/NGS, a strong evidence for the association between them is necessary.

  (…and, it's ususally missing.)

- Karyotyping/omics/NGS are likely to be useful to discuss genetic stability of CTPs, rather than their tumorigenicity. However, their application to the quantitative assessment of the genetic stability needs to be established.

- Of course, karyotyping/omics/NGS are very useful for cell stocks/banks that require high genetic integrity and stability.

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 testing for CTPs.
  - Quality and safety assessments of CTPs are beyond the scope of tumorigenicity tests in WHO-TRS878. So, its application to CTPs would be unreasonable.
- Severely immunodeficient mice may be an option for tumorigenicity testing of CTPs. Standardization of such tumorigenicity testing 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, sponsors should select appropriate tests that meet the criteria for decision-making during development of their CTPs.

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