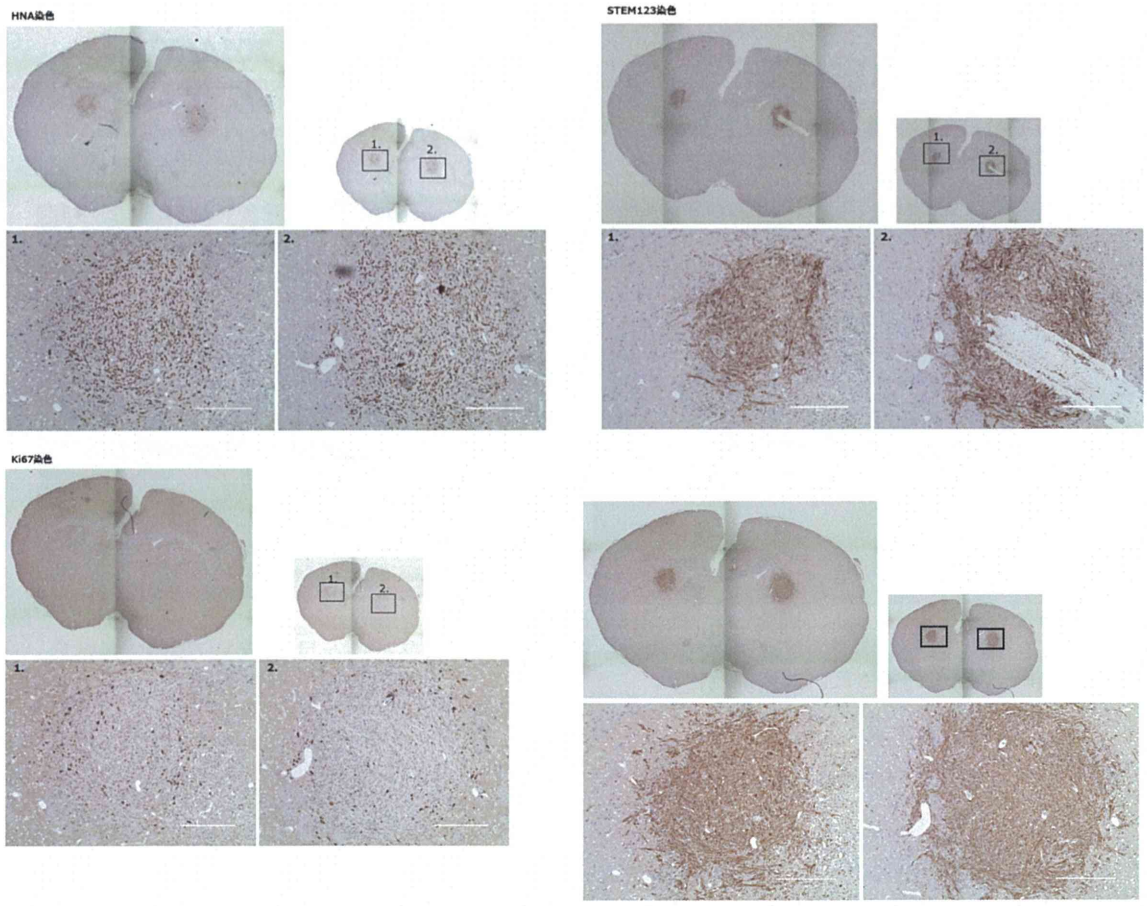

1210B2 株由来神経前駆細胞の移植細胞の組織像。上段左 HNA 染色、右 STEM 123 染色
下段　左　Ki67 染色、右　STEM 123 染色。

細胞label systemの開発

分担研究者：尾上 浩隆
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(独)理化学研究所ライフサイエンス技術基盤研究センター
生命機能動的イメージング部門 イメージング機能研究グループ

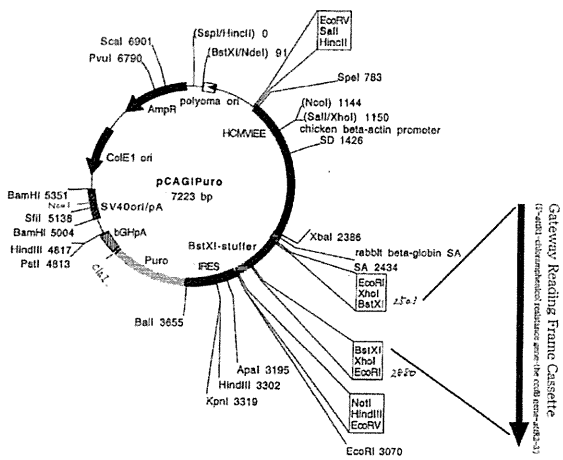
研究要旨

移植後の細胞の移植部位における生着や他の臓器への転移・移動の有無を調べるために、非侵襲的なイメージング技術の開発は必要である。本年度は、移植細胞へルシフェラーゼ遺伝子を導入し、*in vivo* で移植細胞の動態を観察できるシステムの構築を検討した。

ルシフェラーゼ遺伝子発現ベクター（pCAG-Luc-iP,）を構築し、発現ベクターがルシフェラーゼタンパク質を発現すること、およびルシフェラーゼ活性を示すことを確認した。次いで pCAG-Luc-iP ベクターを導入したマウス乳癌由来細胞 4T1 の安定発現株を樹立（4T1-Luc 細胞）し、その細胞を用いてマウス乳癌転移モデルを作製した。現在、in vivo イメージング装置を用いて本細胞の全身分布をどの程度観察できるか検討している。また残留自己 iPS 細胞由来の腫瘍形成がどのように起こるのかを調べる目的で、マウス B6 由来 iPS 細胞株に p CAG-Luc-iP vector を導入し安定株を樹立した。

【研究目的】

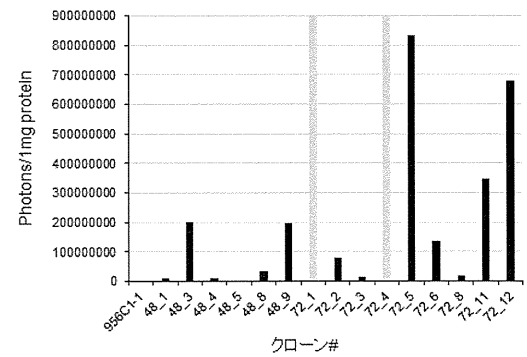
再生医療の細胞移植における安全性について、非侵襲的な評価系すなわちイメージング技術は、最適なツールの一つである。移植後の細胞の移植部位における生着や他の臓器への移動の有無を調べるために、本年度は、移植細胞へルシフェラーゼ遺伝子を導入し、*in vivo*で移植細胞の動態を観察できるシステムの構築を検討した。また細胞膜を改良型PKHで標識することで、簡便な細胞標識法を開発することを目的とした。



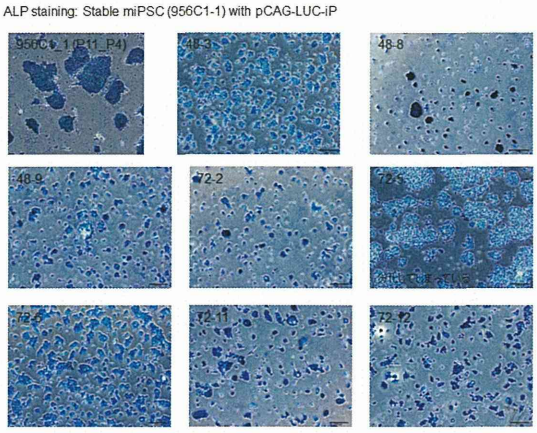
【結果】

二種類のルシフェラーゼ遺伝子発現ベクター（pCAG-Luc-iP, pLenti-Luc-iV）を構築し、ウエスタンブロット法および免疫染色により、両発現ベクターからルシフェラーゼタンパク質が発現することを確認した。また発現したタンパク質がルシフェラーゼ活性を示すかどうか調べるために活性測定を行い、いずれも高い活性が示された。次いで、pCAG-Luc-iPベクターを導入したマウス乳癌由来細胞4T1の安定発現株（4T1-Luc細胞）を樹立するために、遺伝子導入後、2週間、 puromycin 処理を行うことで安定発現株を選択し、その細胞を用いてマウス乳癌転移モデルを作製した。安定株4T1-Luc細胞においてルシフェラーゼが高発現していることも確認された。

またマウスB6由来iPS細胞株にpCAG-Luc-iPベクターを導入した安定細胞株Luc活性を測定して樹立した。



マウスB6 Luc-transfectant株の活性測定



ALP染色による細胞形状の観察

次年度この細胞をB6マウスの尾静脈及び経皮肝臓に注入することで全身での造腫瘍性遠隔転移試験を実施する予定である。また細胞表面をPKH誘導体で標識する試験を実施したが、シグナルの減衰が早いため（２４時間以内)、標識分子及び標識法の改善が考えられた。

【考察】

ルシフェラーゼ高発現ベクターの構築および発現安定株マウスB6－Luc細胞の樹立ができたことで、移植細胞の体内動態の観察システムの運用が可能となった。次年度はこれを用いて移植自家iPS細胞由来分化細胞の体内動態をB6マウスにB6マウスiPS細胞をspikeして行う予定である。

iPS 細胞、および iPS 細胞由来分化細胞の規格化研究

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研究要旨

多能性幹細胞を未分化培養状態で長期間培養すると染色体構造異常が高頻度で出現するので、それを迅速に検査できるシステムの構築が必要である。また検出された変異の意味づけを有意なものにするため、染色体異常と発癌との関係、ヒト癌データベースとの照合も必要である。

H26年度は、先端医療振興財団で樹立したiPS細胞や分化神経幹細胞を用いた解析を行った。custom made CGH arrayのdesignが完成し、その試作品の機能解析を行った。今後は迅速に検査結果が判明するmulticolor BAND (m BAND)やFISH解析と合わせて、総合的に染色体の検査が実施できる基盤を構築した。

【目的】

多能性幹細胞由来分化細胞の移植において最大の懸案は、移植細胞の造腫瘍性評価である。造腫瘍性評価は被検細胞を免疫不全動物に当該臨床部位に移植し長期間観察する試験が必要であるが、移植前の被検細胞の細胞規格とりわけ、①移植細胞中の未分化細胞の検出及び②移植細胞の遺伝子変異の検出と検討が重要である。なぜなら①からは、未分化細胞の細胞特性に起因する腫瘍（奇形腫）の形成が懸念され、②からは、多能性幹細胞の長期培養による染色体構造の変異とそれに基づく造腫瘍能獲得が懸念される。①は、未分化細胞を高感度で検出出来る qRT-PCR 等の技術開発が課題であったが、②については、遺伝子の構造は短期間の培養（1－2 か月）でも不安定になるため、多能性幹細胞由来分化細胞の遺伝子検査を 2-3 日の real time で検出出来る技術の開発が求められている。現在染色体構造検査の主流である G-band 法は検査に約 1 か月要するので、この試験期間を大幅に短縮し in house で検査が可能になるようなシステムの構築が必要である。

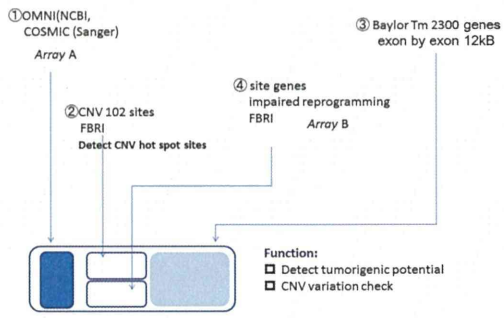
従って、本研究にて遺伝子の染色体構造や copy number variant (CNV)などの変異をほぼ real time（2-3 日）で評価するための技術開発として、新規 CGH array のデザインとこの Array のデザイン機能の検証を行う。合わせて mFISH, mBAND 法の改良を行う。

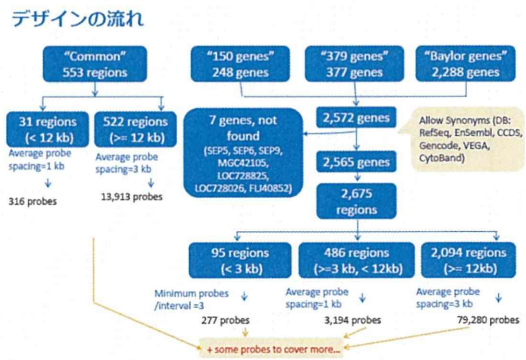
【試験概要】

今年度の研究では、まず custom made CGH array の design を行った。この CGH array と染色体多重の probe で hybrid する mBAND 法の開発と組み合わせ、2-3 日で結果が判明する有用な検査として使用可能であるかの検討を行った。

【試験方法】

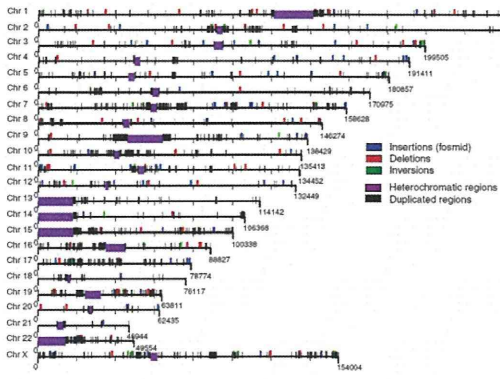
CGH custom array の作成：





Probe の design であるが、まず Baylor University の data base で公知になっている成人の癌責任遺伝子 2300 遺伝子を抽出し array panel に加えた。次に分化過程で分化能が阻害されている iPS 細胞由来分化細胞で発現が増強されていた遺伝子 377 個を抽出し probe を作成した。次に SANGER で公開されている家族性発癌遺伝子 COSMIC の 248 個を加え、probe を作成し、多能性幹細胞で報告されている copy number variants の hot spots 102 領域を選定し、probe を作成した。これらの癌遺伝子や CNV 領域を一つの chip に網羅的にデ

ザインすることで、培養細胞の遺伝子変異を検知できるようにcustome CGHを作成した。

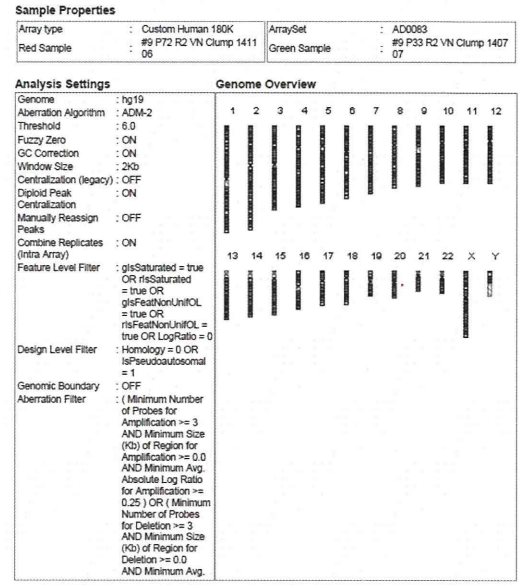


報告されているCNVの一覧表

Stem cell 2012:31 22-27

【試験結果】

このcustom arrayを用いて、作動試験をした。サンプルは、Ripro FF2で培養し,Vitronectin-N上で培養したiPS細胞PFX#9を用いた。Passage 96とpassage 36との遺伝子を比較した。その結果、CNVのhot spotである。Chr 20 q11.20-q11.21でCNVの異常な増幅をP96細胞で検出した、その他の異常は検出されなかった。



Sample Properties

Array type	: Custom Human 180K	ArraySet	: AD0083
Red Sample	: #9 P72 R2 VN Clump 1411 06	Green Sample	: #9 P33 R2 VN Clump 1407 07

Analysis Settings

Genome	: hg19
Aberration Algorithm	: ADM-2
Threshold	: 6.0
Fuzzy Zero	: ON
GC Correction	: ON
Window Size	: 2Kb
Centralization (legacy)	: OFF
Diploid Peak Centralization	: ON
Manually Reassign Peaks	: OFF
Combine Replicates (Intra Array)	: ON
Feature Level Filter	: gIsSaturated = true OR rIsSaturated = true OR gIsFeatNonUnifOL = true OR rIsFeatNonUnifOL = true OR LogRatio = 0
Design Level Filter	: Homology = 0 OR isPseudoautosomal = 1
Genomic Boundary	: OFF
Aberration Filter	: (Minimum Number of Probes for Amplification >= 3 AND Minimum Size (Kb) of Region for Amplification >= 0.0 AND Minimum Avg. Absolute Log Ratio for Amplification >= 0.25) OR (Minimum Number of Probes for Deletion >= 3 AND Minimum Size (Kb) of Region for Deletion >= 0.0 AND Minimum Avg.

Genome Overview

Text Summary Report for Sample AD0083_Set03

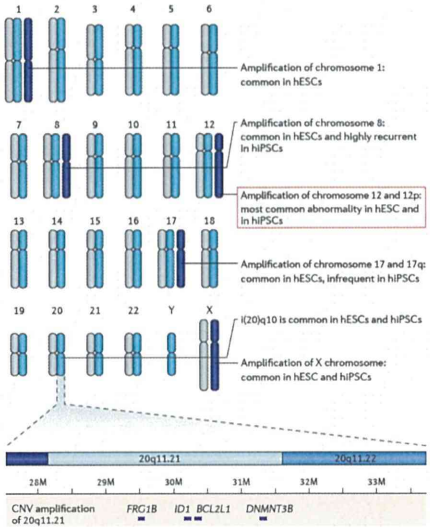
Event No	Chr	Cytoband	#Probes	Amp/Del	P-value	Annotations
1	chr20:31356833-33384831	q11.21 - q11.22	141	0.555020	1.40E-211	DNMT3B, MAPRE1, EFCAB8...

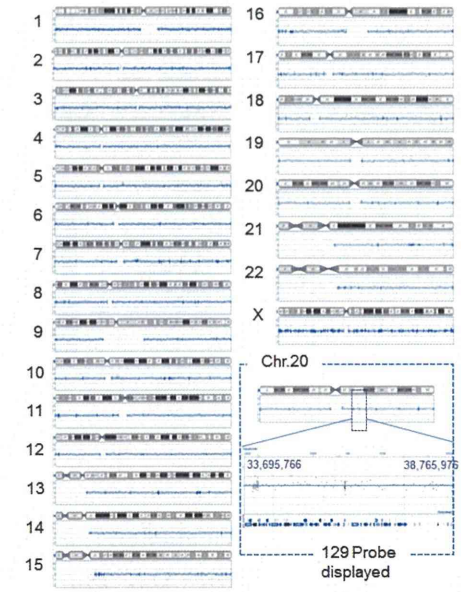
Amp=Amplification
Del=Deletion

AberrationNo	Chr	Cytoband	Start	Stop	#Probes	Amplificati	Deletion	pval	Gene Names	Hs.hg19_CNV_20120403
AD0083_Set03										
1	chr20	q11.21 - q11.22	31356833	33384831	141	0.55502	0	1.40E-211	DNMT3B, MAPRE1, EFCAB8, SUN5, BPIL1, BPIL3, C20orf185, C20orf186, C20orf70, BASE, C20orf71, PLUNC, C20orf114, CDK5RAP1, SNTA1, CBFA2T2, NECAB3, C20orf144, C20orf134, E2F1, PXMP4, ZNF341, CHMP4B, RALY, EIF2S2, ASIP, AHCY, ITCH, MIR644, DYNLRB1, MAP1LC3A, PIGU, TPS3INP2, NCOA6	CNV_5133, CNV_6087, CNV_110398, CNV_6088, CNV_4100, CNV_35918, CNV_73512, CNV_110399, CNV_79177, CNV_90539, CNV_90540, CNV_79178, CNV_79179, CNV_90541, CNV_90539, CNV_9319, CNV_6089, CNV_6090, CNV_6091, CNV_59022, CNV_102955, CNV_114911, CNV_79180, CNV_110400, CNV_114912, CNV_6092, CNV_110401, CNV_23011, CNV_110402, CNV_22513, CNV_61926, CNV_102956, CNV_43531, CNV_23624, CNV_44046, CNV_110404, CNV_102957, CNV_6093, CNV_67681, CNV_59023, CNV_59024

【考察】

これらのことから、今回designしたcustomCGH arrayは、すでに*Nat Rev Gen 2012*:13, 732-744で報告されているCNVのHot Spotを検出ており有用に使用できる可能性が高く、さらに多くの細胞で検討を続ける予定である。課題としては、これらの解析結果を有意なものにするためには、解析結果と造腫瘍性試験の結果とリンクさせ、国内外の発癌data baseとの照合も行い、臨床的に意味のある遺伝子変異を抽出し、総合的な造腫瘍性の検討を行う必要がある。





(*Nat Rev Gen 2012:13, 732-744*)

次年度京都大学CiRAから慶應大学に臨床用のhomo iPS細胞が送付され、神経幹細胞の分化誘導が慶應大学で行われ、当方に送付され造腫瘍性試験に使われるが、その細胞の遺伝子検査も品質規格試験の一環として実施する予定である。またmBANDやmFISHなどの検査がin houseで立ちあがったため、これらの検査も合わせて総合的に遺伝子検査を推進する。

Ⅴ　会議記録

平成26年度　厚生労働科学研究費補助金 再生医療実用化研究事業

「iPS細胞等を用いた移植細胞の安全性データパッケージ構築に関する研究」

日時、場所、参加者、議題 順に記載。

- 2014年 4月30日 14:00～15:00, RIKEN 分子イメージングセンター
  財団： 川真田、西下　理研：尾上、田原
  1. 研究計画会議　（H26年度Imaging Probe開発に関する立案、スケジュール）
- 2014年 5月22日 14:00～18:00, 財団
  財団： 川真田、金村、西下、郷　大阪医療センター：金村
  1. 研究計画会議　（U251移植に関する、protocol策定、スケジュール確認）
- 2014年 7月11日, 14:00～15:00, RIKEN 分子イメージングセンター
  財団： 川真田、金村、理研：尾上、田原
  1. 研究計画会議　（Imaging Probeに進捗確認）
- 2014年 8月26日, TEL/Mail—Meeting, Skipe meeting
  財団： 川真田、西下 慶應：中村、堀、大阪医療センター：金村
  1. 研究計画会議　（H25年度 移植細胞の準備状況確認と移植計画のrevise）
- 2014年11月7日 14:00-15:00, RIKEN 分子イメージングセンター
  財団：川真田、金村　理研：尾上、田原
  1. 研究計画会議　（Imaging probe作成進捗確認）
- 2014年12月15日 14:00-15:00, Skipe meeting
  財団：川真田、金村　慶應：中村、堀、大阪医療センター：金村
  1．研究計画会議　（移植細胞の準備状況確認と移植計画の進捗確認）
- 2015年 3月 21日 9:30～10:00 @横浜再生医療学会
  1. NOG-mouse 線条体 移植スケジュール確認　（財団：田村, 慶應：中村）
  2. 財団—慶應—大阪医療センター（H26年度 研究総括,H27計画）

その他、Mail連絡等は研究実施に伴い逐次連絡できる状況を作成済み。

Ⅵ　研究成果の刊行物・印刷物

平成26年度　厚生労働科学研究費補助金 再生医療実用化研究事業

「iPS細胞等を用いた移植細胞の安全性データパッケージ構築に関する研究」

研究成果の刊行に関する一覧表

Design of a Tumorigenicity Test for induced Pluripotent Stem Cell (iPSC)-derived Cell Products, Shin Kawamata, Hoshimi Kanemura, Noriko Sakai, Masayo Takahashi and Masahiro J. Go, Journal of Clinical Medicine, 2015, 4, 159-171, ISSN 2077-0883

J. Clin. Med. **2015**, *4*, 159-171; doi:10.3390/jcm4010159

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www.mdpi.com/journal/jcm

Review

Design of a Tumorigenicity Test for Induced Pluripotent Stem Cell (iPSC)-Derived Cell Products

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Academic Editor: Michael J. Edel

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Abstract: Human Pluripotent Stem Cell (PSC)-derived cell therapy holds enormous promise because of the cells' "unlimited" proliferative capacity and the potential to differentiate into any type of cell. However, these features of PSC-derived cell products are associated with concerns regarding the generation of iatrogenic teratomas or tumors from residual immature or non-terminally differentiated cells in the final cell product. This concern has become a major hurdle to the introduction of this therapy into the clinic. Tumorigenicity testing is therefore a key preclinical safety test in PSC-derived cell therapy. Tumorigenicity testing becomes particularly important when autologous human induced Pluripotent Stem Cell (iPSC)-derived cell products with no immuno-barrier are considered for transplantation. There has been, however, no internationally recognized guideline for tumorigenicity testing of PSC-derived cell products for cell therapy. In this review, we outline the points to be considered in the design and execution of tumorigenicity tests, referring to the tests and laboratory work that we have conducted for an iPSC-derived retinal pigment epithelium (RPE) cell product prior to its clinical use.

Keywords: PSC-derived cell therapy; iPSC; RPE; tumorigenicity test

1. Introduction

Several notable clinical trials using human Pluripotent Stem Cell (PSC)-derived cell products have been conducted recently. In the first, Geron used embryonic stem cell (ESC)-derived oligodendrocyte progenitor cells (GRNOPC1) for treatment of acute spinal cord injury [1]. Advanced Cell Technology initiated a study in which ESC-derived retinal pigment epithelium (RPE) was used for treatment of Stargardt's disease and dry type Age-related Macular Degeneration (AMD) [2]. More recently, a clinical study for wet type AMD using induced Pluripotent Stem Cell (iPSC)-derived RPE was started at Riken CDB [3–5].

While clinical applications are moving forward, there are concerns that transplantation of differentiated PSC might lead to the formation of tumors in the recipient. Thus, examination of this possible outcome of transplantation is critically important. Cell transplantation or infusion therapy is distinctly different from drug administration. One must consider that transplanted or infused cells can survive for long periods in the host and may form tumors at the site of transplantation or at distal sites. The extent of tumor formation can be influenced by the microenvironment at the transplantation site or the ultimate homing site of the host. Furthermore, once a tumor has formed, it may influence the physical condition of the host through secreted factor(s) [6].

The aforementioned aspects of cell therapy must be addressed with animal transplantation studies prior to clinical use. Tumorigenicity tests that can assess the tumor-forming potential of transplanted cells are particularly important in the case of PSC-based cell therapies. As PSC have "unlimited" proliferation potential as undifferentiated stem cells, they can generate teratomas if they remain in the final product. The chance of generating a teratoma will increase if the procedure uses an autologous iPSC-derived cell product that presents no immunologic barrier. PSC might accumulate chromosomal abnormalities by selecting cells with unusual proliferative advantages over a long culture period. Lund *et al.* reported that some 13% of ESC and iPSC maintained in research labs worldwide demonstrated some type of genetic abnormality [7]. For that reason, the timely assessment of the genetic stability of PSC is of major interest for both research labs and clinical PSC banks. In addition, it is important to assess the potential for differentiation resistance due to incomplete reprogramming or a differentiation bias due to epigenetic memory when iPSC-based therapy is considered. In this context, it is necessary to assess the tumor-forming potential of non-terminally differentiated cells as well.

Information regarding genetic stability, gene expression, differentiation marker expression, cell growth rate and how cells were generated must be collected and evaluated prior to commencement of tumorigenicity testing. Next, it is necessary to have a clear idea about the scope and objective of related safety parameters: toxicology tests, Proof of Concept (POC) tests, biodistribution tests and tumorigenicity tests that can be conducted concurrently.

Toxicology tests can be designed depending on the properties of testing reagents and the purpose of the tests. The Organisation for Economic Cooperation and Development (OECD) Guideline for the

Testing of Chemicals [8] is an internationally recognized test guideline for toxicology testing. They should be conducted in a blinded fashion to minimize the bias of measurement and observation by operators. Short-term and long-term end points are to be defined. Toxicology tests should be conducted by using clinically relevant methods of administration so that they can provide insights into a safe range of therapeutic cell doses. Acute (early) and late phase end points should be established in this test.

POC tests often employ a genetically modified animal that offers a model of the disease in question (e.g., Tg, KI, KO or KD mice) or injured animals to address the potential benefit or efficacy of the investigational therapy and to define the range of the effective dose used in clinical application by escalating the doses. The administration route and the method should be as close as possible to the intended clinical use. Positive and negative events should be clearly defined. In such a POC study, indices such as physiological recovery of lost function or overall survival of transplanted cells that could underlie intended therapeutic use are examined. Measurement of indices should be conducted in a blinded fashion to minimize bias during data acquisition. The size of the test group should be large enough to permit meaningful statistical analysis.

Biodistribution tests should be conducted to address tumorigenic proliferation of transplanted cells at the ectopic site. *Alu* sequence PCR is commonly used to detect human cells in host tissues or organs. While this PCR test detects human cells over a 0.1% frequency in host tissue by DNA ratio [9], greater sensitivity is needed to detect small metastatic colonies. In PET technology, proliferative cell mass is labelled by taking in a metabolic probe such as ^{18}F FLT, providing a distribution of tumorigenic cell proliferation in the animal's body. However to trace the behavior of transplanted cells and their biodistribution over time requires labeling test cells by introducing marker genes by retrovirus or lentivirus that can emit a signal with a high S/N ratio. These approaches are currently under development.

2. Guidelines for Tumorigenicity Tests

Somatic cells with a normal chromosomal structure show limited proliferation potential. Tumorigenicity testing of mesenchymal stem cells may not reveal a serious problem [10]. However, in the case of PSC-derived cell products, the tumor-forming potential should be examined thoroughly because of the "unlimited" proliferation capacity of PSC and their genetic instability. However, there is no internationally recognized guideline for tumorigenicity testing of cells used for cell therapy. WHO TRS 878, "Recommendation for the evaluation of animal cell cultures as substrates for the manufacture of cell banks" [11,12] provides a guideline for animal cell substrates used for the production of biological medicinal products, but not for cells used for therapeutic transplantation into patients. Recently, FDA/CBER commented on the issues to be considered for cell-based products and associated challenges for preclinical animal study [13]. The report stated that when tumorigenicity testing of ESC-derived cellular products is undertaken, the tumorigenicity tests should be designed considering the nature of cell products to be transplanted and the anatomical location or microenvironment of the host animal. Tumorigenic test results from the administration of cells through nonclinical routes are not considered relevant as they would not assess the behavior of transplanted cells in the intended microenvironment to which the cells would be exposed. The study design should

include groups of animals that have received undifferentiated ESCs, serial dilutions of undifferentiated ESCs combined with ESC-derived final products to infer the contamination of undifferentiated ESCs in the final product.

The aforementioned summarizes current discussions of tumorigenicity testing. However, we still need to answer a fundamental question: "How can we extrapolate animal tumorigenicity testing to humans?" The design of tumorigenicity tests should attempt to answer this question. For this, we must first estimate the risk that we will underestimate the incidence of tumor-forming events in humans by conducting an improper or non-informative animal study. So, how do we define such risk? For example, there is a risk that a study is unable to link unexpected tumor formation to genetic abnormalities of test cells presented before transplantation due to inadequate genetic information regarding test cells. In addition, there is a risk of obtaining "false" negative results by transplanting an insufficient dose, using an inadequate monitoring period, using an improper immunodeficient animal model that is insufficient to detect tumor, not transplanting into the right anatomical position, failure of transplantation itself or unexpected early death of transplanted cells in host tissue. We can address the risks by conducting quality control tests of test cells prior to transplantation and small scale pilot studies to determine the design of tumorigenicity tests. The following points should be considered in designing tumorigenicity tests.

1. The history of cell production (cultured in a research lab or Good Manufacturing Practice (GMP) grade cell processing facility).
2. Quality control records of test cells (e.g., phenotype, gene expression, sterility tests, genetic information, passage number and growth rate).
3. The type of immunodeficient animal model used and the route of administration (clinical route or subcutaneous route).
4. The method of transplantation (e.g., embedded with Matrigel or in sheets or in cell suspension).
5. Gender and number of animals to be used.
6. Information about the microenvironment at the transplanting site.
7. Dose of cells to be transplanted.
8. Selection of a positive control cell and definition of positive tumor-forming event.
9. Monitoring periods.
10. Protocol for immunohistochemistry (IHC) to detect transplanted cells in host tissue.
11. Method to detect ectopic tumor formation.

3. Specification of Test Cells

Cells used in tumorigenic tests should be generated in a manner as close as possible to that intended for clinical use. In this context, it is preferable that cells used for all preclinical tests should be generated in a GMP-grade cell processing facility for clinical use. This approach would minimize bias originating from differences in cell production quality. Several types of data, including gene expression profiles obtained from gene chips or qRT-PCR to assess stem cell-like markers and differentiation markers, phenotypic analysis by flow cytometry, sterility tests, mycoplasma tests, exome sequencing, chromosomal stability tests with comparative genomic hybridization (CGH) array and karyotyping by multi-color banding (mBAND) or fluorescent *in situ* hybridization (FISH) would be

valuable. For iPSC-derived cell products, EB formation assays would provide insights into differentiation potential. The results could be used to select "good" clones that demonstrate no differentiation bias or no differentiation resistance. These quality control tests and cell characterization tests are not a part of tumorigenicity testing *per se*. However, the information on starting material should be linked to the results of tumorigenicity testing to render the test results more informative.

In tumorigenicity testing of PSC-derived cell products, one can anticipate several tumor-forming events that include teratoma formation from residual "differentiation-resistant" PSC with normal karyotype, cancer-like progressive tumor formation from cells with abnormal karyotype or acquired genetic variation during culture and tumors with differentiation bias generated from imperfectly reprogrammed cells. To understand the nature of tumor-forming events, the link with results of these quality control tests is indispensable.

4. Selection of an Animal Model

In general, if one were to use "non-immunodeficient" healthy animals or "non-immunodeficient" disease model animals for tumorigenicity testing, one would have to administer a large amount of immunosuppressant for long-term monitoring. However, this approach will not always guarantee satisfactory engraftment of xeno-transplants. Primates can be used for tumorigenicity testing as models representative of humans, but this model is more useful for POC tests, not for tumorigenicity tests. Therefore, immunodeficient healthy rodents are widely used for tumorigenicity testing if human cells (final product) are to be used in the test. Large immunodeficient animals like the SCID pig [14] are also available. However, again, the SCID pig model would be useful to address transplantation efficiency of human cells, such as xeno-bone marrow transplantation of human hematopoietic stem cells as a part of a POC study in large animals. They are not cost-effective large scale statistical studies. To conduct tumorigenicity tests with a sufficient number of immunodeficient animals, a rodent model is a reasonable option for the preparation of test cells. Immunodeficient mice such as nude mice (BALB/cA, JCl-nu/nu), SCID mice (C.B-17/Icr-scid/scid), NOD-SCID mice (NOD/ShiJic-scid) and NOG mice (NOD/ShiJic-scid, IL-2Rγ KO) have been widely used for human cell transplantation studies. Prior to the design of tumorigenicity tests, one needs to evaluate the tumor-generating potential of these immunodeficient mouse strains by transplanting various dose of tumorigenic cell lines subcutaneously.

Another well-known transplantation site in rodents is beneath the testicular capsule space. This transplantation model is mainly used to test for satisfactory engraftment of test cells for POC tests, not for tumorigenicity tests. In our hands, it requires elaborate surgical skills and needs at least 10^4 iPSCs to generate tumors in NOG mice. In addition, tumor formation in the intraperitoneal space is hard to detect from the appearance of mice, thereby preventing statistical studies for tumor-forming events in a timely manner. In our case, the tumorigenic potential of immunodeficient mice was assessed by transplanting various doses of HeLa cells subcutaneously, following recommended procedure stated in WHO TRS 878 [11,12]. The mice were monitored over 12 months, and the TPD50 (minimum dose that can generate a tumor in 50% of transplanted mice) was calculated by the Trimmed Spearman-Karber method for each strain [9]. HeLa cells were used as a representative line of somatic tumorigenic cells with a genetic abnormality. For transplantation, a collagen-based gel lacking

nutrients is sometime used to embed cells and to retain them at the designated transplantation site. Importantly, the gel *per se* does not support growth of the transplanted cells at the site. We have used Matrigel® (BD Biosciences, San Jose, CA, USA) to embed cells and to increase their tumor-forming potential [15]. We obtained the following values for the TPD50 for HeLa cells with Matrigel® via a subcutaneous route: Nude, $10^{3.5}$ (n = 120); SCID, $10^{2.5}$ (n = 24); NOD-SCID, $10^{2.17}$ (n = 24); NOG, $10^{1.1}$ (n = 75). It is notable that during the course of experiments covering 9 months of observation, we also observed spontaneous thymomas with a frequency of some 14% in NOD-SCID mice in agreement with previous reports [16], which makes interpretation of tumorigenicity tests with NOD-SCID mice complicated.

Based on the preceding data, we chose NOG mice for subcutaneous tumorigenicity testing of iPSC-derived RPE, assuming that NOG mice could generate tumors from the lowest number of residual PSC or tumorigenic non-terminally differentiated PSC-derived cells. We then subcutaneously transplanted various doses of iPSC (201B7, Riken CDB) with Matrigel® into NOG mice to determine TPD50 for iPSC. The TPD50 value for iPSC (201B7) via the subcutaneous route was $10^{2.12}$ (n = 20) over 84 weeks of observation [9] (Figure 1). Tumorigenicity tests via a subcutaneous route with NOG mice is a sensitive quality control test to detect a small number of remaining PSC in PSC-derived investigational product regardless of cell type. Of course, the TPD50 for iPSC transplanted via a clinical route can be checked independently. In our case, we used nude rats for tumorigenicity testing via a clinical route, as the subretinal space of mice is very small and transplanting cells via a clinical route requires outstanding technique by a skilled operator. Thus, we needed larger animals to avoid "false" negative results due to failure of transplantation, to transplant a clinically relevant dose of GMP-grade iPSC-derived RPE (without Matrigel) and to confirm that the transplantation of brown colored RPE was in the right position in the albino eye ball of nude rats [9]. We did not use any "AMD" disease model animals [17,18] because they will not recapitulate all the features of human AMD. In human AMD, the macular region is focally affected and the rest of the retinal area is intact. Treatment of human wet-type AMD with an iPSC-derived RPE sheet is conducted by transplanting the RPE sheet into the affected lesion after removal of choroidal neovascularization. Thus, we assumed that a transplanted RPE sheet would receive a trans-effect from the intact retina. For that reason, we transplanted the RPE sheets into nude rats with intact retinal function rather the recapitulate the microenvironment of the clinical setting. Thus, the choice of animal should be made depending on the degree of immunodeficiency, anatomical demands and planned clinical manipulation. The TPD50 value for iPSC or HeLa cells via the clinical route was $10^{4.74}$ (n = 26) or $10^{1.32}$ (n = 37) respectively (Figure 2). The large discrepancy between the TPD50 values for iPSC and that of HeLa prompted us to examine the effect of the microenvironment on iPSC-derived products to better design tumorigenicity tests via the clinical route (see below).

Subcutaneous tumorigenicity test with NOG mice

Cell type	Cell form	Min. dose for tumor formation	Weeks to observe Tumor formation (first to last)	Number of mice	Log_{10} TPD_{50}
iPSC 201B7	cell suspension in Matrigel	1 x 10^1 cells	5 to 40 weeks	30	2.12
Hela	cell suspension in Matrigel	1 x 10^1 cells	5 to 18 weeks	75	1.10

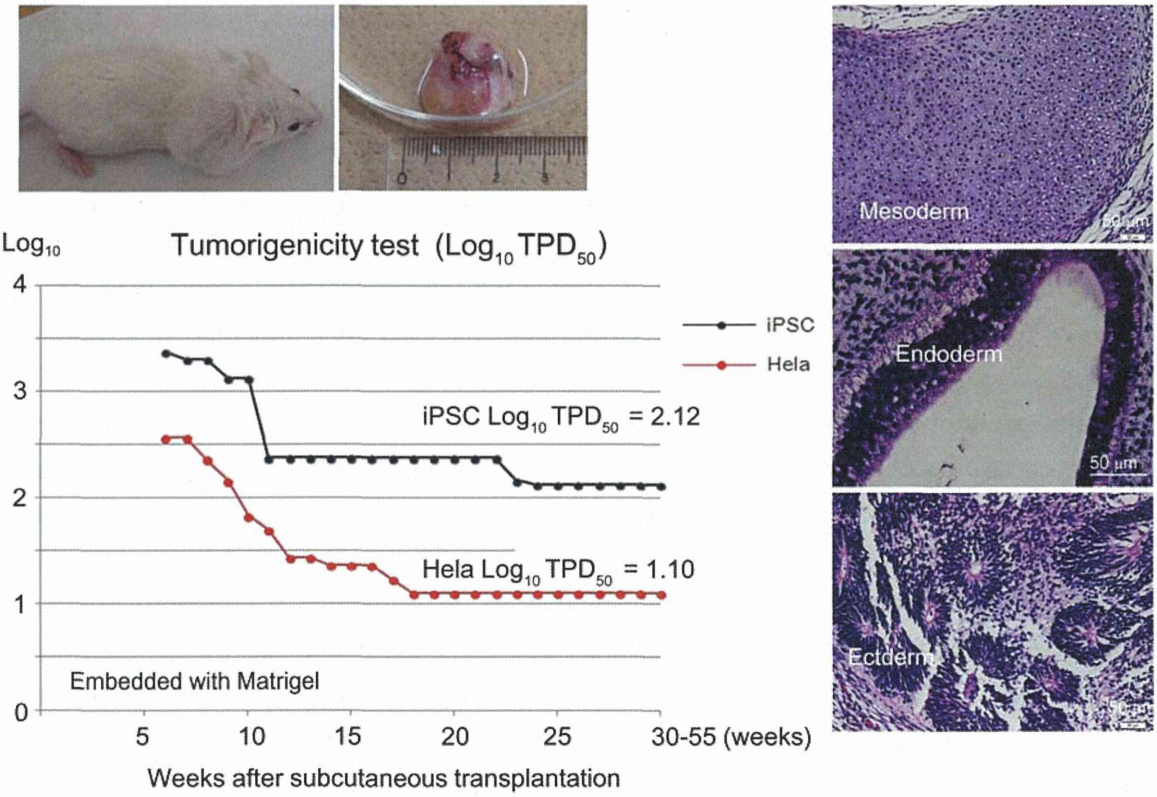

Figure 1. Subcutaneous tumorigenicity test with NOG mice. A table in above showed type of cells used as a positive control for tumorigenicity test (iPSC cell line 201B7 and tumor cell line HeLa), minimum dose for tumor formation and Log_{10} TPD_{50} for them when transplanted subcutaneously with Matrigel®. A line graph showed value for Log_{10} TPD_{50} for iPSC or HeLa at respective monitoring point (0–55 weeks). Photos (clock-wise); NOG mouse with tumor, teraoma from NOG mouse, Slice section of teratoma after HE staining; cartilage (mesoderm), intestinal tissue-like (endoderm) or neural rosette-like (ectoderm) tissue.

Tumorigenicity test via clinical route with Nude rats

Cell type	Cell form	Min. dose for tumor formation	Weeks to observe Tumor formation (first to last)	Number of rats	$Log_{10}TPD_{50}$
iPSC 201B7	cell suspension	1 x 10^4 cells	7 to 33 weeks	20	4.73
Hela	cell suspension	1 x 10^1 cells	5 to 33 weeks	13	1.32

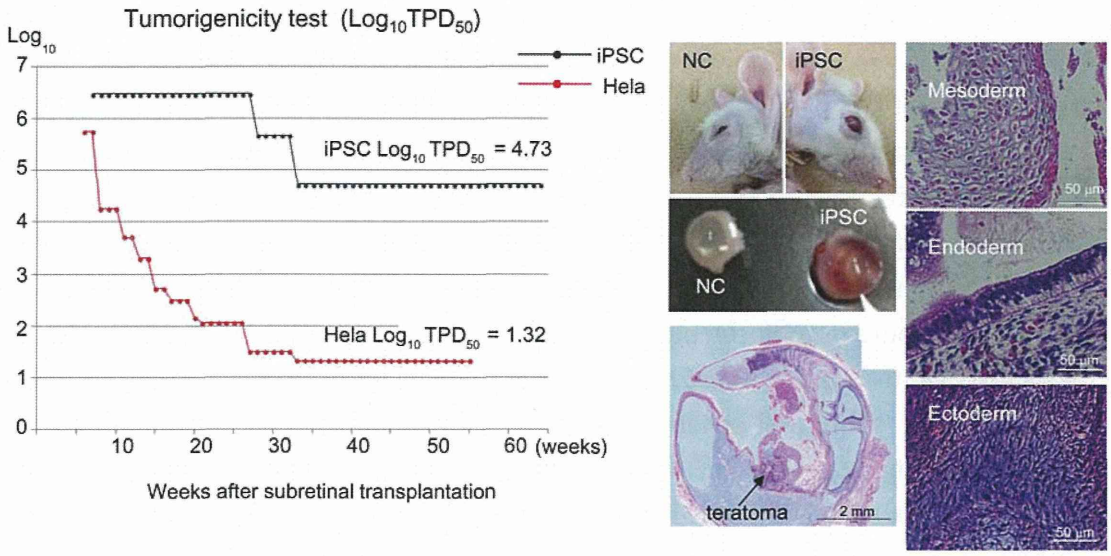

Figure 2. Tumorigenicity test via clinical route with Nude rats. A table in above showed type of cells used as a positive control for tumorigenicity test (iPSC cell line 201B7 and tumor cell line HeLa), minimum dose for tumor formation and Log_{10} TPD_{50} for them when transplanted via clinical route. A line graph showed value for Log_{10} TPD_{50} for iPSC or HeLa at respective monitoring point (0–55 or 64 weeks). Photos (left from top to bottom); NC: non-transplanted control, iPSC: iPSC transplanted mouse. iPSC-transplanted (iPSC) or non-treated control (NC) eye ball. HE staining of slice section of iPSC-transplanted eye ball. Photos (right top to bottom) histology of teratoma formed; cartilage (mesoderm), intestinal tissue-like (endoderm) or neuron-like (ectoderm) tissue.

Another option to address the tumorigenic potential of autologous iPSC-derived products is to transplant rodent cells into a rodent with same genetic background to evade immune rejection associated with xeno-transplantation. Of course, it will be necessary to accumulate sufficient data to demonstrate that rodent cells used in this test are equivalent to human investigational cell products before starting the test.

5. Administration Route and Microenvironment at the Transplantation Site

The administration route should mimic the clinical route as closely as possible to address the tumorigenic potential of investigational cells in the context of the microenvironment at the transplantation site. Therefore, evaluation of the microenvironment of the transplantation site including trans-effects from the microenvironment on investigational cells should be assessed prior to the