

Figure 1. Tumorigenicity testing (TPD₅₀ log₁₀) by subcutaneous transplantation of HeLa cells. Log₁₀TPD₅₀ values (minimal cell doses for 50% of animals to form a tumor) for HeLa cells when transplanted subcutaneously in various immuno-deficient mouse strains (nude, SCID, NOD-SCID, NOG) with or without Matrigel as indicated. Abscissa, weeks after transplantation. Ordinate, Log₁₀ TPD₅₀ values, logarithmic scale. doi:10.1371/journal.pone.0085336.g001

Tumorigenicity Testing of hiPSC-derived RPE (Subcutaneous Transplantation)

To assess the tumorigenic potential of hiPSC-derived RPE cells with high sensitivity, subcutaneous transplantation of a large number of RPE cells would be ideal. However, the maximal number of RPE cells available for transplantation was limited by the culture capacity of the cell processing facility. We hypothesized that transplanting 1×10⁶ hiPSC-derived RPE cells was an acceptable cell number to address the tumorigenic potential of the final cell product when embedded in Matrigel in NOG mice. This hypothesis was based on the facts that we expect to transplant 4–8×10⁴ hiPSC-derived RPE in a clinical setting and as few as 10

undifferentiated hiPSCs or HeLa cells embedded in Matrigel could generate tumors in NOG mice (Table 1, Table 2).

Thus, we subcutaneously transplanted 1×10⁶ hiPSC-derived RPE cells embedded in Matrigel into NOG mice [total n = 42; 59-G3 RPE (n = 14), K21-G18 RPE (n = 13), 101-G25 RPE (n = 15)]. Tumor formation was monitored for more than 70 weeks. Teratoma derived from subcutaneously transplanted iPSCs was analyzed as a positive control for tumor formation event (Figure 3A–3E). The proliferative status of living cells was assessed by HE, Hoechst 33258 and anti-Ki67 antibody staining (Figure 3G–3I). iPSC-derived neural rosette-like human cells were stained by anti-Lamin A antibody to check the specificity of anti-Lamin A antibody for human cells (Figure 3F). We assumed that

Table 1. Incidence of tumor formation after transplanting HeLa cells in various immunodeficient mice.

strain	use of Matrigel	min.dose for tumor formation	weeks to observe Tumor (first to last)	number of mice	Log10TPD50
nude	with	1×10 ⁴ cells	3 to 8	120	3.5
nude	w/o	1×10 ⁴ cells	4 to 12	120	4.9
SCID	with	1×10 ³ cells	3 to 11	24	2.5
SCID	w/o	1×10 ³ cells	3 to 11	24	3.83
NOD-SCID	with	1×10 ² cells	3 to 16	24	2.17
NOD-SCID	w/o	1×10 ³ cells	3 to 14	24	3.5
NOG	with	1×10 ¹ cells	5 to 18	75	1.1
NOG	w/o	1×10 ⁴ cells	3 to 13	105	3.97

Log₁₀TPD₅₀ values for HeLa cells transplanted subcutaneously into various immunodeficient mouse strains with or without Matrigel. Tumor-forming potentials of HeLa cells in nude mice without Matrigel and in NOG mice with Matrigel are highlighted in gray. doi:10.1371/journal.pone.0085336.t001

Table 2. Tumorigenicity testing by subcutaneous transplantation of hiPSC-derived RPE into NOG mice.

hiPSC cell line	cell form	min.dose for tumor formation	weeks to observe Tumor (first to last)	number of mice	Log10TPD50
201B7	Cell suspension in Matrigel	1×10^1 cells	5–40	30	2.12
RPE cell line	cell form	number of cells transplanted	monitor period	number of mice	tumor formation
59-G3(1)	RPE cell suspension in Matrigel	1×10^6 cells	26–84 weeks	9	none
K21-G18	RPE cell suspension in Matrigel	1×10^6 cells	26–74 weeks	8	none
101-G25	RPE cell suspension in Matrigel	1×10^6 cells	23–70 weeks	10	none
59-G3(1)	RPE cell sheet in Matrigel	1×10^6 cells	28–85 weeks	5	none
K21-G18	RPE cell sheet in Matrigel	1×10^6 cells	13–79 weeks	5	none
101-G25	RPE cell sheet in Matrigel	1×10^6 cells	23–79 weeks	5	none
primary RPE	Cell suspension in Matrigel	1×10^6 cells	52 weeks	3	none
primary RPE	Cell suspension w/o Matrigel	1×10^6 cells	52 weeks	2	none
59-G3(2)	RPE cell sheet in Matrigel	1×10^6 cells	26–50 weeks	3	none
RNT10	RPE cell sheet in Matrigel	1×10^6 cells	26–46 weeks	3	none
RNT9	RPE cell sheet in Matrigel	1×10^6 cells	26–38 weeks	3	none
101-EV3	RPE cell suspension in Matrigel	1×10^6 cells	39 weeks	5	none
K11-EV9	RPE cell suspension in Matrigel	1×10^6 cells	39 weeks	3	none
K21-EV15	RPE cell suspension w/o Matrigel	1×10^6 cells	39 weeks	4	none
K11-EV9	RPE cell suspension w/o Matrigel	1×10^6 cells	39 weeks	2	none

Log₁₀TPD₅₀ value for hiPSC 201B7 determined by subcutaneously transplanting cells in Matrigel into NOG was calculated by the Trimmed Spearman-Kärber method (upper panel). Tumor formation from 1×10^6 hiPSC-derived RPE cells prior to making RPE sheets (cell suspension) or after making RPE sheets (cell sheet) transplanted subcutaneously in various conditions into NOG mice. Animals were monitored for 13–85 weeks (lower panel). doi:10.1371/journal.pone.0085336.t002

anti-human Lamin A antibody could stain a wide range of human cell types and was not limited to human RPE. Transplantation of 1×10^6 primary RPE cells embedded in Matrigel ($n = 3$) was used as a transplantation control. No tumor formation was observed from transplanted 1×10^6 hiPSC-derived RPE of several origins in various administration forms. All of the subcutaneous tumorigenicity tests conducted for hiPSC-derived RPE under various conditions using NOG mice are shown in Table 2.

All subcutaneous transplants consisting of RPE cells embedded in Matrigel were excised and subjected to histological examination. The size of transplants in subcutaneous tissue (Figure 4A, 4B) was similar to that of Matrigel without RPE (Figure 4C). Histological and immunohistological study showed that Lamin A-, BEST1- and Hoechst-positively staining RPE cells were present in all the Matrigel transplants (Figure 4F–4M). None of the cells transplanted in Matrigel stained with anti-Ki67 antibody, suggesting the absence of active proliferation in these transplanted cells (Figure 4D, 4E). Human cells derived from transplanted iPSC-derived RPE could be detected by *Alu* PCR at a level of $\geq 0.1\%$ in mouse cells. However, we could not detect human cells in subcutaneous mouse tissue just beneath the transplants, in liver, spleen, kidney or lung by this method (Figure 5A, 5B).

Tumorigenicity Test of hiPSC-derived RPE (Subretinal Transplantation)

Next, we conducted tumorigenicity tests by transplanting test cells into the subretinal space, a procedure that is technically demanding. We chose large albino nude rats to facilitate transplantation and minimize variability of test results. This choice also permitted us to transplant larger doses of human hiPSC-derived RPE cells to the subretinal spaces. First, we

assessed the tumor-formation potential of HeLa and iPSC 201B7 via subretinal transplantation. The TPD₅₀ for HeLa was $10^{1.32}$ ($n = 37$) and $10^{4.73}$ for iPSC ($n = 20$) (Table 3). Teratomas derived from subretinally transplanted iPSC or tumors derived from transplanted HeLa cells were analyzed as positive controls for tumor formation event in the subretinal space (Figure 6A–6I). The proliferative status of living cells was assessed by HE, Hoechst 33258 and anti-Ki-67 antibody staining (Figure 6J–6L). iPSC-derived human cells were stained by anti-Lamin A antibody to check the specificity of anti-human Lamin A antibody to human cells (Figure 6M–6O).

Next, we conducted tumorigenicity tests of iPSC-derived RPE by transplanting 1 mm^2 -sized ($1 \text{ mm} \times 1 \text{ mm}$) iPSC-derived RPE sheets consisting of $0.8\text{--}1.5 \times 10^4$ RPE cells into the subretinal space of nude rats ($n = 26$). The RPE cell number was assessed by cell density and sheet size transplanted. Considering the relative sizes of humans and rats, we estimated that transplanting $0.8\text{--}1.5 \times 10^4$ RPE cells into the subretinal space of nude rats would provide the information required to determine the incidence of tumor formation in humans, as we expect to transplant $4\text{--}8 \times 10^4$ RPE cells in a clinical setting. Thus, we transplanted 5 different hiPSC-derived RPE cell sheets from 5 different patients to minimize individual variations. hiPSC-derived RPE sheets [59-G3 RPE ($n = 9$), K21-G18 RPE ($n = 4$), 101-G25 RPE ($n = 3$), RNT9 RPE ($n = 5$), RNT10 RPE ($n = 5$)] were prepared and transplanted under various conditions. Transplanted nude rats were monitored for tumor formation and physical condition daily for 8 to 82 weeks.

No tumor was found during the period of observation (Table 3). All transplanted eye balls were excised and subjected to histological examination. The location of transplanted RPE sheet

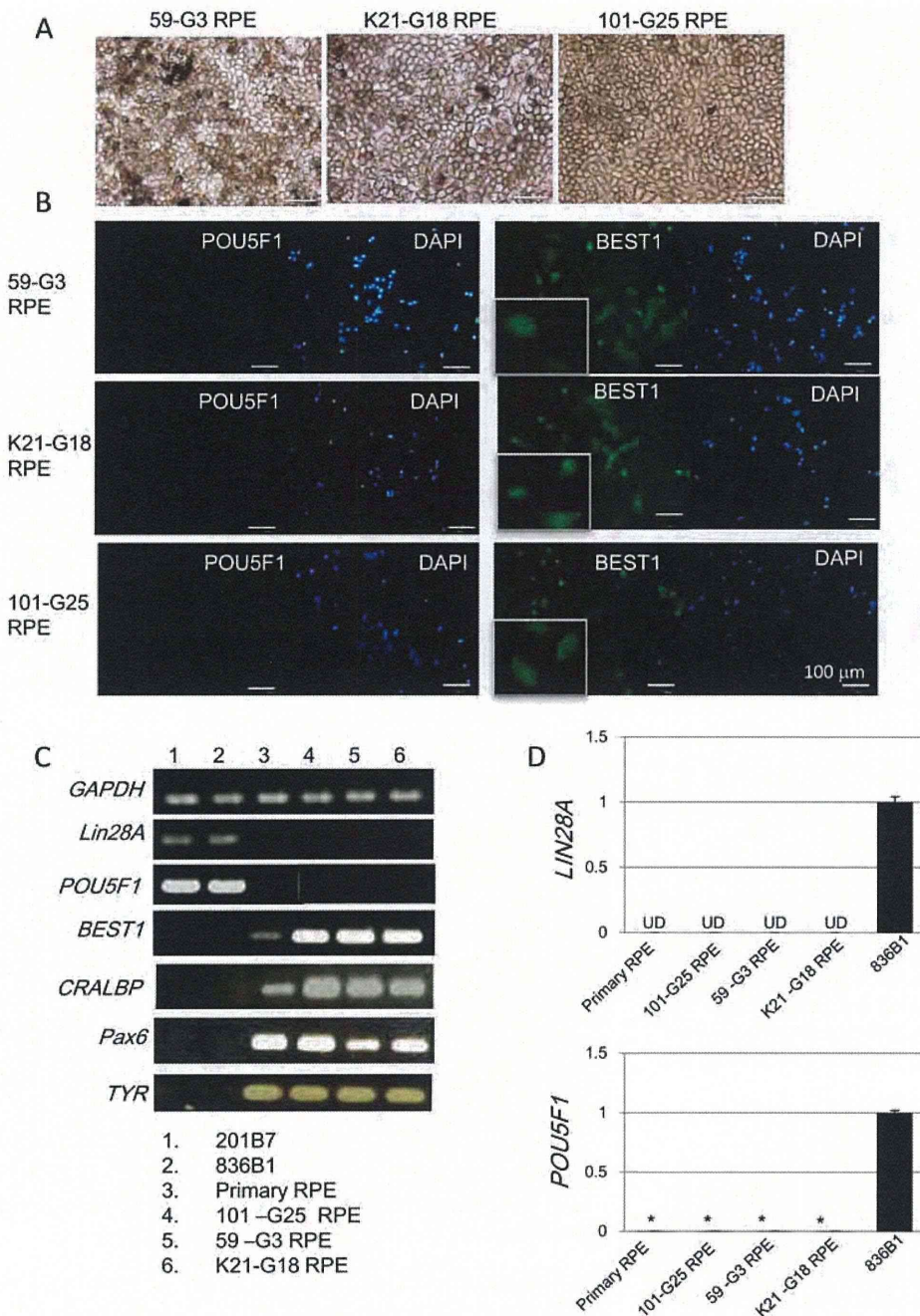


Figure 2. Characterization of hiPSC-derived RPE. A: Phase contrast images of hiPSC-derived RPE cell lines 59-G3 RPE, K21-G18 RPE and 101-G25 RPE. B: Expression of pluripotency-related molecules POU5F1 (OCT3/4, upper panels) and RPE-related molecules BEST1 (lower panels) in lines 59-G3 RPE, K21-G18 RPE and 101-G25 RPE as detected by immunostaining with specific antibodies. Nuclei were stained with DAPI. Magnified photos of BEST1 staining are appended in left lower corners. C: Gene expression profiles of hiPSC-derived RPE cell lines 59-G3-RPE, K21-G18 RPE, 101-G25 RPE. Expression of pluripotent stem cell-related gene markers *LIN28A* and *POU5F1*, or RPE-related makers *BEST1* (bestrophin), *CRALBP*, *PAX6* and *TYR* (tyrosinase) in hiPSC cell lines 201B7 and 836B1, primary RPE (hrPE-1) and hiPSC-derived RPE cell lines 59-G3 RPE, K21-G18 RPE, 101-G25 RPE as determined by RT-PCR (left panel). *GAPDH* was used as an internal control. 50 ng RNA was used for one RT reaction. Gene expression of *LIN28A* (D) or *POU5F1* (E) in hiPSC-derived RPE cell lines 59-G3 RPE, K21-G18 RPE, 101-G25 RPE, hiPSC 836B1 and primary RPE was quantified by qRT-PCR. UD: undetectable level (D). *, $P < 0.005$ (E). P values for primary RPE, 101-G25 RPE, 59-G3 RPE, or K21-G18 RPE versus 836B1 are 0.000153, 0.000177, 0.000432 or 0.000489, respectively. doi:10.1371/journal.pone.0085336.g002

was shown by the brown color of the RPE sheet in albino nude rats (Figure 7A–7B). Histological and immunohistological studies showed that Lamin A- and Hoechst-positively staining or BEST1- and Hoechst-positively staining transplanted RPE cells were

present in the subretinal space (Figure 7H–7O). Although we used serial sections for this staining, we believe more than half of Lamin A positive-human cells were stained with Hoechst, suggesting that these cells were live human transplanted cells at

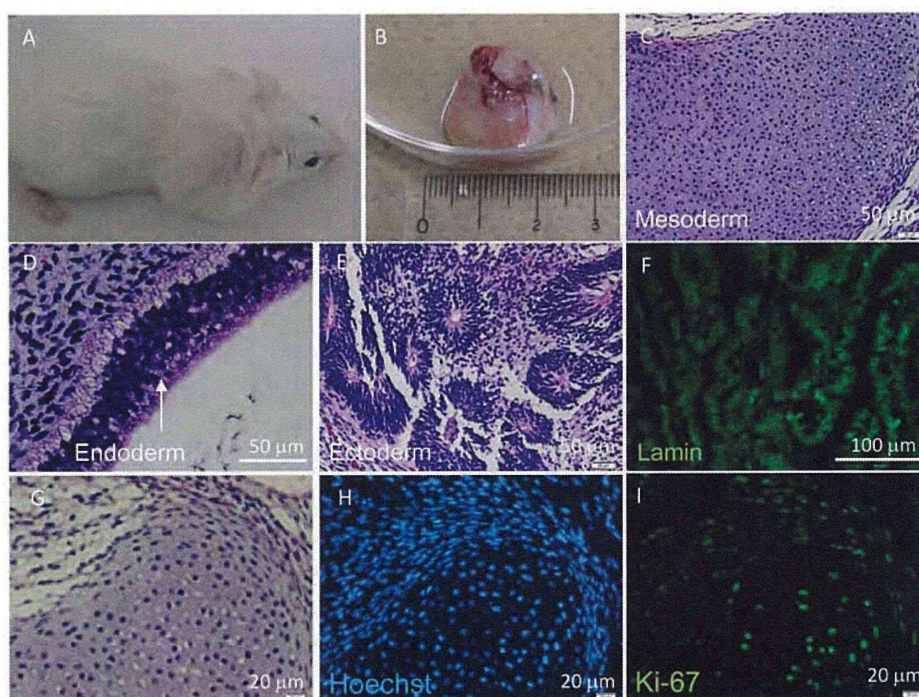


Figure 3. Histological analyses of hiPSCs subcutaneously transplanted into NOG mice. Tumor (teratoma) in NOG mouse was detected 5 weeks after transplanting 1.0×10^4 hiPSCs embedded with Matrigel (A, B). HE staining of sectioned hiPSC-derived teratoma consisted of three germ layers: cartilage-like tissue (mesoderm) (C), intestinal epithelium-like tissue (endoderm) (D) and neural rosette-like tissue (ectoderm) (E). Anti-Lamin A antibody (F) staining of rosette-like tissue. HE (G), Hoechst 33258 (H) and anti-Ki-67 antibody (I) staining of cartilage-like tissue. doi:10.1371/journal.pone.0085336.g003

the end of the experiment (Figure 7H–7K). However, none of the cells in the sub-retinal space was stained with anti-Ki-67 antibody, suggesting that there was no ongoing proliferation in transplanted RPE cells (Figure 7D–7G). Histological analysis of serial sections showed that the shape of hiPSC-derived RPE sheet was maintained after transplantation and no evidence of tissue invasion or destruction of the vicinity of retinal structure was observed (Figure 7C, 7D).

Discussion

Here, we presented the results of nonclinical tests assessing the tumorigenic potential of hiPSC-derived RPE sheets. These studies represent a portion of the nonclinical testing of our scheduled clinical study for the use of autologous hiPSC-derived RPE sheets for the treatment of wet type AMD. The clinical study is scheduled to commence in 2014. The hiPSC-derived RPE cells used in this study were prepared in a GMP-grade cell processing facility using the same procedures that will be used for patient treatment.

Two types of tumorigenicity tests are summarized in this report. The first was a subcutaneous tumorigenicity test in NOG mice using Matrigel and the second was a subretinal tumorigenicity test in nude rats. It is intriguing to compare the objectives and the validity of the 2 tests in regard to hiPSC-derived RPE cell transplantation. Rationales for conducting subcutaneous tumorigenicity test are as follows:

1. Large numbers of test cells can be transplanted without difficulty, and bias-inducing variations in technical skills can be neglected. Moreover, the tumors are easy to detect. Therefore, statistical and endpoint analysis (TPD₅₀ assessment) can be conducted in a timely and accurate manner.

2. It is possible to conduct comparison studies of the tumor-forming potential of different cellular products under the same transplantation conditions.
3. Above all, this test could serve as a substitute for *in vitro* soft agar assays of PSCs. PSCs cannot survive in soft agar, so that mode of testing is not feasible [5]. In contrast, PSCs or PSC-derived cells can survive long-term (more than 12 months) in Matrigel when subcutaneously transplanted in NOG mice. We can detect tumors derived from as few as 10 iPSCs or HeLa cells in this system (Table 1, Table 2). Thus, it provides a highly sensitive tumorigenic test for detecting both residual hiPSCs and tumorigenic transformed cells in hiPSC-derived cell products.

In this context, subcutaneous transplantation testing can be considered a quality control test of final cell products to ensure the absence of tumorigenic cells rather than characterizing the tumorigenic potential of the final cell products at a clinical transplantation site.

Next, we conducted tumorigenicity tests of hiPSC-derived RPE via clinical administration route. Under physiological conditions, the RPE is a monolayer that secretes various cytokines to maintain its structure in the retina. Dimiz et al [3] reported that RPE transplanted in sheets retain better survival than when transplanted in suspension. For clinical application, we plan to transplant hiPSC-derived RPE in a sheet form and our preclinical testing was designed accordingly. Although we do not have RPE cell survival data in suspension form, it would be logical to presume that the transplantation of RPE in a sheet form exerts physiological function more effectively than in suspension, which may also facilitate the adaptation of transplanted cells to the subretinal tissues.

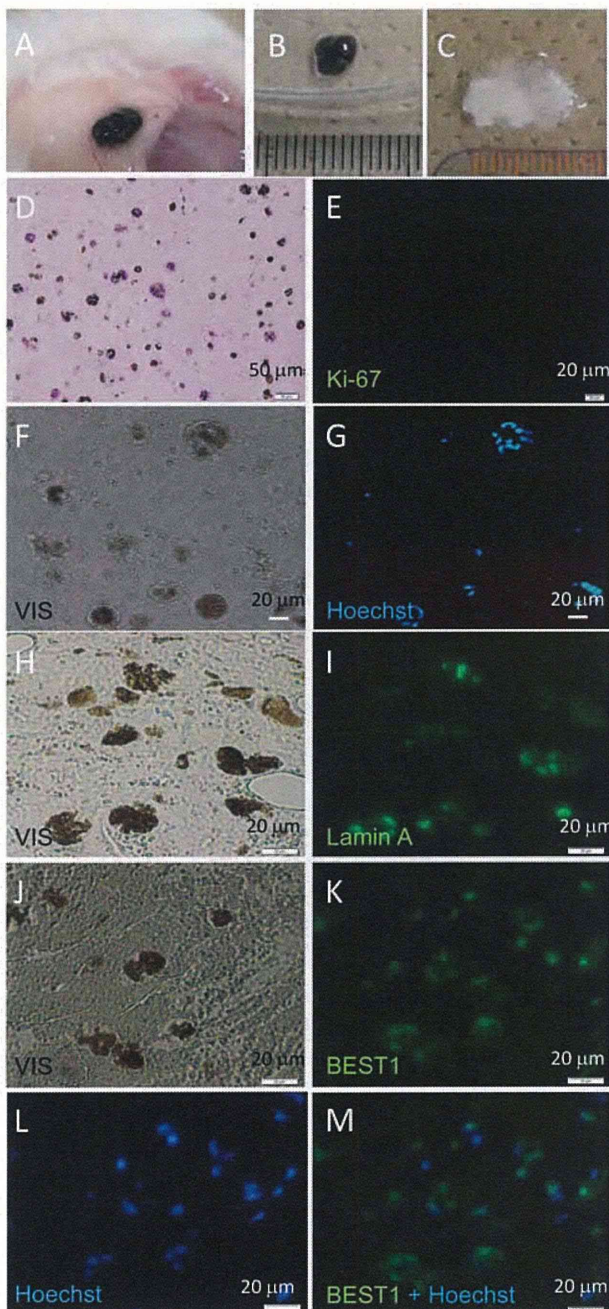


Figure 4. Histological analysis of hiPSC-derived RPE transplanted subcutaneously into NOG mice. NOG mice were examined six months after transplantation of 1.0×10^6 hiPSC-derived RPE cells in Matrigel into subcutaneous tissue. No tumor was detected visually. Site of transplant (A), excised transplant (B), and excised Matrigel only transplant (Matrigel without RPE cells C). Transplants were sectioned and stained with HE (D) and anti-Ki67 antibody (E). Photomicrograph of unstained serial section (F), and section stained with Hoechst 33258 (G). Photomicrograph of unstained serial section (H) or stained with anti-Lamin A antibody (I). Photomicrograph of unstained serial section (J) or stained with anti-BEST1 antibody (K) and Hoechst 33258 (L) and merged (M). Ki-67 positive cells were not observed.
doi:10.1371/journal.pone.0085336.g004

With regard to the tests' ability to detect immature (undifferentiated) hiPSCs in the subretinal space as a growing tumor, we

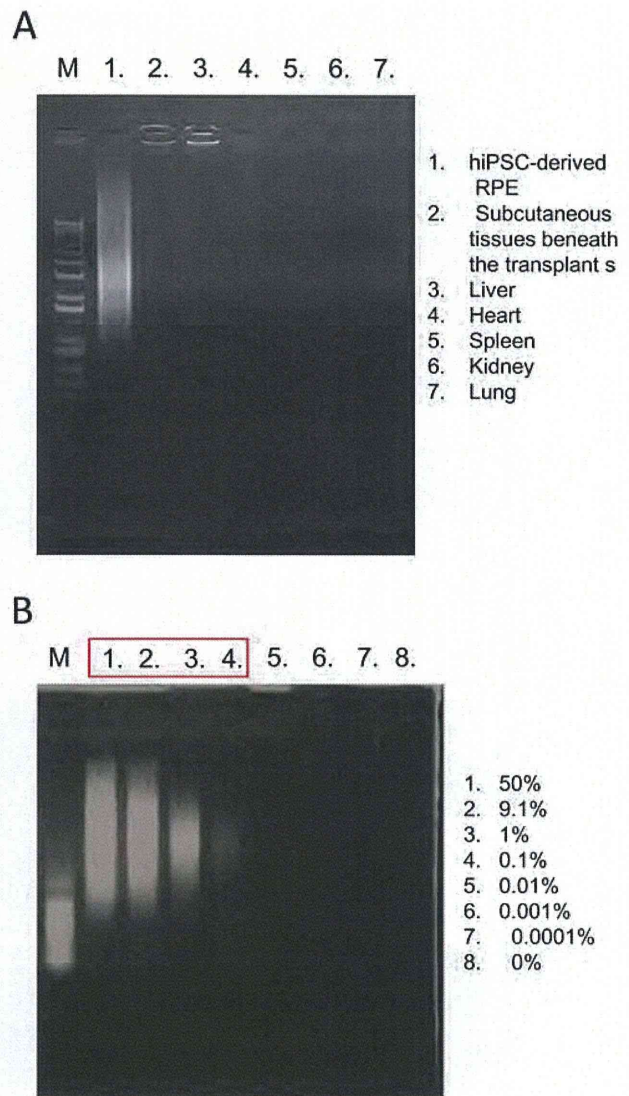


Figure 5. Detection of human cells in host mouse tissue by *Alu* PCR. DNA from hiPSC-derived RPEs (positive control, Lane 1), NOG mouse subcutaneous tissue just beneath the transplants (2), mouse liver (3), mouse heart (4), mouse spleen (5), mouse kidney (6) and mouse lung (7) were used as PCR templates. M: 1 kb marker (A). *Alu* PCR detects $\geq 0.1\%$ human cells included in mouse cells determined by visual assessment of PCR products generated from various ratios of human: mouse DNA template mixtures. Percentage of human DNA in DNA mixture is shown in a respective lane number (1–8) (B). M: 1 kb marker.
doi:10.1371/journal.pone.0085336.g005

demonstrated the trans-effects of RPE on hiPSC in our recent studies. We reported that RPE secreted pigment epithelium derived-factor (PEDF) that markedly induced apoptosis in hiPSC and hESC [21]. hiPSCs or ESCs in culture inserts ceased to survive when co-cultured with RPEs. Further addition of hPEDF induced apoptosis in hiPSCs or ESCs drastically. In fact, when hiPSCs were transplanted into the subretinal space of nude rats, the $\log_{10} TPD_{50}$ value was 4.73 ($n = 20$), whereas the value was only 2.12 ($n = 30$) when hiPSCs alone were transplanted subcutaneously into NOG mice with Matrigel. The 400-fold difference in the TPD_{50} values under these conditions are at least partly explained by an environmental effect related to the subretinal

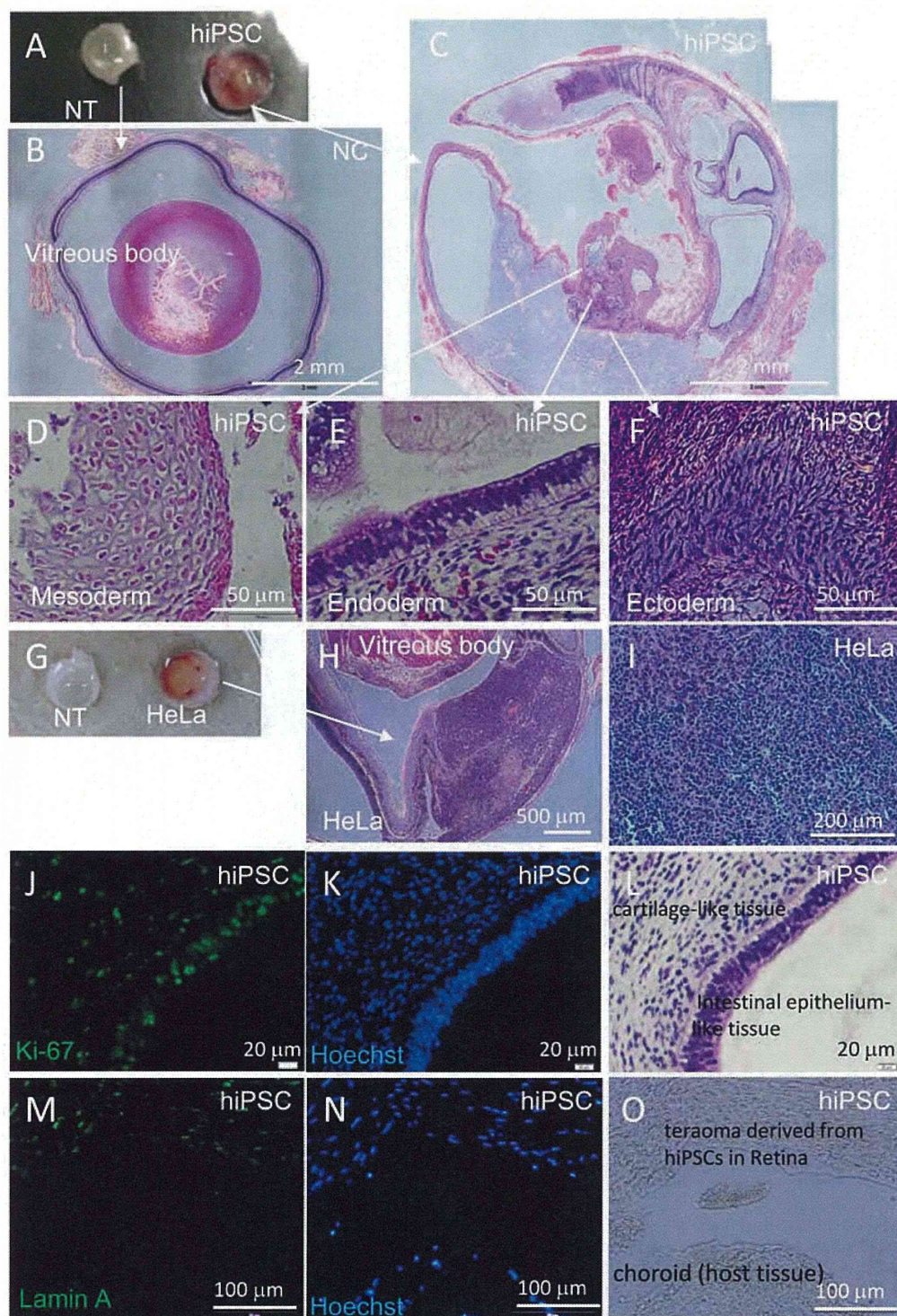


Figure 6. Histological analyses of hiPSCs or HeLa cells transplanted into the subretinal space of nude rats. Eye balls were excised from a nude rat 7 weeks after subretinal transplantation of hiPSC. Non-transplanted right eye ball (NT) and left eye ball transplanted with 1×10^4 hiPSCs (hiPSC) (A). HE staining of cross section of NT eye ball (B) and hiPSC-transplanted eye ball (C). HE staining of hiPSC-derived teratoma with three germ layers: cartilage-like tissue (mesoderm, D), intestinal epithelium-like tissue (endoderm, E) and neuron-like tissue (ectoderm, F) in hiPSC-transplanted eye ball. (G – O) Eye balls were excised from a nude rat 5 weeks after subretinal transplantation of HeLa cells. Non-transplanted right eye ball (NT) and left eye ball transplanted with 1×10^5 HeLa cells (HeLa) (G). HE staining of cross section of HeLa cell-transplanted eye ball (H) and HeLa-derived tumor tissue (I). Anti-Ki-67-antibody (J), Hoechst 33258 (K) and HE staining (L) of serial sections of hiPSC-derived teratoma. Anti-Lamin A antibody (M), Hoechst 33258 (N) staining and microscopic image (O) of serial cross sections containing a boundary of hiPSC-derived teratoma and host rat tissue. Anti-Lamin A antibody specifically recognizes human cells in rat tissue.
doi:10.1371/journal.pone.0085336.g006

Table 3. Tumorigenicity testing by subretinal transplantation of hiPSC-derived RPE in nude rats.

hiPSC cell line	cell form	min.dose for tumor formation	weeks to observe Tumor (first to last)	number of rats	Log ₁₀ TPD ₅₀
HeLa	Cell suspension w/o Matrigel	1×10^1 cells	5–33	37	1.32
hiPSC 201B7	Cell suspension w/o Matrigel	1×10^4 cells	7–33	20	4.73
RPE cell line	cell form	number of cells transplanted	monitor period	number of rats	tumor formation
59-G3 (1)	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	9–82 weeks	4	none
K21-G18	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	9–82 weeks	4	none
101-G25	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	9–82 weeks	3	none
59-G3 (2)	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	8–50 weeks	5	none
RNT10	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	26–47 weeks	5	none
RNT9	RPE cell sheet w/o Matrigel	$0.8\text{--}1.5 \times 10^4$ cells	12–38 weeks	5	none

Log₁₀TPD₅₀ values for HeLa cells or for hiPSC 201B7 cells following subretinal transplantation to nude rats (upper panel). Subretinal tumorigenicity tests conducted using nude rats under various conditions (lower panel).
doi:10.1371/journal.pone.0085336.t003

space, besides the difference in the status of immunodeficiency in these species or use of Matrigel. We suggest that the environmental effects of the subretinal space are mediated by PEDF secreted by RPE. The close protein sequence identity between human PEDF and the rat counterpart support this idea.

As many as 1×10^4 hiPSC cells were required to form tumors in the subretinal space of nude rats. Similar numbers ($0.8\text{--}1.5 \times 10^4$) of hiPSC-derived RPE were transplanted into the subretinal space in the tumorigenicity test. Note that tumorigenicity testing of

iPSC-derived RPE via clinical administration route will always give “negative” results, if we aim to detect a tumor from remaining small number of undifferentiated hiPSCs in final product. In this context, tumorigenicity tests conducted by transplanting serial dilutions of hiPSCs combined with hiPSC-derived RPEs into the subretinal space might not be informative. We suggest that tumorigenicity testing via clinical administration route might be useful to detect tumors from intermediate or incompletely differentiated RPE cells, but not for those relatively rare remaining

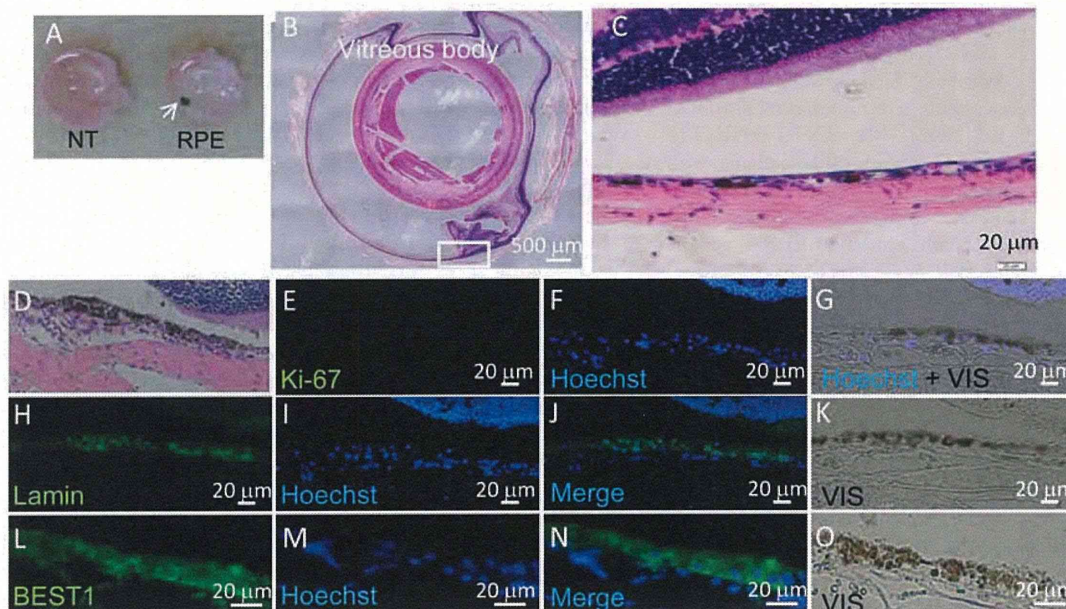


Figure 7. Histological analysis of hiPSC-derived RPE sheets transplanted into the subretinal space of nude rats. (A) Eye balls of nude rat 9 months after subretinal transplantation of $0.8\text{--}1.4 \times 10^4$ hiPSC-derived RPE (in a $1 \text{ mm} \times 1 \text{ mm}$ cell sheet). Left eye ball transplanted with hiPSC-derived RPE (RPE) and non-transplanted right eye ball (NT). (B) HE staining of cross section of left eye ball following transplantation of hiPSC-derived RPE. (C) HE staining of section of eye ball following transplantation of hiPSC-derived RPE, high magnification. HE- (D), anti-Ki67 antibody- (E), and Hoechst 33258-staining (F) and merged (G) images of serial sections of nude rat retina after transplantation of hiPSC-derived RPE. Anti-Lamin A antibody (H), Hoechst 33258 staining (I), merged (J) and micrographic image (K) of serial sections of nude rat retina after transplantation of hiPSC-derived RPE. Anti-BEST1 antibody (L), Hoechst 33258 (M), merged (N) staining and micrographic image (O) of serial sections of nude rat retina following transplantation of hiPSC-derived RPE.
doi:10.1371/journal.pone.0085336.g007

undifferentiated iPSC. For these reasons, we conducted high dose (1×10^6) subcutaneous RPE transplantation in parallel to examine tumor-forming events from rare hiPSC in hiPSC-derived RPE and full dose hiPSC-derived RPE subretinal transplantation without diluting them with hiPSCs. This was the basis for our rationale in designing multiple tumorigenicity tests for iPSC-derived RPE. As the FDA commentary report¹¹ stated, the design of tumorigenicity tests should be tailored for each specific product. We hope our approach will facilitate a further discussion related to tumorigenicity testing of iPSC-derived cell products.

Considering the number of rodents used, the duration of the monitoring period, the sensitivity to detect tumors in immunodeficient rodents via both subcutaneous and subretinal administration routes and the overall incidence of tumor formation from iPSC-derived RPE final cell products in these rodents, we conclude that the tumorigenic potential of the hiPSC-derived RPE cells produced by our methods is negligible. Of course, in considering the overall safety of the procedure in humans, discussion should include the site of transplantation as well as the source of the cells (autologous or allogeneic) and the immunosuppression status of the patients.

Materials and Methods

All the experiments using human samples and animal studies were reviewed and approved by the IRB of the Foundation for Biomedical Research and Innovation (FBRI) and Riken Center for Developmental Biology (Riken CDB), and the committee for animal experiments of the FBRI.

Cell Culture

The human iPSC (hiPSC) line 201B7 [12] established from dermal fibroblast with retroviruses pMXs-*POU5F1*, *-Sox2*, *-c-Myc*, and *-Klf4* (Riken Bio Resource Center, Tsukuba, Japan) was maintained on feeder layers (SNL [13]) in ReproFF2 (ReproCELL) and 5 ng/mL bFGF (Peprotech). Cell line 836B1 (supplied by CiRA Kyoto University) was established from dermal fibroblast of a healthy donor, and 59, K11, K21, 101, RNT9 or RNT10 lines were derived from dermal fibroblast of 6 patients with retinitis pigmentosa (with a photoreceptor-specific gene mutation) after obtaining informed consent from the patients. These fibroblasts were reprogrammed with episomal EBNA vectors carrying integrated *POU5F1*, *SOX2*, *KLF4*, *MYCL*, *LIN28A* and *GLIS1* (59-G, K21-G, 101-G, RNT9 and RNT10) or *POU5F1*, *SOX2*, *KLF4*, *MYCL*, *LIN28A* and *p53shRNA* (101-EV, K11-EV and K21-EV). They were established on autologous fibroblast-derived feeders and were maintained in primate ES medium (ReproCELL) with 5 ng/mL bFGF (Wako) [14]. iPSCs were differentiated into retinal pigment epithelium (RPE) as reported previously [15]. iPSC-derived RPE cell clones (59-G3 RPE, K21-G18 RPE, 101-G25 RPE, RNT9 RPE, RNT10 RPE, 101-EV RPE, K11-EV9 RPE or K21-EV15 RPE) were differentiated from the following parental iPSC clones: 59-G3, K21-G18, 101-G25, RNT9-2-8, RNT10-24, 101-EV3, K11-EV9 or K21-EV15, respectively. They were maintained in RPE maintenance medium [5],[15] [DMEM:F12 (7: 3) (Sigma-Aldrich) containing B-27 supplement (Invitrogen), 2 mM L-glutamine (Sigma), 0.5 nM SB431542 (Sigma-Aldrich) and 10 ng/mL bFGF (Wako)]. Human primary RPE (Lonza) was maintained in Retinal Pigment Epithelial Cell Basal Medium (Lonza Biologics, Basel, Switzerland) containing supplements [L-glutamine, GA-1000, and bFGF (Lonza)]. For transplant studies, hiPSC-derived RPE cells in suspension were collected for subcutaneous transplantation or seeded on collagen gel (collagen gel culture kit, Nitta Gelatin) to

make a collagen-lined RPE monolayer or double layer cell sheet. The RPE cell sheet was maintained in F10 culture medium (Sigma) and 10% FBS for 4 weeks and RPE maintenance medium for 3 weeks and detached from the collagen gel with collagenase-1 (Roche). The RPE cell sheet was then pipetted and mixed with Matrigel for subcutaneous transplantation or dissected with laser micro dissection (LMD, Carl Zeiss) just before retinal transplantation into animals.

Animal Studies

Mouse subcutaneous transplantation. Various doses of HeLa cells either embedded in 200 μ L of MatrigelTM (BD Biosciences) or suspended in 200 μ L of PBS (without Matrigel) were injected into subcutaneous tissue of 7- to 8-week-old female nude mice (BALB/cA, JCl-nu/nu; Clea Japan, Inc. Tokyo), SCID mice (C.B-17/Icr-scid/scid, Jcl; Clea), NOD-SCID mice (NOD/ShiJic-scid, Jcl; Clea) or NOG mice (NOD/ShiJic-scid, IL-2R γ OD/S KO Jic; Clea) using a 1 mL syringe (TERMO) with a 26 G needle. Animals were monitored for 36 weeks. At the end of the experiments, mice were sacrificed and tumors were removed and fixed with 4% PFA. Paraffin sections were stained with hematoxylin and eosin (HE) for histological observation. Various doses of hiPSC 201B7 cells or 1×10^6 hiPSC-derived RPE cells were embedded in 200 μ L of MatrigelTM (BD Bioscience) or suspended in 200 μ L of PBS (without Matrigel) and injected subcutaneously into 7- to 8-week-old female NOG mice using a 1 mL syringe (TERMO) with a 26 G needle and monitored for 6–15 months. At the end of the experiments, mice were sacrificed and all the transplants including RPE embedded in 200 μ L Matrigel were removed with tweezers and fixed with 4% paraformaldehyde (PFA).

Rat subretinal transplantation. Three-week-old female nude rats (F344/NJcl-rnu/rnu; Clea) were anesthetized by intraperitoneal administration of a mixture of ketamine 100 mg/kg; xylazine 10 mg/kg (Daichi-Sankyo). The pupil of the right eye was dilated with mydriatics (0.5% tropicamide and 0.5% phenylephrine hydrochloride, Santen Pharma). A small incision was made at the right eye corner of the sclera with a 27 G needle. Then, various doses of HeLa cells, hiPSCs or 1 mm \times 1 mm hiPSC-RPE cell sheets in 2 μ L DMEM/F12 medium were injected (Hamilton syringe with 33 G needle) into the subretinal space through the previously made incision in the sclera. The cells or the RPE sheet was transplanted just above the subretinal capillary plexus by observing the position of the Hamilton syringe needle through the dilated pupil under a surgical microscope. The subretinal capillary plexus was readily observed in albino nude rats and was used as a landmark of the subretinal space. The transplanted nude rats were monitored for 8–82 weeks. At the end of the experiments, rats were sacrificed and transplanted whole eye balls were removed and fixed with 4% PFA.

RT-PCR and qRT-PCR

Total RNA was isolated with the RNeasy plus Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Contaminating genomic DNA was removed using a gDNA Eliminator spin column. cDNA was generated from 50 ng of total RNA using PrimeScript RT Master Mix (Takara Bio) and PrimeSTAR MAX DNA Polymerase (TaKaRa Bio). Real-time PCR was then performed with an ABI 7000 Sequence Detection System (Applied-Biosystems) and SYBR-green in accordance with the manufacturer's instruction. Gene expression levels were normalized to that of *GAPDH*. qRT-PCR was performed using the QuantiTect Probe one-step RT-PCR Kit (Qiagen). The expression levels of target genes were normalized to those of the

RNase P transcript, which were quantified using TaqMan human RNase P control reagents (Applied Biosystems). All qRT-PCR reactions were run for 45 cycles. The sequences of primers and probes used in the present study are listed in Table S1.

Alu PCR

Alu sequences specific to human cells were used to design the primers. The *Alu* primer 5'-AAGTCGCGGCCGCTTGACAGT-GAGCCGAGAT-3' and 50 ng of DNA template, PrimeSTAR Max DNA Polymerase (Takara) were used for PCR reactions (28 cycles). The DNA templates in various ratios (human HeLa DNA: mouse NIH3T3 DNA) were used to determine the human cell detection sensitivity by *Alu* PCR. PCR products were separated by electrophoresis (MyRun, Cosmobio) with 1% agarose gel (Nacalai), and the image was digitally captured (Bio-Pyramid, Mecan).

Immunohistochemistry

Transplanted tissues were fixed with 4% paraformaldehyde. Paraffin embedded tissue sections were stained with haematoxylin/eosin. Then, the paraffin sections were deparaffinized with xylene and sequential 100%, 95%, 80%, 70% ethanol treatments for 5 min each. The sections were treated with 10 mM citric acid (pH 6) at 95°C for 50 min followed by permeation with 0.4% Triton-X in PBS at room temperature for 30 min. The deparaffinized sections were stained with antibodies against human Lamin-A (1:200; ab108595; Abcam), BEST1 (1:200; ab2182; Abcam) and Ki-67 (1:400; #9449; Cell Signaling). Nuclei were stained with Hoechst 33258 (Dojindo) and DAPI (Dojindo). hiPSC-derived RPE cells were collected in suspension and fixed with 4% paraformaldehyde followed by staining with antibodies against POU5F1 (OCT3/4) (1: 100; sc-5279; Santa Cruz), or BEST1 (1: 200; ab2182; Abcam). Antibodies were visualized with Alexa Fluor 488 goat anti-mouse (1: 1,000; Invitrogen) or Alexa Fluor 488 goat anti-rabbit (1: 1,000; Invitrogen). Fluorescent microscopic images were captured with a fluorescent microscope (Olympus BX51, IX71, Tokyo, Japan).

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Conclusion

We tested the tumorigenic potential of hiPSC-derived RPE using immuno-deficient rodents. These preclinical tests laid the foundation for upcoming clinical studies using autologous hiPSC-derived RPE sheets for treatment of wet type age-related macular degeneration (AMD). One million hiPSC-derived RPE cells were transplanted subcutaneously into 65 NOG mice and $0.8\text{--}1.5 \times 10^4$ hiPSC-derived RPE cells were transplanted into the subretinal space of 26 nude rats. No tumors were found after 6–15 months of monitoring. Considering the number of rodents used, the duration of the monitoring period, the sensitivity to detect tumors in immuno-deficient rodents, we conclude that the tumorigenic potential of the hiPSC-derived RPE cells prepared by our method is negligible.

Supporting Information

Table S1 Primers for RT-PCR and Alu PCR, Probes and Primers for qRT-PCR are listed. (DOCX)

Acknowledgments

We thank Yoji Sato of National Institute of Health Sciences, Tokyo for critical reading of the manuscript, Shin-Ichi Nishikawa of JT BRH and Takao Hayakawa of Kinki University for scientific discussions and Mamoru Ito of CIEA for supplying NOG mice.

Author Contributions

Conceived and designed the experiments: H. Kanemura MJG SK. Performed the experiments: H. Kanemura. Analyzed the data: MS NN. Contributed reagents/materials/analysis tools: NS H. Kamao MM CM MT. Wrote the paper: H. Kanemura SK.

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厚生労働科学研究費補助金
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有害事象発生時の科学的な細胞検証を通じて細胞治療の安全性向上を
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平成26年3月 総括・分担研究報告書

発行 平成 26 年 3 月
発行者 西下 直希・川真田 伸
事務局 〒650-0047 神戸市中央区港島南町1丁目5番地4号
臨床研究情報センター 3階 細胞療法開発事業部門
306号室 (078-306-0681)
印刷所 株式会社トライス
〒650-0016 神戸市中央区橘通 1-1-9
(078-341-2241)