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Original Article

A technique for removing tumourigenic pluripotent stem cells using rBC2LCN lectin

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ABSTRACT

Introduction: Tumourigenesis attributed to residual undifferentiated cells in a graft is considered to be a significant issue in cell therapy using human pluripotent stem cells. To ensure the safety of regenerative medicine derived from pluripotent stem cells, residual undifferentiated cells must be eliminated in the manufacturing process. We previously described the lectin probe rBC2LCN, which binds harmlessly and specifically to the cell surface of human pluripotent stem cells. We report here a technique using rBC2LCN to remove pluripotent cells from a heterogenous population to reduce the chance of teratoma formation.

Methods: We demonstrate a method for separating residual tumourigenic cells using rBC2LCN-bound magnetic beads. This technology is a novel use of their previous discovery that rBC2LCN is a lectin that selectively binds to pluripotent cells. We optimize and validate a method to remove hPSCs from a mixture with human fibroblasts using rBC2LCN-conjugated magnetic beads.

Results: Cells with the potential to form teratoma could be effectively eliminated from a heterogeneous cell population with biotin-labelled rBC2LCN and streptavidin-bound magnetic beads. The efficiency was measured by FACS, ddPCR, and animal transplantation, suggesting that magnetic cell separation using rBC2LCN is quite efficient for eliminating hPSCs from mixed cell populations.

Conclusions: The removal of residual tumourigenic cells based on rBC2LCN could be a practical option for laboratory use and industrialisation of regenerative medicine using human pluripotent stem cells.

1. Introduction

Human pluripotent stem cells (hPSCs), such as human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2], are expected to be applied to cell therapy, disease modelling, and drug development. It is possible to create hiPSCs from various types of somatic cells by the introduction of reprogramming factors, and various research projects using hiPSCs are ongoing.

Clinical trials using hESC-derived retinal pigment epithelium (RPE) and autologous hiPSC-derived RPE have been reported [3–5], and a clinical trial using allogeneic iPSC-derived RPE is underway.

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Another has been initiated for banked allogeneic hiPSCs to treat Parkinson's disease [6]. Further clinical trials have been planned to treat heart failure, spinal cord injury, Parkinson's disease, and aplastic anaemia, using cells created from hiPSCs [7–9]. Although hiPSC-based clinical and industrial applications are becoming realistic, it is still a major safety concern that residual hiPSCs in products for cell therapy could form tumours in transplanted sites [10–12]. The risk of teratoma formation by hPSCs has been reported in various animal studies [13–17]. It is reported that only 100 iPS cells are sufficient to produce a teratoma in a mouse model [13,18]. Therefore, the establishment of a method to detect and eliminate residual hPSCs from cell products without impairing the survival rate and functionality of the cells to be used for cell therapy, disease modelling, and drug development, is required.

Several strategies have been reported for selectively eliminating residual hPSCs from differentiated cells, including expressing a suicide gene into hPSCs [19], or using cytotoxic antibodies [20–22], chemical inhibitors [23–26], and synthetic RNA or peptide [27,28]. Cell sorting methods using hPSC-specific antibodies [20,29,30] and lectins [31] have also been proposed. However, all of these methods have limitations with respect to specificity, throughput, and other issues, and therefore it is still necessary to develop alternative or additional technologies based on different mechanisms.

We have previously reported that a lectin designated recombinant N-terminal domain of BC2L-C lectin derived from *Burkholderia cenocepacia* (rBC2LCN) binds to various types of hiPSCs and hESCs, but not to differentiated somatic cells [32,33]. This lectin binds specifically to the Fuc1-2Gal1-3 motif that is highly expressed on hiPSCs [32,34]. In addition, podocalyxin, a type1 transmembrane protein, was identified as a predominant glycoprotein ligand of rBC2LCN [35]. As its main practical applications, fluorescence-labelled rBC2LCN allows live staining of hESCs/hiPSCs following its addition to the culture medium and is capable of separating live hPSCs by flow cytometry [33]. The staining is specific to undifferentiated cells and rapidly diminishes depending on their differentiation. Furthermore, based on the finding that rBC2LCN was internalised inside hPSCs after binding to the surface of these cells, recombinant lectin-toxin fusion proteins in which rBC2LCN was fused to several domains of *Pseudomonas aeruginosa* exotoxin A was developed for selective elimination of hPSCs [36,37].

In this study, we demonstrate an additional application of rBC2LCN, namely its potential in magnetic bead-based cell separation for reduction of tumourigenic hPSCs from differentiated cell populations. We evaluated cell separation efficiency by flow cytometry and digital PCR analyses. Effective elimination of hPSCs was also verified in a teratoma formation assay in a mouse model.

2. Materials and methods

2.1. Cell culture

The human ES cell line H9 hNanog-pGZ [1] was maintained in mTeSR1 (STEMCELL Technologies, Vancouver, BC, Canada) on a BD Matrigel growth factor reduced (GFR) matrix (BD Biosciences, San Jose, CA, USA) with zeocin, according to the WiCell feeder independent pluripotent stem cell protocols provided by the WiCell Research Institute (www.wicell.org). The human iPS cell line 201B7 [2] was maintained in mTeSR1 (STEMCELL Technologies) on the BD Matrigel hESC-qualified matrix (BD Biosciences), according to the manufacturer's instructions (STEMCELL Technologies). HDF (ATCC PCS-201-012) was maintained in 10% FBS containing DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). HDF cells were treated with 10 μg/ml of Mitomycin C (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) for 120 min to prevent proliferation. The experiments using hiPSCs and hESCs were approved by the National Institute of Advanced Industrial Science and Technology (AIST) (accreditation numbers and hi2016-099).

2.2. Lectin labelling and magnetic cell separation

Recombinant BC2LCN lectin (rBC2LCN) (FUJIFILM Wako Pure Chemical Corporation) was labelled with a Biotin Labeling Kit-NH2 (Dojindo). Biotin-conjugated rBC2LCN (1–100 μg) or biotin-conjugated BSA were incubated with 50 μL of Dynabeads M−280 streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) in 1 ml of MACS buffer [0.5% bovine serum albumin (BSA) and 2 mM EDTA in PBS] on a rotator for 30 min at room temperature. After incubation, the beads were rinsed twice with MACS buffer (rBC2LCN-magnetic bead and BSA-magnetic bead).

Cells (hESCs and hiPSCs) were dissociated with ESGRO Complete Accutase (Merck Millipore, Billerica, MA, USA) and mixed with HDF in a ratio of 1:1. HDF cells were pre-marked with a CellTrace Violet cell proliferation kit according to the manufacturer's protocol (Thermo Fisher Scientific) or with mitomycin C treated for proliferation inhibition, depending on the following analysis. A total of 2×10^6 mixed cells were incubated with 50 μL of the rBC2LCN-magnetic bead, BSA-magnetic bead or magnetic bead alone for 30 min at 4 °C in 1 ml of MACS buffer. The suspensions were placed in a DynaMag magnet (Thermo Fisher Scientific) for 2 min, and the supernatant with untouched cells was collected for flow cytometry, gene expression analysis and teratoma formation assay.

2.3. Flow cytometry

Flow cytometry was performed as described previously [33]. The cells were resuspended at approximately 1×10^6 cells/mL in MACS buffer and incubated with anti−TRA-1-60 antibodies (1:300 dilution; clone TRA-1-60, Merck Millipore) for 1 h at 4 °C. Normal mouse IgM (Merck Millipore) was used as an isotype control. The cells were rinsed with MACS buffer and then incubated with Alexa Fluor 488 goat anti-mouse IgM (1:300 dilution; Thermo Fisher Scientific). After further rinsing, cells were stained with propidium iodide (PI) (Thermo Fisher Scientific), and 20,000 cells were analysed using a Cell Sorter SH800Z (Sony Corporation, Tokyo, Japan). The data were analysed with FlowJo software (BD Biosciences).

2.4. Digital droplet polymerase chain reaction (ddPCR) analysis

Magnetically sorted or unsorted cells were stained with propidium iodide (PI), and PI-negative live cells were collected using a cell sorter SH800Z (Sony Corporation). Total RNA was extracted from frozen cell samples using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) and ddPCR was carried out using a QX200 droplet digital PCR system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Samples were analysed using 2x One-Step RT-ddPCR Supermix (Bio-Rad). The primers and TaqMan probe sequences, their concentrations, and thermal cycling conditions used in the ddPCR methods were according to a previous publication [38]. The probe and primer sequences are as follows: *LIN28* probe, FAM-CGCATGGGGTTCGGCTTCCTGTCC-BHQ1, *LIN28* forward primer, CACGGTGCGGGCATCTG, *LIN28* reverse primer, CCTTCCATGTGCAGCTTACTC; *NANOG* probe, FAM-TGCTGAGGCCTTCTGCGTCACACC-BHQ1, *NANOG* forward primer, CTCAGCTACAAACAGGTGAAGAC, *NANOG* reverse primer, TCCCTGGTGGTAGGAAGAGTAAA. As the internal control, TaqMan GAPDH Control Reagent was used (Thermo Fisher Scientific). Each reaction was performed in duplicate. Total RNA quantities used for the reactions were 0.05, 5, and 50 ng for *GAPDH*, *LIN28*, *NANOG*, respectively. Fluorescence intensities of each droplet in samples were measured using a QX200 droplet reader (Bio-Rad). Positive

and negative droplets were discriminated and counted by applying a threshold determined manually in QuantaSoft software. The same threshold was applied to all the wells for each gene on one PCR plate. We adopted the larger measurement result of accepted droplets among the duplicated wells. The number of adopted droplets was >10,000. The copy number concentration in the sample was calculated using the numbers of positive and accepted droplets. The concentration results in terms of target copies per microliter were provided by QuantaSoft software. The number of target copies per template RNA (ex, copies/50 ng of RNA in the case of *NANOG*) was calculated as concentration (copies/μl) × 20 (μl) × dilution factor of template RNA. The dilution factors were 1 for *NANOG*, 10 for *LIN28* and 1000 for *GAPDH*, respectively. Absolute counts of *NANOG* or *LIN28* were normalised to *GAPDH*. Experiments were performed in triplicate and repeated three times with similar results.

2.5. Teratoma formation assay

Eight-week-old immune-deficient NOD/ShiJic-*scid*Jcl mice (CLEA Japan, Inc., Tokyo, Japan) were used for transplantation. NOD/ShiJic-*scid*Jcl mice were anaesthetised using 2% isoflurane and an animal anesthetizer device (MK-AT210D, Muromachi Kikai Co., Ltd., Tokyo, Japan). The surgical area was disinfected with 70% ethanol. After cutting the centre of the scrotum, a testis was carefully pulled out and injected with 20 μL (~1.0×10^6 cells) of cell suspension with BD Matrigel hESC-qualified matrix (BD Biosciences). The same treatment was applied to the other testis. Cell-injected testes were returned to their original location, and the wound was sutured. The transplanted animals and tumour growth were observed routinely about once a week. They were sacrificed after the development of tumours larger than 2 cm in diameter or following an observation period of about 3 months. Tumours were fixed with 4% paraformaldehyde phosphate buffer Solution (FUJIFILM Wako Pure Chemical Corporation). Paraffin embedding, sectioning, and H&E staining of tumours were performed by UNITECH Co., Ltd (Chiba, Japan). Images were obtained using BZ-X710 microscope (Keyence, Osaka, Japan). This experiment was approved by the National Institute of Advanced Industrial Science and Technology (AIST) (accreditation number A2018-290).

2.6. Statistical analysis

A one-way ANOVA followed by Tukey's honest significant difference (HSD) test was used for analysis of ddPCR and teratoma size. Statistical significance was inferred where $p < 0.05$. The data were analysed with KaleidaGraph v.4.5.2 (Synergy Software, Reading, PA, USA).

3. Results and discussion

3.1. rBC2LCN-bound magnetic beads eliminated hESCs from a heterogeneous cell mixture

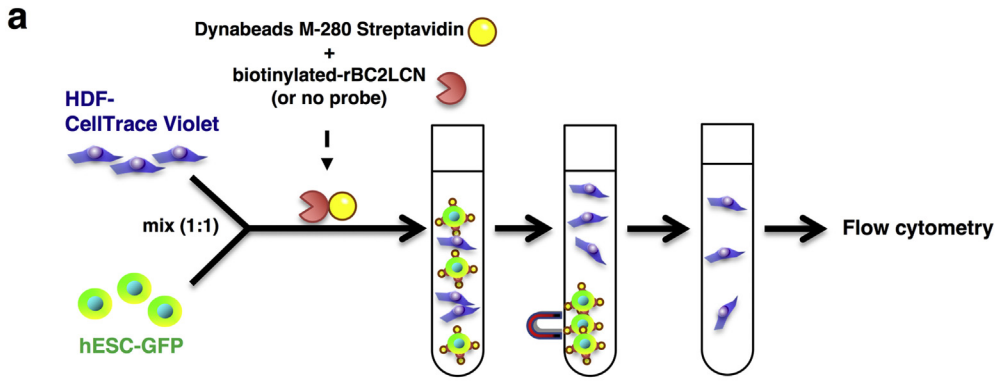
To facilitate the application of hPSC technology, it is important to detect and eliminate residual tumourigenic cells with high sensitivity. As a method to eliminate tumourigenic hPSCs from differentiated cell populations, we applied a magnetic bead-based cell separation system using rBC2LCN. We used hESCs (H9 hNanog-pGZ) [1] and normal human adult dermal fibroblasts (HDF) pre-labelled with CellTrace Violet. Biotin-labelled rBC2LCN was incubated with Dynabeads M−280 Streptavidin (Thermo Fisher Scientific). In this process, rBC2LCN was bound to magnetic beads. We first tested the capability of rBC2LCN-magnetic beads to separate undifferentiated hESCs from a mixed cell population containing hESCs (H9 hNanog-pGZ) expressing *GFP* and HDF pre-labelled with CellTrace Violet at a ratio of 1:1 (each 1×10^6 cells). The mixed cell populations were incubated with rBC2LCN-magnetic beads. The suspensions were treated with a magnet, and the supernatant containing unbound cells was collected for flow cytometry (Fig. 1). The flow of the experiment and details of each sample are shown in Fig. 1a and b, respectively. We tested three concentrations of rBC2LCN (0.1, 1, and 10 μg/ml) (Fig. 1b–h). Residual hESC levels evaluated by the percentage of GFP-positive and CellTrace Violet-negative cells after magnetic sorting were reduced up to 0.092% (Fig. 1h). The reduction in hESCs was correlated with increased rBC2LCN concentration (Fig. 1c–h). Magnetic beads alone as a negative control did not affect hESCs dislodged from the HDF/hESC mixture (Fig. 1e). These results suggest that rBC2LCN-bound magnetic beads efficiently eliminate live pluripotent stem cells from heterogeneous cell mixtures.

3.2. rBC2LCN-bound magnetic beads eliminated hiPSCs from a heterogeneous cell mixture

In order to test more realistic conditions for cell sorting, we next used Mitomycin C-treated HDF (MMC-HDF) and unlabelled hiPSCs (201B7) [2]. The hiPSC/MMC-HDF mixtures were incubated with rBC2LCN-magnetic beads. The subsequent analysis is the same as in Fig. 1, and the collected supernatant was used not only for flow cytometry (Fig. 2) but also for gene expression analysis by ddPCR (Fig. 3) and teratoma formation assay (Fig. 4). We detected hiPSCs as tumour rejection antigens-1–60 (TRA-1–60)-positive cells [39] (Fig. 2). The flow of the experiment and details of each sample are shown in Fig. 2a and b, respectively. The percentage of TRA-1-60-positive cells after magnetic sorting by rBC2LCN (0.19–0.29%) closely matched that of an MMC-HDF sample without iPSCs (0.23%) (Fig. 2c–h). We examined three concentrations of rBC2LCN (1, 10, and 100 μg/ml) (Fig. 2b), which were 10-fold higher than those used in Fig. 1, and no substantial difference in hiPSC removal efficiency was seen among them. The biotin-labelled BSA-magnetic beads as a negative control had no effect on eliminating hiPSCs from an MMC-HDF/hiPSC mixture (Fig. 2e). This result indicated that magnetic sorting using rBC2LCN efficiently functioned to eliminate hiPSCs from cell mixtures, and the separation efficiency was comparable to the limit of detection of flow cytometry with TRA-1-60. In subsequent experiments, we fixed the concentration of rBC2LCN to 10 μg/ml.

3.3. Detection of hiPSCs by droplet digital RT-PCR after magnetic cell separation

We also performed ddPCR analysis, allowing evaluation of the frequency of hiPSCs remaining in the cell suspension after magnetic cell separation using rBC2LCN. For discrimination of dead cells, magnetically sorted or unsorted cells were stained with propidium iodide (PI). The population of PI-negative live cells was collected and analysed (Fig. 3a–d and see Supplementary Tables S1–3 online). Expression levels of *NANOG* (Fig. 3a) and *LIN28* (Fig. 3b) relative to *GAPDH* were dramatically and significantly reduced in the sample sorted by rBC2LCN-magnetic beads, as compared to the sample sorted by BSA-magnetic beads, which was equivalent to the unsorted control sample (*NANOG*: $p < 0.0001$, *LIN28*: $p = 0.0037$). Furthermore, the levels of *NANOG* (Fig. 3c) and *LIN28* (Fig. 3d) mRNAs in sorted cell mixtures were evaluated by comparing to the samples in which total RNA extracted from HDF (HDF RNA) was



b

Sample	1	2	3	4	5	6
cell	HDF	hESC	HDF:hESC=1:1	HDF:hESC=1:1	HDF:hESC=1:1	HDF:hESC=1:1
probe	-	-	-	biotinylated-rBC2LCN	biotinylated-rBC2LCN	biotinylated-rBC2LCN
probe concentration	-	-	-	0.1 μg/ml	1 μg/ml	10 μg/ml
beads	-	-	M-280-SA	M-280-SA	M-280-SA	M-280-SA

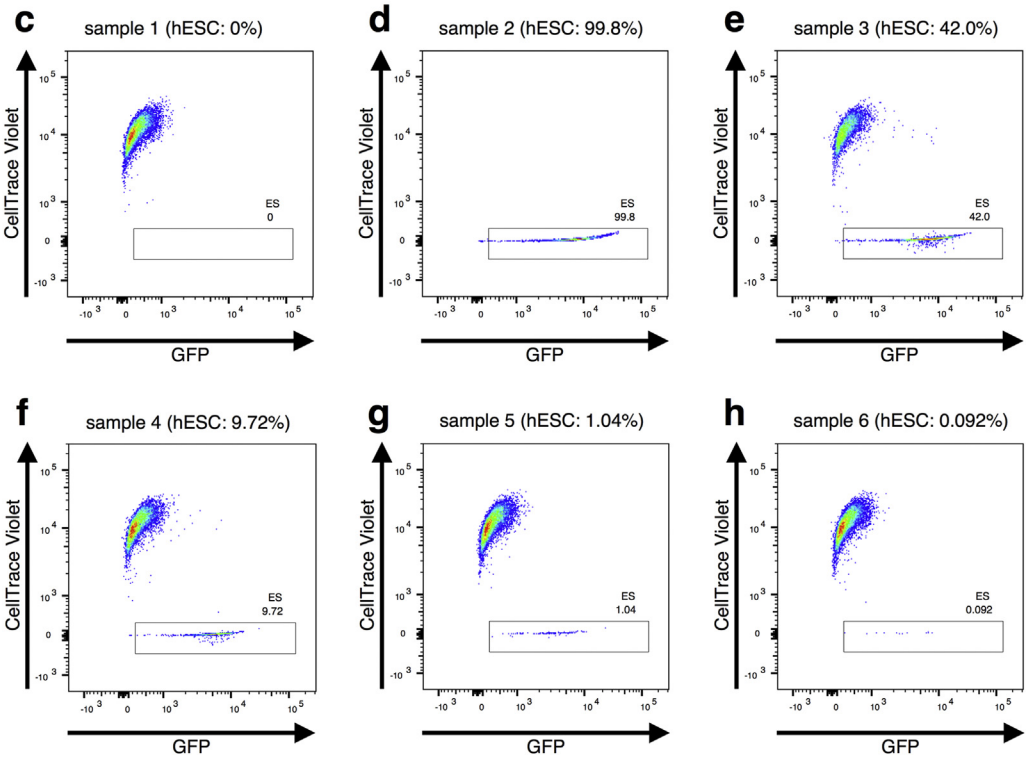
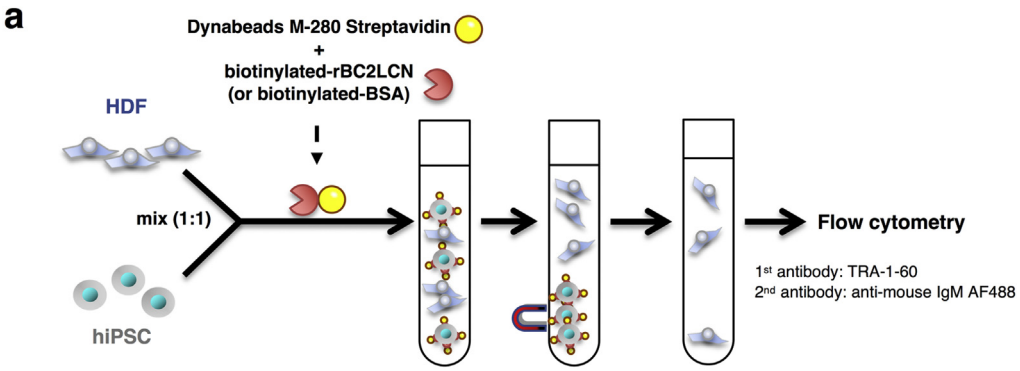


Fig. 1. Magnetic cell separation using rBC2LCN eliminated hESCs from an hESC/HDF mixture. (a) Experimental design to remove hESCs using magnetic beads. HDF labelled with a CellTrace Violet, and hESC line H9 hNanog-pGZ (H9) were mixed in a ratio of 1 to 1. The cells incubated with Dynabeads M−280 Streptavidin (M-280-SA) and biotinylated-rBC2LCN or M-280-SA alone were separated by a magnet and then analysed by flow cytometry. (b) Details of sample preparation (c–h) Flow cytometry of cells selected negatively by magnetic beads. (c) HDF alone showed 0% of GFP-positive and CellTrace Violet-negative cells (sample 1). (d) hESC alone showed 99.8% GFP-positive and CellTrace Violet-negative cells (sample 2). (e) 42.0% of GFP-positive and CellTrace Violet-negative cells were detected in the prepared cells using M-280-SA alone (sample 3; control). Selection using (f) 0.1 μg/ml (sample 4), (g) 1 μg/ml (sample 5), or (h) 10 μg/ml (sample 6) of biotinylated-rBC2LCN reduced the ratio of GFP-positive and CellTrace Violet-negative cells in a rBC2LCN concentration (9.72, 1.04, and 0.092%, respectively)-dependent manner.

spiked with total RNA extracted from hiPSCs (hiPSCs RNA) at a ratio of 0, 0.025, or 0.25%. No statistically significant differences in expression levels of *NANOG* or *LIN 28* were found among HDF RNA, HDF RNA mixed with 0.025% of hiPSCs RNA, or total RNA extracted from the cells sorted by rBC2LCN-magnetic beads. That is, the rBC2LCN magnetic beads removed iPSCs from a 1:1 mixture of iPSCs and HDF to a degree that was not significantly different from HDF alone in *NANOG* and *LIN28* expressions. These results suggested that magnetic cell separation using rBC2LCN is quite efficient for eliminating hPSCs from mixed cell populations.



b

Sample	1	2	3	4	5	6
cell	HDF	hiPSC	HDF:hiPSC=1:1	HDF:hiPSC=1:1	HDF:hiPSC=1:1	HDF:hiPSC=1:1
probe	-	-	biotinylated-BSA	biotinylated-rBC2LCN	biotinylated-rBC2LCN	biotinylated-rBC2LCN
probe concentration	-	-	100 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
beads	-	-	M-280-SA	M-280-SA	M-280-SA	M-280-SA

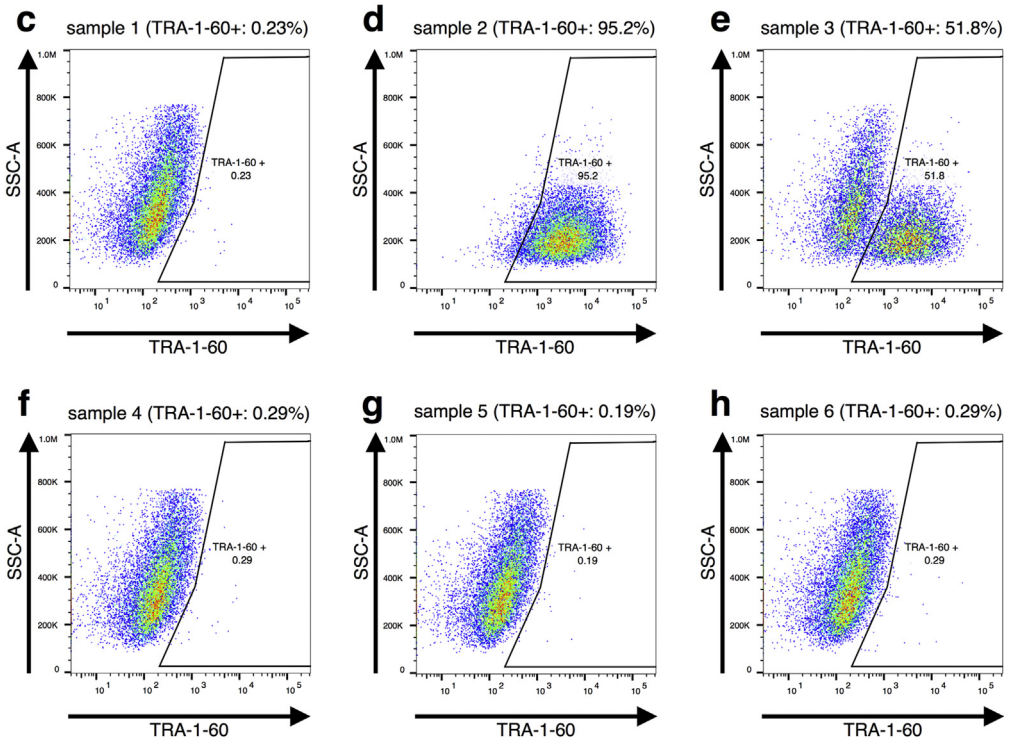


Fig. 2. Magnetic cell separation using rBC2LCN eliminated hiPSCs from an hiPSC/MMC-HDF mixture. (a) Experimental design to remove hiPSCs using magnetic beads. HDF and hiPSCs were mixed in a ratio of 1 to 1. The cells incubated with Dynabeads M−280 Streptavidin (M-280-SA) and either biotinylated-rBC2LCN or biotinylated-BSA, were separated by a magnet, and then analysed by flow cytometry. (b) Details of sample preparation (c–h) Flow cytometry of cells selected negatively by magnetic beads. (c) HDF alone showed 0.23% of TRA-1-60-positive cells (sample 1). (d) hiPSC alone showed 95.2% TRA-1-60-positive cells (sample 2). (e) TRA-1-60-positive cells (51.8%) were detected in the prepared cells using 100 μg/ml of biotinylated-BSA (sample 5; control). Selection using (f) 1 μg/ml (sample 4), (g) 10 μg/ml (sample 5) or (h) 100 μg/ml (sample 6) of biotinylated-rBC2LCN reduced the ratio of TRA-1-60-positive cells to levels approximately equal to that of HDF alone (0.19–0.29% vs 0.23%, respectively).

3.4. Teratomas were not produced by transplantation of negative-sorted cells by rBC2LCN-magnetic beads

The standard method to define pluripotent stem cells capable of generating tumoural structures containing tissues representing the three germ layers is to perform a teratoma formation assay [40–44]. To ensure effective removal of teratoma-forming cells, *in vivo*, we performed a teratoma formation assay in immunodeficient mice. We transplanted either cells magnetically sorted using rBC2LCN-magnetic beads or unsorted hiPSC/MMC-HDF mixtures into the testes of NOD/ShiJic-*scid*Jcl mice and left them to engraft for 3 months (Fig. 4a). If any residual hiPSCs remained after sorting, these cells would form teratomas. In the animals transplanted with sorted cells or Matrigel alone, the rate of teratoma formation was

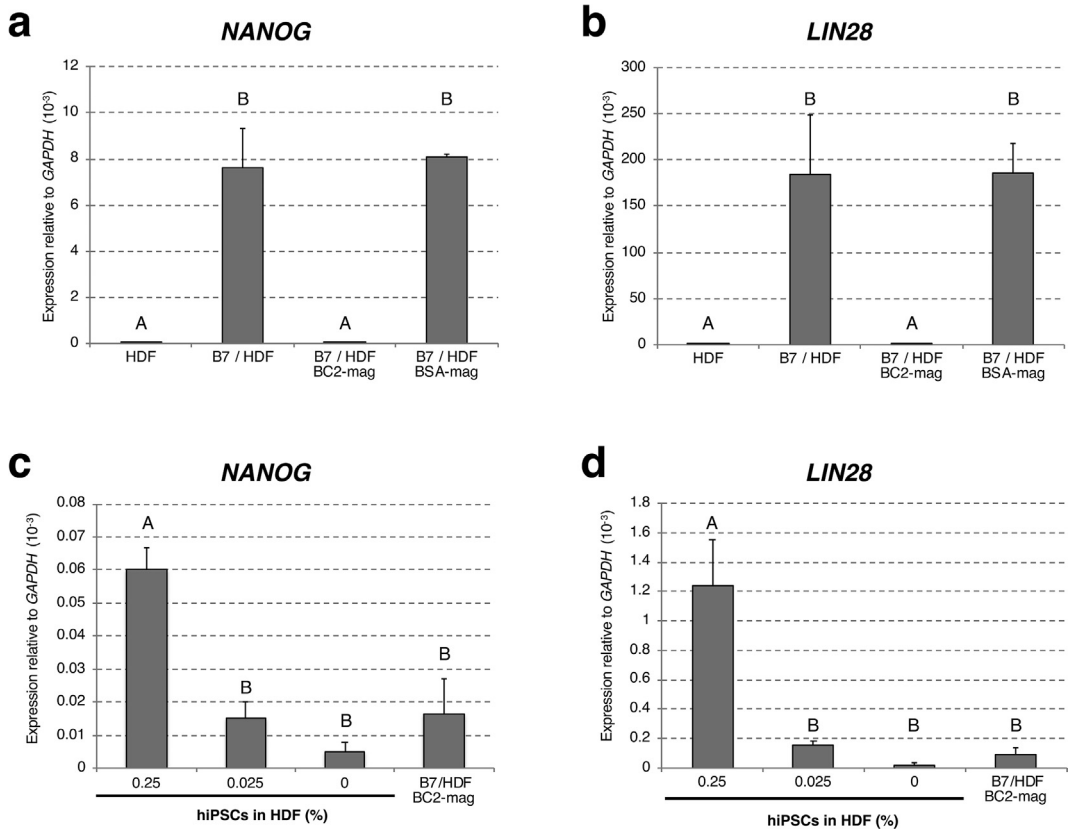


Fig. 3. Detection of hiPSCs by droplet digital RT-PCR. Droplet digital PCR analysis to estimate residual hiPSC frequency after magnetic cell separation by rBC2LCN was performed by evaluating the expression of pluripotent stem cell marker genes, *NANOG* (a, c) and *LIN28* (b, d). After mixing 1×10^6 of iPSCs and HDF at a ratio of 1: 1 respectively, negative-sorted cells by rBC2LCN-magnetic beads were analysed (a–d) Absolute counts were normalised to *GAPDH* (per 1000 copies of *GAPDH*). Results are presented as mean ± standard deviation of independent triplicate experiments (n = 3). Data were analysed by one-way ANOVA followed by Tukey's HSD test (a, b) The relative copy numbers of *NANOG* (a) and *LIN28* (b) mRNAs in 50 ng of total RNA derived from magnetically sorted or unsorted cell mixtures. Significant differences are represented by different letters (Fig. 3a: $p < 0.001$; Fig. 3b: $p < 0.005$) (c, d) Relative copy number comparison between *NANOG* (c) and *LIN28* (d) mRNAs in 50 ng of total RNA derived from magnetically sorted cell mixtures, and comparison samples in which HDF-derived total RNA was spiked with hiPSC-derived total RNA at a ratio of 0, 0.025%, or 0.25% by weight. There are significant differences between different letters ($p < 0.001$). B7: human iPS cell line 201B7. BC2-mag: rBC2LCN-magnetic beads. BSA-mag: BSA-magnetic beads.

0% (n = 8 or four mice, n = 16 or eight testes for the injection of unsorted cells or Matrigel alone, respectively; Fig. 4b and c, and see Supplementary Table S4 online). In contrast, most testes transplanted with unsorted cell mixtures developed teratomas; 14 out of 16 transplanted testes formed large teratomas (≥1.7 cm in maximal diameter) (n = 8 mice; n = 16 testes) (Fig. 4b and c, and see Supplementary Table S4 online). Statistical analysis of maximal diameter of testes revealed significant size differences between unsorted and sorted cell transplantation ($p < 0.0001$, Fig. 4b and see Supplementary Table S5 online). Further microscopic observation by Haematoxylin and Eosin (H&E) staining showed these teratomas contained tissues derived from the three germ layers: ectoderm (immature neuroepithelium), mesoderm (cartilage), and endoderm (glandular epithelium) (Fig. 4c). In agreement with the results from flow cytometry (Fig. 2) and ddPCR (Fig. 3), magnetic-cell separation using rBC2LCN effectively removed hiPSCs and could exclude potential teratoma formation in NOD/ShiJic-*scid*Jcl mice as no teratomas were observed in any of the tested animals (Fig. 4b and c).

3.5. Cell sorting efficiency using rBC2LCN-magnetic beads

To evaluate an elimination efficiency of rBC2LCN magnetic beads, we predicted number of residual iPSCs in cell mixture used in teratoma formation assay from the ddPCR data. By calculating based on *NANOG* and *LIN28* expression, the mean ± 2SD value of residual iPSCs in the transplanted cells in the teratoma assay was about 213 ± 615 and 65 ± 128 cells, respectively (Supplementary Fig. S1). These data indicated that approximately 99.8–100% of iPSCs were eliminated by rBC2LCN-magnetic beads. When using a mixture of differentiated cells and undifferentiated hPSCs in a ratio of 1:1, the separation efficiency of hPSCs by magnetic beads with SSEA4 or 57-C11 antibodies was less than 80% [30,45]. The separation efficiency was up to 99.5% using UEA-1 lectin [31]. Although the experimental conditions were not the same, simple comparisons should be avoided, but this technology showed superior performance compared to existing magnetic bead technology using antibodies and lectins. Our results indicate that even with this technique, trace amounts of hPSCs can remain in the cell products. When used as a quality control technology for cell therapy products, the removal efficiency of this technology is not sufficient for products with a high number of transplanted cells. Previous study reported that an antibody against SSEA-5 glycan expressed on hPSCs is a useful tool to removal of teratoma-forming cells. However, an anti-SSEA-5 antibody alone was insufficient to completely remove teratoma potential and complete removal was achieved only after combining SSEA-5 with two additional pluripotent surface markers (SSEA-5/CD9/CD90 or SSEA-5/CD50/CD200) [29]. Cell sorting by combination of rBC2LCN with other pluripotent surface markers may also improve separation efficiency. Proper combinations of various techniques, including rBC2LCN, to remove residual hPSCs in the manufacturing process are important to ensure the safety of cell therapy products.

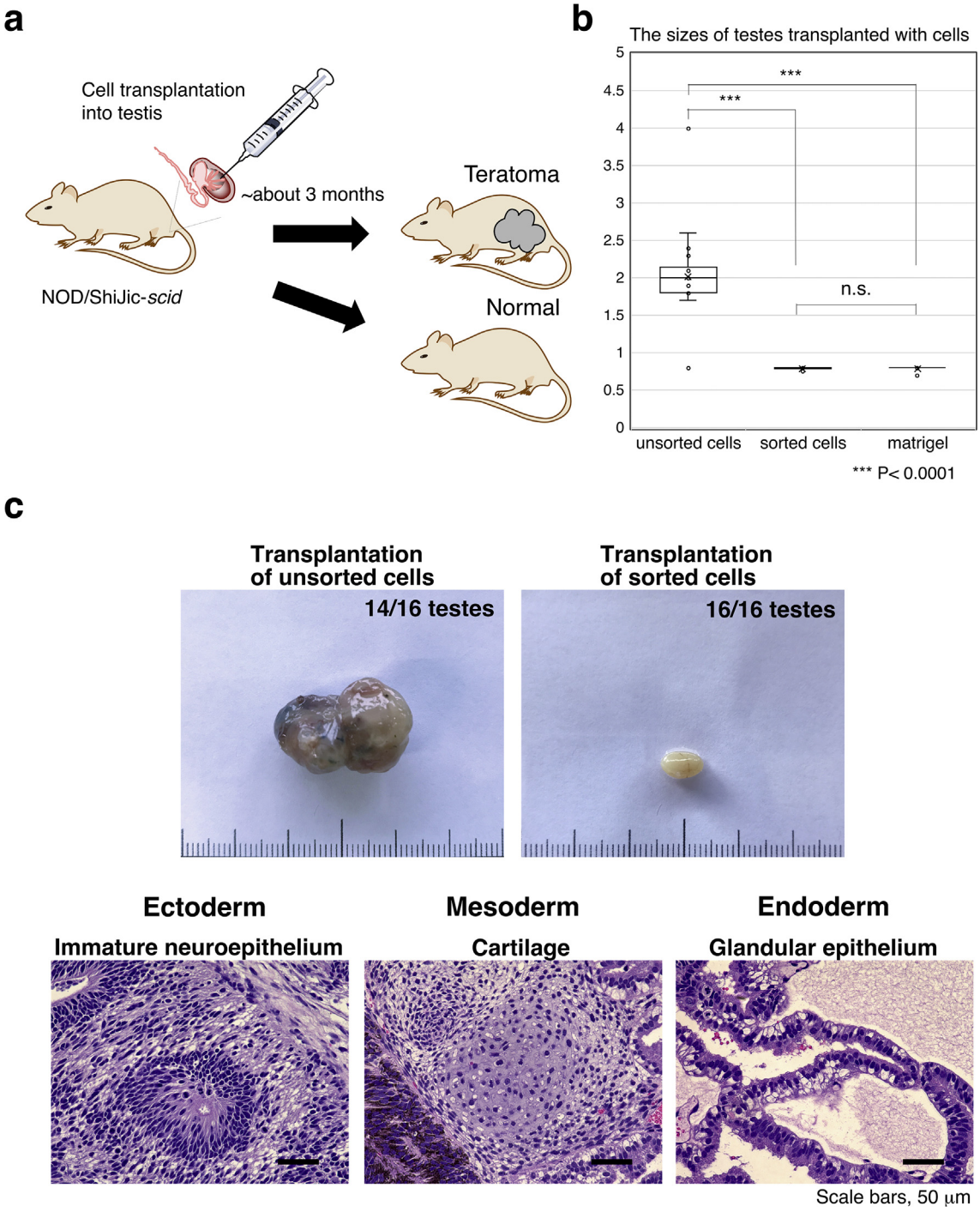


Fig. 4. Teratoma formation assay. (a) Schematic illustration summarising the experimental design. Magnetically sorted cells were resuspended in Matrigel and transplanted by injection into a testis (20 μL volume with $\leq 1.0 \times 10^6$ cells). (b) Box plot analysis of the size of testes in mice transplanted with Matrigel alone (negative control; n = 8), unsorted (positive control; n = 16), or sorted (n = 16) cells. The number of days from transplantation to sampling of testes varied depending on teratoma growth. The sizes of testes transplanted with unsorted cell mixture were significantly larger than those transplanted with sorted cells or Matrigel alone. Data were analysed by one-way ANOVA followed by Tukey HSD test. ***: $p < 0.0001$. (c) The appearance of the testes taken from NOD/ShiJic-*scid*Jcl mice transplanted with unsorted (upper left) or sorted (upper right) cells. Representative H&E-stained sections of testes with teratomas transplanted with unsorted cell mixtures of iPSCs and MMC-HDF, showing the generation of all three germ layers. Teratoma sections contained immature neuroepithelium (ectoderm), cartilage (mesoderm), and glandular epithelium (endoderm). Scale bar: 50 μm.

4. Conclusions

We optimized and validated a method to remove hPSCs from a mixture with human fibroblasts using rBC2LCN-conjugated magnetic beads (Figs. 1–3). Our results suggested that magnetic-cell separation using rBC2LCN effectively removed teratoma-forming pluripotent stem cells from the cell mixture (Fig. 4). We propose that rBC2LCN is a suitable lectin marker for magnetic beads-based cell separation and that this application will contribute to the development of a practical and feasible method for removal of residual undifferentiated cells. As previously reported, the current magnetic activated cell sorting (MACS) technology is insufficient to

meet the purification needs of cell therapy [46]. The relevance and validation of this technique for cellular transplantation will require more detailed experiments. In this study, we eliminated pluripotent stem cells from a fibroblast cell line acutely mixed together. For example, it will be more convincing to demonstrate that pluripotent cells can be separated from pluripotent stem cell-derived cell products or other cellular lineages that have been cultured together in the same conditions over time. It is crucial to properly combine the respective removal techniques according to the application scope. Further experiments are demanded to develop an optimised protocol for removal of residual undifferentiated cells.

Data availability statement

All data analysed during this study are included in this published article and its Supplementary Information files.

Ethics statement

This study was carried out in strict accordance with the National Institute of Advanced Industrial Science and Technology (AIST) guidelines for life science experiments (accreditation numbers hi2016–099 and A2018-290). Human embryonic stem cell experiment was approved by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT). Studies with mice were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments stipulated by the Science Council of Japan.

Author contributions statement

Y.H., Y.O., and Y.I. designed the research. Y.H., Y.O., Y.I., S.M., Y.N., Y.A., K.H., and M.S. carried out the experiments, Y.H., Y.O., and S.M. analysed the data. Y.H. and Y.O. wrote the paper. H.T. and J.H. supervised the research.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.03.017.

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