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rBC2LCN, a new probe for live cell imaging of human pluripotent stem cells

Yasuko Onuma [a], Hiroaki Tateno [b], Jun Hirabayashi [b], Yuzuru Ito [a,*], Makoto Asashima [a]

[a] Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan
[b] Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 2, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan

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ABSTRACT

Cell surface biomarkers have been applied to discriminate pluripotent human embryonic stem cells and induced pluripotent stem cells from differentiated cells. Here, we demonstrate that a recombinant lectin probe, rBC2LCN, a new tool for fluorescence-based imaging and flow cytometry analysis of pluripotent stem cells, is an alternative to conventional pluripotent maker antibodies. Live or fixed colonies of both human embryonic stem cells and induced pluripotent stem cells were visualized in culture medium containing fluorescent dye-labeled rBC2LCN. Fluorescent dye-labeled rBC2LCN was also successfully used to separate live pluripotent stem cells from a mixed cell population by flow cytometry.

1. Introduction

Both positive and negative selection of pluripotent stem cells are key steps for practical applications, such as reducing risk of teratoma formation in transplantation therapy, and maintaining a homogeneous pluripotent cell population in continuous culture. Recent studies have focused on cell surface markers, which are specifically expressed on human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), for visualizing and sorting stem cells by flow cytometry or magnetic cell separation. The conventionally used markers are cell surface glycans specific for pluripotent stem cells, and antibodies for those glycans are well established, such as stage-specific embryonic antigens-3, -4, and-5 (SSEA-3, SSEA-4, SSEA-5), and tumor rejection antigens-1–60 and -1–81 (TRA-1–60, TRA-1–81) [1–5]. Several lectin proteins, which bind to unique glycans, are also useful probes for identifying pluripotent stem cells. UEA-I, which is a lectin isolated through array-based glycomic analysis, has high efficiency to detect human pluripotent stem cells [6]. We also have identified that rBC2LCN, which is a recombinant peptide corresponding to the N-terminal domain of BC2L-C protein, specifically detects undifferentiated iPSCs and ESCs in a lectin microarray system [7,8]. rBC2LCN binds specifically to Fucα1–2Galβ1–3GlcNAc (GalNAc)-containing glycans, such as H type1 (Fucα1–2Galβ1–3GlcNAc), H type3 (Fucα1–2Galβ1–3GalNAc), Lewis b (Fucα1–2Galβ1–3(Fucα1–4)GlcNAc) and Globo H (Fucα1–2Galβ1–3GalNAcβ1–3Galα1–4Galβ1–4Glc) (Supplementary Fig. S1) [7,8]. Globo H is expressed on the surface of undifferentiated hESCs [5], and anti-SSEA-5 antibody is reported to bind to H type1 antigen [4]. In addition, we have demonstrated the presence of α-1–2 linked fucose in both N- and O-linked glycans of iPSCs [9].

In this study, we demonstrate the potential of rBC2LCN as a probe for pluripotent ESCs and iPSCs in both imaging and flow cytometry. Fluorescent dye-labeled rBC2LCN stained human ESCs and hiPSCs and exhibited higher sensitivity upon iPSC differentiation, compared to the established cell surface marker antibodies. Fluorescent dye-labeled rBC2LCN was also capable of visualizing and separating live pluripotent hESCs and hiPSCs by flow cytometry. rBC2LCN is a useful tool for evaluating human ESCs and hiPSCs and holds promise for improving cell sorting efficiency in medical and industrial applications of pluripotent stem cells.

2. Materials and methods

2.1. Cell culture

KhES-1 and KhES-3 cells were maintained as previously described [10]. Human ES cell line, H1, was maintained according to WiCell Feeder Independent Pluripotent Stem Cell Protocols provided by the WiCell Research Institute (http://www.wicell.org). Human iPS cell lines, 201B7 [11] and 253G1 [12], were maintained in DMEM-F12 medium (Invitrogen) supplemented with 20% of KSR (Invitrogen), 0.1 mM of 2-mercaptoethanol (Sigma–Aldrich), MEM non-essential amino acids (Invitrogen), and 10 ng/ml of recombinant human basic FGF (Wako) on mitomycin C-treated mouse embryo fibroblast feeder cells. The 201B7 cell line was cultured in mTeSR1 (STEMCELL Technologies) on BD Matrigel hESC-qualified

* Corresponding author. Fax: +81 29 861 2987.
E-mail address: yuzu-itou@aist.go.jp (Y. Ito).

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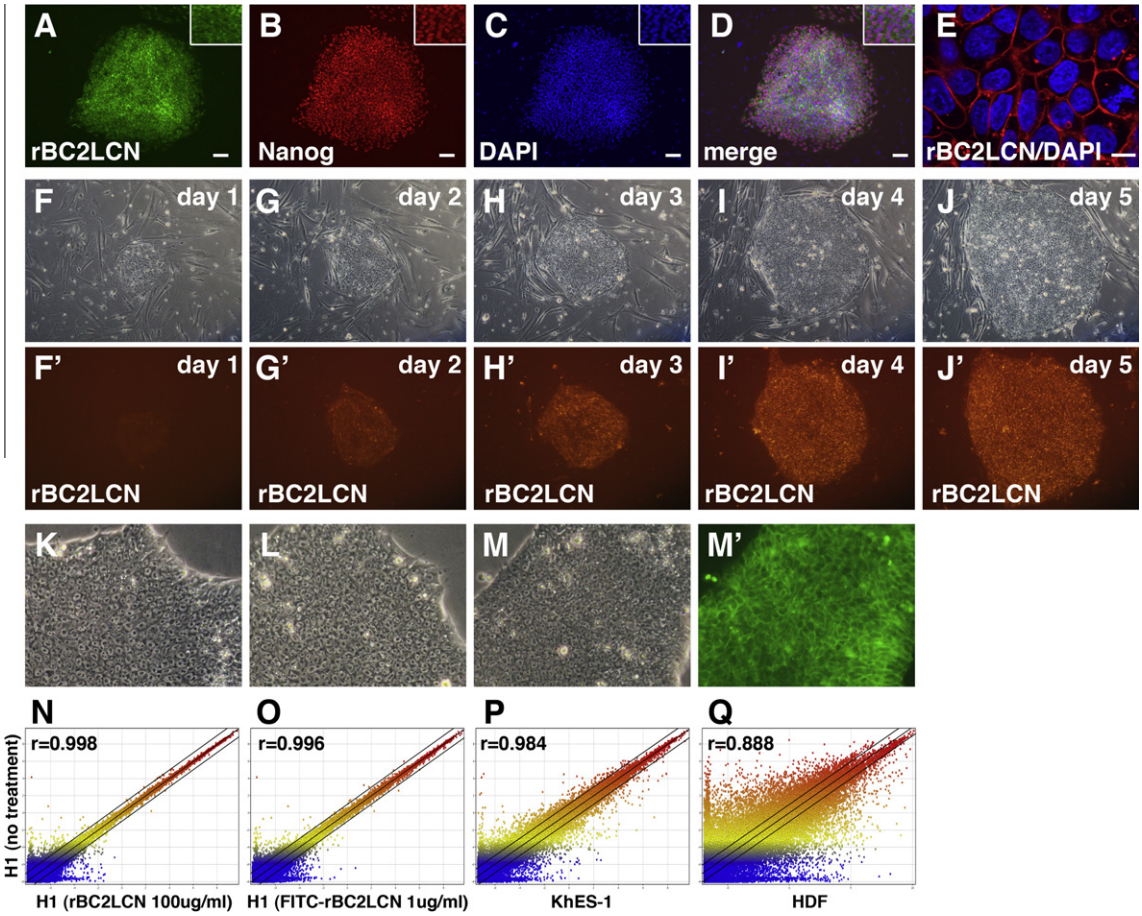

Fig. 1. Fluorescent dye-labeled rBC2LCN directly stains hiPSC colonies. (A–D) A 253G1 iPSC colony stained with FITC-conjugated rBC2LCN (A), anti-Nanog antibody (B) and DAPI (C), and the overlay of A–C images (D). (E) Confocal image of 201B7 cells stained with Cy3-conjugated rBC2LCN and DAPI. rBC2LCN signal can be observed on the cell membrane. (F–J, F′–J′) Chronological live cell imaging of 201B7 iPSC using Cy3-conjugated rBC2LCN in 0.1 μg/mL of final concentration. (K-Q) H1 ESCs were treated with 100 μg/mL rBC2LCN or 1 μg/mL of FITC-rBC2LCN for 3 days. (K–M) Cell morphologies of H1 ESCs without treatment (K), 100 μg/mL of rBC2LCN treatment (L), and 1 μg/mL of FITC-rBC2LCN treatment (M and M′). Treatment with rBC2LCN caused no significant morphological changes. (N–Q) Scatter plot showing $\log_2$ transformed average expression values from gene expression profiles between untreated H1 cells and H1 cells treated with 100 μg/mL of rBC2LCN (N) or 1 μg/mL of FITC-rBC2LCN (O), KhES-1 ESCs (P), or HDF (Q), using arrayed 60 k probe sets. Pearson correlation coefficients (r), using all probes, between untreated and treated cells are indicated. One typical data set of two independent, duplicate examinations is shown. The full array data set was deposited in the GEO databank (GSE42976). Black lines indicate 2-fold up-regulation and down-regulation. Scale bar: 10 μm in (E). 100 μm in others.

matrix (BD) for confocal laser microscopy observation. Normal human adult dermal fibroblast (HDF) cells were cultured in fibroblast basal medium (ATCC) supplemented with fibroblast growth kit-low serum (ATCC).

2.2. Lectin labeling and staining

rBC2LCN protein was prepared according to a previous paper [8]. For fluorescence labeling, rBC2LCN was labeled withCy3-*N*-hydroxysuccinimide (NHS) ester (GE Healthcare), fluorescein-4-isothiocyanate (FITC, Dojindo), and HiLyte Fluor 647 Labeling Kit (Dojindo), respectively. Fixed cell staining was performed as described previously [13]. Briefly, hESCs and hiPSCs were fixed with 4% paraformaldehyde for 10–60 min at 4 °C or room temperature. After rinsing with PBS, cells were incubated with 10 μg/mL of Cy3- or FITC-conjugated rBC2LCN diluted in 1% BSA containing PBS for 1 h at room temperature. The cells were counterstained with DAPI (Dojindo), and then images were collected with a BIOREVO BZ-9000 fluorescence microscope (Keyence) and a FluoView FV1000 confocal laser scanning microscope (Olympus).

Live cell imaging was performed in the regular hESC or iPSC culture medium (with phenol red) by addition of Cy3- or FITC-conjugated rBC2LCN in 0.1–1 μg/mL of final concentration. After 2 h incubation under typical culture conditions, images were collected with an Axiovert 40 CFL inverted microscope (Carl Zeiss Microscopy), without washing. For chronological observations, the same concentration of fluorescent dye-labeled rBC2LCN was added with daily medium changes.

2.3. Immunocytochemistry

Immunocytochemical analysis was performed as described previously [14–16]. Primary antibodies used in this study were the following: anti-SSEA4 (MC-813–70, 1:300 dilution; Millipore), anti-TRA-1–60 (1:300 dilution; Millipore), anti-TRA-1–81 (1:300 dilution; Millipore), anti-Oct-3/4 (1:300 dilution; Santa Cruz Biotechnology), and anti-human-Nanog (1:800 dilution; Cell Signaling Technology). Cells were incubated with a primary antibody diluted in 1% BSA and 5% serum containing PBS at 4 °C overnight. Secondary staining was performed with an appropriate secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 594 (1:300; Invitrogen) for 1 h at room temperature. Cells were counterstained with DAPI (Dojindo). Images were collected with a BIOREVO BZ-9000 fluorescence microscope (Keyence).

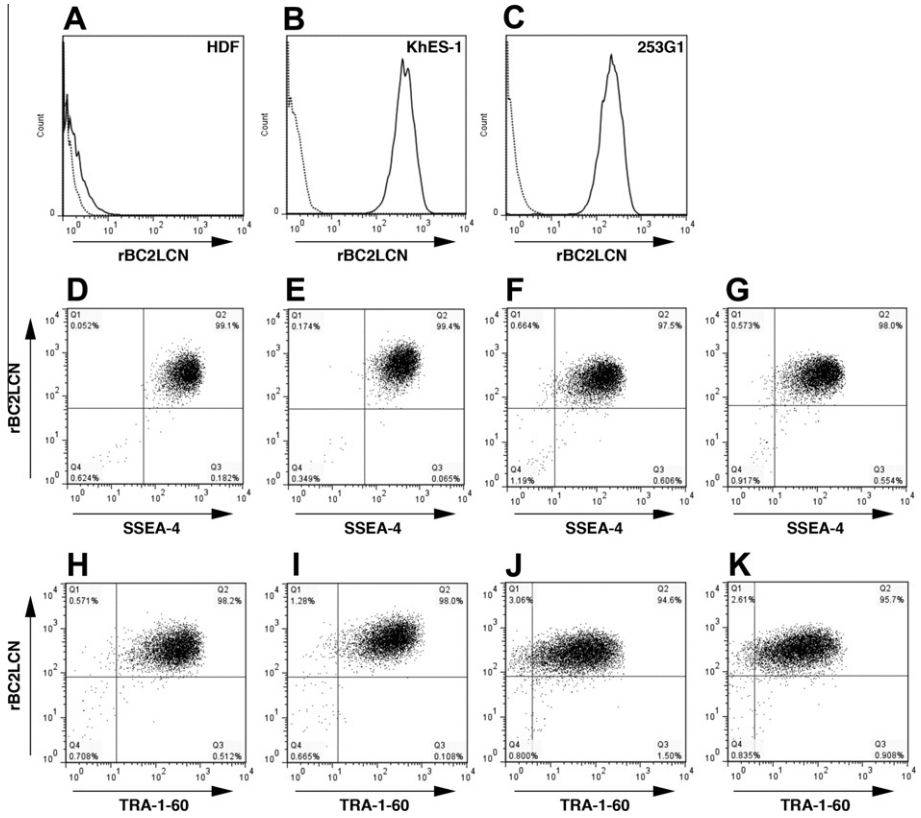

Fig. 2. rBC2LCN is a specific probe for hESCs and hiPSCs in FACS. (A–C) Histogram of representative FACS plots. Non-pluripotent HDF cells (A), KhES-1 hESCs (B), and 253G1 hiPSCs (C) were incubated with HiLyte Fluor 647-conjugated rBC2LCN (solid line) or buffer (dash line). rBC2LCN-binding shifts were observed in both hESCs (B) and hiPSCs (C), but not in HDF cells (A). (D–K) Multi-color flow cytometric analysis of KhES-1 (D, E, H, and I) and 253G1 (F, G, J, and K) with HiLyte Fluor 647-conjugated rBC2LCN and with pluripotent marker antibodies, anti-SSEA-4 (D-G) or anti-TRA-1–60 (H–K). (D, F, H, and J) Cells were first treated with HiLyte Fluor 647-labelled rBC2LCN, and then treated with either anti-SSEA-4 or anti-TRA-1–60 antibody; subsequently, cells were treated with an appropriate secondary antibody. (E, G, I, K) Cells were first treated with either anti-SSEA-4 or anti-TRA-1–60 antibody, and were then further treated with an appropriate secondary antibody. Next, cells were treated with HiLyte Fluor 647-labelled rBC2LCN. Multi-color FACS plots display substantial overlaps of cell populations bound to rBC2LCN, and anti-SSEA-4 and anti-TRA-1–60 antibodies. Values in each quadrant indicate the percentage of cells.

2.4. Fluorescence-activated cell sorting (FACS)

HDF cells were pre-marked with a CellTrace Violet Cell Proliferation Kit according to the manufacturer's protocol (Invitrogen). KhES-1 and 253G1 colonies were treated in a solution that contained 1 mg/mL collagenase IV (Invitrogen), 1 mM $CaCl_2$, 20% KNOCKOUT Serum Replacement (Invitrogen), and 0.25% trypsin (Invitrogen), in a 37 °C incubator. When the edges of the colonies began to dissociate from the bottom of the dish, the solution was removed and the cells rinsed with medium. Colonies were collected and rinsed with PBS to remove feeder cells, and were then dissociated with accutase (Millipore). The dissociated ESCs or iPSCs were resuspended at approximately 1×10^6 cells/mL in MACS buffer (0.5% bovine serum albumin and 2 mM EDTA in PBS), and incubated with primary antibodies or 1 μg/mL of HiLyte Fluor 647-conjugated rBC2LCN for 1 h at 4 °C. Primary antibodies used were the following: anti-SSEA4 (1:300 dilution; Millipore) and anti-TRA-1–60 (1:300 dilution; Millipore). For secondary antibody treatment, cells were rinsed with MACS buffer and then incubated with Alexa Fluor 488 goat anti-mouse IgM or Alexa Fluor 488 anti-mouse IgG (Molecular Probes), depending on the primary antibody. After further rinsing, cells were stained with propidium iodide (PI) and 20,000 cells were counted using a FACS Aria (BD Biosciences). The data were analyzed with FlowJo software (Tree Star, Inc.).

2.5. Differentiation assays

Retinoic acid treatment was carried out as previously described [17,18]. 253G1 cells were treated with the regular maintenance medium, including all-trans retinoic acid at a final concentration of 10^{-5} M for 8 days. Both retinoic acid-treated and untreated cells were fixed at day 5 and 8, respectively, and prepared for lectin staining and immunocytochemistry.

2.6. DNA microarray and analyses

Total RNA was extracted from frozen cell samples using ISOGEN (NIPPON GENE) according to the manufacturer's instructions. Samples were analyzed using an Agilent SurePrint G3 Human GE 8×60 K Microarray Kit (G4851A) and a Low Input Quick Amp Labeling Kit, 1 color (Agilent). Arrays were scanned using a G2505C Microarray Scanner System (Agilent). Raw microarray data were submitted to the Gene Expression Omnibus (GEO) microarray data archive (http://www.ncbi.nlm.nih.gov/geo/) at NCBI (accession number GSE42976).

Data were analyzed using Gene-Spring GX12.0 software (Agilent) after applying two normalization procedures: (1) signal intensities of <1 were set to 1, and (2) each chip was normalized to the 75th percentile of all measurements from that chip. Baseline transformation of these data was not performed.

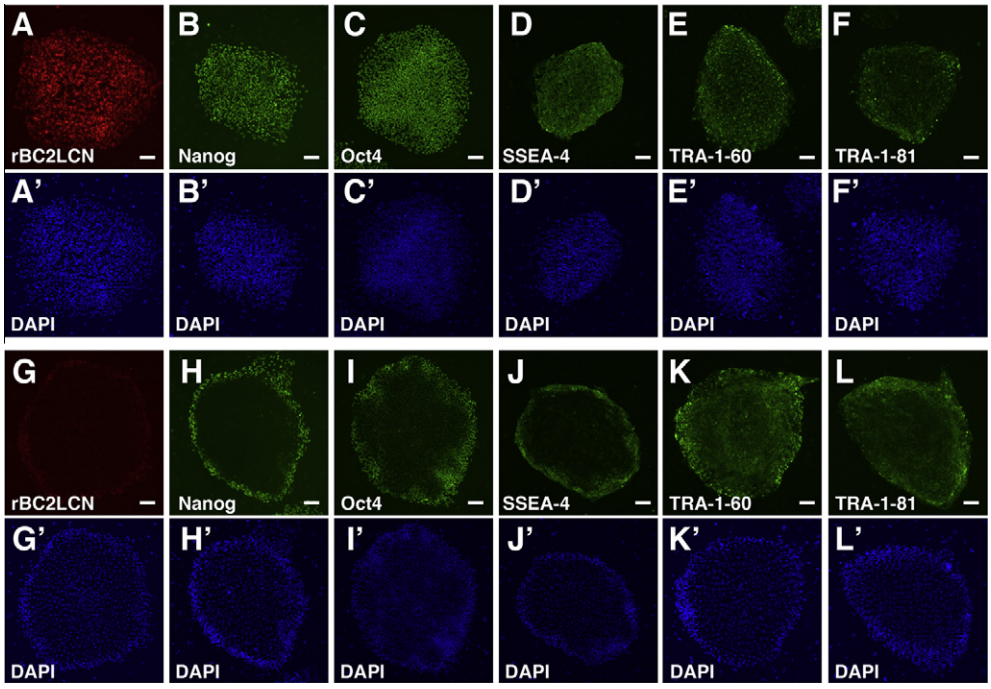

Fig. 3. rBC2LCN staining on differentiating iPS cells. 253G1 iPSCs were treated with retinoic acid for 5 days. (A–F) Control pluripotent iPSC colonies. Signals of rBC2LCN and pluripotent marker antibodies were observed. (G–L) Retinoic acid-treated cells. rBC2LCN signal was significantly reduced by 5 days' treatment (G), compared to treatment with established pluripotent marker antibodies, anti-Nanog (H), anti-Oct4 (I), anti-SSEA-4 (J), anti-TRA-1–60 (K), and anti-TRA-1–81(L). Scale bar: 100 μm.

3. Results and discussion

3.1. rBC2LCN is a specific probe for human pluripotent stem cells

To discover new markers for hESCs and hiPSCs, we performed comprehensive glycan analysis using a high-density lectin microarray and selected a candidate lectin, rBC2LCN [8]. To establish whether rBC2LCN could be used as a probe for pluripotent stem cells, we first performed lectin-staining using fluorescent dye-labeled rBC2LCN (Fig. 1). Strong fluorescence signals were observed for hiPSC colonies (253G1), but no signal was detectable for mouse embryonic fibroblasts (MEF), which were co-cultured as feeder cells (Fig. 1A–D). rBC2LCN staining was observed on the cell membrane of hiPSCs by confocal microscopic observation (Fig. 1E). This was in good agreement with previous reports that rBC2LCN-binding glycans are present on the surface of undifferentiated stem cells [8]. We also examined whether fluorescent dye-labeled rBC2LCN stains live pluripotent stem cell. Chronological observation of 201B7 iPSC cultured in the regular maintenance medium with Cy3-conjugated rBC2LCN, at a final concentration of 0.1 μg/mL, yielded clear live cell images under a conventional florescent microscope (Fig. 1F–J). A similar staining pattern was observed in 3 different hESC lines (KhES-1, KhES-3, and H1; Fig. 1M, Supplementary Fig. S2). These results indicated that fluorescent dye-labeled rBC2LCN stains both fixed and live hESC/hiPSCs on the cell membrane.

We observed no significant changes in colony growth during chronological live cell imaging (Fig. 1F–J). Furthermore, we evaluated the influence of rBC2LCN treatment on pluripotent stem cells. H1 ESCs were cultured in regular maintenance medium with 100 μg/mL of rBC2LCN or 1 μg/mL of FITC-conjugated rBC2LCN for 3 days. The sizes and morphologies of the colonies of the untreated and rBC2LCN or FITC-conjugated rBC2LCN treated cells were not significantly different (Fig. 1K–M).

We further analyzed global gene expression under each condition by DNA microarray (Fig. 1N–Q). The correlation coefficients of gene expression using all probes (about 60 K) between untreated cells and cells treated with 100 μg/mL of rBC2LCN or 1 μg/mL of FITC-conjugated rBC2LCN,were high (0.998 and 0.996, respectively; Fig. 1N–O). Scatter plots also showed very similar gene expression profiles between untreated cells and cells treated with rBC2LCN or FITC-conjugated rBC2LCN. The differences were much smaller than that between H1 ESCs and KhES-1 hESCs ($r = 0.984$; Fig. 1P). These results indicated that hESCs cultured in the presence of rBC2LCN largely maintained normal gene expression profile.

Further analysis of the specificity of rBC2LCN for undifferentiated pluripotent stem cells was carried out by flow cytometry (Fig. 2). rBC2LCN bound selectively to hESCs and hiPSCs, and did not bind to human dermal fibroblast (HDF) cells (Fig. 2A–C). This result indicated that pluripotent cells present glycan markers recognized by rBC2LCN. In multi-color flow cytometric analysis, rBC2LCN-binding cells were also positive for pluripotency markers, TRA-1–60 and SSEA-4 (Fig. 2D–K). When cells were treated with these antibodies either before or after treatment with rBC2LCN, the treatment order did not affect association of either antibody and rBC2LCN (Fig. 2D and E). Most (99.1%) KhES-1 cells were double-positivefor SSEA-4 and rBC2LCN when rBC2LCN treatment occurred first, and 99.4% of cells were double-positive when rBC2LCN followed after antibody labeling (Fig. 2D and E). Anti-TRA-1–60 antibody also bound toKhES-1 cells that were positive for rBC2LCN; 98.2% of cells were double-positive when rBC2LCN-treatment occurred first, and 98.0% when rBC2LCN-treatment occurred last (Fig. 2H and I). Similar results were obtained with the hiPSC line, 253G1 (Fig. 2F, G, J, and K). These results indicated that the sensitivity and specificity of rBC2LCN was comparable to those of established pluripotent marker antibodies.

3.2. rBC2LCN is highly sensitive to cell differentiation

To examine rBC2LCN binding to a range of differentiated cells, we performed rBC2LCN staining on cells differentiated by treat-

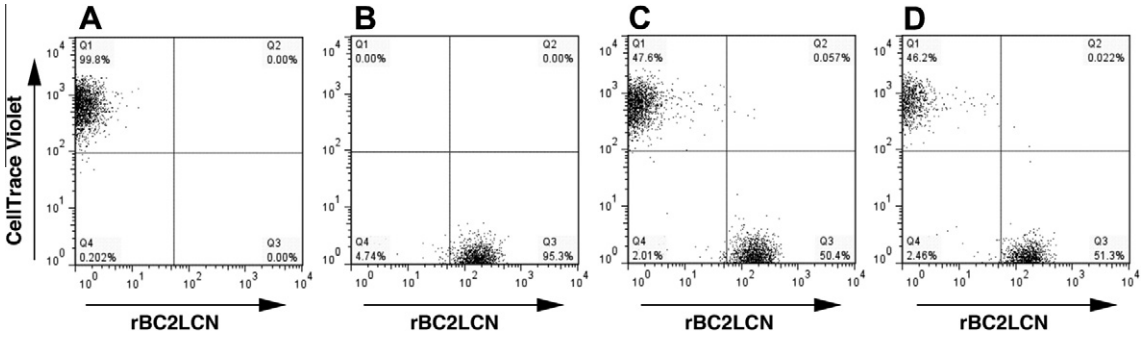

Fig. 4. rBC2LCN facilitates separation of hiPSCs from mixed cell population. Multi-color flow cytometric analysis of 253G1 and HDF. (A) Non-pluripotent HDF cells, which were fluorescently labeled with CellTrace Violet, were incubated with HiLyte Fluor 647-labelled rBC2LCN. (B) 253G1 iPSCs were incubated with HiLyte Fluor 647-labelled rBC2LCN. (C) CellTrace Violet-labeled HDF and 253G1 cells were mixed in a ratio of 1:1, and then the mixed cells were incubated with HiLyte Fluor 647-conjugated rBC2LCN. (D) 253G1 cells were first reacted with HiLyte Fluor 647-conjugated rBC2LCN. 253G1 cells were mixed with CellTrace Violet-labeled HDF cells in a ratio of 1:1, and prepared for Multi-color FACS. Values in each quadrant indicate the percentage of cells. The rates of each quadrant population were similar between (C) and (D).

ment with retinoic acid (Fig. 3). rBC2LCN signals on 253G1 iPSCs were significantly reduced after 5 days of treatment with retinoic acid (Fig. 3G), compared with that of untreated hiPSCs (Fig. 3A). We found that Nanog, Oct4, and SSEA-4 expression was also decreased, particularly in the center of colonies, whereas TRA-1–60 and TRA-1–81 signals remained high throughout colonies after 5 days of treatment with retinoic acid (Fig. 3H–L). TRA-1–60 and TRA-1–81 signals were weak in 253G1 coloniesafter 8 days of treatment with retinoic acid (Supplementary Fig. S3). The simultaneous reduction of rBC2LCN and Nanog expression during differentiation, exhibits the utility of rBC2LCN for monitoring the properties of pluripotent stem cells in culture.

Next, we attempted to use rBC2LCN for separation of hiPSCs from a mixed cell population, also containing non-pluripotent cells, by flow cytometry (Fig. 4). HDFcells were pre-labeled with CellTrace Violet and were mixed with 253G1 cells at a ratio of 1:1. The mixed cell population was incubated with HiLyte Fluor 647-conjugated rBC2LCN and was analyzed by multi-color FACS (Fig. 4C). rBC2LCN$^+$/CellTrace Violet$^-$-cells accounted for 50.4%, and rBC2LCN$^-$/CellTrace Violet$^+$-cells for 47.6%of the total number of cells (Fig. 4C). rBC2LCN$^+$/CellTrace Violet$^+$-cells accounted only for 0.057%of the total cell number. Thus the ratio was approximately 1:1, which corresponded to the proportion of pre-labeled HDF and rBC2LCN-prebound 253G1 in the initial 1:1 mixed cell population (Fig. 4D). These results suggest that rBC2LCN is a useful probe for sorting live pluripotent stem cells from heterogeneous cell cultures.

We identified rBC2LCN as a pluripotency-specific lectin probe, which binds to the cell surface of undifferentiated human ESCs and iPSCs. The binding capacity for both fixed and live cells favored the applicationof rBC2LCN in fluorescence-based imaging and flow cytometry. In addition, rBC2LCN has a higher sensitivity for differentiated cells than do the established cell surface marker antibodies. Further studies will be required to address whether rBC2LCNis effective for cell sorting to eliminate teratoma formation after transplantation of hES or hiPS cell-derived differentiated cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.025.

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