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Podocalyxin-Targeting Comparative Glycan Profiling Reveals Difference between Human Embryonic Stem Cells and Embryonal Carcinoma Cells

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Abstract

Background: Human embryonic stem cells (hESCs) and human embryonal carcinoma cells (hECCs) have been extensively used for stem cell research. Although these cells are known to share many properties including high developmental capability and cell surface antigens, their origins are basically different: hECSs are derived from inner cell mass of blastocysts, while hECCs are from malignant tumors. Thus, the lack of a good method to differentiate these pluripotent cells remains a critical issue to diagnose tumorigenic potential of pluripotent stem cells for their medical applications. In this context, development of specific markers to distinguish hESCs from hECCs is also of clinical value.

Method: In this study, we focused our glycan analysis on a carbohydrate-rich glycoprotein, podocalyxin, known as a carrier of TRA-1-60 and TRA-1-81 antigens, which represent hESC glycan markers. The target glycoprotein semi-quantified by immunoblotting was enriched from the cell extracts by immunoprecipitation, and the glycosylation differences occurring between hESCs and hECCs were systematically analyzed by an advanced technology of lectin microarray, antibody-overlay lectin profiling (ALP). Profiles of human embryonic bodies (hEBs) differentiated from hESCs were also analyzed.

Results and Conclusion: A glycan profile of podocalyxin from hECCs was significantly different from that of hESCs. Lectin signals corresponding to α2-6 linked sialic acid were elevated in the hECCs, and glycosidase digestions further revealed significant difference in the non-reducing terminal and penultimate structures. These results demonstrate that the present procedure with focus on a particular glycoprotein could enhance relatively small but significant differences between closely related cells like hESCs and hECCs at the glycome level. The present finding will be helpful to develop a diagnostic method to distinguish undifferentiated stem cells from differentiated ones used for regenerative therapy.

Keywords: Human embryonic stem cell; Human embryonal carcinoma cell; Lectin microarray; Glycan marker; Podocalyxin

Abbreviations: ALP: Antibody-assisted Lectin Profiling; AP: Alkaline Phosphatase; Gal: Galactose; GlcNAc: *N*-acetylglucosamine; FDR: False Discovery Rate; Fuc: Fucose; iPSC: Induced Pluripotent Stem Cell; hEB: Human Embryoid Body; hECC: Human Embryonal Carcinoma Cell; hESC: Human Embryonic Stem Cell; hIgG: Human Serum Polyclonal IgG; High-Man: High-Mannose; LacNAc: *N*-acetyllactosamine; PBSTx: Phosphate Buffered Saline, containing 1% Triton X-100; pAb: Polyclonal Antibody; PCA: Principal Component Analysis; PVDF: Polyvinylidene Fluoride; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; Sia: Sialic Acid; SSEA-1: Stage-Specific Embryonic Antigen-1; SSEA-3: Stage-Specific Embryonic Antigen-3; SSEA-4: Stage-Specific Embryonic Antigen-4; TBST: Tris-Buffered Saline, containing 1% Tween 20.

Introduction

Human embryonic stem cells (hESCs), which have been established from the inner cell mass of the blastocysts cultured *in vitro*, show high developmental capability and are expected to play important roles in stem cell research. Human embryonal carcinoma cells (hECCs) also show high developmental capability, while they are originally from malignant pluripotent stem cell lines of human germ cell tumor origins [1,2]. Both hESCs and hECCs retain the pluripotency, and in fact hESCs show various properties closely similar to those of hECCs. However, it should be noted that hECCs are teratocarcinoma in the process of differentiation into certainly restricted cell lineages. Hence, effort to distinguish hESCs and hECCs will be helpful for diagnosis of pluripotent stem cells used for regenerative therapy, e.g., ESCs and induced pluripotent stem cells (iPSCs). For their evaluation, it is possible to expect that hESCs and hECCs have some difference representing potency of malignant tumors.

Podocalyxin is a member of the CD-34-related family of sialomucins [3-8]. Podocalyxin was originally cloned from the human kidney as a component of the podocyte cell glycocalyx, while it was also identified on vascular endothelium and hematopoietic cells [3,4,9-11]. Podocalyxin is expressed in hECCs as a heavily glycosylated transmembrane protein with an apparent molecular mass of 200 kDa on cell surface membrane [12,13]. Several functions of podocalyxin have been postulated: as an anti-adhesion molecule to provide structural support for the podocyte filtration slits, and as an adhesion molecule to mediate leukocyte extravasation [14-16]. However, the biological function of podocalyxin in human stem and malignant cells is unclear. A recent study has

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shown that podocalyxin interacts and forms a stable complex with the glucose-3-transporter (GLUT-3), and functions in part within stem and malignant cells for their maintenance and regulation with the cell surface expression. Therefore, it is hypothesized that such transporters have some functional roles in hESC differentiation and embryonic development [17,18]. Another study has demonstrated that a specific monoclonal antibody developed against podocalyxin-like protein-1 on hESCs is cytotoxic to hESCs, but not to differentiated cells [19,20]. On the other hand, as a marker of human pluripotent and multipotent stem cells, podocalyxin is widely used as well as other stem cell markers, such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, K4 and K21 [21-26]. In addition, podocalyxin is also a marker of many cancer types including prostate, breast, kidney and lung [12,27-31]. Various epitopes are known to reside on podocalyxin as carbohydrate antigens. Some of them have Sia residues as a non-reducing terminal component of glycan structures [13,32]. More recently, it was shown that well-known TRA-1-60 and TRA-1-81 antibodies recognize a specific type I LacNAc (Galß1-3GlcNAc) epitope, which is present in hESCs as a part of mucin-type *O*-glycan [33].

Lectins, a wide group of glycan-binding proteins, have long been used as useful tools to characterize cell surface glycans [34,35]. In the 21st century, various new technologies taking advantage of lectins have been developed in the advanced platform of microarray [36-40]. Distinct from conventional physicochemical methods, lectin microarray is innovative in that it enables multiplex glycan profiling in a direct (i.e., without liberation of glycans), rapid and sensitive manner. In the authors' laboratory, as a lectin-based glycan profiling method, a unique lectin microarray system based on an evanescent-field activated fluorescence detection principle was developed [41,42]. With this system, we and other groups successfully profiled distinct sets of glycomes of both somatic and pluripotent stem cells [43-47].

In this report, we challenge discrimination of closely related embryonic cells, i.e., hESC and hECC, NCR-G3, which was established from a testicular embyonal carcinoma, by differential glycan profiling targeting podocalyxin. The results demonstrate that the present procedure with focus on a particular glycoprotein could enhance difference between hESCs and hECCs at the glycome level. The present finding will be helpful to develop a diagnostic method to distinguish undifferentiated stem cells from differentiated ones used for regenerative therapy.

Materials and Methods

Chemicals

Anti-podocalyxin polyclonal antibody (pAb) was purchased from R&D Systems, Inc. (Minneapolis, MN) and the antibody was biotinylated by Biotin Labeling Kit-NH_2 (Dojindo Molecular Technologies, Inc., Tokyo, Japan). Streptavidin-immobilized magnetic beads, Dynabeads MyOne™ streptavidin T1 was from DYNAL Biotech ASA (Oslo, Norway). Alkaline phosphatase (AP)-conjugated streptavidin was from Prozyme Inc. (San Leandoro, CA) and Cy3-labeled streptavidin was from GE Healthcare (Buckinghamshire, UK). Cy3 was purchased from GE Healthcare.

Preparation of cell extracts

Human embryonic stem cell (hESC) line was cultured according to the previous report in Harvard University (Massachusetts). Human embryonal carcinoma cell (hECC) line from a testicular tumor, NCR-G3, was cultured with the G031101 medium (GP Bioscience, Tokyo, Japan) as previously described in National Center for Child Health and Development (Japan) [48,49]. Protein extracts of each cell line were isolated as detergent-soluble or soluble fractions using a CelLytic MEM Protein Extraction kit (Sigma, St. Louis, MO) as previously described [43]. Briefly, approximately 1×10^6 to 10^7 cells were suspended in 300 µl of cold lysis buffer containing 1% protease inhibitor cocktail. The cell suspension was incubated on ice for 10 min, and then centrifuged at 10,000 g at 4°C for 5 min. The supernatant was transferred to a new microcentrifuge tube and partitioned by incubation at 30°C for 5 min. After centrifugation at 3,000 g at 25°C for 5 min, the upper phase solution containing hydrophilic components was collected as a soluble fraction, and then the lower phase solution was suspended with 200 µl of wash buffer. The resultant solution was stored on ice for 10 min, and then partitioned by incubation at 30°C for 5 min. After centrifugation, the resulting lower phase solution was fractionated as a detergent-soluble fraction.

The protein content of the fractions was measured by a Micro BCA™ Protein Assay Reagent kit (Pierce, Rockford, IL).

SDS-PAGE and western blotting

5 µg of the cell extracts from hESCs, hECCs and hEBs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5-20% gradient gel under reducing condition. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 1% non-fat milk (DS Pharma Biomedical Co. Ltd., Osaka, Japan) and was subsequently probed with primary antibody, biotinylated anti-podocalyxin pAb, at 0.2 µg/ml in Tris-buffered saline, containing 1% Tween 20 (TBST). After washing, the membrane was incubated with AP-conjugated streptavidin (1/5,000 diluted in TBST).

Immunoprecipitation

Each cell extract was prepared as a concentration, which was estimated to be almost comparable at a podocalyxin level (i.e. hESC was 40 µg, hECC was 20 µg and hEB was 80 µg) by western blot analysis. 1 µg of pre-conjugate of the biotinylated anti-podocalyxin pAb was added to each cell extract in total volume of 40 µl. After incubation at 4°C overnight, the solution including podocalyxin was immunoprecipitated with 20 µl of streptavidin-immobilized magnetic beads, Dynabeads MyOne™ Streptavidin T1 conjugate, and the solution was further incubated at 4°C for 30 min. After the podocalyxin-captured beads were washed three times with 200 µl of phosphate-buffered saline, containing 1% Triton X-100 (PBSTx), the bound podocalyxin was eluted with 40 µl of the elution buffer (TBS containing 0.2% SDS) by a heat denaturation procedure.

Glycosidase treatments

To remove *N*-glycans, immunoprecipitated extracts of hESCs, hECCs and hEBs were incubated with 4 mU Glycopeptidase F from *Escherichia coli* (Takara Bio Inc. Cat#4450) at 37°C overnight. To remove Sia residues, the extracts were incubated with 0.5 U Sialidase A™ from *Arthrobacter ureafaciens* (ProZyme, Inc. Cat#GK80040) at 37°C for 2 h. To see the presence of non-reducing ß1-3Gal residues, the above desialylated solution was further incubated with 2.5 U ß1-3galactosidase from *Xanthomonas manihotis* (BioLabs, Inc. Cat#P0726L) at 37°C overnight.

Antibody-assisted lectin profiling (ALP)

To evaluate the glycan forms of podocalyxin, an advanced procedure of lectin microarray named antibody-assisted lectin profiling (ALP) was performed as previously described [50]. The

immunoprecipitated extracts and the extracts with a glycosidase treatment were diluted to 60 µl with PBSTx, and then applied to the lectin microarray containing triplicate spots of 42 lectins into each well on glass slide. After incubation at either 20°C (*N*-glycosylation analysis) or 4°C (other analyses) overnight, 20 µg of human serum polyclonal IgG (hIgG) was added to the glass slide, and the reaction was allowed for 30 min. The resultant solution was discarded, and then the glass slide was washed three times with PBSTx. The 20 µg of hIgG and 100 ng of biotinylated anti-podocalyxin pAb solution diluted to 60 µl with PBSTx were applied to the array, and then the solution was incubated at 20°C (Glycopeptidase F analysis) or 4°C (other glycosidase analyses) for 1 h. After washing three times with PBSTx, 60 µl of Cy3-labeled streptavidin (200 ng) solution in PBSTx was applied to the array, and then further incubated for 30 min. The glass slide was added with 10% Indian ink solution and scanned by an evanescent-filed fluorescence scanner, Glycostation™ Reader 1200 (GP Bioscience). All data were analyzed with the Array-Pro analyzer version 4.5 (Media Cybernetics, Inc., MD) or SignalCapture 1.0 and Glycostation™ Tools Pro Suite 1.0 (GP Bioscienc). The net intensity value for each spot was calculated by subtracting a background value from the signal intensity values of three sports. Three spots of the signal intensity values were averaged.

Statistical analysis

The signal intensity was max-normalized as previously described unless otherwise mentioned [43]. For comparison of differences between the arbitrary two data sets, the max-normalized values were calculated for individual lectin signals to obtain the indexes defined by the following formula; i.e., $\{Log_2(hESC/hECC)\}$. For principal component analysis (PCA), the max-normalized data were used for application to a web-based NIA array analysis tool (http://lgsun.grc.nia.nih.gov/ANOVA/) [51-53]. The 2D-biplot format was used, where categorized cell groups and selected lectins were indicated.

Results

Detection of podocalyxin from hESCs and hECCs

In order to confirm the existence of podocalyxin, which has a variety of glycan forms, we first prepared detergent-soluble fractions from hESCs (originally established in Harvard University) and hECCs (NCR-G3), which were subjected to western blot analysis following SDS-PAGE. As a result, a broad band corresponding to podocalyxin at approximately 200 kDa was detected in both hESC and hECC fractions. However, no apparent difference in their sizes attributable to glycan structures was shown between the two cell types. Even when the podocalyxin was enriched from the detergent-soluble fractions with anti-podocalyxin pAb, the band patterns in western blot were almost the same between hESCs and hECCs (Supplementary figure 1A, lanes 2 and 3, respectively). Then, we examined proteins of soluble fractions, because podocalyxin has highly hydrophilic nature by the presence of extensive negative charges of Sia and sulfate residues [54]. In fact, podocalyxin in the soluble fractions was detected in both hESCs and hECCs as broad protein bands over ~200 kDa (Supplementary figure 1B, lanes 2 and 3, respectively), suggesting the presence of a variety of glycan forms. Hence, we performed subsequent experiments using these soluble fractions of hESCs and hECCs.

Characterization of N- and O-glycans of immunoprecipitated podocalyxin from hESCs and hECCs

To investigate the glycan profiles of podocalyxin of hESCs and hECCs, we performed an advanced technology of lectin microarray,

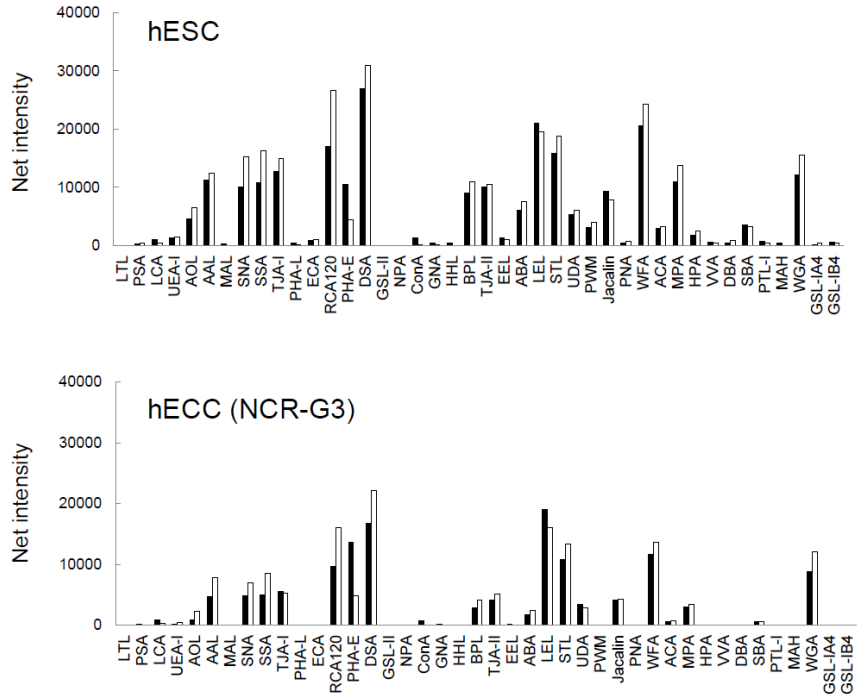

Figure 1: Bar graph representation of 42 lectin signals of hESCs and hECCs (NCR-G3) with or without *N*-glycosidase treatment. Lectin microarray data are shown as net intensity. The signal intensities obtained for soluble extracts from hESC and hECC (NCR-G3) with and without *N*-glycosidase treatment are shown in open and closed bars, respectively.

i.e., antibody-assisted lectin profiling (ALP) [50]. It is known that podocalyxin carries a TRA-series of epitopes on their *O*-glycans [23,32,33]. To confirm this, soluble fractions from both hESCs and hECCs were treated with *N*-glycosidase and were subjected to ALP analysis using anti-podocalyxin pAb as described (Figure 1). The signal intensities of *O*-glycan-binders, such as WFA and HPA (GalNAc-binders) and ABA (Gal-binder) were increased in hESCs possibly by eliminating bulk of *N*-glycans. The signal intensities of other *O*-glycan-binders, ACA and MPA (T-antigen-binders, Galß1-3GalNAcα-Thr/Ser) were also increased, while those of *N*-glycan-binders, such as PHA-L (tri- and tetra-antennary complex type *N*-glycan-binder) and PHA-E (bi-antennary complex type *N*-glycan-binder) were decreased. Similarly, in hECCs (NCR-G3), the signal intensities of these lectins were increased (WFA, ACA and MPA) or decreased (PHA-E). Moreover, the signal intensities of high-mannose-binders (ConA, GNA and HHL) were decreased in both cells. On the other hand, some lectin signals were not observed in hECCs, while they are evident in hESCs, which include HPA, PHA-L, PWM (GlcNAc-binders) and SBA (Gal/GalNAc-binder). These results imply that immunoprecipitated podocalyxin has *O*-glycans associated with various glycan structures and that glycan profiles of hECCs are somewhat different from those of hESCs.

Effect of sialidase treatment on glycan profiles of podocalyxin from hESCs and hECCs

We next investigated more in detail the glycan forms of podocalyxin with focus on Sia residues, because this glycoprotein is characterized as a sialomucin [3,11,14]. Sialidase treatment was performed for the immunoprecipitated podocalyxin from hESCs and hECCs, and their glycan profiles were compared by western blot and ALP. Although large smear protein bands were observed before the sialidase treatment (Figure 2A, lanes 2 and 4, respectively), reaction to anti-podocalyxin pAb was evidently enhanced in both cell preparations after the sialidase treatment. Moreover, while the immunopositive bands remained broad, the intense regions were shifted in a different way between hESCs (<140 and >240 kDa; Figure 2A, lane 3) and hECCs (200 and >240 kDa, lane 5).

To see the effect of sialidase treatment on the glycan profiles of podocalyxin derived from hESCs and hECCs, we performed ALP as described above (for spot patterns on a lectin microarray slide, Supplementary figure 2). Obviously, the signal intensities of glycan profiles in both hESCs and hECCs were greatly increased after the sialidase treatment (Supplementary figure 3). Moreover, signal intensities of hESCs and hECCs became almost the same after this treatment (the highest intensities of hESCs and hECCs are 35,632 and 32,549, respectively), whereas they were significantly different before the treatment: total signal intensities of hESCs were higher than those of hECCs (Figure 2B). This observation indicates that the effect of sialidase digestion is more significant in hECCs than in hESCs. It is also noted that the signal intensities of a series of *O*-glycan-binders, e.g., Jacalin, WFA, ACA, MPA, VVA and SBA, were evidently increased in both cells. These results are consistent with the previous observation that podocalyxin is a heavily glycosylated sialomucin [5,7]. Interestingly to note, among α2-6Sia-binders (SNA, SSA and TJA-I), the signals of only SNA showed decrease by the sialidase treatment, whereas other α2-6Sia-binders did not.

Statistical analysis

As described, podocalyxin contains a number of Sia residues on their *O*-glycans [3,5]. To focus the subsequent analysis on *O*-glycans, lectins, of which signal intensities remained even after the *N*-glycosidase treatment, were used for statistical analysis. The signal intensities of relevant lectins were normalized relative to DSA which showed the highest intensity in each analysis. Thus max-normalized data were used to calculate the indexes defined by the following formula; i.e., Log_2(hESC/hECC). As a result, 24 lectins among 42 lectins were shown to give statistically significant scores (Figure 3A). Among them, 17 showed significant differences in lectin signals between hESCs and hECCs, whereas the others did not. For instance, the signal intensities of UEA-I (Fucα1-2LacNAc-binder), and AOL and AAL (α1-6Fuc-binders) were significantly higher in hESCs than in hECCs, whereas those of TJA-II (α 1-2Fuc-binder) showed no difference. The signal intensities of ECA and RCA120 {type II LacNAc (Galß1-4GlcNAc) complex type *N*-glycan-binders} were higher in hESCs than in hECCs. In contrast, signal intensities of LEL (Poly-LacNAc- or Poly-GlcNAc-binder) were significantly higher in hECCs. Although the signal intensities of UDA, STL and WGA (GlcNAc-binders) in hESCs were lower than in hECCs, opposite is the case for another GlcNAc-binder, PWM. ACA, MPA and HPA signals in hECCs were higher than in hESCs.

In order to extract key lectins, which discriminate most efficiently hESCs and hECCs, the lectin microarray data were applied to a principal component analysis. As a result, hESCs and hECCs were clearly divided into two groups on the 2D-biplot format (FDR<0.05) (Figure 3B). Glycan alterations associated with two cell lines (i.e., hESCs and hECCs) were depicted by double negative- or double positive-correlations of PC1 and PC2, respectively. Two lectins, UEA-I and PWM, were selected in strong association with hESCs. On the other hands, three lectins, ACA, HPA and LEL, were strongly associated with hECCs.

Effect of ß1-3galactosidase treatment

We then examined the effect of ß1-3galactosidase treatment, because the presence of type I LacNAc structure (Galß1-3GlcNAc) was recently shown to be associated with pluripotency [33,55]. Sialidase-treated fractions of both hESCs and hECCs were subjected to further digestion with ß1-3galactosidase, and ALP analysis was performed. The results are shown in supplementary figure 4. Although the signal intensities of GSL-II (GlcNAc-binder) were significantly increased in both hESCs and hECCs, those of other GlcNAc-binders (PWM, STL and UDA) showed no change, or were significantly decreased in hECCs (Figure 4). The signal intensities of ECA, RCA120, DSA (LacNAc-binders) and LEL showed no substantial change in hESCs, but on the contrary they were considerably decreased in hECCs, while PHA-E signal was decreased in both cells. Moreover, the signal intensity of BPL (*O*-glycan- or type I LacNAc complex type *N*-glycan-binder) was decreased in both cells. However, signals of other *O*-glycan-binders, ABA, PNA, ACA and MPA, were increased in hESCs, while they were significantly decreased in hECCs. Thus, glycan structures of podocalyxin are too complex to elucidate completely, but the obtained data of glycan profiling strongly suggest significant difference between hESCs and hECCs. Of particular note is the observation that there was a great difference in sensitivity to ß1-3galactosidase digestion between these cells (Figure 4).

Detection of podocalyxin on hESCs and their differentiated cells

Based on the above observation that glycan profiles of podocalyxin are significantly different between hESCs and hECCs, we wondered whether such difference in glycan structures is also applied to more

differentiated cells. To address this question, we compared glycan profiles of well-characterized three strains of hESCs (previously designated H3, H8 and H9) [56,57] with those of differentiated hEBs.

SDS-PAGE and subsequent western blot analysis revealed the presence of podocalyxin in hESCs and the corresponding hEBs with different mobilities: the podocalyxin was detected in all of the three stains of hESCs (H3, H8 and H9) as a >240 kDa broad band concomitant with a smaller (<140 kDa) minor band (Figure 5A, lanes 2-4, respectively). On the other hand, it was detected as an approximately 240 kDa faint band in the corresponding hEBs (H3_EB, H8_EB and H9_EB; Figure 5A, lanes 5-7, respectively) with a somewhat smaller (<140 kDa) band. However, the podocalyxin bands on all of the hEB preparations are unclear. This agrees with previous reports that the expression of podocalyxin is decreased on differentiated stem cells [23].

Because the above results were relatively vague with the presence of concomitant smaller bands, we performed immunoprecipitation with anti-podocalyxin pAb before western blot analysis. The enriched fractions gave clearer immunostains of podocalyxin in particular in the

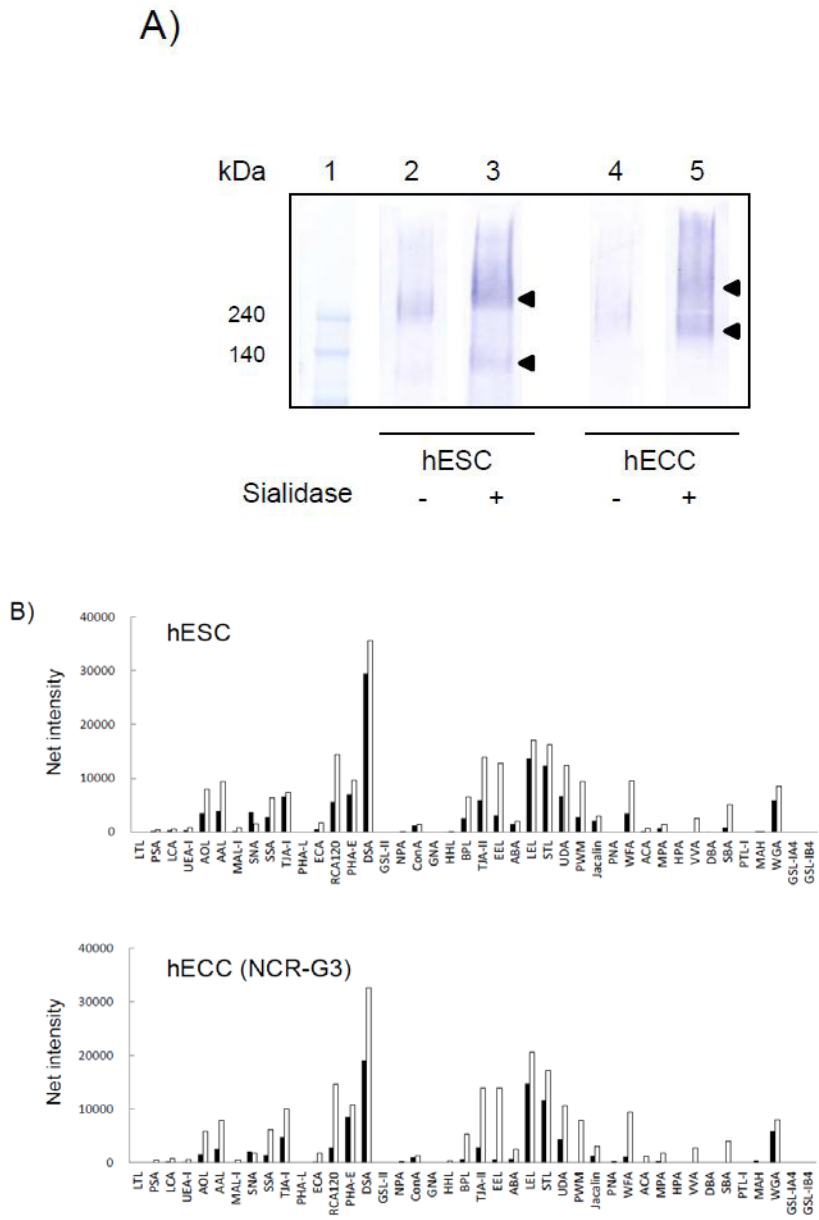

Figure 2: Glycan profiles of immunopurified podocalyxin from hESCs and hECCs.

A) Podocalyxin in each cell extract was immunoprecipitated and subjected to western blot analysis. The immunoprecipitates from hESCs (lane 2) and hECCs (lane 4) were run on SDS-PAGE, and were reacted with anti-podocalyxin pAb. To see the effect of sialylation, the precipitates were treated with sialidase and were analyzed similarly for hESCs (lane 3) and hECCs (lane 5), respectively.

B) Bar-graph representation of 42 lectin signals obtained for hESCs and hECCs by the ALP procedure with or without the sialidase treatment. Lectin microarray data are shown as net intensity without normalization. The data obtained with and without the sialidase treatment are shown as *open* and *closed* bars, respectively.

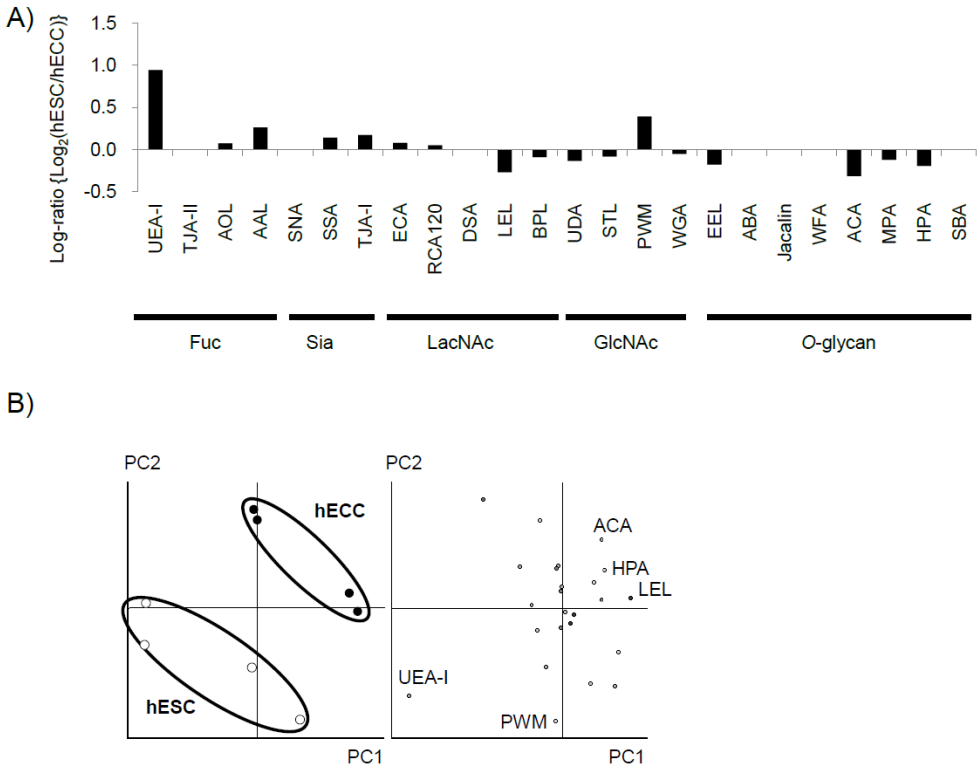

Figure 3: Statistical analysis of the ALP data obtained for podocalyxin from hESCs and hECCs.

A) Lectin microarray data were processed by a max-normalization procedure, and the resultant normalized data of representative lectin signals are shown as the formula "log2(hESC/hECC) for each lectin signal. The signal intensities were categorized into five groups based on the glycan-binding specificities of lectins.

B) Principal component analysis of hESCs and hECCs. Lectin microarray data were max-normalized. hESCs and hECCs were categorized to two groups and five lectins were selected as unique glycan form of cell.

hEB preparations. As a result, difference in apparent molecular mass became evident between hESCs (Figure 5B, lanes 2, 4 and 6) and hEBs (lanes 3, 5 and 7). The former showed a broad band at approximately 240 kDa, whereas the latter showed a broad band at significantly smaller position, i.e., approximately 200 kDa. This observation that apparent molecular mass of podocalyxin in hEBs is smaller than that of hESCs is consistent with previous reports [23].

As a next step, to investigate the glycan profiles of podocalyxin on hESCs and hEBs, we performed ALP analysis. For analysis of the obtained data, the signal intensities of relevant lectins were max-normalized, and the obtained data were used for calculation of log indexes as described above. As a result, 23 significant lectins were found (Figure 5C). Among them, 21 showed the differences in lectin signals between hESCs and hEBs. The signal intensities of two core Fuc-binders, PSA and LCA, were higher in hEBs, whereas those of multiple Fuc-binders, AOL and AAL, were lower. The signal intensities of SNA, SSA and TJA-I (α2-6Sia-binders) in hESCs were higher than in hEBs. Oppositely, the signal intensities of ECA, RCA120 and PHA-E (LacNAC-binders) were lowered in hESCs because sialylation masks the binding sites of these lectins. On the other hand, the type I LacNAc-binder BPL was somewhat higher in hESCs. However, these results are consistent with the recent findings that the presence of both α2-6Sia and type I LacNAc is a characteristic feature of pluripotent stem cells [33,47]. Notably, all of the signal intensities of GlcNAc-binders (UDA, STL and WGA) and *O*-glycan-binders (ABA, Jacalin, WFA, ACA, MPA and SBA) were found to be higher in hEBs. This may suggest that Gal residues on *O*-glycans become more exposed by desialylation as cells are differentiated. Hierarchical cluster analysis clearly categorized hESCs and hEBs into two groups (Figure 5D). Therefore, it is possible to discriminate these cell types by specific glycan profiles.

Discussion

Both hESCs and hECCs show similar properties in high developmental capability. Historically, before the hESCs research began, hECCs had extensively been studied as cells representing pluripotency. Distinct from hESCs, however, hECCs have origins of malignant tumors found in the testis, ovary and other organs. Hence, a risk of malignant transformation in the stem cells including iPSCs resides, and such tumorigenic cells must be eliminated, for which development of useful markers are necessary. Previous studies using protein markers were unsuccessful, while carbohydrate makers should well represent "cell signatures" with different properties. Therefore, the present work will be a model study to see if we can successfully discriminate undifferentiated (e.g., hESCs and iPSCs) and malignant tumor cells (hECCs). For this purpose, it seemed promising to carry out "focused glycomics" on a particular glycoprotein, i.e., podocalyxin, which has been investigated as glycoprotein carrying representative hESC markers, TRA-1-60 and TRA-1-81 [23,32]. Indeed, podocalyxin is characterized as a heavily glycosylated sialomucin. In recent years, it was reported that sialofucosylated podocalyxin-like protein is a selectin ligand on colon carcinoma or pancreatic cancer cells [57,58]. Therefore, these and the present findings on podocalyxin raise a

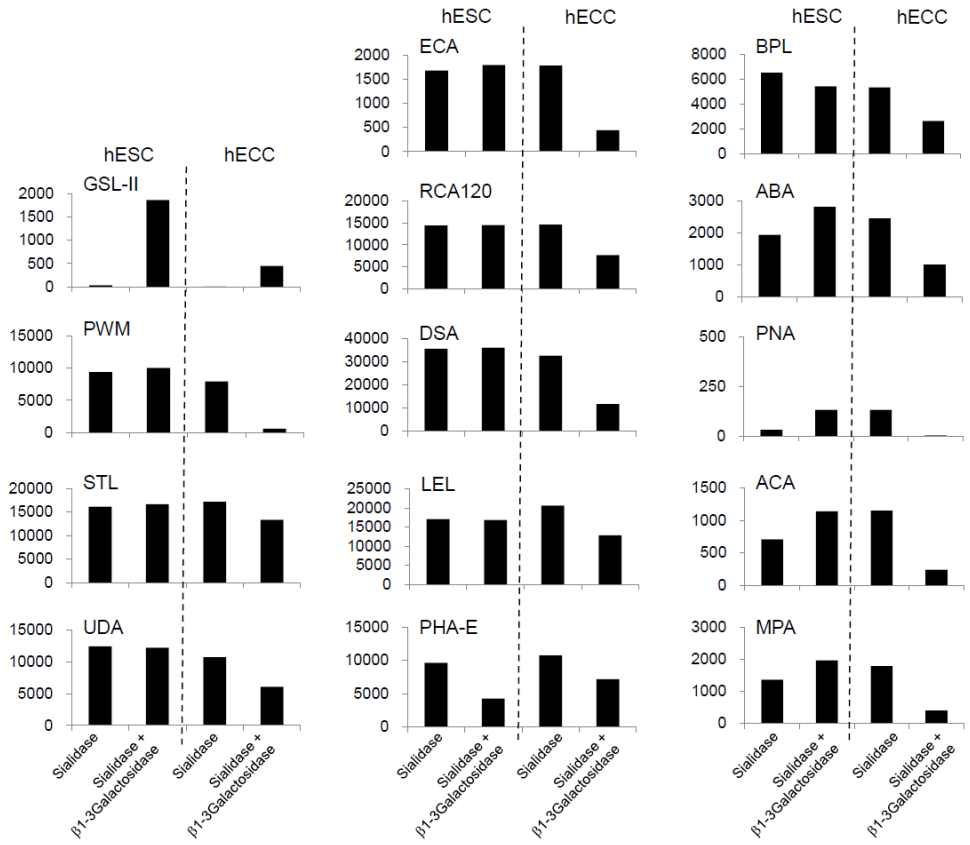

Figure 4: Summary of changes in net signal intensities of lectins after glycosidase treatments. The results of 14 lectins are shown in net intensities, which showed significant difference in either hESCs or hECCs after sialidase and the subsequent ß1-3galactosidase digestion.

possibility that sialylated *O*-glycans of podocalyxin are related to either tumorigenesis or pluripotentcy. There are increasing lines of evidence that show glycosylation alternations are significantly related to malignant transformation. In fact, such examples of tumor-specific glycan markers include CA19-9 and sLe^x [59,60]. Important to note, terminal modifications of Sia and Fuc residues are frequently the cases of critical glycosylation change in important biological phenomena, e.g., differentiation, inflammation and tumorigenesis.

In this study, we detected relatively small but statistically significant differences in glycan profiles between hESCs and hECCs (NCR-G3) with focus on podocalyxin coupled with an advanced lectin microarray technique, ALP [50]. Since the podocalyxin gene was highly expressed in both hESCs and hECCs, the difference between these cells is attributed to significant change in glycan structures of podocalyxin. Although the theoretical molecular mass of podocalyxin is merely 55 kDa, actually immunoprecipitated podocalyxin was found to be >200 kDa, probably with heavily glycosylation consistent with previous reports that the molecular mass of podocalyxin is extremely large representing a feature of sialomucin [5,7]. In our previous works using lectin microarray, we successfully categorized murine and human somatic stem cells into groups based on differentiation potencies [43,44]. Considering potential roles of glycans in cell-cell interactions, it is effective to distinguish hESCs from hECCs based on the glycan profiles targeting a major and heavily glycosylated glycoprotein, i.e., podocalyxin. The changes of the expression level and glycan forms of podocalyxin accompanying cell differentiation have also been shown for hECCs and their differentiated cells [23]. In our observation, podocalyxin of undifferentiated cells (hESCs) was found to be bigger than their differentiated forms (hEBs). In agreement with this, Sia residues, especially of α2-6-linkage, was found to be prominently high in hESCs compared with hEBs in the present study. In case of undifferentiated cells, i.e., hESCs and hECCs, their glycan forms covered with Sia residues were largely the same. This also agrees with the previous report that α2-6sialylated glycan expression on podocalyxin as an anti-TRA-1-60 epitope is higher in undifferentiated cells [32].

In the present study, we detected significant difference between hESCs and hECCs regarding not only direct profiles (non-glycosidase treatment) but also those obtained after sialidase and ß1-3galactosidase digestions. Notably, signals on α2-6Sia-binders (SSA and TJA-I) in hECCs apparently increased even after sialidase digestion, whereas those in hESCs did not. As has been evident in western blot experiments (Figure 2), antigenicity of podocalyxin apparently increased after the sialidase treatment. Therefore, substantial increase in total glycan profiles in both hESCs and hECCs is reasonable. Nevertheless, it remains unclear why the signals on SSA and TJA-I also increased in particular in hECCs. It is possible to speculate that sialomucin is highly resistant to the sialidase digestion, and the resultant product (partially desialylated mucin) shows rather enhanced affinity to some α2-6Sia-binders. It is also known that some Sia-binders recognize sulfated glycans as well (http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp). Moreover, we observed quite different responses between hESCs and hECCs after ß1-3galactosidase digestion: this was particularly evident in signals on GlcNAc-binders (GSL-II, PWM), LacNAc-binders (ECA, RCA120 and DSA) and *O*-glycan (T antigen)-

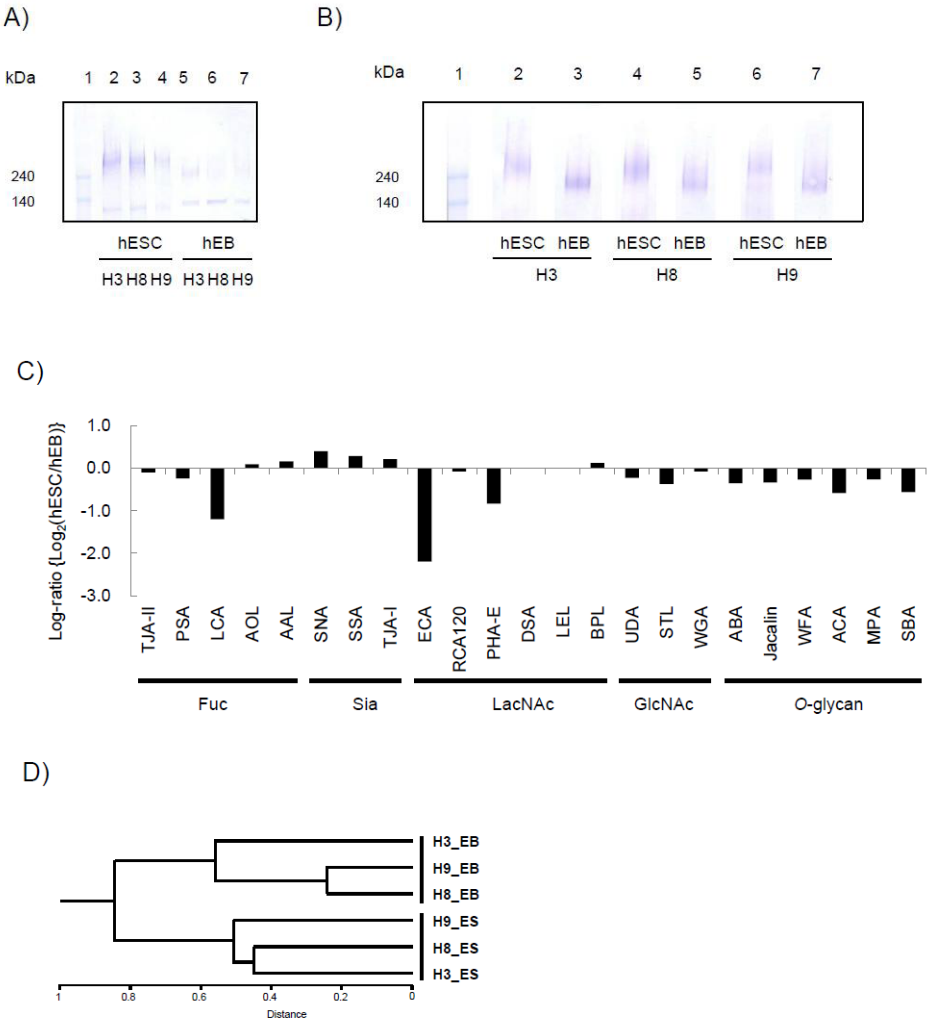

Figure 5: Western blot detection of podocalyxin in extracts from three hESC lines, H3, H8 and H9, and hEBs.

A) The soluble extracts from the three hESCs (H3, H8 and H9) and the corresponding hEBs were directly eluted on lanes 2-4 and 5-7, respectively, and were subjected to western blot analysis using anti-podocalyxin pAb.

B) Immunoprecipitated podocalyxin from hESCs and hEBs were subjected to western blot analysis with anti-podocalyxin pAb. The precipitates from three hESC lines were eluted on lanes 2 (H3), 4 (H8) and 6 (H9), and those from the corresponding hEBs were eluted on lanes 3 (H3), 5 (H8) and 7 (H9).

C) Lectin microarray data were max-normalized, and the log indexes of individual lectins are compared as in figure 3A.

D) Hierarchical clustering analysis showing two clusters of hESCs and hEBs.

binders (ABA, PNA, ACA, MPA). In the last case, signals of all of these T-antigens (Galß1-3GalNAcα)-binders showed increase in hESCs after ß1-3-galactosidase digestion, while they showed decrease in hECCs. This may represent substantial difference in the sialomucin region at the level of "lectin recognition" (e.g., in glycan modification, density, orientation, etc.). Also, a part of GlcNAc-binders, i.e., UDA, STL and PWM, all of which belong to the family of chitin-binding lectins as well as WGA, show similar behaviors after the ß3-galactosidase digestion (Figure 4 and Supplementary figure 4): even though their signals were essentially unchanged in hESCs, they were greatly reduced in hECCs. With this respect, it has been reported that PWM binds to not only GlcNAc residues but also branched Type II LacNAc structures [61], and WGA strongly binds to clustered GalNAc residues, such as Tn-antigen [62]. Because these lectins recognize a cluster of acetyl group, representatively oligo-GlcNAc (chitin), it is reasonable to consider that these lectins bind to a sialomucin region after sialidase digestion in different ways depending of the modification features of Tn (GalNAc) clusters. Further study is necessary to elucidate detailed structures of the sialomucin in podocalyxin.

In the future, a variety of pluripotent cells, such as hESCs and iPSCs, are expected to contribute to a progress in regenerative therapy. For this achievement, however, it is important to distinguish undifferentiated stem cells from malignant tumor cells in a simple and reliable manner. The present approach to differential glycan profiling targeting podocalyxin was successful, at least in part, for discriminating a series of hESCs, hECCs and hEBs, while more definitive classification

is necessary for clinical application of the present findings. In this context, it is convincing that active use of potential glycan markers will greatly help to pioneer an innovative method to diagnose cell signatures targeting both differentiated and undifferentiated cells.

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