Evidences for the involvement of cell surface glycans in stem cell pluripotency and differentiation

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Induced pluripotent stem (iPS) cells are somatic cells that have been reprogrammed to a pluripotent state via the introduction of defined transcription factors. Although iPS is a potentially valuable resource for regenerative medicine and drug development, several issues regarding their pluripotency, differentiation propensity and potential for tumorigenesis remain to be elucidated. Analysis of cell surface glycans has arisen as an interesting tool for the characterization of iPS. An appropriate characterization of glycans at the cell surface of a human embryonic stem (hES) cell and iPS cells might generate crucial data to highlight their role in the acquisition and maintenance of pluripotency. In this study, we characterized the cell surface glycans of iPS generated from menstrual blood-derived mesenchymal cells (iPS-MBMC). We demonstrated that, upon spontaneous differentiation, iPS-MBMC present high amounts of terminal β-galactopyranoside residues, pointing to an important role of terminal-linked sialic acids in pluripotency maintenance. The removal of sialic acids by neuraminidase induces iPS-MBMC and hES cells differentiation, prompting an ectoderm commitment. Exposed β-galactopyranose residues might be recognized by carbohydrate-binding molecules found on the cell surface, which could modulate intercellular or intracellular interactions. Together, our results point for the first time to the involvement of the presence of terminal sialic acid in the maintenance of embryonic stem cell pluripotency and, therefore, the modulation of sialic acid biosynthesis emerges as a mechanism that may govern stem cell differentiation.

Keywords: galactose / glycoconjugate / induced pluripotent stem cells / iPS / sialic acid / stem cells

Introduction

Glycans at the cell surface are referred to as the cell signature, reflecting cellular backgrounds in different biological phenomena like cancer (Li et al. 2010) and embryonic stem (ES) cell differentiation (Muramatsu and Muramatsu 2004; Wearne et al. 2006; Dodla et al. 2011). The most common monosaccharide typically found at the outermost ends of glycans is sialic acids. Sialic acids, a family of nine carbon sugars, confer negative charge to the glycoconjugates which, in turn, mediates attraction/repulsion phenomena. Additionally, the presence of sialic acids can mask underlying structures, for example impeding the binding of Gal-specific receptors of different cell types, hindering their removal from the circulation (Bratosin et al. 1995). The presence of sialic acid is crucial for embryo development, as the absence of enzymes involved in their biosynthetic pathway leads to embryonic lethality (Schwarzkopf et al. 2002). Thus, it is reasonable to associate the presence of terminal sialic acids to many aspects of embryogenesis. ES cells are derived from early stage embryos that give rise to all cell types found in the body via a property termed pluripotency (Evans 2011). In addition, adult cell types can acquire this property of pluripotency after reprogramming them into induced pluripotent stem (iPS) cells, which resemble ES cells in all aspects (Wakao et al. 2012). Previously, iPS cells have been generated from somatic cells of different origin by the ectopic expression of the transcription factor genes Oct3/4, Sox2, c-Myc and Klf4 (Takahashi et al. 2007), opening up a new frontier in regenerative medicine, drug development (Inoue and Yamanaka 2011) and disease models. However, several characteristics of iPS cells regarding their pluripotency, differentiation propensity and potential for tumorigenesis remain to be elucidated. Since different cell lineages express different glycan signatures, which could vary among cell types, stages of development and differentiation (Sjoberg and Varki 1993), an appropriate characterization of glycans on cell surface molecules of ES and iPS cells will generate crucial data to highlight their role in the acquisition and maintenance of pluripotency.

In this study, we characterized the cell surface glycans of iPS generated from menstrual blood-derived mesenchymal cells (iPS-MBMC). We demonstrated that spontaneous differentiation of iPS-MBMC and human ES (hES) cells increased the binding of Peanut agglutinin (PNA), a lectin that recognizes saccharides containing terminal β-galactopyranoside (β-Galp) residues. Enhanced binding to PNA occurs only during ectoderm differentiation. In agreement, the removal of sialic acids by neuraminidase induced cell differentiation, prompting an ectoderm commitment. Our data suggest that terminal galactose...
(β-Gal) masked by sialic acid (Neu5Ac) residues is recognized by β-Gal-binding molecules expressed on the surface of iPS-MBMC and hES cells.

**Results**

**Influence of nuclear reprogramming in the cell glycophenotype**

Recently, MBMC was described as a new cell source for iPS generation (de Carvalho Rodrigues et al. 2012). Therefore, we performed a comparative analysis of MBMC, iPS-MBMC and the ES cell, H9, using a lectin panel that is specific for oligosaccharides that are commonly present in mammalian cells. We selected lectins that are specific to terminal modifications, which change depending on cell phenotype. Table 1 shows a list of the lectins used in this study, the oligosaccharides to which they bind to and the saccharides used as competitive inhibitors to test lectin specificity. Flow cytometry analysis revealed that both iPS-MBMC and hES cells display the same glycophenotype, but they clearly differ from that presented by MBMC (Figure 1A and B). Also, iPS-MBMC and hES cells express high amounts of α2-6-Neu5Ac and α1-3/α1-6-fucose (Fuc), as indicated by strong staining with Aleuria aurantia (AAL) and Sambucus nigra (SNA), respectively, when compared with MBMC. In both cases, lectin specificity was tested by competitive inhibition with its specific saccharide (gray histograms). Both of these cell types demonstrate low binding to Erythrina cristagalli (ECA), Soybean agglutinin (SBA), Solanum tuberosum (STA), PNA and Lotus tetragonolobus (LT). The observed lectin profile allows us to conclude that MBMC reprogramming leads to a reversion of cell glycophenotype to a pluripotent one which presents high amounts of fucose and Neu5Ac on the cell surface, suggesting a role for these saccharides in maintaining pluripotency. In order to confirm whether the glycan profile observed previously is a typical characteristic of pluripotent stem cells, we carried out co-immunocytochemistry assays of iPS-MBMC and the ES cell line H9 with lectins and the pluripotency marker OCT4. Flow cytometry showed that iPS-MBMC and H9 cells present the same glycosignature exhibiting α-mannopyranosyl (α-Man) groups, as indicated by PSA binding, β-GlcNAc residues recognized by STA and α1-3/α1-6-Fuc stained by AAL (Figure 1). On the other hand, cells were negative for SBA (GalNAc) and PNA (β-Gal), corroborating the immunohistochemistry results (Figure 2). The presence of α2-3-Neu5Ac and α2-6-Neu5Ac on the cell surface is clearly demonstrated by staining with the inactive trans-sialidase (iTS) from Trypanosoma cruzi and SNA, respectively. Co-positivity of cells with OCT4 confirmed that cells are undifferentiated in all lectin bindings (Figure 2). In an independent experiment, we compared the amount of sialic acid present in each cell type by applying gas chromatography–mass spectrometry (GC–MS) (Bratosin et al. 2007). The electron impact (EI) mass spectrum of the per-O-trimethylsilylated (per-O-TMS) Neu5Ac is known to be characterized by a prominent fragment at m/z 298 (Kamerling and Gerwig 2006). Figure 3 shows the reconstructed ion chromatogram (i.e. the abundance of fragment m/z 298 as a function of time) of the trimethylsilyl-derivatized mixture from iPS-MBMC, H9 and MBMC cells (Figure 3A–C). These assignments were verified by measuring the GC retention times of per-O-TMS-derivative of authentic Neu5Ac standard in a separate experiment (Figure 3A). The methodology applied allowed the detection of Neu5Ac in the material from iPS-MBMC, H9, but not from MBMC cells, corroborating the above findings using sialic acid-binding lectins.

**Alterations in glycan profile during differentiation**

The observation that overgrown iPS-MBMC cultures consistently contain foci of PNA-positive cells, as verified by immunocytochemistry (Figure 4A and B) or by flow cytometry, indicated a remodeling of the cell surface during cell differentiation. In order to determine whether shifting of cell surface glycan correlates with cell differentiation into any of the three germ layers, cells were prompted to differentiate into embryoid bodies. Figure 5 shows that iPS-MBMC spontaneously differentiated into an Embryoid Body express all three germ layer markers: beta-3-tubulin (ectoderm), NKX 2.5 (mesoderm) and α-fetoprotein (endoderm). Of particular note is that only cells expressing the ectodermal marker beta-3 tubulin are positive for PNA (Figure 5C). Moreover, cells expressing NKX 2.5 (Figure 5G) or α-fetoprotein are negative for PNA (Figure 5K), mesoderm and endoderm germ layer markers, respectively. Together, these evidences suggest that cell differentiation to ectoderm is due to up-regulation of the expression of glycan-containing terminal β-Galp residues or, most probably, due to the decrease of sialic acid expression.

**Influence of sialylated glycan in cell pluripotency**

The presence of high levels of glycans containing β-Gal terminal residues on the surface of ectoderm-differentiated iPS-MBMC prompted us to investigate the role of Neu5Ac in differentiation. Hence, we artificially manipulated the presence of α2-3- and α2-6-Neu5Ac by removing it from the iPS-MBMC surface using a specific neuraminidase. The removal of Neu5Ac from the iPS-MBMC surface by neuraminidase treatment induced phenotypic conversion from cuboid to fibroblast-like cells, which segregated from the colony edge (Figure 6D–F). The neuraminidase treatment for 24, 48 and 72 h culminated in cell separation in a time-dependent manner, indicating a role of Neu5Ac in cell adhesion. No alterations were observed in cells treated with heat-inactivated neuraminidase (Figure 6A–C), confirming that the alterations observed were induced by enzyme activity. Cell viability was assessed by a fluorescence-based method using a fluorogenic esterase substrate (Calcein AM) that

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<th>Lectin</th>
<th>Specificity</th>
<th>Inhibitor</th>
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<tr>
<td>STA</td>
<td>GlcNAc β1-4GlcNAc</td>
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<tr>
<td>PSA</td>
<td>Man</td>
<td>Me-α-Man</td>
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<td>AAL</td>
<td>α1-3/α1-6-Fuc</td>
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<td>SBA</td>
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<td>SNA</td>
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<td>iTS</td>
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Fig. 1. Fluorescence intensity of different lectins in the cell types analyzed in this study. (A) MBMC (green histograms), iPS-MBMC (red histograms) and H9 (blue histograms) lineages were stained with biotinylated lectins and FITC-conjugated avidin. Filled gray histograms show the binding profile of lectins in cells preincubated with their specific saccharides, as labeled. Black empty histogram refers to cells stained with the FITC-conjugated streptavidin. (B) Bar graph showing the differences between three cell types for each lectin. The results shown are one representative out of three experimental replicates.
Fig. 2. Glycophenotype characterization of undifferentiated iP-MBMC by lectin-binding assay. Confocal microscopy analysis shows that pluripotent cells are SBA and PNA negative, whereas highly positive for PSA, STA, SNA, AAL and ITS lectins. All cells were co-stained with OCT4 as a pluripotence marker. Scale bar: 50 µM for AAL and ITS; 100 µM for additional lectins.
is hydrolyzed to a green-fluorescent product (calcein); thus, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Live cells were able to take up calcein and could be analyzed by green-fluorescent light emission. In contrast, ethidium bromide diffuses through the permeable membrane of dead cells and binds to their DNA; thus, dead cells would be detected by a red fluorescent signal. Figure 6 shows that neuraminidase or heat-inactivated neuraminidase treatment does not impact cell viability.

As expected, neuraminidase treatment exposes terminal β-Galp residues that were labeled by PNA (Figure 7). The most remarkable finding was that Neu5Ac removal by neuraminidase treatment induced expression of the ectodermal marker, α-nestin (Figure 7), which co-localized with PNA staining. Cells positive for α-fetoprotein or α-actin were not observed after neuraminidase treatment at the same time points (Figure 7). These results show for the first time that the presence of sialic acid is crucial to the maintenance of embryonic cell pluripotency and, therefore, modulation of sialic acid biosynthesis emerges as a mechanism that may govern stem cell differentiation.

Presence of carbohydrate-binding molecules on H9 and iPS-MBMC surface

Results showing that the expression of terminal β-Galp and Neu5Ac residues plays a role in the fate of iPS-MBMC suggest the presence of a carbohydrate-binding molecule on the cell surface, which is capable of sensing the altered glycan profile and therefore triggering cell differentiation. We hypothesized that the reduced presence of α2-3- and α2-6-Neu5Ac or enhanced terminal β-Galp residues, both induced by neuraminidase treatment, can be sensed by endogenous lectins which, in turn, could result in the induction of the observed differentiation processes. In order to evaluate the presence of possible ligands for these molecules on the surface of iPS-MBMC and H9 cells, cells were incubated with the biotin-conjugated polyacrylamide (PAA) substituted probes (α2-3-sialyllactose-PAA, α2-6-sialyllactose-PAA, and β-Galp-PAA). Flow cytometry analysis revealed that both, iPS-MBMC and H9, present β-Galp-binding molecules (Figure 8A and B, respectively). In contrast, no binding molecules for α2-3-SL (Figure 8C and D) or α2-6-SL (Figure 8E and F) were observed in any cell type. The preincubation of the cells with pure D-Gal prior to Galp-PAA incubation was used to ensure binding specificity (Figure 8B, orange histogram).

Discussion

The advent of iPS cell generation has brought innumerable benefits to the field of developmental biology, disease modeling and, ultimately, a great hope for cell replacement and drug therapies. However, a complete understanding of the biology of pluripotent cells with regard to their phenotypic characterization and their actual differentiation capabilities is still needed to make that hope a reality. In this sense, the proper characterization of the glycophenotype of the iPS cells becomes essential, as even small changes in the cell glycosignature can modulate the IPS interactions with its environment and modulates cell behavior (Howarth and Ting 2006). Here, we demonstrate that iPS-MBMC has remarkable differences regarding the presence of sialylated and fucosylated epitopes at the cell surface when compared with the mesenchymal cells prior to reprogramming. On the other hand, iPS-MBMCs display the same glycophenotype profile as a pluripotent control (hES cells lineage H9). Based on this evidence, it is reasonable to believe that the high levels of sialic acids and fucosylated epitopes...
might be important for maintaining stemness. Indeed, as shown in Figure 2, OCT4-positive iPS cells are highly positive for the presence of α2-3- and α2-6-Neu5Ac (iTS and SNA lectins, respectively) and F α1-3-/α1-6-Fuc (AAL lectin); furthermore, previous studies using other iPS cells also showed a glycophnotype shift (Tateno et al. 2011). Hasehira et al. found that iPS cells display higher amounts of α2-6-sialylation and α1-2-fucosylation when compared with nonpluripotent cells (Hasehira et al. 2012). These observations are only in partial agreement with our findings, since we did not observe any difference in the α1-2-fucosylation (LTl lectin) between iPS-MBMC, H9 and MBMC. On the other hand, we showed a clear shift in the expression of α1-3-/α1-6-Fuc between MBMC, iPS-MBMC and H9 (Figure 1). These divergent results could be explained by the use of different cell types. In both cases, expression analysis of glycosyltransferase genes involved in the synthesis of sialylated and fucosylated epitopes are needed to clarify this ambiguity.

Moreover, the biological meaning of this glycome shift remains to be elucidated in the light of stem cell biology.

We further studied the glycophenotypic changes of spontaneous differentiation foci in overgrown cultures of iPS-MBMC as well as in H9 cells. Interestingly, we observed that cells became PNA positive only in the differentiation focus (Figure 4D–H). This occurs only in cells committed to ectodermal differentiation, as demonstrated by the co-localization of PNA with beta-3-tubulin-positive cells (Figure 5B and C). Dodla and co-workers also showed that differentiated neural precursors are highly PNA positive, which is in contrast to multipotent cells (Dodla et al. 2011). The enhanced expression of Gal-containing glycans could be involved in the induction of the differentiation process. In an attempt to clarify this question, we artificially generated β-Galp-containing epitopes at the iPS-MBMC surface through the removal of sialic acid units. The alterations observed in cell morphology and the enhancement in cell detachment of the colonies after neuraminidase treatment strongly suggest that α2-3- and/or α2-6-Neu5Ac containing glycoconjugates at the cell surface are involved in preserving cell colony morphology (Figure 6). Together with exposition of β-Galp residues (PNA binding), we observed that the removal of sialic acid...
induced early expression of the neural precursor marker nestin (Dahlstrand et al. 1995) (Figure 7). These results suggest that the loss of sialic acid or enhancement in β-Gal-binding molecules might be involved in the induction of iPS-MBMC differentiation. The elucidation of the question of whether sialic acid- or Gal-binding molecules could be involved in the maintenance of pluripotency or if they mediate ES cell differentiation is of great relevance. Also, identification of the glycoconjugates carrying the sialic acid unities is of equal importance. Liang et al. (2011) described suitable changes in the ganglioside structure during the hES cells differentiation into neural precursors and endodermal cells. Interestingly, remarkable differences in the Gal and

Fig. 5. PNA-positive cells express the ectoderm lineage marker. iPS-MBMC subjected to the embryoid body-based spontaneous differentiation protocol were labeled for nuclei (DAPI, A, E and I); for β-Gal terminal residues (PNA, C, G and K); and for the three germ layer lineage markers: ectoderm (beta-3-tubulin, B); mesoderm (NKX 2.5, F) and endoderm (α-fetoprotein, J). It can be seen that only cells positive for the ectoderm lineage marker (beta-3-tubulin) are positive for PNA (C). Scale bar: 100 µm.

Fig. 6. Influence of cell surface sialylation in colony morphology. Light microscopy of iPS-MBMC treated with heat-inactivated neuraminidase during 24, 48 and 72 h (A, B and C, respectively). Cells treated with 0.25 U/mL of neuraminidase for 24 (D), 48 (E) and 72 h (F) segregate from the colony edge. (G–H) Epifluorescence photomicrographs showing the calcein AM uptake (in green) by viable cells treated with the active (G) or heat-inactivated neuraminidase (H). No ethidium bromide (Eth-1; red) representing dead cells was observed. Scale bar, 50 µM.
Fig. 7. Remotion of sialic acid induces early nestin appearance. Confocal microscopy showing cells treated (+Neu) or not (–Neu) with neuraminidase for 5 days. Cells were labeled with FITC-conjugated PNA (first column) and stained with α-nestin, α-fetoprotein and α-actin (second column) and TOPRO (third column).
sialic acid content of the gangliosides were also observed after differentiation. It should be expected that the observed changes in the glycan profile are followed by correlated expression of the glycosyltransferases. However, Nairn and collaborators showed that changes in glycan structures generally, but not uniformly, correlated with alterations in transcript abundance for the corresponding biosynthetic enzymes, suggesting that transcriptional regulation contributes significantly to the regulation of glycan expression (Nairn et al. 2012). The changes in the cell glycophototype could be sensed by a sialic acid- or Gal-binding molecule, which in turn could be responsible for the signal triggering induction of the differentiation process. We used α2-3-SL, α2-6-SL and β-Gal-p-PAA-biotin conjugated in the attempt to find sialic acid- or β-Gal-binding receptors on the surface of iPS-MBMC and H9. Interestingly, both iPS-MBMCs and H9 only display ligands for β-Galp, but both were negative for both isoforms of SL (Figure 8). In the experiment presented in Figure 8, we did not treat the cells with neuraminidase, which could raise the question of whether the receptors are present in the pluripotent state but do not exert any effect in the absence of neuraminidase. The presence of sialic acid could mask ligand binding and modulate differentiation. The results presented here point to the important functions of glycoconjugates in the biology of stem cells, in particular in the reprogramming process to generate iPSCells. In this study, we provide novel evidence that sialic acid might be involved in the iPS-MBMC pluripotency and propose that the expression of Gal-containing glycan can contribute to the differentiation of stem cells into the ectoderm layer. Taken together, our findings provide clues that may help to understand the roles of glycans in the maintenance of pluripotency and induction of the differentiation of ES cells.

Materials and methods

Maintenance of ESC and iPS-MBMC

H9 and iPS-MBMC were cultured on matrigel™ (Stem cell technology) coated 60-mm tissue culture dishes (Techno Plastic Products, Switzerland) and mTeSR1 medium (Stem Cell Technologies Inc., Vancouver, Canada), according to the company’s instructions. The mTeSR1 culture medium was changed every day and the cells passaged every 4–5 days when the colonies became large and began to merge with each other. For passaging, the culture medium was removed, cells were rinsed with DMEM F12 (Gibco Invitrogen) in the absence of FBS, once, and 1 mL of dispase enzyme (1 mg/mL, Stem Cell Technologies Inc.) was added for 7 min. Dispase solution was then removed and the cells were rinsed once with 4 mL of DMEM F12 medium. Fresh mTeSR1 medium was added again and the colonies were scraped off using a 5 mL serological pipette (Techno Plastic Products, Switzerland). The cells were passaged 1:3 or 1:4 depending on the density. The mTeSR1 medium was changed after 2 days and then every other day, until they were ready for passaging again. Human mesenchymal progenitor cells were cultured in mesenchymal medium consisting of DMEM F12 (Gibco Invitrogen) in the absence of FBS, once, and 1 mL of dispase enzyme (1 mg/mL, Stem Cell Technologies Inc.) was added for 7 min. Dispase solution was then removed and the cells were rinsed once with 4 mL of DMEM F12 medium. Fresh mTeSR1 medium was added again and the colonies were scraped off using a 5 mL serological pipette (Techno Plastic Products, Switzerland). The cells were passaged 1:3 or 1:4 depending on the density. The mTeSR1 medium was changed after 2 days and then every other day, until they were ready for passaging again. Human mesenchymal progenitor cells were cultured in mesenchymal medium consisting of DMEM, 10% defined fetal bovine serum (FBS, Hyclone), 1 mM 1-glutamine, 0.5 U/mL penicillin and 0.5 U/mL streptomycin (all from Gibco Invitrogen unless otherwise stated).

Cell lectin binding

To ensure the presence of α2-3-Neu5Ac, we used recombinant iTS (Todeschini et al. 2002). All other lectins used in this study were purchased from Vector Labs (San Diego, USA). iPS-
MBMC and H9, cultured as mentioned before, were passaged into 24-well plates covered with thin slides coated with matri-gel. After cells were grown for 3 days in the pluripotency medium mTeSR, they were washed twice with PBS and freshly fixed for 15 min with PBS containing 2% of paraformaldehyde. Cells were stained with the following biotinylated lectins: STA, PSA, PNA, AAL, LTL, SBA, ECA, SNA and iTS. Cells were incubated with 10 µg/mL of each biotinylated lectin for 30 min, washed twice with PBS and incubated with FITC-conjugated Avidin (Sigma) diluted 1:200. To prove that they are pluripotent, cells were subsequently stained with 1:200 diluted purified anti-OCT4 (Stem Cell Technologies) and with secondary anti-mouse IgG PE-conjugated (Sigma-Aldrich) 1:500. Cells were analyzed by Axiovert 200 coupled to a system LSM 510 META confocal laser scanning microscope (Zeiss, Germany) and photomicrographs digital capture Axiovision.

**GC–MS analysis**

The different cell types, IPS-MBMC, MBMC and H9 cells were plated at confluence of 1 × 10^5 in 6-well plates. After reaching 80% confluence, the cells were harvested and centrifuged at 110 rpm for 5 min. The pellets were lyophilized and stored at ~80°C until analysis. Neu5Ac (10 µg) was used as standard. Samples were methanolized with 0.5 M HCl in methanol for 18 h at 20°C, neutralized with silver carbonate and re-N-acetylated with acetic anhydride for 18 h in the dark at room temperature, before being centrifuged at 2000 rpm for 5 min. The dried residue was trimethylsilylated by the addition of bis(trimethylsilyl)-trifluoroacetamide/pyridine (1:1 v/v) for 1 h (Mattos et al. 2005). The precipitate was removed by centrifugation, and 2 µl of the supernatant was analyzed by gas-liquid chromatography (GC) in a GCMS-QP2010 Shimadzu, using a HP-ULTRA 2 column. In order to optimize visualization of Neu5Ac, a reconstructed m/z 298 ion chromatogram was obtained.

**Immunocytochemistry analysis**

For immunocytochemistry, cells grown on Matrigel-coated cover slips were fixed with 4% paraformaldehyde at room temperature. After three washes in 10 mM PBS (pH 7.4), cells were incubated in PBS with 5% normal goat serum (Invitrogen) and 0.3% Triton-X 100 for 30 min at room temperature. Cells were then incubated overnight at 4°C with anti-OCT4, anti-NANOG, anti-α-SMA, anti-α-fetoprotein and anti-Nestin (1:200; Millipore) antibodies in PBS with 0.3% Triton-X 100. After washing, the secondary antibody Cy3-conjugated goat anti-mouse IgG (1:1000; Jackson, West Grove, PA), in PBS with 0.3% Triton-X 100, was added and the cells were incubated for 2 h at room temperature. Following three washes with PBS, nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole; Sigma) for 3 min and the cover slips were mounted on slides using PPD (2,5-diphenyl-1,3,4-oxadiazole; Sigma). Fluorescence images were recorded using a digital camera attached to an inverted microscope (Carl Zeiss Apotome; Zeiss, GmbH, Germany).

**Flow cytometry analysis**

To analyze the influence of reprogramming on the cell glycophe-notype, MBMC, IPS-MBMC and H9 glycan profiles were compared by flow cytometry. All cell types were harvested using PBS containing 0.05% trypsin EDTA. IPS-MBMC and H9 colonies were disaggregated by pipetting 10 times using P1000 microtips (Gilon). Cells were then fixed with PBS containing 2% paraformaldehyde and stained with 10 µg/mL of each lectin, diluted in PBS, for 30 min. Cells were washed twice and then stained with 1:2500 FITC-conjugated Avidin for 30 min. To ensure lectin specificity, competitive-binding assays were performed preincubating lectins with a related recognized oligosaccharide: 100 mM 1-3-Fuc for AAL and LTL; 200 mM N-acetyl-α-glucosamine (Sigma) for STA; 200 mM methyl-α-D-mannoside (Me-α-Man) for PSA; 200 mM lactose (Galβ1-4Glc) for PNA; 200 mM Galβ1-4GalNAc for ECA; 25 mM Neu5Aco2-6-5L plus 25 mM Galβ1-4Glc for SNA; 25 mM Neu5Aco2-3-5L plus 25 mM Galβ1-4Glc for iTS and benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (Bz-αGalNAc) 200 mM for SBA lectin. In order to determine whether there were any β-Galp or α-2-3/-2-6 Neu5Ac ligands, cells cultivated as above were incubated with 10–40 µg/mL of Neu5Aco2-3SL-PAA-biotin, Neu5Aco2-6SL-PAA-biotin and β-Galp-PAA-biotin (Glycotech, MD) for 30 min, washed twice with PBS and stained with FITC-conjugated Avidin (Sigma) diluted 1:2000. To ensure ligand-oligosaccharide specificity, we preincubated cells with 1 µM of pure D-Gal (Sigma) for 30 min. Cell positivity was analyzed with FACS Aria Illu (BD Biosciences). Histograms were created and median fluorescence intensity were calculated using FlowJo 7.1 software.

**Cell treatment with neuraminidase**

To analyze sialic acid involvement in cell differentiation, H9 and iPS-MBMC were cultured and passaged as described in Maintenance of ESC and iPS-MBMC section. After 2 days, cells were treated daily with 0.25 U/mL of α2-3/2-6 neuraminidase (New England Biolabs) diluted in mTeSR pluripotency medium for 1 or 5 days and maintained at 37°C in 5% CO2. Heat-inactivated neuraminidase (65°C for 15 min) was used as control. Images of cell cultures were taken every day after treatment using light microscopy.
Live/dead cell analysis

The LIVE/DEAD Viability/Cytotoxicity Kit (Molecular probes™) was used following the manufacturer’s instructions. Briefly, iPS-MBMC cells were treated daily with 0.25 U/mL of α2-3/2-6 neuraminidase (New England Biolabs) for 72 h and maintained at 37°C in 5% CO₂. Heat-inactivated neuraminidase (65°C C for 15 min) was used as control. Live cells were able to take up calcein and could be analyzed by green-fluorescent light emission (488 nm). The ethidium bromide homodimer diffuses through the permeable membrane of dead cells and binds to their DNA. Dead cells would be detected by a red fluorescent signal (546 nm). The live/dead assay was analyzed using an AMG EVOS fl LED fluorescence microscope.

Immunohistochemistry of cells treated with neuraminidase

After 5 days of treatment with neuraminidase, cells were incubated with FITC-conjugated PNA diluted 1:100 in PBS, washed twice and subsequently stained with α-nestin, α-fetoprotein or α-actin, diluted 1:200 as ectoderm, endoderm and mesoderm germ layer markers, respectively. The images were taken by confocal fluorescence microscopy (Carl Zeiss Apotome; Zeiss, GmbH, Germany).

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Conflict of interest statement

None declared.

Abbreviations

AAL, Aleuria aurantia; ECA, Erythrina cristagalli; iTS, inactive trans-sialidase; LTL, Lotus tetragonolobus; PNA, Peanut agglutinin; per-O-TMS, per-O-trimethylsilylated; PSA, Pisum sativum; SNA, Sambucus nigra; STA, Solanum tuberosum; SBA, Soybean agglutinin.

References


Absence of N-glycolyneuraminic acid and characterisation of N-acetylneuraminic acid 1,7 lactone. Biochimie. 89:355–359.


