

### Candidates of possible linkages between the inner and outer cellular states

Based on the correspondences between the expression and network signatures and between the expression and glycan signatures, we identified a total of 14 glycosyltransferases, since ST6GAL1 appeared in both sets of correspondences. These glycosyltransferases are potential candidates for the linkage between the inner and outer cellular states in hiPSCs. Interestingly, these glycosyltransferases may be related to the biosynthesis of a glycolipid that is characteristic of hiPSCs (see additional file 13: Knowledge-based relationships between glycosyltransferases and their biosynthetic pathways). Indeed, the allocation of the above glycosyltransferases to the pathways of “Glycan Biosynthesis and Metabolism” in KEGG GLYCAN (Table 1 and see also additional file 14: Locations of the glycosyltransferases detected in the present study in the pathways of “Glycan Biosynthesis and Metabolism”) revealed that the glycosyltransferases identified in the present study are important in the glycolipid biosynthetic pathway. We identified B3GALT5 in the biosynthetic pathway for the carbohydrate chains of the globo-series of glycosphingolipids bearing the well-known SSEA-3 and SSEA-4 epitopes for ESCs and iPSCs [34,35], and although FUT2 is not directly involved in the synthesis of these glycans, it was found in the neighboring pathway that leads to the type IV H antigen. Furthermore, B3GALT1 and GCNT2, in addition to B3GALT5 and FUT2, were found in the extensive biosynthetic pathway of the carbohydrate chains of the lacto- and neolacto-series glycosphingolipids that carry SSEA-1, which is intensively expressed in ESCs, but is absent in cells that have differentiated from ESCs [36]. In addition, the members of the GALNT family,

responsible for the O-glycan biosynthetic pathway of sialyl-T antigen, which is the most abundant glycan in several carcinoma cell lines, and ST6GAL1 were only found in the N-glycan biosynthetic pathway, which is involved in the generation of cell-surface carbohydrate determinants and the differentiation antigens HB-6, CDw75, and CD76 [37]. These analyses identified the glycosyltransferases that are directly and indirectly related to known glycan epitopes, thereby indicating the key molecules and the marker epitopes involved in reprogramming.

### Further remarks on the present study

We analyzed more than 50 hiPSCs that were originally established from parental SCs, and the correspondence between each hiPSC and its parental SC was strictly controlled, which supports the present results based on a comparison with a clear genetic relationship. To further clarify the molecular mechanisms of the pluripotency, embryonic stem cells (ESCs) should be analyzed, following the context of the present study. Indeed, the pluripotency of hiPSCs has been extensively evaluated with reference to that of human ESCs, by various comparisons [38-43]. At present, we have prepared more than 100 hiPSCs with higher passages, and their comparisons with ESCs will be reported in the near future.

As for the experimental measurements, two types of data, gene expression and glycan structure, were analyzed by using microarrays and lectin arrays in the present study. To comprehensively understand the features of hiPSCs, more experimental data should be utilized, such as DNA-methylation and mi-RNA data. In particular, the recent availability of the next-gen sequencer will produce RNA-seq and ChIP-seq data with more

**Table 1 Relationships between glycosyltransferase expression, network, and glycan signature**

Glycosyltransferase	Functions	Glycan structure
ST6GAL1	N-, O-Glycan and glycolipid biosynthesis	Siaa2,6Galb1,4GlcNAc-R
B3GNT3	O-Glycan biosynthesis	core1 extension
GCNT2	N-, O-Glycan and glycolipid biosynthesis	I antigen Siaa2,3Galb1,3GalNAca1-Ser/Thr
ST3GAL1	O-Glycan biosynthesis	Ser/Thr
FUT2	N-, O-Glycan and glycolipid biosynthesis	H antigen
GALNT6	O-Glycan biosynthesis	GalNAca1-Ser/Thr
GALNT8	O-Glycan biosynthesis	GalNAca1-Ser/Thr
GALNT10	O-Glycan biosynthesis	GalNAca1-Ser/Thr
GALNT12	O-Glycan biosynthesis	GalNAca1-Ser/Thr
GALNT14	O-Glycan biosynthesis	GalNAca1-Ser/Thr
GALNTL2	Unknown	
B3GALT5	N-, O-Glycan and glycolipid biosynthesis	Galb1,3GlcNAc-R, SSEA-3
B3GALT1	N-, O-Glycan and glycolipid biosynthesis	Galb1,3GlcNAc-R
B3GNT2	biosynthesis	polylactosamine

The fourteen glycosyltransferases with identified correspondences between expression, network, and glycan signatures were allocated to biosynthetic pathways, using the KEGG GLYCAN database with modifications. The names of the pathways are listed. See also additional file 12 for the detailed pathways of notable glycosyltransferases, according to the KEGG GLYCAN database.

accurate measurements of gene expression and concrete information about the regulated genes. In addition, vast amounts of protein interaction data are accumulating. A comprehensive analysis integrating the various data from more hiPSCs will be reported in the near future.

## Conclusions

The present study is the first to reveal the relationships between gene expression patterns and cell surface changes in hiPSCs, and it reinforces the importance of the cell surface to identify established iPSCs from SCs. In addition, given the variability of iPSCs, which is related to the characteristics of the parental SCs, a glycosyltransferase expression assay should be established that allows more precise definition of hiPSCs and facilitates their standardization, which are important steps towards eventual therapeutic applications of hiPSCs.

## Methods

### Cell experiments

Somatic cell pellets were harvested by scraping. The hiPSCs were incubated at 37°C, in a solution containing 1 mg/ml collagenase IV (Invitrogen, Carlsbad, CA), 1 mM CaCl<sub>2</sub>, 20% KNOCKOUT™ Serum Replacement (KSR), and 10% ACCUMAX (Innovative Cell Technologies, Inc., San Diego, CA). When the edges of the colonies started to dissociate from the bottom of the dish, the collagenase solution was removed and the cells were washed with medium. Colonies were then picked up and collected.

MRC-5 and amniotic mesodermal (AM) cells were maintained in POWEREDBY 10 medium (MED Shira-tori Co., Ltd., Tokyo, Japan). The human placental artery endothelial (PAE) cells were harvested from human placenta. To isolate the arterial endothelium, we used the explant culture method, in which the cells were outgrown from pieces of the placenta's arterial vessels. Briefly, arterial vessels were separated from arteries in the chorionic plate, and chopped into approximately 5-mm<sup>3</sup> pieces. The pieces were washed in endothelial basal medium-2 (EBM-2; Cambrex, Walkersville, MD) and cultured in EGM-2MV medium (Cambrex), which consisted of EBM-2, 5% fetal bovine serum (FBS), and the supplemental growth factors VEGF, bFGF, EGF, and IGF. The arterial vessels attached to the substrata of the culture dishes (BD Falcon; Becton Dickinson, San Jose, CA). Cells migrated out from the surface of the tissues after about 20 days of incubation at 37°C in 5% CO<sub>2</sub>. The cells were harvested in PBS containing 0.1% trypsin and 0.25 mM EDTA, and were re-seeded at a density of 3 × 10<sup>5</sup> cells in a 10-cm dish. Confluent monolayers of cells were subcultured. The culture medium was replaced every 3-4 days. Human uterine endometrium (UtE) was harvested from a patient with endometriosis.

The endometrium was sterilized in PBS and cut into small pieces with dissection scissors. These pieces were placed in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co. St. Louis, MO), supplemented with 10% FBS and an antibiotic-antimycotic (100×) solution (Invitrogen), and incubated for 10-14 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Subconfluent adherent cells were harvested in PBS containing 0.06% trypsin and 0.005% EDTA, and were subcultured. The culture medium was replaced every 4 days. This study was approved by the Ethical Committee of the National Institute for Child Health and Development. The purpose of this study was explained thoroughly to the patients, who gave their written informed consent.

hiPSCs were cultivated on irradiated MEFs in iPSELLON medium (Cardio, Osaka, Japan), supplemented with 10 ng/ml recombinant human bFGF (Wako Pure Chemicals, Osaka, Japan). hiPSCs were established from MRC-5 and AM cells, as previously described [21,22]. In addition, hiPSCs were established from PAE and UtE cells in the present study. Briefly, 1 × 10<sup>5</sup> cells were infected overnight with pooled viral supernatants, obtained by the transfection of HEK293FT cells (TransIT-293 reagent; Mirus, Madison, WI) with the retroviral vector pMXs, which encodes the cDNAs for OCT3/4, SOX2, KLF4, and c-MYC, together with the packaging plasmids pCLGagPol and pHCMV-VEV-G (a gift from T. Kiyono, National Cancer Center Research Institute, Tokyo, Japan). Four days after infection, the cells were split, plated on irradiated MEFs in 100-mm dishes, and maintained in iPSELLON medium until colonies formed.

The immunocytochemical analysis was performed as described previously [22,23]. Human cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C. After washing with 0.1% Triton X-100 in PBS (PBST), the cells were prehybridized in blocking buffer for 1–12 h at 4 °C, and then incubated for 6–12 h at 4°C with the following primary antibodies: anti-SSEA4 (1 : 300 dilution; Chemicon, Temecula, CA), anti-TRA-1–60 (1 : 300; Chemicon), anti-Oct4 (1 : 50; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Nanog (1 : 300; ReproCELL, Tokyo, Japan), and anti-Sox2 (1 : 300; Chemicon). The cells were then incubated with anti-rabbit IgG, anti-mouse IgG or anti-mouse IgM conjugated with Alexa Fluor 488 or Alexa Fluor 546 (1: 500; Molecular Probes, Eugene, OR) in blocking buffer for 1 h at room temperature. The cells were counterstained with DAPI, and then mounted using a SlowFade light anti-fade kit (Molecular Probes).

Teratoma formation was performed as described previously [22,23]. The 1:1 mixtures of the AM-hiPSC suspension and Basement Membrane Matrix (BD Biosciences, San Jose, CA) were implanted subcutaneously, at 1.0 × 10<sup>7</sup> cells / site, into immunodeficient,

non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (CREA, Tokyo, Japan). Teratomas were surgically dissected out 6–10 weeks after implantation, and were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Sections of 10- $\mu$ m thickness were stained with hematoxylin-eosin.

#### Gene expression analysis

Total RNA samples were extracted using ISOGEN (NipponGene). The global gene expression patterns and changes in mRNA levels were monitored using Agilent Whole Human Genome Microarray chips (G4112F) with one-color (Cyanine 3) dye. This microarray chip covers 41,000 well-characterized human genes and transcripts. The raw microarray data were submitted to the GEO (Gene Expression Omnibus) microarray data archive (<http://www.ncbi.nlm.nih.gov/geo/>) at the NCBI (accession number: GSE 20750). After background correction using a Normal plus Exponential convolution model, which adjusts the foreground to the background, we used an offset to dampen the variation of the log-ratios for intensities close to zero.

Among the 41,000 probes, 16,483 representative probes corresponding to MAQC unique genes were used for the following analyses [44]. Global array clustering was performed by the complete linkage method with Euclidean distance, and was visualized using the Java TreeView 1.1.0 software; the gene expression values are displayed as normalized log ratios. Cell line similarities were measured using Pearson correlation coefficients. To further validate whether the global gene expression is different in each origin cell, we evaluated the classification accuracy by leave-one-out cross-validation (LOOCV) on the nearest-neighbor classifier, based on Pearson's correlation distance. To obtain the expression signatures, we performed a differential analysis for each origin cell: differences between the two arbitrary datasets were evaluated by the Student's *t*-test for the expression of each gene. Thereafter, the false discovery rate (FDR) was estimated using the Benjamini–Hochberg procedure. Differentially expressed genes were selected if they satisfied both FDR < 0.05 and a 2.0-fold change in the average values for the cell lines being compared. The gene ontology analysis was performed using the GO Term Finder Perl script [45] (<http://go.princeton.edu/cgi-bin/GOTermFinder>), with EBI human GO annotations and generic GO slim annotations (<http://www.geneontology.org/>).

#### Network screening

Network screening was performed as described previously [21]. This analysis is based on the procedure for estimating the consistency of a network structure (directed acyclic graph) with the measured data for the

constituent variables in the graph. The joint density function for a given network (reference network) was recursively factorized into conditional density functions, according to the parent-child relationship in the graph. The conditional functions were quantified into log-likelihoods, using linear regression for the measured data, with the assumption that the data followed a normal distribution. The probability of the log-likelihood for the network structure (graph consistency probability; GCP) was then estimated from the distribution of log-likelihoods for 2,000 networks, generated under the condition that the networks shared the same numbers of nodes and edges as those of the given network. The significance probability of the given network was set at 0.05 in this analysis.

In the present study, the GCP was estimated for the ensemble of reference networks, to extract the candidate activated networks in the hiPSCs, in a process termed 'network screening'. The reference networks were constructed using the ChIP-on-Chip data and the classification scheme for gene function. The genes bound by four factors were cited from a previous report [20], and were divided into sub-networks according to the functional gene sets previously defined in the Molecular Signatures Database (MSigDB) [24]. The sub-networks that included at least one gene of the expression signature were then selected. The set of selected sub-networks was used as the reference network for network screening.

#### Glycan analysis

We analyzed cell surface glycans with a lectin microarray [31]. The 43 lectins were dissolved at a concentration of 0.5 mg/ml in spotting solution (Matsunami Glass, Osaka, Japan), and were spotted onto epoxysilane-coated glass slides (Nexterion Slide E Epoxysilane-coated Substrate 25 × 75.6 × 1 mm; Schott, Mainz, Germany) attached to a silicone rubber sheet, using a non-contact microarray printing robot (MicroSys 4000; Genomic Solutions, Ann Arbor, MI). The lectins were spotted in triplicate, with a spot diameter of 500  $\mu$ m. The glass slides were incubated at 25°C for 3 h, to allow lectin immobilization. The lectin-immobilized glass slides were then washed with probing buffer (25 mM Tris-HCl [pH 7.5], 140 mM NaCl, 2.7 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1% [v/v] Triton X-100), and incubated with the blocking reagent N102 (NOF, Tokyo, Japan) at 20°C for 1 h. Finally, the lectin-immobilized glass slides were flooded with TBS containing 0.1% NaN<sub>3</sub> and stored at 4°C. The cell membrane fraction was prepared using the CellLytic MEM Protein Extraction Kit (Sigma-Aldrich, Tokyo, Japan), and the protein concentration was determined using the MicroBCA Protein Assay Reagent kit (Thermo Fisher Scientific, Waltham,

MA). After dilution in PBST (10 mM PBS [pH 7.4], 140 mM NaCl, 2.7 mM KCl, 1% Triton X-100), the cell membrane fraction was labeled with Cy3 NHS ester (GE Healthcare Ltd., Buckinghamshire, England). After dilution in probing buffer to the desired concentration, the Cy3-labeled cell membrane fraction was applied to the lectin microarray and incubated at 20°C overnight. After washing with the probing buffer, fluorescence images were acquired using an evanescent-field activated fluorescence scanner (SC-Profiler; GP BioScience, Kanagawa, Japan). The fluorescence signal of each spot was quantified using the Array Pro Analyzer ver. 4.5 software (Media Cybernetics, Bethesda, MD), and the background value was subtracted. The values shown for the lectin signals represent the average of triplicate spots.

### Additional files

There are 14 additional files in the present analysis. For convenience, we provide an overview of the additional files. Additional files 1, 2, 3, 4, 5 are related to the cell classification in Figure 1: the details of the cell lines and their experimental establishment are described in files 1 and 2, and the details of the analyses of the expression data are described in files 3-5. Additional figures 6-8 are related to the gene expression signature in Figure 2: the details of the analyzed data are described in files 6 and 7, and the results obtained by a standard analysis are described in file 8. Additional files 9, 10, 11 are related to the network signature in Figure 3: the methodological aspects of the network screening are described in files 9 and 10, and in file 11, the detailed results are presented. Additional files 12, 13, 14 are related to the glycan signature in Figure 5: all of the information for interpreting the analyzed results is presented in the three files.

### Additional material

**Additional file 1: Cell lines and numbers of passages analyzed in the present study.** The following abbreviations are used for the human somatic cell (SC) and induced pluripotent stem cell (iPSC) sources: AM, amniotic membrane; PAE, placental artery endothelial; UTE, uterine endometrium; and MRC, MRC-5 cell line. The AM and MRC cell lines were named previously [22,23]. The number of passages for each cell line is indicated by the letter 'p' followed by an Arabic number.

**Additional file 2: Generation of iPSCs from human PAE cells.** (A) PAE cells from the arterial endothelium of a human placenta (a), and generation of iPSCs through epigenetic reprogramming by retrovirus infection-mediated expression of OCT4, SOX2, KLF4, and c-MYC (b). (B) Expression patterns of the pluripotent cell markers, TRA-1-60, SSEA-4, NANOG, OCT3/4, and SOX2. The cell nuclei were stained with DAPI. (C) Hematoxylin-eosin staining of sections of teratomas generated by PAE-iPSC implantation. The histological examination revealed that the tumors contain neural tissues (a: ectoderm), cartilage (b: mesoderm), and a gut-like epithelial tissue (c: endoderm).

**Additional file 3: Clustering for all cells by another method.** Another clustering was performed by the WARD method, instead of the complete linkage method of Figure 1, with Euclidean distance, and was visualized using the Java TreeView 1.1.0 software. The gene expression values are

displayed as normalized log ratios. The abbreviations used are the same as those listed in Figure 1 and additional file 1.

**Additional file 4: Correlation coefficient matrix for all cells.** Pearson's correlation coefficients between 51 cells for the expression profiles of all genes were calculated. The abbreviations used are the same as those listed in Figure 1 and additional file 1.

**Additional file 5: Cross-validation of cell classification.** The classification accuracy was evaluated by leave-one-out cross-validation (LOOCV) on the nearest-neighbor classifier, based on the Pearson's correlation distance.

**Additional file 6: Number matrix for common genes.** The numbers of genes that were different between the iPSCs and SCs are listed on the diagonal of the matrix, and those that were shared between the four gene sets that showed expression differences between the iPSCs are listed above the diagonal. The abbreviations used are the same as those listed in Figure 1.

**Additional file 7: List of 2,502 genes in the expression signature, together with the fold-changes in expression levels and FDR values.** The fold-change values are listed for the minimum values among the four sets of comparisons between iPSCs and SCs (+, iPSCs>SCs; -, iPSCs<SCs), and the FDR values shown are the maximum values among these sets.

**Additional file 8: List of enriched GO terms with significant probabilities (FWER < 0.05).**

**Additional file 9: Schematic representation of the procedure used to obtain the network signature.** The procedure for obtaining the network signature from the expression signature is shown schematically. The detailed procedure is as follows: 1) We first prepare the information for the gene sets to which the transcriptional factors bind, as deduced from the ChIP-on-chip experiments [20]; 2) Next, we prepare the information for the gene sets that were classified using knowledge of biological functions [24]; 3) The large gene sets in step 1 are divided into smaller subsets, according to the classification scheme of the gene sets in step 2; 4) If at least one gene in the expression signature is included in each gene subset in step 3, then the subset is regarded as a reference network; 5) In each reference network, the enrichment probability of the genes in the expression signature is tested with a significance probability of 0.05. Thus, we narrow down the network signature from the reference networks, in terms of gene numbers; 6) The significant reference networks identified in step 5 are further tested by calculating the graph consistency probability, which assesses the consistency between the network structure and the expression data for the constituent genes [24]. In this step, we further refine the network signature, in terms of both the network structure and the extent of gene expression; 7) Finally, we define the network signature, using the reference networks that passed the tests in steps 5 and 6.

**Additional file 10: Reference networks and constituent genes.**

**Additional file 11: Details of the network signature.** The characters in the above list are colored, according to the classification of biological function shown in Figure 2A.

**Additional file 12: Lectin-glycan-glycosyltransferase relationships and correlations of lectin array intensities with glycosyltransferase expression patterns.** Lectins with FDR<0.05 are colored red. The glycosyltransferases in the expression signature are indicated by a circle in the column "Expression signature" in "Gene expression". The Pearson's correlation coefficients between the lectin signal intensities and the expression profiles of the corresponding glycosyltransferases are listed, together with the significance probabilities. The original lectin array data can be obtained by request to HT or JH.

**Additional file 13: Knowledge-based relationships between glycosyltransferases and their biosynthetic pathways.**

**Additional file 14: Locations of the glycosyltransferases detected in the present study in the pathways of "Glycan Biosynthesis and Metabolism".** The glycosyltransferases listed in Table 1 were allocated to the pathways in "1.7 Glycan Biosynthesis and Metabolism" of the KEGG GLYCAN program (<http://www.genome.jp/kegg/pathway.html#glycan>). The glycosyltransferases and epitopes related to differentiation are indicated by red-colored boxes and red lines, respectively, in each pathway (see the text for details).

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### Author details

<sup>1</sup>Computational Biology Research Center, National Institute of Advanced Industrial Science Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan. <sup>2</sup>INFOCOM CORPORATION, Sumitomo Fudosan Harajuku Building, 2-34-17, Jingumae, Shibuya-ku, Tokyo, 150-0001, Japan. <sup>3</sup>Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science Technology (AIST), Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan. <sup>4</sup>Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science Technology (AIST), Tsukuba Central 2, 1-1-1 Umezono, Ibaraki 305-8568, Japan. <sup>5</sup>Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. <sup>6</sup>Department of Developmental Biology and Pathology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. <sup>7</sup>Institute for Systems Biology, Shanghai University, Shangda Road 99, Shanghai 200444, China. <sup>8</sup>Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan.

### Authors' contributions

S.S. (computational analysis and manuscript preparation), Y.O. (cell experiments and DNA microarray), Y.I. (DNA microarray and manuscript preparation), H.T. (lectin microarray and manuscript preparation), M.T. (cell experiments and manuscript preparation), H.A. (cell experiments), K.N. (cell experiments), E.C. (cell experiments), Y.F. (cell experiments), Y.M. (vector construction), H.O. (vector construction), N.K. (vector construction), Y.S. (lectin microarray), A.U. (supervision of cell experiments), J.H. (lectin microarray), K.H. (computational analysis and manuscript preparation), and M.A. (project leader and coordination).

### Competing interests

The authors declare that they have no competing interests.

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# Glycome Diagnosis of Human Induced Pluripotent Stem Cells Using Lectin Microarray<sup>\*[S]</sup>

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Hiroaki Tateno<sup>‡</sup>, Masashi Toyota<sup>§</sup>, Shigeru Saito<sup>¶||</sup>, Yasuko Onuma<sup>\*\*</sup>, Yuzuru Ito<sup>\*\*</sup>, Keiko Hiemori<sup>‡</sup>, Mihoko Fukumura<sup>‡</sup>, Asako Matsushima<sup>‡</sup>, Mio Nakanishi<sup>††</sup>, Kiyoshi Ohnuma<sup>††</sup>, Hidenori Akutsu<sup>§</sup>, Akihiro Umezawa<sup>§</sup>, Katsuhisa Horimoto<sup>¶</sup>, Jun Hirabayashi<sup>‡1</sup>, and Makoto Asashima<sup>\*\*††</sup>

From the <sup>‡</sup>Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, the <sup>§</sup>Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan, the <sup>¶</sup>Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology, 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan, <sup>||</sup>Infocom Corporation, Sumitomo Fudosan Harajuku Building, 2-34-17, Jingumae, Shibuya-ku, Tokyo 150-0001, Japan, the <sup>\*\*</sup>Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan, and the <sup>††</sup>Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

Induced pluripotent stem cells (iPSCs) can now be produced from various somatic cell (SC) lines by ectopic expression of the four transcription factors. Although the procedure has been demonstrated to induce global change in gene and microRNA expressions and even epigenetic modification, it remains largely unknown how this transcription factor-induced reprogramming affects the total glycan repertoire expressed on the cells. Here we performed a comprehensive glycan analysis using 114 types of human iPSCs generated from five different SCs and compared their glycomes with those of human embryonic stem cells (ESCs; nine cell types) using a high density lectin microarray. In unsupervised cluster analysis of the results obtained by lectin microarray, both undifferentiated iPSCs and ESCs were clustered as one large group. However, they were clearly separated from the group of differentiated SCs, whereas all of the four SCs had apparently distinct glycome profiles from one another, demonstrating that SCs with originally distinct glycan profiles have acquired those similar to ESCs upon induction of pluripotency. Thirty-eight lectins discriminating between SCs and iPSCs/ESCs were statistically selected, and characteristic features of the pluripotent state were then obtained at the level of the cellular glycome. The expression profiles of relevant glycosyltransferase genes agreed well with the results obtained by lectin microarray. Among the 38 lectins, rBC2LCN was found to detect only undifferentiated iPSCs/ESCs and not differentiated SCs. Hence, the high density lectin microarray has proved to be valid for not only comprehensive analysis of glycans but also diagnosis of stem cells under the concept of the cellular glycome.

Increasing attention has been paid to iPSCs<sup>2</sup> and ESCs in their pluripotency and medical applications (1, 2). However,

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S5 and Figs. S1–S4.

<sup>1</sup> To whom correspondence should be addressed: AIST, Central 2, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan. Tel.: 81-29-861-3124; Fax: 81-29-861-3125; E-mail: jun-hirabayashi@aist.go.jp.

<sup>2</sup> The abbreviations used are: iPSC, induced pluripotent stem cell; AM, amniotic mesodermal; ESC, embryonic stem cell; MEF, mouse embryonic fibro-

establishment of a robust evaluation system of their properties, including differentiation propensity and risk of possible contamination of xenoantigens and even potential of tumorigenesis, has been hampered by the lack of comprehensive methodology directly applicable to target stem cells, although this is an emerging issue essential for the safe use of iPSCs in regenerative medicine. From many aspects, cell surface glycans are considered to be ideal targets for analyzing or identifying the phenotype of each cell in a direct manner by the following reasons (3, 4). (a) Glycans are located at the outermost cell surface. (b) The total repertoire of cell surface glycans varies at every level of biological organization (*i.e.* species, tissues, cell types, and molecules). (c) Global alterations of the cellular glycome also occur during development, cellular activation, differentiation, malignant transformation, and inflammation. The cell surface glycans are therefore referred to as the “cell signature” that closely reflects cellular backgrounds and conditions, probably because they are actually functioning as cell-to-cell mediators in extensive biological phenomena. This fundamental nature of glycans should be understood with the fact that they are not encoded directly in the genome but are generated by a complex system of a number of glycosidases and glycosyltransferases, whose expressions and activities are significantly affected by both intracellular and extracellular environmental changes. Indeed, cell surface molecules, such as stage-specific embryonic antigens (SSEA1 and -3/4) (5) and tumor rejection antigens (Tra-1-60 and Tra-1-81) (6–8) are glycomarkers widely used to evaluate pluripotency. Notably, however, these “representative” glycomarkers have been identified following rather fortuitous development of their specific antibodies, because most carbohydrate structures are poorly antigenic between mammals. In this context, a systematic search is necessary to draw a whole picture of the stem cell glycome and harness its effect on stem cell biology (8, 9). For instance, the growth and directed differentiation of stem cells to specific progeny lineages in cell culture remain problematic. Understanding how stem cells

blast; PAE, placental artery endothelial; SC, somatic cell; UtE, uterine endometrium; rMOA, recombinant MOA; FWER, familywise error rate.



## Glycome Diagnosis of Human Stem Cells

communicate with one another and feeder cells through cell surface glycans may lead to rational design of specific culture systems. However, the glycome is a quite difficult target to predict solely based on any genomic data base because the biosynthetic process of the glycan moieties of glycoproteins is not template-driven and is subject to multiple sequential and competitive enzymatic pathways. In this sense, a rapid and sensitive system enabling direct monitoring of cell surface glycans is essential.

Several methods have been developed for glycan analysis based on physicochemical principles, such as liquid chromatography and mass spectrometry (10–12). Lectin microarray is an alternative technology for structural glycomics, where a panel of lectins with various glycan-binding specificities is printed on a microarray, providing a versatile platform for rapid and high throughput analysis of glycan structures without liberation of glycans (13, 14). Lectins are a class of decoder molecules of cell surface glycans distributed throughout organisms, which mediate various functions through specific glycan recognition. Analytical protocols using lectin microarray have been developed for various sample types: free oligosaccharides (14, 15), tissue sections (16), cell membrane hydrophobic fractions (17, 18), and even whole cells (19, 20). This technology has just begun to be applied to a wide variety of biological researches, including virus profiling (21) and cell profiling (17, 20), and development of cancer glycomarkers (22–24). For cell profiling, less than 100 ng of proteins in hydrophobic fractions are sufficient for each analysis (25, 26). Data processing and normalization procedures were optimized to ensure the proper interpretation of the data (25, 26). More recently, we have demonstrated that lectin microarray is also applicable to stem cells (27, 28), although we have yet to reach a clear conclusion as to how the cellular glycome changes upon induction of pluripotency. Moreover, practical applicability of this technology to the quality control of stem cells has not been attained.

Here, we developed an advanced platform of high density lectin microarray with the increased number of probe lectins (96 lectins) to expand the glycome coverage for more precise comparison of various stem cell glycomes. A systematic survey of the cellular glycome was then performed toward 135 cell types in total, including iPSCs (114 cell types) and ESCs (nine cell types). Through this comprehensive analysis, we obtained strong evidence that all of the four SCs with originally distinct glycan profiles have acquired those similar to ESCs upon induction of pluripotency. We also found structural features common to iPSCs and ESCs, which corresponded well to the results of gene expression analysis of glycosyltransferases. Finally, we demonstrate the applicability of lectin microarray in the stem cell diagnosis of multiple factors, including discrimination between undifferentiated and differentiated cells as well as detection of the contamination of the xenoantigen,  $\alpha$ Gal epitope.

### EXPERIMENTAL PROCEDURES

**Cells**—Endometrium (UtE) (29), placental artery endothelium (PAE) (30), and amnion (AM) (31, 32) were independently established. UtE, AM, and MRC5 cells were maintained in the POWEREDBY10 medium (MED SHIROTORI CO., Ltd.). PAE

cells were cultured in EGM-2MV BulletKit (Lonza) containing 5% FBS. Human iPSCs from UtE, PAE, and AM cells were generated according to the procedures described by Yamanaka and colleagues (1) with slight modification (27, 28, 33). The iPSCs derived from MRC5, UtE, PAE, and AM cells were maintained in iPSELLON medium (Cardio Inc.) supplemented with 10 ng/ml recombinant human basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd.) on irradiated MEF feeder cells. hiPS201B7 and hiPS253G1 cells were maintained in DMEM/F-12 medium (Invitrogen) supplemented with 20% KSR (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), minimum essential medium non-essential amino acids (Invitrogen), and 5–10 ng/ml recombinant human basic FGF (Wako) on mitomycin C-treated mouse embryo fibroblast feeder cells. ESCs were generated and maintained as described previously (34).

**Lectins**—Lectins from natural sources (58 lectins) were purchased from J-OIL MILLS, Vector Laboratories, EY Laboratories, and Seikagaku Corp. (see the lectin list in supplemental Table S2). Recombinant lectins were prepared as follows. Briefly, genes of carbohydrate recognition domains were cloned into pET27b (Stratagene) and were overexpressed in the *Escherichia coli* BL21-CodonPlus (DE3)-RIL strain under the control of isopropyl- $\beta$ -D-thiogalactopyranoside (Fermentas Hanover) at appropriate temperatures. All recombinant lectins were purified by affinity chromatography using appropriate sugar-immobilized Sepharose 4B-CL (GE Healthcare) based on the glycan binding specificity of each lectin. They were then dialyzed against diluted PBS (final concentration 2.5 mM phosphate buffer containing 0.015 M NaCl). The protein concentration was determined by a BCA protein assay (Bio-Rad). Lectins were freeze-dried and stored at 4 °C until use. The purity was checked by SDS-PAGE and gel filtration chromatography on Shodex PROTEIN KW-802.5 (Shodex). The glycan binding activity and specificity were analyzed by hemagglutinating activity using 4% rabbit erythrocytes, frontal affinity chromatography (35), and glycoconjugate microarray (36).

**Lectin Microarray Production**—The lectin microarray was produced as described previously with minor modifications (14, 15). Fifty-eight natural lectins and 38 recombinant lectins (see supplemental Table S2 for a lectin list) were dissolved at a concentration of 0.5 mg/ml in a spotting solution (Matsunami Glass), and spotted onto epoxysilane-coated glass slides (Schott) in triplicate using a non-contact microarray printing robot (MicroSys4000, Genomic Solutions). The glass slides were then incubated at 25 °C overnight to allow lectin immobilization. The lectin-immobilized glass slides were then washed with probing buffer (25 mM Tris-HCl, pH 7.5, 140 mM NaCl (TBS) containing 2.7 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 1% Triton X-100) and incubated with blocking reagent N102 (NOF Co.) at 20 °C for 1 h. Finally, the lectin-immobilized glass slides were washed with TBS containing 0.02% NaN<sub>3</sub> and stored at 4 °C until use. The spot quality and reproducibility of the produced microarrays were checked before use, using a Cy3-labeled test probe containing 250  $\mu$ g/ml asialofetuin (Sigma-Aldrich), 25 ng/ml Sia $\alpha$ 2–3Gal $\beta$ 1–4GlcNAc-BSA (Dextra), 10 ng/ml Fuca1–2Gal $\beta$ 1–3GlcNAc $\beta$ 1–3Gal $\beta$ 1–4Glc-BSA (Dextra), 10 ng/ml  $\beta$ GlcNAc-BSA (Dextra), 10 ng/ml GalNAc $\alpha$ 1–3(Fuca1–2)Gal-BSA (Dextra), 10 ng/ml Gal $\alpha$ 1–3Gal $\beta$ 1–



4GlcNAc-BSA (Dextra), 10 ng/ml Man $\alpha$ 1–3(Man $\alpha$ 1–6)Man-BSA (Dextra), 10 ng/ml  $\alpha$ Fuc-BSA (Dextra), 10 ng/ml  $\alpha$ GalNAc-BSA (Dextra), and 10 ng/ml Sia $\alpha$ 2–6Gal $\beta$ 1–4Glc-BSA (Dextra) dissolved in probing buffer.

**Lectin Microarray Analysis**—Hydrophobic fractions were prepared using CellLytic minimum essential medium protein extraction (Sigma-Aldrich) in accordance with the manufacturer's procedures (25, 27). After protein quantification using a BCA assay (Thermo Fisher Scientific), hydrophobic fractions were fluorescently labeled with Cy3 monoreactive dye (GE Healthcare), and excess Cy3 was removed with Sephadex G-25 desalting columns (GE Healthcare). After adjusting the protein concentration to 2  $\mu$ g/ml with PBST (10 mM PBS, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1% Triton X-100), the hydrophobic fraction was labeled with Cy3 NHS ester (GE Healthcare). After dilution with probing buffer at 0.5  $\mu$ g/ml, the Cy3-labeled hydrophobic fraction was applied to the lectin microarray and incubated at 20 °C overnight. After washing with probing buffer, fluorescence images were acquired using an evanescent field-activated fluorescence scanner (GlycoStation<sup>TM</sup> reader 1200; GP BioSciences). The fluorescence signal of each spot was quantified using Array Pro Analyzer version 4.5 (Media Cybernetics, Bethesda, MD), and the background value was subtracted. The background value was obtained from the area without lectin immobilization. The lectin signals of triplicate spots were averaged and normalized to the mean value of 96 lectins immobilized on the array. An inhibition assay was performed by incubating Cy3-labeled cell membrane fractions of MEF(#1) or MRC5-iPS#25(P22)(#13) with a lectin microarray either in the absence or presence of 100  $\mu$ g/ml of Gal $\alpha$ 1–3Gal $\beta$ 1–4GlcNAc-PAA (catalog no. 01-079, Glycotech) or a negative control PAA (catalog no. 01-000, Glycotech).

**Gene Expression Analysis**—Total RNA was extracted from each sample by using ISOGEN (NipponGene). The global gene expression patterns were monitored using Agilent whole human genome microarray chips (G4112F) with one-color (cyanine 3) dye. This microarray covers 41,000 well characterized human genes and transcripts. Of the 41,000 probes, 16,483 representative probes corresponding to the microarray quality control unique genes were used for the following analyses (37).

**Statistics**—Unsupervised clustering was performed by employing the average linkage method using Cluster 3.0 software. The heat map with clustering was acquired using Java Treeview. Differences between the two arbitrary data sets were evaluated by Student's *t* test to each lectin signal using SPSS Statistics 19 (SPSS). Significantly different lectin signals or the glycosyltransferase expression were selected if they satisfied a familywise error rate (FWER) by the Bonferroni method of <0.001.

**Immunocytochemistry**—Immunocytochemical analysis was performed as described previously (29, 33, 38). Human iPSCs were fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C. After washing with 0.1% Triton X-100 in PBS (PBST), the cells were prehybridized in blocking buffer for 1–12 h at 4 °C and then incubated for 6–12 h at 4 °C with the following primary antibodies: anti-SSEA4 (1:300 dilution; Chemicon), anti-TRA-1-60 (1:300; Chemicon), anti-Oct4 (1:50; Santa Cruz Biotech-

nology, Inc.), anti-Nanog (1:300; ReproCELL), and anti-Sox2 (1:300; Chemicon). The cells were then incubated with anti-rabbit IgG, anti-mouse IgG, or anti-mouse IgM conjugated with Alexa Fluor 488 or Alexa Fluor 546 (1:500; Molecular Probes) in blocking buffer for 1 h at room temperature. The cells were counterstained with DAPI and then mounted using the SlowFade light antifade kit (Molecular Probes).

**Teratomas**—Teratoma formation was performed as described previously (1, 2). The 1:1 mixtures of the human iPSC suspension and basement membrane matrix (BD Biosciences) were implanted subcutaneously at  $1.0 \times 10^7$  cells/site into immunodeficient, non-obese diabetic/severe combined immunodeficiency mice. Teratomas were surgically dissected out 8–12 weeks after implantation and were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Sections of 10- $\mu$ m thickness were stained with hematoxylin-eosin.

**Glycoconjugate Microarray Analysis**—Glycoconjugate microarray production and analysis were performed as described previously (36). Briefly, glycoproteins and glycoside-polyacrylamide conjugates were dissolved in the Matsunami spotting solution at a final concentration of 0.5 and 0.1 mg/ml, respectively. After filtration, they were spotted on the Schott epoxy-coated glass slide using the Microsys non-contact microarray printing robot.

Cy3-labeled lectins dissolved in the probing solution (10 or 1  $\mu$ g/ml) were applied to each chamber of the glycoconjugate microarray (100  $\mu$ l/well) and were incubated at 20 °C overnight. After washing the chambers with the probing solution, fluorescent images were immediately acquired using an evanescent field-activated fluorescence scanner, the GlycoStation<sup>TM</sup> Reader 1200, under Cy3 mode. Data were analyzed with the Array Pro analyzer version 4.5 (Media Cybernetics, Inc.). The net intensity value for each spot was determined by signal intensity minus background value. The lectin signals of triplicate spots were averaged and normalized to the highest signal intensity among 98 glycoconjugates immobilized on the array.

## RESULTS

**Development of High Density Lectin Microarray**—In order to increase glycome coverage and the selection range of lectins suitable for stem cell evaluation, we first increased the number of immobilized lectins from 43 to 96, which is the largest number of immobilized lectins reported (39). For this purpose, lectins with defined structures were first categorized into lectin families with different protein scaffolds. We then selected lectins from various lectin families, intending to cover a wider range of glycan binding specificities. Especially, we increased lectins specific to terminal modifications, such as Sia and Fuc, which often change dramatically depending on cell properties. For production of recombinant lectins, the *E. coli* expression system was chosen to avoid glycosylation of the produced lectins, which might cause nonspecific binding to lectin-like molecules in the objective samples. The recombinant lectins thus produced were purified by affinity chromatography using the most appropriate sugar-immobilized Sepharose. The glycan-binding specificities of 96 lectins used in this study were analyzed by both glycoconjugate microarray (supplemental Fig. S1 and Table S1; also see "Experimental Procedures") (36) and,

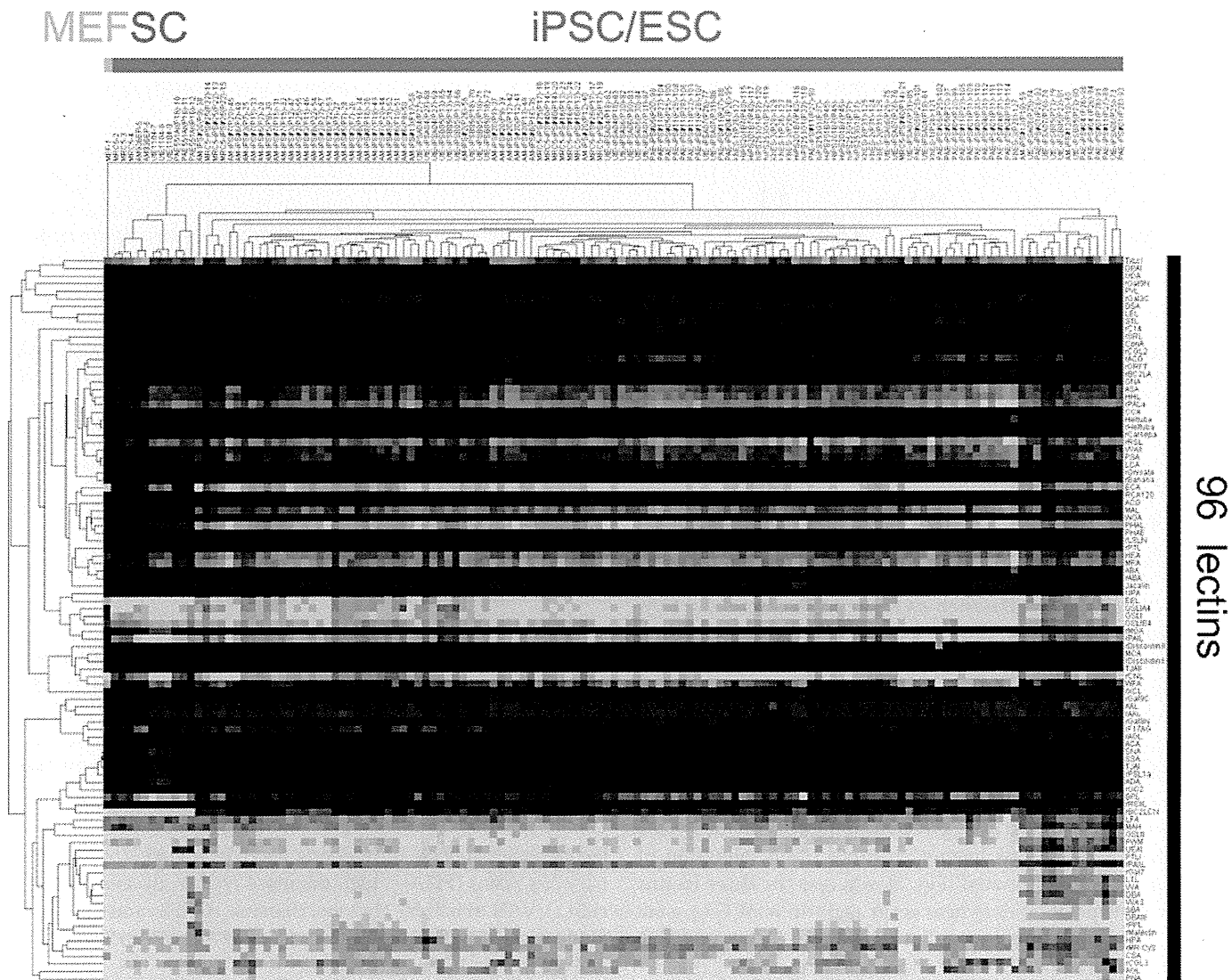


FIGURE 1. **Unsupervised cluster analysis.** Lectin microarray data of iPSCs ( $n = 123$ ), their parental SCs ( $n = 11$ ), ESCs (9), and MEF ( $n = 1$ ) were mean-normalized and log-transformed and then analyzed by Cluster 3.0. The zero value of the lectin signal was converted to 1. Yellow, positive; blue, negative. Clustering method was average linkage. The heat map with clustering was acquired using Java Treeview.

more quantitatively, frontal affinity chromatography (see the Lectin Frontier Database Web page) (35, 40). Their basic specificities evaluated by the above two analytical methods are briefly summarized in supplemental Table S2. The 96 lectins were spotted onto epoxy-activated glass slides by a non-contact spotter (supplemental Fig. S2), and their quality was extensively assessed using a Cy3-labeled test probe (25). Lot-to-lot variance (coefficients of variation) of the developed high density lectin microarray was confirmed to be low (0.14) after mean normalization (25).

*Transcription Factor-induced Reprogramming Leads to a Global Reversion Down to the Pluripotent State at a Cellular Glycome Level as Well*—Using the developed lectin microarray, we have analyzed 135 cell samples in total, including 114 iPSCs, 11 SCs, and nine ESCs, all from human origins, as well as one mouse embryonic fibroblast (MEF). Human iPSCs were generated from four different SC lines: MRC5, AM, UtE, and PAE (supplemental Table S3) (28). We have also analyzed human iPSCs generated from human dermal fibroblasts with four

(201B7) (1) and three transcription factors (253G1) (41) and three cell lines of human ESCs (42). All iPSCs used in this study were morphologically similar to ESCs, and their pluripotency was confirmed by staining with the established undifferentiation markers (SSEA4, Tra1–60, Oct4, Nanog, and Sox2) and DNA microarray (28).

Cell membrane hydrophobic fractions were prepared, and the extracted glycoproteins were then labeled with Cy3-*N*-hydroxysuccinimide ester and analyzed by lectin microarray (25). We have analyzed cell membrane fractions because they can be stored in a freezer until use and are easy to handle, allowing comprehensive analysis of a large number of samples (25, 26).

After being mean-normalized, the obtained data were first analyzed by unsupervised hierarchical clustering (Fig. 1). As a result, differentiated SCs and undifferentiated iPSCs/ESCs were clearly separated into two large clusters, whereas the four SCs were further separated according to their origins. This indicates that SCs (MRC5, AM, UtE, and PAE) with different glycan profiles have acquired profiles quite similar to one

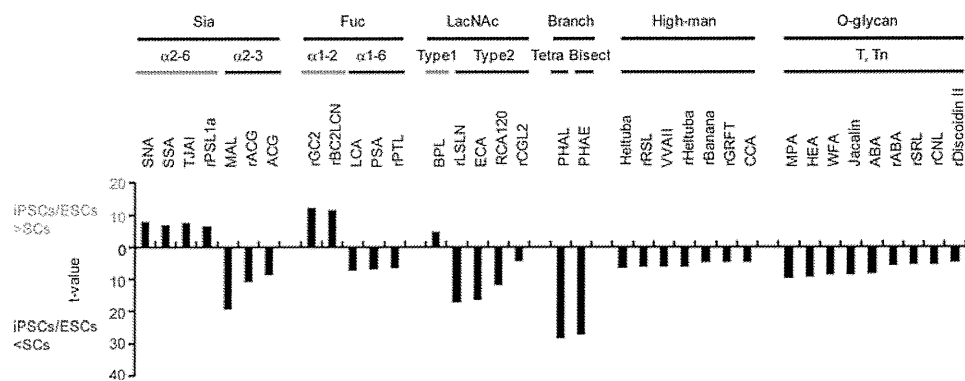


FIGURE 2. **Alterations of the lectin signals upon induction of pluripotency.** Lectin microarray data were mean-normalized and analyzed by Student's *t* test. Lectins with significantly different signals (FWER < 0.001) between undifferentiated iPSCs/ESCs (*n* = 123) and differentiated SCs (*n* = 11) were categorized into six groups based on the glycan binding specificities of lectins. Data are shown with *t* values. Also see supplemental Table S4.

another and even to ESCs upon induction of pluripotency. Thus, transcription factor-induced reprogramming was found to lead to a global reversion down to the pluripotent state at a cellular glycome level as well (27, 28).

**Characteristic Features of Glycome Alteration upon Induction of Pluripotency**—We then examined in more detail how glycan structures altered during the induction of pluripotency. The mean-normalized data were processed by Student's *t* test to select significant probe lectins discriminating between SCs and iPSCs/ESCs (supplemental Table S4). As a result, 38 lectins were selected with FWER of < 0.001. Among them, nine gave higher signals in iPSCs than SCs, whereas 29 exhibited lower signals. Among the 38 lectins, 35 lectins were then categorized into six groups based on their glycan binding specificities, from which glycan alterations having occurred upon induction of pluripotency were estimated (Fig. 2), whereas the three lectins with broader specificities (wheat germ agglutinin (WGA), a Sia binder; rRSIIL and aleuria aurantia lectin (AAL), broad Fuc binders) were not included in this categorization. Here, the lectins with higher signals in iPSCs/ESCs than SCs are shown *below gray lines*, whereas lower signals are shown *below black lines*. The characteristic features of glycan structures of undifferentiated iPSCs/ESCs relative to differentiated SCs are summarized as follows. 1) The signals of  $\alpha$ 2–6Sia-binding lectins (SNA, SSA, Tjai, and rPSL1a) were increased, whereas those of  $\alpha$ 2–3Sia-binding lectins (MAL, rACG, and ACG) were decreased correspondingly (28). This agrees well with the previous report that  $\alpha$ 2–6-sialylated glycan expression is higher in undifferentiated (human ESCs) than differentiated cells (embryoid body) (43). 2) In terms of fucosylation, the signals of  $\alpha$ 1–2Fuc-specific lectins (rGC2 and rBC2LCN) were increased, whereas those of  $\alpha$ 1–6Fuc-specific lectins (LCA, PSA, and rPTL) were decreased. This is consistent with the recent report that human ESCs are stained with anti-Globo H (Fuc $\alpha$ 1–2Gal $\beta$ 1–3GalNAc $\beta$ 1–3Gal $\alpha$ 1–4Gal $\beta$ 1–4Glc) and anti-H type 1 (Fuc $\alpha$ 1–2Gal $\beta$ 1–3GlcNAc), whose antigens contain  $\alpha$ 1–2Fuc (9). 3) The signals of type 1 LacNAc (Gal $\beta$ 1–3GlcNAc)-binding lectins (BPL) were increased, whereas those of type 2 LacNAc (Gal $\beta$ 1–4GlcNAc)-binding lectins (rLSLN, ECA, RCA120, and rCGL2) were decreased. This agrees well with the recent finding that type 1 LacNAc is the glycan epitope recognized by the well known pluripotency markers Tra-1-60 and Tra-1-81 (7). 4) The

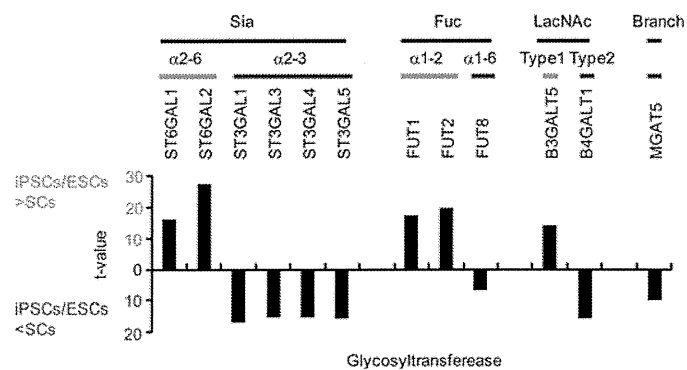


FIGURE 3. **Alterations in the expression of glycosyltransferases upon induction of pluripotency.** Glycosyltransferases related to the lectin signals in Fig. 2 are shown with *t* values. All data are shown in supplemental Table S5.

lectin signals specific to bisecting GlcNAc (PHAE), tetra-antennary *N*-glycans (PHAL), high mannose type *N*-glycans (Heltuba, rRSL, VVAII, rHeltuba, Heltuba, rBanana, rGRFT, and CCA), and O-glycans (MPA, HEA, WFA, Jacalin, ABA, rABA, rSRL, rCNL, and rDiscoidin II) were decreased.

The expression profiles of glycosyltransferases synthesizing glycans agreed well with the results obtained by lectin microarray (Fig. 3 and supplemental Table S5); the expression of  $\alpha$ 2–6-sialyltransferases (ST6GAL1 and -2) (28),  $\alpha$ 1–2-fucosyltransferases (FUT1 and -2), the major glycosyltransferase involved in the synthesis of type 1 LacNAc (B3GALT5) (28), and MGAT5, a glycosyltransferase involved in the synthesis of tetra-antennary *N*-glycans, was increased, whereas that of  $\alpha$ 2–3-sialyltransferases (ST3GAL1, -3, -4, and -5),  $\alpha$ 1–6-fucosyltransferase (FUT8), and the major glycosyltransferase related to the synthesis of type 2 LacNAc (B4GalT1) was decreased correspondingly in iPSCs/ESCs relative to SCs. Based on the results obtained by lectin and DNA microarrays, it is conceivable that the expression of  $\alpha$ 2–6-sialylation,  $\alpha$ 1–2-fucosylation, and type 1 LacNAc is increased, whereas that of  $\alpha$ 2–3-sialylation and tetra-antennary *N*-glycans is decreased upon induction of pluripotency (Fig. 4).

**Selection of the Best Lectin Probe to Discriminate Pluripotency**—We then addressed the challenge to develop a lectin-based procedure to discriminate between differentiated SCs and undifferentiated iPSCs/ESCs, which could be utilized to monitor the state of differentiation. As described, rGC2, rBC2LCN, SNA, Tjai, SSA, rPSL1a, rRSIIL, BPL, and AAL gave

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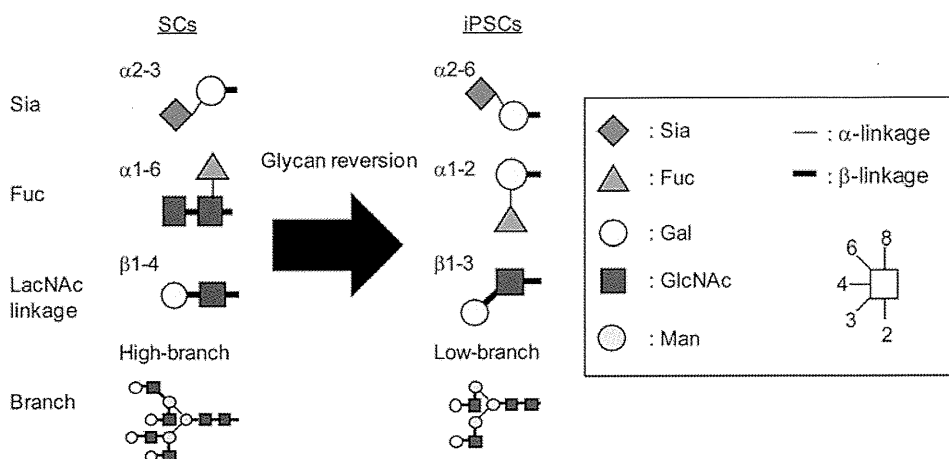


FIGURE 4. Schematic representation of the putative glycan alterations upon induction of pluripotency.

**TABLE 1**  
Selection of the best lectin probe to evaluate pluripotency among 96 lectins

Lectins with significantly higher signals (FWER < 0.001) for iPSCs/ESCs than SCs are shown. Also see supplemental Fig. S4.

Lectin	SC (mean, $n = 11$ )	SC (S.D.)	iPSC/ESC (mean, $n = 123$ )	iPSC/ESC (S.D.)	FWER (Bonferroni)
rGC2	125	31	304	47	1E-21
rBC2LCN	1	1	23	6	2E-19
SNA	69	43	124	18	4E-11
TJAI	96	58	170	28	9E-10
SSA	97	55	156	22	9E-09
rPSL1a	61	32	107	21	2E-07
rRSIIL	78	23	109	20	3E-04
BPL	9	5	20	8	7E-04
AAL	518	77	677	114	1E-03

significantly higher signals in iPSCs/ESCs than SCs with FWER < 0.001 (Table 1). Among them, rBC2LCN showed the best performance as a probe to detect only undifferentiated iPSCs/ESCs but never reacted with differentiated SCs and MEF, whereas other lectins also reacted with SCs (Table 1). Namely, although rGC2 showed a better score in terms of FWER ( $1 \times 10^{-21}$ ) than rBC2LCN ( $2 \times 10^{-19}$ ), the former reacted strongly with MEF (Fig. 5). Similarly, SNA ( $4 \times 10^{-11}$ ), a representative  $\alpha 2-6$ Sia-binding lectin, showed significant cross-reactivity with a part of SCs derived from PAE in addition to MEF (Fig. 5).

**Monitoring the Contamination of the Xenantigen,  $\alpha$ Gal Epitope**—From a practical viewpoint, monitoring possible contamination by xenotransplantation antigens in iPSCs/ESCs is essential for their safe use in regenerative medicine. A recombinant MOA (rMOA) recognizes the xenotransplantation antigen Gal $\alpha 1-3$ Gal $\beta 1-4$ GlcNAc (44) present in most cells from New World monkeys and non-primate mammals, including mice, but not in humans. Indeed, rMOA strongly bound to MEFs but not to any human SCs (Fig. 6). Therefore, rMOA signals should not be detected in human iPSCs. However, triplicate samples of the two cell lines MRC5-iPS#25(P22)(#13–15) and UtE-iPSB05(P13)(#64–66) exhibited significant signals on rMOA. In order to validate whether the binding of rMOA is mediated by a carbohydrate recognition domain of rMOA, we then performed inhibition assay. As shown in Fig. 6B, the binding of rMOA to cell membrane fractions of MEF and MRC5-iPS#25(P22,#13) were abolished in the presence of 100  $\mu$ g/ml Gal $\alpha 1-3$ Gal $\beta 1-4$ GlcNAc-PAA (Fig. 6B), but no inhibitory effect was observed for 100  $\mu$ g/ml of a negative control (PAA

without sugar moiety), indicating that the binding is due to specific interactions via the rMOA carbohydrate recognition domain. As expected, no inhibitory effect of Gal $\alpha 1-3$ Gal $\beta 1-4$ GlcNAc-PAA on a Fuc-binding lectin, rAAL, was observed. These data unambiguously reflect contamination by the xenoantigen  $\alpha$ Gal epitope, in the above two cell lines, which were most probably contaminated with MEF.

## DISCUSSION

Using the developed high density lectin microarray, we performed a systematic analysis of cell surface glycans of a large set of human iPSCs (114 cell types) and ESCs (nine cell types). As a result, a basis for a rational stem cell evaluation system was established, which can reveal both the state of undifferentiation and inclusion of  $\alpha$ Gal epitope (a representative xenoantigen). Such a comprehensive glycome analysis targeting iPSCs and ESCs has never been carried out so far. There are at least three key advantages in using a lectin microarray. 1) An overall glycan profile of each cell type is readily obtained using a relatively small number of cells ( $\sim 1 \times 10^3$ ), and thus, the method is widely applicable to stem cells. 2) The proposed evaluation system includes selection of the best probe by a statistical strategy among a number of lectins, which are immobilized on the array. As candidate probes, carbohydrate-binding antibodies developed so far could also be included. 3) Various properties of stem cells can be assessed simultaneously (*i.e.* with “one-chip” technology). Using the same strategy described in this study, lectin-based evaluation methods targeting tumorigenesis and the differentiation propensity of stem cells could also be developed.

Based on the lectin signals and the expression profiles of glycosyltransferases, we concluded that the expression of  $\alpha 2-6$ -sialylation,  $\alpha 1-2$ -fucosylation, and type 1 LacNAc increases, whereas that of  $\alpha 2-3$ -sialylation and tetra-antennary *N*-glycans decreases correspondingly upon the induction of pluripotency. These changes are consistent with the recent reports that relevant glycans (*i.e.* the expression of Globo H and H type 1 with an  $\alpha 1-2$ Fuc and  $\alpha 2-6$ Sia in human ESCs) are higher than that in differentiated embryoid body (9, 43). Interestingly, the increased expression of  $\alpha 1-2$ Fuc and type 1 LacNAc, which are synthesized by the action of FUT1/2 and B3GalT5, respectively, is closely related to the synthesis of the well known pluripo-

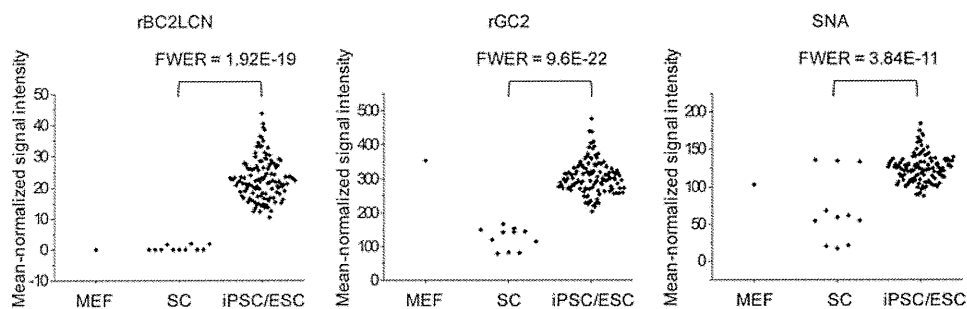


FIGURE 5. **Selection of the best lectin probe to discriminate pluripotency.** The mean-normalized signal intensities of rGC2, rBC2LCN, and SNA to MEF ( $n = 1$ ), SCs ( $n = 11$ ), and iPSCs/ESCs ( $n = 123$ ) are shown.

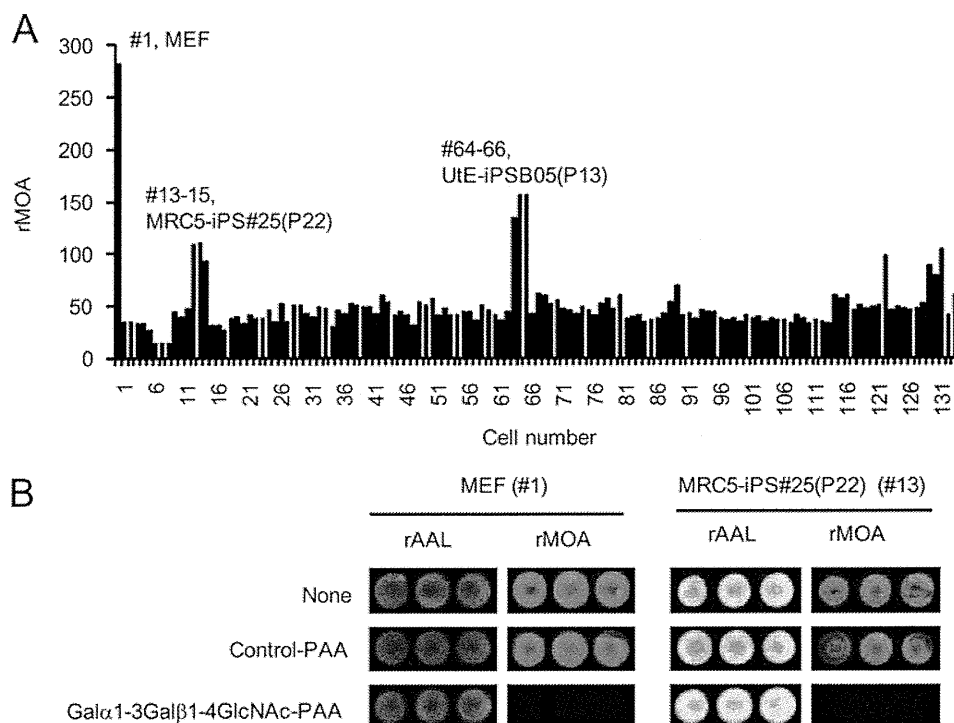


FIGURE 6. **Monitoring the contamination of the xenoantigen  $\alpha$ Gal epitope using the rMOA lectin.** A, mean-normalized lectin microarray data are represented by a bar graph. Numbers correspond to cell types described in supplemental Table S3. B, inhibition assay. Cy3-labeled cell membrane fractions of MEF(#1) or MRC5-iPS#25(P22)(#13) were incubated with lectin microarray either in the absence (*None*) or presence of 100  $\mu$ g/ml Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-PAA or negative control PAA without sugar moiety. Data shown were obtained at gain 110 for MEF and gain 120 for MRC5-iPS#25(P22).

tency markers, SSEA3/4 and Tra-1-60/81 (for a scheme, see supplemental Fig. S4).

In this study, rBC2LCN was selected as the best lectin probe to evaluate pluripotency among the 96 lectins. BC2LCN is a TNF-like lectin molecule identified from a Gram-negative bacterium *Burkholderia cenocepacia* (45). Glycoconjugate microarray analysis revealed that rBC2LCN binds specifically to Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc (GalNAc)-containing glycans, such as H type 1 (Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc), H type 3 (Fuc $\alpha$ 1-2Gal $\beta$ 1-3GalNAc), and Lewis b (Fuc $\alpha$ 1-2Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc), which include the two structural characteristics related to the pluripotency ( $\alpha$ 1-2Fuc and type 1 LacNAc) as described above (supplemental Fig. S3). This observation is consistent with the previous report (45) in which Sulak *et al.* studied the glycan-binding specificity of BC2LCN in detail using glycan microarray and titration microcalorimetry. They also demonstrated that this lectin also binds to Globo H (Fuc $\alpha$ 1-2Gal $\beta$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc), which

was recently proposed as a glycosphingolipid type pluripotency marker (supplemental Fig. S4) (9, 45). These results explain the mechanism of how this lectin could be used as the probe to discriminate pluripotency. rBC2LCN could be used to probe glycoproteins and possibly all glycoconjugates carrying Fuc $\alpha$ 1-2Gal $\beta$ 1-3GlcNAc (GalNAc), whereas anti-SSEA3 and anti-SSEA4 specifically target glycosphingolipids. From a practical viewpoint, rBC2LCN is cost-effective because it can be produced in large amounts by the conventional *E. coli* expression system (84 mg/liter). Thus, this lectin could be a versatile probe to evaluate pluripotency.

In contrast, Globo H has also been reported to be overexpressed in epithelial cell tumors (46). Furthermore,  $\alpha$ 2-6Sia up-regulated in iPSCs/ESCs has been reported to be overexpressed in many types of human cancers, and its high expression positively correlates with tumor metastasis and poor prognosis (47). Thus, the glycan alterations upon induction of pluripotency observed in this study are apparently similar to

## Glycome Diagnosis of Human Stem Cells

those occurring during malignant transformation, as was implied recently (9). Although the reason for this similarity remains to be elucidated, the characteristic glycan changes should be related to the ability of eternal cell proliferation and maintenance, properties common to both cancer cells and pluripotent stem cells.

Glycans are located at the outermost cell surface, where various events take place on the basis of cell-to-cell recognition and interactions. Endogenous lectins, major counterpart molecules of glycans, should play crucial roles in the events (e.g. by regulating several signaling pathways). In this context, interactions occurring between cell surface glycans and endogenous lectins are considered to be essential for the maintenance of pluripotency, self-renewal, and differentiation of iPSCs/ESCs (48). Indeed, heparan sulfate proteoglycans were reported to regulate self-renewal and pluripotency of embryonic stem cells (49). Moreover, reduced sulfation on heparan sulfate and chondroitin sulfate were demonstrated to direct neural differentiation of mouse ESCs and human iPSCs (50). Recently, synthetic substrates recognizing cell surface glycans were reported to facilitate the long term culture of pluripotent stem cells (48). Thus, global analysis of the cellular glycomes of iPSCs and ESCs performed in this study will be necessary to provide the basis to explore the functions and applications of the stem cell glycobiochemistry. They include rational design of the effective substrates and culture conditions to support the long term propagation of ESCs and iPSCs (48). Of course, the results obtained in this study could also be readily applied to staining (specification of the place the event occurs), enrichment (e.g. lectin-aided capturing of necessary cells), and targeting of specific cells (e.g. elimination of unwanted undifferentiated cells). In this regard, stem cell glycoengineering with the aid of a lectin microarray is a key issue in realization of regenerative medicine in the near future.

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## Biomaterials for the Feeder-Free Culture of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells

Akon Higuchi,<sup>\*,†,‡,§</sup> Qing-Dong Ling,<sup>§,||</sup> Yi-An Ko,<sup>||</sup> Yung Chang,<sup>⊥</sup> and Akihiro Umezawa<sup>‡</sup>

<sup>†</sup>Department of Chemical and Materials Engineering, National Central University, Jhongli, Taoyuan, 32001 Taiwan

<sup>‡</sup>Department of Reproduction, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

<sup>§</sup>Cathay Medical Research Institute, Cathay General Hospital, No. 32, Ln 160, Jian-Cheng Road, Xi-Zhi Dist., New Taipei City, Taiwan

<sup>||</sup>Institute of Systems Biology and Bioinformatics, National Central University, No. 300, Jhongda RD., Jhongli, Taoyuan, 32001 Taiwan

<sup>⊥</sup>Department of Chemical Engineering, R&D Center for Membrane Technology, Chung Yuan Christian University, 200 Chung-Bei Rd., Jhongli, Taoyuan 320, Taiwan

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### 1. INTRODUCTION

Human embryonic stem cells (hESCs) are derived from the inner cell mass of 3- to 5-day-old blastocysts.<sup>1–5</sup> hESCs are characterized by a high nucleus-to-cytoplasm ratio, prominent nucleoli, and distinct colony morphology.<sup>6</sup> Recently, pluripotent stem cells that are similar to ESCs were derived from an adult somatic cell by the “forced” expression of certain pluripotency genes,<sup>7–11</sup> such as Oct3/4, Sox2, c-Myc, and klf-4, or their proteins<sup>12</sup> and microRNAs.<sup>13</sup> These cells are known as induced pluripotent stem cells (iPSCs). iPSCs are believed to be similar to ESCs in many respects, including the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time,

embryoid body formation, teratoma formation, viable chimera formation, potency, and differentiability. However, the full extent of their similarities to ESCs is still under investigation.<sup>7,10</sup>

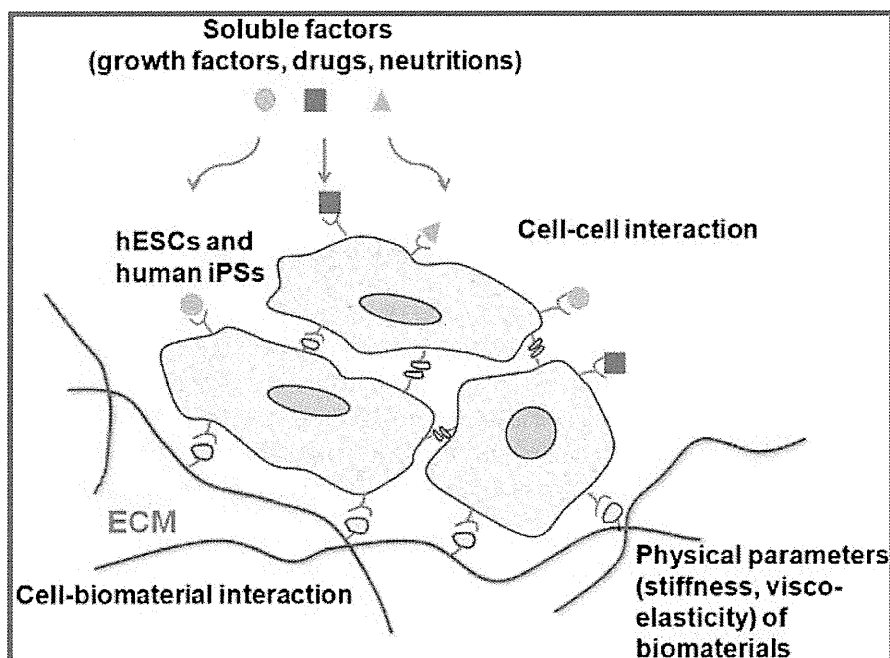
hESCs and human iPSCs have significant potential in therapeutic applications for many diseases because they have the specific ability to differentiate into all types of somatic cells.<sup>14</sup> For example, hESCs and human iPSCs that have been differentiated into nerve cells that secrete dopamine or  $\beta$  cells that secrete insulin can be transplanted for the treatment of Parkinson’s disease<sup>15–17</sup> and diabetes,<sup>18–20</sup> respectively. The pluripotent nature of these cells could permit the development of a wide range of potential stem cell-based regenerative therapies and possible drug discovery platforms.<sup>14</sup>

However, the tentative clinical potential of hESCs and human iPSCs is restricted by the use of mouse embryonic fibroblasts (MEFs) as a feeder layer. While the addition of the leukemia inhibitory factor (LIF) to the culture medium can allow mouse ESCs to proliferate and remain undifferentiated in the absence of a feeder layer of MEFs, this method is not effective for hESCs.<sup>1,2</sup> The addition of LIF to the culture medium is insufficient to maintain the pluripotency and self-renewal of hESCs in a feeder layer-free culture.<sup>6</sup> The possibility of xenogenic contamination during culture restricts the clinical use of transplanted hESCs and human iPSCs. Furthermore, the process of culturing hESCs and human iPSCs using feeder layers is elaborate and costly, limiting the large-scale culture of those cells. The variability of MEFs between laboratories and across batches also affects the characteristics and differentiation abilities of hESCs and human iPSCs. The development of feeder-free cultures using synthetic polymers or biomacromolecules as stem cell culture materials will offer more reproducible culture conditions and lower the cost of production without introducing xenogenic contaminants. These improvements will increase the potential clinical applications of differentiated hESCs and human iPSCs.<sup>6</sup>

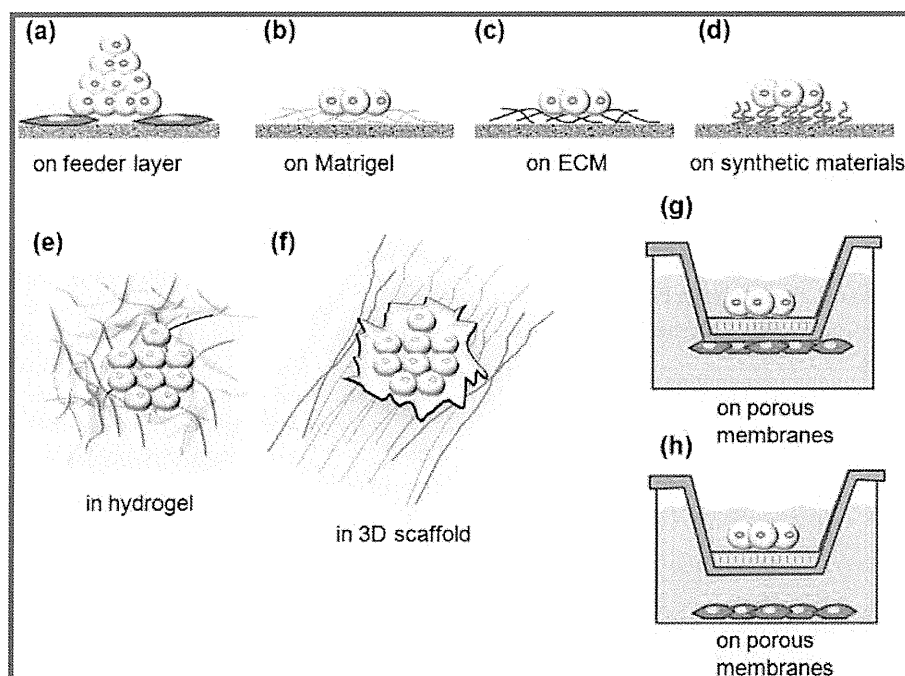
Several factors in the microenvironment and niches of stem cells influence their fate: (1) several soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (2) cell–cell interactions; (3) cell–biomacromolecule (or biomaterial) interactions; (4) and physical factors, such as the rigidity of the environment (Figure 1). Mimicking the stem cell microenvironments and niches

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**Figure 1.** Schematic representation of the microenvironment and niches of hESCs and human iPSCs and their regulation by the following factors: (1) several soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (2) cell–cell interaction; (3) cell–biomacromolecule (or biomaterial) interaction; and (4) physical factors, such as rigidity, of the environment.



**Figure 2.** Schematic representation of different culture methods for hESCs and human iPSCs. hESCs and human iPSCs have been cultured (a) on MEF, (b) on Matrigel, (c) on 2D materials coated with ECM or other biomacromolecules, (d) on 2D materials prepared from synthetic materials, (e) in hydrogels made from glycosaminoglycan or other biomacromolecules, (f) on a 3D scaffold, and (g,h) on porous polymeric membranes.

using biomacromolecules and/or synthetic materials will facilitate the production of large numbers of stem cells and specifically differentiated cells needed for *in vitro* regenerative medicine.<sup>21</sup>

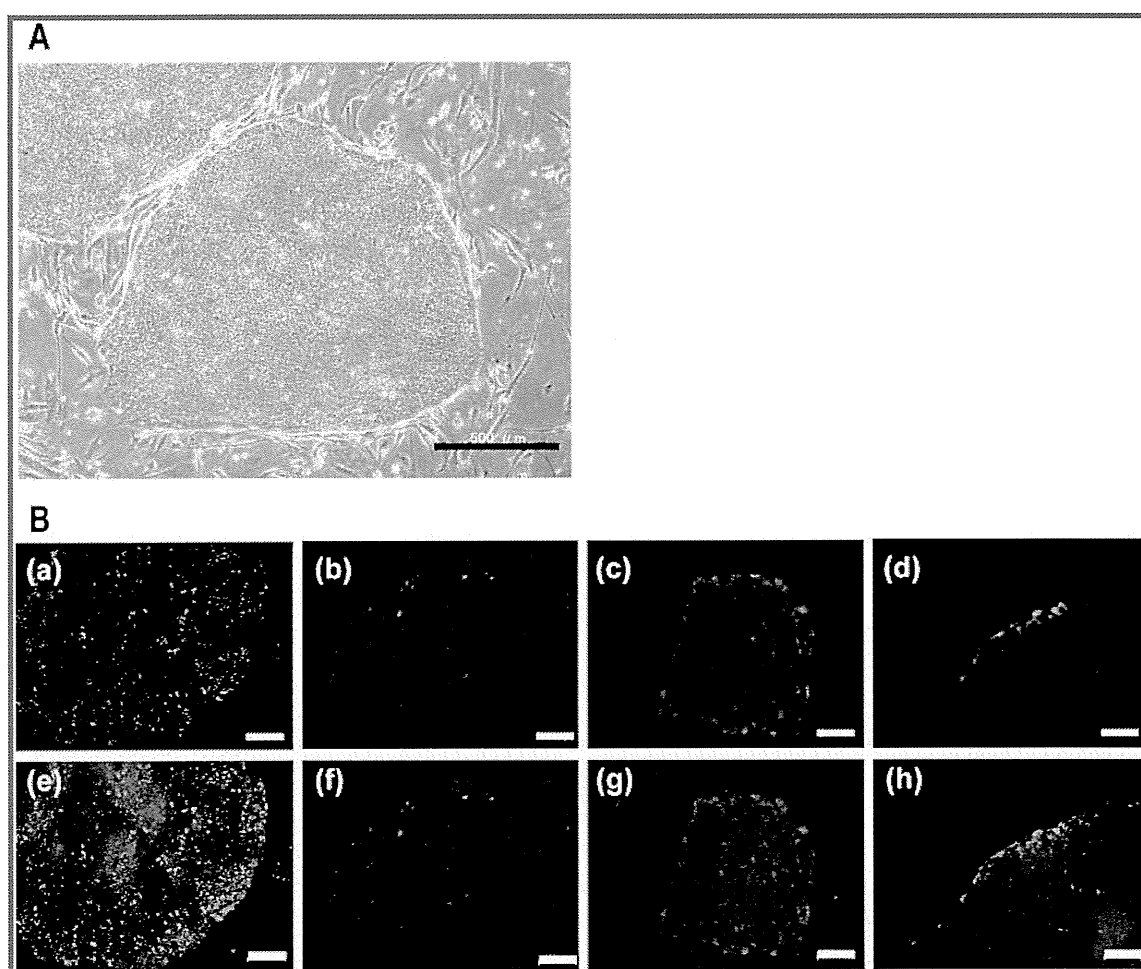
Tissue-specific stem cell niches provide crucial cell–cell contacts and paracrine signaling.<sup>21,22</sup> The extracellular matrix (ECM) keeps stem cells in the niche and serves to initiate signal transduction,<sup>23,24</sup> while locally concentrated glycosaminoglycans (GAGs) provide soluble growth factors or cytokines. Both

*in vitro* and *in vivo*, the niche is established by supportive cells, the ECM and soluble factors, which regulate stem cell fate *via* complementary mechanisms, including the presentation of immobilized signaling molecules, the modulation of matrix rigidity, and the creation of cytokine gradients.<sup>21</sup> Thus, it would be highly beneficial to design, construct, and reproduce the microenvironment and niche of pluripotent stem cells *in vitro* with biomacromolecules and synthetic polymers.

Table 1. Characterization of Pluripotent ESCs and iPSCs<sup>a</sup>

characterization	specification (examples)	ref
1. morphology		
cell morphology	colony formation	2, 14, 31, 34, 39, 45, 47, 50, 76
2. protein level		
surface marker analysis	<b>Oct-4, Oct-3/4, Nanog, TRA-1-60, Tra-1-81, SSEA-3, and SSEA-4</b>	6, 28–30, 37, 38, 40, 50, 72, 75, 95
immunohistochemical analysis	<b>Oct3/4, Oct-4, Sox-2, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and Nanog</b> alkaline phosphatase (AP) SSEA-1 (negative staining)	28–35, 38, 46, 48–50, 68, 71
3. gene level	<b>Oct3/4, Oct-4, Sox-2, Nanog, TDGF-1, UTF-1, REX1, hTERT, ABCG2, DPPAS, CRIPTO, FOXD3, Tert1, Rex2, and DPPAS</b>	31, 37, 39, 40, 44, 46, 48, 68
4. differentiation ability	<b>embryonic body formation in vitro (EB)</b> <b>teratoma formation In Vivo</b>	2, 5, 31, 34, 45, 47, 68, 72, 75, 96, 127

<sup>a</sup> Bold genes and proteins are frequently analyzed for the characterization of hESCs and human iPSCs.



**Figure 3.** Morphology and expression of pluripotent markers in human iPSCs grown on a MEF feeder layer. Human iPSCs were derived from MRC-5 cells injected with Oct-4, Sox2, Klf4, and c-Myc using a retroviral vector. (A) A human iPSC colony grown on MEF. (B) Human iPSCs stained with antibodies and/or dye for (a) Oct3/4 (green), (b) Sox2 (red), (c) SSEA-4 (red), (d) TRA-1-60 (green), (e) Oct3/4 (green) + Nanog (red) + DAPI (blue), (f) Sox2 (red) + DAPI (blue), (g) SSEA-4 (red) + DAPI (blue), and (h) TRA-1-60 (green) + DAPI (blue).

Recently, several articles from both material scientists and molecular biologists have discussed the effect of culture materials on the fate of stem cells.<sup>14,25–27</sup> This review describes and discusses the use of culture materials derived from biomacromolecules and synthetic polymers that support the propagation of hESCs and human iPSCs while maintaining their pluripotency.

Figure 2 shows a schematic representation of the culture methods discussed in detail in this review: (a) cells cultured on two-dimensional (2D) materials coated with ECM or other biomacromolecules, (b) cells cultured on 2D materials prepared from synthetic materials, (c) cells cultured in hydrogels from glycosaminoglycan or other biomacromolecules, (d) cells cultured

on porous polymeric membranes, and (e) cells cultured on three-dimensional (3D) materials. In addition, this review discusses the design and importance of cell culture materials that maintain the pluripotency of hESCs and iPSCs.

## 2. ANALYSIS OF THE PLURIPOTENCY OF HESCS AND HUMAN IPSCS

hESCs and human iPSCs display high telomerase activity and express several pluripotency surface markers, such as glycolipid stage-specific embryonic antigen 4 (SSEA-4), [28–30] tumor rejection antigen 1–60 (Tra-1–60), keratan sulfate-related antigen,<sup>28,31–33</sup> and tumor rejection antigen 1–81 (Tra-1–81),<sup>31,33–35</sup> but not glycolipid stage-specific embryonic antigen 1 (SSEA-1). SSEA-1 is expressed on mouse ESCs.<sup>1,2,6,31</sup> hESCs also show high expression levels of specific pluripotency genes, such as Oct3/4,<sup>32,36,37</sup> Oct-4 (POUSF1, POU domain transcription factor),<sup>28,38,39</sup> Nanog,<sup>39,40</sup> Sox-2,<sup>40,41</sup> Rex-1,<sup>39,42</sup> and hTERT, the catalytic component of telomerase.<sup>39,43,44</sup> Table 1 summarizes the characteristics of pluripotent hESCs.

The pluripotency of hESCs and human iPSCs is evaluated based on (a) the colony morphology by microscopy,<sup>14,31,34,45</sup> (b) the expression of pluripotency genes by RT-PCR and qRT-PCR measurements,<sup>31,40,44,46</sup> and (c) the expression of pluripotent proteins by flow cytometry<sup>6</sup> and immunofluorescence analyses<sup>31,38</sup> (Table 1). Pluripotent hESCs and human iPSCs generate colonies with spherical cells. Figure 3 shows a typical example of a colony of iPSCs. Differentiated hESCs and human iPSCs have small, coagulated, or fibroblast-like morphologies.<sup>47</sup> Once hESCs and human iPSCs have differentiated, the cells expand from the differentiated stem cells and cannot be used as a source of stem cells in clinical or research applications.

The expression of pluripotency genes, such as Oct3/4, Oct-4, Sox-2, Nanog, Rex-1, hTERT, Tra-1-60, Tra-1-81, SSEA-3, and SSEA-4, is generally analyzed by RT-PCR and/or qRT-PCR methods (Table 1). The expression of pluripotency proteins, including Oct-4, alkaline phosphatase (AP),<sup>48–50</sup> SSEA-4, Tra-1-60, and Tra-1-81, is analyzed by immunofluorescence or flow cytometry analysis with specific antibodies. The mRNA expression level of a pluripotency gene does not directly relate to the expression level of the corresponding pluripotency protein due to regulation by interference RNA, such as microRNA (miRNA).<sup>51,52</sup> Figure 3 also shows the typical expression patterns of pluripotency proteins in human iPSCs.

The analysis of pluripotency genes and proteins through RT-PCR and/or qRT-PCR analysis and immunofluorescence and/or flow cytometry analysis, respectively, is important to verify the pluripotency of hESCs and human iPSCs. The difference between hESCs and human iPSCs and adult or fetal stem cells, such as bone marrow-derived stem cells (mesenchymal stem cells),<sup>53,55</sup> adipose-derived stem cells,<sup>56,57</sup> and amniotic fluid stem cells,<sup>58,59</sup> is the ability to differentiate into cells of all three germ layers (endoderm, mesoderm, and ectoderm).<sup>30,47,60,61</sup> Mesenchymal stem cells and other adult and fetal stem cells primarily differentiate into cells from the mesoderm, such as osteoblasts, chondrocytes, and adipocytes, although several exceptions have been reported.<sup>62–65</sup> Therefore, the ability to differentiate into cells from all three germ layers is also used to evaluate the pluripotency of hESCs and human iPSCs. hESCs and human iPSCs can generate embryonic bodies (EB) when cultured on untreated polystyrene dishes in differentiation medium, which includes three germ layers or tissue.<sup>35,45,66</sup> The formation of teratomas that include all three germ layers is also

used to evaluate the pluripotency of hESCs and human iPSCs by injecting hESCs and human iPSCs into immunodeficient mice, such as mice with severe combined immunodeficiency (SCID).<sup>2,45,67</sup> Table 2 summarizes the characterization methods used to analyze the ability of cells to differentiate into all three germ layers in both EB and teratomas.

The ability to differentiate into cells from all three germ layers in EB and teratomas is analyzed in several ways: (1) observation of tissue that includes all three germ layers [the epithelial component (endoderm (E)), renal tissue (E), intestinal mucosa (E), cartilage (mesoderm (M)), bone (M), muscle (M), chondrocyte (M), mesenchymal tissue (M), the neural component (ectoderm (EC)),<sup>201</sup> and the epidemial component (EC)],<sup>2,5,31,34,47,68</sup> (2) expression of differentiated genes, including endoderm genes [ $\alpha$ -fetoprotein (AFT), SOX17, amylase, albumin, FOXA1 (HNF3 $\alpha$ ), GATA6, and PDX1], mesoderm genes (Brachyury T,  $\beta$ -globin, MIX-LIKE-1, Hand1, and Msx1), and ectoderm genes [ $\beta$ III-tubulin, SOX1, neurofilament heavy chain (NFH), keratin 15, neural progenitor markers PAX6 and NeuroD, and Nestin)], by RT-PCR and/or qRT-PCR,<sup>38,40,49,50,69,70</sup> and (3) the expression of differentiated proteins, including endoderm-related proteins [AFP, cytokeratin 19 (CK19), glucagons, and albumin], mesoderm-related proteins (actin,  $\alpha$ -actinin, cTnI, and Brachyury T), and ectoderm-related proteins [ $\beta$ -III tubulin, enolase, nestin, and glial fibrillary acidic protein (GFAP)].<sup>30,38,40,71,72</sup> Figure 4 shows typical examples of the histochemical analysis of teratomas with cells from all three germ layers.

hESCs proliferate continuously under the appropriate conditions and are able to differentiate into all types of somatic cells from all three germ layers *in vivo* and *in vitro*.<sup>2</sup>

## 3. CELL-FREE CULTURE OF HESCS ON BIOMATERIALS MAINTAINS PLURIPOTENCY

hESCs and human iPSCs are currently cultured on MEFs as a feeder layer to maintain the pluripotency and self-renewing characteristics. hESCs and human iPSCs can be cocultured with MEFs for extended periods of time without undergoing differentiation. However, if the MEFs are removed and hESCs and human iPSCs are cultured under normal culture conditions, differentiation into many somatic cell types is triggered.<sup>2,14,73</sup>

However, concerns over the cross-species transfer of viruses<sup>14,28,35,74</sup> and immunogenic epitopes, such as *N*-glycolylneuraminic acid (Neu5Gc),<sup>28,74</sup> have prompted the investigation of xeno-free culture and cell-free culture in recent years. As an alternative to cocultures of hESCs with MEFs, several isolated ECM or cell adhesion molecules that support hESC attachment and proliferation have been evaluated. Tables 3 and 4 summarize the feeder layer-free culture of hESCs and human iPSCs on biomacromolecules and synthetic polymers. The addition of a high concentration of basic FGF (bFGF, FGF-2) is necessary for the culture of hESCs and human iPSCs in the absence of a feeder layer and/or without a conditioned medium from MEFs. It has also been suggested that inhibition of the BMP signaling pathway plays a significant role in the molecular mechanism of hESC self-renewal.<sup>14,75,76</sup> FGF-2 signaling is critical for the self-renewal of hESCs, and the transforming growth factor beta (TGF- $\beta$ ) signaling pathways are necessary for preventing differentiation.<sup>77</sup> Therefore, hESCs and human iPSCs require FGF-2 for self-renewal. At the same time, it is necessary to block BMP signaling to maintain the phenotype.<sup>77</sup> The addition of FGF-2 and Activin A/Nodal to serum-free media increases the expression of pluripotency markers compared with Activin A/Nodal alone, while FGF-2 alone is

Table 2. Characterization of Differentiation Ability to Three Germ Layers in EB and Teratoma<sup>a</sup>

characterization	specification (examples)	ref
1. morphology	Von Kossa staining (calcification)	30, 68
	Picrosirius staining for collagen, blood vessels, etc.	
	Alizarin Red staining (calcification)	
	Alcian blue/Van Giesson's staining haematoxylin and eosin staining	
	(a) endoderm differentiation columnar epithelia with goblet cell, primitive epithelium stained with cytokeratin 18 antibody, respiratory epithelium, gut epithelium, epithelial, intestinal mucosa, intestinal epithelium, pigmented epithelium, and renal tissue	2, 5, 31, 34, 45, 47, 68, 75, 96
	(b) mesoderm differentiation hyaline cartilage, muscle, cartilage, bone, smooth muscle, striated muscle, mesenchymal tissue, smooth muscle stained with actin antibody, and chondrocytes	2, 5, 31, 34, 45, 47, 68, 75, 127
	(c) ectoderm differentiation neural rosettes, neural epithelium, neuroectoderm, neuronal tissue stained with neurofilament 200 K antibody, peripheral Schwann cells, embryonic ganglia, stratified squamous epithelium, epithelium, and neural tubes	2, 5, 31, 34, 47, 68, 72, 75, 127
	2. protein level immunohisto chemical analysis	(a) endoderm differentiation AFP, Glucagon, pdx-1, HNF3 $\beta$ , CK19, glucagon, NFH, GFAP, IFABP, albumin, Titf1, TTF-1, and FOXa2
(b) mesoderm differentiation FOXA2, cTnN, $\alpha$ -SMA, brachyury, vimentin, $\alpha$ -actin, $\alpha$ -actinin, muscle actin, actin, BMP-4, and cTnI		28, 30, 38, 71, 72, 88, 127
(c) ectoderm differentiation NCAM, Tuj1, neurofilament, $\beta$ III-tubulin, GFAP, enolase, and nestin		28, 30, 38, 40, 48, 71, 72, 75, 128
surface marker		VEGFR2 (mesoderm), PDGFR $\alpha$ (mesoderm), and CXCR4 (endoderm)
3. gene level	(a) ectoderm gene expression SOX-1, PAX6, Nestin, NES, Tuj1, MAP2, NeuroG1, TUBB3, $\beta$ III-tubulin, NeuroD, NOG, NEFL, keratin, Keratin 8, Keratin 18, Keratin 15, NFH, and neurofilament (NF)-68	28, 32, 33, 33, 38, 40, 46, 48, 50, 69, 70, 76, 88
	(b) endoderm gene expression AFP, cerberus, GATA3, GATA4, GATA6, SOX17 (G3, G16, A17, A14, A1), ONECUT1, FOXA2 (A17G101), IPF1, FOXA1, PROX1, HHEX, ALB, HNF3b, HNF4a, Albumin, PDX1, amylase, TTF-1, IFABP, and Titf1	28, 33, 38, 46, 48, 50, 69, 70, 73, 76, 88
	(c) mesoderm gene expression brachyury T, Hand1, IGF2, FLK1, MIXL1, MESP1, EOMES, PAX3, MYOD1, PECAM1, NKX2, GATA1, GATA2, GATA4, KDR, BMP4, SIL, HOXB4, MyoD, Msx1, C-actin, $\beta$ -globin, $\alpha$ -cardiac actin, cardiac actin, VE-cadherin, enolase, MtoD, and CD31	28, 32, 33, 38, 46, 48, 50, 69, 70, 88
	(d) cardiomyocyte differentiation Nkx2.5, GATA-4, MYH-6, TNNT2, TBX-5, Mlc2a, MLC-2 V, tropomyosin, cTnI, ANP, desmin, $\alpha$ -MHC, $\beta$ -MHC, cTnT, Isl-1, and Mef2c	33, 40, 47
(e) hepatocyte differentiation	AFP, albumin, and TAT	
(f) neural differentiation	Nestin, Musashi 1, Tuj1, astrocytes (GFAP), and oligodendrocytes (myelin basic protein)	

<sup>a</sup> NCAM, neural cell adhesion molecule; cTnT, cardiac Troponin-T; FOXA2, forkhead box 2;  $\alpha$ -SMA, alpha smooth muscle actin (K15); pdx-1, pancreatic marker; Tuj1,  $\beta$ -III-tubulin (neuronal marker); AFP,  $\alpha$ -fetoprotein; NFH, neuro-filament heavy chain; GFAP, glial fibrillary acidic protein; cTnI, cardiac troponin I.

insufficient to maintain pluripotency marker expression.<sup>77</sup> FGF-2 induces the expression of hESC supportive factors, and Smad2/3

activation (TGF- $\beta$  pathway) is required for hESCs to maintain pluripotency.<sup>77</sup> In addition to soluble factors in the culture medium,