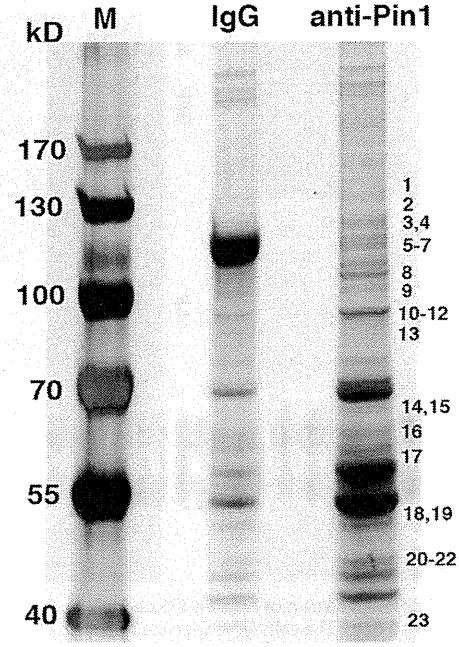
A




B

| No. | Accession No. | Gene description | Predicted size |
|---|---|---|---|
| 1 | ADCY5_HUMAN | Adenylate cyclase type 5 | 138818 |
| 2 | CCD40_HUMAN | Coiled-coil domain-containing protein 40 | 130033 |
| 3 | PK3CA_HUMAN | Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha | 124203 |
| 4 | ZEB1_HUMAN | Zinc finger E-box-binding homeobox 1 | 123997 |
| 5 | VINC_HUMAN | Vinculin | 123722 |
| 6 | RADIL_HUMAN | Ras-associating and dilute domain-containing protein | 117351 |
| 7 | UBP2L_HUMAN | Ubiquitin-associated protein 2-like | 114465 |
| 8 | DSG1_HUMAN | Desmoglein-1 | 113644 |
| 9 | ENPP3_HUMAN | Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 | 100059 |
| 10 | ZN337_HUMAN | Zinc finger protein 337 | 86819 |
| 11 | NASP_HUMAN | Nuclear autoantigenic sperm protein | 85186 |
| 12 | ZY11B_HUMAN | Protein zyg-11 homolog B | 83921 |
| 13 | MPEG1_HUMAN | Macrophage-expressed gene 1 protein | 78587 |
| 14 | FA13C_HUMAN | Protein FAM13C | 65687 |
| 15 | VPS45_HUMAN | Vacuolar protein sorting-associated protein 45 | 65036 |
| 16 | ANR53_HUMAN | Ankyrin repeat domain-containing protein 53 | 59493 |
| 17 | RPA34_HUMAN | DNA-directed RNA polymerase I subunit RPA34 | 54951 |
| 18 | VIME_HUMAN | Vimentin | 53619 |
| 19 | KCAB1_HUMAN | Voltage-gated potassium channel subunit beta-1 | 46534 |
| 20 | PRS8_HUMAN | 26S protease regulatory subunit 8 | 45597 |
| 21 | FKBP8_HUMAN | Peptidyl-prolyl cis-trans isomerase FKBP8 | 44534 |
| 22 | PO5F1_HUMAN | POU domain, class 5, transcription factor 1 (OCT4) | 38571 |
| 23 | THAP1_HUMAN | THAP domain-containing protein 1 | 24928 |

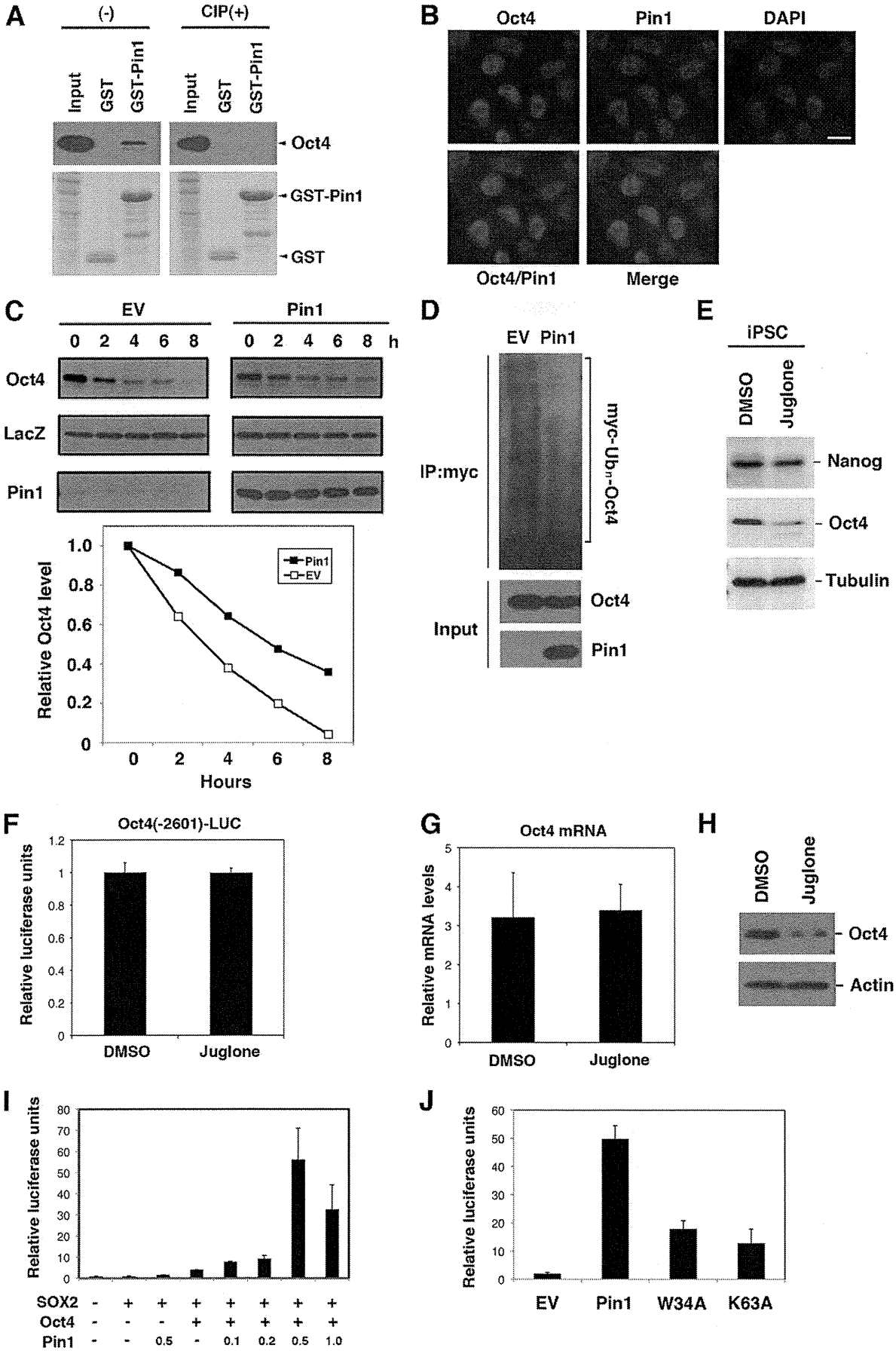
FIGURE 5. **Identification of Pin1-binding proteins in human iPS cells.** *A* and *B*, lysates of human iPS cells were subjected to immunoprecipitation with either non-immunized control mouse IgG (IgG) or mouse anti-Pin1 monoclonal antibodies. Proteins bound to protein A/G-agarose beads were isolated, resolved by SDS-PAGE, and detected by silver staining (*A*). *M* indicates protein marker. Excised gel bands were digested with trypsin and analyzed on a linear ion trap (LTQ) Orbitrap hybrid mass spectrometer followed by peptide mass fingerprinting with the Mascot and Aldente search algorithms (*B*).

that Pin1 can bind only phosphorylated Ser/Thr-Pro motifs (17, 27) of which only one ($Ser^{12}$-Pro) exists between residues 1 and 34 in the Oct4 protein. Interestingly, this motif is conserved between various species including human, mouse, rat, and rabbit (Fig. 7*B*). We generated an Oct4 site-directed mutant at this site by substituting serine 12 for alanine (S12A). GST pulldown analysis subsequently revealed that Pin1 binds wild-type Oct4, but not its S12A mutant (Fig. 7*C*). These results confirm that Pin1 indeed bind the phosphorylated $Ser^{12}$-Pro motif of Oct4.

To further examine the functional interactions between Pin1 and Oct4 on this site, we next investigated the nature of the S12A mutant in terms of its protein expression in the presence of Pin1. HeLa cells were transfected with either wild-type Oct4 or its S12A mutant and co-transfected with Pin1. This was followed by immunoblotting analysis. We found that Pin1 increased the expression levels of wild-type Oct4, but not the S12A mutant (Fig. 7*D*).

## DISCUSSION

In our present study, we report that Pin1 is an essential regulator of the self-renewal and maintenance of pluripotent stem cells. We further found the following: 1) Pin1 is induced upon the induction of human iPS cells; 2) the co-expression of Pin1 with defined reprogramming factors significantly enhances the



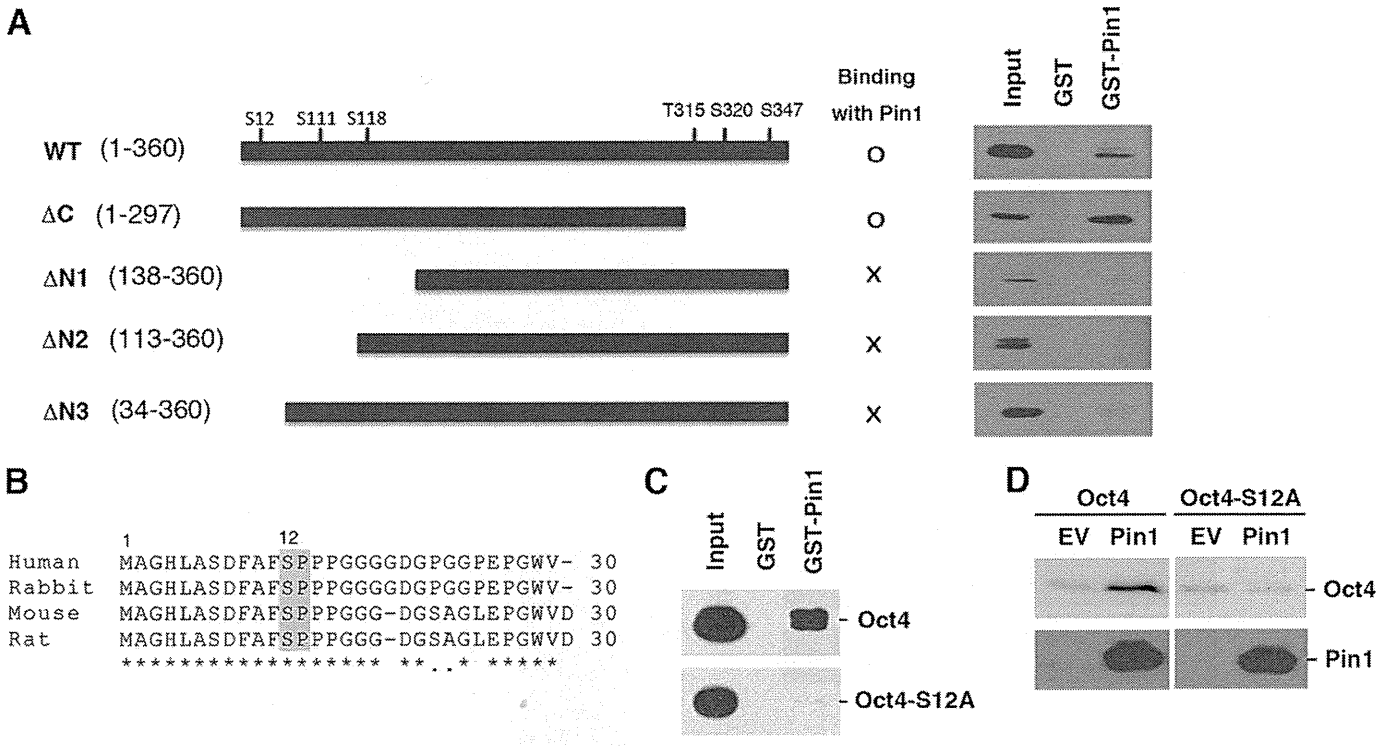



FIGURE 7. **Pin1 interacts with the Ser[12]-Pro motif of Oct4.** *A*, schematic representation of the Oct4 deletion mutants generated in this study (*left panel*). HeLa cells were transfected with the indicated Oct4 deletion mutants for 24 h. Cell lysates were then prepared and subjected to GST pulldown analysis with either GST or GST-Pin1 followed by immunoblotting analysis with Oct4 antibodies (*right panel*). *B*, amino acid sequence alignment of the human, rabbit, mouse, and rat Oct4 proteins. The conserved Ser[12]-Pro motifs are *boxed*. *C*, HeLa cells were transfected with the Oct4 site-directed mutant Oct4-S12A and subjected to GST pulldown analysis. *D*, HeLa cells were transfected with wild-type Oct4 or its S12A mutant with or without Pin1. After 24 h, the cells were subjected to immunoblotting analysis with an anti-Oct4 antibody.

frequency of iPS cell induction; 3) the blockade of Pin1 significantly inhibits the colony formation of dissociated human iPS cells and murine ES cells; 4) Pin1 inhibition leads to the aberrant cell differentiation in human iPS cells and murine ES cells after forming colonies; 5) Oct4 is a putative Pin1 substrate in human iPS cells; and 6) Pin1 interacts with Oct4 at its Ser[12]-Pro motif and facilitates its stability and enhanced transcriptional activity. Our findings thus uncover a novel role of Pin1 as a putative regulator of the self-renewal and survival of pluripotent stem cells via Oct4 function.

Our current results add to previous findings indicating that Pin1 is a multifunctional protein that mediates various phosphorylated proteins involved in divergent cellular processes (17). This implicates Pin1 as a modulator of multiple signaling pathways depending on the cell type and biological context. Indeed, we demonstrate in our present study that Pin1 is a crucial regulator of the phosphorylation-dependent intracellular signaling network that controls cellular stemness and pluripotency. Moreover, iPS cells induced by the expression of four Yamanaka factors (Oct4, SOX2, Klf4, and c-Myc) led to a high expression level of Pin1, and these cells were found to be dependent on Pin1 function. This suggests that Pin1 could be one of the crucial factors in the induction of iPS cells from somatic cells that functions by cooperating with reprogramming transcription factors.

FIGURE 6. **Pin1 interacts with phosphorylated Oct4 and enhances its transcriptional activity.** *A*, human iPS cell lysates treated or untreated with calf intestine alkaline phosphatase were subjected to GST pulldown analysis with either GST or GST-Pin1, followed by immunoblotting analysis with anti-Oct4 antibody (*upper panel*). Coomassie staining for the GST or GST-Pin1 used in the assay is shown in the *lower panel*. *B*, human iPS cells were fixed with 4% paraformaldehyde and then co-immunostained with monoclonal antibodies against Oct4 (*green*) and polyclonal antibodies against Pin1 (*red*). Cells were then analyzed by confocal microscopy. *Scale bar*, 10 $\mu$m. *C*, HeLa cells transfected with the indicated vectors and HA-LacZ cells were treated with cycloheximide and harvested at the indicated time points. This was followed by immunoblotting analysis with Oct4, Pin1, and HA antibodies (*upper panel*). Quantitative data are shown in the *lower panel*. *D*, HeLa cells were transfected with Myc-tagged ubiquitin, Oct4, and co-transfected with either empty vector (*EV*) or Pin1. Cells were then treated with MG-132 for 12 h, and lysates were prepared and immunoprecipitated with anti-Myc antibody followed by immunoblotting analysis with anti-Oct4 antibody. Total cell lysates prior to immunoprecipitation (input) were immunoblotted with anti-Pin1 or anti-Oct4 antibody. *E*, human iPS cells were plated on Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (20 $\mu$M) for 24 h. Cell lysates were then processed for immunoblotting analysis with anti-Nanog, anti-Oct4, or anti-tubulin antibodies. *F*, a plasmid containing the luciferase (*LUC*) gene flanked with 2601 bp of the Oct4 5′-upstream region was transfected into murine ES cells. The resulting cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 $\mu$M) for 24 h, and analyzed by gene reporter assay. *G*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 $\mu$M) for 24 h. Total RNAs were then extracted and reverse-transcribed. These preparations were then subjected to quantitative RT-PCR analysis for Oct4. The transcript levels were normalized using GAPDH. *H*, murine ES cells were cultured in Matrigel-coated feeder-free dishes and treated with either DMSO or juglone (10 $\mu$M) for 24 h. Cell lysates were then processed for immunoblotting analysis with either anti-Oct4 or anti-$\beta$-actin antibody. *I*, HeLa cells were transiently transfected with plasmids encoding Oct4, SOX2, or Pin1 and co-transfected with Oct-SOX reporter gene and pRL-CMV. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay. *J*, HeLa cells were transiently transfected with an Oct-SOX reporter gene and co-transfected with plasmids encoding wild-type Pin1 or its W34A or K63A mutants, together with Oct4 and SOX2. At 24 h post-transfection, the cells were collected and subjected to a gene reporter assay.

The molecular mechanisms underlying the regulation of Pin1 in the induction and maintenance of pluripotency are likely to be highly complex given that Pin1 interacts with multiple substrates in pluripotent stem cells, as revealed by our proteomics analysis. However, our current findings also indicate that Pin1 is involved in the growth and maintenance of pluripotency in stem cells through its phosphorylation-dependent prolyl isomerization of substrates such as Oct4. In this regard, a recent report by Moretto-Zita *et al.* (30) has demonstrated that Pin1 can also associate with another pluripotent transcription factor, Nanog, in murine ES cells and sustain the self-renewal and teratoma formation of these cells in immunodeficient mice. These results indicate that Pin1 is a crucial modulator of the transcription factor network governing cellular stemness. It is possible also that Pin1 could regulate this process by modulating the function of other substrates. Further studies of Pin1 function in stem cells at various stages might shed new light on the underlying molecular pathways and factors that control self-renewal and multipotency.

It has been demonstrated that Pin1 knock-out mice develop normally but display some proliferation abnormalities, including a decreased body weight, retinal degeneration, and impaired mammary gland development (31, 32). Pin1 knock-out mice also exhibit testicular atrophy with a significantly impaired proliferation of primordial germ cells and the progressive loss of spermatogenic cells (33). These phenotypes can now be attributed to the impaired maintenance and proliferation of germ-related stem cells due to the loss of Pin1 function.

In many circumstances, Pin1 acts as either a repressor or an enhancer of the degradation of substrate proteins (15–17, 34). Our current data now additionally demonstrate that Pin1 can also prolong the protein half-life of Oct4, thereby enhancing its transcriptional activity. Oct4 has been shown to be regulated by post-translational modifications such as SUMOlylation (35). Our current findings reveal that Oct4 is also regulated by phosphorylation and subsequent prolyl isomerization. Identification of the kinase(s) responsible for the association of Pin1 and Oct4 will enhance our understanding of the regulatory pathways that operate during and after the induction of pluripotency.

It is desirable to utilize pluripotent stem cells such as iPS cells for future regenerative medicine applications. However, there are already concerns surrounding the use of iPS cells in a clinical setting because prior studies have suggested that they are likely to develop cancers (4, 36). Our current findings suggest, however, that the Pin1 inhibition could effectively block the proliferation of iPS cells in an undifferentiated state. Pin1 could therefore act as a molecular switch that can reversibly control the proliferation and survival of iPS cells, thereby reducing the risk of cell transformation and tumor formation.

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Genes to Cells

# Lectin microarray analysis of pluripotent and multipotent stem cells

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**Stem cells have a capability to self-renew and differentiate into multiple types of cells; specific markers are available to identify particular stem cells for developmental biology research. In this study, we aimed to define the status of somatic stem cells and the pluripotency of human embryonic stem (hES) and induced pluripotent stem (iPS) cells using a novel molecular methodology, lectin microarray analysis. Our lectin microarray analysis successfully categorized murine somatic stem cells into the appropriate groups of differentiation potency. We then classified hES and iPS cells by the same approach. Undifferentiated hES cells were clearly distinguished from differentiated hES cells after embryoid formation. The pair-wise comparison means based on 'false discovery rate' revealed that three lectins -*Euonymus europaeus* lectin (EEL), *Maackia amurensis* lectin (MAL) and *Phaseolus vulgaris* leucoagglutinin [PHA(L)]- generated maximal values to define undifferentiated and differentiated hES cells. Furthermore, to define a pluripotent stem cell state, we generated a discriminant for the undifferentiated state with pluripotency. The discriminant function based on lectin reactivities was highly accurate for judgment of stem cell pluripotency. These results suggest that glycomic analysis of stem cells leads to a novel comprehensive approach for quality control in cell-based therapy and regenerative medicine.**

## Introduction

Stem cells produce almost every tissue of the human body. In general, they have the ability to divide and self-renew and to differentiate into various cell types. Stem cells have varying degrees of differentiation potential: (i) totipotency (ability to form the embryo and the trophoblast of the placenta) like fertilized eggs (zygotes); (ii) pluripotency (ability to differentiate into almost all cells that arise from the three germ layers) like human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells; (iii) multipotentiality (capability of producing a limited range of differentiated cell lineages upon their location) like most tissue-based stem cells; and (iv) unipotentiality (ability to generate one cell type) like cells such as the epidermal stem cells and the spermatogonial cells of the testis. That is, a hierarchy of stem cells exists. In addition, human ES cell lines show variation in differentiation propensity (Osafune *et al.* 2008). iPS cells, another type of pluripotent stem cell, have been generated from somatic cells of different origin by retroviral transduction of four transcription factors (Takahashi *et al.* 2007; Yu *et al.* 2007). The established iPS cells have a wider variety of differentiation ability and gene expression when compared to ES cells (Aoi *et al.* 2008; Lee *et al.* 2009; Kaichi *et al.* 2010). However, a small proportion of these stem cells sometimes show spontaneous differentiation during serial passage. Therefore, to realize the potential for iPS cells to be utilized for cell therapy and as a valuable tool for drug discovery, it is necessary to monitor the status of these stem cells and to define

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their exact stage during processes of growth and/or differentiation.

Glycosylation is a critical post- or co-translational modification found in more than 50% of eukaryotic proteins (Budnik *et al.* 2006). Thus, the glycome, which represents the total set of glycans expressed in a cell, is believed to be information-rich, as it varies among cell types, stages of development and differentiation, and even in the malignant transformation processes (Varki 1993). Lectins have long been used as tools to characterize cell surface glycans, such as for blood-group typing, tissue staining, lectin-probed blotting and flow cytometry (Sharon & Lis 2004). The use of lectins in glycan profiling provides considerable advantages. A modern technology to discriminate glycan profiling is lectin microarray analysis, which is an emerging technology that enables ultrasensitive detection of multiplex lectin–glycan interactions (Angeloni *et al.* 2005; Kuno *et al.* 2005; Pilobello *et al.* 2005). The system developed by Kuno *et al.* (2005) is based on a unique principle, that is, the evanescent-field fluorescence-detection principle, which has been used extensively for biosensors to study real-time binding events on the glass slide surfaces. Thus, the evanescent-field methods have greater advantage to analyze relatively weak interactions between lectins and glycoproteins in a liquid phase at equilibrium. Furthermore, this method is applicable for the analysis of the physiological and pathological status of crude glycoproteins extracted from mammalian cells (Ebe *et al.* 2006; Kuno *et al.* 2008) and cell surfaces (Tateno *et al.* 2007). Although the number of probes in lectin microarray is much smaller than in mRNA expression arrays, lectin microarray analysis enables high-throughput and sensitive analysis of a large set of biological samples and provides a snapshot of cell profiling. In this study, we further developed lectin microarray technology to define the status of somatic and pluripotent stem cells. The glycan-based comprehensive approach promises to be of great value, complementing more established methods such as gene expression analysis and epigenetic analysis.

## Results

### Lectin microarray analysis of mouse mesenchymal cells

Mesenchymal stem cells are multipotent and therefore may be useful in cell-based therapy along with ES cells and iPS cells. Mesenchymal stem cell (MSC) lines [(9-15c), osteoblasts (KUSA-A1), chondroblasts (KUM5) and preadipocytes (H-1/A)] were established from mouse bone marrow and were shown to retain potency both *in vivo* and *in vitro* (Umezawa *et al.* 1991; Matsumoto *et al.* 2005; Sugiki *et al.* 2007). To investigate their carbohydrate structures, we carried out a lectin microarray analysis of the cell membrane proteins. We quantified lectin signal using 'Array-Pro Analyzer' software and calculated the average net intensities of three spots for each lectin on the chip (Fig. 1A). Experiments with each cell line were performed in triplicate or quadruplicate. Four mesenchymal cell lines with different potencies showed differential lectin reactivities. 9-15c MSCs showed strong reactivity to wheat germ agglutinin (WGA), *Lycopersicon esculentum* lectin (LEL), concanavalin A (ConA), *Sambucus nigra* agglutinin (SNA) and *Ricinus communis* agglutinin I (RCA120) (Fig. 1A and Fig. S1 in Supporting Information). These signal intensities by lectin microarray were consistent with mean fluorescent intensities by flow cytometric analysis (Fig. 1B). We then performed hierarchical clustering analysis and principal component analysis (PCA) on the signal values of each lectin (Fig. 1C, D). H-1/A preadipocytes can be distinguished by KUM5 chondroblasts by lectin reactivities of GSL1A4, GSL1B4, BPL, PWM and MPA (PC1 axis), and 9-15c MSCs can be distinguished by KUSA-A1 osteoblasts by SNA. These cell types were reproducibly categorized into independent distinct groups.

### Lectin microarray analysis of human mesenchymal cells

Human MSCs harvested from a variety of tissues have the capability to differentiate into numerous tissue lineages despite the fact that they may have tissue-specific characteristics. To clarify relationship between the tissue-specific characters of mesenchymal cells and glycomics, we performed lectin microarray analysis (LecChip™: Fig. S1 in Supporting Information) of mesenchymal cells derived from various tissues (Fig. 2A). Signal intensities by lectin microarray were consistent with the mean fluorescent intensities analysis determined by flow cytometric analysis (Fig. 2B). Hierarchical clustering analysis showed that human embryonic carcinoma NCR-G3 cells were reproducibly categorized into an independent group (red color in Fig. 2C), which is distinct from a group of mesenchymal cells derived from a variety of tissues (green color in Fig. 2C). In mesenchymal cells, bone marrow-, placenta- and extra finger-derived mesenchymal cells were categorized into distinct groups labeled in yellow, orange and blue, respectively (Fig. 2C).

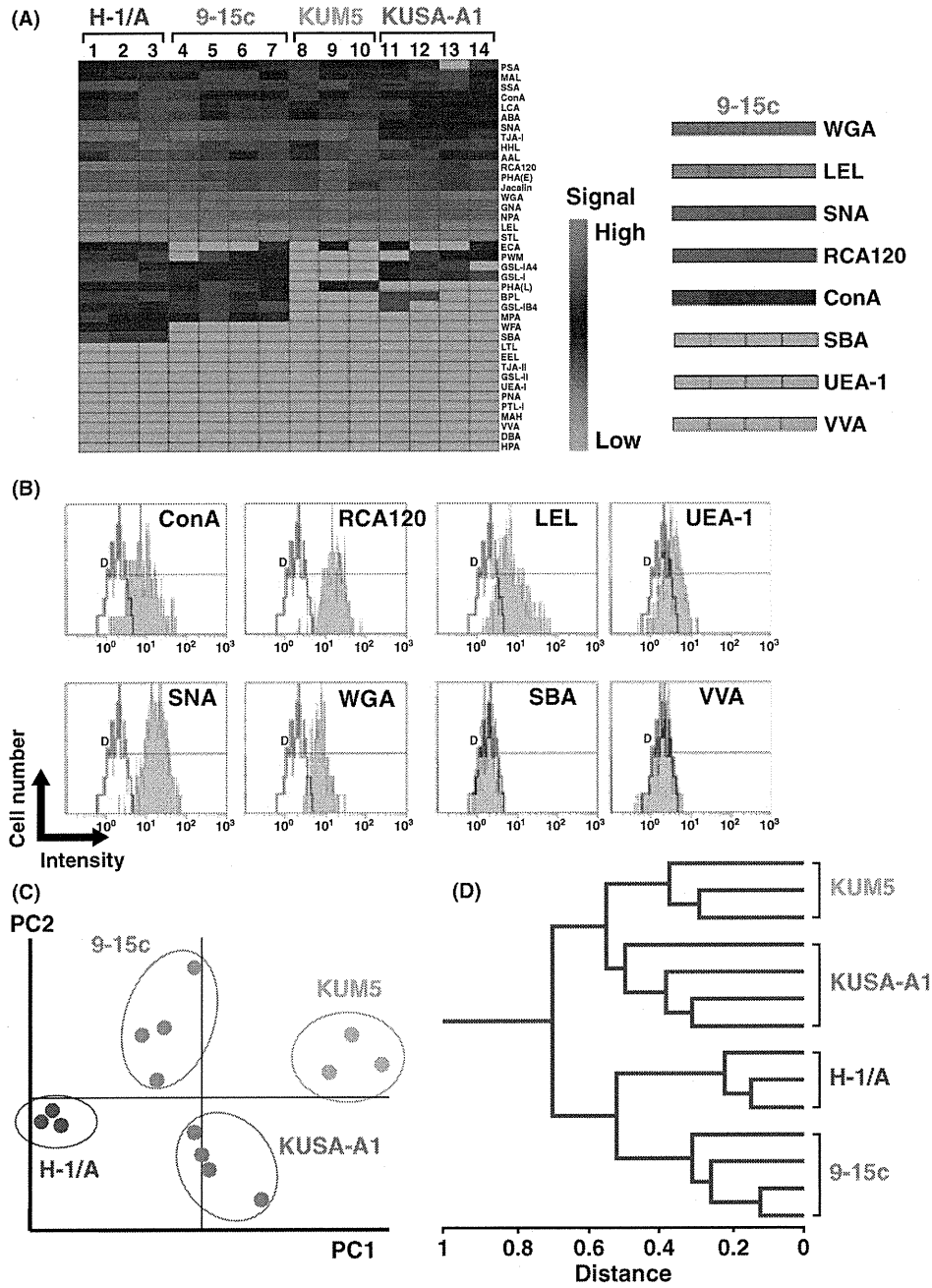
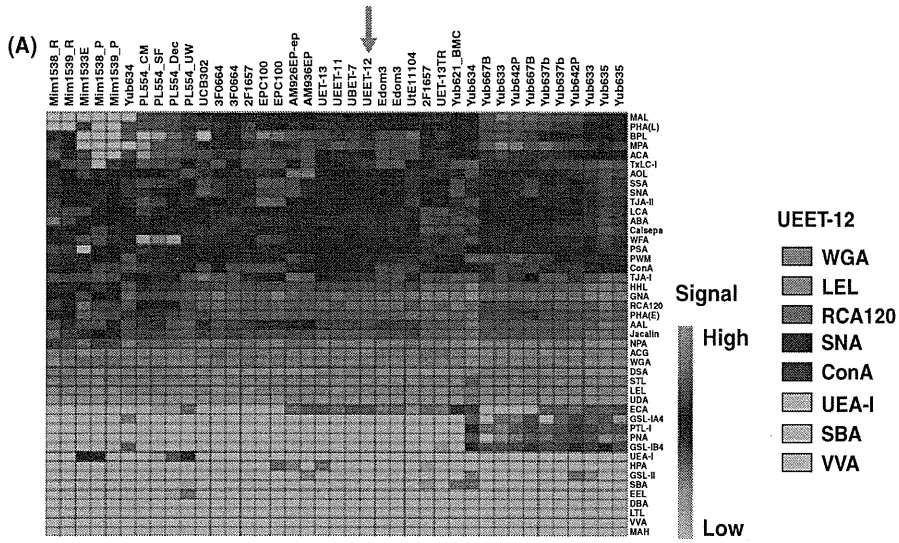
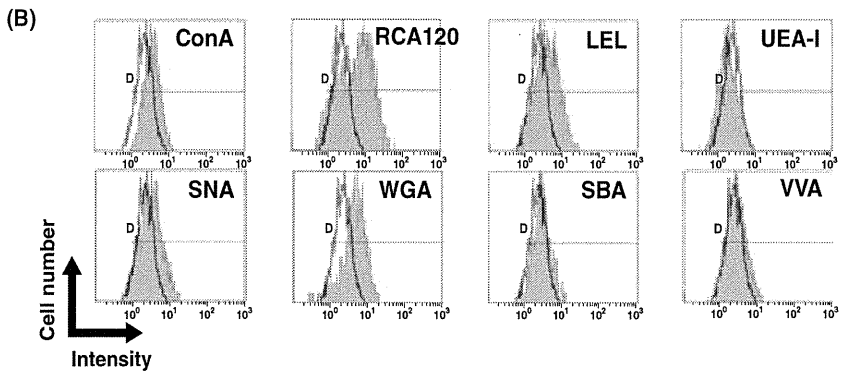
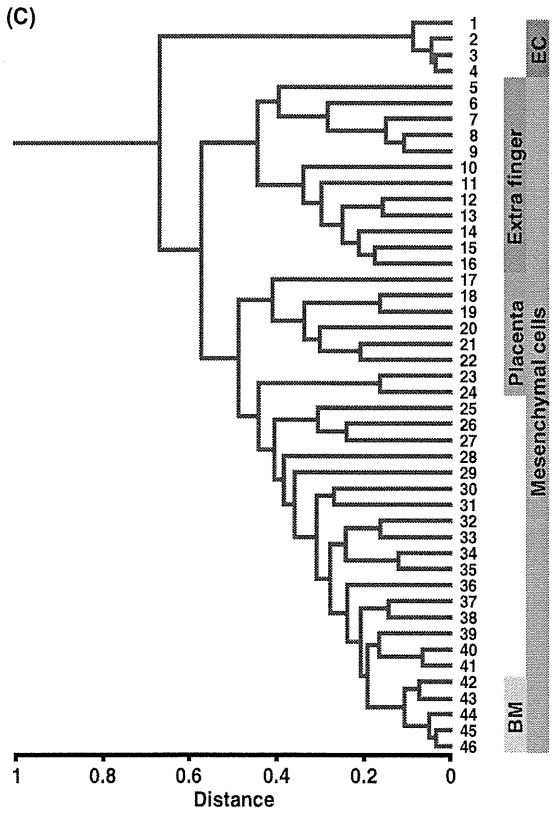



**Figure 1** Lectin microarray analysis of mouse mesenchymal cells. (A) Heat map of 9-15c multipotent cells, KUSA-A1 osteoblasts, KUM5 chondroblasts and H-1/A preadipocytes. (B) Flow cytometric analysis of 9-15c multipotent cells using each lectin probe. Mean fluorescent intensities by flow cytometric analysis are consistent with signal intensities by lectin microarray. Nonshaded and shaded areas indicate reactivity of antibodies for isotype controls and that of antibodies for cell surface markers, respectively. (C) Principal component analysis of lectin microarray on mouse bone marrow–derived mesenchymal cells. Each cell is reproducibly subcategorized into groups of mesenchymal cell types. (D) Hierarchical clustering analysis of lectin microarray on mouse bone marrow–derived mesenchymal cells.

Human mesenchymal cells reacted to (i) *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Aspergillus oryzae* lectin (AOL) and *Aleuria aurantia* lectin (AAL) that bind to Fucα1-6GlcNAc; (ii) SNA, *Sambucus sieboldiana* agglutinin (SSA) and *Trichosanthes japonica* agglutinin I (TJA-I) that bind to




| Cell | Source |
|---|---|
| 1 NCRG3 | Human embryonal carcinoma |
| 2 NCRG3 | Human embryonal carcinoma |
| 3 NCRG3 | Human embryonal carcinoma |
| 4 NCRG3 | Human embryonal carcinoma |
| 5 Yub634 | Extrafinger |
| 6 Yub667B | Cortical bone (extrafinger) |
| 7 Yub667B | Cortical bone (extrafinger) |
| 8 Yub642P | Periosteum (extrafinger) |
| 9 Yub633 | Extrafinger |
| 10 Yub634 | Extrafinger |
| 11 Yub637B | Cortical bone (extrafinger) |
| 12 Yub635 | Extrafinger |
| 13 Yub635 | Extrafinger |
| 14 Yub637B | Cortical bone (extrafinger) |
| 15 Yub642P | Periosteum (extrafinger) |
| 16 Yub633 | Extrafinger |
| 17 PL554_CM | Villous chorion (placenta) |
| 18 PL554_SF | Chorionic plate (placenta) |
| 19 PL554_CM | Villous chorion (placenta) |
| 20 PL554_Dec | Decidua (placenta) |
| 21 PL554_SF | Chorionic plate (placenta) |
| 22 PL554_Dec | Decidua (placenta) |
| 23 PL554_UW | Decidua and uterine wall (placenta) |
| 24 PL554_UW | Decidua and uterine wall (placenta) |
| 25 UET-13TR | Marrow stromal cells |
| 26 Yub621_BMC | Bone marrow (extrafinger) |
| 27 2F1657 | Marrow stromal cells |
| 28 UCB302 | Umbilical cord blood |
| 29 EPC100 | Endometrium |
| 30 AM936EP | Amniotic membrane, epithelium |
| 31 AM926EP-ep | Amniotic membrane, epithelium |
| 32 EPC100 | Endometrium |
| 33 2F1657 | Marrow stromal cells |
| 34 3F0664 | Marrow stromal cells |
| 35 3F0664 | Marrow stromal cells |
| 36 UET-13 | Marrow stromal cells |
| 37 UET-13TR | Marrow stromal cells |
| 38 2F1657 | Marrow stromal cells |
| 39 UtE1104 | Endometrium |
| 40 Edom3 | Menstrual blood |
| 41 Edom3 | Menstrual blood |
| 42 UEET-12 | Marrow stromal cells |
| 43 UBET-7 | Marrow stromal cells |
| 44 UBET-11 | Marrow stromal cells |
| 45 UBET-11 | Marrow stromal cells |
| 46 UBET-11 | Marrow stromal cells |

**Figure 2** Lectin microarray analysis of human mesenchymal cells. (A) Heat map on human cells derived from extra finger (auricular cartilage), bone marrow, umbilical cord blood, amnion, menstrual blood and endometrium. (B) Flow cytometric analysis of UEET-12 marrow stromal cells using each lectin probe. Nonshaded and shaded areas indicate reactivity of antibodies for isotype controls and that of antibodies for cell surface markers, respectively. (C) Hierarchical clustering analysis was performed based on the results of lectin microarrays. Human embryonic carcinoma cells (NCR-G3) and mesenchymal cells are discriminated by color bars (EC: red, mesenchymal cells: green, bone marrow (BM): yellow, placenta: orange, extra finger: blue).

Siaα2-6Gal/GalNAc; (iii) *Narcissus pseudonarcissus* agglutinin (NPA), ConA, *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum hybrid* lectin (HHL), that bind to high-mannose structures; (iv) *Datura stramonium* agglutinin (DSA), LEL, *Solanum tuberosum* lectin (STL), *Urtica dioica* agglutinin (UDA), Pokeweed mitogen (PWM) and WGA that bind to GlcNAcβ1-4GlcNAc. Osteoblasts specifically reacted to *Griffonia simplicifolia* lectin I, isolectin (GSL I) A4 and its isolectin B4 that bind to α-GalNAc and α-Gal, respectively, Peanut agglutinin (PNA) that binds to Galβ1-3GalNAc and *Psophocarpus tetragonolobus* lectin I (PTL I) that binds to α-GalNAc (Fig. S1 in Supporting Information). These results suggested the lectin microarrays are a practical tool for glycan-based category of human mesenchymal cells, and that each cell type in the various cell lineages have specific carbohydrate structures.

## Lectin microarray analysis of hES cells

To study glycans during differentiation of hES cells, we performed lectin microarray analysis with extracts from undifferentiated hES cells (hES-3, 8, 9 provided from Harvard University) and differentiated hES cells after embryoid body formation (EB) (Fig. S2 in supporting Information). The lectin microarray data after statistical analysis show that undifferentiated hES cells and differentiated cells (EB) were clearly categorized (Fig. 3A). To select lectins to discriminate between ES (pluripotent) and EB (nonpluripotent) cells, we analyzed lectin signals using 'pair-wise comparison means' based on FDR (False Discovery Rate) statistics. Three lectins [MAL, PHA(L) and EEL that bind to Siaα2-3Galβ1-4GlcNAc, tri/tetra-antennary complex-type N-glycan and Galα1-3Gal, respectively] could discriminate between the individual cell populations (FDR <0.05, fold-change >2.0) (Fig. 3B). The signals of MAL and PHA(L) in hES population were lower than those in EB, whereas the EEL signal in ES was higher than that in EB (Fig. 3C, D).

## Lectin microarray analysis of iPS cells

We generated human iPS cell lines from MRC-5 embryonic lung fibroblasts (Makino *et al.* 2009) (Table S4 in Supporting Information) and performed

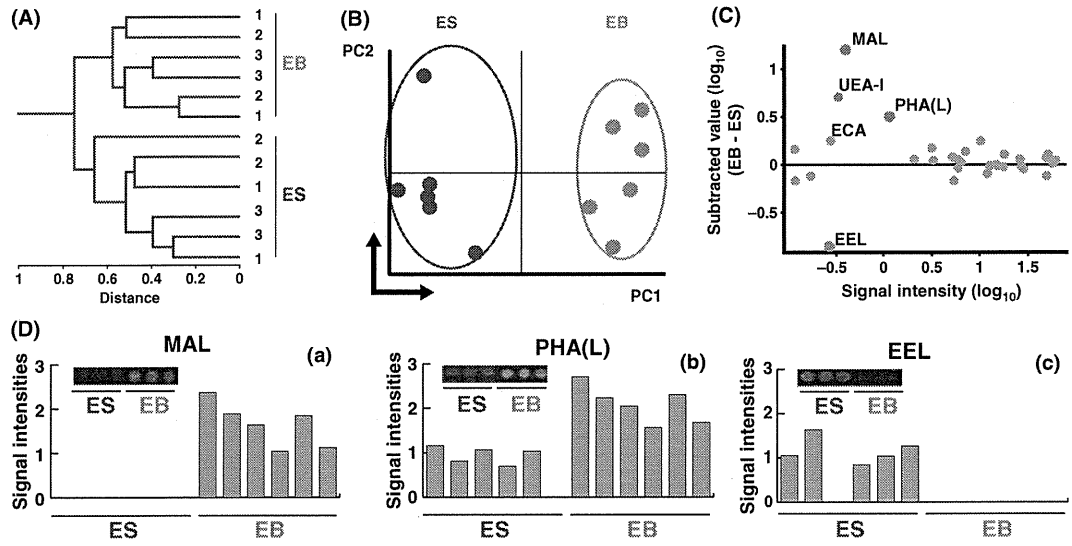



**Figure 3** Lectin microarray analysis of human embryonic stem cells. (A) Hierarchical Clustering analysis of undifferentiated and differentiated ES cells. (B) Principal component analysis of lectin microarray analysis on undifferentiated and differentiated ES cells. (C) Signal value for *Maackia amurensis* lectin (MAL) processed by a max-normalization procedure after a gain-merging process. (D) Images of signal spots and signal intensities for MAL (a), PHA(L) (b), and *Euonymus europaeus* lectin (EEL) (c).

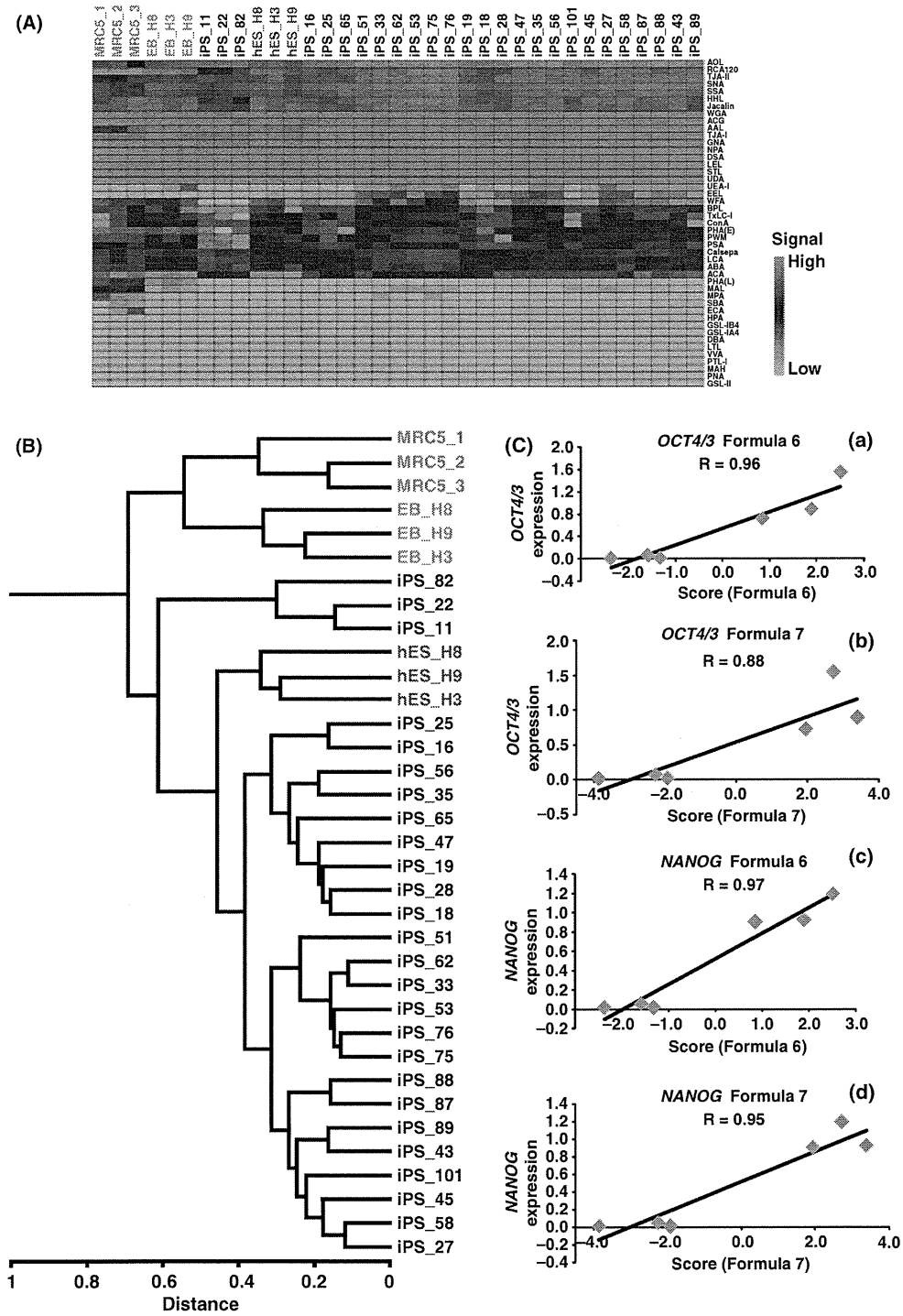



**Figure 4** Lectin microarray analysis of human-induced pluripotent stem (iPS) cells. (A) Heat map of lectin microarray with MRC-5 and MRC-5-derived iPS cells. MRC-5 and iPS cells are discriminated by letter color: red, MRC-5; blue, hES cells; green, embryoid body (EB) cells; black, iPS cells. (B) Hierarchical Clustering analysis of MRC-5 and MRC-derived iPS cells. MRC-5 and iPS cells are discriminated by letter color: red, MRC-5; blue, hES cells; green, EB cells; black, iPS cells. (C) The correlation between expression of *OCT4/3* or *NANOG* and scores calculated from each formula. The correlation factors (R) are shown in each panel.

lectin microarray analysis of these cells and their parental MRC-5 cells. The iPS cell lines were clearly distinguishable from their parental cell MRC-5 (Fig. 4A,B). We then performed the lectin microarray analysis on iPS lines and their differentiated forms. All differentiated ES cells (EB; EB_H8, EB_H9 and EB_H3) were categorized into the group including MRC-5 parental cells, and undifferentiated iPS cells were categorized into the same group with hES cells (Fig. 4B). These results suggest that glycomic analysis using lectin microarray presents a specific lectin profile for pluripotency.

### Generation of discriminant functions for pluripotency of human stem cells

To define pluripotency of human ES and iPS cells, we constructed seven formulas with the combination of the selected three lectins, MAL, PHA(L) and EEL (Table 1), using the lectin microarray data of 3 hES cells and 3 differentiated cells (EB) as a training set (Table S1 in Supporting Information). The criterion for classifying undifferentiated and differentiated from of pluripotent cells is as follows: if *Score value* is >0 or equal to 0, cells are categorized into 'pluripotent' cell population, and if *Score value* is <0, cells are categorized into 'nonpluripotent/differentiated' cell population. To evaluate the accuracy of these functions, we used the lectin microarray data of MRC-5-derived iPS cells and MRC-5 parental cells as a test set (Table 2A and Table S2 in Supporting Information). Linear discriminant function with the combination of PHA(L) and EEL (Formula 6: $F = -1.75 \times PHA(L) + 1.28 \times EEL + 1.92$) shows the highest accuracy (100%) of determination of pluripotency, followed by that of MAL and EEL (Formula 5: $F = -2.45 \times MAL + 1.23 \times EEL + 1.45$) (97%), whereas the discriminant function with the combination of three lectins (Formula 7: $F = -2.98 \times MAL + 0.75 \times PHA(L) + 1.44 \times EEL + 0.70$) and MAL and PHA(L) (Formula 4: $F = -2.81 \times MAL + 0.03 \times PHA(L) + 2.29$) shows 86.6% and 55.2%, respectively. Determination with single lectins shows 94.0% (Formula 2: $F = -2.38 \times PHA(L) + 3.46$), 55.2% (Formula 1: $F = -2.78 \times MAL + 2.32$) and 89.6% (Formula 3: $F = 2.59 \times EEL + 1.25$) accuracy. We then analyzed lectin profiles on iPS cells derived from amniotic mesoderm (Nagata *et al.* 2009) (Table 2B, Tables S3 and S5 in Supporting Information). Formula 6 with PHA(L) and EEL as variants generated the highest accuracy (100.0%) among the formulas generated. These results suggest that two lectins, EEL and PHA(L), are most suitable to determine pluripotency of stem cells. To investigate if scores calculated from each formula are correlated with 'pluripotency', we performed RT-PCR analysis of stem cell-specific genes. Positive correlations were observed between the scores and expression of the *OCT4/3* and *NANOG* genes (Fig. 4C).

**Table 1** Discriminant functions

| No. | Combination of lectins | Formula |
|---|---|---|
| 1 | MAL | $F = -2.78 \times MAL + 2.32$ |
| 2 | PHA(L) | $F = -2.38 \times PHA(L) + 3.46$ |
| 3 | EEL | $F = 2.59 \times EEL + 1.25$ |
| 4 | MAL, PHA(L) | $F = -2.81 \times MAL + 0.03 \times PHA(L) + 2.29$ |
| 5 | MAL, EEL | $F = -2.45 \times MAL + 1.23 \times EEL + 1.45$ |
| 6 | PHA(L), EEL | $F = -1.75 \times PHA(L) + 1.28 \times EEL + 1.92$ |
| 7 | MAL, PHA(L), EEL | $F = -2.98 \times MAL + 0.75 \times PHA(L) + 1.44 \times EEL + 0.70$ |

**Table 2** Evaluation of discriminant functions

| Formula number | Sensitivity (%) | Specificity (%) | Accuracy (%) |
|---|---|---|---|
| (A) MRC-derived iPS cells | | | |
| 1 | 50 | 100 | 55.2 |
| 2 | 93.3 | 100 | 94 |
| 3 | 93.3 | 57.1 | 89.6 |
| 4 | 50 | 100 | 55.2 |
| 5 | 96.7 | 100 | 97 |
| 6 | 100 | 100 | 100 |
| 7 | 85 | 100 | 86.6 |
| (B) AM-derived iPS cells | | | |
| 1 | 0 | 100 | 16.7 |
| 2 | 10 | 100 | 25 |
| 3 | 100 | 50 | 91.7 |
| 4 | 0 | 100 | 16.7 |
| 5 | 60 | 100 | 66.7 |
| 6 | 100 | 100 | 100 |
| 7 | 70 | 100 | 75 |

$$\text{Sensitivity} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{number of false negatives}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{Number of false positives}}$$

$$\text{Accuracy} = \frac{\text{Number of true positives} + \text{Number of true negatives}}{\text{Number of positives} + \text{Number of negatives}}.$$

## Discussion

The goal of this study was to distinguish oligosaccharide structures that are increased in pluripotent and

multipotent cell types. Categorization using lectin probes enabled us to distinguish between different stem cell potencies or to discriminate between undifferentiated and differentiated forms. These results could lead to the use of lectin profiling as a tool for the better understanding of cell identity. To date, global glycan profiles have been preferentially analyzed by mass spectrometry (Satomaa *et al.* 2009; Wollscheid *et al.* 2009). Specifically, high- resolution mass spectrometry is the primary technique for characterizing the structures of individual glycans in most glycomic studies (Satomaa *et al.* 2009; Alvarez-Manilla *et al.* 2010). Mass spectrometry can also be employed to define sites of attachment of glycans to the underlying protein scaffold. A major benefit of mass spectrometry is the detailed information it provides regarding the structure of a glycan. A drawback, however, is its relatively low throughput and the need for different experimental protocols for each glycan subtype. In contrast, lectin microarray can be employed to interrogate the glycome with much higher throughput and provide global information about the types of glycan epitopes that are present in the sample (Kuno *et al.* 2005; Yue & Haab 2009; Porter *et al.* 2010). The high-throughput platform as well as satisfactory sensitivity allows rapid comparison of multiple glycomes in search of global changes that might motivate further mass spectrometry studies.

### Glycan-based quality control for cell therapy—Defining the states of pluripotent stem cells

In cell-based therapy, lectin microarray is a practical tool for the quality control of stem cell products. Flow cytometric analysis and immunocytochemical analysis with single probes have been used in this regard, but the lectin microarray technique with multiple probes provides an opportunity to address this issue in a simple, inexpensive and fast manner (Katrlik *et al.* 2010). Cell identity needs to be validated after each step of cell processing, i.e., isolation, *in vitro* propagation, harvesting and transfer because cells may be modified or changed after either of these steps and should thus be monitored by the most trustworthy method. Human ES and iPS cells for potential use as donor cells in cell-based therapy need to be validated for maintenance of the 'undifferentiated' state during *in vitro* propagation and while stored in master and working cell banks (Wobus & Boheler 2005; Yamanaka 2009). Lectin microarray techniques for precise monitoring of the undifferentiated or differentiated state are indeed sensitive and only a small number of cells ($1 \times 10^3$) are sufficient to obtain reproducible results. This feature of the technology, to define diverse cell identities, also leads to high-throughput screening for drug discovery and toxicology and safety testing.

### Glycan profile to determine cell identity

Hematopoietic stem cells were originally defined by GlcNAc-specific wheat germ agglutinin (WGA), one of the most common plant lectins (Spangrude *et al.* 1988), and human and murine endothelial cells were defined by another lectin, α1-2Fuc-specific *Ulex europaeus* agglutinin I (UEA-I) (Jackson *et al.* 1990). Neural stem cells were also defined by the glycolipid antigen LeX/SSEA-1 (Capela & Temple 2002). Furthermore, human ES and iPS cells have been previously evaluated by the presence of carbohydrate markers. The International Stem Cell Initiative characterized 59 human ES cell lines from 17 laboratories worldwide. Human ES cell lines are characterized by carbohydrate markers such as the glycolipid antigens SSEA3 and SSEA4, and the keratan sulfate antigens TRA-1-60, TRA-1-81, GCTM2 and GCT343 as well as the protein antigens (Adewumi *et al.* 2007; Wright & Andrews 2009). In addition to detection of carbohydrate markers by lectins and antibody probes, comprehensive glycan analysis serves as another method to detect and define cell identities. In this study, we found the pluripotent stem cells have the specific glycan structure, Galα1-3Gal, recognized by EEL (Fig. S1 in Supporting Information). Their major specific N-glycosylation feature in hES cells is complex fucosylation (Satomaa *et al.* 2009), whereas PHA(E) ligands are signs of hES cell differentiation (Venable *et al.* 2005; Wearne *et al.* 2006). This study suggests that glycan profiling by lectin microarray is more sensitive, compared with any other analysis. Further analysis of stem cell glycan may also lead to establishing new glycan structures as stem cell markers in addition to the commonly used SSEA and TRA glycan structures.

Glycans function as ligands for specific glycan receptors and modulate the activity of their carrier proteins and lipids (Imperiali & O'Connor 1999; Zanetta & Vergoten 2003). More than half of all proteins in a human cell are glycosylated. Consequently, a global change in protein-linked glycan biosynthesis can simultaneously modulate the properties of multiple proteins. It is likely that drastic changes during differentiation of human stem cells have major influences on a number of cellular signaling cascades and affect biological processes within the cells (Xu *et al.* 2005; Sasaki *et al.*

2008). Thus, glycan profiling can be useful for validation of cell identity (Satomaa *et al.* 2009). Categorization of stem cells by lectin microarray analysis can become another fundamental method in addition to immunocytochemistry and flow cytometric analysis. Microarray technologies currently enhance our understanding of gene expression, genomic stability and epigenetics, are commonly used in research laboratories and clinics today, and will likely play important roles in advancing stem cell research. In the future, analysis of stem cell glycan structure may be useful for establishing new markers beyond the lectin markers that already play a major role in the rapidly evolving world of stem cell biology.

## Experimental procedures

### Cells and cell culture

9-15c (uncommitted stem cells), H-1/A (preadipocytes), KUM5 (chondroblasts) and KUSA-A1 (osteoblasts) are available through cell banks (JHSF cell bank: http://www.jhsf.or.jp/English/index_gc.html; RIKEN cell bank: http://www.brc.riken.go.jp/lab/cell/english/). 9-15c (Yamada *et al.* 2007), H-1/A (Umezawa *et al.* 1991), KUM5 (Sugiki *et al.* 2007) and KUSA-A1 cells (Umezawa *et al.* 1992) were cultured using methods described previously. The cells were maintained in POWEREDBY10 medium (MED SHIROTORI CO., Ltd, Tokyo, Japan) or Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/mL)/streptomycin (100 μg/mL)/amphotericin B (250 ng/mL) at 33 °C with 5% $CO_2$. Human mesenchymal cells were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 100 μg/mL penicillin, 100 IU/mL streptomycin and 10% fetal calf serum at 37 °C in a $CO_2$ incubator. Human embryonal carcinoma cell line NCR-G3, from a testicular tumor, was cultured in G031101 medium (Med Shirotori, Tokyo, Japan) as previously described (Maruyama *et al.* 1996; Umezawa *et al.* 1996). Human iPS cells were cultured in Valuegen medium (Med Shirotori, Tokyo, Japan) (Makino *et al.* 2009; Nagata *et al.* 2009).

### Extraction of membrane fractions and lectin microarray analysis

Cells ($0.1–1 \times 10^6$) were washed with PBS and collected with a cell scraper. Cell pellets of hES-3, -8, and -9 cells (Osafune *et al.* 2008) were kindly obtained from Dr Douglas Melton (Harvard University). Cell membrane fractions were extracted from the cell pellets using a CelLytic MEM Protein Extraction kit (Sigma, St Louis, MO, USA). Lectin microarray analysis was performed as previously described (Kuno *et al.* 2005, 2008). Briefly, a small aliquot of protein fraction (200 ng) was labeled with Cy3-succinimidyl ester (designated as Cy3-labeled glycoprotein). The lectin chip with 43 lectins (Kuno *et al.* 2005) for mouse cells or LecChip™ with 45 lectins (GP Bio-Sciences, Kanagawa, Japan) for human cells was incubated with the Cy3-labeled glycoprotein solution (100 μL) at a concentration of 0.25 and 0.5 μg/mL in probing buffer (TBS containing 0.05% Triton X-100) at 4 °C until binding reached equilibrium. Lectins are well known as glycan recognizers and are classified into several categories, for instance, fucose, sialic acid, asialo-form, agalacto-form, high mannose, O-glycan and branching structure recognizers (Fig. S1 in Supporting Information). We calculated the net intensity value for each spot by subtracting a background value from signal intensity and then averaged the signal net intensity values of three spots. Lectin microarray data on each cell type were processed by the microarray system using a max-normalization procedure after a gain-merging process (Kuno *et al.* 2008).

### Hierarchical clustering analysis and principal component analysis

To analyze the lectin microarray data, we used agglomerative hierarchical clustering and principal component analysis (PCA) (Sharov *et al.* 2005). The hierarchical clustering techniques classify data by similarity and their results are represented by dendrograms. PCA is a multivariate analysis technique that finds major patterns in data variability.

### Discriminant analysis of pluripotency in human pluripotent stem cells

Coefficients and constants of each formula were defined, using the lda function in the MASS library of the statistical package R [http://www.r-project.org/, (Venables & Ripley 2002), (Ripley 1996)].

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## Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

**Figure S1** List of lectins on LecChip™ and their specificity.

**Figure S2** Signal intensities of each lectin on LecChip™.

**Table S1** Scores of ES and EB cells by each formula

**Table S2** Scores of iPS cells and their parental cells (MRC-5) by each formula

**Table S3** Scores of iPS cells and their parental cells (AM936EP) by each formula

**Table S4** Cell name of MRC-derived iPS cells

**Table S5** Cell name of AM-derived iPS cells

Additional Supporting Information may be found in the online version of this article.

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REVIEW

# Investigating cellular identity and manipulating cell fate using induced pluripotent stem cells

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**Abstract**

Induced pluripotent stem (iPS) cells, obtained from reprogramming somatic cells by ectopic expression of a defined set of transcription factors or chemicals, are expected to be used as differentiated cells for drug screening or evaluations of drug toxicity and cell replacement therapies. As pluripotent stem cells, iPS cells are similar to embryonic stem (ES) cells in morphology and marker expression. Several types of iPS cells have been generated using combinations of reprogramming molecules and/or small chemical compounds from different types of tissues. A comprehensive approach, such as global gene or microRNA expression analysis and whole genomic DNA methylation profiling, has demonstrated that iPS cells are similar to their embryonic counterparts. Considering the substantial variation among iPS cell lines reported to date, the safety and therapeutic implications of these differences should be thoroughly evaluated before they are used in cell therapies. Here, we review recent research defining the concept of standardization for iPS cells, their ability to differentiate and the identity of the differentiated cells.

## The potential of stem cells and reprogramming

During mammalian development, cells in the developing fetus gradually become more committed to their specific lineage. The cellular differentiation process specializes to achieve a particular biological function in the adult, and the potential to differentiate is lost. Cellular differentiation has traditionally been thought of as a unidirectional process, during which a totipotent fertilized zygote becomes pluripotent, multipotent, and terminally differentiated, losing phenotypic plasticity (Figure 1). However, recent cloning experiments using nuclear transplantation have demonstrated that the epigenetic constraints imposed upon differentiation in mammalian oocytes can be released and the adult somatic nucleus restored to a totipotent embryonic state [1]. This process, a rewinding of the developmental clock, is termed nuclear reprogramming.

Embryonic stem (ES) cells derived from the inner cell mass of the mammalian blastocyst, an early-stage embryo, were first established from mice by Evans and Kaufman in 1981 [2]. Approximately two decades later, a human ES (hES) cell line was established by Thomson and colleagues [3]. ES cells possess a nearly unlimited capacity for self-renewal and pluripotency: the ability to differentiate into cells of three germ layers. This unique property might be useful to generate a sufficient amount of any differentiated cell type for drug screening or evaluations of drug toxicity and for cell replacement therapy. In addition, pluripotent stem cells provide us with an opportunity to understand early human embryonic development and cellular differentiation. Pluripotent ES cells are spun off directly from pre-implantation embryos [2-5]. To induce the somatic cell back to a pluripotent state, a strategy such as nuclear transplantation is fraught with technical complications and ethical issues. Thus, the direct generation of pluripotent cells without the use of embryonic material has been deemed a more suitable approach that lends itself well to mechanistic analysis and has fewer ethical implications [6].

In a breakthrough experiment, Takahashi and Yamanaka [7] identified reprogramming factors normally expressed in ES cells, Oct3/4, Sox2, c-Myc, and Klf4, that were sufficient to reprogram mouse fibroblasts to become pluripotent stem cells closely resembling ES cells. Because they were induced by the expression of defined factors, these cells were termed induced pluripotent stem (iPS) cells [7]. Since this landmark report in 2006, the technology has been rapidly confirmed among a number of species, including humans [8,9], rhesus monkeys [10], rats [11,12], rabbits [13], pigs [14] and two endangered primates [15]. In addition, mouse iPS (miPS) cells can be derived from various cell types, including fibroblasts [7,16], neural cells [17,18], liver cells [19], pancreatic β

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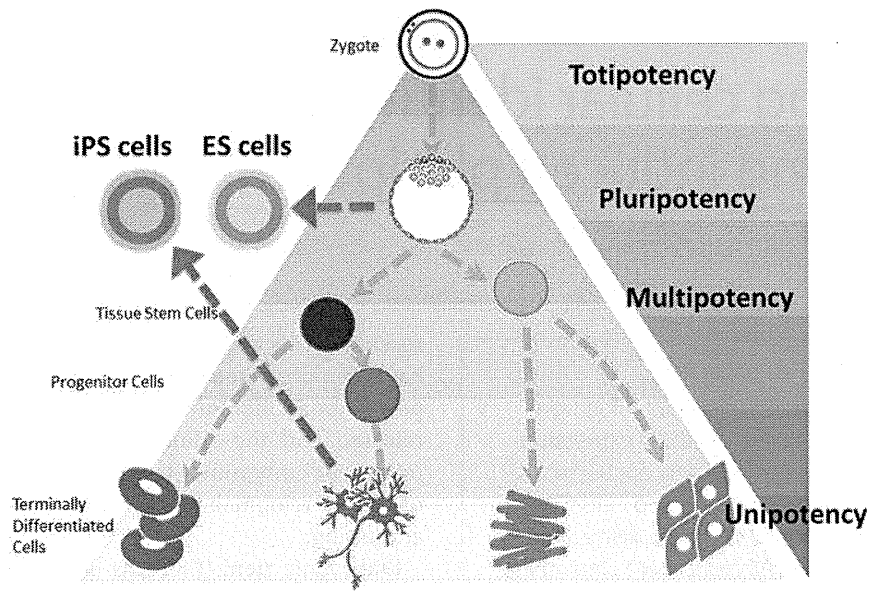


**Figure 1. Hierarchical potential of stem cell development.** A totipotent cell, such as a zygote and a blastomere of an early pre-implantation embryo, can give rise to all of the cell types in the whole body and the extraembryonic tissues. During mammalian development, pluripotent cells of the inner cell mass differentiate to give rise to lineage-committing stem cells and progenitor cells, and finally terminally differentiated cells by losing differential potential. Embryonic stem (ES) cells are spun off directly from the inner cell mass of blastocysts and induced pluripotent stem (iPS) cells are generated by reprogramming differentiated cells back to the pluripotent state. ES cells and iPS cells seem to have highly similar pluripotential properties.

cells [20], and terminally differentiated lymphocytes [21,22]. Subsequently, human iPS (hiPS) cells have been derived from various readily accessible cell types, including skin fibroblasts [8,9], keratinocytes [23], gingival fibroblasts [24], peripheral blood cells [25,26], cord blood cells [27,28] and hair follicle cells [29].

These products and systems for this state-of-the art technology provide useful platforms for disease modeling and drug discovery, and could enable autologous cell transplantation in the future. Given the methodologies for studying disease mechanisms, disease- and patient-specific iPS cells can be derived from patients. For applying novel reprogramming technologies to biomedical fields, we need to determine the essential features of iPS cells. In this review, we summarize the functional and molecular properties of iPS cells in comparison to ES cells in the undifferentiated state and with regard to differentiation efficiency. We also review evaluation for the types of differentiated cells derived from of iPS and ES cells and compare the functions of these.

## Reprogramming methods and factors

Although the establishment of iPS cells from somatic cells is technically easier and simpler compared with nuclear transplantation, several variables should be considered due to variations in the reprogramming process, including the reprogramming factors used, the combinations of factors and the types of donor-parent cells. Each method has advantages and disadvantages, such as efficiency of reprogramming, safety, and complexity, with the process used affecting the quality of the resultant iPS cells. Initial generations of miPS and hiPS cells employed retroviral and lentiviral vectors [7-9] (Table 1), carrying the risk of both insertional mutagenesis and oncogenesis due to misexpression of the exogenous reprogramming factors, Oct3/4, Sox2, c-Myc, and Klf4. In particular, reactivation of c-Myc increases tumorigenicity in the chimeras and progeny mice, hindering clinical applications.

Since the initial report of iPS cell generation, modifications to the reprogramming process have been made in order to decrease the risk of tumorigenicity and increase reprogramming efficiency [30-32]. Several small molecules and additional factors have been reported to enhance the reprogramming process and/or functionally replace the role of some of the transcription factors (Table 1). Small molecules are easy to use and do not result in permanent genome modifications, although iPS generation using only a set of small molecules has not been reported. Combining small molecule compounds with reprogramming factors would enhance reprogramming efficiency. Integration-free hiPS cells have been established using Sendai virus [33,34], episomal plasmid vectors [35,36], minicircle vectors [37], and direct protein

**Table 1. Various methods used for reprogramming**

| Method | Factors[a] | Sources | Enhancement factors |
|---|---|---|---|
| Adenovirus | OSKM | Mouse fibroblast and liver cells [77], human embryonic fibroblast cells [78] | |
| Bacteriophage | OSKM | Mouse embryonic fibroblasts, human amniocytes [79] | |
| Episomal vector | OSKMNL | Human foreskin fibroblasts [36] | SV40LT |
| | | Human fibroblasts, adipose stem cells, cod blood cells [80] | SV40LT, LIF, MEK/GSK3b/TGFBR inhibitor, HA-100/human |
| | OSKM*L | Human dermal fibroblasts [81] | p53 shRNA |
| Lentivirus | OSKM | Mouse pancreatic b cells [20] | |
| | | Human adult fibroblasts [82] | p53 siRNA, UTF1 |
| | | Mouse B lymphocytes [21] | C/EBPa or Pax5 shRNA |
| | OSNL | Human newborn foreskin [9] | |
| | | Human fibroblasts [83] | SV40LT |
| | OSKMNL | Human fibroblasts [84] | |
| | OSN | Gut mesentery-derived cells [85], human amnion-derived cells [86] | |
| | O | Human epidermal keratinocytes [87] | TGFBR/MEK1 inhibitor, PDK1 activator, sodium butyrate |
| Minicircle vector | OSNL | Human adipose stromal cells [37] | |
| microRNA | miR-200c, 302a/b/c/d, 369-3p/5p | Human and mouse adipose stromal cells [64] | |
| mRNA | OSNL | Human fibroblasts [88] | |
| | OSKM(L) | Primary human neonatal epidermal keratinocytes [40] | |
| piggyBAC | OSKM | Human and mouse embryonic fibroblasts [89,90] | |
| Plasmid | OSKM | Mouse embryonic fibroblasts [35,91] | |
| | OSNL | Human foreskin fibroblasts [92] | MEK inhibitor |
| Protein | OSKM | Mouse embryonic fibroblasts [38] | VPA |
| | OSKM | Human fibroblasts [39] | |
| Retrovirus | OSKM | Human fibroblasts [8], mouse fibroblasts [7], human keratinocytes [23], human peripheral blood cells [25] | |
| | | Human fibroblasts, adipose stem cells [93] | Vitamin C, VPA |
| | OSK | Adult human dermal fibroblasts [30] | |
| | | Mouse embryonic fibroblasts [94] | Wnt3a |
| | | Rat liver progenitor cells [11] | MEK/ALK5/GSK3b inhibitor |
| | | Mouse embryonic fibroblasts [93] | Vitamin C |
| | | Mouse and human fibroblasts [32] | GLIS1 |
| | | Mouse embryonic fibroblasts [95] | mmu-miR-106a/18b/20b/19b/92a/363 or 302a/302b/302c/302d/367 |
| | | Human fibroblasts [96] | hsa-miR-302b or 372 |
| | OK | Mouse embryonic fibroblasts [97] | BIX01294, BayK8644 |
| | | Neonatal human epidermal keratinocytes [98] | GSK3b inhibitor |
| | O | Mouse neural stem cells [99] | |
| | | Mouse fibroblasts [100] | GSK3b inhibitor, vitamin C, BMP4 |
| | hsa-miR-302a/b/c/d | Human skin cancer cells [101] | |
| Sendai virus | OSKM | Human fibroblasts [33], human cord blood [102] | |

[a]O, OCT3/4; S, SOX2; K, KLF4; M, C-MYC; M*, L-MYC; N, NANOG; L, LIN28. ALK, anaplastic lymphoma kinase; BayK8644, L-type calcium channel agonist; BIX01294, histone methyltransferase inhibitor; BMP, bone morphogenetic protein; GSK, glycogen synthase kinase; GLIS, GLI (MIM 165220)-related Kruppel-like zinc finger; LIF, leukemia inhibitory factor; PDK, pyruvate dehydrogenase kinase; shRNA, short hairpin RNA; siRNA, small interfering RNA; TGFBR, transforming growth factor beta receptor; UTF, undifferentiated transcription factor; VPA, valproic acid (histone deacetylase inhibitor).

[38,39] or mRNA [40] delivery (Table 1). However, direct delivery of proteins or RNA requires multiple transfection steps with reprogramming factors compared to other viral integration methods.

## iPS cells appear indistinguishable from ES cells

The key to generating iPS cells is to revert somatic cells to a pluripotent state that is molecularly and functionally equivalent to ES cells derived from blastocysts (Table 2). Reprogrammed iPS cells express endogenous transcription factors that are required for self-renewal and maintenance of pluripotency, such as OCT3/4, SOX2, and NANOG, and for unlimited proliferation potential, such as TERT [8,9]. Telomeres were elongated in iPS cells compared to the parental differentiated cells in both humans and mice [41,42]. In addition, cellular organelles such as mitochondria within hiPS cells were morphologically and functionally similar to those within ES cells [43]. The establishment of an ES cell-like epigenetic state is a critical step during the reprogramming of somatic cells to iPS cells and occurs through activation of endogenous pluripotency related genes. Bisulfite genomic sequencing has shown that the promoter regions of the pluripotency markers NANOG and OCT3/4 are significantly demethylated in both hiPS and hES cells [8,44], and the heterogeneity of X chromosome inactivation in hiPS cells is similar to that in ES cells [45].

In terms of multilineage differentiation capacity, miPS cells from various tissue types have been shown to be competent for germline chimeras [19,32,46]. It was shown that miPS cells generated viable mice via tetraploid complementation [47,48]. In the mouse system, iPS cells retain a developmental pluripotency highly similar to that of mouse ES cells according to the most stringent tests. Although it has been generally assumed that autologous cells should be immune-tolerated by the recipient from whom the iPS cells were derived, Zhao and colleagues [49] reported that the transplantation of immature miPS cells induced a T-cell-dependent immune response even in a syngeneic mouse. This is an unexpected result but some issues need to be considered: the influence of the cell type of origin on the immunogenic properties of resultant iPS cells must be explored; undifferentiated iPSCs should never be used for medical applications; and the mechanism of aberrant gene expression should be determined [50].

To functionally assay hiPS cells, teratoma formation and histological analysis to confirm the presence of structures derived from all three germ layers are currently regarded as the most rigorous ways to prove pluripotency of human stem cells. Recently, Müller and colleagues [51] proposed the use of PluriTest, a bioinformatics assay for the prediction of stem cell pluripotency using microarray data. Such microarray-based gene expression and DNA methylation assays are low cost, save time and have been used to evaluate the differentiation efficiency of individual cell lines [52].

## ES and iPS cells differ in their epigenetic signatures

Epigenetic modification of the genome ensures proper gene activation for maintaining the pluripotency of stem cells and also differentiation into proper functional cells [1]. It will be important to assess the epigenetic state of hiPS cells compared to donor parent cells and embryo-derived hES cells. Analyzing epigenetic states, such as histone modifications and DNA methylation of selected key pluripotency genes, showed the chromatin state of iPS cells to be identical to that of ES cells upon reprogramming (reviewed in [53]).

Genome-wide analyses of histone methylation patterns have demonstrated that iPS cells were clearly distinguished from their origin and similar to ES cells in the mouse [54]. All of these analyses, however, reported some differentially methylated regions (DMRs) between ES and iPS cells. Recent studies found that miPS cell lines retained the residual signatures of DNA methylation of the parental cells [55,56]. Additionally, some of the hyper-methylated regions in hiPS cells are also hyper-methylated in the original cells, meaning that an epigenetic memory is inherited during the reprogramming process through early passaging [57]. Parental cell-related DMRs and incomplete promoter DNA methylation contributed to aberrant gene expression profiles in iPS cells to some extent [58]. The other remaining DMRs appeared to be aberrantly methylated regions established in iPS cells during reprogramming that differ from both the parental cells and the ES cells. Nishino and colleagues [57] compared methylation profiles of six hiPS cell lines and two hES cell lines and reported that approximately 60% of DMRs were inherited and 40% were iPS-specific. Interestingly, most aberrant DMRs were hyper-methylated in iPS cell lines [57,59]. Lister and colleagues [60] also compared methylation profiles in five hiPS cell lines and two hES cell lines and found that the hiPS cells shared megabase-scale DMRs proximal to centromeres and telomeres that display incomplete reprogramming of non-CpG methylation, and differences in CpG methylation and histone modifications in over a thousand DMRs between hES and hiPS cells. Although lots of studies have detected several DMRs shared between iPS and ES cells, no DMRs were found in all iPS cell lines.

microRNAs (miRNAs), which are also epigenetically regulated, play critical roles in gene regulation by targeting specific mRNAs for degradation or by suppressing their translation. Several studies recently reported the presence of unique clusters of miRNAs, such as the human and mouse miR-302 cluster in ES and iPS cells [61,62]. These miRNAs enhance the transcription factor-mediated

**Table 2. Characteristics of human induced pluripotent stem cells compared to human embryonic stem cells**

| Variable factor | Characteristics | Characteristics of hiPS cells |
|---|---|---|
| Cell source | | Without the use of embryonic material<br>Enable autologous cell transplantation |
| Technique for the generation of iPS cells | | Simply trans-activating several transcription factors and/or exposure to several chemical components<br>Variables due to reprogramming methods and/or donor-parental cells |
| Morphology | | Flat and tightly packed colony identical to hES cells |
| Proliferation potency | | Unlimited self-renewal identical to hES cells |
| Pluripotency | Genes | OCT3/4, NANOG, SOX2 expression identical to hES cells |
| | Gene promoter | OCT3/4, NANOG demethylation identical to hES cells |
| | Cell surface antigens | SSEA3, SSEA4, TRA-1-60, TRA-1-81 positive identical to hES cells |
| | Teratoma formation | Differentiation into three germ layers similar to hES cells |
| X chromosome inactivation (XCI) | | Heterogeneity (complete XCI, partial XCI, pre-XCI) similar to hES cells |
| Mitochondria | Genome | Accumulated mtDNA mutations transmitted from parental cells<br>Genetic mutations during reprogramming |
| | Morphology | Globular shape with only small christae similar to hES cells and ES cell-like distribution |
| | Function | Expression of nuclear factors involved in mitochondrial biogenesis |
| Telomere | | Telomere elongation and ES cell-like telomerase activity |
| Epigenetic profile | | Retention of somatic memory and aberrant methylation during the reprogramming process |
| microRNAs | | Up-regulation of miR-302 cluster identical to hES cells |

ES, embryonic stem; hES, human embryonic stem; hiPS, human induced pluripotent stem; iPS, induced pluripotent stem; mtDNA, mitochondrial DNA; XCI, X chromosome inactivation.

reprogramming process (Table 1). Furthermore, two independent groups generated human and mouse iPS cells by adding only miRNAs in the absence of any additional protein factors [63,64]. Two reports have described a small number of differences in miRNA expression patterns between hiPS and hES cells [62,65], although our preliminary analysis showed that miR-372 and miR-373 are expressed at similar levels in both hiPS and hES cells and they were not detected in parental cells.

## Changes of epigenetic profiles in iPS cells during culture

It is possible that iPS cells vary in their epigenetic profiles and degree of pluripotency due to differential levels of reprogramming. Nishino and colleagues [66] investigated the effect of continuous passaging on DNA methylation profiles of seven hiPS cell lines derived from five cell types. Although *de novo* DMRs that differ between hES and hiPS cells appeared at each passage, their number decreased and they disappeared with passaging; therefore, the total number of DMRs that differ between ES and iPS cells decreased with passaging. Thus, continuous passaging of the iPS cells diminished the epigenetic differences between iPS and ES cells, implying that iPS cells lose the characteristics inherited from the parental cells and develop to very closely resemble ES cells over time [66]. They also confirmed that the transgenes were silenced at each passage examined, indicating that the number of DMRs that differed between ES and iPS cells decreased during the transgene-independent phase. This is consistent with a study by Chin and colleagues [67], who found that the gene expression profile of hiPS cells appeared to become more similar to that of hES cells upon extended passaging. Although comprehensive DNA methylation profiles have recently been generated for hiPS cells, it seems harder to determine common DMR sites during iPS reprogramming. There are three possible explanations for the many inconsistent results regarding iPS cell-specific DMRs: hiPS cells have only been analyzed at a single point of passage in almost all studies; inherited methylation from parental cells is non-synchronous and stochastic, much like aberrant methylation, rather than deterministic [66]; and the aberrant hypermethylation at DMRs in iPS cells occurs 'stochastically' throughout the genome during passaging [66].

## Genetic changes during reprogramming and extended culture

Genomic stability is critical for the clinical use of hiPS cells. The occurrence of genetic changes in hES cells is now well known as well as that the karyotypic changes observed are nonrandom and commonly affect only a few chromosomes [68]. Recent studies revealed that the