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

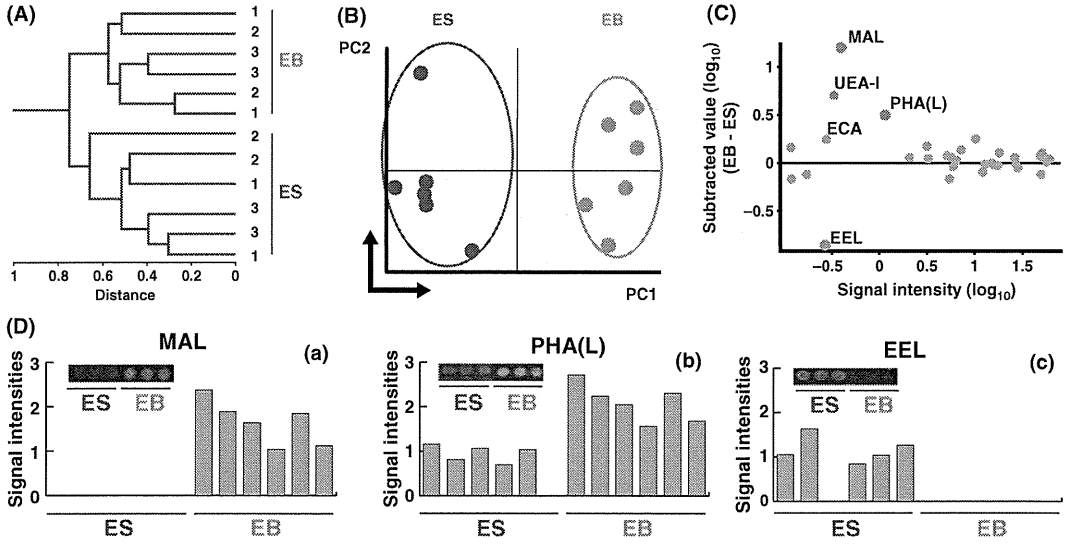

Figure 3 Lectin microarray analysis of human embryonic stem cells. (A) Hierarchical Clustering analysis of undifferentiated and differentiated ES cells. (B) Principal component analysis of lectin microarray analysis on undifferentiated and differentiated ES cells. (C) Signal value for *Maackia amurensis* 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).

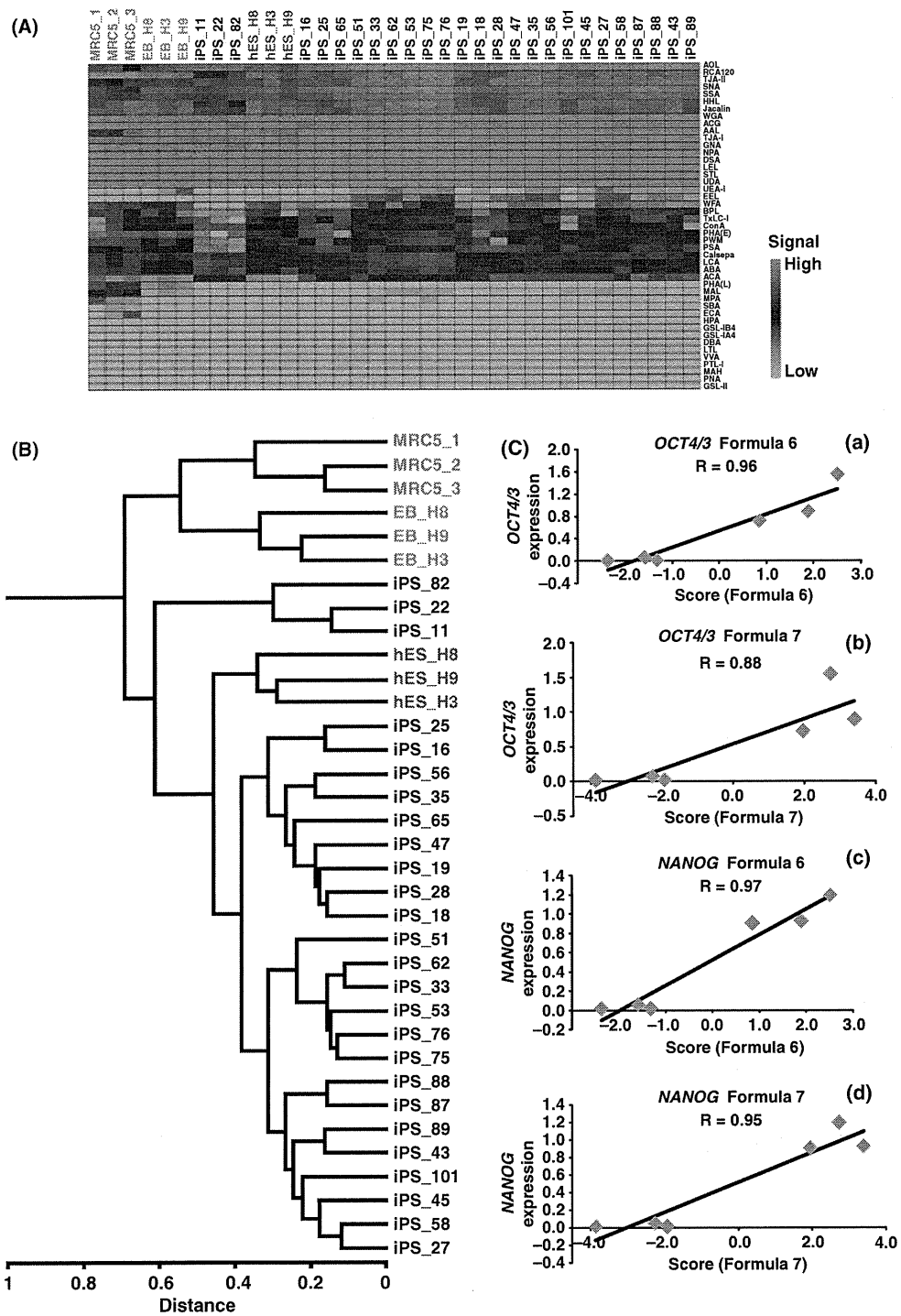

Figure 4 Lectin microarray analysis of human-induced pluripotent stem (iPS) cells. (A) Heat map of lectin microarray with MRC-5 and MRC-5-derived iPS cells. MRC-5 and iPS cells are discriminated by letter color: red, MRC-5; blue, hES cells; green, embryoid body (EB) cells; black, iPS cells. (B) Hierarchical 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 (1×10^3) 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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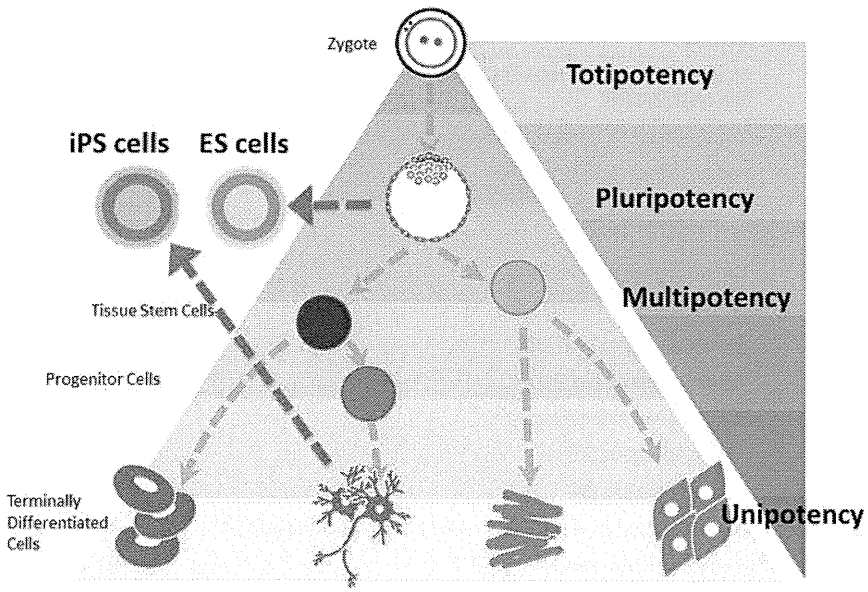

Figure 1. Hierarchical potential of stem cell development. A totipotent cell, such as a zygote and a blastomere of an early pre-implantation embryo, can give rise to all of the cell types in the whole body and the extraembryonic tissues. During mammalian development, pluripotent cells of the inner cell 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 Enable autologous cell transplantation
Technique for the generation of iPS cells		Simply trans-activating several transcription factors and/or exposure to several chemical components 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 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

reprogramming process and subsequent culture of iPS cells *in vitro* can induce genetic changes. Three types of genomic abnormalities were seen: aberrations of somatic cell origin, aberrations present in early passages but not of apparent somatic cell origin, and aberrations acquired during passaging. Notably, the high incidence of chromosome 12 duplications observed by Mayshar and colleagues [69] caused significant enrichment for cell cycle-related genes, such as *NANOG* and *GDF3*. Another study reported that regions close to pluripotency-associated genes were duplicated in multiple samples [70]. Selection during hiPS cell reprogramming, colony picking and subsequent culturing may be factors contributing to the accumulation of mutations.

Impact of epigenetic differences on pluripotency

One of the goals of using hiPS cells is to generate functional target cells for medical screening and therapeutic applications. For these applications, it must be evaluated thoroughly whether small DMRs among ES and iPS cells affect the competency, differentiation propensities, stability and safety of iPS cells. It remains to be elucidated how the degree of these differences contributes to the variance in pluripotency among ES and iPS cells. Analysis of iPS cells obtained from mouse fibroblasts and hematopoietic and myogenic cells demonstrated that cellular origin influences the potential of miPS cells to differentiate into embryoid bodies and different cell types *in vitro*. In a related study, Kim and colleagues [56] compared the ability to differentiate to blood lineages of iPS cells derived from fibroblasts, neural cells, hematopoietic cells and ES cells in the mouse system, and demonstrated consistent differences in blood-forming ability - that is, blood derivatives showed more robust hematopoiesis *in vitro* than neural derivatives. Therefore, low-passage iPS cells derived from different tissues harbor residual DNA methylation signatures characteristic of their somatic tissue of origin, which favors their differentiation along lineages related to the parental cell, while restricting alternative cell fates. Similarly, Miura and colleagues [71] demonstrated that differences in gene expression in miPS cells derived from different types of parental cells result in variations in teratoma formation. These studies demonstrate that reprogramming to generate iPS cells is a gradual process that modifies epigenetic profiles beyond the acquisition of a pluripotent state.

Prediction for pluripotency and differentiation preference

Significant variation has been also observed in the differentiation efficiency of various hES cell lines [72]. Incomplete DNA methylation of somatic cells regulates the efficiency of hiPS cell generation [58], and selection of parental cell types influences the propensity for differentiation [73,74]. Such differences must be better understood before hES and hiPS cell lines can be confidently used for translational research. To predict a cell line's propensity to differentiate into the three germ layers, Bock and colleagues [52] performed DNA methylation mapping by genome-scale bisulfite sequencing and gene expression profiling using microarrays and quantified the propensity to form multiple lineages by utilizing a non-directed embryoid bodies formation assay and high-throughput transcript counting of 500 lineage marker genes in embryoid bodies using 20 hES cells lines and 12 hiPS cell lines over passages 15 to 30. They bioinformatically integrated these genomic assays into a scorecard that measures the quality and utility of any human pluripotent cell line. The resulting lineage scorecard pinpoints quantitative differences among cell-line-specific differentiation propensities. For example, one hES cell line that received a high score for endoderm differentiation performed well in directed endoderm differentiation, and other hES cell lines that received high scores for neural lineage differentiation efficiently differentiated into motor neurons. In addition, two hiPS lines that the scorecard predicted to have a low propensity to differentiate into the neural lineage were impaired in motor neuron-directed differentiation. On the other hand, other hiPS lines that the scorecard predicted to have a high propensity to differentiate into ectodermal and neural lineages were found to differentiate well into motor neurons. Therefore, the scorecard can detect lineage-specific differences in the differentiation propensities of a given cell line [52].

Functional assay for differentiated cells from iPS and ES cells

Although the propensity for differentiation could be predicted, it remains to be elucidated whether iPS cell-derived cells are functionally and molecularly the same as ES cell-derived cells. To address this issue, two studies conducted functional assays comparing differentiated neural cells derived from iPS cells to those derived from ES cells by marker gene expression and action potential measurements [75,76]. There was some variation in efficiency and quantitative differences in motor neuron generation among the lines, but the treatment of neuroepithelial cells from pluripotent stem cells with retinoic acid and sonic hedgehog resulted in the generation of iPS and ES cell lines with a neuronal morphology that expressed TUJ1. In addition, electrophysiological recordings using whole-cell patch clamping showed inward and outward currents, and it was concluded that ES cell- and iPS cell-derived neurons are similarly functional at a physiological level. These studies demonstrated that the temporal course and gene-expression pattern during

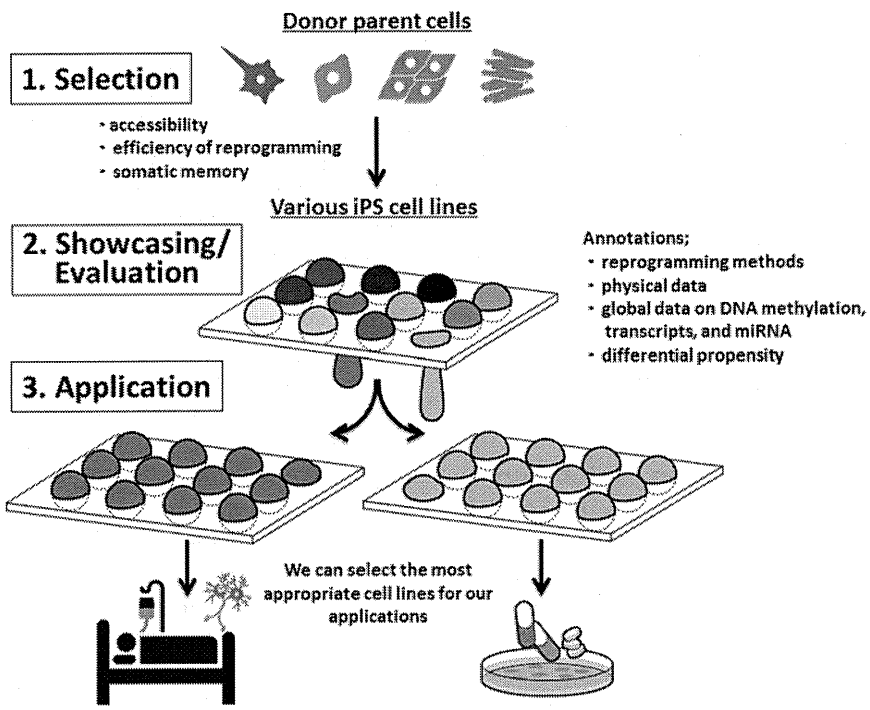

Figure 2. Workflow for human iPS cell applications. 1. Selection: choosing donor parent tissue considering accessibility, efficiency of reprogramming, and differential propensity. It would be useful to evaluate the expression of somatic memory genes, such as *C9orf64*, which reduces the efficiency of induced pluripotent stem (iPS) cell generation [58]. 2. Showcasing/evaluation: provides annotated information on reprogramming methods, culture conditions, physical data on stem cells, and global data on DNA methylation, transcription and microRNAs (miRNAs). It is very informative to integrate the genetic and epigenetic and biological data, such as differential propensity [52,76]. 3. Application: using annotation data, we can select the most appropriate iPS cell lines for our applications. Various hiPS cell lines (shown as differently shaded spheres) would be listed before further processing of the application. Valid cell lines (colored purple and blue) could be functionally and molecularly selected for appropriate applications, such as cell replacement therapy and/or drug screening.

neuroepithelial cell differentiation and production of functional neurons were nearly identical between ES and iPS cells, regardless of the reprogramming method, cellular origin, and differences between iPS and ES cells. These findings raise hopes of applying human iPS cells to the modeling of diseases and potential autologous cell transplantation.

It is important to acquire scientific information on pluripotential stem cells for further applications, such as industrial and clinical uses. Pluripotent stem cells, including disease-specific stem cells, could be showcased with useful annotation data and the most appropriate cell lines could be selected (Figure 2).

Conclusion

Many issues have yet to be resolved before the results of stem cell research can benefit the public in the form of medical treatments. In this review, we have discussed the substantial variation observed among pluripotent stem cells, including transcriptional and epigenetic profiles in the undifferentiated state, the ability to differentiate into various types of cells, and the functional and molecular nature of embryoid body or stem cell-derived differentiated cells. These results suggest that most, but not all, iPS cell lines are indistinguishable from ES cell lines, even though there is a difference between the average ES cell and the average iPS cell. Thus, ES and iPS cells should not be regarded as one or two well-defined points in the cellular space but rather as two partially overlapping point clouds with inherent variability among both ES and iPS cell lines [52,76]. Notably, human iPS cells seemed to be more variable than human ES cells. No single stem cell line may be equally powerful for deriving all cell types *in vitro*, implying that researchers would benefit from identifying the best cell lines for each application. Furthermore, for clinical use in the future, it is important to use both ES and iPS cells in research, and to standardize reprogramming methods, culture equipment and techniques and to optimize differentiation methods and evaluate the functions and tumorigenicity of differentiated cells.

This article is part of a review series on *Induced pluripotent stem cells*. Other articles in the series can be found online at http://stemcellres.com/series/ipsc

Abbreviations
DMR, differentially methylated region; ES, embryonic stem; hES, human embryonic stem; hiPS, human induced pluripotent stem; iPS, induced pluripotent stem; miPS, mouse induced pluripotent stem; miRNA, microRNA.

Competing interests
The authors declare that they have no competing interests.

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剤形	外用剤
規格・含量	0.5mL製剤：人フィブリノゲン40mg　人血液凝固第XIII因子37.5単位 アプロチニン液500KIE　日本薬局方　トロンビン125単位 日本薬局方　塩化カルシウム水和物2.95mg 1mL製剤：人フィブリノゲン80mg　人血液凝固第XIII因子75単位 アプロチニン液1,000KIE　日本薬局方　トロンビン250単位 日本薬局方　塩化カルシウム水和物5.9mg 2mL製剤：人フィブリノゲン160mg　人血液凝固第XIII因子150単位 アプロチニン液2,000KIE　日本薬局方　トロンビン500単位 日本薬局方　塩化カルシウム水和物11.8mg 3mL製剤：人フィブリノゲン240mg　人血液凝固第XIII因子225単位 アプロチニン液3,000KIE　日本薬局方　トロンビン750単位 日本薬局方　塩化カルシウム水和物17.7mg 5mL製剤：人フィブリノゲン400mg　人血液凝固第XIII因子375単位 アプロチニン液5,000KIE　日本薬局方　トロンビン1,250単位 日本薬局方　塩化カルシウム水和物29.5mg
一般名	和　名：人フィブリノゲン、人血液凝固第XIII因子、アプロチニン液、トロンビン、塩化カルシウム水和物 洋　名：Human fibrinogen, Human antihemophilic factor XIII fraction, Aprotinin Solution, Thrombin, Calcium Chloride Hydrate
製造販売承認年月日 薬価基準収載年月日 発売年月日	製造販売承認年月日：2009年 6月23日 薬価基準収載年月日：2009年 9月25日 発売年月日：1991年11月28日
開発・製造販売・発売・提携・販売会社名	製造販売：一般財団法人　化学及血清療法研究所 販　売：アステラス製薬株式会社
医薬情報担当者の連絡先・電話番号・FAX番号	

本IFは2010年4月改訂の添付文書の記載に基づき改訂した.

IF利用の手引きの概要

― 日本病院薬剤師会 ―

1. 医薬品インタビューフォーム作成の経緯

当該医薬品について製薬企業の医薬情報担当者(以下，MRと略す)等にインタビューし，当該医薬品の評価を行うのに必要な医薬品情報源として使われていたインタビューフォームを，昭和63年日本病院薬剤師会(以下，日病薬と略す)学術第2小委員会が「医薬品インタビューフォーム」(以下，IFと略す)として位置付けを明確化し，その記載様式を策定した．そして，平成10年日病薬学術第3小委員会によって新たな位置付けとIF記載要領が策定された．

2. IFとは

IFは「医療用医薬品添付文書等の情報を補完し，薬剤師等の医療従事者にとって日常業務に必要な医薬品の適正使用や評価のための情報あるいは薬剤情報提供の裏付けとなる情報等が集約された総合的な医薬品解説書として，日病薬が記載要領を策定し，薬剤師等のために当該医薬品の製薬企業に作成及び提供を依頼している学術資料」と位置付けられる．
しかし，薬事法の規制や製薬企業の機密等に関わる情報，製薬企業の製剤意図に反した情報及び薬剤師自らが評価・判断・提供すべき事項等はIFの記載事項とはならない．

3. IFの様式・作成・発行

規格はA4判，横書きとし，原則として9ポイント以上の字体で記載し，印刷は一色刷りとする．表紙の記載項目は統一し，原則として製剤の投与経路別に作成する．IFは日病薬が策定した「IF記載要領」に従って記載するが，本IF記載要領は，平成11年1月以降に承認された新医薬品から適用となり，既発売品については「IF記載要領」による作成・提供が強制されるものではない．また，再審査及び再評価(臨床試験実施による)がなされた時点ならびに適応症の拡大等がなされ，記載内容が大きく異なる場合にはIFが改訂・発行される．

4. IFの利用にあたって

IF策定の原点を踏まえ，MRへのインタビュー，自己調査のデータを加えてIFの内容を充実させ，IFの利用性を高めておく必要がある．
MRへのインタビューで調査・補足する項目として，開発の経緯，製剤的特徴，薬理作用，臨床成績，非臨床試験等の項目が挙げられる．また，随時改訂される使用上の注意等に関する事項に関しては，当該医薬品の製薬企業の協力のもと，医療用医薬品添付文書，お知らせ文書，緊急安全性情報，Drug Safety Update(医薬品安全対策情報)等により薬剤師等自らが加筆，整備する．そのための参考として，表紙の下段にIF作成の基となった添付文書の作成又は改訂年月を記載している．なお適正使用や安全確保の点から記載されている「臨床成績」や「主な外国での発売状況」に関する項目等には承認外の用法・用量，効能・効果が記載されている場合があり，その取扱いには慎重を要する．