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

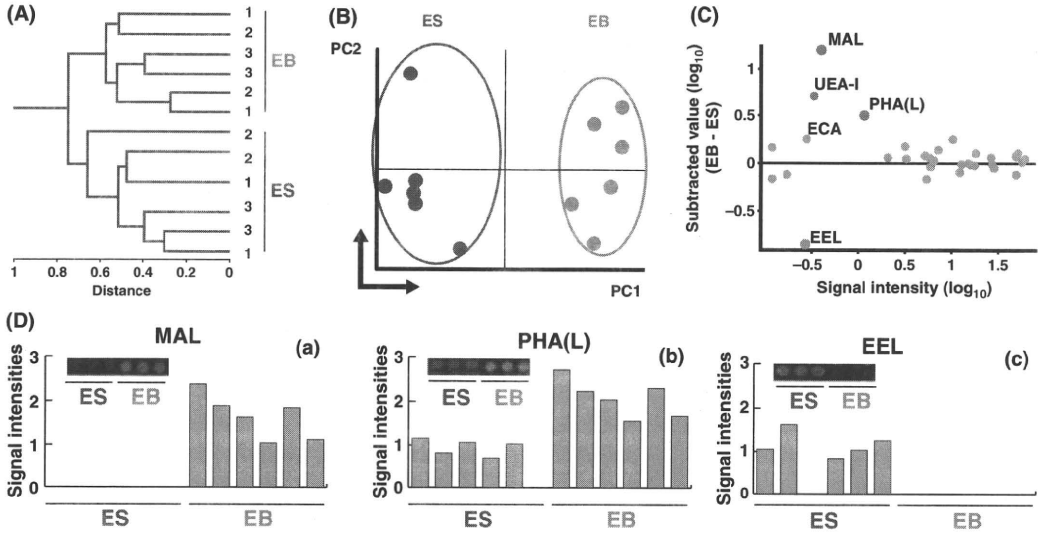

Figure 3 Lectin microarray analysis of human embryonic stem cells. (A) Hierarchical Clustering analysis of undifferentiated and differentiated ES cells. (B) Principal component analysis of lectin microarray analysis on undifferentiated and differentiated ES cells. (C) Signal value for *Maackia amurensis*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

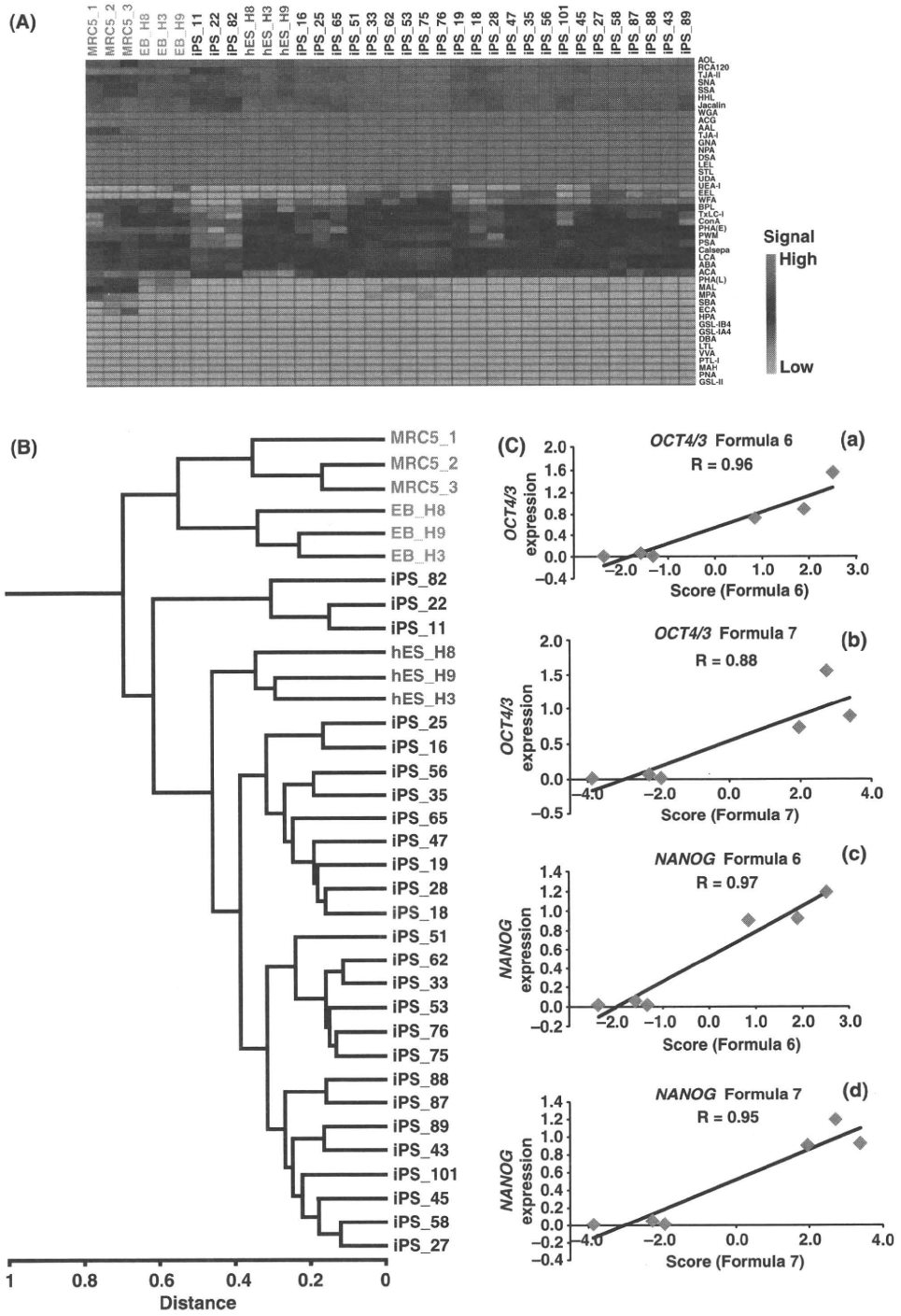

Figure 4 Lectin microarray analysis of human-induced pluripotent stem (iPS) cells. (A) Heat map of lectin microarray with MRC-5 and MRC-5-derived iPS cells. MRC-5 and iPS cells are discriminated by letter color: red, MRC-5; blue, hES cells; green, embryoid body (EB) cells; black, iPS cells. (B) Hierarchical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

REVIEW

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

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CONTENTS

1. INTRODUCTION

Human embryonic stem cells (hESCs) are derived from the inner cell mass of 3- to 5-day-old blastocysts.[1−5] hESCs are characterized by a high nucleus-to-cytoplasm ratio, prominent nucleoli, and distinct colony morphology.[6] Recently, pluripotent stem cells that are similar to ESCs were derived from an adult somatic cell by the "forced" expression of certain pluripotency genes,[7−11] such as Oct3/4, Sox2, c-Myc, and klf-4, or their proteins[12] and microRNAs.[13] These cells are known as induced pluripotent stem cells (iPSCs). iPSCs are believed to be similar to ESCs in many respects, including the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, potency, and differentiability. However, the full extent of their similarities to ESCs is still under investigation.[7,10]

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

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

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

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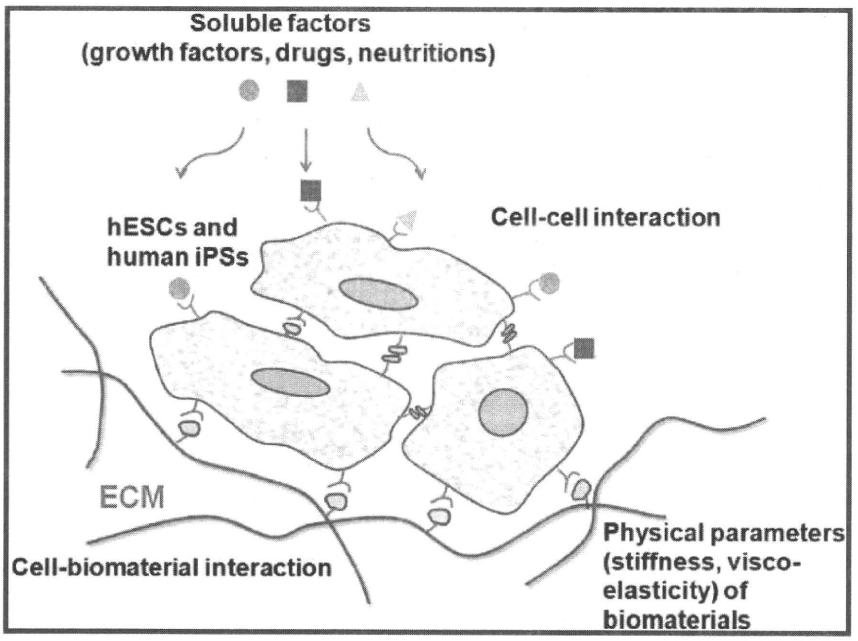

Figure 1. Schematic representation of the microenvironment and niches of hESCs and human iPSCs and their regulation by the following factors: (1) several soluble factors, such as growth factors or cytokines, nutrients, and bioactive molecules; (2) cell−cell interaction; (3) cell−biomacromolecule (or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

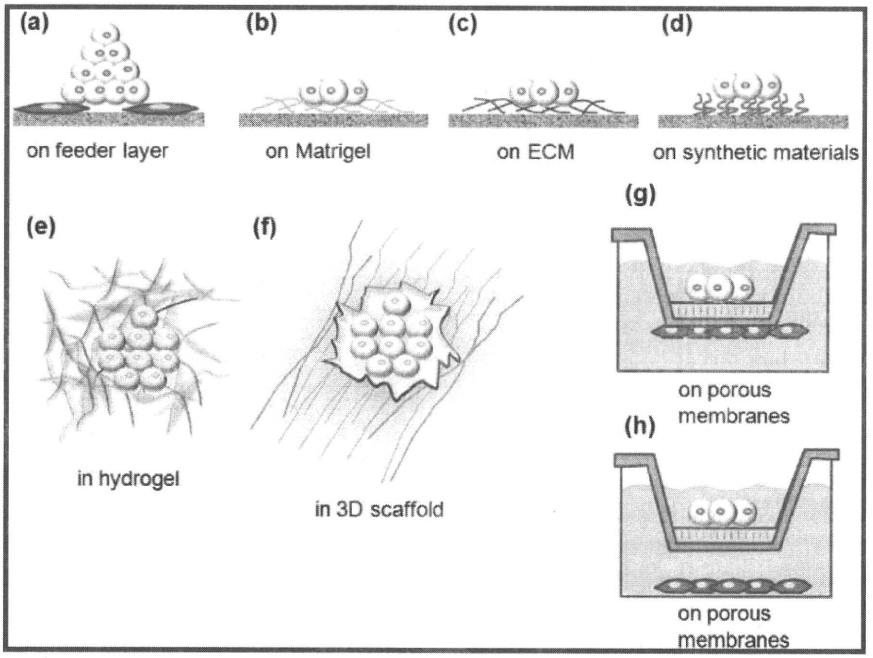

Figure 2. Schematic representation of different culture methods for hESCs and human iPSCs. hESCs and human iPSCs have been cultured (a) on MEF, (b) on Matrigel, (c) on 2D materials coated with ECM or other biomacromolecules, (d) on 2D materials prepared from synthetic materials, (e) in hydrogels made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

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

Tissue-specific stem cell niches provide crucial cell−cell contacts and paracrine signaling.[21,22] The extracellular matrix (ECM) keeps stem cells in the niche and serves to initiate signal transduction,[23,24] while locally concentrated glycosaminoglycans (GAGs) provide soluble growth factors or cytokines. Both *in vitro* and *in vivo*, the niche is established by supportive cells, the ECM and soluble factors, which regulate stem cell fate *via* complementary mechanisms, including the presentation of immobilized signaling molecules, the modulation of matrix rigidity, and the creation of cytokine gradients.[21] Thus, it would be highly beneficial to design, construct, and reproduce the microenvironment and niche of pluripotent stem cells *in vitro* with biomacromolecules and synthetic polymers.

Table 1. Characterization of Pluripotent ESCs and iPSCs[a]

characterization	specification (examples)	ref
1. morphology		
cell morphology	colony formation	2, 14, 31, 34, 39, 45, 47, 50, 76
2. protein level		
surface marker analysis	**Oct-4, Oct-3/4, Nanog, TRA-1-60, Tra-1-81, SSEA-3,** and **SSEA-4**	6, 28−30, 37, 38, 40, 50, 72, 75, 95
immunohistochemical analysis	**Oct3/4, Oct-4, Sox-2, SSEA-3, SSEA-4, TRA-1-60, TRA-1−81,** and Nanog **alkakine phosphatase (AP)** **SSEA-1** (negative staining)	28−35, 38, 46, 48−50, 68, 71
3. gene level	**Oct3/4, Oct-4, Sox-2,** Nanog, TDGF-1, UTF-1, REX1, hTERT, ABCG2, DPPA5, CRIPTO, FOXD3, Tert1, Rex2, and DPPA5	31, 37, 39, 40, 44, 46, 48, 68
4. differentiation ability	**embryonic body formation in vitro (EB)** **teratoma formation In Vivo**	2, 5, 31, 34, 45, 47, 68, 72, 75, 96, 127

[a] Bold genes and proteins are frequently analyzed for the characterization of hESCs and human iPSCs.

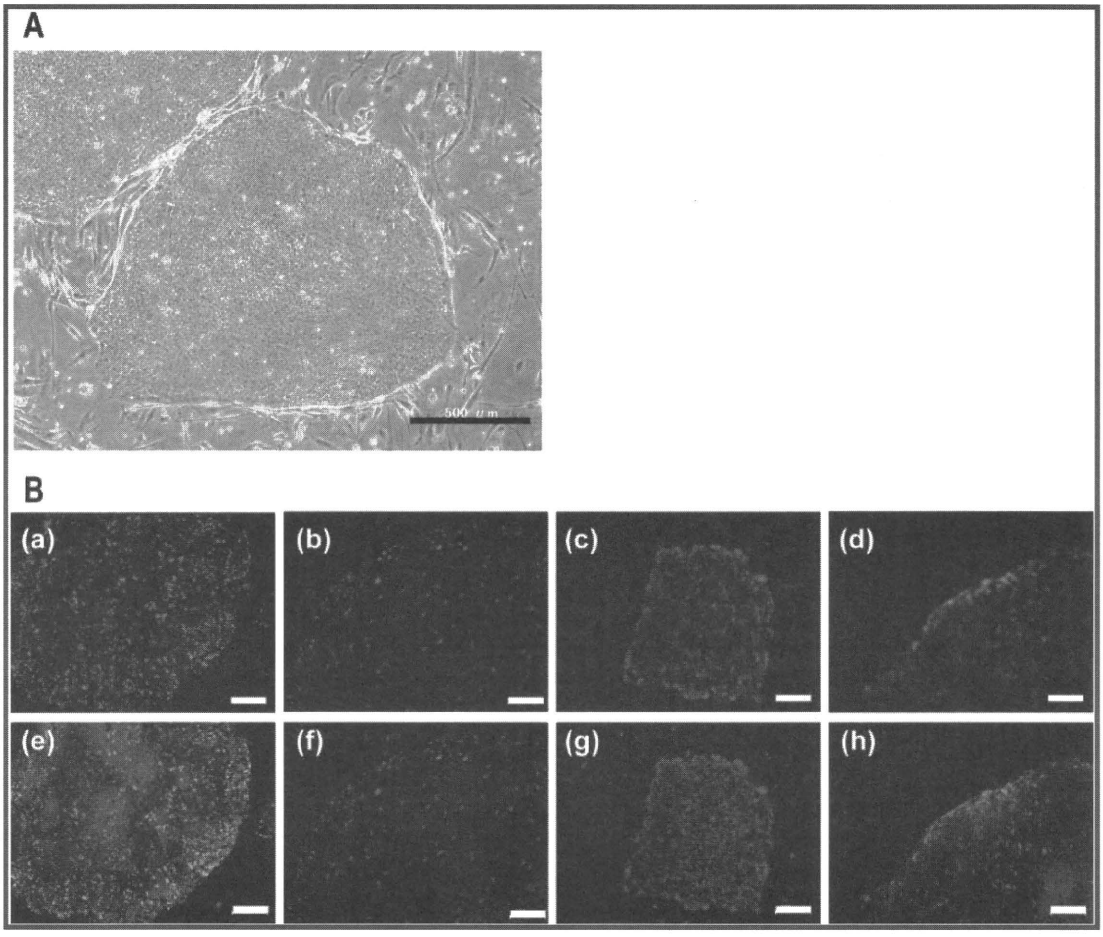

Figure 3. Morphology and expression of pluripotent markers in human iPSCs grown on a MEF feeder layer. Human iPSCs were derived from MRC-5 cells injected with Oct-4, Sox2, Klf4, and c-Myc using a retroviral vector. (A) A human iPSC colony grown on MEF. (B) Human iPSCs stained with antibodies and/or dye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

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

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

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

2. ANALYSIS OF THE PLURIPOTENCY OF HESCS AND HUMAN IPSCS

hESCs and human iPSCs display high telomerase activity and express several pluripotency surface markers, such as glycolipid stage-specific embryonic antigen 4 (SSEA-4), [28−30] tumor rejection antigen 1−60 (Tra-1−60), keratan sulfate-related antigen,[28,31−33] and tumor rejection antigen 1−81 (Tra-1−81),[31,33−35] but not glycolipid stage-specific embryonic antigen 1 (SSEA-1). SSEA-1 is expressed on mouse ESCs.[1,2,6,31] hESCs also show high expression levels of specific pluripotency genes, such as Oct3/4,[32,36,37] Oct-4 (POU5F1, POU domain transcription factor),[28,38,39] Nanog,[39,40] Sox-2,[40,41] Rex-1,[39,42] and hTERT, the catalytic component of telomerase.[39,43,44] Table 1 summarizes the characteristics of pluripotent hESCs.

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

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

The analysis of pluripotency genes and proteins through RT-PCR and/or qRT-PCR analysis and immunofluorescence and/or flow cytometry analysis, respectively, is important to verify the pluripotency of hESCs and human iPSCs. The difference between hESCs and human iPSCs and adult or fetal stem cells, such as bone marrow-derived stem cells (mesenchymal stem cells),[53,55] adipose-derived stem cells,[56,57] and amniotic fluid stem cells,[58,59] is the ability to differentiate into cells of all three germ layers (endoderm, mesoderm, and ectoderm).[30,47,60,61] Mesenchymal stem cells and other adult and fetal stem cells primarily differentiate into cells from the mesoderm, such as osteoblasts, chondrocytes, and adipocytes, although several exceptions have been reported.[62−65] Therefore, the ability to differentiate into cells from all three germ layers is also used to evaluate the pluripotency of hESCs and human iPSCs. hESCs and human iPSCs can generate embryonic bodies (EB) when cultured on untreated polystyrene dishes in differentiation medium, which includes three germ layers or tissue.[35,45,66] The formation of teratomas that include all three germ layers is also used to evaluate the pluripotency of hESCs and human iPSCs by injecting hESCs and human iPSCs into immunodeficient mice, such as mice with severe combined immunodeficiency (SCID).[2,45,67] Table 2 summarizes the characterization methods used to analyze the ability of cells to differentiate into all three germ layers in both EB and teratomas.

The ability to differentiate into cells from all three germ layers in EB and teratomas is analyzed in several ways: (1) observation of tissue that includes all three germ layers [the epithelial component (endoderm (E)), renal tissue (E), intestinal mucosa (E), cartilage (mesoderm (M)), bone (M), muscle (M), chondrocyte (M), mesenchymal tissue (M), the neural component (ectoderm (EC)),[201] and the epidemial component (EC)],[2,5,31,34,47,68] (2) expression of differentiated genes, including endoderm genes [α-fetoprotein (AFT), SOX17, amylase, albumin, FOXA1 (HNF3α), GATA6, and PDX1], mesoderm genes (Brachyury T, β-globin, MIX-LIKE-1, Hand1, and Msx1), and ectoderm genes [βIII-tubulin, SOX1, neurofilament heavy chain (NFH), keratin 15, neural progenitor markers PAX6 and NeuroD, and Nestin)], by RT-PCR and/or qRT-PCR,[38,40,49,50,69,70] and (3) the expression of differentiated proteins, including endoderm-related proteins [AFP, cytokeratin 19 (CK19), glucagons, and albumin], mesoderm-related proteins (actin, α-actinin, cTnI, and Brachyury T), and ectoderm-related proteins [β-III tubulin, enolase, nestin, and glial fibrillary acidic protein (GFAP)].[30,38,40,71,72] Figure 4 shows typical examples of the histochemical analysis of teratomas with cells from all three germ layers.

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

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

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

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

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Table 2. Characterization of Differentiation Ability to Three Germ Layers in EB and Teratoma[a]

characterization	specification (examples)	ref
1. morphology	Von Kossa staining (calcification)	30, 68
	Picrosirius staining for collagen, blood vessels, etc.	
	Alizarin Red staining (calcification)	
	Alcian blue/Van Giesson's staining	
	haematoxylin and eosin staining	
	(a) endoderm differentiation	
	columnar epithelia with goblet cell, primitive epithelium stained with cytokeratin 18 antibody, respiratory epithelium, gut epithelium, epithelial, intestinal mucosa, intestinal epithelium, pigmented epithelium, and renal tissue	2, 5, 31, 34, 45, 47, 68, 75, 96
	(b) mesoderm differentiation	
	hyaline cartilage, muscle, catilage, bone, smooth muscle, striated muscle, mesenchymal tissue, smooth muscle stained with actin antibody, and chondrocytes	2, 5, 31, 34, 45, 47, 68, 75, 127
	(c) ectoderm differentiation	
	neural rosettes, neural epithelium, neuroectoderm, neuronal tissue stained with neurofilament 200 K antibody, peripheral Schwann cells, embryonic ganglia, stratified squamous epithelium, epithelium, and neural tubes	2, 5, 31, 34, 47, 68, 72, 75, 127
2. protein level immunohisto chemical analysis	(a) endoderm differentiation	
	AFP, Glucagon, pdx-1, HNF3β, CK19, glucagon, NFH, GFAP, IFABP, albumin, Titf1, TTF-1, and FOXa2	28, 30, 31, 40, 71−73, 75, 88
	(b) mesoderm differentiation	
	FOXA2, cTcN, α-SMA, brachyury, vimentin, α-actin, α-actinin, muscle actin, actin, BMP-4, and cTnI	28, 30, 38, 71, 72, 88, 127
	(c) ectoderm differentiation	
	NCAM, Tuj1, neurofilament, βIII-tubulin, GFAP, enolase, and nestin	28, 30, 38, 40, 48, 71, 72, 75
surface marker	VEGFR2 (mesoderm), PDGFRα (mesoderm), and CXCR4 (endoderm)	128
3. gene level	(a) ectoderm gene expression	
	SOX-1, PAX6, Nestin, NES, Tuj1, MAP2, NeuroG1, TUBB3, βIII-tubulin, NeuroD, NOG, NEFL, keratin, Keratin 8, Keratin 18, Keratin 15, NFH, and neurofilament (NF)-68	28, 32, 33, 33, 38, 40, 46, 48, 50, 69, 70, 76, 88
	(b) endoderm gene expression	
	AFP, cerberus, GATA3, GATA4, GATA6, SOX17 (G3, G16, A17, A14, A1), ONECUT1, FOXA2 (A17G101), IPF1, FOXA1, PROX1, HHEX, ALB, HNF3b, HNF4a, Albumin, PDX1, amylase, TTF-1, IFABP, and Titf1	28, 33, 38, 46, 48, 50, 69, 70, 73, 76, 88
	(c) mesoderm gene expression	
	brachyury T, Hand1, IGF2, FLK1, MIXL1, MESP1, EOMES, PAX3, MYOD1, PECAM1, NKX2, GATA1, GATA2, GATA4, KDR, BMP4, SIL, HOXB4, MyoD, Msx1, C-actin, β-globin, α-cardiac actin, cardiac actin, VE-cadherin, enolase, MtoD, and CD31	28, 32, 33, 38, 46, 48, 50, 69, 70, 88
	(d) cardiomyocyte differentiation	
	Nkx2.5, GATA-4, MYH-6, TNNT2, TBX-5, Mlc2a, MLC-2 V, tropomyosin, cTnI, ANP, desmin, α-MHC, β-MHC, cTnT, Isl-1, and Mef2c	33, 40, 47
	(e) hepatocyte differentiation	
	AFP, albumin, and TAT	
	(f) neural differentiation	
	Nestin, Musashi 1, Tuj1, astrocytes (GFAP), and oligodendrocytes (myelin basic protein)	

[a] NCAM, neural cell adhesion moleule; cTnT, cardiac Troponin-T; FOXA2, forkhead box 2; α-SMA, alpha smooth muscle actin (K15); pdx-1, pancreatic marker; Tuj1, β-III-tubulin (neuronal marker); AFP, α-fetoprotein; NFH, neuro-filament heavy chain; GFAP, glial fibrillary acidic protein; cTnI, cardiac troponin I.

insufficient to maintain pluripotency marker expression.[77] FGF-2 induces the expression of hESC supportive factors, and Smad2/3 activation (TGF-β pathway) is required for hESCs to maintain pluripotency.[77] In addition to soluble factors in the culture medium,

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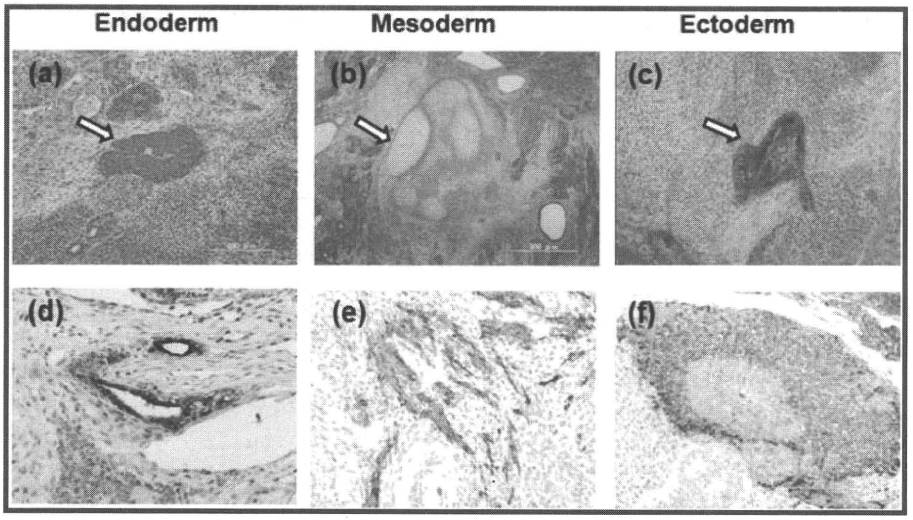

Figure 4. *In vivo* differentiation (teratoma formation) of human iPSCs, and histological and immunochemistry analysis of teratomas. The arrow indicates (a) hepatocytes (endoderm), (b) cartilage (mesoderm), and (c) retinal pigment epithelium (ectoderm). Differentiation markers representing the three germ layers, (d) AFP (endoderm), (e) smooth muscle actin (mesoderm), and (f) TuJ1 (ectoderm) were also observed.

the interaction between cells and their environment is also important and can influence the pluripotency of hESCs and human iPSCs. Figure 5 summarizes literature reports of different culture materials (substrates) in the feeder layer-free culture of hESCs.

3.1. hESC Culture on Matrigel

Matrigel is typically used as a substrate in feeder layer-free culture of many hESC lines. Matrigel is composed of isolated components from the sarcomas of Engelbreth−Holm−Swarm mice,[78,79] including laminin, collagen IV, heparan sulfate proteoglycans, enactin, and growth factors (e.g., TGF-β, EGF, and FGF),[14,80] which have been reported to support the pluripotency of many hESC lines.[28]

The effect of Matrigel and its individual components, such as collagen IV, laminin, and fibronectin, on the self-renewing capacity of hESCs has been investigated.[45] Collagen IV, one of the components of Matrigel, could not maintain the pluripotency and self-renewing capability of hESCs, while laminin, which is also a component of Matrigel, successfully maintained and supported long-term hESC culture in undifferentiated states using MEF-conditioned medium (MEF-CM).[45] MEF-CM is a culture medium that has been used to culture MEFs and contains several growth factors, extracellular matrix (ECM) and cell binding molecules, which are secreted by MEFs. It has been reported that the successful maintenance of hESCs without MEF-CM requires a culture medium supplemented with high concentrations of FGF-2, TGF-β1, and LIF and the use of fibronectin-coated dishes. In this culture system, FGF-2 maintains hESC culture,[81] TGF-β1 supports hESC proliferation,[45] fibronectin promotes cell adhesion,[82] and LIF activates the JAK/STAT3 pathway and supports the self-renewal of hESCs.[35,83] However, there are several reports that neither LIF nor activation of the STAT3 pathway contributes to the self-renewal of hESCs.[2,84]

The first feeder cell-free culture was reported by Xu et al.[45] They reported that hESCs cultured on Matrigel attached and formed small colonies that were less compact than hESC colonies on MEF feeder layers.[45] Differentiated hESCs appeared between colonies after a few days. hESCs on Matrigels were, therefore, reported to be dense, undifferentiated colonies surrounded by differentiated cells. This group was able to culture hESCs on Matrigel in MEF-CM and maintained the undifferentiated hESCs for over 130 population doublings (>180 days).[45] In contrast, hESCs seeded onto gelatin in MEF-CM had a low survival rate, and the cells tended to differentiate within the first passage.[45] Furthermore, it was observed that only a few appropriate hESC colonies existed in cultures on Matrigels with conditioned media from STO (an immortal mouse embryonic fibroblast cell line) or BJ5ta (a human foreskin fibroblast cell line immortalized with telomerase) cells after passage 39. Only conditioned medium from specific cells seems to support hESC growth. hESCs on Matrigels in MEF-CM maintained a normal karyotype and a stable proliferation rate (a doubling time of 31−33 h, similar to that for hESCs grown on a MEF feeder layer) and displayed high telomerase activity.[45]

The hESCs on Matrigels showed successful expression of pluripotency genes, including Oct-4 and hTERT, alkaline phosphatase activity (AP), and the surface markers of pluripotency proteins, including SSEA-4, Tra-1-60, and Tra-1-81, after 53 passages.[45] hESCs generated embryonic bodies (EB) with heterogeneous morphologies, including beating cells *in vitro* and teratomas in SCID/beige mice, which differentiated into cells from all three germ layers.[45] It should be noted that hESCs can be maintained on Matrigel in MEF-CM, but hESCs on Matrigel in nonconditioned hESC medium completely differentiated after two passages. Thus, culture on Matrigels is not sufficient to maintain the pluripotency of hESCs. Several soluble factors, such as growth factors and ECM components secreted by MEFs, are also required.

Ullmann et al. reported the successful culture of hESCs on Matrigel-coated plates using MEF-CM and conditioned medium from human fetal skin fibroblasts.[34] They were unable to maintain the pluripotency of feeder-free hESCs on Matrigel for more than 37 passages, whereas the maintenance of hESCs on MEFs maintained pluripotency for more than 100 passages.[34,85] Immunochemistry analysis showed that cells at the periphery of the hESC colonies in the feeder-free culture on Matrigel were negative for E-cadherin expression and positive for vimentin expression, which is indicative of the epithelial−mesenchymal transition (EMT). It was suggested that the feeder-free culture conditions using Matrigels forced the hESC colonies to undergo early differentiation into an EMT process.[34,60]

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Table 3. Feeder Layer-free Culture of hESCs and Human iPSCs Keeping Their Pluripotency on Biomaterials[a]

hESCs (cell lines)	cell culture substrates	culture medium	longest time in culture	pluripotency evaluation	ref (year)
H1, H7, H9, H14	matrigel	MEF-CM	6 months	proteins, genes, EB, teratoma	45 (2001)
H1, H7, H9, H14	matrigel	MEF-CM	88 passages	proteins, genes	37 (2003)
BG03	matrigel	MEF-CM	24 passages	proteins, genes, EB	46 (2004)
H1, H7, H9	matrigel	MEF-CM	70 passages	proteins, genes, teratoma	72 (2004)
H1	matrigel	HEF1-CM	12 passages	proteins, genes, EB	86 (2004)
H7, H9	matrigel	SFM	15 passages	proteins, EB, teratoma	44 (2005)
H1, H9	matrigel	SFM	10 passages	genes, EB, teratoma	76 (2005)
H1	matrigel	MEF-CM or SFM	5 passages	proteins, genes, Diff	88 (2005)
H1, BGN1, BGN2	matrigel	MEF-CM or SFM	5 passages	proteins, genes, EB	87 (2005)
SA002, AS038, SA121	matrigel	SFM	35 passages	proteins, teratoma	68 (2005)
H14	matrigel	SFM	35 passages	morphology	60 (2006)
VUB01, VUB03_DM1, VUB04_CF	matrigel	MEF-CM	37 passages	protein	34 (2007)
VUB01, VUB03_DM1, VUB04_CF	matrigel	hF-CM	37 passages	proteins	34 (2007)
HUES7, NOTT-1, HESC-NL-1	matrigel	MEF-CM	20 passages	proteins, genes	89 (2008)
HS401	matrigel	SFM	30 passages	proteins, genes, EB	29 (2009)
H1, H7, H9, H14	laminin	MEF-CM	6 passages	morphology, genes	45 (2001)
H1	human laminin	SFM	11 passages	proteins, genes, EB, teratomas	75 (2005)
HSF6	laminin	SFM	20 passages	proteins, genes, teratomas	96 (2005)
H9, H13	laminin	SFM	15 passages	protein, EB	95 (2006)
KhES-1, KhES-2, KhEs-3	laminin	MEF-CM	10 passages	protein, EB	50 (2008)
BG03	fibronectin	MEF-CM	24 passages	proteins, genes, EB	46 (2004)
I-3, I-6, H-9	fibronectin	SFM	30 passages	proteins, EB, teratoma	35 (2004)
HS360	fibronectin	SFM	2 passages	proteins, genes, EB	29 (2009)
MAN1, HUES7, HUES1	fibronectin	SFM	10 passages	proteins, genes, EB	38 (2009)
HUES1, HES2, HESC-NL3	vitronectin	SFM	8 passages	proteins	71 (2008)
HUES-1, Shef1	collagen (type 1)	SFM	24 passages	proteins, genes, EB	32 (2008)
H1, H7, H9, H14	gelatin	MEF-CM	few passages	morphology	45 (2001)
H9, ACT-14	MEF-ECM	SFM	30 passages	proteins, EB, teratoma	127 (2005)
hES2, hES3, hES7	hMSC-derived matrix	hMSC-CM	30 passages	proteins	49 (2008)
HS360, HS401	hECM mixture	SFM	6−7 passages	proteins, genes, EB	29 (2009)
H9	recombinant E-cadherin	SFM	35 passages	proteins, genes, teratoma	103 (2010)
HS360	FBS	SFM	10 passages	proteins, genes, EB	29 (2009)
HS360	human serum	SFM	2 passages	proteins, genes, EB	29 (2009)
H1, hES-NCL1	human serum	hES-dF-CM	27 passages	proteins, genes, EB, teratoma	39 (2005)
hESCs	hyaluronic acid hydrogels	MEF-CM	20 days	proteins, EB	113 (2007)

[a] MEF-CM, mouse embryonic fibroblast-conditioned medium; SFM, serum free medium; hMSC-CM, human mesenchymal stem cell-conditioned medium; hMSC-derived matrix, extracellular matrix derived from human mesenchymal stem cell; hECM mixture, mixture of human extracellular matrix, proteins, surface marker analysis, and immunohistochemical analysis of pluripotency of hESCs; genes, gene expression analysis of pluripotency of hESCs; EB, embryonic body analysis; teratoma, teratoma analysis; Diff, differentiation analysis.

3.2. hESC Culture on Serum-Coated Dishes

Matrigel[37,45,86−89] has been frequently used for the feeder-free growth of hESCs in undifferentiated states because it supports the attachment and growth of undifferentiated hESCs in MEF-CM. The use of Matrigel is, however, not ideal for potential medical applications of hESCs due to the risk of xenogenic pathogens. Stojkovic et al. reported the maintenance of undifferentiated hESC cultures on human serum-coated dishes for several passages using conditioned medium from fibroblasts derived from differentiated hESCs (hES-dF-CM).[39] hESCs grown on human serum-coated dishes under these conditions maintained undifferentiated characteristics after prolonged culture (>27 passages), while hESC cultured on uncoated dishes formed embryoid bodies or attached to the plates, leading to spontaneous differentiation. hESCs cultured on human serum-coated dishes expressed cell surface and intracellular hESC markers typical of undifferentiated cells: SSEA-4, Oct-4, TRA-1-60, TRA-1-81, and alkaline phosphatase.[39] When hESCs were cultured on human serum-coated dishes in the absence of hES-dF-CM, hESCs tended to differentiate spontaneously within 48 h, indicating that hES-dF-CM is a key factor for the maintenance of undifferentiated hESCs in this system. Furthermore, hESCs cultured on human serum-coated dishes had the potential to differentiate into tissues from all three embryonic germ layers *in vivo* and *in vitro* (e.g., cartilage, muscle, primitive neuroectoderm, neural ganglia, kidney, secretory epithelia, connective tissues, etc.) and maintained a normal karyotype.[39] It has been reported that hESCs can maintain pluripotency when cultured on dishes coated with different types of human serum from different batches or prepared from patients with type I diabetes.[39]

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Table 4. Feeder Layer-free Culture of hESCs and Human iPSCs Keeping Their Pluripotency on Synthetic Polymers[a]

hESCs (cell lines)	cell culture substrates	culture medium	longest time in culture	pluripotency evaluation	ref (year)
CHA-hES3, H9	porous PET membranes	SFM +STO feeder cells	25 passages	protein, teratoma	[114](2007)
HUES7, NOTT1	oxygen plasma etched TCPS	MEF-CM	14 passages	proteins, genes, Diff.	[28](2009)
hESCs	TMA-PSt microcarriers	SFM	6 passages	proteins, EB	[40](2008)
HS237	PDTEC	hES-CM	1 passages	proteins, genes, EB	[29](2009)
HS237	PLDLA	hES-CM	1 passages	proteins, genes, EB	[29](2009)
H1	calcium alginate hydrogels	SFM	260 days	proteins, genes	[48](2008)
BG01 V	chitosan and alginate 3D scaffolds	SFM	21 days	proteins, genes, teratoma	[30](2010)
SA167, AS034.1	TCPS	hF-CM	43 passages	proteins, teratoma	[31](2008)
BG01, WIBR3	FBS-coated acrylate copolymer	MEF-CM	10 passages	proteins, genes, teratoma	[117](2010)
BG01, WIBR3	human serum-coated acrylate copolymer	SFM	5 passages	proteins, genes, teratoma	[117](2010)

[a] MEF-CM; mouse embryonic fibroblast-conditioned medium, SFM; serum free medium; proteins; hES-CM; human foreskin fibroblast-conditioned medium, hF-CM; human fiblobrasts-conditioned medium, FBS; fetal bovine serum, proteins; surface marker analysis and immunohistochemical analysis of pluripotency of hESCs, genes; gene expression analysis of pluripotency of hESCs, EB; embryonic body analysis, teratoma; teratoma analysis, Diff; differentiation analysis, PET, polyethylene terephthalate; TMA-PSt, trimethylammonium-coated polystyrene microcarriers; PDTEC; poly-(desaminotyrosyl-tyrosine-ethyl ester carbonate), and PLDLA; poly-L.D-lactide.

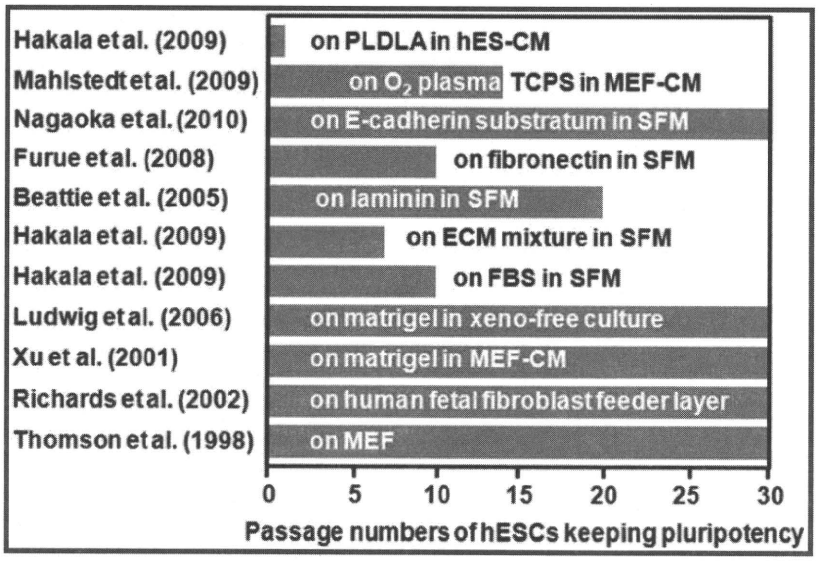

Figure 5. Use of different culture materials for the feeder layer-free culture of hESCs reported in the literature.

This indicates that different soluble growth factors, adhesion molecules, and ECM components that support the maintenance of undifferentiated hESCs are common and consistently present in different batches of human serum prepared from both normal donors and patients with type I diabetes. However, Hakala et al. reported that hESCs cultured on human serum-coated dishes could not maintain pluripotency in a chemically defined medium or conditioned medium prepared from human foreskin fibroblasts.[29] This indicates that the soluble factors in the culture medium of hESCs can also determine the fate of undifferentiated hESCs cultured on human serum-coated dishes.

3.3. hESC Culture on ECM-Coated Dishes

Recombinant or natural collagen IV, laminin, fibronectin, and vitronectin, which are components of the ECM, have been used instead of Matrigel or serum as coating materials for the feeder-free growth of undifferentiated hESCs and human iPSCs. Feeder cell-free and serum-free hESC culture on human fibronectin-coated dishes was investigated in a culture medium containing KnockOut Serum Replacement (Ko-SR) together with TGF-β1 and FGF-2 (Table 3).[29,90]

The feeder layer-free and serum-free culture of hESCs (I-3, I-6, and H-9) on fibronectin-coated dishes showed low differentiation percentages in medium containing Ko-SR, TGF-β1, and FGF-2, while hESCs cultured on gelatin showed complete differentiation on day 8.[35] With regard to the growth rates of hESCs, the colony forming efficiency on human fibronectin was lower but similar to that on MEFs, while the colony forming efficiency of hESCs cultured on bovine fibronectin was dramatically lower than that on MEFs and on human fibronectin.[35] Amit et al. reported that more than 50 passages were possible for hESC culture on fibronectin without differentiation of the cells.[35] However, it should be noted that another study[29] reported that hESCs cultured on fibronectin-coated dishes did not maintain pluripotency under the same conditions described by Amit et al.[35] The hESCs quickly differentiated and attached poorly beyond the second passage. Furthermore, Xu et al. found that hESCs cultured on collagen IV and fibronectin in MEF-CM did not contain as many undifferentiated colonies as those cultured on Matrigels or laminin.[45]

Gelatin is thermally denatured collagen derived from animal skin and bones. Mouse ESCs can be maintained on gelatin-coated

dishes in a culture medium supplemented with LIF for extended periods of time.[91−93] The binding of the LIF receptor β/gp130 heterodimer and activation of the JAK/STAT3 signaling pathway have been implicated in the self-renewal of mouse ESCs.[94] However, gelatin was not able to support undifferentiated hESCs. hESCs seeded onto gelatin in MEF-CM had low survival rates, and the cells tended to differentiate within the first passage.[45]

Laminin is the first extracellular matrix protein expressed in two- to four-cell stage mouse embryos and is a major component of the extracellular matrix of basal laminae in all vertebrates.[45,95,96]

hESCs cultured on laminin could be maintained in an undifferentiated state for more than 42 days.[45] The hESCs cultured on laminin successfully expressed pluripotency genes (Oct-4 and hTERT) and had high telomerase activity. hESC culture on recombinant human laminin (rh laminin) in MEF-CM has also been reported.[50] Recombinant human ECM is abundantly available and a well-characterized source of human-derived proteins produced in an *in vitro* system. Laminin, which is a major component of Matrigel, plays an important role in cellular adhesion and consists of three distinct subunits: α_1 to α_5, β_1 to β_3, and γ_1 to γ_3.[50,97] More than 15 laminin isoforms have been identified, and the expression of these isoforms is specifically regulated by different types of cell surface receptors during embryonic development.[98] Four laminin-binding types ($\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$) among 24 unique integrin isoforms have been identified.[98] Consistent with Xu et al.,[45] Miyazki et al. found that hESCs primarily expressed integrin $\alpha_6\beta_1$, which binds predominantly to laminin-111, −332, and −511/−521.[50] When hESCs were cultured on rh laminin in MEF-CM, the cells adhered well to rh laminin-332-coated plates but did not adhere to rh laminin-511- and rh laminin-111-coated plates.[50] The hESCs proliferated on these three rh laminin-coated plates in MEF-CM for several passages while maintaining pluripotency.[50] These results show that rh laminin-111, -332, and -511 are able to expand undifferentiated hESCs due to their high affinity for integrin $\alpha_6\beta_1$, which is expressed on hESCs; however, it is unknown whether hESC pluripotency can be maintained for extended passages.[50] In addition, because the hESCs were cultured in MEF-CM, the hESC culture on rh laminin is feeder-free but not xeno-free.[50]

hESC culture on laminin-coated dishes using serum-free medium that contains human-derived and recombinant proteins supplemented with recombinant growth factors has also been reported.[75] The hESCs maintained an undifferentiated morphology and expressed pluripotency genes (SSEA-4, Tra-1-60, and Cripto) similarly to cells cultured in MEF-CM. These hESCs were also able to generate teratomas in SCID/beige mice, indicating that the cells from all three germ layers differentiated when the hESCs were cultured for less than 14 passages.[75] hESCs cultured in xeno-free conditions must be evaluated for longer periods (>20−30 passages) for bioengineering applications.

Several reports have suggested that dishes coated with individual proteins from the ECM are inadequate for culturing and maintaining undifferentiated hESCs.[29,60] Therefore, Ludwig et al. used a combination of four human ECM proteins (collagen IV, vitronectin, fibronectin, and laminin) and a defined, xenogenic component-free culture medium to investigate xeno-free hESC culture.[99] The derived hESC lines, however, were karyotypically abnormal.[60] Therefore, a modified, more reasonable xenogenic protein-containing medium (mTeSR1) combined with Matrigel was used for hESC culture and has been offered commercially by their group.[60] Hakala et al. also tried hESC (HS237, HS360, and HS401) culture on a mixture of human ECM components.[29] They were able to culture hESCs for a maximum of seven passages in conditioned medium or xeno-free culture medium (TeSR1), after which all cells showed differentiated morphologies and lost the expression of Oct3/4, a marker of undifferentiated hESCs.[29] The human ECM mixture and xeno-free culture medium did not support maintenance of undifferentiated hESCs beyond the early passages and led to cell detachment and the loss of pluripotency markers.

These results suggest that it is difficult to culture several cell lines of hESCs in feeder layer-free conditions on ECM-coated dishes for multiple passages without using Matrigel containing undefined and animal-derived components.

3.4. hESC Culture on a Recombinant E-cadherin Substratum

E-cadherin is a Ca^{2+}-dependent cell−cell adhesion molecule[100,101] and is essential for intercellular adhesion and colony formation of ESCs.[34,102] Undifferentiated ESCs are expressing a high amount of E-cadherin. Nagaoka et al. prepared a fusion protein consisting of an E-cadherin extracellular domain and the IgG Fc domain (E-cad-Fc), and they investigated the hESC culture on the recombinant E-cadherin substratum in MEF-CM and in serum free medium (mTeSR1).[103] The hESCs thus cultured could maintain pluripotency for >35 passages and could generate embryonic body in vitro and teratoma *in vivo* where histological analysis revealed the presence of cells from all three germ layers.

Integrin-mediated cell-ECM interactions have been considered essential for maintenance of stem cell pluripotency and viability.[104,105] Eventually, as found in previous sections, significant efforts have been devoted to finding a suitable ECM component that can maintain pluripotency of hESCs with interaction between hESCs and integrin receptors on ECMs. Integrin-ECM interactions activate signaling pathways of integrin-linked kinase (ILK) or focal adhesion kinase (FAK) as well as PI3K/Akt and MAPK pathways,[106] while E-cadherin-mediated adhesion of hESCs is typically associated with β-catenin signaling and also stimulates PI3K/Akt signaling.[103,107,108] Especially Akt signaling pathways are considered to be important for maintenance of pluripotency of hESCs.[109,110] It was suggested that trans-homodimerization between E-cadherin on hESCs and the E-cadherin domain presented on the recombinant E-cadherin substratum could promote and maintain the pluripotency of hESCs by activation of the PI3K/Akt signaling pathway.[103]

Mouse ESCs on the recombinant E-cadherin substratum did not form aggregated colonies and were scattered with a spindle-like morphology.[111] In contrast, hESCs retained their ability to form the colonies, as is typically observed on MEF or Matrigels.[103] The pluripotency of mouse ESCs should be maintained by LIF signaling pathways under no aggregated colony formation, where the aggregated colony formation is important to maintain the pluripotency of human ESCs.

3.5. hESC Culture on Glycosaminoglycan

Hyaluronic acid (HA) is a linear polysaccharide of $\beta(1-4)$-D-glucuronic acid and $\beta(1-3)$-*N*-acetyl-D-glucosamine found in the ECM of undifferentiated cells during early embryogenesis. Differentiated cells have reduced expression of HA.[14,112] A synthetic hydrogel matrix of HA has been used for the long-term culture of hESCs with self-renewing capabilities.[113] To prepare hESCs encapsulated in HA gel, hESCs were added to a 2% methacrylated HA and 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone solution. The solution was poured into a mold to generate discs that were 3 mm in diameter and 2 mm thick. These discs were photopolymerized with 10 mW/cm^2 of ultraviolet light for 10 min.[113] hESCs encapsulated in dextran gels were also prepared using a similar method. hESCs encapsulated within HA hydrogels and grown in MEF-CM remained undifferentiated for 20 days, while

hESCs cultured on a monolayer of HA or encapsulated in dextran hydrogels did not maintain pluripotency. Differentiation could be induced within HA hydrogels by addition of different soluble factors to the culture media. hESCs were released from the HA hydrogel by treatment with hyaluronidase to digest the HA hydrogels.[14,113] The HA hydrogels provide a unique microenvironment for the self-renewal and differentiation of hESCs. However, it is difficult to keep the hESCs cultured on HA hydrogels undifferentiated for more than 10 passages.

3.6. hESC Culture on Synthetic Polymers

Biomacromolecules, such as Matrigel and several kinds of ECM, are costly and have limited shelf lives. The development of completely synthetic substrates is desirable for the culture of hESCs and human iPSCs.[28] The use of synthetic polymers that maintain pluripotency and the self-renewing capabilities of the cells has been reported (Table 4).[28−31,40,48,114]

3.6.1. hESC Culture on 2D Synthetic Polymers. hESCs have been cultured on oxygen plasma etched tissue culture polystyrene (PE-TCPS) using MEF-CM. This synthetic culture surface was stable at room temperature for at least a year. hESCs (HUES7 and NOTT1) cultured on PE-TCPS expressed stem cell marker proteins (Oct-4, TRA1-60, and SSEA-4) and showed a stable karyotype after 10−14 passages.[28] The differentiation of HUES7 and NOTT1 cells cultured on PE-TCPS was evaluated by inducing the aggregation of defined numbers of hESCs into EB. qRT-PCR showed that markers of early germ layer formation, Brachyury T (mesoderm), SOX17 (endoderm), and SOX1 (ectoderm), were similarly expressed in hESCs cultured on PE-TCPS and Matrigels.[28] α-Actin (mesoderm), alkaline phosphatase (AFP, endoderm), and β-III tubulin (ectoderm), which are present during the late stage differentiation, were also detected by immunostaining analysis. NOTT1 cells were also induced to differentiate into cardiomyocytes. Beating outgrowths were mechanically isolated from the main body of EBs and seeded onto Matrigel-coated microelectrode arrays (MEAs). The extracellular field potentials of the cell clusters were analyzed with microelectrode arrays.[28] The cell clusters showed a beating rate of 100 per min at rest, while the beating rate increased significantly to 151 per min when the cell clusters were treated with 1 μM isoprenaline, a β-adrenoceptor agonist that is known to have a positive chronotropic effect on the human heart. The pharmacological response observed suggested that the clusters of beating cells contained cardiomyocytes that differentiated from the hESCs because contraction of skeletal or smooth muscle cells would be inhibited or unaffected by isoprenaline.[28]

Harding et al. investigated the use MEF-CM for the culture of hESC on PE-TCPS.[28] hESCs cultured solely on synthetic polymers without the use of xeno-derived biomacromolecules were attempted by Hakala et al.[29] In their study, poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTEC) and poly-L,D-lactide (PLDLA) were used for hESC culture.[29] PLDLA is commonly used for hESC differentiation,[115] while PDTEC is used for guided bone regeneration in animal models.[116] However, the hESCs did not attach to the synthetic polymers in a xeno-free and chemically defined medium. Furthermore, the hESCs did not attach to PLDLA in human foreskin fibroblast-conditioned medium, while some of the hESCs attached to PDTEC, as well as Ti, TiO_2, and ZrO_2 surfaces, in human foreskin fibroblast-conditioned medium.[29] The hESC colonies were very fragile on the synthetic polymers and the Ti, TiO_2, and ZrO_2 surfaces. PLDLA and PDTEC without ECM failed to support hESC culture and did not maintain undifferentiated hESCs, even in human foreskin fibroblast-conditioned medium. These results demonstrate the difficulty of culturing hESCs solely on synthetic polymers in xeno-free cultures.

It is inefficient to evaluate synthetic polymers for the culture of hESCs without the concept of design of the biomaterials. Therefore, Mei et al. developed a combinatorial technique to evaluate the biomaterials using microarrays for the culture of hESC and iPSC maintaining their pluripotency.[117] The microarrays were prepared from 22 acrylate monomers with diversified hydrophobicity−hydrophilicity (water contact angle) and cross-linking densities. The microarrays were prepared by copolymerization between each of 16 "major" monomers and each of six "minor" monomers at six different ratios [100:0, 90:10, 85:15, 80:20, 75:25, 70:30 (v/v)]. Therefore, microarrays with 496 [16 + (16 × 5 × 6)] different combinations of copolymers were evaluated, consisting of the major monomer (70−100%) and minor monomer (0−30%). Water contact angle, surface topography, surface chemistry [analysis of functional group by time-of-flight secondary-ion mass spectroscopy (ToF-SIMS) analysis], and indentation elastic modulus of polymeric substrates were quantified using high-throughput methods to develop structure−function relationships between material properties and pluripotency of hESCs cultured on the polymeric substrates coated with fetal bovine serum (FBS) in the microarray.[117]

Proteins such as ECM and growth factors, and glycosaminoglycans from FBS can adsorb onto the material surface used for cell culture.[118] The surface properties of cell-culture substrates are regulated by both the amount and the conformation of adsorbed proteins and glycosaminoglycans, which interact with cell surface receptors to initiate signal transduction and alter cell behavior. Therefore, the synthetic polymeric materials having no specific binding sites for hESCs can be converted into the materials having specific binding sites after the materials were adsorbed with FBS and culture medium, because components and amount of proteins and glycosaminoglycans on the materials depend on the surface chemistry and physics of the materials.[117]

The colony-formation frequency was defined and investigated as the number of polymer spots on which hESC colonies (expressing Oct-4 and SSEA-4) formed divided by the total number of replicate spots of the same kind of polymer on each array. The surface roughness of the substrate in air, in PBS, and in culture medium after FBS adsorption did not correlate strongly with colony-formation frequency, although it was reported to affect the cell growth and attachment of adult somatic and stem cells.[26,119] A positive correlation was observed between the indentation elastic modulus of hydrated polymeric substrate and colony-formation frequency. However, it was found that the polymeric substrate exhibiting a low indentation elastic modulus also exhibited a low water contact angle in their polymer substrates. The optimum wettability (65° < water contact angle < 80°) of copolymer showed high colony-formation frequency over a broad range of polymer stiffness. Especially, polymers with a moderate water contact angle generated from multiple-acrylate-group-containing monomers performed the best colony-formation frequency in their experiments.[117]

The hit arrays were further evaluated for their capacity to maintain the pluripotency of hESCs after more than 2 months of culture (>10 passages). HSCs were found to maintain an undifferentiated state with evidence from expression of pluripotent markers, Oct-4, Nanog, Tra-1-60, and SSEA-4 after prolonged culture. The differentiation of these hESCs into all three germ layer lineages was also confirmed.[117]

3.6.2. hESC Culture on Porous Polymeric Membranes. hESCs cultured on a conventional MEF-feeder layer must be

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treated with enzymes, such as collagenase or Dispase, when the hESCs are transferred to new culture dishes for isolation or expansion. Continuous exposure to enzymes can cause cytogenetic aberrations in the hESCs, although enzyme treatment is advantageous for the large-scale bulk expansion of hESCs over a short time period with laborious and time-consuming steps. An interesting hESC culture method was reported by Kim et al.; in this study, porous polymeric membranes (1, 3, and 8 μm pore sizes) were used to separate hESCs and feeder cells.[114] The feeder cells were seeded and attached to the bottom of the porous membranes of trans-well inserts. hESCs were then cultured on top of the membranes (Figure 2). This method allowed the hESCs to be successfully cultured and effectively separated from the feeder cell layer without enzyme treatment. hESCs were placed onto the feeder cells through the porous membrane barrier without displacing the feeder cells, while the hESCs seeded on the feeder cells began to push the feeder cells away and then attach and grow on the culture dish.[114]

hESCs on the membranes interacted with the feeder cells through the pores of the membranes. The interaction was dependent on the pore size of the porous membranes used. The number of attached hESC colonies was dependent on the cell density of the feeder cells on the bottom of the membranes. On the other hand, hESC colonies did not attach to the porous membranes when the feeder cells were located on the bottom of the culture dish instead of on the porous membrane.[114]

hESC attachment on 3-μm and 8-μm porous membranes was higher compared to that on the 1-μm porous membranes. However, porous membranes with greater than 3-μm pore sizes allowed feeder cells on the bottom of the porous membranes to migrate upward, which generated contamination of the hESC colonies. The 1-μm pore membranes rarely permitted migration of the feeder cells.[114] hESCs cultured on the 1-μm pore size membrane failed to maintain the hESCs for more than 15 passages, while hESCs on the 3-μm pore size membranes sustained the culture for more than 25 passages.[114]

The hESCs cultured on the porous membranes not only exhibited the expression of several undifferentiated markers and a normal karyotype but also formed teratomas that consisted of all three germ layers *in vivo*. This indicates that cell−cell contacts through the membrane pore and/or a close distance between the hESCs and feeder cells are important for maintaining the undifferentiated states of hESCs. Although the hESCs were cultured under xeno-containing conditions, culturing hESCs on porous membranes would be a useful method to exclude enzyme treatment and prevent contamination from feeder cells.

3.7. hESC Culture on 3D Biomaterials

The culture of hESCs on a 3D porous polymeric scaffold composed of chitosan and alginate and without the support of feeder cells or conditioned medium has been reported.[30] The pluripotency of the hESCs was maintained in the serum-free medium for 21 days. The hESCs expressed the expected gene profile for undifferentiated hESCs, including Oct-4, Nanog, SSEA-4, TERT, and AFP. The hESCs also formed teratomas in SCID mice that included derivatives of all three germ layers. However, this study[30] did not determine whether the pluripotency of hESCs could be maintained for >30 days.

hESC culture and expansion on microcarriers has also been reported. Phillips et al. reported the successful feeder-free 3D suspension culture of hESCs (ESI-017) on trimethyl ammonium-coated polystyrene microcarriers in serum-free medium.[40] The hESCs were maintained through six passages with a 14-fold increase in cell number. The cells expressed several undifferentiated markers, including Oct-4 and Tra-1-81,[40] showing that the suspension-based expansion of hESCs on microcarriers was possible under feeder layer-free conditions. Using directed differentiation protocols, it was possible to induce the hESCs cultured on the microcarriers after six passages to express the pancreatic marker, pdx-1, and neuronal marker, Tuj1 (β-III-tubulin). The hESCs expressed cardiomyocyte markers, such as α-actin, Nkx2.5, Mlc2a, and tropomyosin.[40] Thus, the hESCs retained their capacity to differentiate into the pancreatic (endoderm), neuronal (ectoderm), and cardiomyocyte (mesoderm) lineages. However, the pluripotency for the hESCs was not determined for passages >10.

One of the difficulties of 3D culture using microcarriers or porous materials is the detachment of hESCs during the passage of the cells.[120] In general, hESCs were tightly adhered in tortuous environment, and it was difficult to recover the hESCs efficiently, even with an enzymatic treatment.

In another report, hESCs were maintained in a feeder layer-free and xeno-free environment by encapsulation in hydrogels.[48] hESCs were encapsulated in calcium alginate hydrogels and grown in a serum-free medium for up to 260 days. The encapsulated hESCs formed aggregates that increased in number and size without loss of the cells from the hydrogel. The aggregates were tightly and homogeneously packed with defined spherical borders. The hESCs retained their pluripotency and differentiated into cells of all three germ layers when they were subsequently cultured in differentiation medium.[48] Immunohistochemistry and RT-PCR experiments showed that the hESC aggregates expressed pluripotent proteins and genes, including Oct-4, Nanog, SSEA-4, TRA-1-60, and TRA-1-81. The hESCs were arranged in closely packed clusters and showed no cytoplasmic organelles, suggesting that they were in an undifferentiated state.[48] This study showed that encapsulation in the appropriate hydrogels allows the hESCs to maintain an undifferentiated state without passaging, EB formation, or xenogenic contamination. Furthermore, hESCs encapsulated in alginate hydrogels were easily recovered from the hydrogels using a dissolution buffer.

Although hESCs encapsulated in HA maintained a undifferentiated state only for 10 passages,[113] encapsulation (3D culture) of hESCs in the appropriate materials seems to support long-term maintenance in the undifferentiated state without the need for feeders or passaging.

The 3D microenvironments that hESCs encounter *in vivo* have a combination of biological, chemical, physical, and mechanical cues, which can be mimicked by hydrogels, while traditional 2D culture is conducted on flat and rigid substrates of tissue culture polystyrene dishes (TCPS).[14,121] During embryogenesis, cells in the inner cell mass are embedded in a 3D matrix, which regulates both their self-renewal and differentiation.[122]

It is important to establish a 3D culture system using hydrogels in which hESCs can be maintained as undifferentiated cells and then induced to differentiate by external signals, such as soluble growth factors or chemicals in the culture medium.

4. CONCLUSIONS

Human feeder cells, including human fetal fibroblasts and human bone marrow cells, have been developed for culturing hESCs.[5,114,123−125] However, it is difficult to achieve high passage numbers and to produce sufficient hESCs for clinical therapy with human feeder cells because human feeder cells are unable to maintain continuous, undifferentiated hESCs as well as animal feeder cells, such as STO and MEF.[114,123]

The development of feeder cell-free hESC culture would substantially reduce the labor and cost of hESC culture, and would increase

the production of hESCs for potential clinical applications.[29] Currently, no synthetic polymers, with or without ECM and growth factors, have been able to maintain the pluripotency and undifferentiated state of hESCs in a xeno-free culture medium for an extended period of time. Matrigel-coated plates combined with a chemically defined culture medium containing xenogenic proteins support long-term undifferentiated hESC culture, and this would be the most adequate conditions for feeder layer-free culture. Mouse ESC culture is simpler because the culture medium containing LIF and/or a LIF-immobilized surface sufficiently supports mouse ESCs in a feeder layer-free culture.[6,126] The replacement of Matrigel with LIF and/or mixed mouse or human ECMs[127] is insufficient to maintain the pluripotency of hESCs. Matrigel is known to contain several soluble factors, including ECM and growth factors. Some currently unknown key growth factors or soluble factors might be required for the maintenance of undifferentiated hESCs, and identification of these factors would contribute to the development of feeder layer-free hESC culture in xeno-free and chemically defined culture medium.

The relationship between the surface chemistry of culture materials and the maintenance of the pluripotency of hESCs and iPSCs is not still clear tentatively, although the surface roughness affects the cell growth and attachment of MSCs and also the elasticity of culture materials can direct MSCs into specific cell lineages (e.g., soft culture materials that mimic brain are neurogenic, stiffer culture materials that mimic muscle are myogenic, and rigid culture materials that mimic collagenous bone prove osteogenic).[26] It is only reported that the optimal wettability of the culture materials where FBS coated is maintaining the pluripotency of hESCs and iPSCs.[117] If we design the culture dishes prepared with different synthetic polymers (having different roughness, elasticity, and wettability) and immobilized with ECMs, the culture dishes having different surface chemistry can be prepared where the same ECMs are immobilized. The combination of surface chemistry of the culture materials and specific interaction between human ECM proteins and hESCs or iPSCs would improve the maintenance of undifferentiated hESCs and iPSCs in a xeno-free culture for a longer time.

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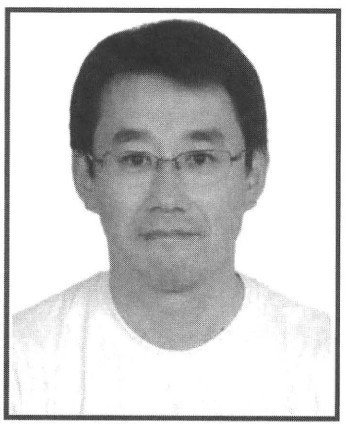

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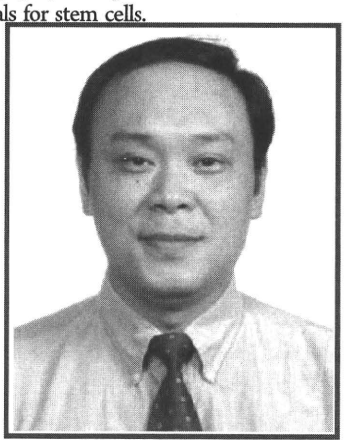

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