

stem cells into trophoblast and placenta during early mammalian development.

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Growth factor-defined culture medium for human mesenchymal stem cells

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ABSTRACT Human bone marrow-derived mesenchymal stem cells (hMSCs) are potential cellular sources of therapeutic stem cells as they have the ability to proliferate and differentiate into a wide array of mesenchymal cell types such as osteoblasts, chondroblasts and adipocytes. hMSCs have been used clinically to treat patients with graft vs. host disease, osteogenesis imperfecta, or alveolar cleft, suggesting that transplantation of hMSCs is comparatively safe as a stem cell-based therapy. However, conventional culture medium for hMSCs contains fetal bovine serum (FBS). In the present study, we developed a growth factor-defined, serum-free medium for culturing hMSCs. Under these conditions, TGF- β 1 promoted proliferation of hMSCs. The expanded hMSC population expressed the human pluripotency markers SSEA-3, -4, NANOG, OCT3/4 and SOX2. Furthermore, double positive cells for SSEA-3 and a mesenchymal cell marker, CD105, were detected in the population. The potential to differentiate into osteoblasts and adipocytes was confirmed. This work provides a useful tool to understand the basic biological properties of hMSCs in culture.

KEY WORDS: *mesenchymal stem cell, serum-free culture, TGF- β 1*

Introduction

Bone marrow-derived cells can differentiate into osteoblasts *in vitro* and *in vivo* (Friedenstein *et al.*, 1966) and thus are considered a useful source of stem cells for bone regeneration. Recently, many studies have reported that human bone marrow contains a distinct cell fraction referred to as multipotent mesenchymal stem cells (hMSCs) which can give rise to a wide array of mesenchymal cell types, including bone, fat, and cartilage (Pittenger *et al.*, 1999). However, hMSCs can differentiate along some ectodermal and endodermal cell lineages such as neuronal cells and liver cells (Pittenger *et al.*, 1999; Dezawa *et al.*, 2004; Dezawa *et al.*, 2005). Further, a recent study reported that hMSCs have the ability to generate the multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*,

2010). It has been estimated that hMSCs comprise about 0.001 to 0.01% of total bone marrow mononuclear cells (Pittenger *et al.*, 1999). For use in cell-based therapies, hMSC populations require extensive *in vitro* expansion to obtain sufficient numbers. The conventional culture medium for hMSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Haynesworth *et al.*, 1992; Lennon DP, 1996). Although these traditional culture conditions provide robust undifferentiated hMSC expansion, the ill-defined components of FBS is undesirable for clinical applications and also hampers analysis of the cell biological mechanisms that control cell behavior.

Abbreviations used in this paper: hES cells, human embryonic stem cells; hMSCs, human mesenchymal stem cells.

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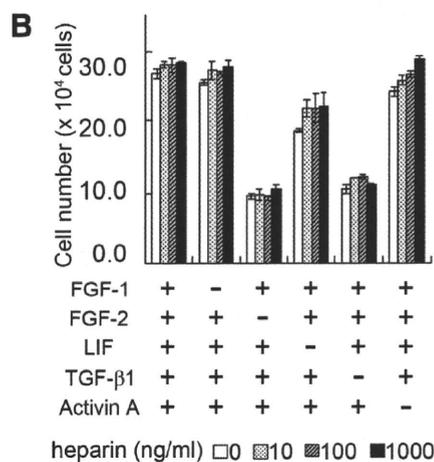
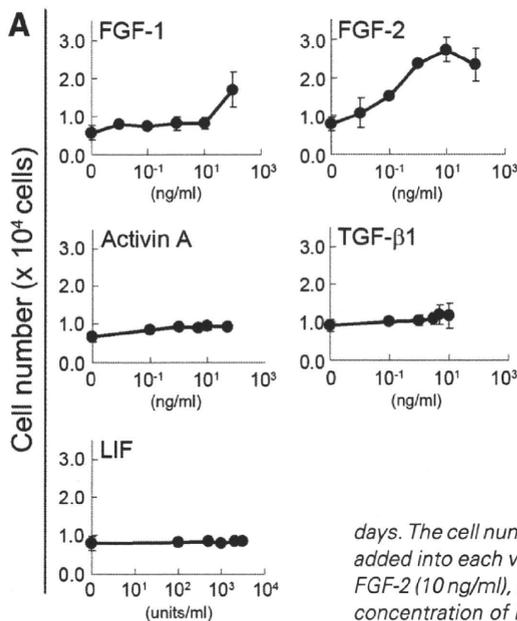
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We and others previously described serum-free media consisting of minimum essential components suitable to propagate and accurately analyze the characteristics of differentiated cells (Hayashi and Sato, 1976; Furue and Saito, 1998; Sato *et al.*, 2002; Furue *et al.*, 2005; Furue *et al.*, 2008; Hayashi *et al.*, 2010). One of these media, hESF9, supports the serial cultivation of undifferentiated human embryonic stem (hES) cells in the absence of feeder cells and thus provides an experimental system for elucidating cellular responses to specific environmental stimuli (Furue *et al.*, 2008; Na *et al.*, 2010). For example, either FGF-2 or heparin promotes proliferation of hES cells in a concentration-dependent manner although these effects were not detected under conventional culture conditions. Thus, a defined serum-free medium consisting of minimum essential components should be useful in elucidating hES/iPS cell responses to specific cues that control self-renewal, differentiation, and lineage selection (Furue *et al.*, 2010).

Because hMSCs have multipotent properties similar to hES cells, we speculated that hMSCs should be able to grow in similar culture conditions as hES cells. In the present study, we demonstrated that addition of TGF- β 1 to the defined serum-free medium for hES cells supports the robust proliferation of hMSCs. The hMSC population expanded in the absence of serum expressed the mesenchymal cell markers CD44, CD73, CD90, and CD105. Further, they expressed human pluripotency surface markers, SSEA-3, -4, TRA-2-54, and also the transcription factors of *NANOG*, *OCT3/4*, and *SOX2*. We show that the serum-free expanded hMSCs can differentiate into osteoblasts and adipocytes. This work sets the stage for serum-free hMSC cell culture and thereby provides a useful tool to understand the basic biological characteristics of hMSCs.

Results

In this study we used a human bone marrow-derived hMSC line designated UE7T-13 (JCRB 1154). The life span of these



days. The cell numbers were counted by Coulter Counter. (A) Each growth factor at indicated concentration was added into each well individually. The values are the mean \pm SD ($n=3$). (B) All five factors of FGF-1 (100 ng/ml), FGF-2 (10 ng/ml), LIF (2000 unit/ml), TGF- β 1 (5 ng/ml), and activin A (10 ng/ml) or four factors of them with varying concentration of heparin were added into each well. The values are the mean \pm SD ($n=3$).

cells was prolonged by infecting them with a retrovirus containing human papillomavirus E7 and telomerase reverse transcriptase (hTERT) cDNAs (Mori *et al.*, 2005; Shimomura *et al.*, 2007; Ishii *et al.*, 2008; Takeuchi *et al.*, 2007). We first tested the ability of hESF9 medium, which we had developed for use with hES cells, to support the growth of UE7T-13 cells. The cells were harvested using trypsin/EDTA, from cultures in conventional medium containing 10% FBS (POWERDBY10) and transferred to 0.1% gelatin-coated dishes in hESF9 medium. However, UE7T-13 cell growth was quite slow. We then investigated the effects of various growth factors on proliferation of the cells. UE7T-13 cells were seeded on 0.1% gelatin in hESF9 in the absence of FGF-2 and heparin (hESF9(-/-)), containing increasing concentrations of FGF-1, FGF-2, TGF- β 1, activin A, or leukemia inhibitory factor (LIF) (Fig. 1). Both FGF-1 and FGF-2 promoted UE7T-13 proliferation in a dose-dependent manner, and the greatest effect was seen at 10 ng/ml FGF-2. Neither LIF nor activin A affected on UE7T-13 cell proliferation, but TGF- β 1 slightly stimulated UE7T-13 proliferation. Next all five factors (FGF-1, FGF-2, TGF- β 1, activin A, and LIF) or four factors with increasing concentrations of heparin were added to UE7T-13 cultures (Fig. 2). When either FGF-2 or TGF- β 1 was withdrawn from the cultures, the cell numbers decreased significantly. Heparin promoted cell proliferation in a dose-dependent manner. This result suggested that addition of FGF-2 and TGF- β 1 to hESF9(-/-) medium, is critical for UE7T-13 proliferation, and heparin also enhanced cell growth. hESF9 medium supplemented with TGF- β 1 was designated hESF10.

L-ascorbic acid-2-phosphate (Asc 2-P) in hESF9 medium supported hES cells. However, it is known to promote hMSC cell differentiation into osteoblasts. Therefore, we examined whether the presence of Asc 2-P in hESF10 medium promoted osteoblastic differentiation of UE7T-13 cells. We analyzed the expression of *bone sialoprotein (IBSP)*, *osteocalcin (BGP)*, *osteonectin (SPOCK2)*, and *osteopontin (SPP1)* in UE7T-13 cell cultured in hESF10 with or without Asc 2-P and in conventional medium (Fig. 2). These osteoblast genes were expressed at significantly lower levels in cells cultured in the serum-free media than in those cultured in the conventional medium. These results suggest that the serum-free medium is suitable for hMSC maintenance. *IBSP* gene expression was higher in the cells cultured in the

Fig. 1. Effect of growth factors on UE7T-13 cell proliferation in defined serum-free culture conditions. After the UE7T-13 cell grown in the conventional culture conditions (POWERDBY10) were cultured in hESF9(-/-) overnight, the cells were seeded in a 24-well plate coated 0.1% gelatin in hESF9(-/-) at 1×10^4 cells per well and cultured for 6

presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3⁺/CD105⁺ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrin β 1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

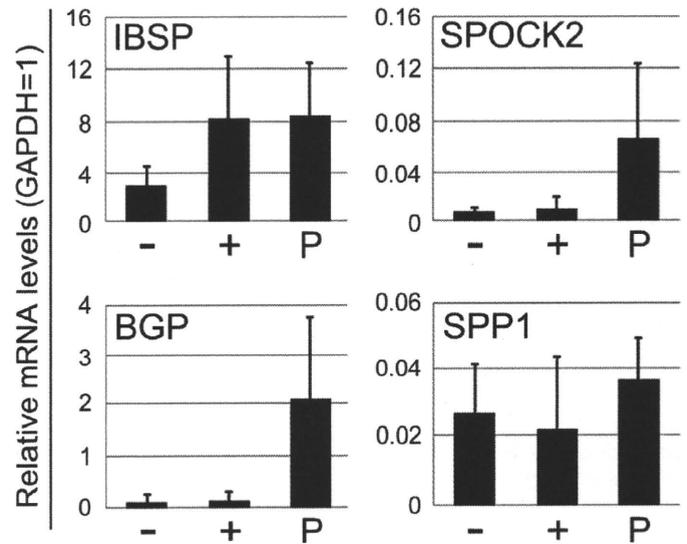


Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of GAPDH. The values are the mean \pm SD ($n=3$).

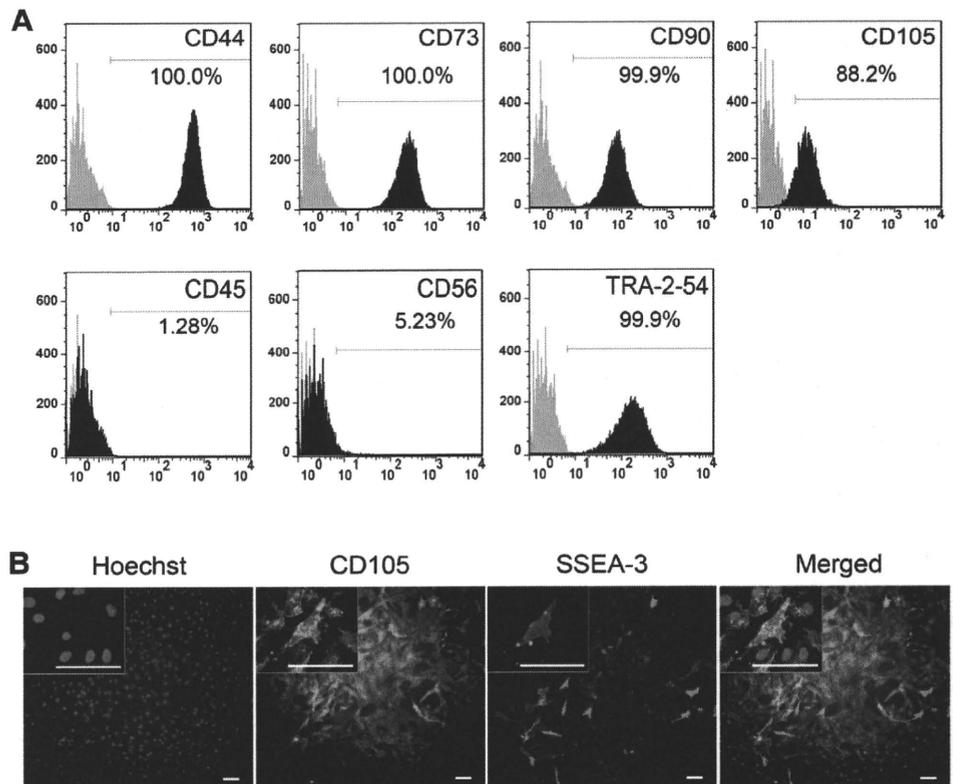


Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells. (B) Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μ m.

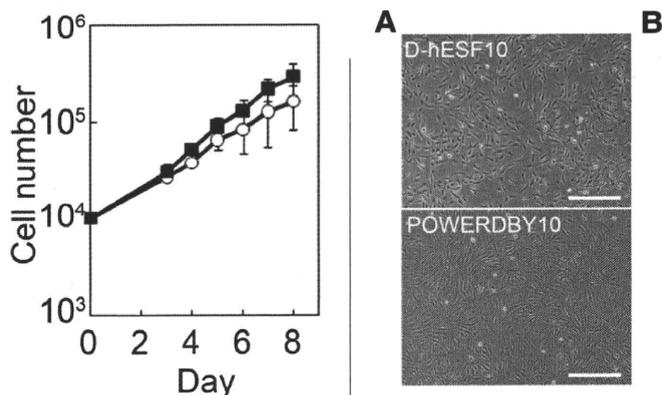


Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1×10^4 cells per well. Cell numbers were counted every day. The values are the mean \pm SD ($n=3$).

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF- β 1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to

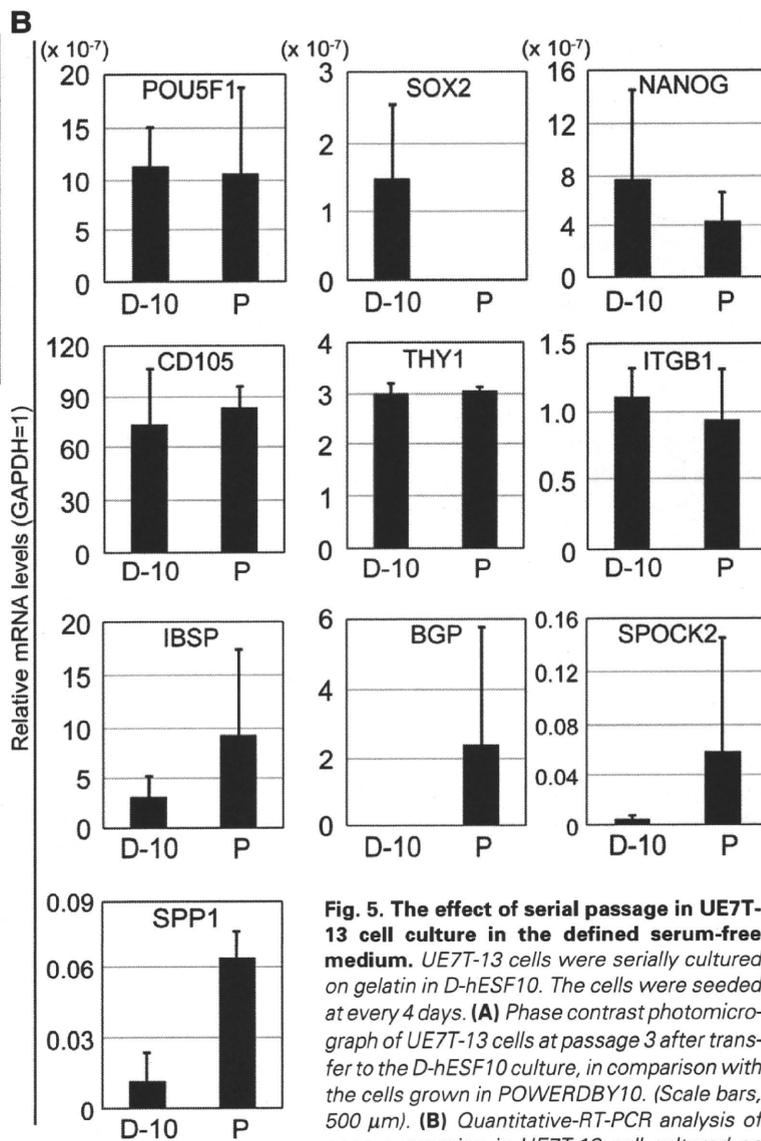


Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days. (A) Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μ m). (B) Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to GAPDH. The values are the mean \pm SD ($n=3$).

support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

The TGF- β 1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF- β 1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF- β 1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF- β 1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so

they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF- β 1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

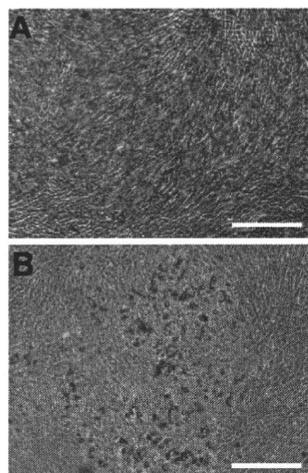
Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of 1×10^4 cells/well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium. (A) Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red). (B) Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μ m.



Antigen expression

For *in situ* immunocytochemistry, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the binding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

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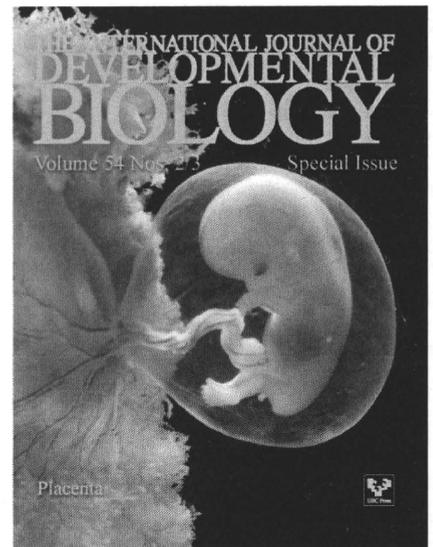
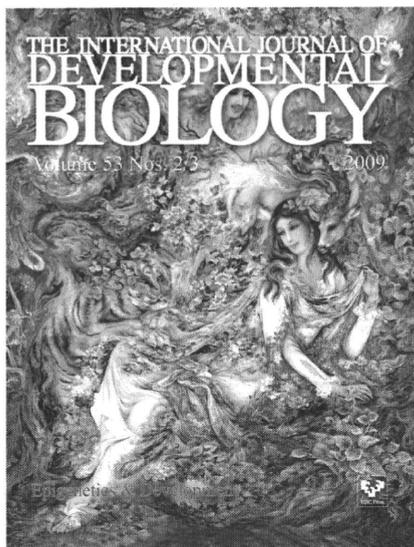
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Gaetana A. Tonti and Ferdinando Mannello
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Lectin microarray analysis of pluripotent and multipotent stem cells

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

Introduction

Stem cells produce almost every tissue of the human body. In general, they have the ability to divide and self-renew and to differentiate into various cell types. Stem cells have varying degrees of differentiation potential: (i) totipotency (ability to form the embryo and the trophoblast of the placenta) like fertilized eggs (zygotes); (ii) pluripotency (ability to differentiate into almost all cells that arise from the three germ layers) like human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells; (iii) multipotentiality (capability of producing a limited range of differentiated cell lineages upon their location) like most tissue-based stem cells; and (iv) unipotentiality (ability

to generate one cell type) like cells such as the epidermal stem cells and the spermatogonial cells of the testis. That is, a hierarchy of stem cells exists. In addition, human ES cell lines show variation in differentiation propensity (Osafune *et al.* 2008). iPS cells, another type of pluripotent stem cell, have been generated from somatic cells of different origin by retroviral transduction of four transcription factors (Takahashi *et al.* 2007; Yu *et al.* 2007). The established iPS cells have a wider variety of differentiation ability and gene expression when compared to ES cells (Aoi *et al.* 2008; Lee *et al.* 2009; Kaichi *et al.* 2010). However, a small proportion of these stem cells sometimes show spontaneous differentiation during serial passage. Therefore, to realize the potential for iPS cells to be utilized for cell therapy and as a valuable tool for drug discovery, it is necessary to monitor the status of these stem cells and to define

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

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

Results

Lectin microarray analysis of mouse mesenchymal cells

Mesenchymal stem cells are multipotent and therefore may be useful in cell-based therapy along with ES cells and iPS cells. Mesenchymal stem cell (MSC) lines [(9–15c), osteoblasts (KUSA–A1), chondroblasts (KUM5)

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

Lectin microarray analysis of human mesenchymal cells

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

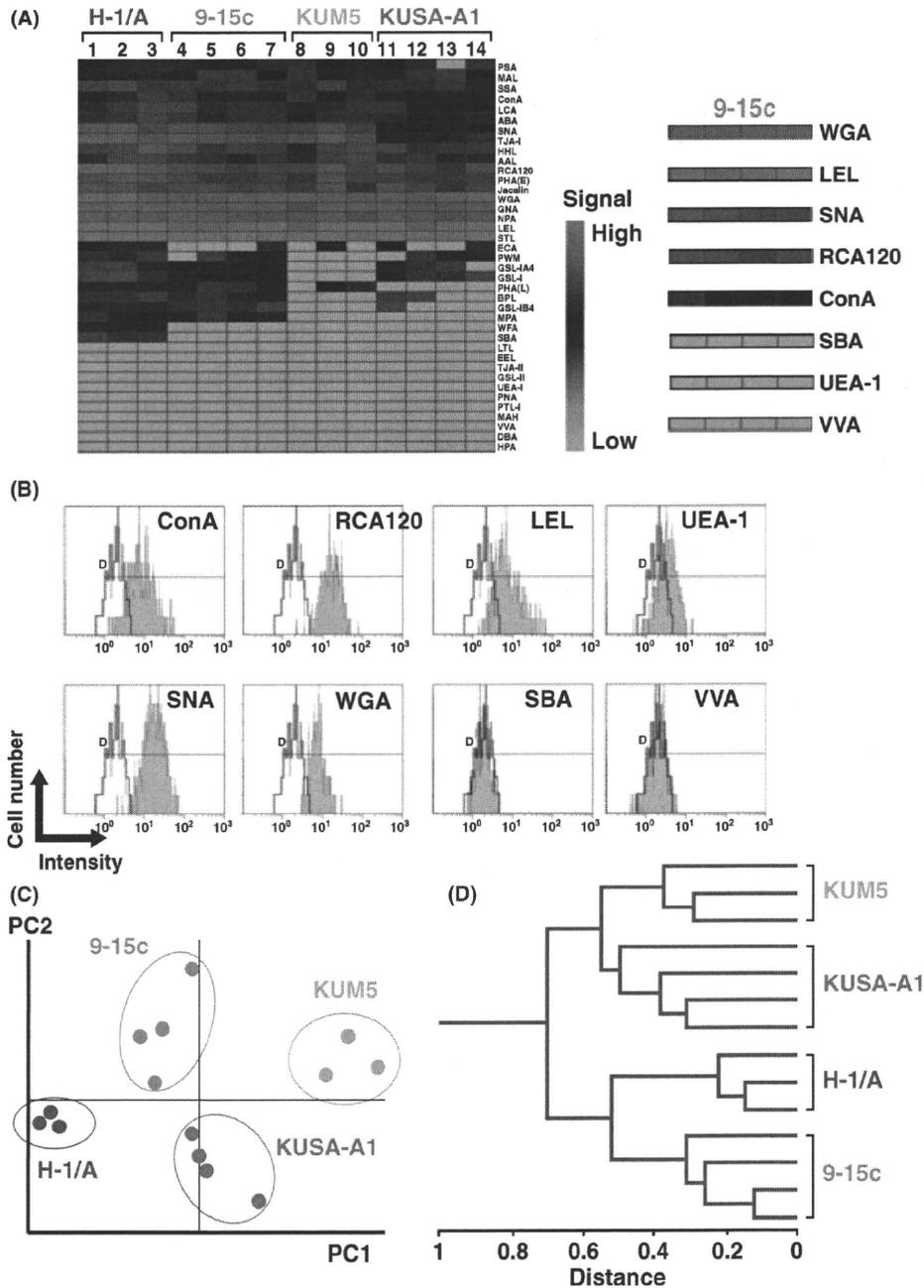


Figure 1 Lectin microarray analysis of mouse mesenchymal cells. (A) Heat map of 9-15c multipotent cells, KUSA-A1 osteoblasts, KUM5 chondroblasts and H-1/A preadipocytes. (B) Flow cytometric analysis of 9-15c multipotent cells using each lectin probe. Mean fluorescent intensities by flow cytometric analysis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

Human mesenchymal cells reacted to (i) *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Aspergillus oryzae* lectin (AOL) and *Aleuria aurantia*

lectin (AAL) that bind to Fuc α 1-6GlcNAc; (ii) SNA, *Sambucus sieboldiana* agglutinin (SSA) and *Trichosanthes japonica* agglutinin I (TJA-I) that bind to

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).

Sia α 2-6Gal/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 GlcNAc β 1-4GlcNAc. 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 Gal β 1-3GalNAc 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 Sia α 2-3Gal β 1-4GlcNAc, tri/tetra-antennary complex-type N-glycan and Gal α 1-3Gal, 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

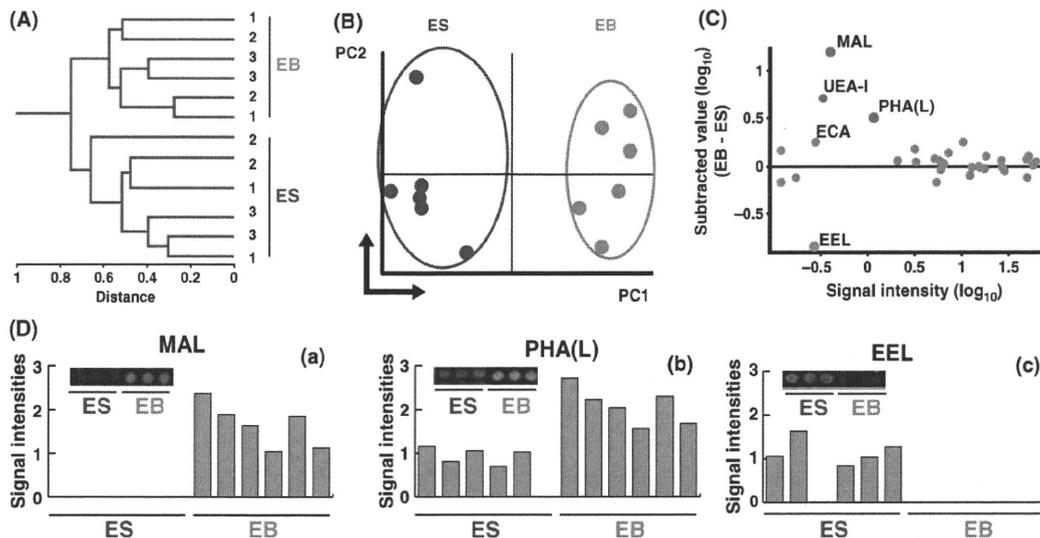


Figure 3 Lectin microarray analysis of human embryonic stem cells. (A) Hierarchical Clustering analysis of undifferentiated and differentiated ES cells. (B) Principal component analysis of lectin microarray analysis on undifferentiated and differentiated ES cells. (C) Signal value for *Maackia amurensis*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

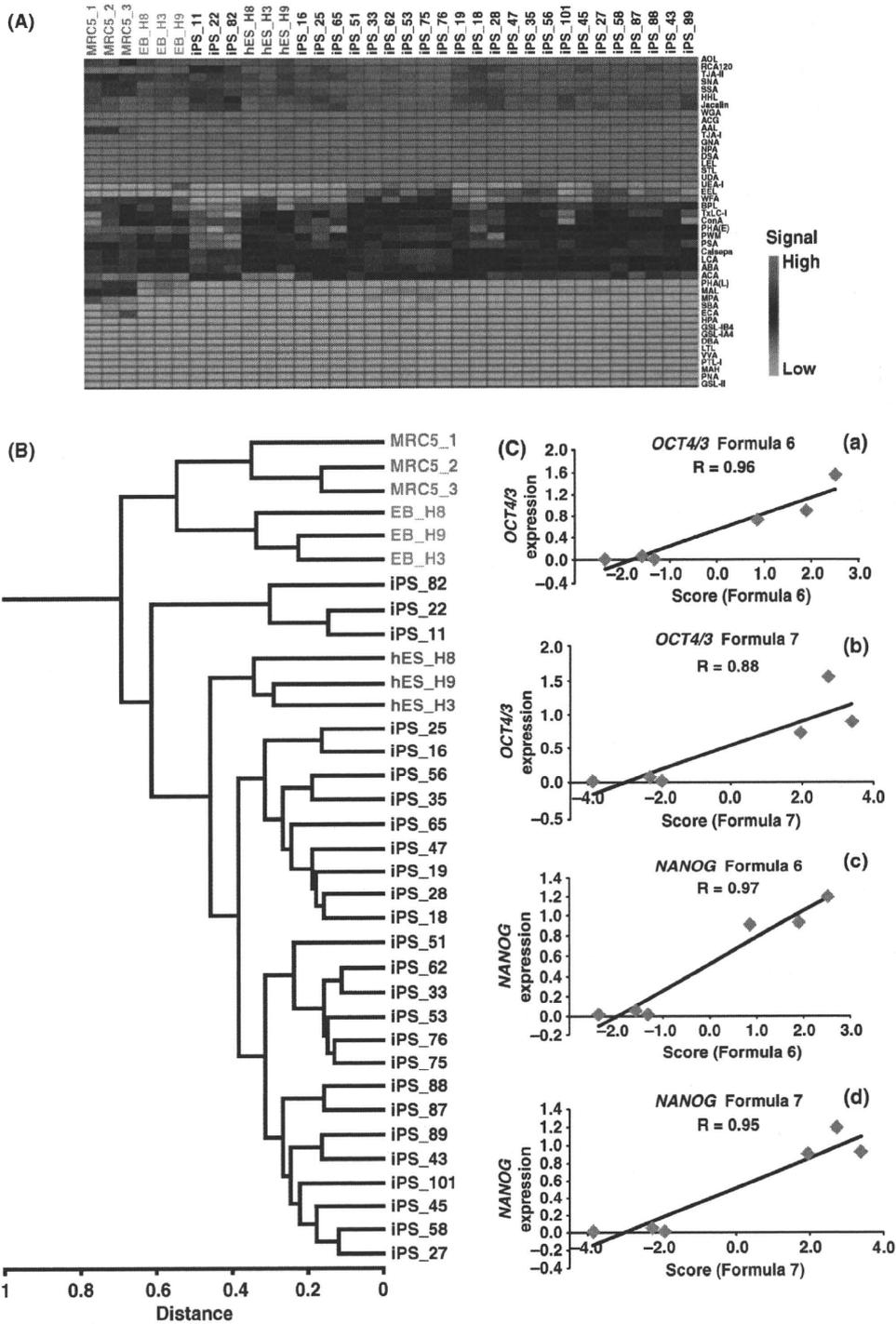


Figure 4 Lectin microarray analysis of human-induced pluripotent stem (iPS) cells. (A) Heat map of lectin microarray with MRC-5 and MRC-5-derived iPS cells. MRC-5 and iPS cells are discriminated by letter color: red, MRC-5; blue, hES cells; green, embryoid body (EB) cells; black, iPS cells. (B) Hierarchical 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 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: $F = -1.75 \times \text{PHA(L)} + 1.28 \times \text{EEL} + 1.92$) shows the highest accuracy (100%) of determination of pluripotency, followed by that of MAL and EEL (Formula 5: $F = -2.45 \times \text{MAL} + 1.23 \times \text{EEL} + 1.45$) (97%), whereas the discriminant

Table 1 Discriminant functions

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

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

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

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

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

function with the combination of three lectins (Formula 7: $F = -2.98 \times \text{MAL} + 0.75 \times \text{PHA(L)} + 1.44 \times \text{EEL} + 0.70$) and MAL and PHA(L) (Formula 4: $F = -2.81 \times \text{MAL} + 0.03 \times \text{PHA(L)} + 2.29$) shows 86.6% and 55.2%, respectively. Determination with single lectins shows 94.0% (Formula 2: $F = -2.38 \times \text{PHA(L)} + 3.46$), 55.2% (Formula 1: $F = -2.78 \times \text{MAL} + 2.32$) and 89.6% (Formula 3: $F = 2.59 \times \text{EEL} + 1.25$) 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).

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 (Katrlík *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, α 1-2Fuc-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, Gal α 1-3Gal, 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% CO₂. 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 CO₂ 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 Valugen 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 CellLytic 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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