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Adipogenic differentiation of human induced pluripotent stem cells: Comparison with that of human embryonic stem cells

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ABSTRACT

Induced pluripotent stem (iPS) cells were recently established from human fibroblasts. In the present study we investigated the adipogenic differentiation properties of four human iPS cell lines and compared them with those of two human embryonic stem (ES) cell lines. After 12 days of embryoid body formation and an additional 10 days of differentiation on Poly-L-ornithine and fibronectin-coated dishes with adipogenic differentiation medium, human iPS cells exhibited lipid accumulation and transcription of adipogenesis-related molecules such as C/EBPα, PPARγ2, leptin and aP2. These results demonstrate that human iPS cells have an adipogenic potential comparable to human ES cells.

1. Introduction

Pluripotent embryonic stem (ES) cells have been considered potent candidates for regenerative medicine as an unlimited source of cells for the transplantation therapy and a useful tool for the investigation of cell development/differentiation, especially after establishment of human ES cells [1]. We previously clarified the differentiation process of mouse, monkey and human ES cells into vascular cells [2–4] and demonstrated that transplantation of vascular cells derived from human ES cells may constitute a novel strategy for vascular regeneration [4,5]. A number of immunological and ethical problems remain to be overcome before clinical application of the ES cells, however. Recently, novel ES cell-like pluripotent cells, termed induced pluripotent stem (iPS) cells, were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into mouse skin fibroblasts [6], and soon thereafter iPS cells were also generated from human skin fibroblasts [7,8]. Since then, a new generation of human iPS cells has been generated by introducing into fibroblasts just three of the aforementioned transcription factors (c-Myc was omitted) [9]. By overcoming the immunological and ethical problems associated with ES cells, iPS cells open a new avenue for cell transplantation-based regenerative medicine and provide a powerful new tool with which to investigate organ development/differentiation in specific disease states, especially in inherited diseases.

Generalized lipodystrophy consists of congenital and acquired types characterized by the lack of the whole adipose tissue, which leads to severe insulin-resistant diabetes, hypertriglyceridemia and fatty liver. We previously analyzed genes and phenotypes of congenital generalized lipodystrophic Japanese [10] and also demonstrated the long-lasting efficacy and safety of the leptin-replacement therapy in these patients [11–13]. Since metabolic abnormality in the mouse model is known to be cured by mature

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adipocytes transplantation, the regeneration therapy of the adipose tissue with human iPS cells-derived adipocytes is the ideal goal for lipodystrophic patients. Moreover, in vitro adipogenic differentiation system of human iPS cells will contribute to elucidate the pathogenesis of congenital generalized lipodystrophy when iPS cell lines are established from patients with lipodystrophy. In the present study we have investigated the adipogenic differentiation of human iPS cells and compared with that of human ES cells.

2. Materials and methods

2.1. Cells and culture

Four human iPS cell lines (201B6, 201B7, 253G1 and 253G4) were investigated. The 201B6 (B6) and 201B7 (B7) lines were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into human skin fibroblasts while the 253G1 (G1) and 253G4 (G4) lines were generated using only three factors (c-Myc was omitted) [9]. These iPS cell lines were maintained as previously described [7]. Two human ES cell lines (H9 and KhES-1) were used and maintained as previously described [1,14].

2.2. Adipogenic differentiation

For embryoid body (EB) formation, iPS and ES colonies were digested with 1 mg/ml collagenase type IV (GIBCO, CA, USA) and plated onto non-adherent bacterial culture dishes, where they were allowed to aggregate in maintenance medium without bFGF. Retinoic acid (Sigma–Aldrich, Japan) was added to the medium to a concentration of 100 nM from day 2 to day 5. After 12 days, EBs were transferred to 6-well plates coated with a combination of 30 μg/ml Poly-L-ornithine (Sigma–Aldrich) and 2 μg/ml fibronectin (Sigma–Aldrich). To induce adipocyte differentiation from iPS and ES cells, we applied a modification of a procedure described previously for use with mouse and human ES cells (Fig. 1) [15–19]. Differentiation was induced for 10 days using medium consisting of DMEM-F12, 10% KSR, and an adipogenic cocktail (0.5 mM IBMX, 0.25 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin and 1 μM pioglitazone).

2.3. Immunocytochemistry

Immunocytochemistry was carried out as previously described [7]. The anti-human primary antibodies included Nanog (R&D Systems, MN, USA) and Alexa 488-conjugated SSEA-4 (Santa Cruz Biotechnology Inc., CA, USA) and TRA1-60 (CHEMICON, LA, USA). The TRA1-60 antibody was labeled using an Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Molecular Probes, OR, USA). Alexa 546-conjugated donkey anti-sheep IgG (Molecular Probes, OR, USA) served as the secondary antibody. Alkaline phosphatase activity was detected using a BCIP/NBT substrate system (Dakocytomation, CA, USA).

2.4. Oil Red O staining and microscopic analysis of adipocytes

Cells were washed with phosphate-buffered saline (PBS) twice, fixed in 3.7% formaldehyde for 1 h and then stained with 0.6% (w/v) Oil Red O (Nacalai Tesque, Japan) solution (60% isopropanol, 40% water) for 2 h at room temperature. The cells were then washed with water to remove unbound dye. Subsequently, the bound Oil Red O was eluted with isopropanol.

After staining with Oil Red O, each EB was examined microscopically for the presence of adipocyte colonies, and the percentage of EBs with outgrowths showing adipocyte positivity was determined as previously described [15]. EBs in which adipocytes accounted for more than half of their circumference were considered adipocyte-positive. The percent area of Oil Red O staining (+) was determined at 20× magnification by counting the number of pixels exhibiting Oil Red O positivity in selected microscope fields (449 × 338 pixels). Four randomly selected fields were examined in each well of a 6-well plate, and the percent area was calculated as the average for the four fields. Six independent experiments were performed for each cell line.

Table 1
Primers for reverse-transcription polymerase chain reaction.

Gene		Sequence
Nanog	Sense	CAGCCCCGATTCTTCCACCAGTCCC
	Antisense	CGGAAGATTCCCAGTCGGGTTCACC
PPARγ2	Sense	ATTGACCCAGAAAGCGATTC
	Antisense	CAAAGGAGTGGGAGTGGTCT
C/EBPα	Sense	GCAAACTCACCGCTCCAATG
	Antisense	TTAGGTTCCAAGCCCCAAGTC
aP2	Sense	AACCTTAGATGGGGGTGTCCTG
	Antisense	TCGTGGAAGTGACGCCTTTC
Leptin	Sense	GAACCCTGTGCGGATTCTTGTG
	Antisense	CGTTTCTFFAAGGCATACTGGTGAG
GAPDH	Sense	ACCACAGTCCATGCCATCAC
	Antisense	TCCACCACCCTGTTGCTGT
PPARγ2 (real-time RT-PCR)	Sense	GATACACTGTCTGCAAACATATCACAA
	Antisense	CCACGGAGCTGATCCCAA
	Probe	AGAGATGCCATTCTGGCCCACCAACTT

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA was extracted using TRizol Reagent (Invitrogen, CA, USA) and treated with RNase-Free DNase Set (QIAGEN, Germany) to remove any contaminating genomic DNA. For RT-PCR, cDNA was synthesized using a PrimeScript RT reagent Kit (Takara Bio Inc., Japan), after which RT-PCR was run using ExTaq (Takara Bio Inc.). For quantitative real-time PCR, TaqMan PCR was carried out using a Step One Plus Real-Time PCR System as instructed by the manufacturer (Applied Biosystems, CA, USA). Levels of mRNA were normalized to those of 18S mRNA. The primers used are listed in Table 1.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA for comparison among six groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Adipogenic differentiation of human iPS and ES cells

Morphological phenotypes, immunoreactivities of Nanog, SSEA-4 and TRA-1-60, and ALP activity of human iPS cells did not differ

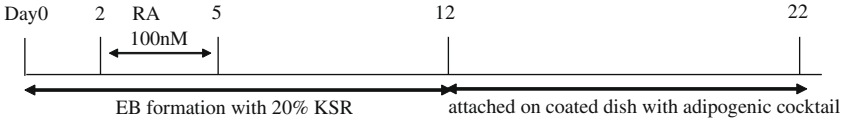

Fig. 1. Schematic diagram of the experimental protocol used for adipocyte differentiation from human ES and human iPS cells. EB: embryoid body. Adipogenic cocktail: 0.5 mM IBMX, 0.25 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin and 1 μM pioglitazone.

from those of human ES cells (Fig. 2). In order to assess their potential for adipogenic differentiation, the human iPS cells were subjected to adipogenic induction culture. After 12 days of EB formation, EBs derived from human iPS cells were attached to coated dishes to induce differentiation. Several kinds of coating for the dishes, including gelatin, collagen IV and fibronectin were compared, and the efficiency of EB attachment and adipogenic differentiation were the best on dishes coated with a combination of Poly-L-ornithine and fibronectin. On day 15, after 3 days of adipogenic differentiation following the EB formation, differentiated cells containing small cytoplasmic lipid droplets were observed spreading outward from the attached EBs. On day 22, the lipid accumulation was evaluated by staining the cells with Oil Red O.

To evaluate the adipogenic potential of individual iPS cell lines, the percentage of EB outgrowths having adipocyte colonies and the percent area of Oil Red O staining (+) were determined. For each of iPS and ES cell lines tested, 40–60% of EBs formed adipocyte colonies (Table 2). In all of the iPS cell lines, lipid accumulation was similar to that seen in human ES cell lines (Fig. 3), though the B7 line showed stronger lipid accumulation than the other cell lines.

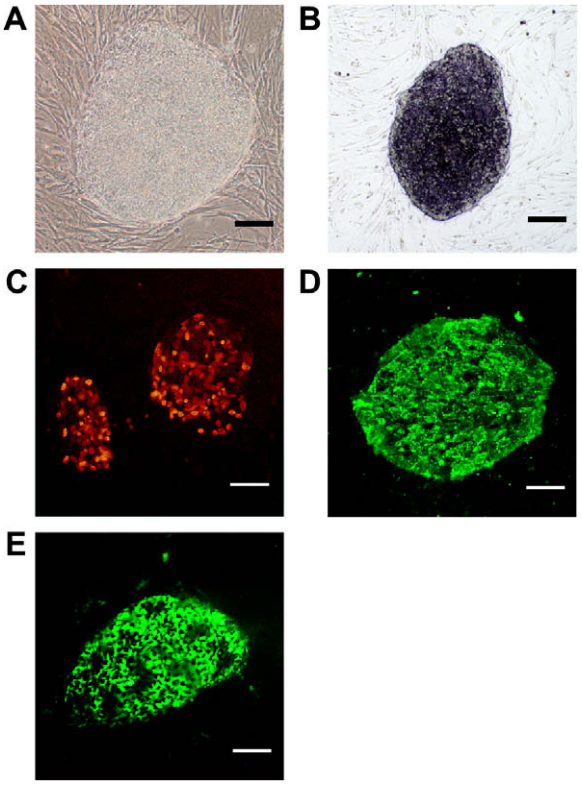

Fig. 2. Morphology of undifferentiated human iPS cells (G4). (A) Phase-contrast photomicrograph of an undifferentiated colony. (B) Alkaline phosphatase activity. (C) Immunofluorescent staining with Nanog. (D) Immunofluorescent staining with SSEA-4. (E) Immunofluorescent staining with TRA1-60. Scale bar = 100 μM.

Table 2
% of EBs with adipocyte colonies.

Cell line	[Number of EBs with adipocytecolonies/total number of EBs]
201B6	54.1% [40/74]
201B7	59.7% [46/77]
253G1	50.0% [35/70]
253G4	56.4% [44/78]
H9	48.8% [39/80]
KhES-1	45.5% [35/77]

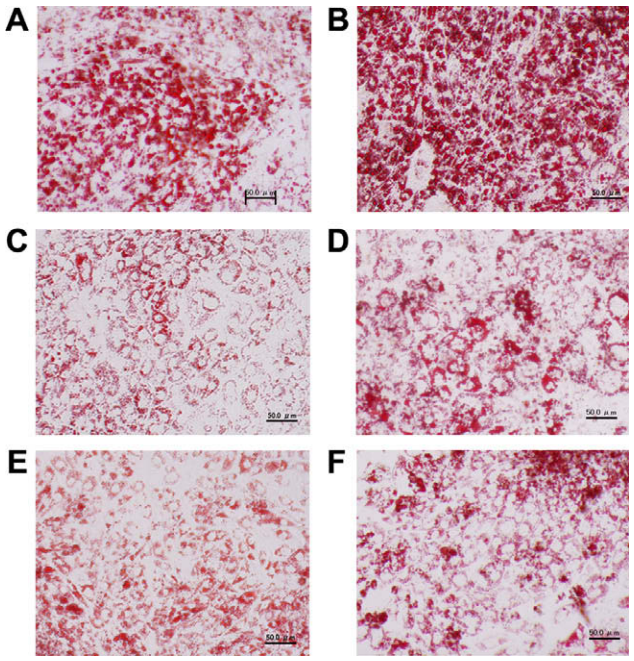

Fig. 3. Oil Red O staining of adipocytes derived from human iPS cells (A–D) and ES cells (E, F) on day 22. B6 (B) B7 (C) G1 (D) G4 (E) H9 (F) KhES-1. Scale bar = 50 μM.

Statistical analysis of the percent area of Oil Red O staining (+) showed no significant differences among the cell lines (Fig. 4).

3.2. Expression of adipogenesis-related molecules

Using RT-PCR, transcription of adipogenic markers was investigated on days 0 and 22 of differentiation (Fig. 5A). Though not detected at day 0, mRNAs encoding the adipogenic transcription factors C/EBPα (CCAAT/enhancer binding protein α) and PPARγ2 (peroxisome proliferator-activated receptor γ2) were detected on day 22. In contrast, expression of Nanog mRNA was strongly suppressed on day 22, as compared with its expression on day 0. Expression of the mature adipocyte markers leptin and aP2 (adipocyte fatty acid binding protein) was also clearly detected on day 22. All of the human iPS cell lines expressed mRNAs encoding adipogenesis-related molecules at levels that were comparable to the levels seen in human ES cell lines (Fig. 5A). In Quantitative real-time PCR analysis, expression of PPARγ2 mRNA differed somewhat among the iPS and ES cell lines. The differences between the B7 line and the two ES cell lines were significant, but other differences were not significant (Fig. 5B).

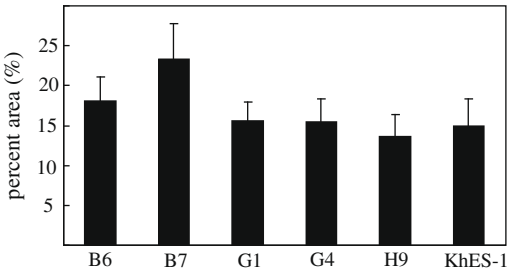

Fig. 4. Percent area of Oil Red O staining. Results are means of six independent experiments. No significant differences were observed among the iPS and ES cell lines.

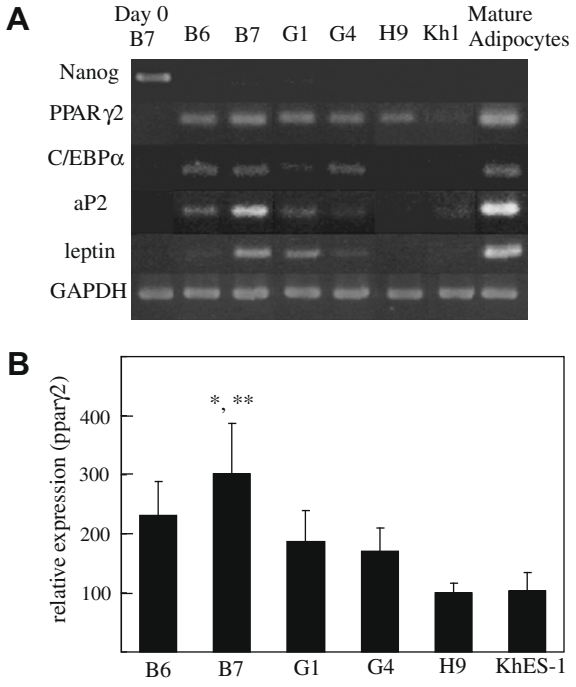

Fig. 5. (A) Transcription of the adipocyte-specific markers PPARγ2, CEBP/α, aP2 and leptin. RNA samples from undifferentiated human iPS cells (B7, day 0) and differentiated stage iPS cells (B6, B7, G1, G4) and human ES cells (H9, KhES-1), as well as mature human adipocytes differentiated from human adipose-derived mesenchymal stem cells (positive control), were analyzed by RT-PCR. Nanog is an undifferentiated human ES cell marker. GAPDH served as an internal standard for RT-PCR. Kh1: KhES-1. Adipose: human mature adipocytes differentiated from human adipose-derived mesenchymal stem cells. (B) Relative levels of PPARγ2 mRNA expression are shown as means ± S.E.M. of 4–6 independent experiments and normalized to those of 18S. The levels are expressed as percentages of the expression in the H9 cell line. $^{*}P < 0.05$ vs. H9. $^{**}P < 0.05$ vs. KhES-1.

4. Discussion

The present study demonstrates that human iPS cells have adipogenic potential comparable to human ES cells. Four human iPS cell lines of two generations were investigated. The B6 and B7 were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into human skin fibroblasts while the G1 and G4 were generated using only three factors (c-Myc was omitted) [9]. After 12 days of embryoid body formation and an additional 10 days of differentiation on Poly-L-ornithine and fibronectin-coated dishes with adipogenic differentiation medium, all human iPS cell lines of both generations exhibited lipid accumulation and transcription of such adipogenesis-related molecules as C/EBPα, PPARγ2, leptin and aP2. We also compared differentiation efficiency between human iPS and ES cells using two lines of human ES cells and found no apparent difference between human iPS and ES cells in properties of adipogenic differentiation including the time course and potential. In terms of lipid accumulation and transcription of adipogenesis-related molecules, human iPS-derived adipocytes appear to reach at least the same level of maturity as those derived from human ES cells. The B7 line tended to show stronger adipogenic potential than the other five iPS lines and the ES cell lines, but the difference in terms of percent area of Oil Red O staining (+) was not significant. The B7 line also showed significantly stronger expression of PPARγ2 than the two ES cell lines tested, but PPARγ2 expression varied among the different iPS cell lines, despite their having the same genetic background. We conclude that the adipogenic potential of iPS cells did not essentially differ from ES cells, though their adipogenic potentials were rather varied in each line.

Despite the prevalence of obesity, systems for research into human adipocyte biology remain underdeveloped, in part because of a lack of available human adipocyte cell lines. There are significant differences between adipocyte development in humans and mice [20]. The established in vitro adipocyte differentiation system using human iPS cells in the present study should make it possible to dissect out the cellular mechanisms underlying human adipocyte differentiation. It should also contribute to the better understanding of adipocyte biology and serve as a basis for advances in research into obesity and adipotoxicity, which has been proposed as the sum of the negative effects associated with obesity [21].

Adipogenesis is largely divided into two phases: the early phase consisting of the lineage commitment of adipocytes from pluripotent stem cells and the late phase consisting of the terminal differentiation of preadipocytes into adipocytes [22]. The molecular mechanism underlying the terminal adipocyte differentiation has been identified through analysis of the differentiation process in immortalized mouse preadipocyte cell lines (e.g., 3T3-L1 and 3T3-F442A cells) [22–24], but the differentiation from pluripotent stem cells during the early stage of adipogenesis must await further clarification. The establishment of adipocyte differentiation system with human iPS cells should facilitate that line of research.

In contrast to human ES cells, iPS cells can be induced from any human being irrespective of their genetic make-up. Consequently, the study of iPS cells should contribute to the identification of new susceptibility genes associated with obesity and metabolic syndrome, and to the clarification of the functions of those genes. The establishment of iPS cell lines from patients with inherited diseases presenting adipocyte abnormality should enable clarification of their pathogenesis. And because they overcome the immunological and ethical problems associated with human ES cells, iPS cell systems should also contribute to the development of novel regenerative therapies for reconstruction of soft tissue defects after tumor resections, extensive deep burns and lipodystrophy. The induced cells obtained with our protocol are not a homogeneous population. Consequently, at this stage human iPS cells may not yet have as much adipogenic potential as adipose-derived stem cells (ADSCs), which are derived from the stromal vascular fraction of human adipose tissue and are thought to be a safe and useful tool in adipose regenerative medicine [25]. About 80% of ADSCs differentiate into adipocytes under suitable conditions [26]. The next issue we plan to address will be the establishment of an improved differentiation protocol that includes a purification process such as cell sorting.

In conclusion, the present study demonstrates that human iPS cells have adipogenic potential that is generally equal to that of human ES cells. The use of iPS cells will contribute to the development of regenerative therapies of adipose tissue for lipodystrophy. This work should also contribute to our understanding of human adipogenesis and to the clarification of the pathogenesis and pathophysiology of obesity and metabolic syndrome, potentially leading to the development of new drug therapies.

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