Yamanaka [29]. Human ES cells (H9 and KhES-1) were cultured as described previously [29]. H9 was purchased from Wicell Research Institute, Inc. KhES-1 was kindly provided by Norio Nakatsuji [30]. W12 as well as B7 were generated from human dermal fibroblasts from a 36-year-old Caucasian woman (Cell Applications, Inc.) by introducing four factors, such as Oct3/4, Sox2, Klf4, and c-Myc. Expression of human ES cell markers and pluripotency were examined by immunocytochemistry, in vitro differentiation, and teratoma formation (Supplementary Fig. S1A–K; Supplementary Data are available online at www.liebertpub.com/scd) according to the methods described previously [1]. Standard G-banding chromosome analysis was performed in the Nihon Gene Research Laboratories, Inc. (Supplementary Fig. S1L). Human bone marrow-derived mesenchymal stem cells were purchased from Lonza. Adipogenic differentiation was induced according to the manufacturer's instruction.

Adipogenic differentiation of human iPS and ES cells

Human iPS and ES cells were differentiated into the adipocyte lineage via embryoid body (EB) formation using a modified version of the protocol, which we previously described [13]. Briefly, adipogenic differentiation was initiated by aggregation of iPS and ES cells to form EBs. From day 2–5, retinoic acid (SIGMA-Aldrich) with the concentration of 100 nM was supplemented. From day 8–11, 1 μg/mL insulin (Roche Diagnostics) and 1 μM pioglitazone (SIGMA-Aldrich) were supplemented. After 11 days of EB culture, EBs were transferred to plates coated with type IV collagen (BD Biosciences). Adipogenic differentiation was induced for additional 3–5 days of culture using 10% FBS/DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque), 0.25 μM dexamethasone (Nacalai Tesque), 1 μg/mL insulin, and 1 μM pioglitazone.

Oil Red O staining

Differentiated human iPS and ES cells were washed with phosphate-buffered saline (PBS) twice, fixed in 3.7% formaldehyde for 1 h, and then stained with a 0.6% (w/v) Oil Red O solution (60% isopropanol and 40% water) for 2 h at room temperature. Cells were then washed with water to remove unbound dye. Optical sections were obtained with BZ-9000 (KEYENCE). Oil Red O-stained areas were calculated as the percentage of the area divided by the total area.

Immunocytochemistry

Differentiated human iPS and ES cells were washed with PBS and fixed for 10 min with 4% paraformaldehyde. Then, the cells were permeabilized by 0.1% Triton X in PBS for 5 min and incubated by Protein Block (DAKO) for 30 min at room temperature. The cells were immunostained with a 1:200 dilution of a primary antibody against peroxisome proliferator-activated receptor γ (PPARγ; Cell Signaling Technology). After washed with PBS, Alexa 546-conjugated anti-rabbit IgG (Molecular Probes) was used as a secondary antibody with the concentration of 10 μg/mL. Then, after being washed with PBS, BODIPY 493/503 (Molecular Probes) was added for 15 min for lipid staining. The cells were mounted in the medium with DAPI after washed with PBS twice (Vector Labs). Then, human iPS cells were stained with primary antibodies against Nanog (1:20; R&D Systems), TRA-1-60 (1:100; Millipore), SSEA-4 (1:100; Santa Cruz Biotechnology, Inc.), β3-tubulin (1:100; Millipore), α-smooth muscle actin (pre-diluted; DAKO), and α-fetoprotein (1:100; Millipore) according to the protocol described previously [20]. Alexa Fluor 488-conjugated anti-mouse IgG, Alexa Fluor 546-conjugated anti-goat IgG, and Alexa Fluor 546-conjugated anti-mouse IgG antibodies (Molecular Probes) were used as secondary antibodies with a concentration of 10 μg/mL. An alkaline phosphatase activity was detected using a BCIP/NBT substrate system (DAKO). 3T3-L1 cells and human mesenchymal stem cells were stained with a primary antibody against vimentin (1:100; DAKO). Alexa Fluor 546-conjugated anti-mouse IgG was used as a secondary antibody. Optical sections were obtained with BZ-9000.

Lipolysis assays

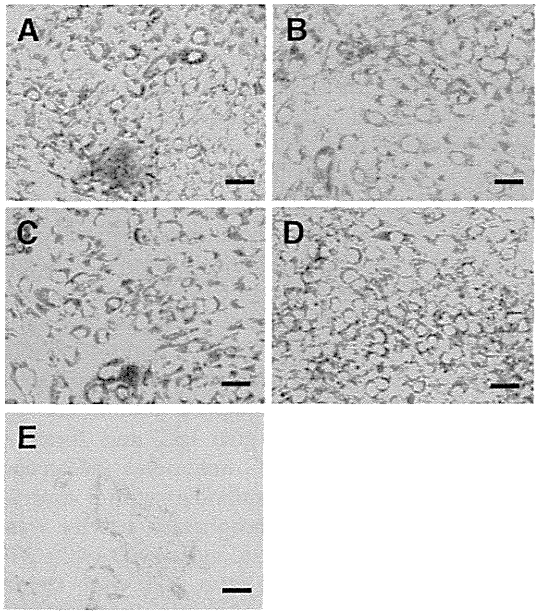
Before lipolysis assays, differentiated human iPS and ES cells were incubated in the serum-free DMEM with 0.5% fatty acid-free bovine serum albumin for 4 h. For lipolysis assays, differentiated cells were stimulated with 10 μM forskolin for 6 h. The medium was then collected and established procedures were used to quantify the glycerol as

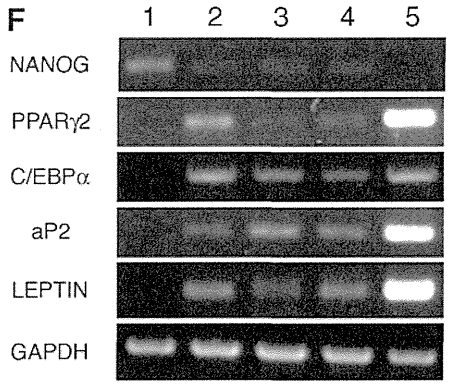
FIG. 1. Adipogenic differentiation of human iPS and ES cells in vitro. **(A–E)** Oil Red O staining of differentiated human iPS cells **(A:** G4, **B:** B7, **C:** W12**)**, differentiated human ES cells **(D:** H9**)**, and undifferentiated human iPS cells **(E:** W12**)**. Scale bar = 25 μm. **(F)** Gene expression of adipocyte markers in differentiated human iPS and ES cells. Lane 1: undifferentiated iPS cells (B7), lane 2: differentiated G4, lane 3: differentiated B7, lane 4: differentiated H9, lane 5: adipocytes derived from human bone marrow-derived mesenchymal stem cells. **(G–O)** Subcellular localization of PPARγ in differentiated human iPS cells (W12), differentiated human ES cells (H9), and undifferentiated human iPS cells (W12). Lipid staining by BODIPY 493/503 and immunostaining with PPARγ in differentiated iPS cells **(G)**, differentiated ES cells **(J)**, and undifferentiated iPS cells **(M)**. Nuclear staining with DAPI in differentiated iPS cells **(H)**, differentiated ES cells **(K)**, and undifferentiated iPS cells **(N)**. Merge sections in differentiated iPS cells **(I)**, differentiated ES cells **(L)**, and undifferentiated iPS cells **(O)**. Scale bar = 20 μm. **(P)** Forskolin-stimulated lipolysis in undifferentiated or differentiated human iPS (W12) and ES (H9) cells. Glycerol release in the culture medium was measured. Data are expressed as mean ± SE from duplicate experiments ($n = 3–5$). $^{*}p < 0.05$; $^{**}p < 0.01$ compared with vehicle-treated groups. hiPSCs: undifferentiated human iPS cells, hESCs: undifferentiated human ES cells, dhiPSCs: differentiated human iPS cells, dhESCs: differentiated human ES cells, FK: forskolin. **(Q)** Insulin-induced AKT phosphorylation in undifferentiated or differentiated human iPS cells (W12) and ES cells (H9). Cells were stimulated with 100 nM or 1 μM insulin for 5 min. hiPSCs: undifferentiated human iPS cells, hESCs: undifferentiated human ES cells, dhiPSCs: differentiated human iPS cells, dhESCs: differentiated human ES cells. iPS cells, induced pluripotent stem cells; ES cells, embryonic stem cells; PPARγ, peroxisome proliferator-activated receptor γ.

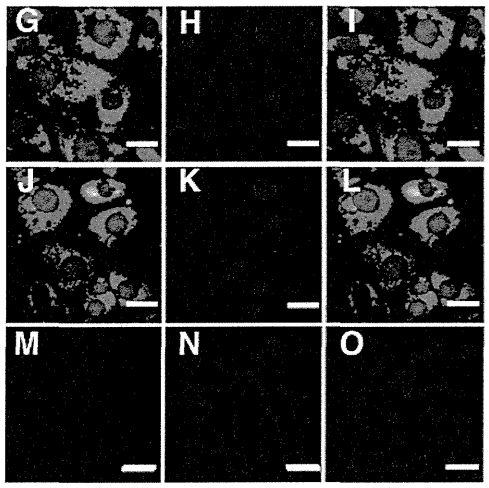
instructed by the manufacturer (SIGMA-Aldrich). The protein content was determined using Protein Assay (BIORAD).

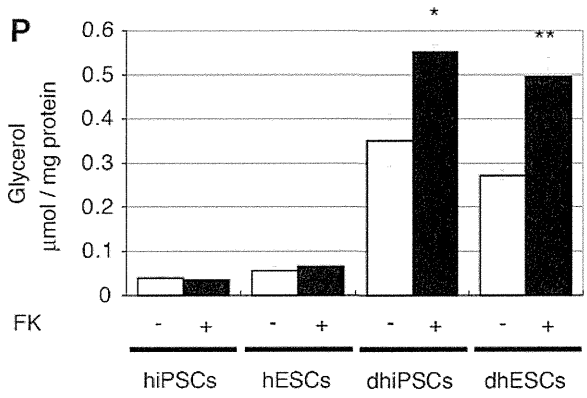
Western blot analysis

Preparation of total cell lysates and western blot analysis were performed as we previously described [31]. Briefly, cells were harvested in the lysis buffer. For western blot analysis, proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with the primary antibodies against AKT and phospho AKT (Ser 473) (Cell Signaling Technology). Membranes were reacted with the secondary antibody (GE Healthcare) and developed with ECL plus (GE Healthcare) as instructed by the manufacturer. The signal on the blot was detected with ImageQuant LAS 4000 System (GE Healthcare).









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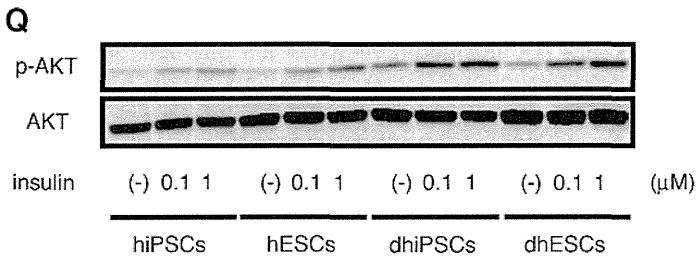

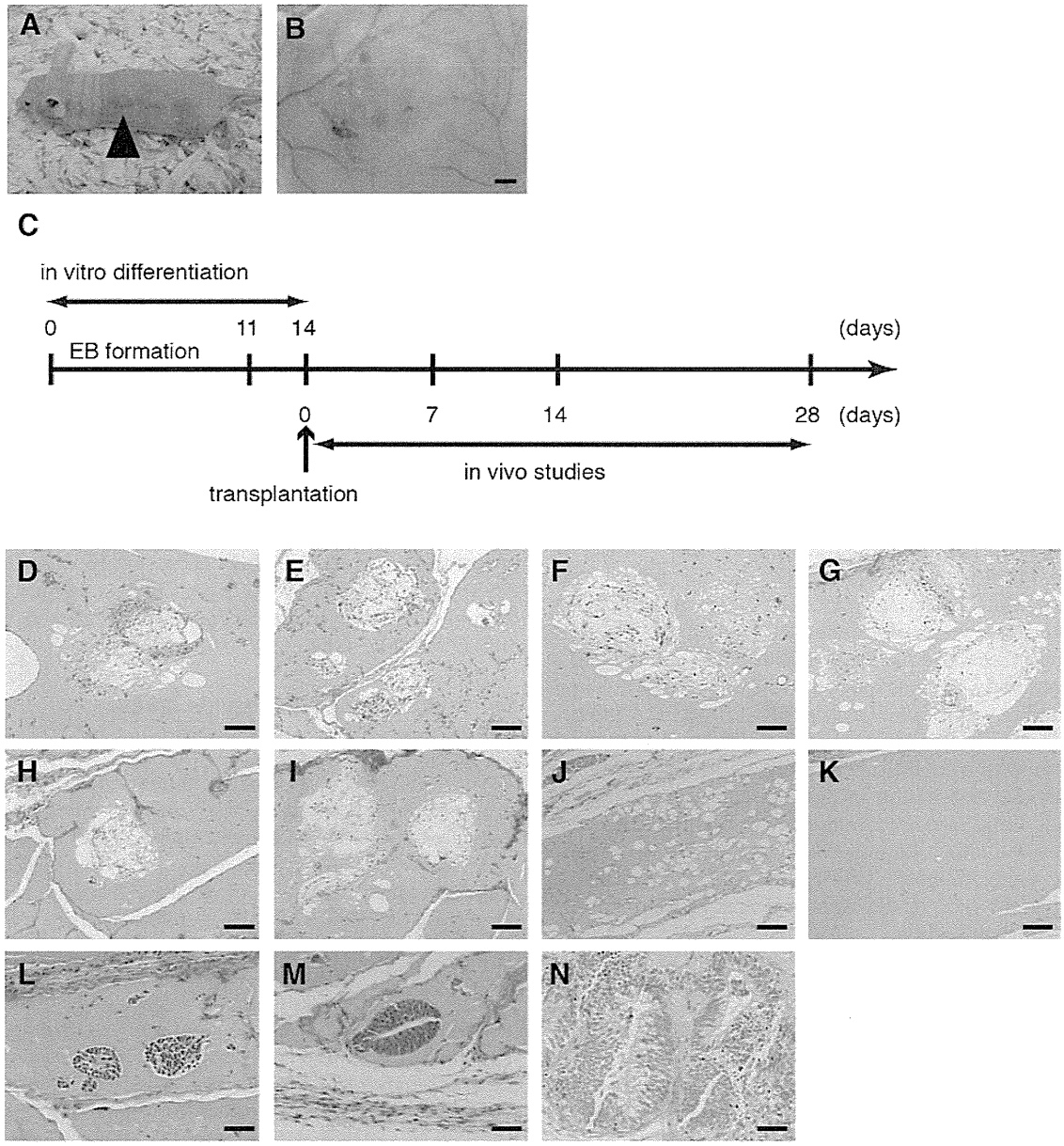

FIG. 2. Transplantation of adipocytes derived from human iPS cells and ES cells. **(A)** Appearance of a BALB/cA nude mouse into which Matrigel containing differentiated human iPS cells was transplanted. The *arrowhead* indicates the transplantation site. **(B)** Appearance of the mouse subcutaneous tissue collected at 2 weeks after transplantation of Matrigel containing differentiated human iPS cells. Scale bar = 2 mm. **(C)** Timeline of transplantation study. After 14 days of in vitro differentiation, differentiated human iPS and ES cells were transplanted. Implanted Matrigel containing differentiated human iPS and ES cells was collected at the indicated time points. **(D–I)** Transplanted cells were stained with hematoxylin and eosin. Morphological features of implanted Matrigel containing differentiated G4 **(D, F, H)** and H9 **(E, G, I)** after 1–4 weeks. **D, E:** 1 week, **F, G:** 2 weeks, **H, I:** 4 weeks. Scale bars = 100 μm. **(J, K)** Morphological features of implanted Matrigel containing adipocytes derived from human mesenchymal stem cells **(J)** and implanted cell-free Matrigel **(K)** after 4 weeks. Scale bars = 100 μm. **(L–N)** Morphological features of implanted Matrigel containing undifferentiated G4 after 1–4 weeks. **L:** 1 week, **M:** 2 weeks, **N:** 4 weeks. Scale bars = 50 μm.

◀4C

Flow cytometric analysis

At 3 days after attachment of EBs and supplementation of adipogenic cocktails, cells were harvested. Single-cell suspensions were labeled for 30 min on ice with the mouse fluorescence-conjugated antibodies against PE-CD73 and PE-CD105 (eBioscience). The cells were analyzed by FACS-Aria II (BD Biosciences).

Transplantation of derivatives from human iPS cells and ES cells

This study was performed after approval of the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (No. 824 and ES6). All animal experiments were performed in strict accordance with the guidelines for animal experiments of Kyoto University. Matrigel (BD Biosciences) was

mixed with a suspension of differentiated or undifferentiated human iPS and ES cells harvested from a confluent 100-mm dish. Matrigel incorporating 2×10^7 differentiated or 1×10^7 undifferentiated PS cells was then carefully implanted into the subcutaneous tissue on the backs of 8-week-old male BALB/cA nude mice (CLEA Japan) using a syringe with a 21-gauge needle. Samples of skin tissue, including the implanted Matrigel were harvested for further studies at 1 day, 1 week, 2 weeks, and 4 weeks after transplantation. Matrigel incorporating adipocytes derived from 2×10^6 human bone marrow-derived mesenchymal stem cells was also implanted into the subcutaneous tissue on the backs of the BALB/cA nude mice as a positive control study.

Immunohistochemistry

Human adipose tissue biopsy was performed after approval of the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (No. 553). Each tissue specimen was fixed in 10% neutralized formalin solution, embedded in paraffin, sectioned at the central portion of implanted Matrigel, and followed by staining with hematoxylin and eosin (HE) or immunohistochemical studies. The paraffin sections were immunostained using the Polymer Immunocomplex System (DAKO) with a 1:50 dilution of a primary antibody against human vimentin (DAKO). Phosphohistone H3 was stained with a 1:100 dilution of the primary antibody (Cell Signaling Technology). Cryosections from the samples were fixed with a 10% neutralized formalin solution before embedding in optimal cutting temperature compound. Lipid accumulation in transplanted cells was assessed by Oil Red O staining. Morphometric analyses were performed using BZ-9000. The relative adipocyte area was expressed as the ratio of the adipocyte area to the total Matrigel area in each section.

RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen). For RT-PCR assay, cDNA was synthesized by iScript (BIORAD). Semiquantitative PCR was carried out using GeneAmp PCR System 9700 as instructed by the manufacturer (Applied Biosystems). TaqMan PCR was performed using Step One Plus Real-Time PCR System as instructed by the manufacturer (Applied Biosystems). Relative levels of mRNA were normalized to the mRNA level of GAPDH. The primers and probes used were listed in Supplementary Fig. S2.

Statistical analysis

The data are presented as mean ± SE. The Student's *t*-test was used as the statistical analysis. The level of significant difference was the p-value < 0.05.

Results

Adipogenic differentiation of human iPS and ES cells in vitro

Human iPS (G4, B7, and W12) and ES (H9) cells were differentiated into the adipocyte lineage in vitro. Differentiated iPS and ES cells exhibited prominent lipid accumulation by Oil Red O staining, while lipid accumulation was rarely observed in undifferentiated cells (Fig. 1A–E). After differentiation in vitro, gene expression of a set of adipocyte markers, such as PPARγ2, CCAAT/enhancer-binding protein (C/EBP) α, fatty acid-binding protein-4 (aP2), and leptin, was detected by RT-PCR analysis. Gene expression of Nanog in differentiated cells was markedly lower than in undifferentiated cells (Fig. 1F). Immunohistochemical analysis showed that the PPARγ protein was present within the nuclei of a portion of the differentiated iPS and ES cells containing lipid droplets, while the PPARγ protein was not detected in undifferentiated cells (Fig. 1G–O). Further, these differentiated cells were examined whether they have lipolytic responses and insulin responsiveness. In differentiated cells from both iPS and ES cells, 10 μM forskolin significantly enhanced glycerol release into the culture medium (~1.6-fold and ~1.8-fold increase, respectively), as compared to vehicle-treated groups. In undifferentiated iPS and ES cells, 10 μM forskolin did not significantly increase glycerol release (Fig. 1P). In addition, 100 nM or 1 μM insulin remarkably increased AKT phosphorylation in differentiated iPS and ES cells as compared to vehicle-treated groups. AKT phosphorylation was also slightly enhanced in undifferentiated iPS and ES cells (Fig. 1Q). These findings suggest the presence of adipocytes with functional properties in differentiated iPS and ES cells.

Transplantation of adipocytes derived from human iPS and ES cells

After 14 days of in vitro differentiation, Matrigel containing differentiated human iPS (G4) or ES (H9) cells was transplanted into the subcutaneous tissue on the backs of 8-week-old BALBc/A nude mice (Fig. 2A). Matrigel containing undifferentiated iPS cells or cell-free Matrigel was transplanted as a negative control, while Matrigel containing adipocytes derived from human bone marrow-derived mesenchymal stem cells was transplanted as a positive control. At 1 day, 1 week, 2 weeks, and 4 weeks after transplantation, samples of skin tissue containing the Matrigel were harvested. Grossly, small blood vessels could be seen distributed in Matrigel (Fig. 2B). Timeline of the transplantation study was demonstrated (Fig. 2C). Preparation of histological sections and HE staining of specimens collected at 1 week after transplantation revealed that the differentiated iPS or ES cells possess adipocyte-like features, including thin rims of cytoplasm surrounding the vacuole and flattened nucleus (Fig. 2D, E). Similar histological findings were observed at 2 and 4 weeks after transplantation of differentiated iPS and ES cells (Fig. 2F–I). These cells were also noted at 4 weeks after transplantation of adipocytes derived from human mesenchymal stem cells (Fig. 2J), but no adipocytes described above were observed in cell-free Matrigel at 4 weeks after transplantation (Fig. 2K). By contrast, adipocytes derived from human iPS and ES cells were rarely seen at 1 day after transplantation (data not shown), which may indicate the loss of lipid droplets caused by the transplantation procedure and insufficient vascularization in the Matrigel at that time. Histological findings also showed that Matrigel with undifferentiated human iPS cells mainly contained immature neuroectodermal cells such as neural tube cells at 1, 2, and 4 weeks after transplantation (Fig. 2L–N). No adipocytes described above were detected throughout the transplantation period.

Characterization of transplanted cells derived from human iPS and ES cells

We next calculated the relative areas of the adipocytes by dividing the adipocyte area by the total Matrigel area in sections of skin tissue collected at 1 day, 1 week, 2 weeks, and 4 weeks after transplantation of differentiated human iPS and ES cells. The areas were 0.63%±0.10% (G4) and 0.42%±0.17% (H9) on day 1 after transplantation. At 1 week after transplantation, the relative areas were 2.27%±1.19% (G4) and 9.39%±1.36% (H9), and by 2 weeks after transplantation, they had increased to 5.26%±0.46% (G4) and 12.9%±5.32% (H9). However, the areas had declined to 2.17%±1.28% (G4) and 6.97%±1.66% (H9) at 4 weeks after transplantation (Fig. 3A). Thus, the adipocytes were clearly present at 1–4 weeks after transplantation of differentiated iPS or ES cells, and relative adipocyte areas were maximal at 2 weeks after transplantation.

To assess the proliferative capacity of the transplanted cells, the cells were immunostained with an antibody against phosphohistone H3 at 2 weeks after transplantation. Adipocytes derived from human iPS and ES cells exhibited little or no proliferative capacity (Fig. 3B, C), whereas the immature neuroectodermal cells exhibited a high-proliferative capacity in transplantation of undifferentiated human iPS cells (Fig. 3D).

The origin of the adipocytes in the Matrigel was studied by immunostaining with an antibody against human vimentin. According to the manufacturer's guide and an earlier report [32], the antibody used does not cross react with mouse vimentin. We also evaluated the cross reactivity of the antibody with mouse vimentin. Vimentin was stained with the antibody in human mesenchymal stem cells, while it was not stained in mouse 3T3-L1 cells (Supplementary Fig. S3A–D). Moreover, human subcutaneous adipose tissue was stained with the antibody (Supplementary Fig. S3E), but mouse subcutaneous adipose tissue was not (Supplementary Fig. S3F).

When we then assessed the human vimentin immunoreactivity of the cells within the Matrigel, we found that adipocytes derived from both iPS and ES cells were labeled by the anti-vimentin antibody at 2 and 4 weeks after transplantation (Fig. 4A–H). Further, lipid accumulation in the cells was demonstrated by staining frozen sections with Oil Red O at 2 and 4 weeks after transplantation (Fig. 4I–L). Similar histological findings were observed at 2 weeks after transplantation of adipocytes derived from human bone marrow-derived mesenchymal stem cells (Fig. 4M–O).

Gene expression of a set of adipocyte markers in transplanted differentiated iPS and ES cells was then investigated by PCR analyses. Gene expression of PPARγ2, C/EBPα, aP2, and leptin was detected at 2 and 4 weeks after transplantation (Fig. 5A, B). Quantitative real-time PCR analyses carried out with the samples after transplantation revealed that leptin and PPARγ2 were expressed at 1, 2, and 4 weeks after transplantation. In both iPS and ES cells, mRNA levels of leptin at 1 and 2 weeks after transplantation were higher than those at 4 weeks after transplantation (Fig. 5C). Meanwhile, mRNA levels of PPARγ2 at 4 weeks after transplantation were maintained in both cell lines as compared with those at 2 weeks (Fig. 5D).

Transplantation of other human PS cell lines

When we similarly transplanted Matrigel containing other differentiated human iPS cell line (B7 and W12) and another human ES cell line (KhES-1), adipocytes described above were observed at 2 weeks after transplantation, suggesting that adipocytes derived from other PS cells can survive after transplantation (Fig. 6A–C). At that time, the relative areas of adipocytes derived from B7, W12, and KhES-1 were 1.11%±0.57%,

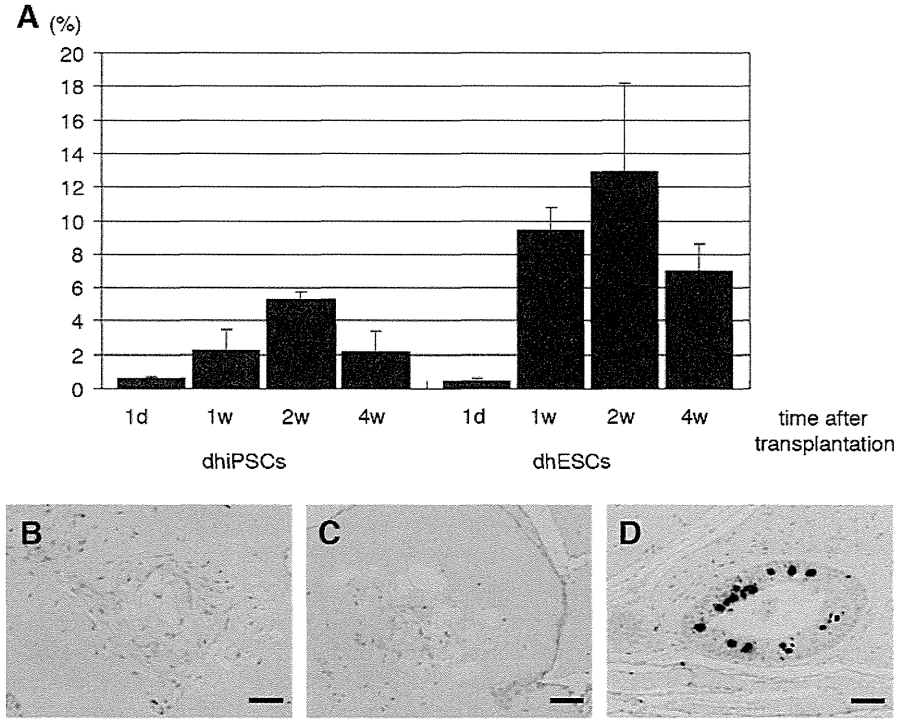

FIG. 3. Quantification of adipocyte areas and proliferative capacity of transplanted cells. **(A)** Relative adipocyte areas at 1 day, 1 week, 2 weeks, and 4 weeks after transplantation of differentiated G4 (dhiPSCs) and differentiated H9 (dhESCs) were shown. Data are expressed as mean±SE. At least, six sections from three samples were analyzed for each group. 1d: 1 day, 1w: 1 week, 2w: 2 weeks, 4w: 4 weeks. **(B–D)** Proliferative capacity of transplanted cells after 2 weeks. Immunostaining with an antibody against phosphohistone H3 of transplanted cells. Implanted Matrigel containing differentiated G4 **(B)**, differentiated H9 **(C)**, and undifferentiated G4 **(D)**. Scale bars = 50 μm.

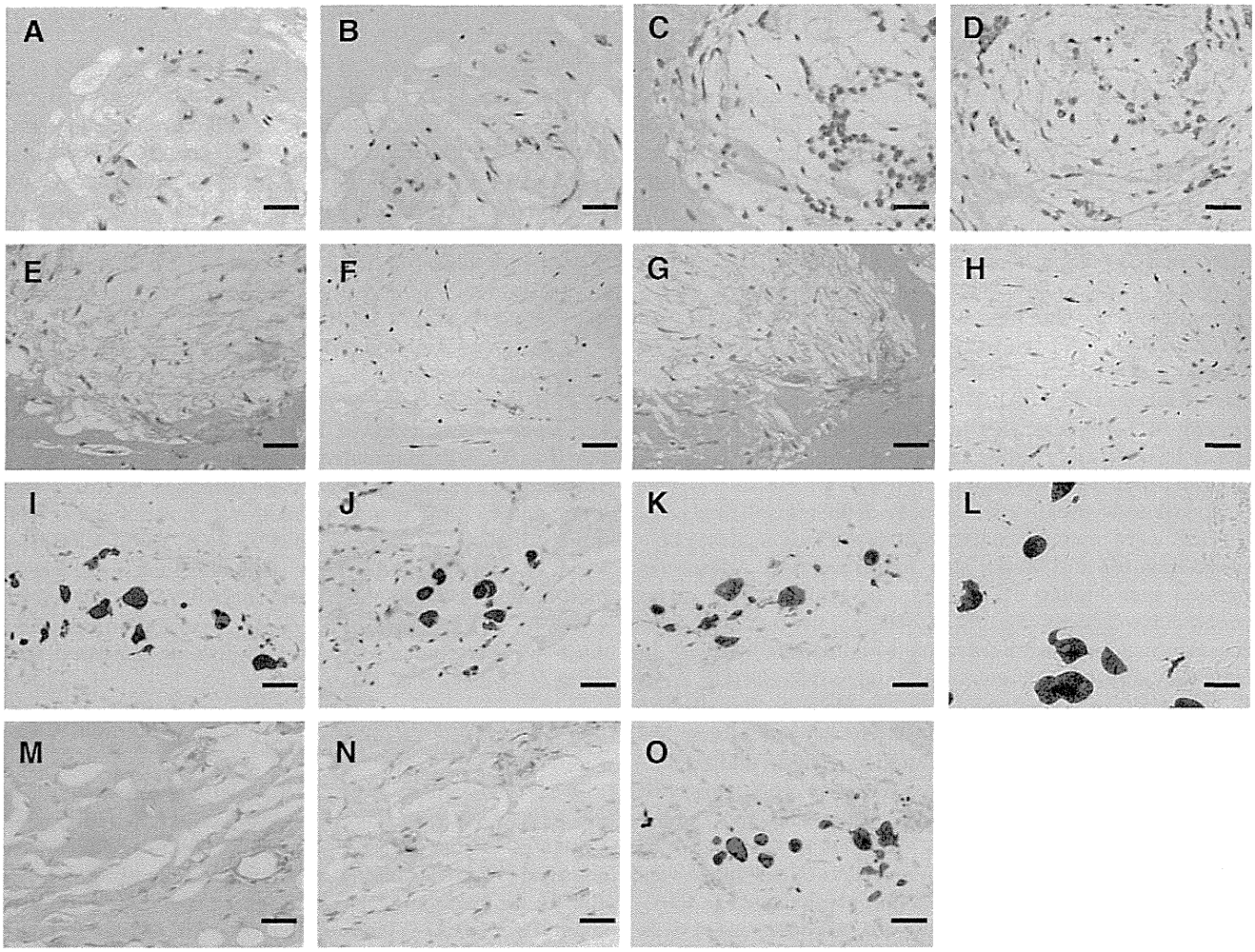

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FIG. 4. Human origin and lipid accumulation in transplanted cells. **(A–H)** HE staining **(A, C, E, G)** and immunostaining with an antibody against human vimentin **(B, D, F, H)** of serial sections of transplanted cells after 2 weeks **(A, B:** differentiated G4; **C, D:** differentiated H9**)** and 4 weeks **(E, F:** differentiated G4; **G, H:** differentiated H9**)**. Scale bars = 20 μm. **(I–L)** Oil Red O staining of the frozen sections of transplanted cells after 2 weeks **(I**: differentiated G4, **J:** differentiated H9**)** and 4 weeks **(K**: differentiated G4, **L:** differentiated H9**)**. Scale bars = 50 μm. **(M, N)** HE staining **(M)** and immunostaining with an antibody against human vimentin **(N)** of serial sections of transplanted adipocytes derived from human bone marrow-derived mesenchymal stem cells after 2 weeks. Scale bars = 20 μm. **(O)** Oil Red O staining of the frozen sections of transplanted adipocytes derived from human bone marrow-derived mesenchymal stem cells after 2 weeks. Scale bar = 50 μm. HE, hematoxylin and eosin.

1.45% ± 0.48%, and 2.41% ± 1.42%, respectively (Fig. 6D), much less than were seen with G4 and H9. To examine the adipogenic differentiation rate of in vitro differentiated iPS and ES cells before transplantation, we quantified Oil Red O-stained areas of the differentiated iPS and ES cells. The areas of differentiated G4 and H9 were 23.89% ± 1.28% and 26.51% ± 1.29%, respectively. Moreover, those of differentiated B7, W12, and KhES-1 were 20.92% ± 2.77%, 22.92% ± 0.56%, and 21.25% ± 2.17%, respectively (Supplementary Fig. S4), smaller than those of differentiated G4 and H9.

SF4 ▶

Discussion

The present study demonstrates that human iPS and ES cells can differentiate into adipocytes with functional properties in vitro and that adipocytes derived from human iPS and ES cells can survive and maintain the differentiated properties in vivo for at least 4 weeks after transplantation.

We and others have reported that human iPS [13,33] and ES [34–40] cells have adipogenic potential in vitro. However, the functional properties of adipocytes derived from human iPS cells have not yet been fully characterized. Lipid storage and lipolysis are considered as major functions of mature adipocytes. Adipocytes store triacylglycerol when energy is in excess, and hydrolyze triacylglycerol to release fatty acids for utilization in other cells in response to energy demand. Triacylglycerol synthesis occurs in various tissues, while lipolysis predominantly occurs in adipose tissue [41]. The present study shows that PPARγ is localized in the nuclei of differentiated human iPS cells containing lipid droplets, and that these cells exhibit forskolin-stimulated lipolytic responses and insulin-induced AKT phosphorylation, suggesting that adipocytes with functional properties, such as lipid storage, lipolysis, and insulin responsiveness, can be differentiated from human iPS cells.

We also confirmed the presence of adipocytes after transplantation of differentiated human iPS and ES cells. We

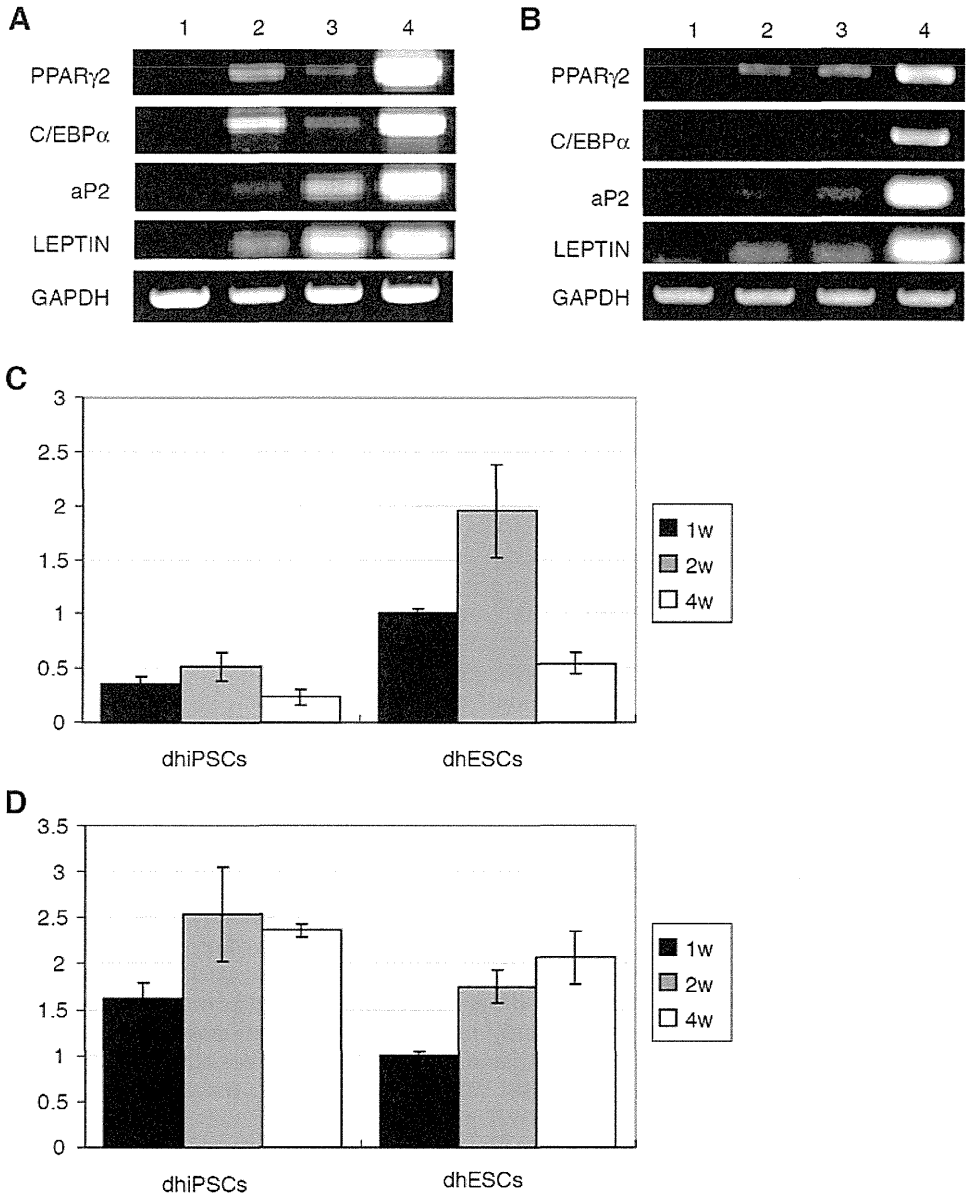

FIG. 5. Gene expression of adipocyte markers in transplanted cells. **(A, B)** Total RNA from transplanted cells (differentiated G4 and differentiated H9) was analyzed by RT-PCR at 2 **(A)** and 4 **(B)** weeks after transplantation. Lane 1: undifferentiated iPS cells (G4), lane 2: differentiated G4, lane 3: differentiated H9, lane 4: adipocytes derived from human bone marrow-derived mesenchymal stem cells. **(C, D)** Gene expression of human leptin **(C)** and PPARγ2 **(D)** was assessed by quantitative real-time PCR. Relative mRNA levels at 1, 2, and 4 weeks after transplantation of differentiated G4 (dhiPSCs) and differentiated H9 (dhESCs) were shown in black, gray, and white bars, respectively. Data represent ratios of the mRNA levels in samples of interest to the level in samples of differentiated H9 at 1 week after transplantation, respectively. Data are expressed as mean±SE from duplicate experiments ($n=5$). RT-PCR.

studied whether in vitro-differentiated adipocyte-derived human PS cells survived or not. When human iPS and ES cells are subjected to in vitro adipogenic induction via EB formation, the derivatives make up a heterogeneous population that includes adipocytes, preadipocytes, residual undifferentiated cells, and other cell types. Thus, adipocytes in transplanted Matrigel could be derived from any of these cell types after transplantation.

Time course analysis showed that the presence of adipocytes was confirmed at 1 week after transplantation of differentiated iPS and ES cells. Then, relative adipocyte areas peaked at 2 weeks. The adipocytes at 2 weeks after transplantation exhibited little or no proliferative capacity compared to immature neuroectodermal cells derived from undifferentiated iPS cells. Adipocytes were still present at 4 weeks. These findings indicate that adipocytes survive and maintain the differentiated state for 4 weeks after transplantation. Further, adipocytes described above were not seen in transplantation of undifferentiated iPS cells.

In that context, we hypothesize that the presence of adipocytes in transplanted Matrigel reflects the survival and maintenance of iPS and ES cells differentiated in vitro, although in vivo differentiation of preadipocytes may also contribute to the adipocytes seen in Matrigel after transplantation. Adipocytes as well as skeletal muscle cells, osteocytes, and chondrocytes are thought to be derived from mesenchymal progenitor cells [42,43]. Human mesenchymal stem cells have a characteristic surface antigen profile. Mesenchymal progenitor cells derived from human PS cells are enriched by sorting CD73- or CD105-positive cells [14,33,34,38]. We investigated the presence of mesenchymal progenitor cells in differentiated iPS and ES cells with adipogenic cocktail just before the transplantation. The expression of these surface antigens was analyzed in differentiated iPS and ES cells after the treatment with adipogenic cocktail. However, these representative surface antigens of human mesenchymal stem cells were not detected in differentiated iPS and ES cells (Supplementary Fig. S5), suggesting that

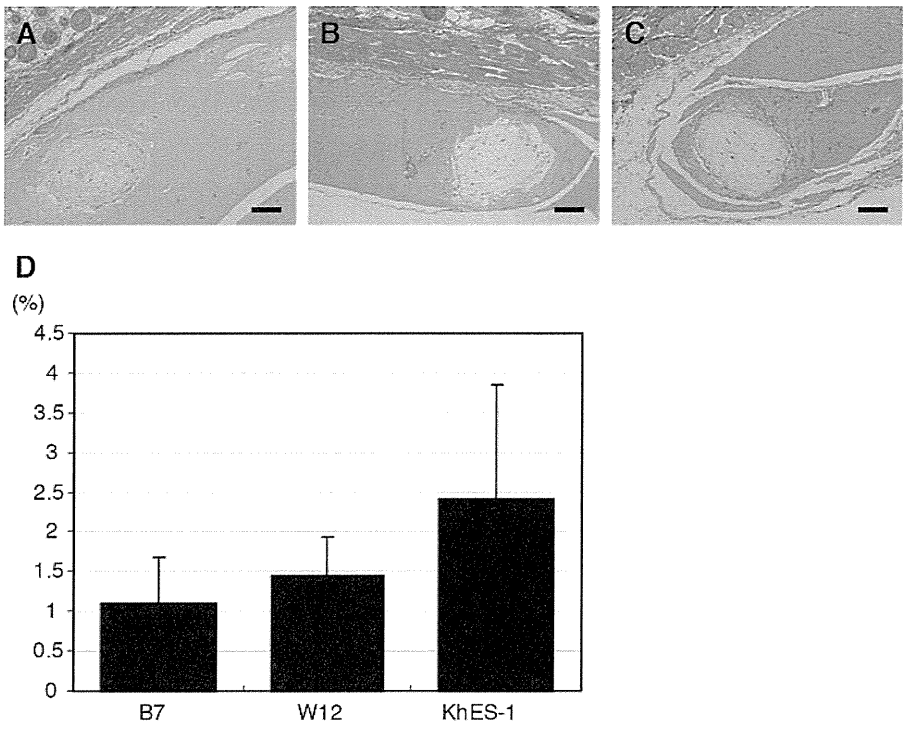

FIG. 6. Transplantation of adipocytes derived from other human pluripotent stem cells. **(A–C)** Transplanted cells were stained with HE. Morphological features of implanted Matrigel containing differentiated B7 **(A)**, W12 **(B)**, and KhES-1 **(C)** after 2 weeks. Scale bars = 100 μm. **(D)** Relative adipocyte areas at 2 weeks after transplantation of differentiated B7, W12, and KhES-1. Data are expressed as mean ± SE. At least six sections from three samples were analyzed for each group.

mesenchymal progenitor cells are rare populations in differentiated iPS and ES cells after adipogenic induction at that point although mesenchymal progenitor cells may reside in derivatives from human PS cells at an earlier time point of this differentiation protocol.

Transplantation of mature adipocytes often results in graft loss caused by direct reduction of the number of viable adipocytes [22]. Transplantation of mature adipocytes together with adipose-derived stem cells significantly improves survival times and graft volumes, as compared with transplantation of mature adipocytes alone [21,44]. Thus, cotransplantation of mature adipocytes and preadipocytes derived from human iPS and ES cells may be advantageous for graft survival. Establishing a lineage-specific adipocyte differentiation protocol and the methods for the purification of adipocyte progenitors derived from human PS cells will be essential for the success of future cell therapies using adipocytes derived from human iPS cells.

Adipose tissue is now known to be a bona fide endocrine organ, which secretes a variety of adipocytokines, including leptin. Generalized lipodystrophy is caused by a profound deficiency in adipose tissue, which leads to diabetes with marked insulin resistance, hypertriglyceridemia, and ectopic lipid accumulation. We and others have established the efficacy and safety of long-term leptin replacement therapy for generalized lipodystrophy [45–50], but this therapy does not rescue these patients from their generalized lack of adipose tissue. The only complete cure for these patients would be replenishment of adipose tissue or adipocytes. Indeed, transplantation of adipose tissue or adipocyte progenitors has been demonstrated to ameliorate metabolic disorders in animal models of lipodystrophy [20,51]. Generalized lipodystrophy is classified into two types, congenital and acquired lipodystrophy. In the case of congenital lipodystrophy, we need to repair gene mutation of the patient-specific iPS cells for cell therapy. On the other hand, in the case of acquired lipodystrophy, iPS cells are expected to differentiate into adipocytes. However, successful engraftment of adipocytes derived from human iPS cells may be affected by host factors. Then, in consideration of allogeneic transplantation, iPS cell banking is now discussed, and some groups proposed clinical application of iPS cells from HLA homologous donors [52,53]. Transplantation using those allogeneic iPS cells can decrease or minimize the risk of immune rejection. Human iPS cell-derived adipocytes from patients or HLA homologous donors are a new strategy for the treatment of lipodystrophy.

In our transplantation studies, adipocytes derived from differentiated iPS (G4) and ES (H9) cells were clearly observed, whereas adipocytes derived from other human iPS cell lines (B7 and W12) or another human ES cell line (KhES-1) were observed less frequently. This suggests there is diversity among these cell lines with respect to the survival and maintenance of adipocytes. It was previously reported that there are marked differences in differentiation propensity among human ES cell lines [54]. One possible explanation is that these differences are attributable to the difference of in vitro adipogenic differentiation potential among these cell lines caused by their genetic backgrounds, sites of transgene integration, and epigenetic states. Further studies will be needed to clarify the mechanism underlying the observed differences.

In summary, the present study demonstrates that human iPS and ES cells can differentiate into adipocytes with functional properties and that adipocytes derived from human iPS and ES cells can survive and maintain the differentiated properties in vivo for at least 4 weeks after transplantation. Establishment of refined adipocyte differentiation protocol of human iPS and ES cells and the transplantation method of adipocytes derived from human iPS and ES cells will contribute to understanding the pathophysiology of metabolic

diseases such as obesity and lipodystrophy as well as to future therapeutic applications.

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Author Disclosure Statement

The authors declare no potential conflict of interests.

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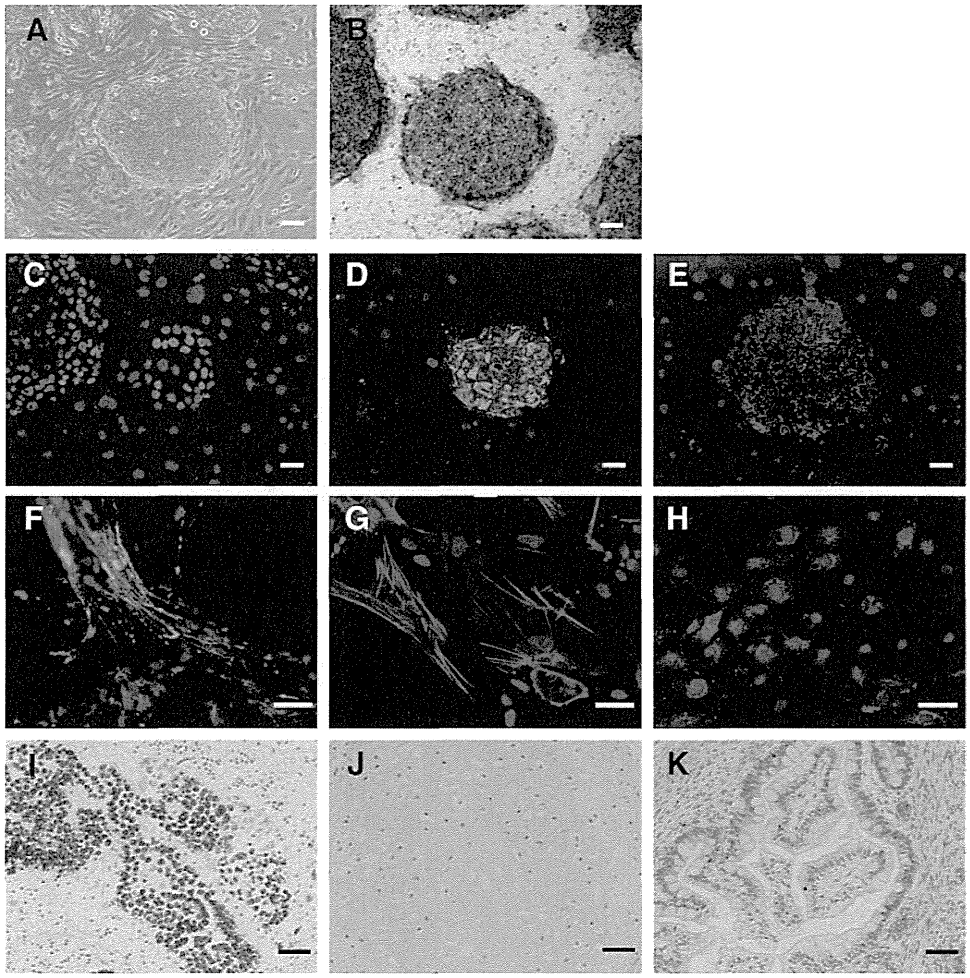
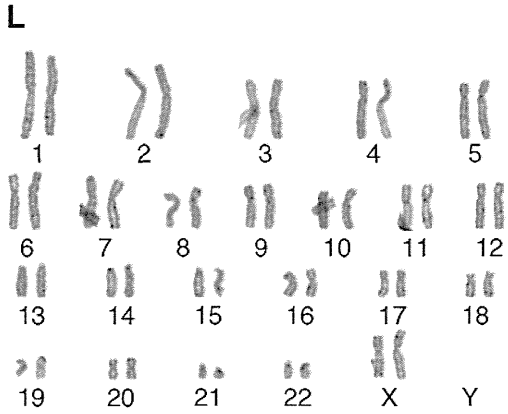
Supplementary Data



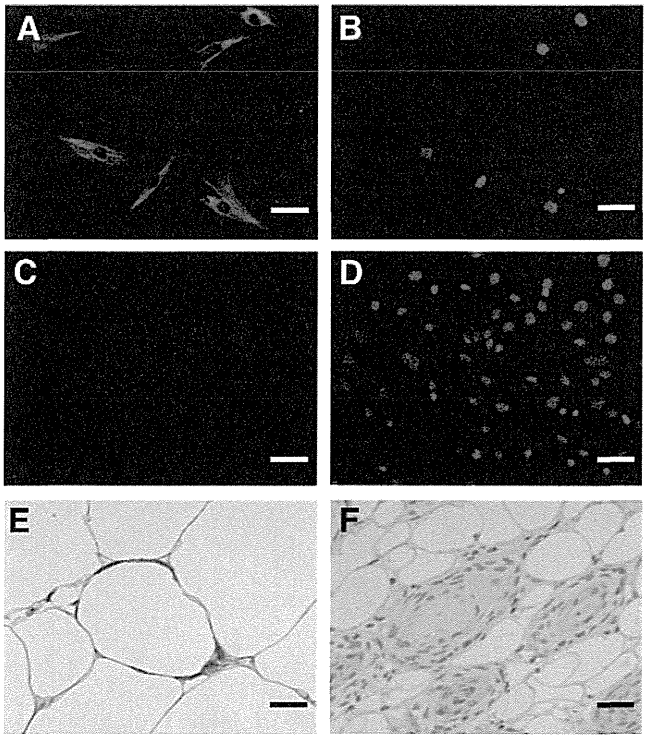


SUPPLEMENTARY FIG. S1. Characterization of human iPS cells (W12). **(A)** Phase-contrast image of an iPS colony. **(B)** Alkaline phosphatase activity. **(C–E)** Immunofluorescent staining with human ES cell markers: Nanog **(C)**, TRA-1-60 **(D)**, and SSEA-4 **(E)**. W12 exhibited characteristics similar to human ES cells. **(F–H)** EB-mediated in vitro differentiation of human iPS cells. Immunofluorescent staining with β3-tubulin (ectoderm) **(F)**, α-smooth muscle actin (mesoderm) **(G)**, and α-fetoprotein (endoderm) **(H)**. **(I–K)** Teratoma formation assay. Pigment cells (ectoderm) **(I)**, cartilage (mesoderm) **(J)**, and gut-like cells (endoderm) **(K)** W12 could differentiate into three germ layers in vitro and in vivo. **(L)** Karyotype. The cells showed normal karyotype. Scale bars = 50 μm. iPS cell, induced pluripotent stem cell; ES cell, embryonic stem cell; EB, embryoid body.

Gene		Sequences (5'→3')
PPARγ2	Sense primer	TTCCATGCTGTTATGGGTGA
	Anti-sense primer	ACCCTTGCATCCTTCACAAG
C/EBPα	Sense primer	AGAAAGGGGTGGAAACATAGG
	Anti-sense primer	GAAAGCTGAGGGCAAAGG
aP2	Sense primer	TGCAGCTTCCTTCTCACCTT
	Anti-sense primer	TGGTTGATTTTCCATCCCAT
Leptin	Sense primer	GGCTTTGGCCCTATCTTTTC
	Anti-sense primer	GCTCTTAGAGAAGGCCAGCA
Nanog	Sense primer	CAGCCCTGATTCTTCCACCAGTCCC
	Anti-sense primer	TGGAAGGTTCCCAGTCGGGTTCACC
GAPDH	Sense primer	AGCCGCATCTTCTTTTGCGTC
	Anti-sense primer	TCATATTTGGCAGGTTTTTCT
PPARγ2	Sense primer	GATACACTGTCTGCAAACATATCACAA
	Anti-sense primer	CCACGGAGCTGATCCC
	Probe	AGAGATGCCATTCTGGCCCACCAACTT
Leptin	Sense primer	TCACCAGGATCAATGACATTTCACA
	Anti-sense primer	GCCCAGGAATGAAGTCCAAACC
	Probe	ACGCAGTCAGTCTCCTCCAAACA
GAPDH	Sense primer	TGAAGCAGGCGTCGGAGG
	Anti-sense primer	GCTGTTGAAGTCAGAGGAGACC
	Probe	CCTCAAGGGCATCCTGGGCTACACTG

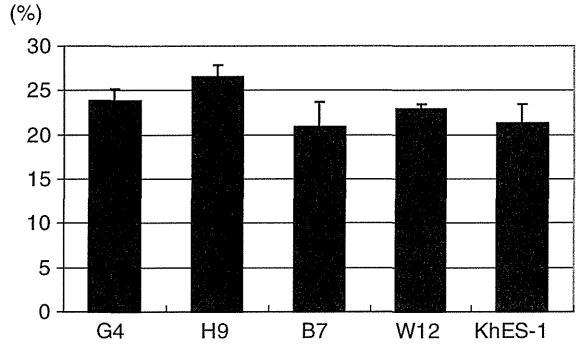
SUPPLEMENTARY FIG. S2. Sequences of primers and probes for reverse transcription PCR.

◀AU3

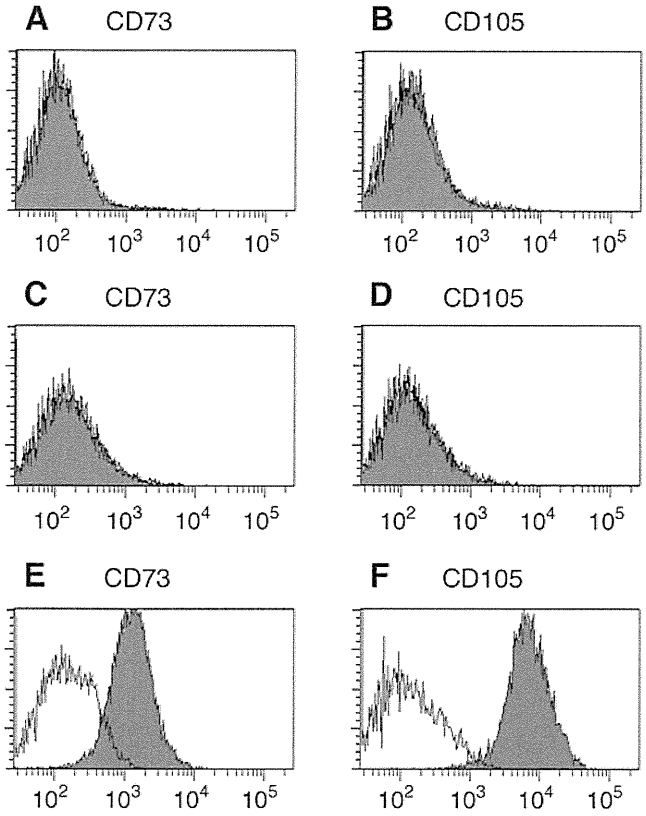


SUPPLEMENTARY FIG. S3. Cross reactivity of antibody against human vimentin. **(A–D)** Immunofluorescent staining with the vimentin antibody **(A)** and DAPI **(B)** in human bone marrow-derived mesenchymal stem cells. Immunofluorescent staining with the vimentin antibody **(C)** and DAPI **(D)** in mouse 3T3-L1 cells. Scale bars = 50 μm. **(E, F)** Immunostaining with an antibody against vimentin of human **(E)** and mouse **(F)** adipose tissue. Scale bars = 20 μm. DAPI.

AU4 ▶



SUPPLEMENTARY FIG. S4. Quantification of Oil Red O-stained areas of differentiated human iPS and ES cells. After 14 days of in vitro differentiation, differentiated pluripotent stem cells (G4, H9, B7, W12, and KhES-1) were stained by Oil Red O. The percentage of the Oil Red O-stained area divided by the total area was shown. Data are expressed as mean ± SE (n=4–5).



SUPPLEMENTARY FIG. S5. Expression of mesenchymal stem cell markers in differentiated human iPS and ES cells. **(A–F)** Flow cytometric analysis of representative human mesenchymal stem cell markers such as CD73 and CD105 in differentiated iPS cells (B7) **(A, B)**, differentiated ES cells (H9) **(C, D)**, and human bone marrow-derived mesenchymal stem cells **(E, F)**.

AUTHOR QUERY FOR SCD-2013-0113-VER9-NOGUCHI_1P

AU1: Please note that gene symbols in any article should be formatted per the gene nomenclature. Thus, please make sure that gene symbols, if present in this article, are italicized.
AU2: Please review all authors' surnames for accurate indexing citations.
AU3: Please expand FBS, DAPI, RT-PCR, and SE.
AU4: Please define DMEM, RT-PCR, and DAPI.

Original Article
CLINICAL TRIALS: BEHAVIOR, PHARMACOTHERAPY, DEVICES, SURGERY

Obesity

Early Changes of Abdominal Adiposity Detected with Weekly Dual Bioelectrical Impedance Analysis during Calorie Restriction

Midori Ida[1], Masakazu Hirata[1], Shinji Odori[1], Eisaku Mori[1], Eri Kondo[1], Junji Fujikura[1], Toru Kusakabe[1], Ken Ebihara[1], Kiminori Hosoda[1,2] and Kazuwa Nakao[1]

Objective: To elucidate early change of intra-abdominal fat in response to calorie restriction in patients with obesity by weekly evaluation using a dual bioelectrical impedance analysis (Dual BIA) instrument.
Design and Methods: For 67 Japanese patients with obesity, diabetes, or metabolic syndrome, intra-abdominal fat area (IAFA), initially with both Dual BIA and computed tomography (CT), and in subsequent weeks of calorie restriction, with Dual BIA were measured.
Results: IAFA by Dual BIA (Dual BIA-IAFA) correlated well with IAFA by CT (CT-IAFA) in obese patients ($r = 0.821$, $P < .0001$, $n = 67$). Ten males and 9 females (age 49.0 ± 14.4 years, BMI 33.2 ± 7.3 kg/m^2) lost more than 5% of baseline body weight (BW) in 3 weeks, and their Dual BIA-IAFA, BW, and WC decreased by 18.9%, 5.3%, and 3.8%, respectively ($P < .05$, ANCOVA).
Conclusion: Dual BIA instrument could detect the weekly change of Dual BIA-IAFA under calorie restriction in obese patients and demonstrated a substantially larger change of IAFA compared with changes of BW and WC in early weeks. This observation corroborates the significance of evaluating IAFA as a biomarker for obesity, and indicates the clinical usefulness of the Dual BIA instrument.

Obesity (2013) **21**, E350-E353. doi:10.1002/oby.20300

Introduction

Abdominal adiposity is associated with development of obesity and metabolic abnormalities in obesity-related diseases (1-3). The adipose tissue distribution has been quantitatively evaluated by computed tomography (CT) (4) or magnetic resonance imaging (MRI) (5), and intra-abdominal fat area (IAFA) is used as a clinical parameter of abdominal adiposity (6). Although waist circumference (WC) is casually employed to evaluate abdominal adiposity (7), WC is known to reflect both the intra-abdominal and the subcutaneous abdominal adiposity. In addition, the correlation of WC with intra-abdominal adiposity is influenced by age and sex as shown in epidemiological studies (5). Thus, WC does not necessarily provide the precise information about abdominal fat distribution. Therefore, a new practical method for detecting early change in abdominal adiposity is needed to elucidate its consequence during acute phase of calorie restriction in obesity treatment (8). There have been a few proposals of methods (9,10) that assess IAFA as alternatives to CT (4) or MRI (5). However, there has been no report on clinical application of these methods analyzing the weekly change of IAFA during calorie restriction. We have developed the dual bioelectrical impedance analysis (Dual BIA) instrument that can determine IAFA by measuring truncal impedance and surface impedance at the abdomen separately, each of which reflects the truncal adiposity and the subcutaneous adiposity respectively (11-13). The Dual BIA instrument has been optimized with aims at robustness for use in a wide range of human variation by analyzing the size of effect that each parameter, such as age and gender, can have on the calculation outcomes utilizing information technology (11-13). In this study, we report on application of the Dual BIA instrument to compare the weekly change in IAFA and body weight (BW) of obese patients with the metabolic syndrome or diabetes mellitus resulting from calorie restriction.

Methods

Dual BIA method and instrumentation

Dual BIA instrument calculates the cross-sectional area of intra-abdominal fat at the level of umbilicus based on the measurement of electrical potentials resulting from applying small electrical currents in two different body space. Principles of IAFA determination by Dual BIA instrument have been described previously (11-13) in detail. Briefly, the Dual BIA instrument consists of bioelectrical impedance

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component that measures truncal and surface impedance of the body, and a device that measures physical size of the abdomen. The two sets of electrodes are for limb and truncal placement. The limb electrodes consist of four clip-on electrodes placed on wrists and ankles. The truncal electrodes are eight pairs of electrodes 6 cm apart longitudinally that are fixed to a belt where four pairs each for front and back are positioned at an equal inter-electrode distance. The belt is adjustable so that the electrodes are positioned centered on mid-sagittal line at the level of umbilicus in supine position. The truncal impedance is measured by applying electrical currents between upper and lower limb leads and reading voltage from the electrodes around the abdominal circumference. The surface impedance is measured by applying and reading voltage from the abdominal circumferential electrodes. IAFA by Dual BIA (Dual BIA-IAFA) is calculated as follows.

$$\text{Dual BIA} - \text{IAFA} = \alpha_1 A + \alpha_2 B^2 - \alpha_3 \left(A^2 + B^2\right)^{1/2} Z_s - \alpha_4 / Z_t + \varepsilon \quad (1)$$

A: abdominal antero-posterior diameter, B: abdominal transverse diameter, Z_s: surface impedance, Z_t: truncal impedance, ε: residual constant.

There was a good agreement of Dual BIA-IAFA and IAFA measured by CT (CT-IAFA) with the correlation coefficient of 0.888 ($n = 98$, $P < .001$) (13).

Patient selection

The study was performed according to the protocol approved by Kyoto University Medical Ethics Review Board (no. 080116). The patient gave a written consent to participate in this study which took place at the endocrinology and metabolism ward of Kyoto University Hospital. We collected data from 67 Japanese patients (36 males and 31 females; mean ± SD age, 54.7 ± 14.7 years, BMI 29.3 ± 6.5 kg/m^2) with obesity ($n = 56$), diabetes mellitus ($n = 45$), or the metabolic syndrome ($n = 38$) who were hospitalized for calorie restriction therapy or diet education, and had measurement of IAFA by both Dual BIA method and CT method at the start of calorie restriction. Obesity was diagnosed as BMI 25.0, and metabolic syndrome was diagnosed according to 2005 Japanese criteria of metabolic syndrome (14). Average daily calorie intake was 1437.3 ± 201.4 kcal/day (19.3 ± 4.3 kcal/ideal BW). Out of 67 patients, 35 patients could be followed for longer than 3 weeks, while the other patients were discharged earlier after examination of complications and diet and lifestyle education. Total daily energy was varied individually during hospitalization based on consultation between the patient, a dietician, and a physician. Out of 35 patients who had their Dual BIA-IAFA monitored every week for at least 3 weeks (four times), 19 patients lost more than 5% of baseline BW, and were included in the analysis of weekly change in Dual BIA-IAFA, WC, and BW during weight reduction.

Measurement of Dual BIA, CT, and anthropometric parameters

Dual BIA-IAFA was measured every week in the morning before breakfast depending on individual patient's treatment schedule (Figure 1A). Abdominal CT was performed for calculation of CT-IAFA within 7 days before the initial Dual BIA-IAFA measurement. CT-IAFA was calculated at umbilical level by the software, Virtual Place Lexus (AZE of Japan, Ltd). BW was measured to the nearest 0.1 kg in the morning of the Dual BIA-IAFA measurement.

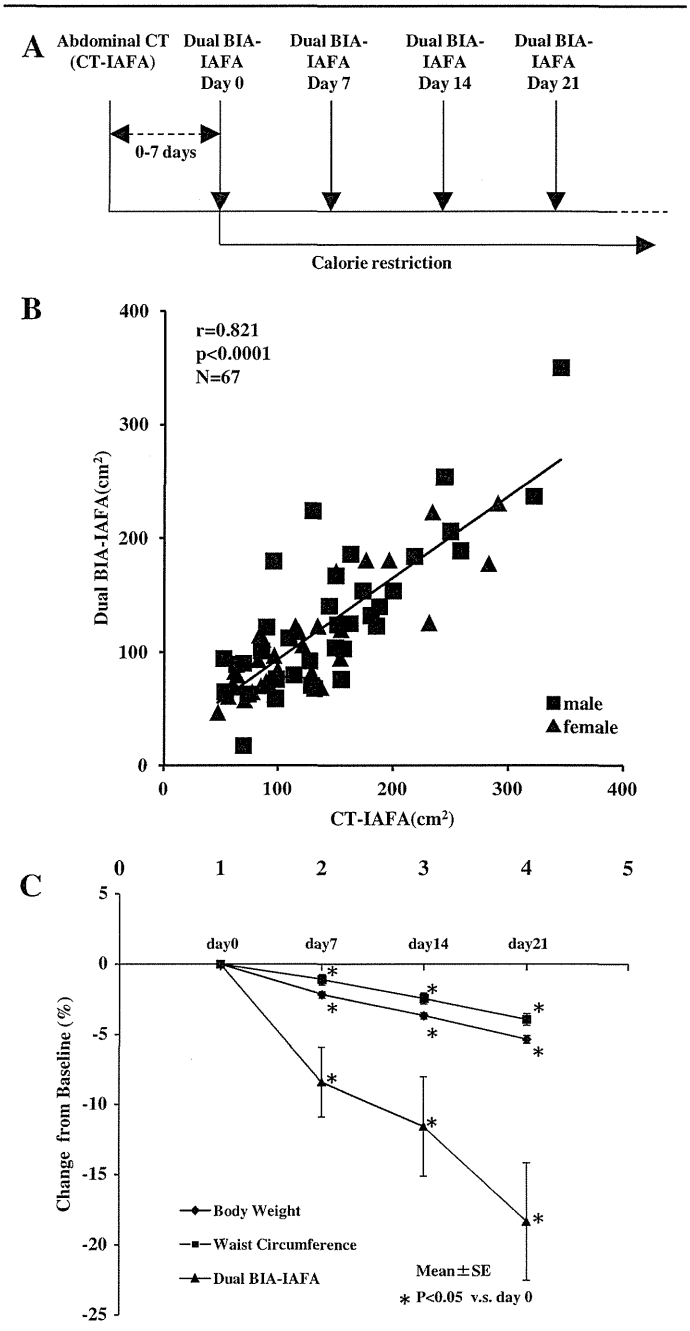

FIGURE 1 A: Diagram of IAFA assessment schedule during the calorie restriction. Patients started fixed calorie diet within 7 days of taking the abdominal CT image. Dual BIA-IAFA assessment took place in the morning before meal every week. CT imaging took place either in the morning or in the afternoon. **B**: Correlation between CT-IAFA and BIA-IAFA in 67 patients who were with obesity-related disorders. Square symbols: male, Triangle symbols: female. $r = 0.821$, $P < .001$ by Pearson's analysis. **C**: Weekly change of Dual BIA-IAFA plotted along with BW and WC during weight loss. Nineteen patients who underwent the calorie restriction and had abdominal CT examined at baseline were monitored for their anthropometric parameters and Dual BIA-IAFA weekly for at least 3 weeks. They lost more than 5% of BW during the period. Size of the change from baseline values (mean ± SE) is expressed as %. *$P < .05$ by Student's paired t-test.

WC was measured at the level of the umbilicus to the nearest 0.1 cm in the standing position at the end of expiration while breathing gently at the time of Dual BIA measurement.

Statistical methods

Correlation between values obtained by Dual BIA and CT were evaluated using Pearson's correlation analysis. Weekly values of Dual BIA-IAFA, BW, and WC were compared with the baseline values of day 0 by Student's paired *t*-test. Analysis of covariance was applied for comparison of Dual BIA-IAFA, BW, and WC at week 3.

Results

In 67 patients with obesity and related conditions, Dual BIA-IAFA correlated well with CT-IAFA ($r = 0.821$, $P < .0001$) (Figure 1B).

Thirty-five (17 males and 18 females) out of 67 patients were monitored with Dual BIA for longer than 3 weeks, and 19 (10 males and 9 females) out of 35 patients achieved weight loss of more than 5% of the initial BW. In order to elucidate the change in IAFA during weight loss, Dual BIA-IAFA, BW, and WC of the 19 patients were analyzed. Baseline characteristics of the 19 patients were (mean ± SD); age, 49.0 ± 14.4 years, height 163.0 ± 10.5 cm, BMI 33.2 ± 7.3 kg/m^2, and CT-IAFA 143.6 ± 47.4 cm^2. BW, WC, and Dual BIA-IAFA at baseline and at week 3 were: 89.2 ± 26.2 kg and 84.5 ± 25.1 kg, 110.6 ± 14.1 cm and 106.0 ± 14.2 cm, and 150.4 ± 73.7 cm^2 and 124.3 ± 70.3 cm^2, respectively.

Figure 1C shows the weekly change of Dual BIA-IAFA, BW, and WC in 19 patients whose BW decreased more than 5% during the 3 weeks of monitoring. Dual BIA-IAFA, BW and WC showed a significant reduction after 1 week during the calorie restriction compared with the baseline values ($P < .05$). Dual BIA-IAFA decreased every week for the initial 3 weeks and the average reduction in Dual BIA-IAFA was 18.9%, which was larger than in BW (5.3%) and WC (3.8%) (ANCOVA, $P < .05$).

Discussion

The present study demonstrates that the weekly change in IAFA can be detected with the Dual BIA instrument during the calorie restriction. Due to the practical limitations such as instrumentation and cost, CT and MRI are unsuitable for weekly monitoring of change in IAFA. There is also a problem of X-ray exposure in CT scanning. Consequently, it has been impractical to monitor IAFA weekly or frequently, in clinical follow-up period with CT or MRI. There have been several attempts to evaluate the IAFA by BIA (9-13). They include calculation from whole body impedance and from measuring abdominal impedance by the electrodes placed on the abdomen (9,10). Some of the estimates of IAFA incorporate gender and age of the subject in order to attain high correlation with CT (9,10). In contrast, Dual BIA, which is a method that is not dependent on external variables, such as gender or age, had shown a good correlation between Dual BIA-IAFA and CT-IAFA (11-13). In the present study, we confirmed the good correlation of Dual BIA-IAFA and CT-IAFA in obese patients. The correlation coefficient for the Dual BIA-IAFA and CT-IAFA was 0.821 ($n = 67$) with our subjects whose average BMI was 29.3. This indicates that Dual BIA produced reliable measurements with obesity patients and the result was comparable to the correlation coefficient of 0.888 obtained with subjects whose average BMI was around 25 (13). It must be noted that CT-IAFA and Dual BIA-IAFA was not measured on the same day in the present study, unlike the previous report in which Dual BIA- and CT-IAFA was taken on the same day (13), and therefore direct comparison has its limitations. By applying Dual BIA to monitoring the weekly change of individual body component during the calorie restriction, we could detect the characteristic change of IAFA. The significant decrease in Dual BIA-IAFA, BW, and WC at week 1 supports the suitability of selecting 5% of BW change at week 3 as a criterion for including in weekly analysis of these parameters.

On average, IAFA showed a larger reduction than BW and WC during the initial 3 weeks of calorie restriction. The rapid response of intra-abdominal adipose tissue to calorie restriction has been suggested in an ultrasonography study that examined a portion of peritoneal fat thickness (15). The larger decrease of Dual BIA-IAFA observed is also in agreement with a study which showed larger reduction in IAFA evaluated with MRI than that of BW up to 12 weeks on very low calorie diet (16). Together with these results, the present study established that the intra-abdominal fat decreases rapidly in the initial period of calorie restriction by measuring Dual BIA-IAFA, and demonstrates the usefulness of monitoring the change in IAFA during the treatment of obesity and its related disorders.

Weakness of our study is that its design was not of a prospective weight reduction where every participant was prescribed daily calorie that could produce predetermined level of weight loss within the study period. Instead we selected participants that had their weight decreased by at least 5% in order to illustrate the change in abdominal adiposity on weekly basis. It is also of note that the BW and Dual BIA-IAFA at week 1 may be affected by salt restriction and loss of body water that is observed early in calorie restriction. Because of the small sample size, the observed change in Dual BIA-IAFA could be larger than actual change. It also depends on the precision of the instrument. In a separate population, the coefficient of variation was 7.6% (Ida, M. manuscript in preparation).

In conclusion, the present study demonstrated that Dual BIA instrument can be used to measure IAFA in obese patients, allows frequent measurement, and is useful for detecting the early change in IAFA during calorie restriction. Information thus obtained along with other changes in metabolic parameters will be indispensable for understanding the role of abdominal adiposity, and especially useful as a diagnostic marker for monitoring obesity and its related disorders (1). In addition, the instrument's safety and convenience could be suitable for large population studies.**O**

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