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Abstract

White mature adipocytes give rise to so-called dedifferentiated fat (DFAT) cells that spontaneously undergo multilineage differentiation. In this study, we defined stem cell characteristics of DFAT cells as they are generated from adipocytes and the relationship between these characteristics and lineage differentiation. Both mouse and human DFAT cells, prepared from adipose tissue and lipoaspirate, respectively, showed evidence of pluripotency, with a maximum 5–7 days after adipocyte isolation. The DFAT cells spontaneously formed clusters in culture, which transiently expressed multiple stem cell markers, including stage-specific embryonic antigens, and Sca-1 (mouse) and CD105 (human), as determined by real-time polymerase chain reaction, fluorescence-activated cell sorting, and immunostaining. As the stem cell markers decreased, markers characteristic of the three germ layers and specific lineage differentiation, such as α-fetoprotein (endoderm, hepatic), Neurofilament-66 (ectoderm, neurogenic), and Troponin I (mesoderm, cardiomyogenic), increased. However, no teratoma formation was detected after injection in immunodeficient mice. A novel modification of the adipocyte isolation aimed at ensuring the initial purity of the adipocytes and avoiding ceiling culture allowed isolation of DFAT cells with pluripotent characteristics. Thus, the adipocyte-derived DFAT cells represent a plastic stem cell population that is highly responsive to changes in culture conditions and may benefit cell-based therapies. Stem Cells Translational Medicine *2014;3:161–171*

Introduction

Mature adipocytes and their potential role in regenerative medicine have recently gained increased attention [1–3]. Previous studies have demonstrated that white adipocytes are able to reversibly change their phenotype and undergo reprogramming into other lineages, by transitioning through cells that are referred to as dedifferentiated (DFAT) cells [4–7]. The DFAT cells initially lack expression of CD34, CD31, CD146, CD45, and pericyte markers, distinguishing them from progenitor cells previously identified in the adipose stroma [2], and have been shown to differentiate into multiple mesenchymal lineages, including cardiovascular, osteochondrogenic, and adipogenic lineages [2, 4, 8–11], suggesting a significant degree of multipotency or pluripotency.

Pluripotent stem cells have the ability to proliferate and differentiate into cells from all three germ layers. Three major types of pluripotent stem cells have to date been identified: embryonic stem cells (ES), induced pluripotent (iPS) cells, and multilineage-differentiating stress-enduring cells, so-called Muse cells, derived from mesenchymal human tissues [12]. A recent report suggested that a low percentage

(∼0.4%–1.2%) of rat DFAT cells express embryonic stem cell markers when assessed after 2 or more weeks in culture [13]. However, it is not clear whether newly generated DFAT cells are a significant source of pluripotent cells and, if so, how the pluripotency relates to lineage differentiation.

In this study, we provide evidence that both mouse DFAT (mDFAT) and human DFAT (hDFAT) cells have significant pluripotent characteristics, including formation of clusters with expression of multiple stem cell markers associated with ES signatures. The pluripotency gradually decreases as the DFAT cells spontaneously undergo differentiation with gene expression characteristics of the three germ layers and specific cell lineages in vitro and in vivo. However, no teratomas were detected after injection of DFAT cells in immunodeficient mice. In addition, simple modifications of the DFAT cell preparation, which helped ensure the purity of the adipocyte and avoided ceiling culture, enhanced the pluripotent characteristics of the DFAT cells.

Together, the results suggest that the adipocyte-derived DFAT cells are a cell model with transient pluripotency and significant flexibility that may be adapted for use in stem

cell research, regenerative medicine, and autologous tissue engineering.

MATERIALS AND METHODS

Collection of Adipose Tissue

For collection of mouse adipose tissue for cell isolation, transgenic or wild-type C57BL/6 mice were euthanized at 8–10 weeks of age by inhalation of isoflurane (5%–30%), and adipose tissues were collected postmortem. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Samples of fresh human subcutaneous lipoaspirate were used for this study, but the investigators were blinded to the identities, characteristics, and the medical histories of the human subjects.

Isolation of Adipocytes and Culture of DFAT Cells

Lipid-filled mature adipocytes and adipose stromal cells (ASCs) were isolated from 2 g of mouse subcutaneous adipose tissue or human fresh lipoaspirate, as previously described [4, 9, 14]. Before adipocyte isolation, the lipoaspirate was washed repeatedly with phosphate-buffered saline (PBS) until the PBS washes were clear. After the adipocytes had been isolated, they were washed three times in culture medium (Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum [HyClone, Logan, UT, http://www.thermoscientific.com/hyclone] and 0.5% of antibiotic antimycotic solution [Mediatech, Manassas, VA, http://www.cellgro.com]) before they were used for further analysis or culture. If the adipocytes were used for generation of DFAT cells, they were preincubated (floated) on top of medium in culture dishes or 50-ml plastic tubes with loosened caps for 24 hours to allow for any remaining nonadipocytes to detach and sink to the bottom. Adipocytes (30–50 μl of the top creamy layer) were then added to culture medium in six-well plates fitted with 70-μm filters and incubated for 5 days. DFAT cells generated from the adipocytes passed through the filters and attached to the bottom of the dishes (Fig. 1, schematic outline). After 5 days, the filters with remains of the adipocytes were removed. This method of preparing DFAT cells did not include attachment of the adipocytes to plastic surfaces or ceiling culture, as previously described [4]. In addition, our method allowed the separation of the DFAT cells from the adipocytes as soon as they passed through the filter and attached to the bottom of the dish. We regularly collected up to 10,000 hDFAT cells during the collection time of 5 days.

RNA Analysis

Real-time polymerase chain reaction (PCR) and reverse transcription PCR were performed, as previously described [2, 15]. The primers and probe used for real-time PCR for mouse and human POU homeodomain protein Oct3/4, mouse and human SRY (sex-determining region Y)-box 2 (SOX2), mouse and human homeobox protein Nanog, mouse c-Kit (CD117), mouse stem cell antigen 1 (Sca1), mouse bone morphogenetic protein 4 (BMP4), human c-Myc, human Kruppel-like factor 4 (Klf4), human α-fetoprotein, human Neurofilament-66, human Nestin, human Troponin I, human peroxisome proliferator-activated receptor γ (PPARγ), human CCAAT/enhancer-binding protein (C/EBP)α, human CD31, human VE-Cadherin, human Osteopontin, human Osterix, and human Aggrecan were predesigned and obtained from Applied Biosystems (Grand Island, NY, http://www.lifetechnologies.com/us/en/home/brands/applied-biosystems.html) as part of TaqMan gene expression assays. Previously prepared cDNA from human embryonic cell line HFS-1 and mouse ES was used as control. The primers used for RT-PCR are listed in supplemental online Tables 1 and 2. The products from RT-PCR were analyzed by 2% agarose gel electrophoresis.

Immunohistochemistry and Immunocytochemistry

Immunostaining was performed, as previously described in detail [2]. Briefly, cells grown in chamber slides were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 10% goat serum and 1% bovine serum albumin (BSA) in PBS, and incubated overnight at 4°C with the appropriate primary antibodies or nonspecific immunoglobulin G (IgG) control antibodies, diluted 1:200 in 1% BSA in PBS. The next day, cells were incubated with secondary AF-488-conjugated (green fluorescence) or AF-594–conjugated (red fluorescence) goat anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR, http://probes.invitrogen.com). The cells were washed with PBS, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and visualized by confocal or regular fluorescence microscopy. The nonspecific IgG control antibodies showed no staining and are not included in the figures.

We used the following antibodies for immunostaining: rat anti-stage-specific embryonic antigen (SSEA)-3, mouse anti-SSEA-4, rabbit anti-Neurofilament-66, and mouse anti-human mitochondria (all from Millipore/Chemicon, Billerica, MA, http://www.millipore.com); rabbit anti-human α-fetoprotein and mouse anti-human CD31 (both from Dako, Glostrup, Denmark, http://www.dako.com); goat anti-BMP4 and rabbit anti-c-Kit (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com); goat anti-mouse Sca-1/Ly6 (both from R&D Systems, Minneapolis, MN, http://www.rndsystems.com); rabbit anti-CD133, mouse anti-Nanog, and mouse anti-Perilipin (all three from Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com); mouse anti-human Nestin (Neuromics Antibodies, Edina, MN, http://www.neuromics.com); rabbit anti-Islet-1 (Abcam, Cambridge, U.K., http://www.abcam.com); mouse anti-human Nanog (all from Sigma-Aldrich); mouse anti-mouse Oct3/4 and rabbit anti-mouse SOX2 (both from StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com); goat anti-human Oct3/4 and mouse anti-human/mouse SSEA-1 (both from R&D Systems); mouse anti-TRA-1-60 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com); rabbit anti-Klf4 and rabbit anti-c-Myc (both from Abgent, San Diego, CA, http://www.abgent.com); and rabbit anti-VE-Cadherin, rabbit anti-Troponin I, and rabbit anti-SOX9 (all three from Santa Cruz Biotechnology).

Flow Cytometric Analysis

The size of the isolated adipocytes was assessed by fluorescence-activated cell sorting (FACS) using the lipophilic fluorescent dye AdipoRed (Lonza, Walkersville, MD, http://www.lonza.com), as previously described [9, 16]. For characterization of the phenotypes of DFAT cells and ASCs, FACS analysis was performed after the first passage, as previously described [2], using fluorescein isothiocyanate-, phycoerythrin-, or Alexa Fluor 488 (AF-488)-conjugated
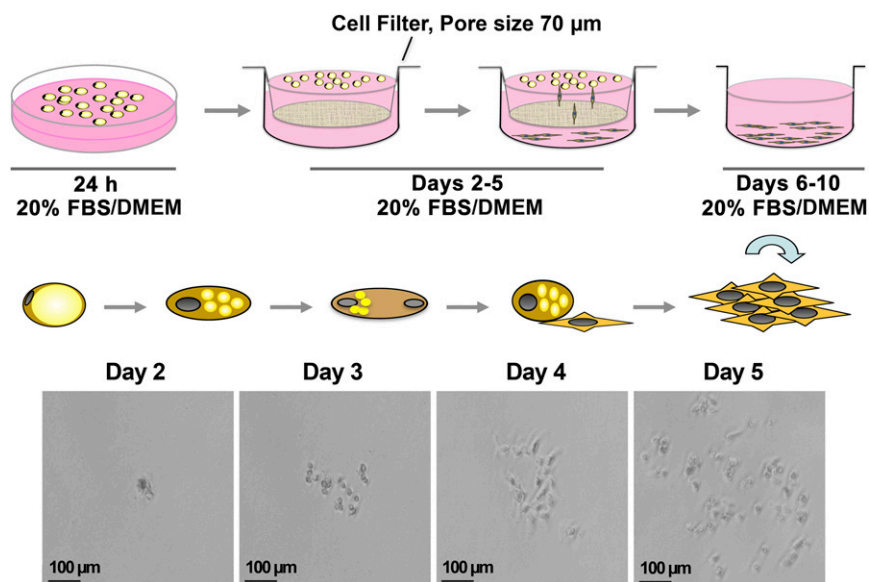
Figure 1. Schematic drawing of dedifferentiated fat (DFAT) cell preparation using preincubation and filters. Isolated adipocytes were incubated for 24 hours on culture medium before transfer to a new dish with filter, where the adipocytes remained for 5 days before the filter was removed. DFAT cells were allowed to sink through the filter to the bottom of the dish. The photos show the DFAT cells on the bottom of the dish on days 2–5. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

anti-mouse or anti-human antibodies against human CD14, CD34, CD105, SSEA-3, and SSEA-4 (all 1:200; BD Pharmingen, and eBioscience, San Diego, CA http://www.bdbiosciences.com). Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls.

Teratoma Formation

The teratoma formation analysis was performed by Applied Stem-Cell (Menlo Park, CA, http://www.appliedstemcell.com) using their standardized protocols. First- and second-passage DFAT cells were used for teratoma formation analysis. The cells were cultured in TeSR2 iPS medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com), which maintained a similar expression of pluripotency markers (Oct3/4, SOX2, Klf4, and Nanog) to the one observed on day 5 after filter removal. No mycoplasma was detected in the cells. Male Fox Chase severe combined immune deficiency (SCID)-beige (6 weeks old) were injected in kidney capsules and testes; 1.5–2 million cells in 30% Matrigel (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) were injected per site. Three injected kidneys and three injected testes were collected 96 days after injections of the hDFAT cells, or the control embryonic stem cells, and were further analyzed by Applied StemCell and our laboratory.

Alkaline Phosphatase and Lipid Staining

The alkaline phosphatase (ALP) staining was performed using an ALP detection kit (Millipore), as per the manufacturer's instructions. Lipids were stained with AdipoRed, as per the manufacturer's instructions, before immunofluorescence.

Histochemical Staining

Alizarin Red S staining was performed, as previously described [17], to visualize calcium deposition. Alcian Blue staining (0.05% Alcian Blue GX in 0.025 M acetate buffer solution, containing 0.025 M

$MgCl_2$, final pH 5.8, for 18 hours) was performed to visualize cartilage proteoglycans.

Sections From Injured Mouse Myocardium

Sections of injured myocardium were obtained from experiments previously performed and described in detail [2]. Briefly, ligation of the left anterior descending artery in wild-type C57BL6/J male mice was performed, and green fluorescent protein (GFP)-expressing DFAT cells prepared from GFP-transgenic mice were injected into the infarct zone. The cells were unpassaged and used on day ~10. The mice were sacrificed 2–6 weeks after the procedure, and the heart was fixed for histological analysis. Cardiac sections from 6 weeks after the procedure were used in the current study.

Statistical Analysis

Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey's analysis using the GraphPad Instat 3.0 software (GraphPad Software, La Jolla, CA, http://www.graphpad.com). p values less than .05 were considered significant. All experiments were repeated a minimum of three times.

RESULTS

Evidence of Pluripotency in Mouse DFAT Cells

Based on our previous findings that DFAT cells readily differentiate into endothelial cells and cardiomyocytes [2, 18], we hypothesized that the DFAT cells would have significant pluripotent characteristics that may have been underestimated previously. To address this question, we isolated adipocytes from the dorsal fat pads (inguinal fat depots) and prepared mouse DFAT (mDFAT) cells, as outlined in Materials and Methods. We first explored expression of pluripotency markers in the
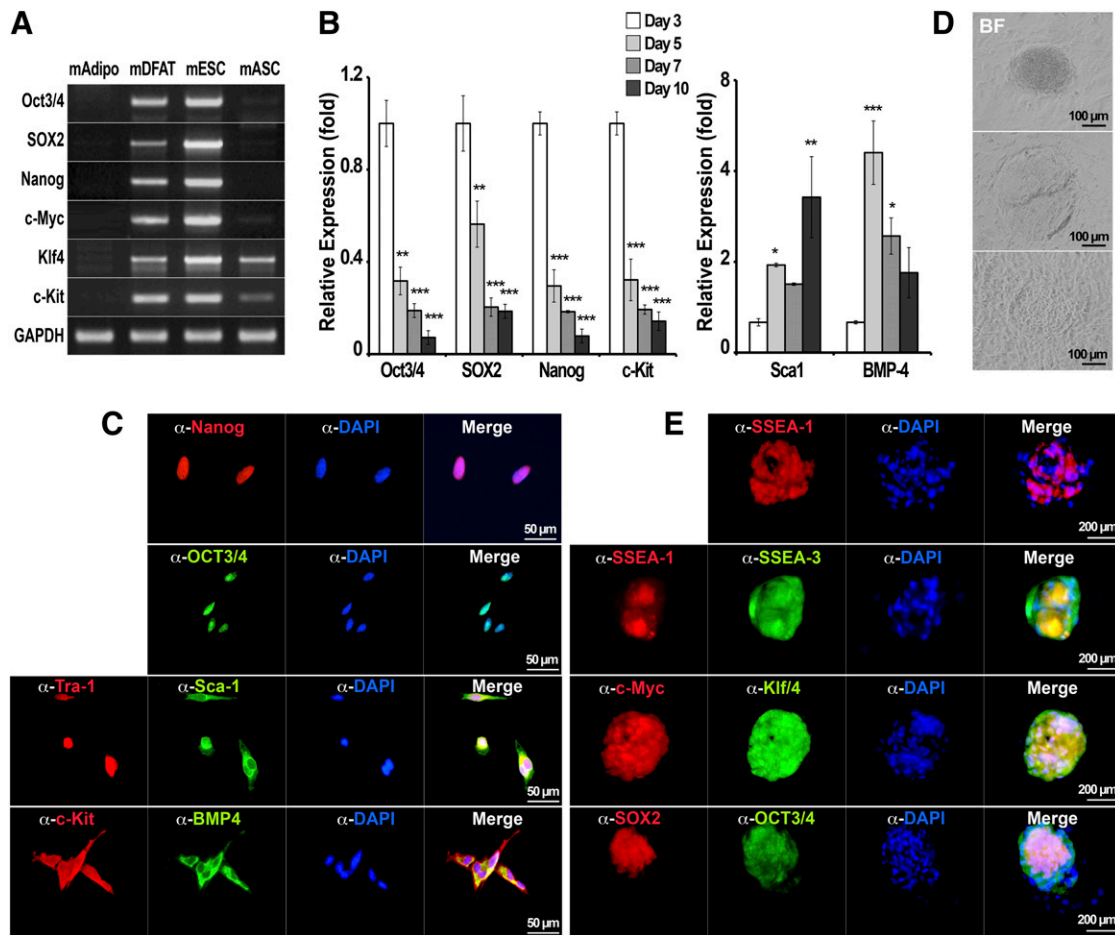
Figure 2. Expression of pluripotency markers in mDFAT cells. **(A):** Expression of Oct3/4, SOX2, Nanog, c-Myc, and Klf4, pluripotency markers, and c-Kit, a mesenchymal stem cell marker, was detected in mDFAT cells by reverse transcription polymerase chain reaction (PCR) on day 3. Isolated mouse adipocytes and mouse embryonic stem cells were used as negative and positive controls, respectively, and mouse adipose stromal cells are shown for comparison. GAPDH was used as RNA control. **(B):** Expression of Oct3/4, SOX2, Nanog, and c-Kit decreased in mDFAT cells between day 3 and day 10, whereas expression of Sca1, a mesenchymal stem cell marker, and BMP4, a stem cell renewal factor, increased after 3 days, as determined by real-time PCR. Asterisks indicate a statistically significant difference as compared with day 0: ∗, $p < .05$; ∗, $p < .01$; ∗∗∗, $p < .001$; Tukey's test. **(C):** Immunolocalization of Nanog, Oct3/4, Tra-1, Sca1, c-Kit, and BMP4 in single mDFAT cells on day 0. DAPI (blue) was used to visualize nuclei. **(D):** Characteristic mDFAT cell clusters formed spontaneously in mDFAT cell cultures on day 3 and later, as visualized by reverse-phase microscope (bright field). **(E):** Immunolocalization of SSEA-1, SSEA-3, SOX2, Oct3/4, c-Myc, and Klf4 in cell clusters on days 5–7. Abbreviations: BF, bright field; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAdipo, mouse adipocytes; mASC, mouse adipose stromal cells; mDFAT, mouse dedifferentiated fat; mESC, mouse embryonic stem cells; SOX2, sex-determining region Y-box 2; SSEA, stage-specific embryonic antigen.

mDFAT cells. We collected RNA from DFAT cells on days 3, 5, 7, and 10 after the day of filter removal (Fig. 1), which was counted as day 0, unless otherwise indicated. Expression of Oct3/4, SOX2, Nanog, c-Myc, and Klf4, as well as c-Kit, a mesenchymal stem cell marker, was easily detected by RT-PCR on day 5 (Fig. 2A) and resembled the expression in mouse ESCs. Mouse ASCs prepared from the same fat tissue as the adipocytes and used within the first passage, however, expressed mainly Klf4 and c-Kit. None of the markers was expressed in the adipocytes. Real-time PCR revealed that the expression of Oct3/4, SOX2, Nanog, and c-Kit progressively decreased between day 3 and day 10 (Fig. 2B), whereas expression of Sca1, a mesenchymal stem cell marker, and BMP4, essential for cell renewal, increased progressively after day 3 (Fig. 2B). We also compared mDFAT cells prepared by ceiling culture [4] and mDFAT cells prepared by our current

protocol. We found that the expression of Oct3/4, SOX2, and Nanog was approximately 6- to 14-fold higher on day 5 in the mDFAT cells prepared by our method compared with ceiling culture (supplemental online Fig. 1A).

Immunofluorescence confirmed expression of Nanog, OCT3/4, Sca-1, c-Kit, BMP4, and tumor rejection antigen 1 (Tra1), an embryonic stem cell marker, in individual cells on day 0 (Fig. 2C). On day 3 and later, the mDFAT cells formed cell clusters (Fig. 2D), which were transferred to chamber slides and allowed to attach for subsequent immunofluorescence. The immunofluorescence confirmed expression of Oct3/4, SOX2, c-Myc, and Klf4 (Fig. 2E). In addition, the clusters were positive for SSEA-1 and SSEA-3, embryonic stem cell markers (Fig. 2E). Together, the results suggested that mDFAT cells exhibited transient signs of pluripotency before the previously reported lineage differentiation [2, 4, 8–11].
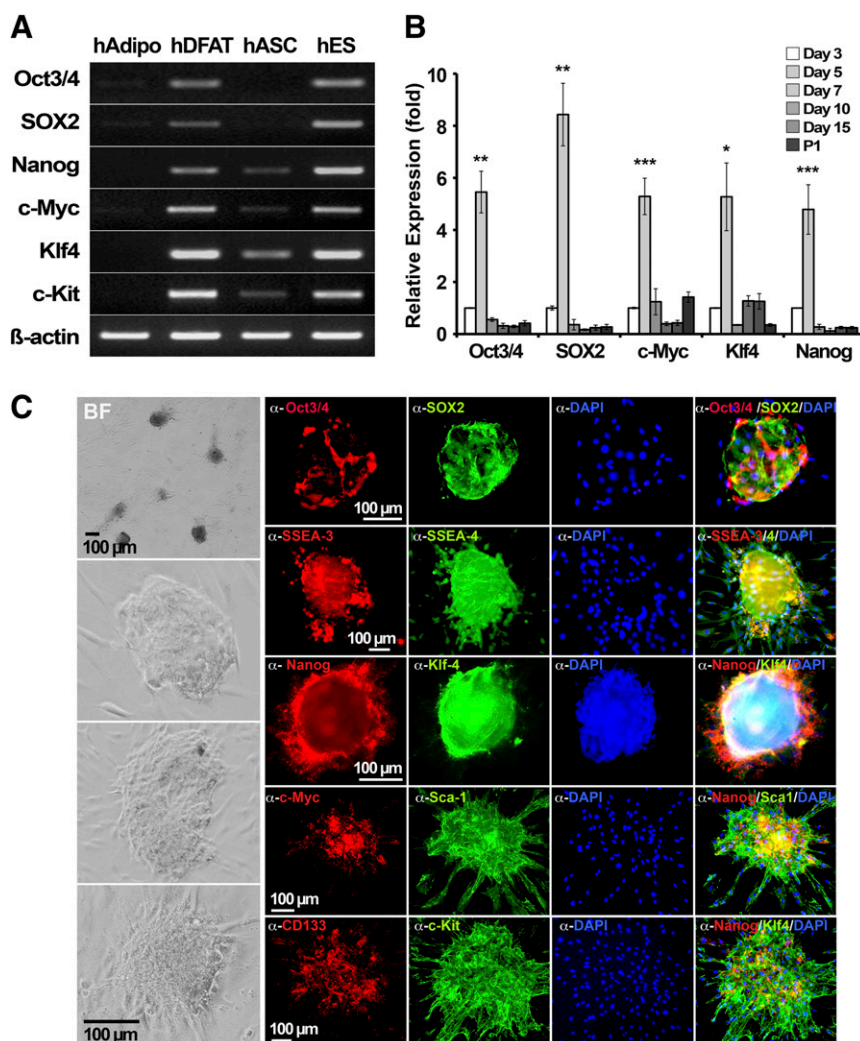
Figure 3. Expression of pluripotency markers in hDFAT cells. **(A):** Expression of Oct3/4, SOX2, Nanog, c-Myc, Klf4, and c-Kit was detected in hDFAT cells by reverse transcription polymerase chain reaction (PCR) on day 3. Isolated human adipocytes and human embryonic stem cells were used as negative and positive controls, respectively, and human adipose stromal cells are shown for comparison. β-Actin was used as RNA control. **(B):** Expression of Oct3/4, SOX2, c-Myc, Klf4, and Nanog decreased between day 3 and day 15 in hDFAT cells as determined by real-time PCR. hDFAT cells after P1 were included for comparison. Asterisks indicate a statistically significant difference as compared with day 0: *, $p < .05$; **, $p < .01$; ***, $p < .001$; Tukey's test. **(C):** Characteristic hDFAT cell clusters formed spontaneously in 7–10 days in hDFAT cell cultures, as visualized by reverse-phase microscopy (BF). Images show the immunolocalization of Oct3/4 and SOX2, SSEA-3 and SSEA-4, Nanog and Klf4, c-Myc and Sca1, and CD133 and c-Kit in cell clusters. DAPI (blue) was used to visualize nuclei. Abbreviations: BF, bright field; DAPI, 4′,6-diamidino-2-phenylindole; hAdipo, human adipocytes; hASC, human adipose stromal cells; hDFAT, human dedifferentiated fat; hES, human embryonic stem cells; P1, passage 1; SOX2, sex-determining region Y-box 2; SSEA, stage-specific embryonic antigen.

Evidence of Pluripotency in Human DFAT Cells

We then examined the human DFAT (hDFAT) cells for evidence of pluripotency. We obtained 10 de-identified human subcutaneous lipoaspirates. We isolated the adipocytes and divided them into three size fractions, 0–70 μm, 70–100 μm, and 100–150 μm, as determined by FACS after AdipoRed staining (supplemental online Fig. 2). The hDFAT cells were prepared using the same methods as for the mDFAT cells. Adipocytes from the two larger fractions lost the fat and gave rise to DFAT cells without detectable differences over 5 days. However, the smallest fraction (0–70 μm) gave rise to very few cells that failed to proliferate, suggesting that this fraction consisted mostly of debris and lipid droplets.

The hDFAT cells were cultured for up to 15 days after filter removal, which was counted as day 0. RNA samples were prepared on days 3, 5, 7, 10, and 15 from DFAT cells collected from the bottom of the dishes (Fig. 1). Expression of Oct3/4, SOX2, c-Myc, Klf4, Nanog, and c-Kit was easily detected in hDFAT cells by RT-PCR on day 5 (Fig. 3A), and was higher than in human ASCs (hASCs, used within the first passage), which showed some expression of Nanog, c-Kit, c-Myc, and Klf4, but no detectable expression of Oct3/4 and SOX2. The original adipocytes showed no detectable marker expression, whereas the hESC expressed all markers (Fig. 3A). Again, we compared marker expression on day 5 in hDFAT cells prepared by ceiling culture [4] and hDFAT cells prepared by our protocol, and found that expression of Oct3/4, SOX2, and Nanog was approximately three- to sixfold higher in the hDFAT cells prepared by our method (supplemental online Fig. 1B).
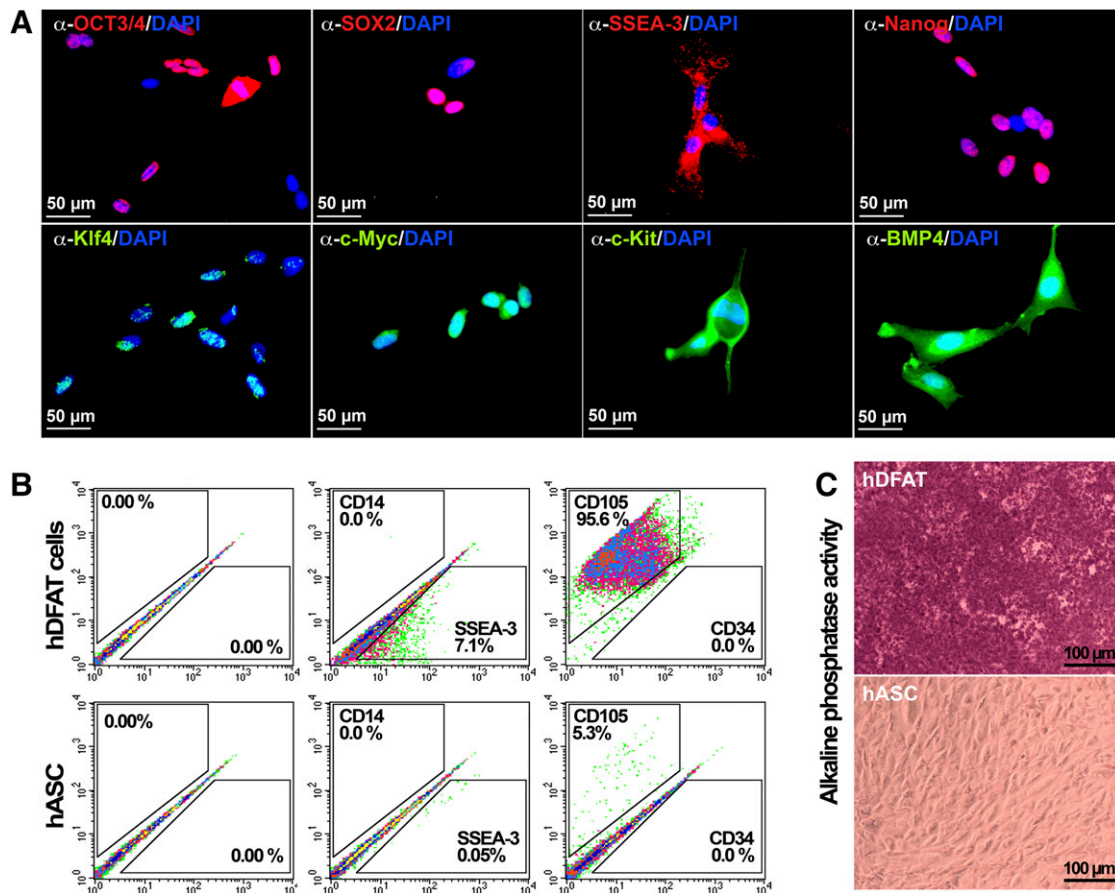
Figure 4. Expression of pluripotency markers in hDFAT cells. **(A):** Immunolocalization of Oct3/4, SOX2, SSEA-3, Nanog, Klf4, c-Myc, c-Kit, and BMP4 in single hDFAT cells after dissociation of clusters on days 7–8. DAPI (blue) was used to visualize nuclei. **(B):** hDFAT cells and hASCs positive for CD14, CD105, stage-specific embryonic antigen-3, and CD34 as determined by fluorescence-activated cell sorting on day 5. **(C):** hDFAT cells and hASCs stained for alkaline phosphatase activity on day 7. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; hASC, human adipose stromal cell; hDFAT, human dedifferentiated fat; hES, human embryonic stem; SOX2, sex-determining region Y-box 2; SSEA, stage-specific embryonic antigen.

Real-time PCR revealed that expression of Oct3/4, SOX2, c-Myc, Klf4, and Nanog progressively decreased between day 3 and 15 (Fig. 3B). Furthermore, expression of Oct3/4, SOX2, and Nanog was significantly higher than in the original adipocytes (P1) (Fig. 3B). The hDFAT cells formed clusters in 7–10 days (Fig. 3C, left), but the density of the clusters varied depending on which human sample was used. Clusters continued to form for up to eight passages, but the number of clusters decreased and the number of single cells increased with each passage. The clusters were examined by immunofluorescence, which demonstrated expression of Oct3/4, SOX2, SSEA-3, SSEA-4, Nanog, Klf4, c-Myc, Sca1, CD133 (hematopoietic stem cell marker), and c-Kit (Fig. 3C). Cell clusters, even as they grew sparser, continued to be positive for these markers. This was reflected in the overall expression of Oct3/4, SOX2, and Nanog, which decreased as the number of passages increased, as determined by real-time PCR (supplemental online Fig. 3).

We also examined marker expression in individual cells after dissociation of the clusters. Again, immunofluorescence showed expression of Oct3/4, SOX2, SSEA-3, Nanog, Klf4, c-Myc, and c-Kit, as well as BMP4 (Fig. 4A). FACS showed that 7.1% and 95.6% of the hDFAT cells were positive for SSEA-3 and CD105, respectively, on day 5 (Fig. 4B). By comparison, only 0.05% and 5.3% of the hASCs

were positive for SSEA-3 and CD105, respectively (Fig. 4B). Lastly, the hDFAT cells stained uniformly for alkaline phosphatase (Fig. 4C), further supporting pluripotency.

Pluripotency Marker Expression in hDFAT Cells Collected From Adipocyte Culture on Different Days

To examine the expression of pluripotency marker expression during each of the 5 days when hDFAT cells were generated from the adipocytes, we collected hDFAT cells on days 2, 3, and 5 after the initial adipocyte isolation, respectively, as well as for the combined days 1–4 for comparison (see Fig. 5A for schematic outline). The different cell populations were compared with hDFAT cells prepared by the standard ceiling culture. Expression of Oct3/4, SOX2, Nanog, c-Myc, and Klf4 was highest on day 5 and in cells collected on days 1–4, as compared with hDFAT cells prepared by ceiling culture (Fig. 5B). FACS analysis further showed that approximately 2.2%–5.5% of the hDFAT cells expressed SSEA-3 or SSEA-4 on days 3, 5, and 1–4 (Fig. 5C), and >95% of the cells expressed CD105 in all three samples (Fig. 5C). No expression of CD14, a marker of monocytic cells, was detected. The results supported that a period of 5 days was reasonable for collecting hDFAT from adipocytes for experiments.
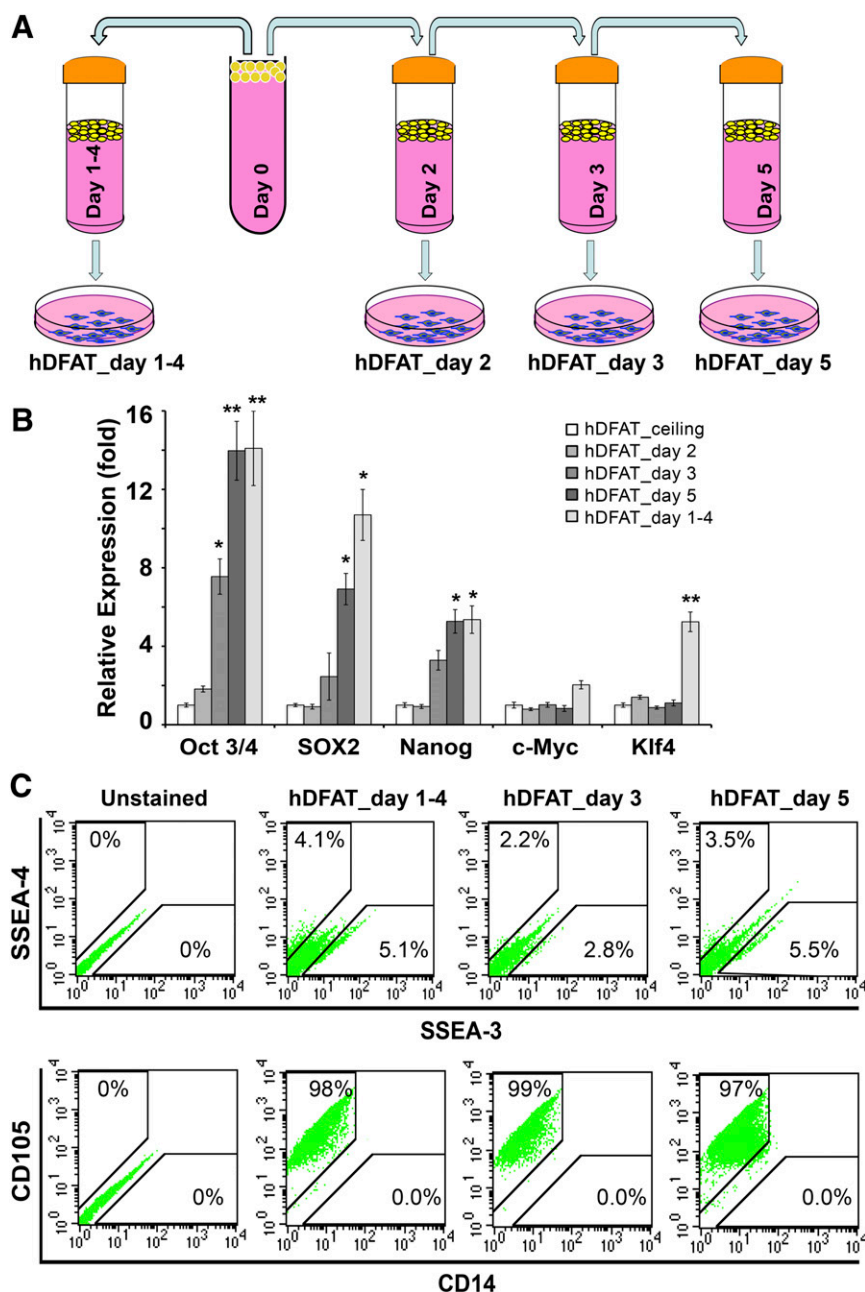
Figure 5. Expression of mulitpotent markers in hDFAT cells collected on separate days. **(A):** Schematic drawing of experiment in which hDFAT cells were collected from the isolated adipocytes for 4 days (days 1–4) or for 24 hours on days 2, 3, and 5. **(B):** Expression of Oct3/4, SOX2, Nanog, c-Myc, and Klf4 in hDFAT cells prepared by ceiling culture, or collected from floating adipocytes, as described above, as determined by real-time polymerase chain reaction. $*$, $p < .05$; $**$, $p < .01$; Tukey's test. **(C):** Expression of SSEA-3, SSEA-4, CD105, and CD14 in hDFAT cells collected, as described above, as determined by FACS. Abbreviations: hDFAT, human dedifferentiated fat; SOX2, sex-determining region Y-box 2; SSEA, stage-specific embryonic antigen.

Lineage Differentiation in hDFAT Cells

To examine lineage differentiation in hDFAT cells, we generated hDFAT cells from three separate human lipoaspirates and allowed them to remain in culture for up to 20 days. We then determined expression of lineage markers by RT-PCR, real-time PCR, and immunofluorescence. RT-PCR on day 5 revealed expression of the neural marker Nestin (ectodermal origin) and the ectodermal marker microtubule-associated protein 2 (MAP-2), the mesodermal markers Nkx2.5 and T-box transcription factor 5 (Tbx5), and

the endodermal markers α-fetoprotein and GATA-binding protein 6 (GATA6) in all three hDFAT cell preparations (Fig. 6A), suggesting that the cells undergo differentiation characteristic of the three germ layers. No expression was detected in the original adipocytes, and minimal expression was detected in hASCs isolated at the same time as the adipocytes (hASC3 sample shown, isolated at the same time as hDFAT3).

Real-time PCR revealed an increase in several markers, including Neurofilament-66 and Nestin (ectodermal origin), Troponin I,
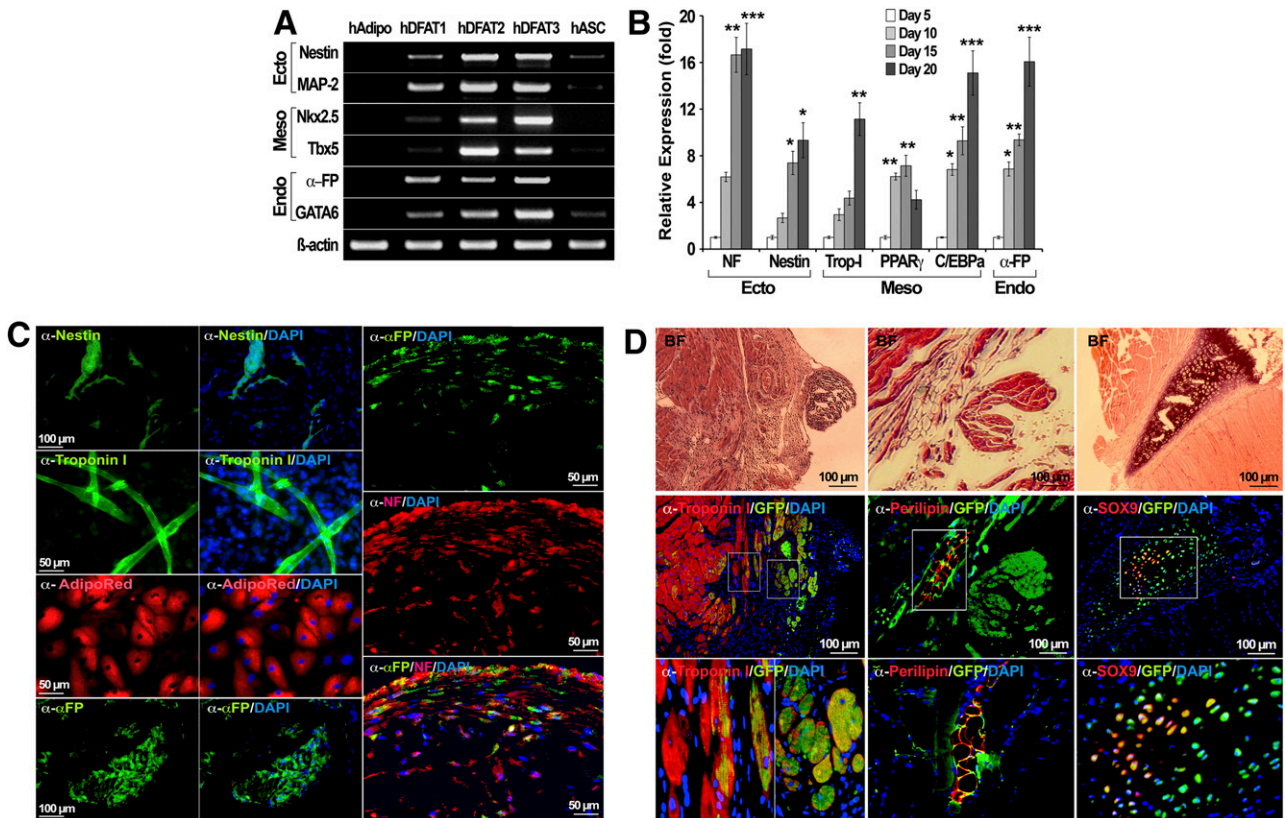
Figure 6. Expression of lineage markers in hDFAT cells. **(A):** Expression of the ectodermal markers Nestin and microtubule-associated protein-2, the mesodermal markers Nkx2.5 and Tbx5, and the endodermal markers α-fetoprotein and GATA6 in three different preparations of hDFAT cells as determined by reverse transcription polymerase chain reaction (PCR). Isolated human adipocytes showed no expression of these markers. hASCs are shown for comparison. β-Actin was used as RNA control. **(B):** Expression of the endodermal marker α-fetoprotein, the neural markers Neurofilament and Nestin, and the mesodermal markers Troponin I, PPARγ, and C/EBPα between day 0 and day 15, as determined by real-time PCR. Asterisks indicate a statistically significant difference as compared with day 0. $*$, $p < .05$; $**$, $p < .01$; $***$, $p < .001$; Tukey's test. **(C):** Immunostaining in dedifferentiated fat (DFAT) cells for the neural markers Nestin and Neurofilament-66, the cardiomyocyte marker Troponin I, AdipoRed-positive lipid droplets characteristic of adipocytes, and the endodermal marker α-fetoprotein, as visualized by immunofluorescence. **(D):** Colocalization of GFP and Troponin I, Perilipin, and SOX9 in infarcted mouse myocardium 6 weeks after injection of GFP-positive DFAT cells. DAPI (blue) was used to visualize nuclei. Nonspecific immunoglobulin G control showed no staining (data not shown). Abbreviations: BF, bright field ; C/EBPa, CCAAT/enhancer-binding protein α; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; hAdipo, human adipocytes; hASC, human adipose stromal cells; hDFAT, human dedifferentiated fat; PPARγ, peroxisome proliferator-activated receptor γ; SOX9, sex-determining region Y-box 9.

peroxisome proliferator-activated receptor (PPAR)γ and CCAAT/enhancer-binding protein (C/EBP) α (mesodermal origin), and α-fetoprotein (endodermal origin) (Fig. 6B). The markers increased over 15–20 days in contrast to pluripotency markers, which decreased over the same time period.

Several of the markers are characteristic of specific lineage differentiation and were further studied by immunofluorescence on day 20 (Fig. 6C). Nestin and Neurofilament-66 are neural markers, and Troponin I is a late cardiomyocyte marker, which was found in a striated pattern typical of cardiomyocytes. The presence of lipid accumulation, as seen by AdipoRed staining, was consistent with the increase in the adipogenic markers PPARγ and C/EBPα, and the presence of α-fetoprotein suggested hepatic lineage differentiation (Fig. 6C). In addition, hDFAT cells readily differentiated into endothelial-like cells, as shown by increased expression of the endothelial markers CD31 and VE-Cadherin, by real-time PCR and immunofluorescence (supplemental online Fig. 4A, 4B), which is consistent with our previous results [2]. Furthermore, the DFAT cells underwent ostechondrogenic differentiation, as evidenced by expression of the osteogenic markers

Osteopontin and Osterix and the chondrogenic marker Aggrecan (supplemental online Fig. 4A, 4C). In addition, Alizarin Red and Alcian Blue staining revealed calcium accumulation and cartilage mucopolysaccharides, respectively (supplemental online Fig. 4C). This suggests that hDFAT cells have significant differentiation capability.

Differentiation of mDFAT Cells in Injured Mouse Myocardium

In our previous studies [2], we found that GFP-expressing mDFAT cells expressed endothelial cell markers after injection of infarcted mouse myocardium. To explore whether other types of differentiation could be detected in the injured myocardium, we used cardiac sections from the previous experiments, in which mice had been sacrificed 6 weeks after injection. We found that the injection of GFP-mDFAT cells had resulted in the coexpression of GFP with Troponin I, Perilipin (an adipogenic marker), and SOX9 with GFP (Fig. 6D), which supports the idea that DFAT cells have differentiation capability.

Teratoma Formation in Nude Mice

The potential for teratoma formation of the hDFAT cells was tested by injection of expanded first-passage hDFAT in kidneys and testes in nude mice. The hDFAT cells were cultured in iPS medium, which was added at the time of filter removal. This maintained high expression of pluripotency markers and cluster formation as compared with regular culture medium (supplemental online Fig. 5). The injected kidneys and testes were collected 96 days after injections and examined. No teratoma formation was detected in the tissues injected with hDFAT cells, whereas teratomas were detected in control tissues injected with ES, as determined by visual inspection and hematoxylin and eosin staining (Fig. 7A, 7B). To determine whether human gene expression could be detected, we costained the tissues for human mitochondria and Neurofilament-66 for the kidneys and α-fetoprotein for the testes. The staining showed positive staining for human mitochondria, which partially colocalized with Neurofilament-66 and α-fetoprotein staining (Fig. 7C), suggesting that the hDFAT cells had been incorporated into the mouse tissue.

DISCUSSION

In this study, we demonstrated that DFAT cells that were generated from highly purified adipocytes had pluripotent characteristics, using a protocol that did not involve ceiling culture. We report that the DFAT cells formed clusters and expressed genes associated with pluripotency. The expression of pluripotency marker decreased with time and correlated inversely with the expression of lineage markers from the three germ layers, suggesting a transient phase of pluripotency. Furthermore, the DFAT cells showed significant differentiation capability but did not form teratomas in nude mice. The generation of DFAT cells is unlike the generation of iPS cells, in which simultaneous overexpression of Oct4, SOX2, c-Myc, and Klf4 leads to the generation of a pluripotent, ESC-like state in murine fibroblasts [19]. A previous study showed very low levels of pluripotency markers in DFAT cells that had been cultured for longer than 2 weeks [13]. A lingering expression of pluripotency markers after such a time period would be consistent with our results. It is possible that the early expression of pluripotency markers in DFAT cells was missed in previous investigations in which specific lineages were studied [4, 7, 14], causing underestimation of early pluripotency in DFAT cells.

The adipocytes used for DFAT preparation were highly purified and screened, as previously described [2]. We also introduced additional steps to enhance the DFAT preparation. We allowed the adipocytes to float for 24 hours on medium before transferring them to new dishes with filters, in which the DFAT cells separated from the floating adipocytes and passed through the filters on their way to the bottom of the dish. In this way, we avoided stretching of the adipocytes when they attach to the ceiling and the potential attachment of contaminating cells to the ceiling, both of which have been concerns in the past [20]. These additional steps, and probably the separation of the DFAT cells from adipocytes during preparation, significantly increased the expression of pluripotency markers.

Several investigators have reported low levels of expression of pluripotency markers in human adipose-derived stem cells [21–24]. Zuk [21] reported expression of Oct4 and SOX2 in hASCs of passages 2 and 3, although the author had concerns about the antibodies used in the study. Sun et al. [22] reported expression of c-Myc, Klf4, and c-Kit in the human ASCs within the two first passages, which was similar to our findings. However, our cells also expressed low levels of Nanog, SSEA-3, and CD105, whereas Sun et al. [22] found low levels of Oct3/4. In addition, Sachs et al. [23] and Guasti et al. [24] reported expression of several multipotent markers in human and human pediatric ASCs, including Sox2, Oct-4, Nanog, c-Myc, Klf4, and DNA (cytosine-5)-methyltransferase 3β, although it is not clear what passage cells were used in the two studies. Altogether, the studies support the presence of pluripotent cells in fat tissue.

Differences in expression of pluripotency markers in different preparations of DFAT and ASCs may be the result of a number of factors that can differ between preparations and laboratories. Factors that may lower pluripotency include a high number of passages and significant expansion before use, high confluence of the cells when analyzed, and poor cluster formation. Differences between the human lipoaspirates are likely to be important as well. Heterogeneity within a cell population could easily be induced by variations in the microenvironment and be amplified as the cells grow. For example, local variations in cell density, local buildup of extracellular matrix, and local cell death may trigger some cells to start differentiating and lose all or some pluripotency markers. This could explain why not all cells express the same set of pluripotency markers. As the cells are expanded, certain cells may have a higher rate of proliferation, causing them to dominate the culture.

In this study, we allowed the DFAT cultures to spontaneously differentiate in a basic medium to show the simultaneous decrease of pluripotency markers and increase of specific lineage markers. However, we used the iPS medium to maintain pluripotency before teratoma formation assays. For induction of specific lineage differentiation, treatments could start shortly after adipocyte isolation to catch the cells before any heterogeneity develops. Alternatively, our results suggest that defined media, such as iPS medium, can be used to defer differentiation. In a clinical setting, it may be possible to extract and isolate adipocytes, treat the cells with inducers of specific lineages, and reinject within the span of a few hours to days. Our method can easily be scaled up to generate large number of cells by increasing the sizes of filters and culture dishes or by using custom-built devices. This would be preferable to extensive expansion of a small number of cells that would risk losing important characteristics in the process. Another attractive possibility would be if fat cells in and around organs could be manipulated in situ, omitting the ex vivo step. Thus, adipocyte-derived multipotent cells may provide a readily available alternative in regenerative therapies.

Other studies have shown that human subcutaneous tissue and bone marrow may have small amounts of pluripotent cells that are capable of differentiation characteristic of all three germ layers without the need to introduce exogenous genes, so-called Muse cells [12], which set the precedent for pluripotent cells in mesenchymal tissues like fat tissue. Similar to our DFAT cells, the Muse cells did not induce teratoma formation in nude mice [12].

Dedifferentiation in fully mature cells may be triggered by stress; for example, heart regeneration after injury in zebrafish may occur through dedifferentiation and proliferation of existing adult cardiomyocytes [25]. In our case, the dedifferentiation may be triggered by lipid loss or stress because of loss of external cues, which causes the adipocytes to revert to an immature state and gain pluripotency.

In this study, we did not have access to information about the location from which the fat was aspirated or the characteristics of
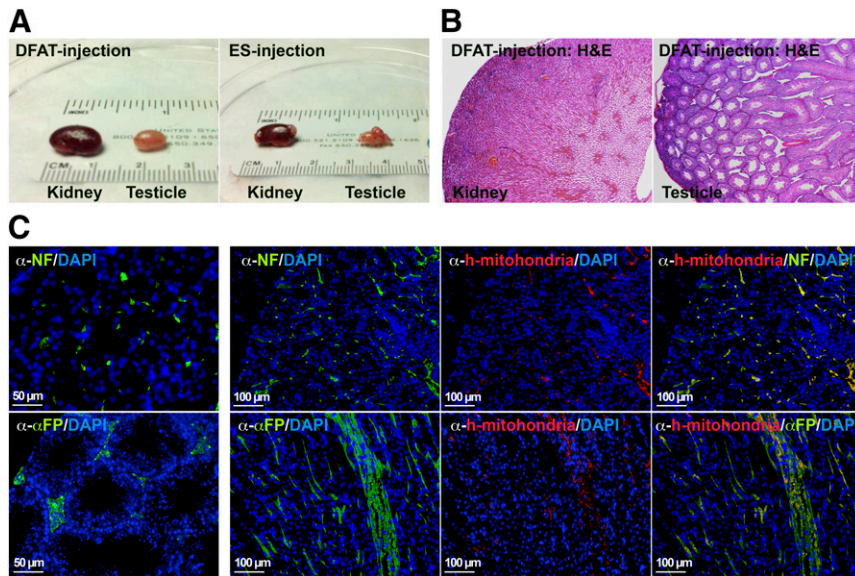
Figure 7. Human dedifferentiated fat (hDFAT) cells do not form teratomas in nude mice. **(A):** No evidence of teratoma in kidneys or testes after hDFAT cell injection by visual inspection (left) compared with injection of embryonic stem cell control (right). **(B):** No evidence of teratoma in kidneys or testes after hDFAT cell injection by inspection of hematoxylin and eosin-stained tissues. **(C):** Staining for the neural marker NF and the endodermal marker αFP in kidney injected with hDFAT cells (left panels). NF and αFP staining colocalized with staining for human mitochondria (right four panels). Abbreviations: αFP, α-fetoprotein; DAPI, 4′,6-diamidino-2-phenylindole; DFAT, dedifferentiated fat; ES, embryonic stem cell; h, human; H&E, hematoxylin and eosin; NF, Neurofilament-66.

the human subjects such as age, gender, body mass index, or presence of diabetes mellitus. The influence of such factors on the human DFAT characteristics will be important in future studies and could advance the use of DFAT cells in tissue regeneration as an individual source of progenitor cells for tissue generation and drug testing or as a diagnostic tool. The mouse system is attractive in that it can generate homogenous populations of DFAT cells from mice with particular genetic defects for mechanistic studies without the use of small interfering RNA or expression vectors.

CONCLUSION

Together, our data suggest that the DFAT cells represent a readily available cell model that undergoes a transient phase of significant pluripotency, during which interventions could induce specific lineages desired for various applications.

AUTHOR CONTRIBUTIONS

M.J.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; R.A, A.L., M.R.C., L.J.S., and S.H.-H.: collection and/or assembly of data; D.A.D.: financial support; Y.Y.: data analysis and interpretation; K.I.B.: conception and design, data analysis and interpretation, financial support, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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