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Human Adipose Tissue Possesses a Unique Population of Pluripotent Stem Cells with Nontumorigenic and Low Telomerase Activities: Potential Implications in Regenerative Medicine

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Abstract

In this study, we demonstrate that a small population of pluripotent stem cells, termed adipose multilineage-differentiating stress-enduring (adipose-Muse) cells, exist in adult human adipose tissue and adipose-derived mesenchymal stem cells (adipose-MSCs). They can be identified as cells positive for both MSC markers (CD105 and CD90) and human pluripotent stem cell marker SSEA-3. They intrinsically retain lineage plasticity and the ability to self-renew. They spontaneously generate cells representative of all three germ layers from a single cell and successfully differentiate into targeted cells by cytokine induction. Cells other than adipose-Muse cells exist in adipose-MSCs, however, do not exhibit these properties and are unable to cross the boundaries from mesodermal to ectodermal or endodermal lineages even under cytokine inductions. Importantly, adipose-Muse cells demonstrate low telomerase activity and transplants do not promote teratogenesis in vivo. When compared with bone marrow (BM)– and dermal-Muse cells, adipose-Muse cells have the tendency to exhibit higher expression in mesodermal lineage markers, while BM- and dermal-Muse cells were generally higher in those of ectodermal and endodermal lineages. Adipose-Muse cells distinguish themselves as both easily obtainable and versatile in their capacity for differentiation, while low telomerase activity and lack of teratoma formation make these cells a practical cell source for potential stem cell therapies. Further, they will promote the effectiveness of currently performed adipose-MSC transplantation, particularly for ectodermal and endodermal tissues where transplanted cells need to differentiate across the lineage from mesodermal to ectodermal or endodermal in order to replenish lost cells for tissue repair.

Introduction

Mesenchymal stem cells (MSCs) derived from adipose tissue are multipotent stromal cells that can differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts in vitro and undergo differentiation in vivo [1]. MSCs are currently being applied in a number of clinical studies that target numerous diseases because of their accessibility, nontumorigenicity, and powerful trophic effects [2,3].

MSCs derived from adipose tissue (adipose-MSCs) provide an abundant and minimally invasive source of cells [4]. Adipose-MSCs can be maintained in culture for extended periods of time and can be induced in vitro to differentiate into all mesenchymal cell lineages [1,4]. Moreover, adipose-MSCs can be safely and efficiently transplanted to autologous hosts, and they are currently being used successfully for a variety of regenerative therapies [2,3].

Although not in high ratio, adipose-MSCs also have the capacity to differentiate into neuronal cells [5,6], Schwann cells [7], beta cells [8], and hepatocytes [9,10] in the presence of specific cell differentiation media. Thus adipose-MSCs may cross the oligolineage boundaries from mesodermal to ectodermal or endodermal lineages. Adipose-MSCs exhibit

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a wide variety of triploblastic differentiation not only in vitro, but also in vivo. At a low ratio, they may spontaneously differentiate into hepatocyte-marker-positive cells in the damaged liver [11,12], neuronal- and glial-marker-positive cells in ischemic brain injury [13,14], and cardiomyocytes in acute myocardial infarction [15] after homing to damaged sites. The low rate of adipose-MSC differentiation into ectodermal and endodermal cell lineages could be explained in part by the presence of a small population of stem cells within the adipose-MSC population that have the ability to differentiate to any type of cells, much like pluripotent stem cells. Isolation of such stem cells could have a critical impact in regenerative medicine and cell therapy.

Recently, a novel population of stem cells with pluripotent characteristics has been isolated from mesenchymal tissues, such as human skin fibroblasts and bone marrow (BM). These cells, termed multilineage-differentiating stress-enduring (Muse) cells, are of mesenchymal origin, comprise several percentages of human dermal fibroblasts and BM-MSCs, and are highly resistant to cellular stress. They are double positive for CD105, an MSC marker, and the stage-specific embryonic antigen-3 marker (SSEA-3), well known for the characterization of undifferentiated human embryonic stem (ES) cells. Muse cells can differentiate into cells of ectodermal, endodermal, and mesodermal lineages both in vitro and in vivo, and have the ability to self-renew [16]. Advantageously, Muse cells do not produce teratomas in vivo, nor do they induce immunorejection in the host upon autologous transplantation [16,17]. In addition, Muse cells are shown to home into the damaged site in vivo and spontaneously differentiate into tissue-specific cells according to the microenvironment to contribute to tissue regeneration when infused into the blood stream [16].

In the present study, we isolated Muse cells derived from human adipose tissue (adipose-Muse cells) using SSEA-3-cell-sorting techniques. Further characterization indicates that SSEA-3(+) adipose-Muse cells express general mesenchymal markers CD105, CD90, and CD29 [18,19]. They express the pluripotent stem cell markers Nanog, Oct3/4, PAR4, Sox2, and TRA-1-81 and can spontaneously differentiate into cells representative of all three germ layers from a single cell. Conversely, alternate cells in adipose-MSCs, SSEA-3 (−) adipose-MSCs (adipose-non-Muse cells), can only differentiate into mesenchymal but not into ectodermal and endodermal cell lineages even under the presence of cytokine induction. Further, adipose-Muse cells are negative for CD34 and CD146, known as classical adipose-derived stem cell (ADSC) markers [4]. While core properties of Muse cells among BM, dermis, and adipose tissues, namely, triploblastic differentiation, self-renewal, and non-tumorigenicity, are the same, BM- and dermal-Muse cells show higher expression of ectodermal and endodermal lineage markers while adipose-Muse cells show a tendency for higher expression of mesodermal markers, and preferentially differentiate into mesodermal cell lineages, suggesting that the propensity for differentiation is in accordance with the source of tissue from which Muse cells are derived.

In contrast to ES and induced pluripotent stem (iPS) cells, adipose-Muse cells have low telomerase activity and do not produce teratomas in vivo, which may alleviate one of the primary concerns with the use of pluripotent stem cells in the clinical arena. Adipose-Muse cells could be an ideal source of pluripotent stem cells with the potential to have a critical impact on regenerative medicine.

Materials and Methods

Cell source

Two different sources of adipose-MSCs were used in this study: adipose-MSCs commercially purchased from Lonza (LA-MSCs) and freshly isolated adipose-MSCs from subcutaneous adipose tissue (AT-MSCs). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium high-glucose (DMEM; Gibco) containing 15% fetal bovine serum (FBS) and 0.1 mg/mL kanamycin sulfate (Gibco) in an atmosphere containing 5% CO_2.

The use of human subcutaneous adipose tissue was approved by the Ethics Committee for Animal Experiments at the Tohoku University Graduate School of Medicine. Subcutaneous adipose tissue was provided by Department of Dermatology, Tohoku University Graduate School of Medicine with informed consent. Isolation of AT-MSCs from adipose tissue was done according to the method previously reported by Estes et al. with minor modification [20]. In brief, adipose tissue was minced into small pieces and incubated in equal volume of phosphate-buffered saline (PBS, without calcium chloride and magnesium chloride) containing 1 mg/mL Collagenase Type I (Worthington Biochemical) and 1% bovine serum albumin (Nacalai) at 37°C for 1 h with mild shaking. Digested material was then centrifuged at 300 *g* for 5 min to obtain a cell pellet. The pellet was resuspended and filtered through a 100-μm nylon mesh filter (Becton Dickinson) and centrifuged again at 300 *g* for 5 min. The pellet was resuspended in DMEM containing 15% FBS and 0.1 mg/mL kanamycin sulfate and cultured. Cells were plated in adherent dishes at density of 3.5×10^4 cells/cm^2 and cultured after reaching ~90% confluence, exhibiting a fibroblast-like shape. The doubling time of the cells was 0.9–1.3 days/cell division.

Mouse ES cells (TT2 cells) were cultured at 37°C in DMEM containing 15% FBS, 0.1 mg/mL kanamycin sulfate, 0.1 mM MEM non-essential amino acid solution (NEAA; Gibco), 1 mM sodium pyruvate solution (SP; Gibco), 1000 U/mL leukemia inhibitory factor (Merk), and 100 μM 2β-mercaptoethanol on mitomycin-C-treated mouse embryonic fibroblast feeder cells established from 12.5-day embryos of C57BL/6 mice.

Fluorescence-activated cell sorting

Confluent adipose-MSCs (two to eight passages) were used for cell sorting. Cells were collected by trypsin-EDTA (0.25%) treatment, centrifuged, and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.5% BSA and 2 mM EDTA) [21] at a concentration of 1×10^6 cells/100 μL. Cells were incubated in FACS buffer containing 15% human serum for 20 min. After two successive washes by FACS buffer, cells were incubated with anti-SSEA-3 antibody (1:50; Millipore) for 1 h at 4°C. Cells were then washed three times with FACS buffer, followed by FITC-conjugated anti-rat IgM (1:100; Jackson ImmunoResearch) for 1 h at 4°C. After three consecutive washes in FACS buffer, cells were sorted for SSEA-3(+) and SSEA-3(−) cells (adipose-Muse and -non-Muse cells)

by Special Order Research Products FACSAriaII (Becton Dickinson) using a low stream speed. This ensured a high level of cell survival and the highest purity of the sorted cells, via the four-way purity sorting mode, as previously described [21]. SSEA-3(+)-adipose-Muse cells (labeled with FITC) were analyzed by flow cytometry for the expression of cell surface antigens CD29 [labeled with phycoerythrin (PE)], CD90 (PE), CD105 (Pacific Blue), CD34 (PE), and CD146 (PE) (Becton Dickinson).

Single-cell suspension culture

Adipose-Muse cells were cultured as floating cells using poly-HEMA-coated dishes as previously described [21]. Each single cell was plated in an individual well on 96-well plates after limiting dilution with alpha-MEM medium containing 15% FBS. The actual number of cells deposited in each well was determined by visual inspection using a phase-contrast microscope, and empty wells or wells with more than one cell were excluded from analysis.

Spontaneous differentiation of clusters in vitro

After 7–10 days of single-cell suspension culture, single clusters of adipose-Muse cells were picked up with a glass micropipette and transferred onto a gelatin-coated culture dish or cover glass. After another 7–10 days of incubation, clusters were subjected to immunocytochemistry and reverse-transcription polymerase chain reaction (RT-PCR).

Immunocytochemistry

Immunocytochemistry was performed as previously described [21]. Clusters of adipose-Muse cells were fixed with 4% paraformaldehyde in 0.01 M PBS, embedded in OCT compound, and then cut into 8-μm-thick cryosections. Differentiated cells derived from adipose-Muse cell cluster were grown in gelatin-coated dishes. Cells were fixed using the same fixative described earlier. Antibodies used in this study included Nanog (1:100; Millipore), Oct3/4 (1:100; Santa Cruz Biotechnology), Sox2 (1:1000; Millipore), PAR4 (1:100; Santa Cruz Biotechnology), TRA-1-81 (1:100; Santa Cruz Biotechnology), smooth muscle actin (SMA, 1:100; Lab Vision, Thermo Fisher Scientific), neurofilament-M (1:100; Millipore), cytokeratin 7 (CK7, 1:100; Millipore), alpha-fetoprotein (α-FP, 1:100; DAKO), fatty acid-binding protein 4 (FABP-4, 1:100; R&D Systems), human hepatocyte paraffin-1 (HepPar1, 1:200; Dako), delta-like protein-1 (DLK1, 1:100; Santa Cruz), human albumin (1:100; Bethyl Laboratories), and neuronal class III β-tubulin (Tuj-1, 1:1000; Covance). All primary antibodies were diluted 1:200 in PBS/0.1% BSA solution and incubated overnight at 4°C. Following treatment with primary antibodies, cells were washed three times with PBS and incubated for 1 h at R/T with PBS/0.1% BSA containing secondary immunofluorescent antibodies. These antibodies included FITC-, Alexa-488-, or Alexa-568-labeled conjugated anti-rabbit IgG, anti-mouse IgG, anti-mouse IgM, or anti-rat IgM (1:100; Jackson ImmunoResearch). Nuclei were identified by 4′,6-diamidino-2-phenylindole (DAPI) staining (1:1000; Sigma). Cells were then washed three times with PBS. Images were acquired with a confocal laser scanning microscope (CS-1; Nikon).

RT-PCR

Total RNA was extracted from cells and purified using NucleoSpin RNA XS (Macherey-Nagel). First-strand cDNA was generated using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The PCRs were performed using Ex Taq DNA polymerase using standard temperature cycling conditions (TaKaRa Bio). The primers used were (1) β-actin sense 5′-AGGCGGACTATGACTTAGTTGCGTTACACC-3′ and antisense 5′-AAGTCCTCGGCCACATTGTGAACTTTG-3′, (2) Nkx2.5 sense 5′-GGGACTTGAATGCGGTTCAG-3′ and antisense 5′-CTCCACAGTTGGGTTCATCTGTAA-3′, (3) α-FP sense 5′-CCACTTGTTGCCAACTCAGTGA-3′ and antisense 5′-TGCAGGAGGGACATATGTTTCA-3′; (4) microtubule associated protein-2 (MAP-2) sense 5′-ACTACC AGTTTCACACCCCCTTT-3′ and antisense 5′-AAGGGT GCAGGAGACACAGATAC-3′, and (5) GATA6 sense 5′-CCTGCGGGCTCTACAGCAAGATGAAC-3′ and antisense 5′-CGCCCCTGAGGCTGTAGGTTGTGTT-3′.

Evaluation for cell self-renewal

Cell self-renewal of adipose-Muse cell clusters was performed as previously described [21]. Briefly, adipose-Muse cells isolated by FACS were grown in single-cell suspension after limiting dilution to generate the first-generation cluster. After 7–10 days of single-cell suspension culture, first-generation clusters were transferred onto an adherent culture for expansion. After another 7 days of incubation of first-generation clusters in adherent culture, expanded cells were collected by trypsinization and returned to single-cell suspension culture after limiting dilution to form second-generation cluster. This cycle was repeated up to third-generation clusters. In each generation step, samples were subjected to RT-PCR.

Test for teratoma formation in immune-deficient mice testes

Adipose-Muse cells (1×10^5 cells) were suspended in PBS and injected using glass micropipette into the testes of 8-week-old CB17/Icr-Prkdc scid/CrlCrlj(SCID) mice ($n = 6$). Mice were sacrificed for analysis 6 months after injection. For negative control, testes were injected with PBS ($n = 2$) and, for positive control, 5×10^5 mouse ES cells ($n = 4$) were injected, and were sacrificed 8 weeks after injection. Tissues were fixed with 4% paraformaldehyde in 0.01 M PBS and 3-μm-thick paraffin sections and analyzed by HE staining.

Telomerase activity

Telomerase activity was detected using TRAPEZE XL telomerase detection kit (Millipore) and Ex Taq polymerase (TaKaRa Bio). Fluorescence intensity was measured with a microplate reader (infinite M1000; Tecan) as described by Wakao et al. [17].

In vitro differentiation into adipocytes, hepatocytes, and neuronal cells

Experiments were repeated three times for each differentiation. Adipose-Muse cells and -non-Muse cells were

incubated in adipogenic differentiation medium (R&D Systems) for 14 days. Formation of new adipocytes was detected using the human MSC functional identification kit (R&D Systems) [19]. For hepatocyte induction, adipose-Muse cells (at a density of 2.0×10^4 cells/cm^2) were cultured on collagen-coated dishes for 14 days in DMEM supplemented with 10% FBS, insulin-transferrin-selenium (Gibco), 10 nM dexamethasone (Sigma), 100 ng/mL hepatocyte growth factor (R&D Systems), and 50 ng/mL fibroblast growth factor-4 (R&D Systems) [9]. Neuronal differentiation was performed according to the method reported by Boulland et al. [6]. Briefly, cells were induced by culture in the Neurobasal medium (Invitrogen) containing 1% FCS, 1×B27 supplement, 0.5 mM 1-methyl-3 isobutylxanthine, 1 mM dexamethasone, 0.2 mM 8CPTcAMP, 10 mM valproic acid, and 10 mM forskolin for 7 days. Comparatively, adipose-non-Muse, SSEA-3 (−) cells obtained from the same adipose tissue were used as controls in all these cell differentiation studies.

Quantitative PCR (q-PCR)

Adipose-Muse cells were induced to neuronal differentiation and total RNA was extracted as described previously. Customized primers for Tuj-1 were purchased from SA Biosciences. Total RNA of BM- (Lonza) and dermal-Muse cells (Lonza) and adipose-Muse cells from LA-MSCs was collected using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the RT2First Strand Kit (SA Biosciences). In both experiments, DNA was amplified with the Applied Biosystems 7300 real-time PCR system according to the manufacturer's instructions. Data were processed using the ΔΔCT method [22].

Comparative analysis of gene expression

Total RNA of Muse cells derived from BM (Lonza), normal human dermal fibroblasts (Lonza), and LA-MSCs was extracted and purified using NucleoSpin RNA XS (TaKaRa Bio). The poly-A RNA molecules were further purified from total RNA using poly-T oligo-attached magnetic beads, and then fragmented and converted into cDNA using Illumina TruSeq RNA Sample Prep Kit (Illumina) to make libraries. The quality of libraries was determined with Agilent 2100 Bioanalyzer. The libraries were analyzed by Illumina HiSeq2000 sequencing (Illumina) according to standard procedure. Paired-end 100-bp reads were generated and subjected to data analysis with the use of the platform provided by DNAnexus.

Results

Culture of adipose-MSCs

This study utilized two sources of human adipose-MSCs; five lots of commercially available LA-MSCs that are widely used as adipose-derived MSCs, and four lots of adipose-MSCs established from human subcutaneous adipose tissue, namely, AT-MSCs. For AT-MSCs, volumes, culture duration, and total number of MSCs obtained from the four samples are shown in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/scd). On average, a culture of 15 cm^3 of adipose tissue for 3 weeks yielded $\sim 3 \times 10^7$ adipose-MSCs. There were no significant differences observed in morphology or doubling time between LA-MSCs and AT-MSCs (Supplementary Fig. S1).

Characterization of adipose-Muse cells in LA-MSCs and AT-MSCs

We previously reported the presence of Muse cells in the adult human BM and dermis isolated by cell sorting using SSEA-3, a pluripotent stem cell marker for undifferentiated ES cells [16,17]. FACS analysis revealed the presence of SSEA-3-positive cells in LA-MSCs (3.8% ± 0.9%) and AT-MSCs (8.8 ± 1.3), which are termed as adipose-Muse cells in the following descriptions (Fig. 1A, B and Supplementary Table S1).

Surface marker expression was further analyzed in these adipose-Muse cells. They expressed general mesenchymal markers; all of SSEA-3(+)-adipose-Muse cells expressed CD105 (100%) and CD90 (100%), and in a lesser ratio (60%–70%) with CD29 (Fig. E–H). Adipose tissue is generally known to contain so-called ADSCs that express both CD34 and CD146 markers [23]. Adipose-Muse cells isolated from both LA-MSCs and AT-MSCs were, however, negative for these markers, suggesting that they are a distinct population from ADSCs (Fig. 1I, J).

When adipose-Muse cells were transferred to a single-cell suspension culture, each cell began to proliferate and form a cluster that is similar to the human-ES-cell-derived embryoid body formed in suspension culture at days 7–10 (Fig. 1C, D). On an average, adipose-Muse cells derived from LA-MSCs and AT-MSCs formed clusters in a single-cell suspension at a ratio of 31.3% ± 2.8% and 40.9% ± 6.8%, respectively (Supplementary Table S1). Importantly, none of the SSEA-3 (−) adipose-MSCs, namely, adipose-non-Muse cells, obtained from both LA-MSCs and AT-MSCS formed clusters in a single-cell suspension.

Adipose-Muse cell clusters both from LA-MSCs and AT-MSCs expressed pluripotency markers Nanog, Oct3/4, PAR4, Sox2, and TRA-1-81 and were positive for alkaline phosphatase reaction, one of the indicators of ES cells (Fig. 2). When these single-cell-derived clusters were individually transferred onto gelatin-coated dish and cultured adherently for 10–14 days, cells expanded from the cluster and proliferated. Among the expanded cell population, cells positive for α-FP (endodermal marker), SMA (mesodermal), and neurofilament (ectodermal) were recognized (Fig. 3A–D). Cells expanded from clusters of adipose-Muse cells were collected and analyzed by RT-PCR, and gene expression was detected for *NKX2-5* (mesodermal), *GATA6* (endodermal), *MAP2* (ectodermal), and *α-FP* (endodermal) (Fig. 3E). Expression of these genes further indicated that adipose-Muse cells, from either LA-MSCs or AT-MSCs, may have the ability to spontaneously generate cells representative of all three germ layers from a single cell.

To examine the potential for self-renewal, adipose-Muse cells from LA-MSCs and AT-MSCs were subjected to single-cell suspension culture in order to obtain first-generation clusters. Half of the clusters were transferred individually onto gelatin culture, maintained, and analyzed by RT-PCR for the expression of endodermal, mesodermal, and ectodermal markers, as described previously. The rest of the

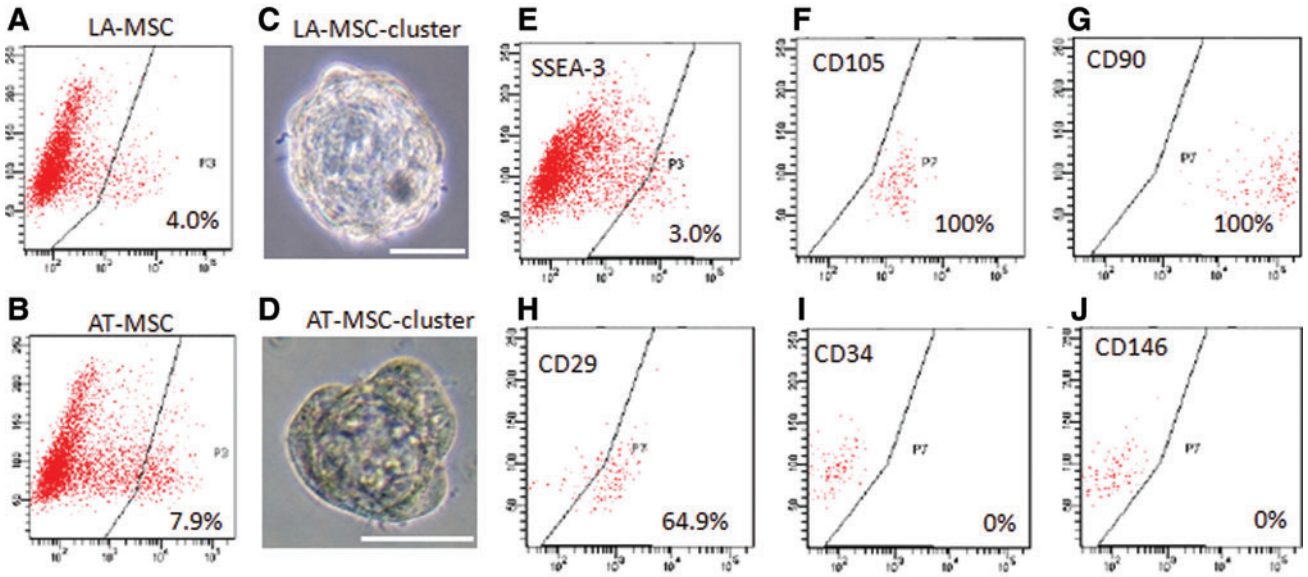

FIG. 1. Characterization of SSEA-3(+) cells in LA-MSCs and AT-MSCs. **(A, B)** An example of SSEA-3(+) cells in LA-MSC **(A)** and AT-MSC **(B)** FACS analysis showed the presence of SSEA-3(+) cells in both populations. **(C, D)** Clusters were formed in single-cell suspension culture from LA-MSC- **(C)** and AT-MSC-SSEA-3(+) cells **(D)**. Scale bars = 50 μm. **(E–J)** Expression of mesenchymal and ADSC markers in LA-MSC-SSEA-3(+) cells. Cells positive for SSEA-3 **(E)** were positive for CD105 **(F)**, CD90 **(G)**, and CD29 **(H)** but were negative for CD34 **(I)** and CD146 **(I)**. AT-MSCs, adipose-MSCs from subcutaneous adipose tissue; LA-MSCs, adipose-MSCs commercially purchased from Lonza. Color images available online at www.liebertpub.com/scd

clusters were individually transferred to adherent culture and allowed to proliferate for 7–10 days, after which they underwent a second round of single-cell suspension in culture to generate second-generation clusters (Fig. 4). This experimental cycle was repeated three times and clusters from each step were analyzed by RT-PCR. Again, gene expression of *MAP2*, *GATA6*, *α-FP*, and *NKX2.5* was detected in first-, second-, and third-generation clusters, demonstrating that adipose-Muse cells maintain self-renewal as well as triploblastic differentiation ability up to the third generation (Fig. 4).

Telomerase activity and in vivo transplantation of adipose-Muse cells

Being a strong indicator of tumorigenicity, telomerase activity was examined in adipose-Muse cells from LA-MSCs and AT-MSCs. High telomerase activity was observed

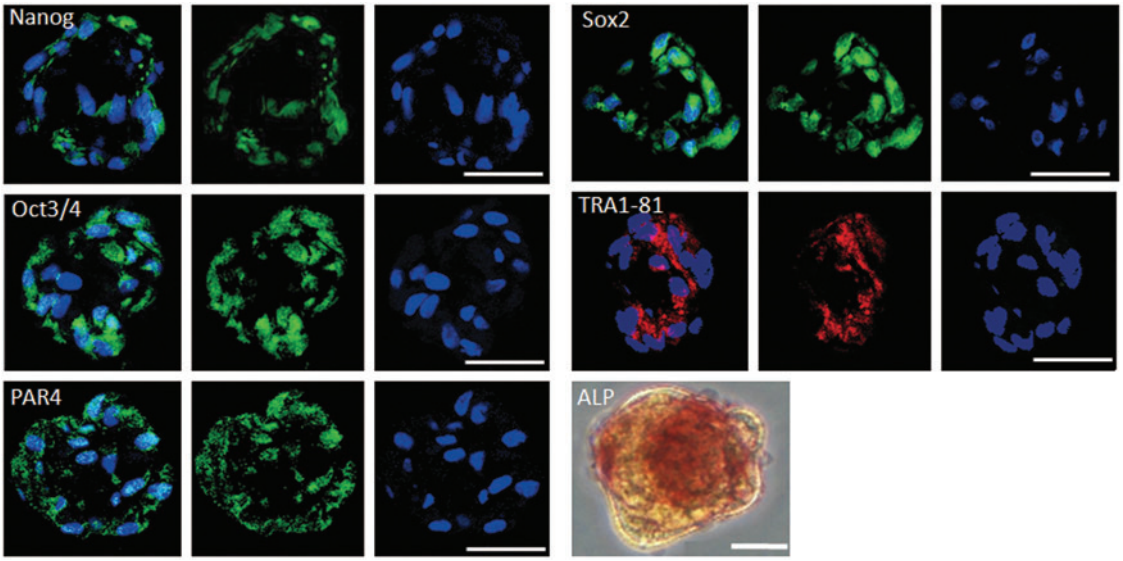

FIG. 2. Immunostaining of clusters formed from adipose-Muse cells in single-cell suspension culture. Clusters were positive for Nanog, Oct3/4, PAR4, Sox2, and TRA1-81, as well as for reactive alkaline phosphatase (ALP). Nanog, Oct3/4, and TRA-1-81 were from AT-MSC-SSEA-3(+) cells and PAR4, Sox2, and ALP from LA-MSC-SSEA-3(+) cells. Scale bars = 25 μm. Color images available online at www.liebertpub.com/scd

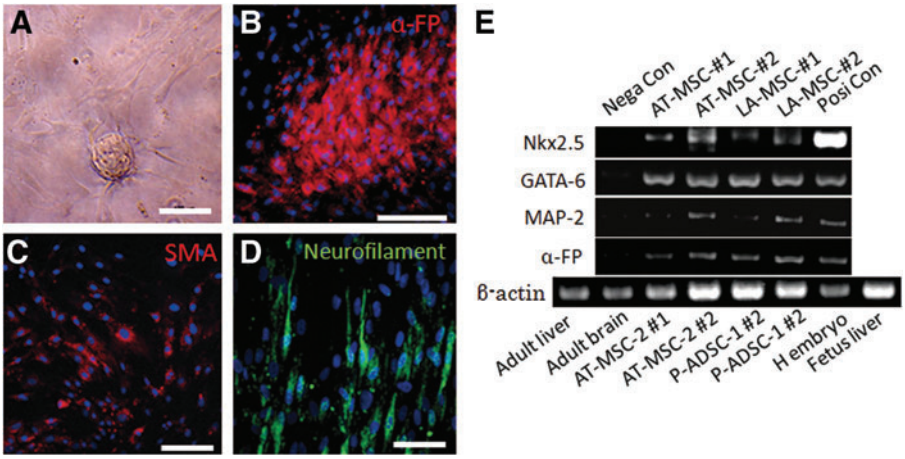

FIG. 3. Differentiation of single-Muse-cell-derived cluster into endodermal, mesodermal, and ectodermal lineages. Clusters formed from adipose-Muse cells of LA-MSCs in single-cell suspension culture were transferred onto gelatin-coated culture **(A)** allowing the cells to expand and differentiate spontaneously, expressing alpha-fetoprotein (α-FP: endodermal) **(B)**, smooth muscle actin (SMA: mesodermal) **(C)**, and neurofilament (ectodermal) **(D)**. Scale bars = 100 μm. RT-PCR analysis of cells expanded from single adipose-Muse cell cluster detected signals for *Nkx2.5* (mesodermal), *GATA-6* (endodermal), *MAP-2* (ectodermal), and *α-FP* (endodermal) in both AT-MSCs and LA-MSCs **(E)**. Positive controls for *Nkx2.5, MAP-2,* and *α-FP* were human whole embryo (H embryo) and for *GATA-4* was human fetus liver (Fetus liver). Negative controls for *Nkx2.5* and *MAP-2* were human adult liver (Adult liver) and for *GATA-6* and *α-FP* were human adult brain (Adult brain). RT-PCR, reverse transcription-polymerase chain reaction. Color images available online at www.liebertpub.com/scd

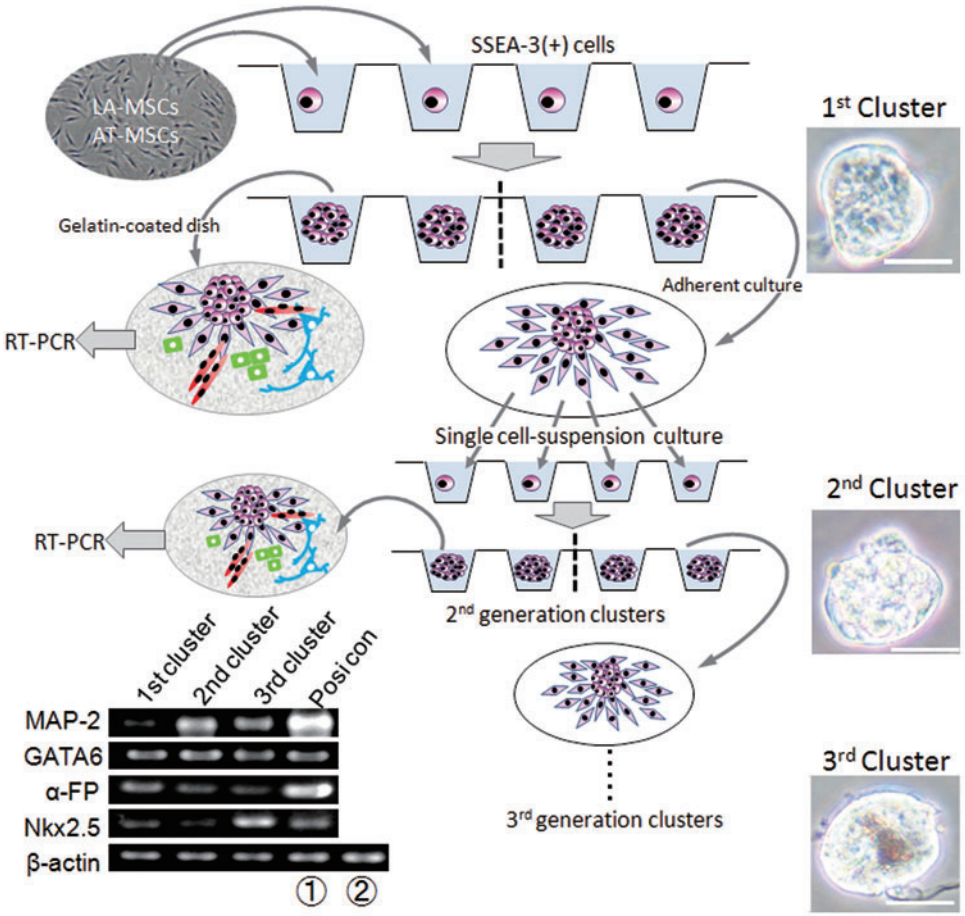

FIG. 4. Adipose-Muse cells demonstrate the capacity for self-renewal. Schematic diagram outlines experiments that validate self-renewal ability of adipose-Muse cells. RT-PCR data are from AT-MSC-derived adipose-Muse cells as an example. *MAP-2* (ectodermal), *GATA-6* (endodermal), *α-FP* (endodermal), and *Nkx2.5* (mesodermal) gene expression was detected in RT-PCR from cells expanded from each of clusters from first to third generations. Adipose-Muse cells from LA-MSCs showed basically same data (not shown). Positive controls for *MAP-2, α-FP,* and *Nkx2.5* were human whole embryo and for *GATA-4* was human fetus liver. 1 and 2 in *β-actin* are from human whole embryo (1) and human fetus liver (2), respectively. Scale bars = 25 μm. Color images available online at www.liebertpub.com/scd

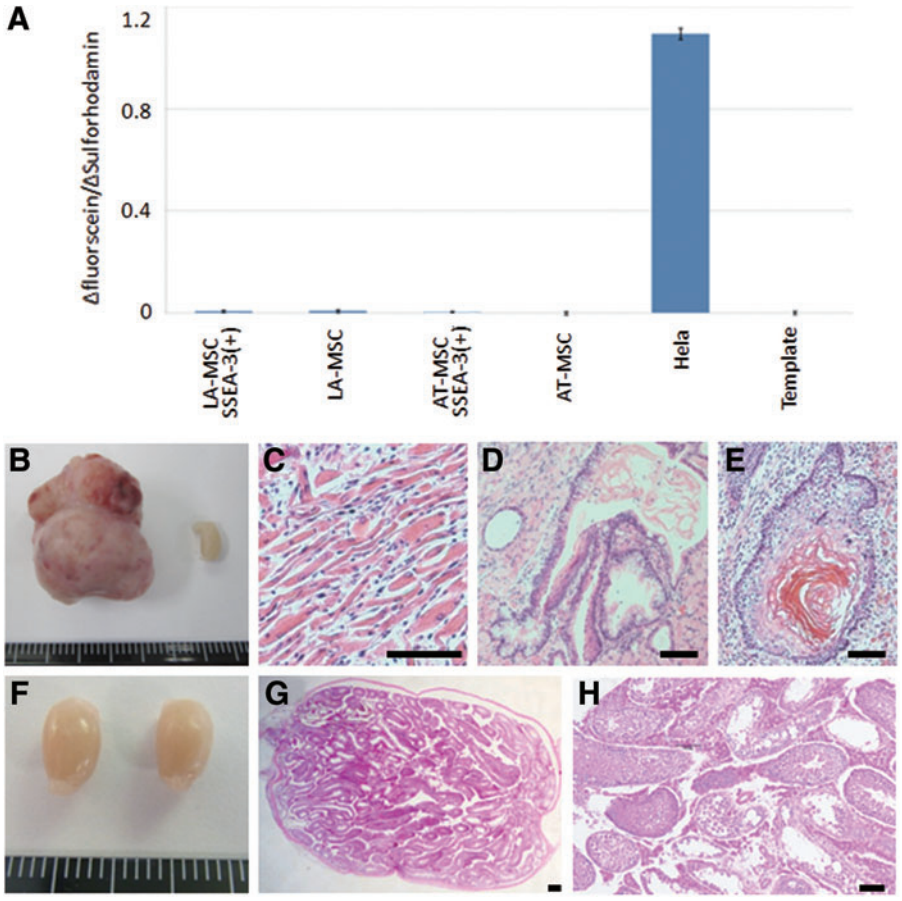

FIG. 5. Nontumorigenicity of adipose-Muse cells. **(A)** Telomerase activity in LA-MSCs, AT-MSCs, as well as adipose-Muse cells [SSEA-3(+)] from both populations. Hela cells (Hela) were used as positive control and template for negative control. **(B–E)** Teratoma formation in mouse testis with mouse ES cell transplantation (8 weeks after) **(B)**. Histological analysis showed that the teratoma contained muscle tissue **(C)**, intestine-like structure **(D)**, and keratinized skin formation **(E). (F–H)** Transplantation of adipose-Muse cells from LA-MSCs into Nog mouse testis did not form teratoma even after 6 months **(F)** and maintained normal testis structure **(G, H)**. Scale bars = 100 μm. Color images available online at www.liebertpub.com/scd

in Hela cells, while adipose-Muse cells both from LA-MSCs and AT-MSCs were at nearly the same reduced level as cells from the original LA-MSC and AT-MSC populations (Fig. 5A).

Next, cell transplantation was performed in the testes of immune-deficient mice. When mouse ES cells were transplanted, large teratomas, consisting of mesodermal, endodermal, and ectodermal tissues, were formed by 8–10 weeks (Fig. 5B–E), while even after 6 months, the adipose-Muse cells transplanted in the mouse testes never formed teratomas. Normal testicular tubes were maintained in these testes (Fig. 5F–H).

All of the characteristics present in adipose-Muse cells, namely, expression both of pluripotency and mesenchymal markers, generation of embryoid-body-like clusters in suspension, triploblastic differentiation from a single cell, self-renewal, and nontumorigenicity, were consistent with previously reported BM- and dermal-Muse cells [16,17,21].

Cytokine induction into endodermal, ectodermal, and mesodermal lineages

Adipose-Muse and -non-Muse cells were treated with cocktails of cytokines and reagents for adipocyte (mesodermal), hepatocyte (endodermal), and neuronal cell (ectodermal) differentiation. In adipocyte induction, both adipose-Muse and -non-Muse cells generated cells with lipid droplets that were positive for Oil Red-O staining. Immunolabeling of FABP-4, however, resulted in 72.4% ± 3.4% of adipose-Muse cells that display positivity, while adipose-non-Muse cells were only 34.4% ± 2.9% positive, suggesting that adipose-Muse cells have higher potential to become adipocytes than adipose-non-Muse cells (Fig. 6A–D).

Hepatocyte induction demonstrated that cells positive for hepatic stem cell marker human DLK1, and hepatocyte markers human HepPar1 and human albumin were induced from adipose-Muse cells but not from adipose-non-Muse cells. The positivity for human albumin in adipose-Muse cells was 13.7% ± 1.6% while it was undetectable in adipose-non-Muse cells (Fig. 6E–H).

Neuronal induction in adipose-Muse cells resulted in generation of cells positive for Tuj-1 with neuron-like morphology. These cells were generated from adipose-Muse cells but not from adipose-non-Muse cells. These results were also confirmed by q-PCR of Tuj-1 expression (Fig. 6I–M).

These results suggest that both adipose-Muse and -non-Muse cells are capable of differentiating into mesodermal lineage cells, such as adipocytes; however, higher efficiency is anticipated in adipose-Muse cells rather than -non-Muse cells. In contrast, differentiation from mesodermal to ectodermal or endodermal lineages was only possible in adipose-Muse cells.

Comparison among BM-, dermal-, and adipose-Muse cells

Muse cells collected as SSEA-3(+) cells from human BM-MSCs (BM-Muse), dermal fibroblasts (dermal-Muse),

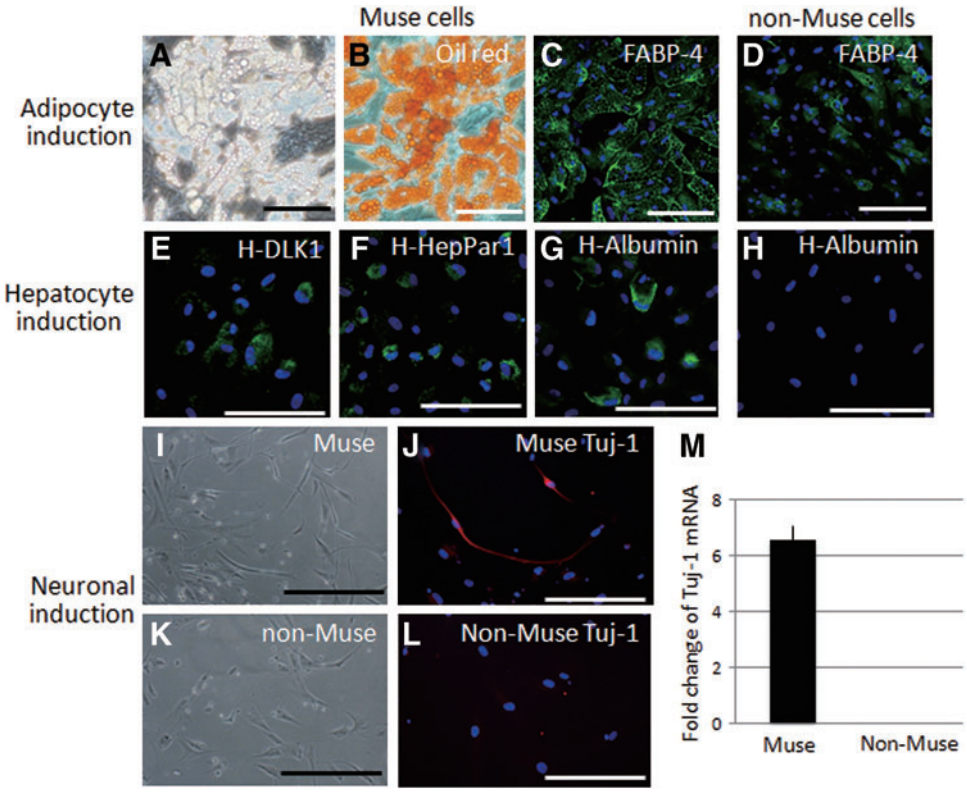

FIG. 6. Induced differentiation of adipose-Muse and -non-Muse cells. **(A–D)** Muse **(A–C)** and non-Muse **(D)** cells from LA-MSCs were subjected to adipocyte induction. Cells with lipid droplets **(A)** that are stained with Oil Red-O **(B)** were detected in adipose-Muse cells. Those cells were also positive for the adipocyte marker FABP-4. Adipose-non-Muse cells also contained cells positive for FABP-4 but with lower ratio **(D). (E–H)** After hepatocyte induction, Muse cells were positive for liver stem cell marker human-DLK1 **(E)** and hepatocyte markers human HepPar1 **(F)** and human albumin **(G)**, while non-Muse cells lacked these expression. An example of non-Muse cells was shown in human albumin expression **(H). (I–M)** After neuronal induction, Muse cells demonstrated a morphology similar to neuronal cells **(I)**, and some were also positive for Tuj-1 **(J)**. However, adipose-non-Muse cells were not like neuronal cells **(K)** and all cells were Tuj-1 negative **(L)**. Q-PCR consistently detected *Tuj-1* signal only in Muse cells and not in non-Muse cells **(M)**. Scale bars = 100 μm. Color images available online at www.liebertpub.com/scd

and LA-MSCs (adipose-Muse) were subjected to next generation sequencing to compare expression levels of genes related to endodermal, mesodermal, and ectodermal differentiation (Table 1). Analysis of mesodermal lineage expression revealed that osteogenic, adipogenic, and myogenic genes were generally higher in adipose-Muse rather than BM-Muse or dermal-Muse cells, and some of the factors, such as SP7, osteogenic factor, and Pax7 muscle stem cell marker, were only detected in adipose-Muse cells. Different from mesodermal factors, endodermal factors were more predominantly expressed in BM-Muse cells than in adipose-Muse cells. However, cholesterol 7, alpha-hydroxylase (CYP7A1), insulin gene enhancer binding protein (ISL1), and hepatocyte nuclear factor 4 alpha (HNF4A) were only expressed in adipose-Muse cells and not in BM-Muse or dermal-Muse cells (Table 1). Ectodermal genes that relate to neuronal differentiation were higher in both BM-Muse and dermal-Muse cells than in adipose-Muse cells while factors such as genes encoding for musashi RNA-binding protein 1 (MSI1), ISL1, and myelin transcription factor 1-like (MYT1L) were not expressed in BM-Muse or dermal-Muse cells, but only in adipose-Muse cells (Table 1).

Discussion

In this study, we demonstrate that both adult human subcutaneous adipose tissue and commercially available adipose-MSCs contain a small percentage of stem cells with the capacity for triploblastic differentiation and self-renewal. These cells do not undergo tumorigenic proliferation in vitro, nor do they elicit teratomas when transplanted in vivo. These characteristics match those of previously reported Muse cells that were isolated from the BM and dermis [16,17,21], indicating that adipose tissue also contains Muse cells.

In adipose tissue, single-cell-derived cluster formation in suspension was unique to adipose-Muse cells; however, cluster formation ratio did not always reach 100% (Supplementary Table S1). This may be in part because of cellular damage caused by laser irradiation in the process of FACS isolation. Alternatively, cells might have been in an inactive dormant state, such that they did not proliferate. This property of Muse cells requires further study.

While MSCs are known to provide trophic and anti-inflammatory effects, these effects are temporary and do not

Table 1. Comparison of Gene Expression Related to the Differentiation of Endodermal, Mesodermal, and Ectodermal Lineages Among Adipose-Muse Cells Versus Dermal- and BM-Muse Cells

Mesodermal		
	Adipose / Dermal	Adipose / BM
PPARG	1.8939	3.0752
CEBPA	1.6335	0.8092
CEBPB	1.4336	1.0564
CEBPD	0.8261	0.5665
KLF15	4.2002	7.8730
LEP	0.8650	0.7210
ADIPOQ	2.3432	1.2264
AP2B1	0.7541	0.9276
FOXO1	1.9200	1.4321
SLC2A4	1.2509	1.0284
RUNX2	0.4624	0.7576
FOS	0.7956	0.3422
JUN	0.6616	0.4266
STAT1	0.7229	0.7139
SMAD1	1.0395	1.3637
SP7	Adipose only	Adipose only
ALPL	10.6316	1.5629
PAX3	0.0563	Adipose only
PAX7	Adipose only	Adipose only
MEF2C	0.4435	1.2908
TBX5	0.2465	Adipose only
KDR	2.8002	Adipose only
CXCR4	ND	BM only
NKX2-5	Dermal only	ND

Endodermal		
	Adipose / Dermal	Adipose / BM
AFP	2.0655	0.5843
ALB	ND	BM only
CD44	1.0116	1.3584
CDH1	5.3242	1.3244
CDH2	5.8358	1.4388
CTNNA1	0.9106	0.7770
CTNNB1	0.8035	0.7119
CXCR4	ND	BM only
CYP7A1	Adipose only	Adipose only
FN1	0.9322	0.8965
HNF1A	1.3105	0.9468
HNF1B	1.3378	0.4668
HNF4A	Adipose only	0.3549
HTATSF1	0.9685	1.0462
ISL1	Adipose only	0.2334
ITGA6	0.1835	0.4860
ITGB1	0.7992	0.8514
KRT7	0.7876	0.9359
NRP2	1.0134	1.0528
OTX1	0.2428	2.8003
SYP	1.0983	0.7001
THY1	0.7876	0.9118
TTR	Dermal only	ND
GATA6	4.3770	1.3704

Ectodermal		
	Adipose / Dermal	Adipose / BM
SOX2	0.2190	0.4993
NEUROG2	ND	BM only
HES1	1.8092	0.2084
HES5	0.3344	0.2169
ASCL1	Dermal only	ND
ZNF521	1.2807	13.9544
NES	0.3800	4.6525
MSI1	Adipose only	0.7347
OLIG2	ND	BM only
ISL1	Adipose only	0.2334
ISL2	0.1484	0.1060
GFAP	0.8829	Adipose only
POU3F2	1.0385	3.6332
MYT1L	Adipose only	Adipose only
NR4A2	0.5149	0.0536
DLX1	1.9342	0.2784
DLX2	6.9419	0.4366
MAP2	0.3737	0.5471
TP63	0.1497	0.0956
CRABP2	0.1967	1.2002
FN1	0.9322	0.8965
NOTCH1	0.8426	1.3712
NGFR	0.2069	0.3905
S100B	Dermal only	BM only

Expression level in adipose-Muse cells that is higher than that in dermal- or BM-Muse cells is indicated by red, whereas lower is indicated by blue colors.

BM, bone marrow.

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directly contribute to cell replacement or tissue regeneration [24,25]. In the true sense of functional recovery, replenishment of functional cells is essential; however, the major consensus of the primary efficacy of adipose-MSC transplantation is also attributed to trophic effects [25]. This could be explained, in part, by the small percentage of adipose-Muse cells within adipose-MSC population. However, if the ratio of adipose-Muse cells could be increased, then there may be an improvement in the curative effect of adipose-MSC transplantation. Recently, Muse cells derived from adipose tissue were reported to have been efficiently enriched from human lipoaspirated fat by long-term incubation with collagenase. Such a simple approach would be an extremely practical strategy to increase the overall yield of Muse cells for stem cell therapy [26].

BM, dermis, and adipose tissue are representative mesenchymal sources for cell-based therapy because of their easy accessibility and versatility. Even though core properties of Muse cells, namely, triploblastic differentiation, self-renewal, nontumorigenicity, and surface marker expression, are the same among those three sources, Muse cells are not the same in their gene expression that relate to endodermal-, mesodermal-, and ectodermal-lineage differentiation. Adipose-Muse cells exhibited the tendency toward expressing mesodermal lineage genes more highly than BM- and dermal-Muse cells. Conversely, genes related to endodermal and ectodermal lineages were lower in adipose-Muse cells than in those two sources. Therefore, the source for Muse cells should be selected in accordance with target tissues.

Our data show that expression of human peroxisome proliferator-activated receptor gamma (PPARγ), a gene of mesodermal lineage, in adipose-Muse cells exceeds that of dermal- and BM-Muse cells. Considering that PPARγ expression is highly sensitive to the host environment, it may play a role in the unique adipose-Muse cell response to highly stressful conditions [27]. Other mesodermal genes that are elevated in adipose-Muse cells include Krüppel-like factor 15 (KLF15) and adiponectin (ADIPOQ), which encode prominent factors in adipocyte function, further supporting the preferential adipose-Muse cell differentiation to adipocytes [26].

Genes that are downregulated in adipose-Muse cells include FOS and JUN, genes that function paradoxically in both oncogenesis and tumor suppression depending on the cell type and its differentiation state and tumor stage [28]. CDH1, which encodes Cadherin-1, was expressed more highly in adipose-Muse cells than in BM- and dermal-Muse cells. Low expression of Cadherin-1 can support tumor progression, which may allude to the absence of tumorigenesis in CDH1-rich adipose-Muse cells [29]. Further, alpha-6 integrin (ITGA6) that plays a role in mammary tumorigenesis is decreased in adipose-Muse cells as compared with dermal- and BM-Muse cells [30]. Together with low telomerase activity and nontumorigenicity, this gene expression pattern may support the safety of adipose-Muse

cells for autologous transplantation. The uniqueness in the nontumorigenicity of Muse cells has been reported previously [16,17]. Gene analysis has shown that Muse cells exhibit extremely low expression of Lin28 [17], a gene that plays a pivotal role in both maintaining pluripotency and tumorigenesis that prevail in ES and iPS cells [31]. While Muse cells retain their pluripotent capacity in the absence of a Lin28 influence [17], they reap the benefits in their insusceptibility to tumor formation. Lin28 is likely only one of the many genes that simultaneously play a role in pluripotency as well as tumorigenesis, as these two prominent aspects of stem cell character have been repeatedly described to go hand-in-hand [26,31].

It is of significance to note that ectodermal genes are primarily downregulated in adipose-Muse cells as compared with dermal- and BM-Muse cells. Important genes in neural stem cell differentiation, including HES5 and achaete-scute homolog 1 (ASCL1), are poorly expressed in adipose-Muse cells. This may indicate, perhaps, a decrease in susceptibility to neural differentiation as compared with dermal- and BM-Muse cells. Interestingly, POU domain, class 3, transcription factor 2 (POU3F2), a gene that is imperative to differentiation of pluripotent stem cells into neural cells [32], is increased in adipose-Muse cells, which suggests a capacity of adipose-Muse cells to, if not a susceptibility to, neuronal differentiation.

There exists a cohort of genes that are present only in adipose-Muse cells, and not in dermal- and BM-Muse cells. Along the mesodermal lineage, adipose-Muse cells express SP7, which encodes transcription factor Sp7, and PAX7, which encodes paired box protein Pax7. SP7 regulates osteogenic differentiation [33] and the presence of SP7 in adipose-Muse cells but not in dermal- and BM-Muse cells suggests the high capacity of adipose-Muse cells to form bone cells. It has been shown that PAX7 plays a critical role in stem cell commitment to the myosatellite cell fate, which represents a niche for a population of quiescent stem cells that have the capacity for the regeneration of muscle tissue [34,35]. PAX7 could therefore be a critical factor in the maintenance of adipose-Muse cells to remain in quiescence [26]. Along the endodermal lineage, adipose-Muse cells express CYP7A1, which encodes cholesterol 7 alpha-hydroxylase and plays a major role in maintaining hepatocyte function [36]. This supports adipose-Muse cell aptitude for differentiation into functional hepatocytes. Along the ectodermal lineage, adipose-Muse cells express MYT1L, which encodes myelin transcription factor 1-like, and thus also support the possibly of differentiation into myelin-forming cells, such as oligodendrocytes [37].

Recently, a rare population of Lin−/CD75+/CD90− pluripotent stem cells was isolated from normal human breast tissue. Similar to adipose-Muse cells, this cell population has low telomerase activity [38]. While Lin−/CD75+/CD90− cells have low tumorigenicity, adipose-Muse cells have nontumorigenic activity. This difference may be attributable in part to the expression of CD90 in adipose-Muse cells. CD90, also known as THY1, is a classical marker for mesenchymal stem cells. The role of CD90 in promoting or suppressing tumorigenesis is still controversial likely depending on the tissue target analyzed [39,40].

Several reports have indicated the presence of a population of very small cells termed very small embryonic-like stem cells (VSELs) in BM or in circulation, which like Muse cells have been described to have pluripotent potential [41]. However, other labs have failed to replicate this data, with only one lab demonstrating that VSELs could differentiate to lung epithelium [42,43]. While Muse cells do not share morphologic or molecular markers with VSELs, the current controversy in VSELs regarding the reproducibility emphasizes the importance of having both simple and reproducible protocols as an essential aspects for the utilization of cells.

Since AT-MSCs exhibited a higher concentration of Muse cells and higher propensity for cluster formation than in LA-MSCs, Muse cells can be obtained from adipose tissue rather than commercially available adipose-MSCs. Based on our results, $\sim 15\,cm^3$ human adipose tissue (eg, $4 \times 9.5\,cm^2$ subcutaneous adipose tissue) yields $\sim 3 \times 10^7$ MSCs by week 3, which contain nearly $\sim 3 \times 10^6$ of adipose-Muse cells (corresponding to nearly 9% of total adipose-MSCs; see Supplementary Fig. S1 and Table 1). Granted that one million Muse cells are required for one-time treatment, the same volume of $\sim 15\,cm^3$ adipose tissue for 1 week culturing is estimated to be necessary. Interestingly, from 1 to 2 mL of BM, $\sim 3 \times 10^7$ MSCs can be obtained after 3 weeks that contain $\sim 0.3 \times 10^6$ BM-Muse cells ($\sim$1% of BM-MSCs). From these calculations, adipose-Muse cells can be considered a realistic cell source for regenerative medicine as with BM-Muse cells. Cell safety is the most important issue for the treatment of human disorders. Adipose-Muse cells do not require additional gene transfer or artificial modifications. They are naturally preexisting stem cells in adult human adipose tissue that account for a small percentage of adipose-MSCs, which have already been applied in clinical studies. Both the capacity for differentiation and lack of teratoma formation make adipose-Muse cells an attractive source for use in the clinical setting. However, there are still several hurdles that must be overcome on the way to making these cells a viable clinical resource, beyond what is already observed in adipose stem cells utilized in the clinical setting. Therefore, future experiments must include rigorous in vivo studies that explore the functional capability and nontumorigenicity of transplanted adipose-Muse cells, as well as further evidence of consistent and predictable controlled differentiation for various directed lineages.

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Author Disclosure Statement

All authors state that they have no competing financial interests.

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