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

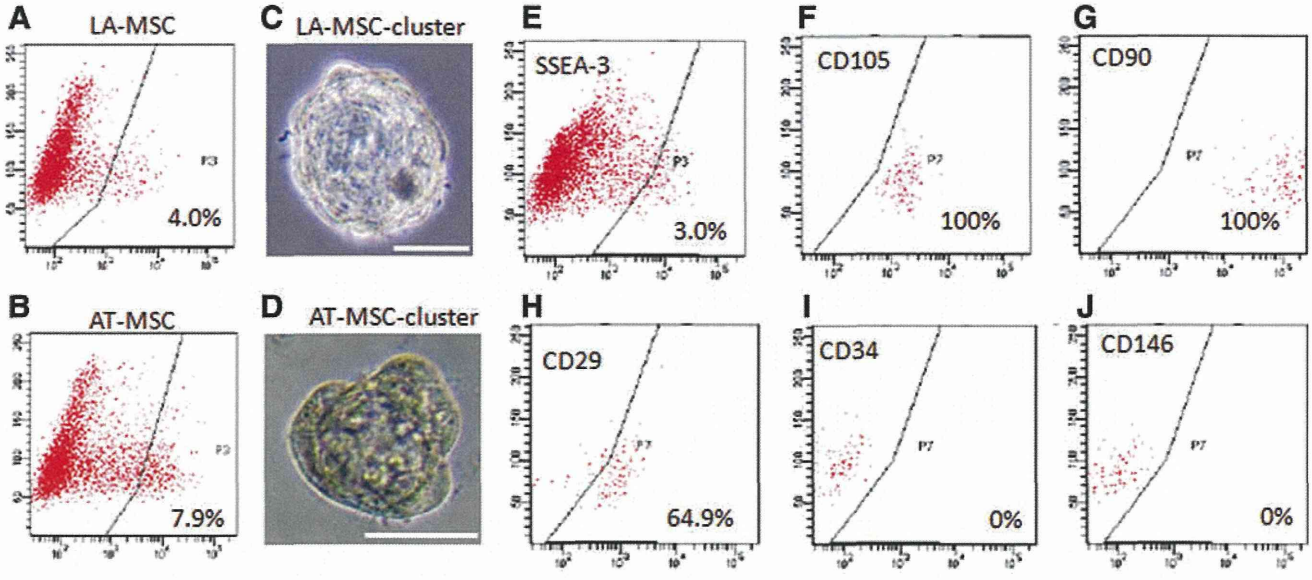

FIG. 1. Characterization of SSEA-3(+) cells in LA-MSCs and AT-MSCs. **(A, B)** An example of SSEA-3(+) cells in LA-MSC **(A)** and AT-MSC **(B)** FACS analysis showed the presence of SSEA-3(+) cells in both populations. **(C, D)** Clusters were formed in single-cell suspension culture from LA-MSC- **(C)** 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

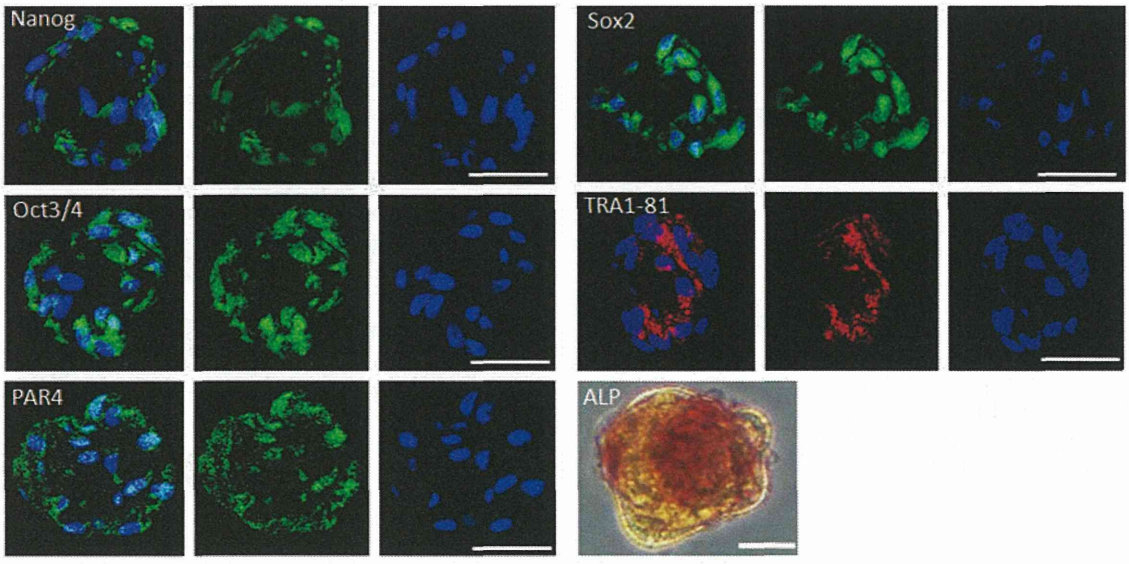

FIG. 2. Immunostaining of clusters formed from adipose-Muse cells in single-cell suspension culture. Clusters were positive for Nanog, Oct3/4, PAR4, Sox2, and TRA1-81, as well as for reactive alkaline phosphatase (ALP). Nanog, Oct3/4, and TRA-1-81 were from AT-MSC-SSEA-3(+) cells and PAR4, Sox2, and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

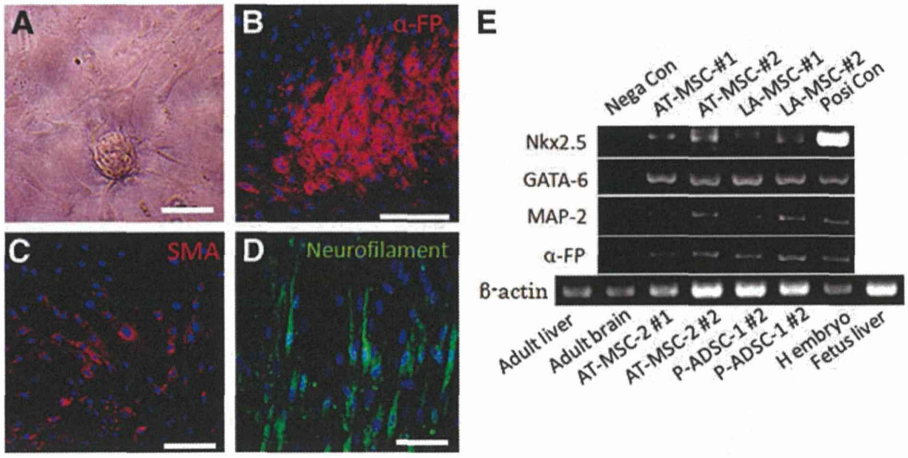

FIG. 3. Differentiation of single-Muse-cell-derived cluster into endodermal, mesodermal, and ectodermal lineages. Clusters formed from adipose-Muse cells of LA-MSCs in single-cell suspension culture were transferred onto gelatin-coated culture **(A)** allowing the cells to expand and differentiate spontaneously, 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

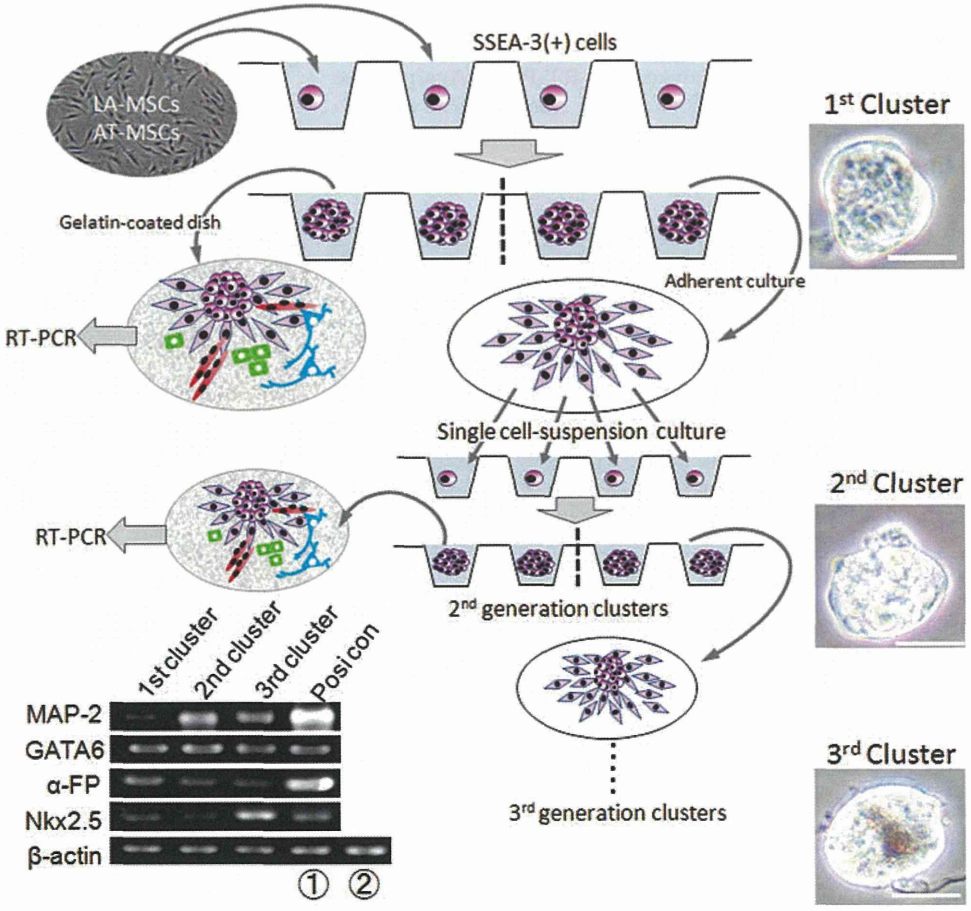

FIG. 4. Adipose-Muse cells demonstrate the capacity for self-renewal. Schematic diagram outlines experiments that validate self-renewal ability of adipose-Muse cells. RT-PCR data are from AT-MSC-derived adipose-Muse cells as an example. *MAP-2* (ectodermal), *GATA-6* (endodermal), *α-FP* (endodermal), 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

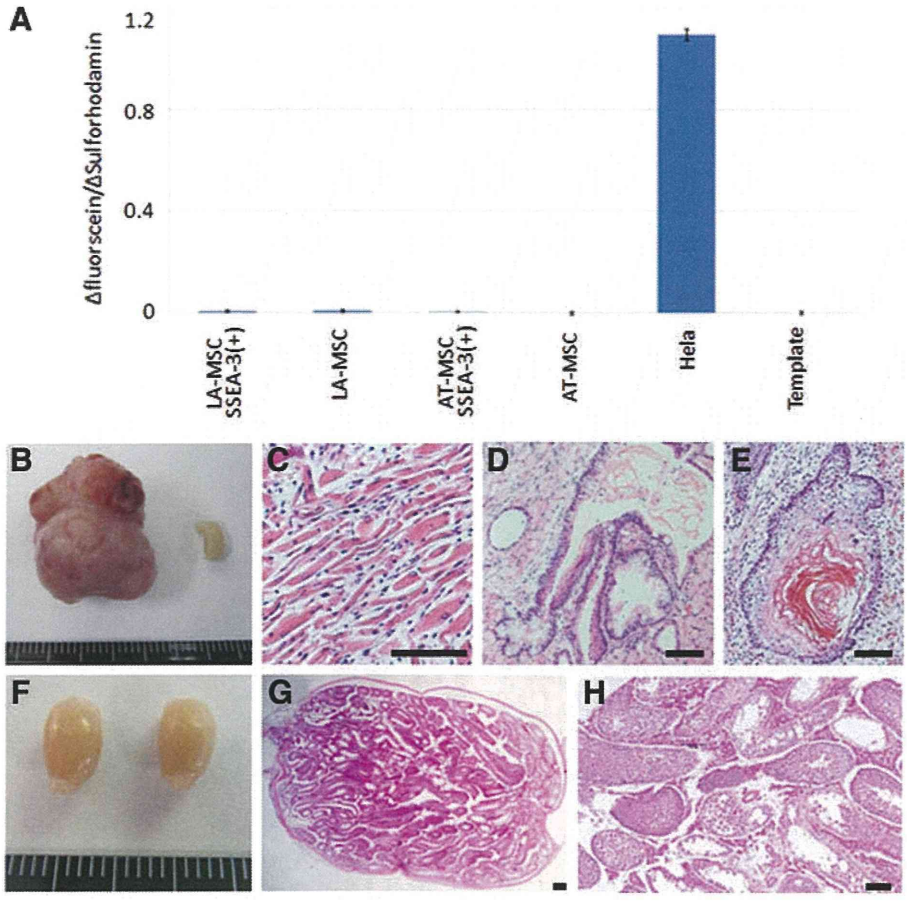

FIG. 5. Nontumorigenicity of adipose-Muse cells. **(A)** Telomerase activity in LA-MSCs, AT-MSCs, as well as adipose-Muse cells [SSEA-3(+)] from both populations. Hela cells (Hela) were used as positive control and template for negative control. **(B–E)** Teratoma formation in mouse testis with mouse 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),

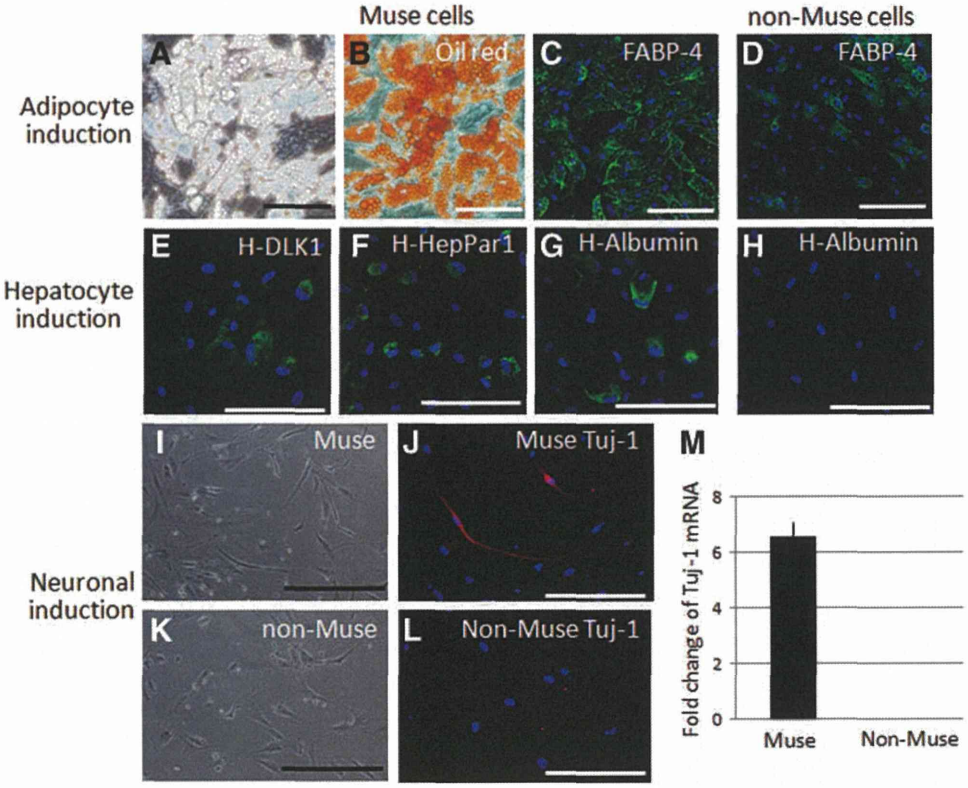

FIG. 6. Induced differentiation of adipose-Muse and -non-Muse cells. **(A–D)** Muse **(A–C)** and non-Muse **(D)** cells from LA-MSCs were subjected to adipocyte induction. Cells with lipid droplets **(A)** that are stained with Oil Red-O **(B)** were detected in adipose-Muse cells. Those cells were 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.
Color images available online at www.liebertpub.com/scd

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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Isolation, culture and evaluation of multilineage-differentiating stress-enduring (Muse) cells

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Multilineage-differentiating stress-enduring (Muse) cells are distinct stem cells in mesenchymal cell populations with the capacity to self-renew, to differentiate into cells representative of all three germ layers from a single cell, and to repair damaged tissues by spontaneous differentiation into tissue-specific cells without forming teratomas. We describe step-by-step procedures for isolating and evaluating these cells. Muse cells are also a practical cell source for human induced pluripotent stem (iPS) cells with markedly high generation efficiency. They can be collected as cells that are double positive for stage-specific embryonic antigen-3 (SSEA-3) and CD105 from commercially available mesenchymal cells, such as adult human bone marrow stromal cells and dermal fibroblasts, or from fresh adult human bone marrow samples. Under both spontaneous and induced differentiation conditions, they show triploblastic differentiation. It takes 4–6 h to collect and 2 weeks to confirm the differentiation and self-renewal capacity of Muse cells.

INTRODUCTION

Mesenchymal stem cells (MSCs) are tissue stem cells that reside in mesenchymal tissues and that have attracted attention because of their unique properties. Generally, tissue stem cells are known to differentiate into the cell types of the tissue in which they reside; neural stem cells differentiate into neurons and glial cells, and hematopoietic stem cells give rise to all blood cell types[1,2]. Distinct from these tissue stem cells, MSCs have been reported to have the broad-ranged differentiation ability that crosses the oligolineage boundaries between the mesodermal and ectodermal or endodermal lineages[3,4]. In particular, the differentiation ability of bone marrow stromal cells (BMSCs), which are cultured as adherent cells from bone marrow aspirates, has been studied from the late 1990s up to the present time. BMSCs *in vitro* have been reported to give rise to cells not only in the same mesodermal lineage (osteocytes, chondrocytes, adipocytes[5,6], skeletal muscle cells[7–9], cardiac muscle cells[10,11] and endothelial cells[12,13]) but also in ectodermal (neuronal cells[14,15], Schwann cells[16,17]) and endodermal (hepatocytes[18,19], insulin-producing pancreatic beta cells[20,21]) lineages. In addition, when they are grafted into animals with tissue damage, BMSCs have been shown to spontaneously differentiate into and function as tissue-specific cells that replenish lost cells *in vivo*; therefore, BMSCs are presumed to act as the 'repairing cells'. This *in vivo* spontaneous differentiation has been demonstrated in skeletal muscle cells[22], cardiac muscle cells[23], neuronal cells[24], retinal cells[25], keratinocytes[26] and hepatocytes[27] after grafting, but as the efficiency of integration and differentiation is generally low, it is believed that only a particular subset of BMSCs can lead to such reparative effects. The similar differentiation abilities of mesenchymal cell populations have also been reported in adipose tissue[28], synovial tissue[29], dental pulp[30], umbilical cord[31] and dermis[32].

The above findings suggested that MSCs include a particular subset of stem cells that have the triploblastic differentiation ability that can give rise to various types of cells of all three germ layers and that can function as tissue-repairing cells *in vivo*. However, the 'stemness' of such cells has not been fully elucidated. The term 'MSCs' is generally used to represent cells that are collected from mesenchymal tissues by adherent culture, and therefore they are not composed of a single cell type but of crude heterogeneous cell populations. Classification as stem cells requires the presence of two fundamental properties: differentiation and self-renewal[33]. The triploblastic differentiation ability of MSCs mentioned above has largely been demonstrated by studies using crude heterogeneous MSCs, whose self-renewal property has not clearly been demonstrated[34,35]. Although there are some reports demonstrating the self-renewal property of a particular stem cell population of the MSCs, the differentiation ability presented in these studies was not triploblastic[36,37]. For these reasons, it has long been debated whether a specific stem cell population that shows both triploblastic differentiation and self-renewal capacity exists among MSCs[4,38].

Properties of Muse cells

Recently, we used a clonal analyis to identify a specific cell population that has a capacity for self-renewal and triploblastic differentiation similar to that of pluripotent stem cells in adult human mesenchymal cell populations[39]. We refer to this specific stem cell fraction as Muse cells because of their particular broad-ranged differentiation ability and stress tolerance. They can be collected as cells that are double-positive for the pluripotency marker SSEA-3 and the mesenchymal cell marker CD105 from commercially available mesenchymal cell populations, such as adult human BMSCs and dermal fibroblasts, or they can be collected from the mononuclear cell fraction of fresh adult human bone marrow aspirates. These cells express other pluripotency markers and have the property of self-renewal. A single Muse cell is able to give rise to not only mesodermal-lineage cells such as osteocytes, chondrocytes, adipocytes, smooth muscle cells and skeletal muscle cells but also ectodermal and endodermal cells such as neurons, epidermal cells, hepatocytes and biliary cells. Although these capacities of Muse cells are considered similar to those of other pluripotent stem cells, it is notable that, in contrast to embryonic stem (ES) cells or iPS cells, Muse cells are not immortal in culture or tumorigenic *in vivo*.