

# Distinct populations of adipogenic and myogenic Myf5-lineage progenitors in white adipose tissues<sup>S</sup>

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**Abstract** Brown adipose tissues (BAT) are derived from a myogenic factor 5 (Myf5)-expressing cell lineage and white adipose tissues (WAT) predominantly arise from non-Myf5 lineages, although a subpopulation of adipocytes in some WAT depots can be derived from the Myf5 lineage. However, the functional implication of the Myf5- and non-Myf5-lineage cells in WAT is unclear. We found that the Myf5-lineage constitution in subcutaneous WAT depots is negatively correlated to the expression of classical BAT and newly defined beige/brite adipocyte-specific genes. Consistently, fluorescent-activated cell sorting (FACS)-purified Myf5-lineage adipo-progenitors give rise to adipocytes expressing lower levels of BAT-specific *Ucp1*, *Prdm16*, *Cidea*, and *Pparg1a* genes and beige adipocyte-specific *CD137*, *Tmem26*, and *Tbx1* genes compared with the non-Myf5-lineage adipocytes from the same depots. Ablation of the Myf5-lineage progenitors in WAT stromal vascular cell (SVC) cultures leads to increased expression of BAT and beige cell signature genes. Strikingly, the Myf5-lineage cells in WAT are heterogeneous and contain distinct adipogenic [stem cell antigen 1 (Sca1)-positive] and myogenic (Sca1-negative) progenitors. The latter differentiate robustly into myofibers in vitro and in vivo, and they restore dystrophin expression after transplantation into mdx mouse, a model for Duchenne muscular dystrophy. These results demonstrate the heterogeneity and functional differences of the Myf5- and non-Myf5-lineage cells in the white adipose tissue.—Shan, T., X. Liang, P. Bi, P. Zhang, W. Liu, and S. Kuang. **Distinct populations of adipogenic and myogenic Myf5-lineage progenitors in white adipose tissues.** *J. Lipid Res.* 2013. 54: 2214–2224.

**Supplementary key words** brown adipose tissue • lineage tracing • progenitor cell • Cre/LoxP • diabetes • regeneration

Adipose tissues play important roles in energy metabolism and life span of mammals. In mice, adipocytes can be broadly divided into white adipocytes, brown adipocytes, and beige (brite) adipocytes (1, 2). A white adipocyte contains a single, large lipid droplet that stores triglycerides as

chemical energy; by contrast, a brown adipocyte contains multiple small lipid droplets and numerous mitochondria that metabolize triglycerides through  $\beta$ -oxidation and oxidative respiration to generate heat to defend against hypothermia and obesity (3). The mitochondrial inner membranes of brown adipocytes uniquely express the uncoupling protein 1 (UCP1) that dissipates the proton gradient produced by the electron transfer chain, thus generating heat instead of ATP (3). A beige adipocyte is an adaptive thermogenic adipocyte found within white adipose tissues (WAT) induced by cold exposure (4, 5) and hormonal stimulation (6, 7). A number of key gene regulatory factors have been shown to induce browning of white adipocytes (8–11). Like the brown adipocytes, beige adipocytes express UCP1 and respond to cyclic AMP stimulation. However, beige adipocytes are distinct from the white and brown adipocytes, and they can be identified by their unique expression of several markers, including CD137, transmembrane protein 26 (Tmem26), and T-box 1 (Tbx1) (12).

The myogenic factor 5 (Myf5) is a gene expressed during embryonic myogenesis and one of the core transcriptional factors involved in muscle development (13, 14). Genetic-lineage tracing indicates that the classical brown adipocytes and skeletal muscle are derived from Myf5-expressing progenitors, while the white and beige adipocytes are predominately from the non-Myf5-lineage progenitors

Abbreviations: asWAT, anterior subcutaneous WAT; BAT, brown adipose tissue; bFGF, basic fibroblast growth factor; Cadh15, cadherin 15; Cav3, caveolin 3; Cidea, cell death-inducing DFFA-like effector a; DAPI, 4',6-diamidino-2-phenylindole; Des, desmin; DEXA, dexamethasone; DMEM, defined minimal essential medium; DT, diphtheria toxin; DTR, DT receptor; eMHC, embryonic myosin heavy chain; eWAT, epididymal WAT; FACS, fluorescent-activated cell sorting; IBMX, 3-isobutyl-methylxanthine; ingWAT, inguinal WAT; Myf5, myogenic factor 5; Myog, myogenin; PGC1 $\alpha$ , peroxisome proliferator-activated receptor coactivator 1 $\alpha$ ; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; Prdm16, PR domain containing 16; qPCR, quantitative real-time PCR; RFP, red fluorescent protein; Sca1, stem cell antigen 1; SAT, subcutaneous adipose tissue; SVF, stromal-vascular fraction; T3, triiodothyronine; TA, tibialis anterior; Tbx1, T-box 1; Tmem26, transmembrane protein 26; UCP1, uncoupling protein 1; WAT, white adipose tissue; WT, wild-type.

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(15). However, a recent study by Sanchez-Gurmaches and colleagues provides evidence that a subset of white adipocytes is derived from the Myf5-lineage mesenchymal progenitors (16). The relative contribution of Myf5-lineage cells to WAT appears to vary among different WAT depots (16). Interestingly, Pax3 (an upstream regulator of Myf5 during myogenesis) lineage cells also contribute to a subset of white adipocytes in different depots (17). Due to the Myf5-lineage origin of brown adipocytes, it is plausible to hypothesize that the Myf5-lineage progenitors are more likely to give rise to the adaptive beige adipocytes. However, until now the functional implication of Myf5-lineage and non-Myf5-lineage adipocytes has not been explored.

It has been widely accepted that WAT contains an abundant and accessible source of adult stem cells with multiple differentiation potentials (18–20). Cells from the stromal vascular fraction (SVF) of WAT can differentiate into adipocytes, myocytes, osteocytes, chondrocytes, cardiomyocytes, hepatocytes, epithelial cells, endothelial cells, and even neuron-like cells (20–24). It has been shown that adipose tissue-derived progenitors have myogenic potential *in vitro* and *in vivo* (25, 26). Our previous study further demonstrates that the myogenic progenitors are enriched in the non-aP2-lineage population of SVF cells in WAT (27). A longstanding question is whether the multilineage potential of WAT SVF cells results from a multipotent mesenchymal stem cell population or from separate subpopulations of unipotent progenitors. If the latter is true, what are the unique markers for each subpopulation of progenitor cells?

In this study, we used cell-lineage labeling, cell ablation, fluorescence-activated cell sorting (FACS), and cell transplantation to demonstrate the phenotype and function of the Myf5-lineage and non-Myf5-lineage progenitors in various depots of subcutaneous adipose tissue (SAT), a tissue that has tremendous plasticity in cold-induced browning. We showed that the Myf5-lineage SVF cells contain subpopulations of adipogenic and myogenic progenitors that can be prospectively isolated based on Sca1 expression. We found that the Myf5-lineage adipocytes expressed lower levels of brown and beige adipocyte markers than the non-Myf5-lineage adipocytes within the same SAT depots. We further analyzed the Myf5-lineage myogenic progenitors of the SAT SVF cells and found that the Myf5-lineage progenitors are the only population that has the myogenic differentiation potential. The FACS-purified, Myf5-lineage myogenic progenitors are capable of restoring dystrophin expression in mdx mice and form exclusively slow fibers after transplantation into wild-type (WT) recipient mice. These results provide new insights into the roles of Myf5-lineage cells in white adipose tissue and suggest that Myf5-lineage cells in the BAT and WAT are functionally distinct.

## MATERIAL AND METHODS

### Animals

All procedures involving mice were performed in accordance with Purdue University's Animal Care and Use Committee. Mice were housed in the animal facility with free access to standard rodent chow and water. All mice were from Jackson Laboratory

(Bar Harbor, ME) under these stock numbers: Myf5-Cre (stock #007893), Rosa26-iDTR (stock #007900), Rosa26-tdTomato (stock #007905), and mdx mice (stock #001801). The PCR genotyping was done using protocols described by the supplier.

### Adipose SVF cell isolation and culture

The white adipose SVF cells were isolated using collagenase digestion followed by density separation (17, 27). Briefly, the anterior subcutaneous WAT (asWAT), inguinal WAT (ingWAT), and epididymal WAT (eWAT) were collected and minced into 2–5 mm<sup>2</sup> pieces. The WAT pieces were then digested in 1.5 mg/ml collagenase at 37°C for 1.5–2 h. The digestions were terminated with DMEM containing 10% fetal bovine serum (FBS), and then filtered through 100 µm filters to remove connective tissues and undigested trunks of tissues. Cells were then centrifuged at 450 *g* for 5 min to separate the SVF cells in the sediment and the lipid-containing adipocytes in the floating layer. The freshly isolated SVF cells from the WAT were seeded and cultured in growth medium containing DMEM, 20% FBS, 1% penicillin/streptomycin (P/S) at 37°C with 5% CO<sub>2</sub> for three days, followed by feeding with fresh medium every two days. For adipogenic differentiation, the cells were induced with induction medium containing DMEM, 10% FBS, 2.85 µM insulin, 0.3 µM dexamethasone (DEXA), and 0.63 mM 3-isobutyl-methylxanthine (IBMX) for three days upon confluence, and then differentiated in differentiation medium containing DMEM, 10% FBS, 200 nM insulin, and 10 nM T3 for four days until adipocytes matured. For myogenic differentiation, the cells were induced with DMEM, 2% horse serum, 1% P/S for six days upon confluence. To ablate Myf5-lineage cells in culture, the SVF cells from WAT and BAT of the Myf5-Cre/Rosa26-iDTR mice were treated with diphtheria toxin (DT, 200 ng/ml) for 48 h. To avoid the effect of cell density on adipogenic or myogenic differentiation, the control and DT-treated cells were induced to differentiate when they reached 90% confluence.

### Muscle myoblast isolation and culture

Myoblast cells were isolated using type I collagenase and dispase B digestion (28). Briefly, the skeletal muscles near the asWAT from the Myf5-Cre/Rosa26-tdTomato mice were collected, minced, and digested. The digestions were stopped with F-10 Ham's medium containing 20% FBS, and then the cells were filtered through 70 µm and centrifuged at 450 *g* for 5 min. The pelleted cells were seeded on collagen-coated dishes and cultured in growth medium containing F-10 Ham's medium with 20% FBS, 4 ng/ml basic fibroblast growth factor (bFGF), and 1% P/S at 37°C with 5% CO<sub>2</sub>. The medium was refreshed every two days. Cells were trypsinized with 0.25% trypsin under close monitoring only to lift off myoblasts (but not fibroblasts) during passages. Enriched myoblasts were used for transplantation (5 × 10<sup>4</sup> cells per muscle).

### FACS

SVF cells were isolated from SAT tissues of Myf5-Cre/Rosa26-tdTomato mice as described above. The red fluorescent protein (RFP)-positive (tdTomato<sup>+</sup>) and RFP-negative (tdTomato<sup>-</sup>) SVF cells represent Myf5-lineage and non-Myf5-lineage cells, respectively. Freshly isolated SVF cells were labeled with lineage markers CD45, CD31 TER-119, and CD11b (Lin) conjugated with PE-Cy7, and stem cell antigen 1 (Sca1) conjugated with FITC, as described (27). All antibodies were purchased from eBioscience. After staining, SVF cells were isolated and filtered through 30 µm filter before sorting. Nonlabeled SVF cells from wild-type (WT) mice were used as negative control for gating purposes. From the Lin-negative cells, we sorted four populations (RFP<sup>+</sup>Sca1<sup>+</sup>, RFP<sup>+</sup>Sca1<sup>-</sup>, RFP<sup>-</sup>Sca1<sup>+</sup>, and RFP<sup>-</sup>Sca1<sup>-</sup>) of SVF cells based on RFP expression and Sca1

(FITC) signal. Sorted cells were cultured and differentiated to examine their adipogenic and myogenic potential.

### Total RNA extraction, cDNA synthesis, and real-time PCR

Total RNA extraction, cDNA synthesis and real-time PCR were performed as described (17, 27). Briefly, total RNA was extracted from cells using Trizol Reagent according to the manufacturer's instructions. RNA was treated with RNase-free DNase I to remove contaminating genomic DNA. The purity and concentration of total RNA were measured by a spectrophotometer (Nanodrop 3000, Thermo Fisher) at 260 nm and 280 nm. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. Then 5  $\mu$ g of total RNA were reverse transcribed using random primers and MMLV- reverse transcriptase. Real-time PCR was carried out in a Roche Lightcycler 480 PCR System with SYBR Green Master Mix and gene-specific primers. Primer sequences are from published papers (12, 17, 27). Ct value of 18S rRNA was used as internal control and  $2^{-\Delta\Delta Ct}$  method was used to analyze the relative expression levels of various genes.

### Protein extraction and Western blot analysis

The protein extraction and Western blot were conducted as previously described (27). Briefly, total protein was isolated from cells using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. Protein concentrations were determined using Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA), and incubated with the primary antibodies overnight. The Myog and GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and MF20 was from the Developmental Studies Hybridoma Bank (Developmental Studies Hybridoma Bank, Iowa City, IA). The secondary antibody (anti-rabbit IgG or anti-mouse IgG, Santa Cruz Biotechnology) was diluted 8,000-fold. Immunodetection was performed using enhanced chemiluminescence (ECL) Western blotting substrate (Pierce Biotechnology, Rockford, IL) and detected with a Gel Logic 2200 imaging system (Carestream).

### Immunostaining and image acquisition

Immunostaining was performed as previously described (27). Cells or tissue sections were fixed with 4% PFA, and the fixed cells or sections were blocked with blocking buffer containing 5% goat serum, 2% BSA, 0.2% triton X-100, and 0.1% sodium azide in PBS for 1.5 h. Then the samples were incubated with primary antibodies diluted in blocking buffer overnight. After washing with PBS, the samples were incubated with secondary antibodies and DAPI for 45 min at room temperature. Fluorescent images were captured using a Leica DM 6000B fluorescent microscope.

### Transplantation

The SVF cells and the FACS-purified Sca1<sup>-</sup>RFP<sup>+</sup> cells from asWAT of Myf5-Cre/Rosa26-tdTomato mice were mixed with 1 ng/ml bFGF and injected into tibialis anterior (TA) muscles of 6- to 8-week-old WT or mdx mice. After 5 or 21 days, the recipient mice were euthanized, and the TA muscles were collected and examined for RFP expression using a fluorescent microscope and staining. The myoblasts from the skeletal muscles near the asWAT were also transplanted into WT mice as the control.

### Data analysis

All experimental data are presented as means  $\pm$  SEM. Comparisons were made by unpaired two-tailed Student *t*-test or

one-way ANOVA, as appropriate. Effects were considered significant at  $P < 0.05$ .

## RESULTS

### Myf5 lineage contributes to both mature adipocytes and immature SVF cells

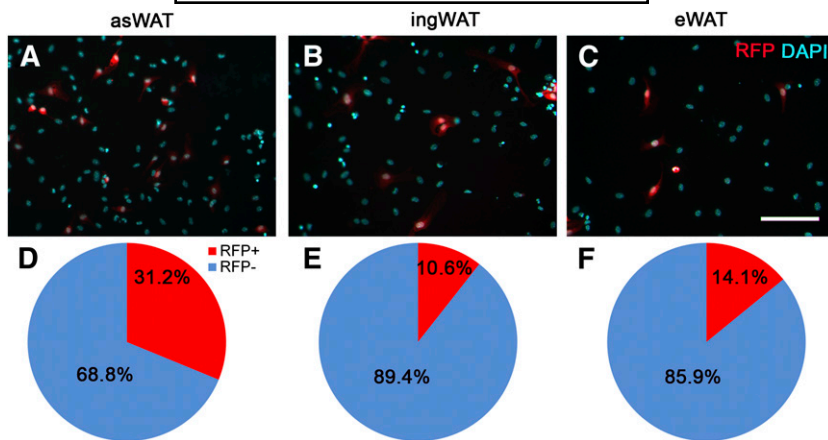
To investigate the progeny of Myf5-lineage cells in various tissues, we conducted lineage-tracing experiments using Myf5-Cre driver and Rosa26-tdTomato reporter mice (29, 30), in which Myf5-lineage cells were labeled by tdTomato, an improved RFP. Consistent with previous reports (15), the vast majority of cells in muscle and BAT cross-sections were RFP-positive (supplementary Fig. 1, A, B), suggesting that the Myf5 lineage contributes to these tissues. This observation also demonstrates the effectiveness of our lineage tracing model. RFP-positive cells were also detected in the heart, brain, and spleen, but not in the liver, lung, or kidney (supplementary Fig. 1, C–H). Importantly, RFP-positive cells can be detected in depots of the asWAT, ingWAT, and eWAT (supplementary Fig. 1, I–K). Costaining with adipocyte marker aP2 confirmed that the RFP-positive cells in these adipose depots are bona fide adipocytes (supplementary Fig. 1, L–N). These results demonstrate that a subpopulation of adipocytes in various WAT depots is derived from the Myf5-lineage progenitors.

To further characterize whether the Myf5 lineage gives rise to adipose progenitors in WAT, we isolated the SVF cells and found RFP-positive cells in the SVF cells (Fig. 1A–C). Quantitative analysis indicated that there are about 31, 11, and 14% RFP-positive cells in the SVF of asWAT, ingWAT, and eWAT, respectively (Fig. 1D–F). To examine whether at least some of the RFP-positive SVF cells are adipogenic progenitors, we first examined the expression of Sca1, an established marker for adipose stem cells (31, 32). FACS analysis (refer to Fig. 3 for details) indicated that 13, 4, and 4% of freshly isolated Lin-negative SVF cells in asWAT, ingWAT, and eWAT were double-positive for RFP<sup>+</sup>Sca1<sup>+</sup>, respectively (supplementary Fig. 2). We next induced the SVF cells to undergo adipogenic differentiation. After induction and differentiation, about 45% and 6% of mature adipocytes were RFP-positive in the asWAT (Fig. 2A, B) and ingWAT (Fig. 2C, D), indicating that Myf5-lineage SVF cells have the adipogenic differentiation potential in vitro. Together, these results suggest that a subpopulation of WAT adipocytes and SVF cells are derived from Myf5-lineage precursors.

### Myf5-lineage adipocytes express lower levels of BAT and beige markers in subcutaneous WAT

Given that BAT adipocytes are derived from the Myf5 lineage (15), we hypothesized that Myf5-lineage adipocytes in WAT should express higher levels of BAT and beige signature genes. To test this hypothesis, we first examined whether the relative abundance of Myf5-lineage adipocytes in different WAT depots affects the expression of BAT signature genes, including *Ucp1*, *Prdm16*, *Cidea*, and *Ppargc1a*. Contrary to what we expected, we found that



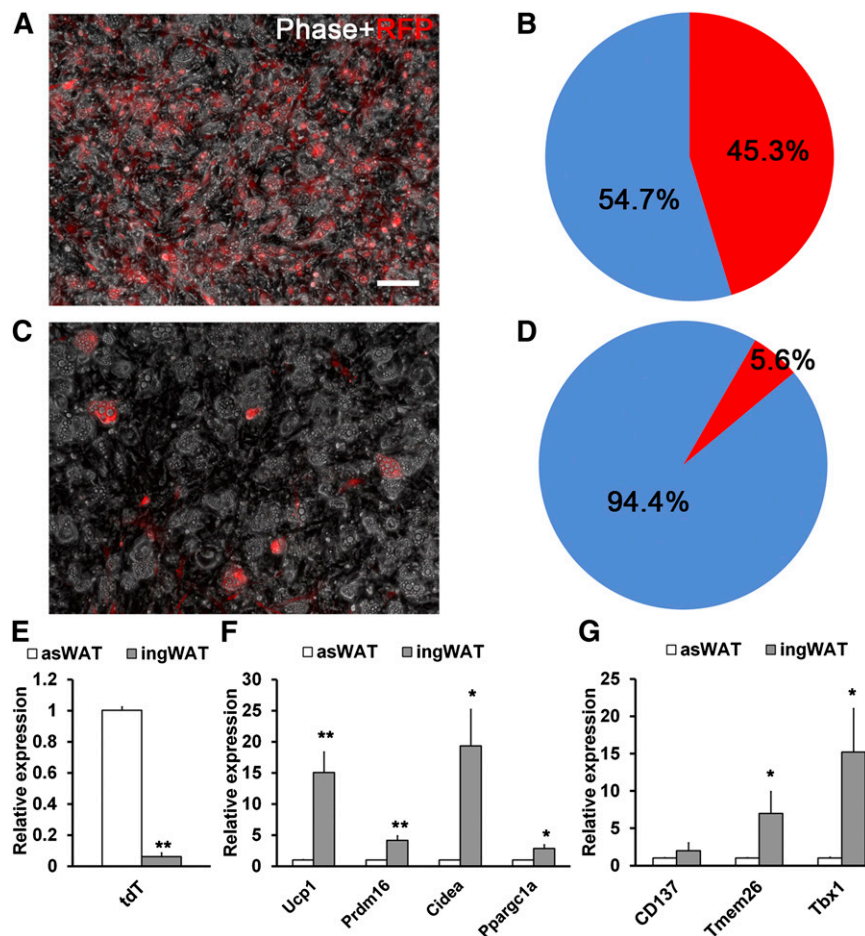


**Fig. 1.** Myf5-lineage tracing in white adipose SVF cells. (A–C) RFP expresses in SVF cells from asWAT (A), ingWAT (B), and eWAT (C). RFP (red); DAPI (blue). (D–F) Percentage of RFP-positive cells in SVF from asWAT (D), ingWAT (E), and eWAT (F). Scale bars: 100  $\mu$ m.

the abundance of RFP-positive adipocytes in the asWAT and ingWAT was inversely correlated to the mRNA levels of the BAT marker genes (Fig. 2E, F). Likewise, the expression level of beige adipocyte markers *Tmem26* and *Tbx1*

was inversely correlated to the abundance of RFP-positive adipocytes in the asWAT and ingWAT (Fig. 2G).

The inverse correlation between the abundance of Myf5-lineage adipocytes and BAT/beige gene expression



**Fig. 2.** The number of Myf5-lineage adipocytes correlates negatively to BAT and beige marker genes expression in various SAT depots. (A, C) Microscopic image of the asWAT and ingWAT SVF after differentiation. (B, D) RFP-positive and RFP-negative adipocyte quantification of the asWAT and ingWAT SVF after differentiation. (E–G) qPCR analysis of tdTomato (E), brown adipocyte markers (*Ucp1*, *Prdm16*, *Cidea*, and *Ppargc1a*) (F), and beige cell markers (*CD137*, *Tmem26*, *Tbx1*) (G) in the asWAT and ingWAT. Error bars represent SEM, n = 4. \* $P < 0.05$ , \*\* $P < 0.01$ . Scale bars: 100  $\mu$ m.

in different WAT depots may be due to depot-specific differences other than Myf5-lineage contribution. To address this possibility, we examined whether Myf5-lineage and non-Myf5-lineage adipocytes within the same WAT depot expressed different levels of BAT/beige marker genes. We purified four populations from the asWAT by FACS (RFP<sup>+</sup>Sca1<sup>+</sup>, RFP<sup>+</sup>Sca1<sup>-</sup>, RFP<sup>-</sup>Sca1<sup>+</sup>, and RFP<sup>-</sup>Sca1<sup>-</sup>; all Lin<sup>-</sup>) of SVF cells (Fig. 3A, B). We then induced them to undergo adipogenic differentiation and examined the expression of BAT/beige markers in the differentiated adipocytes. Only Sca1-positive cells had adipogenic potential, and the RFP<sup>-</sup>Sca1<sup>+</sup> and RFP<sup>+</sup>Sca1<sup>+</sup> cells had similar adipogenic potency (Fig. 3C, D), as is also evident from their identical expression levels of *Adipoq* and *Leptin* (Refer to Fig. 3G). Interestingly, the RFP<sup>+</sup>Sca1<sup>-</sup> cells gave rise to numerous myotubes after differentiation under adipogenic conditions (Fig. 3F), whereas the RFP<sup>-</sup>Sca1<sup>-</sup> cells failed to form adipocytes or myotubes (Fig. 3E). Consistent with our earlier observations, the RFP<sup>+</sup>Sca1<sup>+</sup> SVF gave rise to adipocytes expressing significantly lower levels of BAT marker genes *Ucp1*, *Prdm16*, *Cidea*, and *Ppargc1a* compared with the RFP<sup>-</sup>Sca1<sup>+</sup> descendant adipocytes (Fig. 3G). Additionally, the RFP<sup>+</sup>Sca1<sup>-</sup> descendant adipocytes expressed significantly lower levels of beige marker genes *CD137*, *Tmem26*, and *Tbx1* compared with the RFP<sup>-</sup>Sca1<sup>-</sup> derived adipocytes (Fig. 3H). Similar BAT/beige marker expression patterns were observed in Myf5-lineage and non-Myf5-lineage adipocytes derived from ingWAT (supplementary Fig. III, A–D). Together, these data provide compelling evidence that the Myf5-lineage adipocytes are less brown than the non-Myf5-lineage adipocytes within the same WAT depot.

To further confirm these results, we established the Myf5-Cre/Rosa26-iDTR mouse model (17, 33). In this model, Myf5-Cre induces the expression of DT receptor (DTR), which is normally not expressed by murine cells, and renders the Myf5-lineage cells sensitive to DT. Thus, DT treatment should selectively ablate all Myf5-lineage cells but not the non-Myf5-lineage cells. SVF cells cultured from Myf5-Cre/Rosa26-iDTR mice were treated with DT to ablate the Myf5-lineage cells, then grown to confluence and induced to undergo adipogenic differentiation. Ablation of Myf5-lineage SVF cells did not affect the accumulation of lipids and the expression of the mature adipocyte markers *Adipoq* and *Leptin*, but it significantly upregulated the mRNA levels of BAT markers *Ucp1*, *Prdm16*, *Cidea*, *Ppargc1a*, and *Ppara* (Fig. 3I). The beige cell markers *Tmem26* and *Tbx1* were also increased significantly after ablation of Myf5-lineage SVF cells (Fig. 3J). Taken together, the inter-depot comparison, intradepot analysis of Myf5-lineage and non-Myf5-lineage adipocytes and lineage ablation studies demonstrate that Myf5-lineage adipocytes express lower levels of brown and beige adipocyte markers than the non-Myf5-lineage adipocytes.

#### Myf5-lineage progenitors are necessary for the myogenic potential of SVF cells

Adipose-derived stem cells have the potential to differentiate into multiple lineages, including adipogenic, chondrogenic, and myogenic differentiations (23). Our FACS

analysis demonstrated that the adipogenic and myogenic activities were not shared by a common stem cell population but rather resided in distinct progenitor cell populations (Fig. 3C–F). We attempted to further examine the lineage origin of myogenic progenitors in WAT depots. We isolated SVF cells from the asWAT of Myf5-Cre/Rosa26-tdTomato mice and induced them to undergo myogenic differentiation. More than 95% of the differentiated myotubes were RFP-positive (Fig. 4A, B), indicating that they have been predominantly derived from the Myf5-lineage cells.

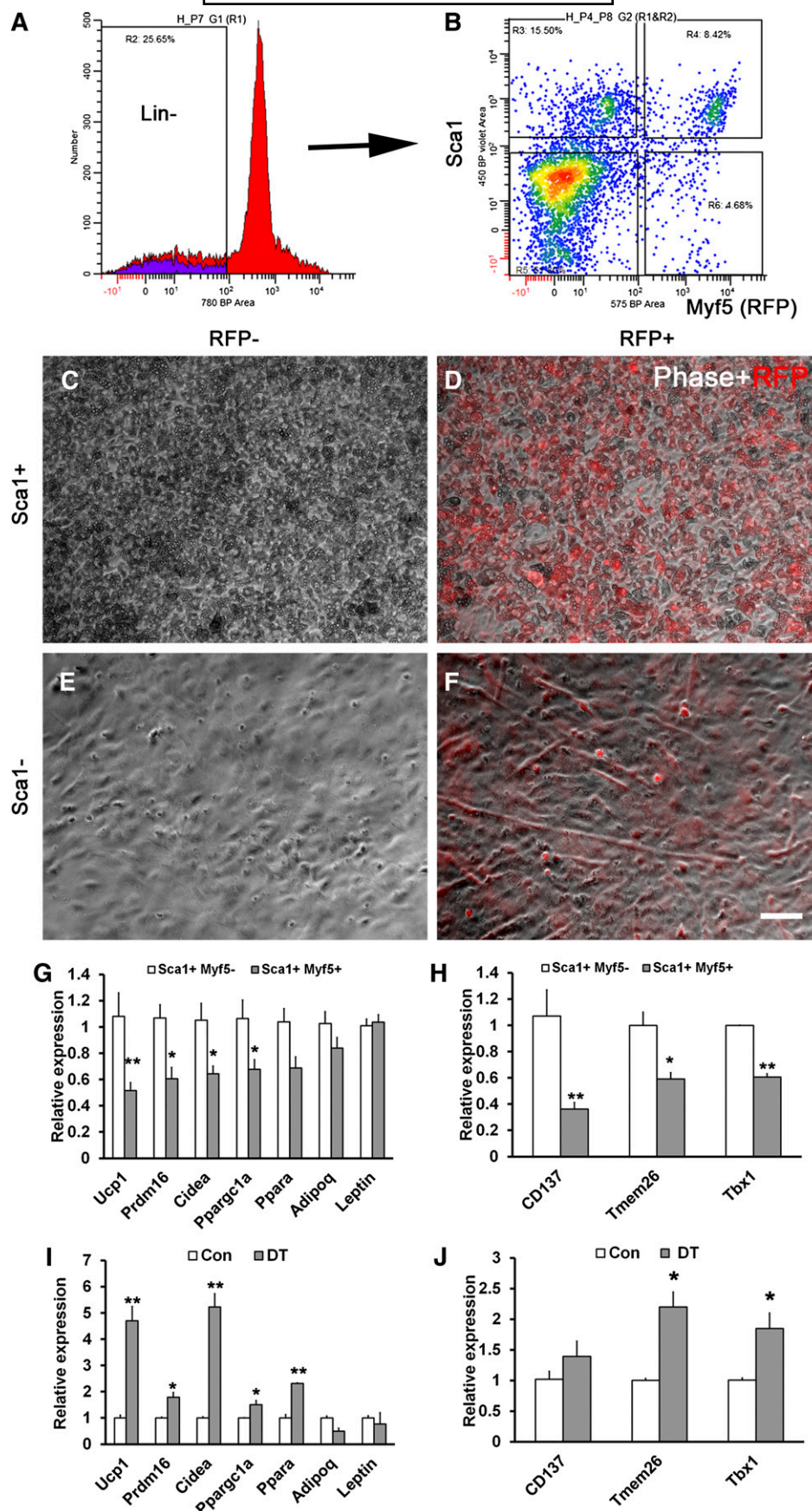
To verify whether the Myf5-lineage cells were necessary for the myogenic potential of WAT SVF cells, we conducted lineage ablation. SVF cells cultured from asWAT of Myf5-Cre/Rosa26-iDTR mice were treated with DT to ablate the Myf5-lineage cells, then grown to confluence and induced to undergo myogenic differentiation. Ablation of Myf5-lineage SVF cells nearly abolished the emergence of myotubes (Fig. 4C, D). In the control groups, numerous myosin heavy-chain-expressing (MF20-positive) myotubes were readily detectable after differentiation (Fig. 4E). By contrast, DT treatment nearly eliminated the MF20-positive myotubes (Fig. 4F). The mRNA levels of myogenic markers, such as *Myod*, *Myog*, *eMHC*, *Myf5*, *Myf6*, *Des*, *Cav3*, and *Cadh15* were also significantly decreased after DT treatment (Fig. 4G). Western blot confirmed that the protein levels of sarcomeric myosin heavy chain and Myog were almost decreased to undetectable levels after DT treatment (Fig. 4H). Similar results were observed in ingWAT SVF cells after the Myf5-lineage ablation (supplementary Fig. IV, A–C). These results indicate that Myf5-lineage cells are essential for the myogenic differentiation of the WAT SVF cells.

We further confirmed these results by FACS analysis. When RFP<sup>+</sup>Sca1<sup>+</sup>, RFP<sup>+</sup>Sca1<sup>-</sup>, RFP<sup>-</sup>Sca1<sup>+</sup>, and RFP<sup>-</sup>Sca1<sup>-</sup> cells were grown to confluence, only the RFP<sup>+</sup>Sca1<sup>-</sup> cells spontaneously formed small myotubes (Fig. 5A–D). Six days after serum withdrawal induced differentiation, long myotubes were readily detectable in the RFP<sup>+</sup>Sca1<sup>-</sup> fraction, with a few short myotubes in the RFP<sup>+</sup>Sca1<sup>+</sup> fraction and no myotubes in the two other fractions (Fig. 5E–H). Consistently, the expression levels of myogenic genes *Myod*, *Myog*, and *eMHC* were more than 100 times higher in the RFP<sup>+</sup>Sca1<sup>-</sup> fraction than in the RFP<sup>-</sup>Sca1<sup>+</sup> fraction (Fig. 5I). Together, these results demonstrate that the myogenic differentiation potential of the WAT SVF cells resides in the Myf5-lineage progenitors.

#### Myf5-lineage SVF cells efficiently differentiate into muscles in vivo after transplantation

To examine the myogenic potential of Myf5-lineage SVF cells in vivo, we first transplanted the RFP<sup>+</sup>Sca1<sup>-</sup> SVF cells from the asWAT of Myf5-Cre/Rosa26-tdTomato mice to cardiotoxin (CTX)-injured TA muscle of WT mice. Five days after transplantation, we detected RFP-positive myofibers in the recipient mice (Fig. 6A–C). Moreover, cultured Lin<sup>-</sup>RFP<sup>+</sup>Sca1<sup>-</sup> SVF cells predominantly expressed MyoD and Pax7 (Fig. 6D–I), two well-established myogenic progenitor-cell markers (34, 35). These results provided strong evidence that the Myf5-lineage SVF cells represent





**Fig. 3.** Myf5-lineage adipocytes express lower levels BAT genes and beige cell markers than the non-Myf5-lineage cells within the same SAT depot. (A, B) Four populations ( $Sca1^+Myf5^-$ ,  $Sca1^+Myf5^+$ ,  $Sca1^-Myf5^-$ ,  $Sca1^-Myf5^+$ ) were isolated by FACS from the asWAT SVF cells of Myf5-Cre/Rosa26-tdTomato mice using RFP

a population of myogenic progenitors capable of differentiating into mature myofibers in vivo.

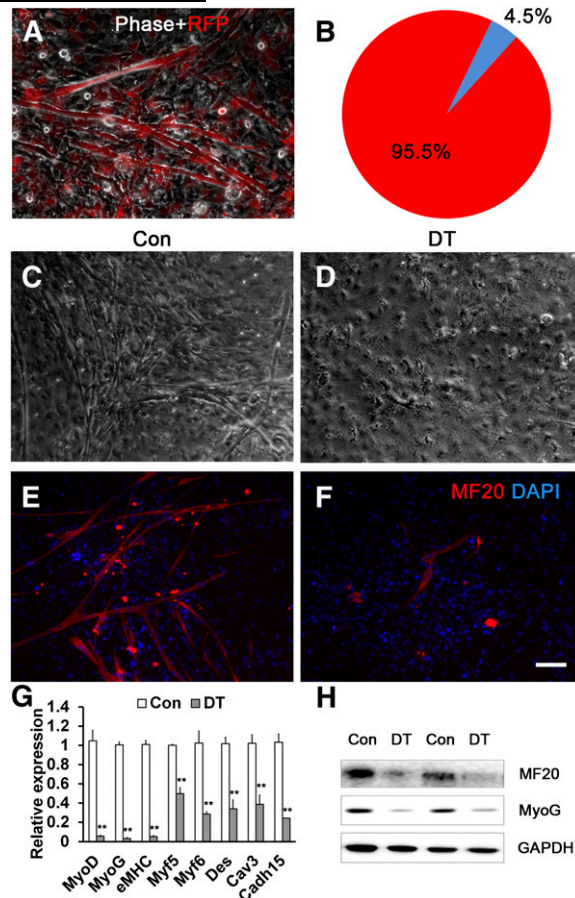
Interestingly, the transplanted the RFP<sup>+</sup>Sca1<sup>-</sup> cells differentiated into myofibers exclusively expressing slow myosin heavy chain (NOQ-positive) in the host TA muscle consisted of predominantly fast (NOQ-negative) myofibers (supplementary Fig. V, A–F). By contrast, myoblasts isolated from skeletal muscles adjacent to the asWAT exclusively formed myofibers expressing fast (My32-positive), but not slow, myosin heavy chain (supplementary Fig. V, G–L). These results indicate that WAT-derived Myf5-lineage myogenic cells are phenotypically different from the skeletal myoblasts in myosin heavy-chain gene expression.

To demonstrate the potential clinical applications of the WAT-derived RFP<sup>+</sup>Sca1<sup>-</sup> myogenic progenitors, we transplanted the RFP<sup>+</sup>Sca1<sup>-</sup> cells into mdx mice that lack the *dystrophin* gene, thus representing a model for Duchenne muscular dystrophy. We grafted freshly sorted RFP<sup>+</sup>Sca1<sup>-</sup> cells into TA muscles of mdx mice (50,000 cells/mouse). After 21 days, dystrophin-positive fibers were detected in the recipient mice, while there were nearly no dystrophin-positive fibers in the sham-operated contralateral TA muscles (Fig. 6J–L). These results demonstrate that the RFP<sup>+</sup>Sca1<sup>-</sup> cells represent a myogenic progenitor population that can be used to treat muscular dystrophy diseases.

## DISCUSSION

In this study, we dissected the phenotypic and potential functional differences of Myf5-lineage and non-Myf5-lineage adipocytes in WAT depots. We discovered that the Myf5-lineage adipocytes in WAT express lower levels of BAT and beige marker genes than do the non-Myf5-lineage adipocytes. In addition, we showed that the Myf5-lineage SVF cells in WAT contain two distinct subpopulations of adipogenic and myogenic progenitors, and we provided proof-of-principle evidence that the adipose-derived myogenic progenitors can be used to restore muscle function in an animal model of muscular dystrophy.

One important question in developmental biology is whether lineage origin underlies cell function. The analysis of Myf5-lineage origin provides mixed answers to this question. Our results clearly indicate that among the subcutaneous WAT depots and within single WAT depots, the answer is “Yes”. Through lineage tracing and ablation, FACS, differentiation assay, and gene expression analysis, we provided compelling evidence that the Myf5-lineage and non-Myf5-lineage WAT adipocytes have distinct phenotype and function. On the other hand, our finding that the Myf5-lineage WAT adipocytes are less brown than the non-Myf5-lineage WAT adipocytes is inconsistent with the

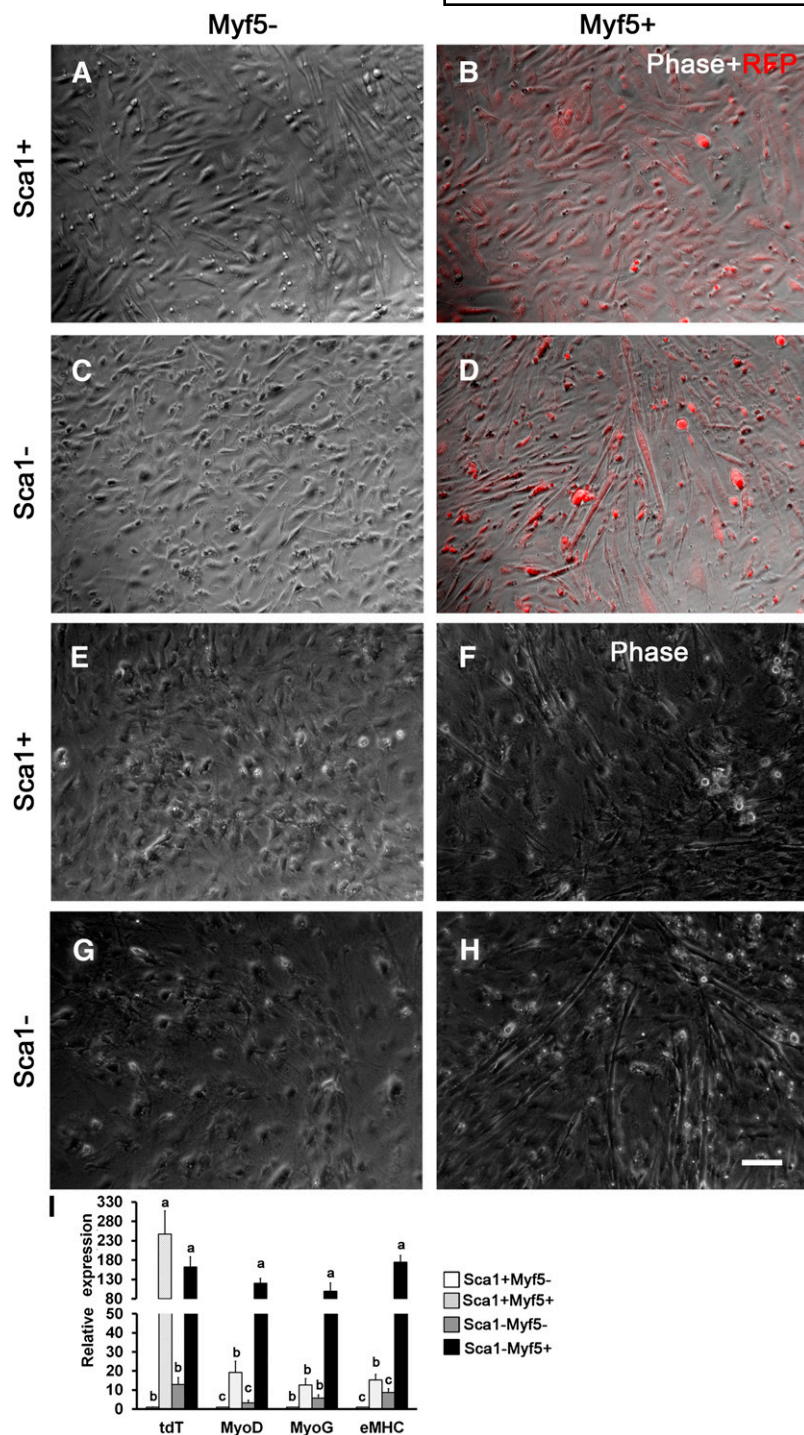


**Fig. 4.** Myf5-lineage cells are necessary for the myogenic differentiation of the WAT SVF cells. (A) Representative images of the myogenic differentiated asWAT SVF cells from the Myf5-Cre/Rosa26-tdTomato mice. (B) Quantification of the RFP-positive myotubes in the myogenic differentiated asWAT SVF cells. (C, D) Phase contrast images of the control (Con) and DT-treated SVF cells after myogenic differentiation. (E, F) The Con and DT-treated SVF cells were stained with MHC antibody MF20 after myogenic differentiation. (G) qPCR analysis of the myogenic markers in Con and DT-treated SVF cells after myogenic differentiation. (H) The Western blot results of the MHC and MyoD in Con and DT-treated SVF cells after myogenic differentiation. Error bars represent SEM, n = 6. \*\*P < 0.01. Scale bars: 100  $\mu$ m.

well-established dogma that the classical intrascapular BAT adipocytes are derived from the Myf5 lineage and the WAT is predominantly from the non-Myf5 lineage (15). Therefore, the Myf5-lineage adipocytes in the phenotypically and functionally distinct BAT and WAT appear to have drastically different gene expression and function. This observation suggests that local environments in different tissues can override the intrinsic/default property of a common precursor cell population. Alternatively, the Myf5-lineage

and Sca1 antibody. (C–F) Representative images of the sorted cells after adipogenic differentiation. (C, E) are phase contrast and (D, F) are phase contrast and red fluorescence merged images. (G, H) The expression of BAT and beige marker genes in the sorted adipocyte progenitors (Sca1<sup>+</sup>Myf5<sup>-</sup> and Sca1<sup>+</sup>Myf5<sup>+</sup> SVF cells) after differentiation. (I, J) The expression of BAT and beige marker genes in control (Con) and DT treated SVF cells after induced differentiation. Error bars represent SEM, n = 6. \*P < 0.05, \*\*P < 0.01. Scale bars: 100  $\mu$ m.





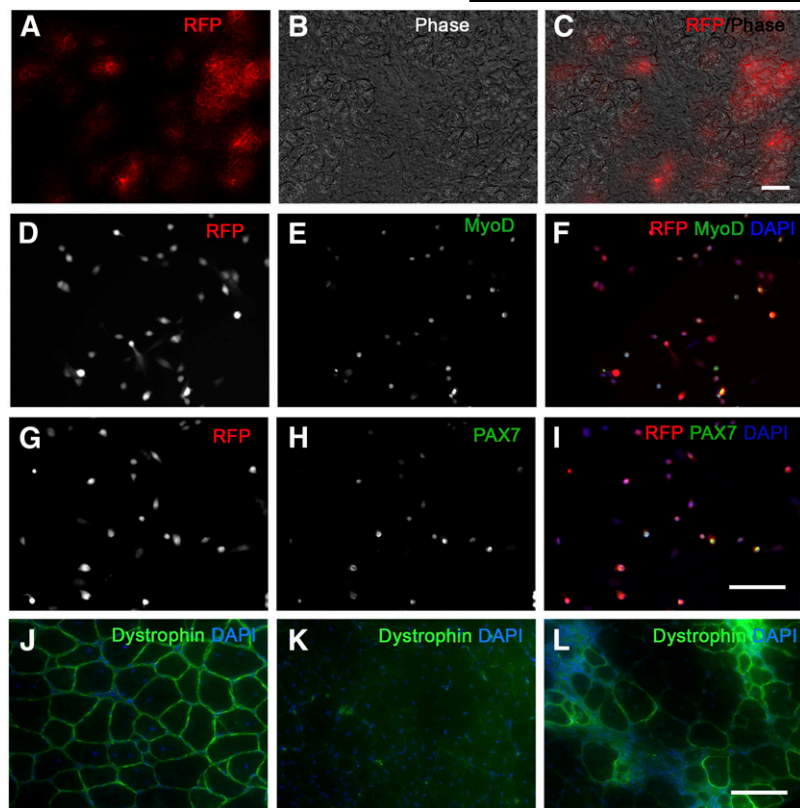
**Fig. 5.** The myogenic differentiation of the FACS sorted SVF cells. (A–H) Representative images of the FACS sorted cells before induction (A–D) and after induction (E–H). (I) The Myogenic genes expression after myogenic induction. Error bars represent SEM,  $n = 6$ . Different letters means  $P < 0.05$ . Scale bars: 100  $\mu\text{m}$ .

adipocytes in BAT and WAT may have originated from two distinct populations of Myf5-expressing progenitors that are phenotypically and functionally unrelated. In addition, as Myf5 is expressed by several mesodermal and neural cell lineages during development (15, 36–40), it is possible that the Myf5-lineage cells in BAT and WAT are from spatially unrelated embryonic primordial structures. Using the highly sensitive Rosa26-TdTomato reporter that contains a strong CAG promoter knocked into the ubiquitously expressed Rosa26 gene locus (30), we found that Myf5-lineage cells contribute to the skeletal muscle, adipose, brain, heart, and spleen, but not to liver, lung, or

kidney. The identification of Myf5-lineage adipocytes in both subcutaneous and visceral WAT is consistent with the recent observation by Sanchez-Gurmaches and colleagues (16). Together, our results suggest that lineage origin does underscore cell function within the same tissue but that cells derived from the same lineage marker may have distinct function in different tissues/organs.

As brown adipocytes are derived from the Myf5-cell lineage (15), we hypothesized that Myf5-lineage adipocytes in WAT may represent the adaptive beige adipocytes that share physiological function and gene expression with BAT. However, our data show that the Myf5-lineage adipocytes in WAT





**Fig. 6.** Myf5-lineage progenitors form new fibers in wild-type mice and rescue dystrophin expression in the mdx mice after transplantation. (A–C) The SVF cells from the asWAT of the Myf5-Cre/Rosa26-tdTomato mice formed RFP-positive myofibers in vivo five days after transplantation. (D–F) The FACS-sorted Scα1<sup>+</sup>Myf5<sup>+</sup> cells were staining with myogenic marker MyoD (D–F) and Pax7 (G–I). (J–L) Dystrophin expression in TA muscle sections of wild-type control without cell transplantation (J), mdx mice without cell transplantation (K), and mdx mice transplanted with  $5 \times 10^4$  adipose-derived myogenic progenitors (L). Scale bars: 100 μm.

express lower levels of BAT and beige adipocyte marker genes. We draw this conclusion through multiple lines of evidence. First, the higher abundance of Myf5-lineage adipocytes in the asWAT than in ingWAT is inversely correlated to the relative expression levels of BAT/beige adipocyte marker genes. Second, Myf5-lineage and non-Myf5-lineage adipocytes isolated by FACS from the same WAT depot expressed different levels of BAT/beige marker genes, with higher expression levels in the non-Myf5-lineage adipocytes. Third, ablation of the Myf5-lineage adipocytes resulted in elevated expression of BAT/beige-specific genes. Our results are in line with the observation by Seale et al. that the adrenergic stimulation-induced adaptive beige adipocytes in the subcutaneous WAT are derived from non-Myf5 lineage (15). Although a previous study has shown that both Myf5-positive and Myf5-negative precursors in WAT respond to  $\beta_3$ -adrenoreceptor stimulation (16), our current results suggest that the Myf5-lineage WAT adipocytes are perhaps less adaptive/recruitable, as they express lower levels of BAT and beige adipocyte-specific genes. Taken together, these data indicate that Myf5-lineage cells contribute less significantly to beige adipocytes or browning of WAT.

We identified two subpopulations of Myf5-lineage progenitors in WAT: the Scα1-positive adipogenic and Scα1-negative myogenic progenitors. Our results are consistent with recent reports demonstrating Scα1 as a positive selection marker for adipose stem cells and a negative selection marker for muscle stem cells (31, 32, 41, 42). The myogenic potential of Myf5-lineage SVF cells in WAT is not surprising given that Myf5-lineage precursors give rise to muscle tissues (40). Importantly, we show that the myogenic potential of adipose progenitors resides only in the

Myf5-lineage SVF cells, as more than 95% of myotubes found in WAT SVF cultures arose from the Myf5 lineage. In addition, ablation of the Myf5-lineage SVF cells abolished the myogenic potential of WAT SVF cells. The in vitro and in vivo myogenic differentiation potential of adipose-derived cells has also been reported (25, 26). However, what has been unclear is whether the multilineage differentiation potential of the so-called “adipose-derived stem cells” (ADSC) resides in a bona fide multipotential stem-cell population or mixed subpopulations of unipotent progenitors. Our FACS analysis results showing that the Scα1-positive subpopulation was adipogenic and Scα1-negative subpopulation was myogenic support the notion that adipose SVF cells consist of mixed populations of unipotent progenitor cells with adipogenic, myogenic, and other lineage potentials.

Although it has been shown that myogenic cells can be derived from adipose tissues, whether the adipose-derived myogenic progenitors are equivalent to skeletal myoblasts is unclear. We have identified several common features but also differences between adipose- and skeletal muscle-derived myogenic cells. The adipose-derived myogenic cells express common skeletal myoblast markers Pax7 and MyoD (34, 35). Like the skeletal myoblasts, the adipose-derived myogenic cells can efficiently differentiate into myotubes in vitro and in vivo, and they can restore dystrophin expression in mdx mice. However, the adipose-derived (asWAT) myogenic cells differentiated exclusively into slow myofibers, whereas the skeletal myoblasts isolated from the adjacent muscles differentiated exclusively into fast myofibers. The slow myofiber-specific differentiation of adipose-derived progenitors may represent an

advantage in therapeutic applications. It has been well established that slow myofibers are more resistant to damage under stress conditions, such as muscular dystrophy and diabetes (43–45). In addition, slow myofibers express high levels of the mitochondrial biogenesis-determinant genes Pgc1 $\alpha$  and PPAR $\delta$ ; therefore, they are more sensitive to insulin-regulated glucose uptake and may provide beneficial metabolic effects (46–49). Expression of CD34 or Sca1, two stem-cell markers, is associated with the myogenic potential of progenitor cells derived from bone marrow, blood, and embryonic vasculature (50–53). In addition, expression of CD34 defines the majority of quiescent satellite cells in adult skeletal muscle (54). However, our results from the FACS-sorted cells show that the Sca1-positive SVF cells were predominantly adipogenic, with little or nearly no myogenic activity. Strikingly, the RFP<sup>+</sup>Sca1<sup>-</sup> SVF cells form myotubes spontaneously, suggesting that the myogenic progenitors in WAT SVF cells are negative for Sca1. Consistent with our results, previous studies have shown that the ability to spontaneously form skeletal myotubes in vitro resides in the Sca1-negative SVF cells (25). Other groups have also shown that noncultured, adipose-derived, CD45-negative side-population cells were enriched with progenitors that gave rise to myofibers in vivo (55). Our recent data further demonstrate that the myogenic progenitors in WAT SVF cells were from the non-aP2-lineage cells (27). Taken together, these results indicate that the Myf5-lineage Sca1-negative cells are the myogenic progenitors in WAT SVF and that these cells are distinct from the other myogenic cells derived from regenerating muscle, bone marrow, or other tissues that have been reported to express Sca1.

In conclusion, we demonstrate in this study that Myf5-lineage progenitors in subcutaneous WAT give rise to both adipogenic and myogenic lineages. In addition, the Myf5-lineage adipocytes express lower levels of BAT and beige cell markers than do the non-Myf5-lineage adipocytes. Furthermore, the Myf5-lineage myogenic progenitors in WAT can efficiently differentiate into myofibers in vitro and in vivo, but they are distinct from the myogenic progenitors from skeletal muscles in differentiation potential toward slow myofibers. These results provide novel insights into the adipogenic and myogenic differentiation potential of Myf5-lineage cells in WAT. Such knowledge may be useful for treating metabolic and muscle diseases. **FIG**

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