Supporting Information

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SI Experimental Procedures

Animals. All animal procedures have been approved by the Institutional Animal Care and Use Committee (IACUC). C57BL/6NTAC mice (The Jackson Laboratory) aged 6 to 10 wk were used throughout the study unless otherwise indicated. Transgenic GFP mice were on the C57BL/K_a-Thy1.1 genetic background, as previously described (1, 2). Myf5-Cre–expressing animals (a kind gift of P. Soriano, Mount Sinai School of Medicine, New York, NY) (3), were crossed to Rosa26-YFP reporter mice (The Jackson Laboratory) for myf5 lineage-tracing experiments. The 129S1/SvEvTac mice (The Jackson Laboratory) were obtained age-matched to the C57BL/6 mice for studies involving genetic background. For injections of cells isolated from GFP-transgenic mice (GFP^+) into GFP^- wild-type littermates, cells were pretreated with bone morphogenetic protein 7 (BMP7) in vitro, as described above. Cells were then harvested and injected at 1 million cells per injection site in a total volume of 25 μL of F10 nutrient mixture (Invitrogen) with the addition of 2% matrigel (BD Biosciences). Cells were injected into subcutaneous locations on the back of the animal, gastrocnemius muscle, or the subcutaneous or epididymal fat pads, as indicated. Animals were killed 10 d after injection for monitoring engraftment of GFP^+ cells. Mice were treated with daily intraperitoneal injections of 1 μg/g bodyweight CL316,243 for 8 d before dissection. Body temperature was assessed as described (4). For BMP7-injection studies, mice aged 4 wk were maintained on a high-fat diet (60% kcal% fat; Research Diets) for 6 wk. Recombinant human BMP7 (provided by Stryker Biotech) was administered by daily intraperitoneal injections at a concentration of 300 ng/g body weight. BMP7 was provided as a 0.9 mg/mL stock solution which was further diluted in a buffer containing 10 mM lactate and 9% wt/vol trehalose, which was also used as vehicle control for injections. Similarly, CL316,243 was dissolved and injected as described above.

Cell Sorting. Sca-1^+ progenitor cells (ScaPCs) were isolated from mouse skeletal muscle using FACS, as previously described (5, 6), with some modifications. Similarly, the stromovascular fraction was isolated form white and brown fat depots, as described (4). For isolation of ScaPCs from muscle, pooled limb skeletal muscles (including extensor digitorum longus, tibialis anterior, gastrocnemius, quadriceps, soleus, and triceps brachii) were harvested and subjected to a two-step digestion protocol, as described before, to dissociate single myofibers and liberate the fraction of myofiber-associated cells. Interstitial cells were removed before the second digestion step by repeated washes. For isolation of ScaPCs from adipose-tissue depots, depots were dissected and minced in PBS containing 3.5% BSA and 2 mg/mL collagenase. Digestion was performed at 37 °C for up to 25 min. The stromovascular fraction was collected by centrifugation and further filtration through 40-μm filter before FACS. Live cells were isolated by positive selection for calcein blue staining (1:1,000, stock of 1 mg in 215 μL DMSO; Invitrogen) and negative selection for propidium iodide staining (1 μg/mL; Sigma-Aldrich). Antibodies used were anti-mouse CD45 [1:200, Phycoerythrin (PE) conjugate, clone M1/70], anti-mouse Mac-1 (CD11b) (1:200, PE conjugate, clone 30-F11), anti-mouse Sca-1 [Ly-6A/E, 1:200, Allophycocyanin (APC) conjugate, clone D7] (all from eBioscience). These antibodies were used in all isolations of ScaPCs using positive staining for Sca-1-APC and negative staining for CD45-PE and Mac-1-PE. Sca-1^- were obtained similarly by negative selection for PE and collection of both APC^+ and APC^- cells. Other antibodies used were anti-mouse CD29 (β1-integrin, 1:200, PE-Cy7 conjugate, clone eBioHMb1-1), anti-mouse CD117 (c-Kit, 1:100, PE-Cy7 conjugate, clone 2B8), anti-mouse CD31 (PECAM-1, 1:100, FITC conjugate, 1:100, clone 390), anti-mouse CD34 (1:100, FITC conjugate, clone RAM34) (all from eBioscience), and anti-mouse CD34 (1:100, biotin conjugate, clone MEC14.7), with secondary streptavidin-FITC conjugate (1:100), anti-mouse-CD140a (PDGFRα, 1:50, APC conjugate) and anti-mouse-CD140b (PDGFRβ, 1:50, APC conjugate) (all from Biolegend). All antibody incubations were performed for 20 min on ice in HBSS (Invitrogen) containing 2% FBS (Sigma-Aldrich). For cell sorting, either a BD FACSAria (Becton Dickinson) or Cytomation Moflo (Cytomation) instrument was used. FACS data were collected using DIVA (Becton Dickinson) or Summit (Cytomation) software and analyzed offline using FlowJo software (Tree Star, Inc., Macintosh version 8.1.1).

Cell Culture. Cells were grown as described before with some modifications (7). Specifically, a medium containing 60% DMEM with low glucose (Invitrogen), and 40% MCDB201 (Sigma-Aldrich), supplemented with 100 U/mL penicillin and 1,000 U/mL streptomycin (Invitrogen) was prepared. Additionally, the medium was supplemented with 2% FBS, 1× insulin-transferrin-selenium mix, 1× linoleic acid conjugated to BSA, 1 nM dexamethasone, and 0.1 mM L-ascorbic acid 2-phosphate (all from Sigma-Aldrich). Before use, the following growth factors were added to the medium: 10 ng/mL epidermal growth factor (PeproTech), 10 ng/mL leukemia inhibitory factor (Millipore), 10 ng/mL platelet-derived growth factor BB (PeproTech), and 5 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich). The bFGF was added daily throughout the culture period except where stated otherwise. ScaPCs were washed three times in growth medium after sorting and initially plated at 50,000 cells per well in 24-well cell culture plates. Cells were expanded for 6 or 7 d, until they reached ≈90% to 95% confluence. For most assays, cells were seeded at 20,000 cells per well in 48-well plates. Cells were treated with 3.3 nM BMP7 (R&D Systems). The treatment was for 3 d in basal growth medium with growths factors (termed as day 3). Medium was then replaced by differentiation medium without addition of growth factors. For adipogenic conditions, cells were treated with an adipogenic induction mixture in growth medium for 48 h (termed as day 5), containing 5 μg/mL human insulin (Roche Applied Science), 50 μM indomethacin, 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 1 nM 3,3′,5-triiodo-L-thyronine (T3) (all from Sigma-Aldrich), followed by further differentiation in growth medium with the addition of T3 and insulin only for 7 d (termed as day 12). Myogenic medium was prepared from DMEM with low glucose (Invitrogen) with the addition of 2% horse serum (Atlanta Biologicals). Osteogenic conditions were used as described previously (7), with the addition of 50 ng/mL L-thyroxine (Sigma-Aldrich). Rosiglitazone (Cayman Chemicals) was added to the cells as indicated, either replacing or in combination with BMP7 at a concentration of 1 μM.

To assess uncoupled mitochondrial respiration, a Seahorse Extracellular Flux Analyzer (Seahorse Bioscience Inc.) was used to quantify oxygen consumption rates of ScaPCs. Cells were seeded on 24-well format plates and allowed to adhere overnight, before BMP7-treatement was initiated. After 3 d, BMP7 was removed and adipogenesis was induced as described. After adipogenic induction for 48 h, oxygen consumption rates were analyzed. To measure oxygen consumption rates independent of oxidative phosphorylation, 0.5 μM oligomycin (EMD Chemicals Inc.) was

added to the cells. Subsequently, rotenone and antimycin A (Sigma-Aldrich) were added to measure basal rates of nonmitochondrial respiration. The difference between these two stages was calculated as respiration of the uncoupling proteins (UCPs).

Cultivation and differentiation of human preadipocytes was performed as described (8–10). Cultures from different depots from the same subjects were studied in parallel. Cells were exposed continuously to 3.3 nM BMP7 for the entire period of differentiation.

Lipid accumulation was monitored by staining cells with the lipid specific dye Oil Red O, as previously described (4).

RNA and Protein Expression. Protein and RNA from cultured cells were harvested at the indicated time points and prepared as described previously (4). Primer sequences used for quantitative real-time PCR are listed in Table S2. Western blot analysis for UCP1 protein was performed using a polyclonal antibody directed against murine UCP1 (Santa Cruz Biotechnology).

Immunohistochemistry. For detection of GFP, formalin-fixed tissues were embedded in paraffin blocks and 4-μm sections of the entire block were made. Every 10th to 12th section of the tissue sample was used to detect GFP by immunohistochemistry. After rehydration of the sections, slides were treated with 3% H_2O_2 for 10 min to quench endogenous peroxidase activity and washed three times with PBS. Blocking was performed in two subsequent steps with unconjugated avidin and biotin (Avidin/Biotin Blocking Kit; Vector Laboratories) for 1 h at room temperature followed by PBS washes. Sections were then blocked with TNB blocking buffer (TSA Kit; PerkinElmer) for 1 h. A polyclonal rabbit anti-GFP antibody was applied overnight at 4 °C at a dilution of 1:500 (ab6556; Abcam). After three washes with PBS, slides were incubated with rabbit anti-biotin (Jackson ImmunoResearch) at a dilution of 1:400 for 1 h at room temperature. Following subsequent washes with PBS, horseradish peroxidase-coupled streptavidin was applied at 1:100 for 30 min. After PBS washes, DAB solution was applied for 2 to 5 min (ABC Vectastain Kit; Vector Laboratories), and washed with ddH_2O. A similar protocol was used for detection of perilipin on sections adjacent to those previously stained for GFP expression, except that rabbit anti-perilipin A/B (Sigma-Aldrich) was used as the primary antibody.

Immunofluorescence. Sections were deparaffinzed and immediately boiled in Dako Antigen Retrieval Solution (Dako North America, Inc.) in a water bath for 30 min. Sections were then incubated in 200 μL of a 0.3% Sudan Black solution in 70% ethanol for 30 min at room temperature to block autofluorescence. Slides were rinsed with immunohistochemical rinse buffer (Millipore) and then placed in a humid chamber for incubation with Blocking Reagent (Millipore) for 20 min at 37 °C. Positive and negative controls were run to detect autofluorescence and any nonspecific binding. Primary antibodies were diluted in Antibody Diluent (Dako), and a volume of 100 μL was pipetted on each tissue section for a 48-h incubation at 4 °C in a humid chamber. Primary antibody concentrations were 1:50 for anti-mouse UCP1 (rabbit polyclonal, #53936; AnaSpec), and 1:100 for anti-GFP (chicken polyconal, ab13970, chicken polyclonal; Abcam). After primary antibody incubation, the sections were rinsed and incubated in appropriate secondary antibody at a 1:200 dilution for 10 min. After secondary antibody incubation, sections were rinsed with Wash Buffer (Millipore) and then distilled water, and mounted with Light Diagnostics Mounting Fluid (Millipore). Sections were kept in the dark after secondary antibody incubation and immediately analyzed by confocal microscopy on a Zeiss LSM-410 Invert Laser Scan Microscope (Carl Zeiss MicroImaging). Yellow color in the merged images of the green channel (GFP) and the red channel (UCP1) was considered as colocalization.

Microarray. Analysis of gene expression using GeneChip Mouse Genome 430A 2.0 Array (Affymetrix) was performed on ScaPCs following 72 h of treatment with 3.3 nM BMP7. Normalized expression data were used for analysis with the Gene Set Enrichment Analysis program (Broad Institute, Cambridge, MA) (11, 12).

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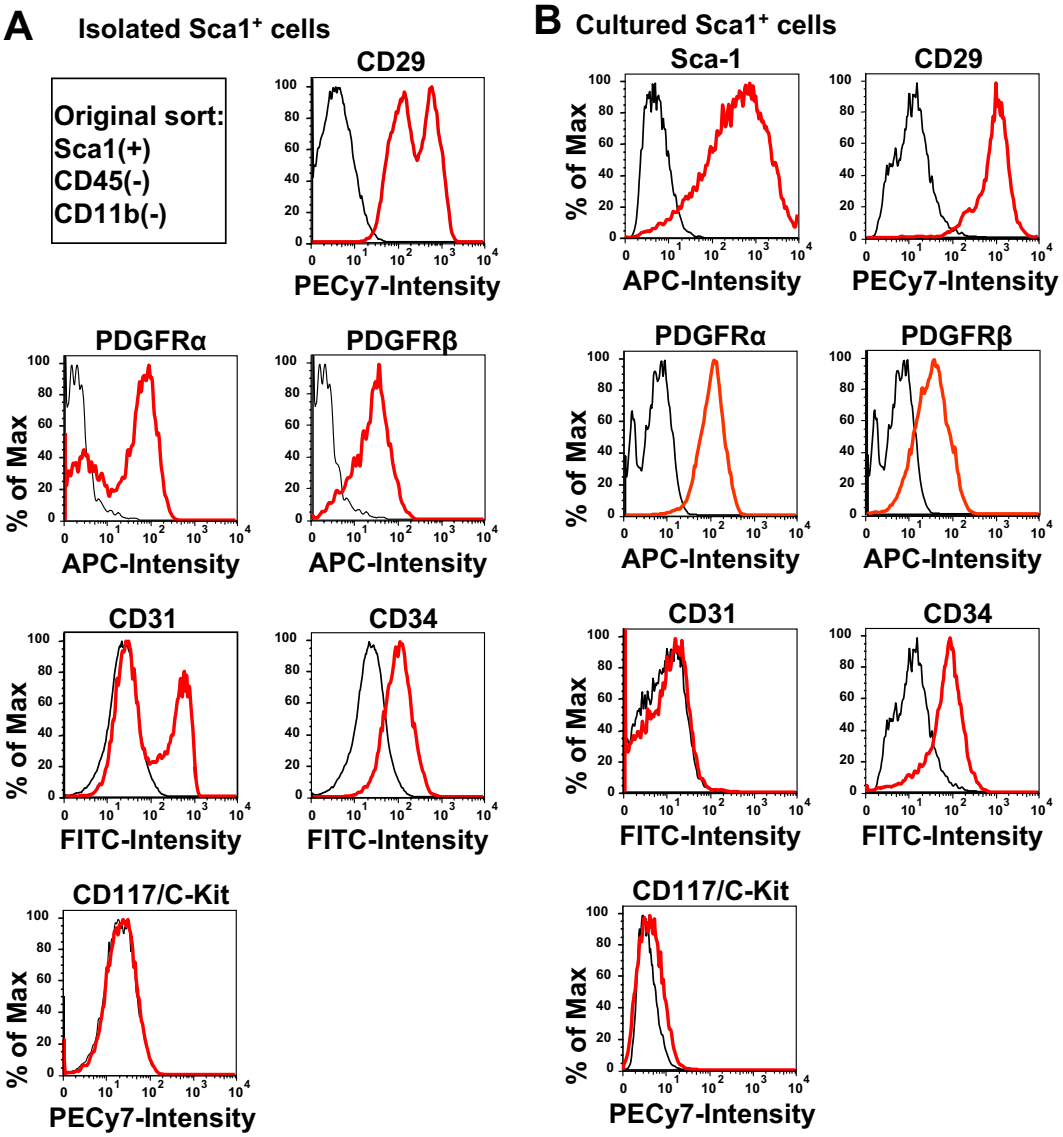

Fig. S1. Surface marker expression pattern and ScaPCs. Expression of cell-surface markers was determined in Sca1$^+$ cells (*A*) directly after isolation and (*B*) after 6 to 7 d of culture. In the fresh isolates, only Sca1$^+$ cells were used for the analysis. Presence of Sca1, CD29 (integrin β1), CD34, CD140a (PDGFRα), CD140b (PDGFRβ), CD31 (PECAM), and CD117 (c-Kit) was determined. Experiments were repeated three times. CD29, CD34, and PDGFRβ were expressed on all Sca1$^+$ cells directly after isolation; CD117 (c-Kit) was not expressed. PDGFRα and CD31 were expressed in 70% and 45% of the freshly isolated Sca-1$^+$ cells, respectively. In cultured cells, all cells expressed PDGFRα, but CD31 was no longer detected. Further subfractionation of CD31$^+$ and CD31$^-$ cells revealed that only the CD31$^-$/Sca-1$^+$ cells could attach to tissue culture plastics, suggesting that in vitro culture of the ScaPCs may provide an additional selection to enrich populations of adipogenic progenitors.

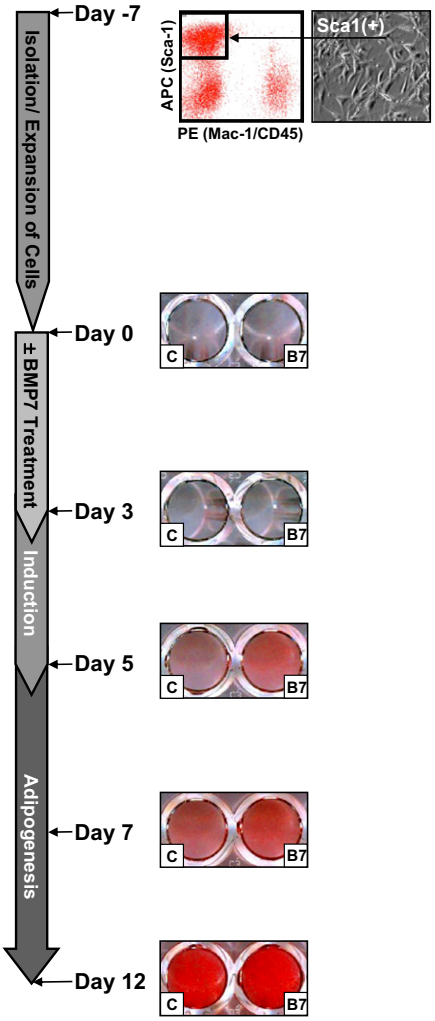

Fig. S2. Time course of ScaPC differentiation conditions. Cells were harvested (day −7) and allowed to expand to near-confluency for 6 to 7 d, before they were seeded for actual experiments. Cells were seeded and allowed to attach for 16 h (top panel is day −7). Initiation of bone morphogenetic protein 7 (BMP7) treatment is the starting point of all experiments (termed: day 0). Cells were then pretreated with 3.3 nM BMP7 for 3 d (d3), after which BMP7 was removed and growth medium replaced with adipogenic induction medium for 48 h (d5). Cells were then allowed to differentiate for another 7 d before the final time point was harvested (d12). Oil Red O staining of lipid-accumulating cells in both control and BMP7 pretreated cells at indicated time points (day 0 to day 12).

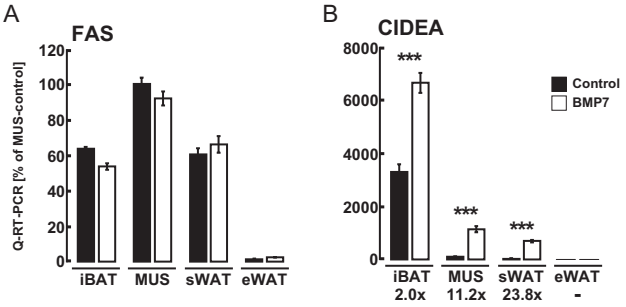

Fig. S3. Gene expression pattern of adipocyte markers in three adipose-tissue depots and skeletal muscle. Quantitative RT-PCR of (*A*) fatty acid synthase (FAS) and (*B*) Cell death-inducing DFFA-like effector A (CIDEA) in differentiated ScaPCs from all four tissue types with and without BMP7 pretreatment. Tissues used were interscapular brown adipose tissue (iBAT), skeletal muscle (MUS), subcutaneous white fat (sWAT), and epididymal white fat (eWAT). Values given under each tissue type represent fold-changes of expression over respective control. Black bars indicate control conditions; white bars indicate BMP7-pretreatment. Data are presented as mean ± SEM (n = 3). Asterisks denote significant differences between control and BMP7-pretreated cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

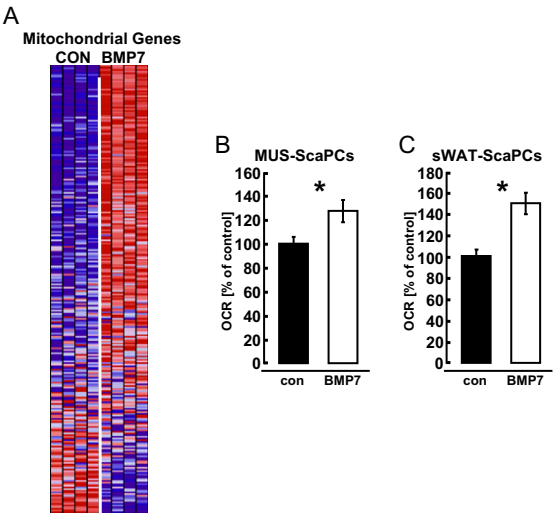

Fig. S4. BMP7 Treatment induces mitochondrial activity and uncoupling in ScaPCs. (*A*) Expression profiles of ScaPCs treated with 3.3 nM BMP7 or vehicle for 72 h. Gene Set Enrichment Analysis revealed a significant ($P < 0.001$, false-discovery rate q-value <0.001) enrichment of genes involved in many aspects of mitochondrial physiology. The complete list of genes is summarized in Table S1. Each column indicates an individual chip using RNA of ScaPCs isolated from one mouse. There are four replicates for each condition. Red indicates expression higher than the mean and blue indicates expression lower than the mean. Uncoupled respiration of ScaPCs derived from (*B*) MUS and (*C*) sWAT. Cells were treated with 0.5 μM oligomycin to assess state IV respiration and subsequently with a combination of 0.5 μM rotenone and 0.5 μM antimycin A to measure nonmitochondrial respiration by inhibiting mitochondrial electron transport. The difference between these two stages is considered the uncoupled respiration by uncoupling proteins. Asterisks denote significant differences between control and BMP7-treated cells (*$P < 0.05$).

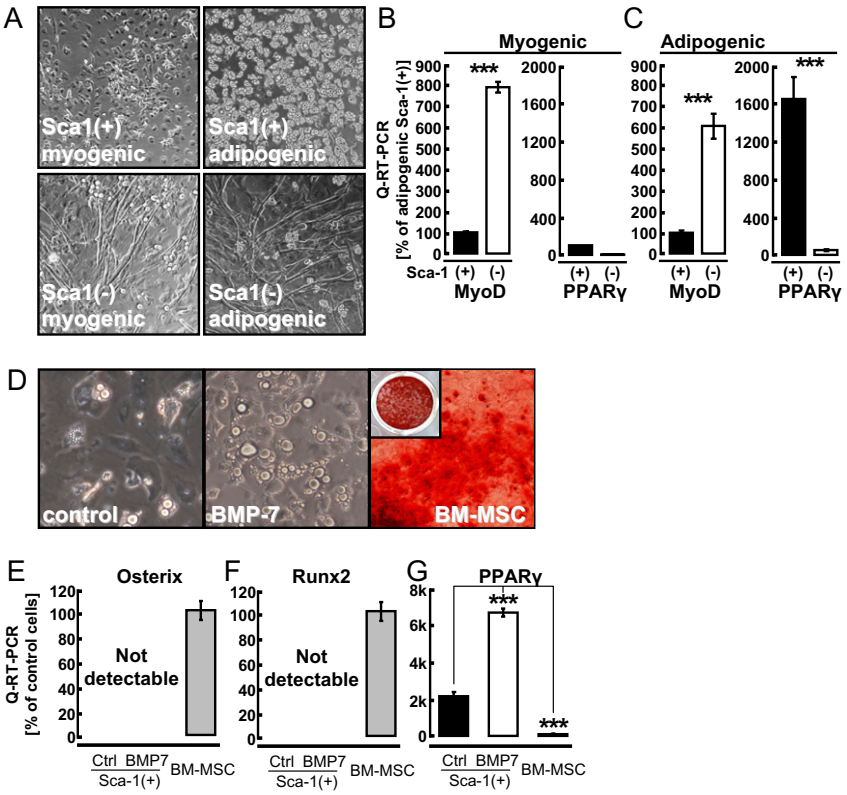

Fig. S5. Muscle-derived ScaPCs possess no myogenic or osteogenic capacities. (*A*) Morphological differences of nonhematopoietic cells selected for Sca-1 expression (*Upper*: Sca-1$^+$ cells; *Lower*: Sca-1$^-$ cells) under conditions of either myogenic (*Left*) or adipogenic (*Right*) lineage differentiation. (*B*) Quantitative RT-PCR analysis of gene expression of the myogenic marker myoD and (*C*) the adipogenic marker peroxisome proliferator-activated receptor-γ (PPARγ) in Sca-1$^+$ or Sca-1$^-$ cells under myogenic (*Left*) or adipogenic (*Right*) conditions. Black bars represent gene expression in Sca-1$^+$ cells and white bars reflect gene expression levels of Sca-1$^-$ cells. Data are presented as mean ± SEM ($n = 3$; ***$P < 0.001$ between Sca-1$^+$ and Sca-1$^-$ cells). (*D*) ScaPCs treated with BMP7 or vehicle for 3 d (*Left*), and bone marrow-derived mesenchymal stem cells (BM-MSCs, *Right*) were grown in osteogenic conditions for 21 d, then stained with Alizarin S Red for mineralization. (*E–G*) Quantitative RT-PCR analysis of osteogenic transcription factors (*E*) osterix and (*F*) runx2, and (*G*) PPARγ in ScaPCs ± BMP7 and BM-MSCs. Data are presented as mean ± SEM ($n = 3$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

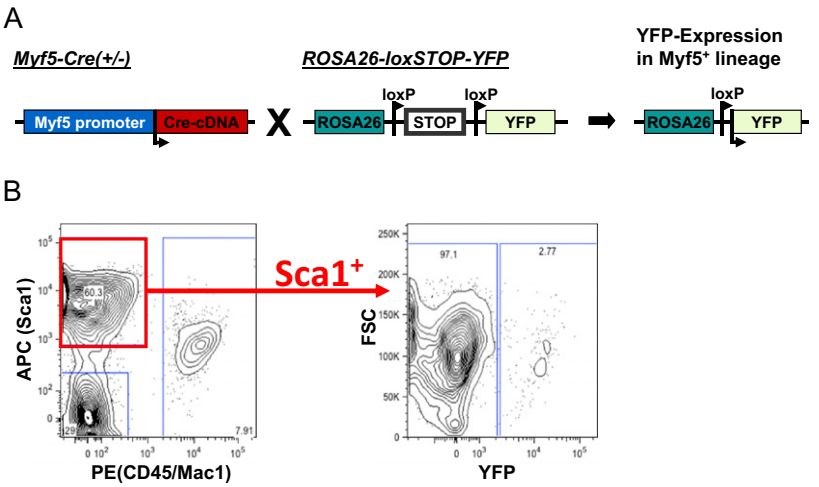

Fig. S6. ScaPCs are not derived from a myf5-expressing lineage. (*A*) Breeding scheme for mice expressing Cre recombinase under control of the myf5-promoter crossed to ROSA26-YFP reporter mice. Cre-mediated combination removes a transcriptional stop cassette in the ROSa26 locus and thus allows expression of YFP in all cells descending from the myf5-expressing progenitor. (*B*) Myofiber-associated cells were isolated from double transgenic mice and the Sca1$^+$ population was analyzed for expression of YFP (n = 2, 2.8 ± 0.01, mean ± SD).

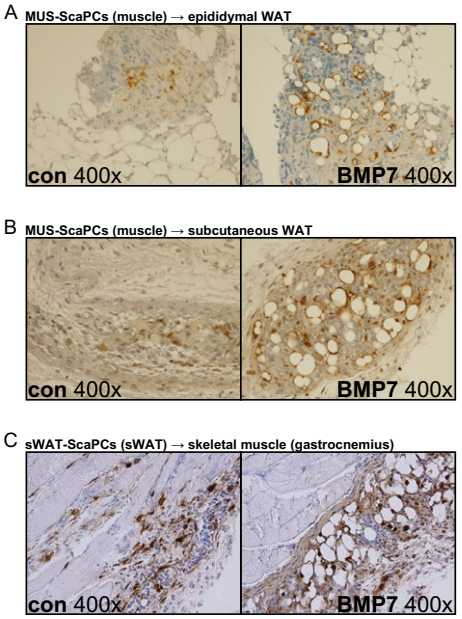

Fig. S7. Engraftment and adipogenic differentiation of ScaPCs depends on BMP7. Immunodetection of GFP$^+$ cells following injection into recipient mice. Muscle-derived ScaPCs were injected into (*A*) eWAT and (*B*) sWAT. One fat pad received untreated control cells, and the fat pad on the other side was injected with cells treated with BMP7. (*C*) Cells derived from sWAT were injected into MUS. Analogously, one side each received treated or untreated cells. Cells were left in recipient mice for 10 to 14 d before collection of tissues. Tissues were collected and examined for GFP$^+$ cells using light microscopy. Areas with detectable fluorescence were then dissected and processed for immunohistochemistry.

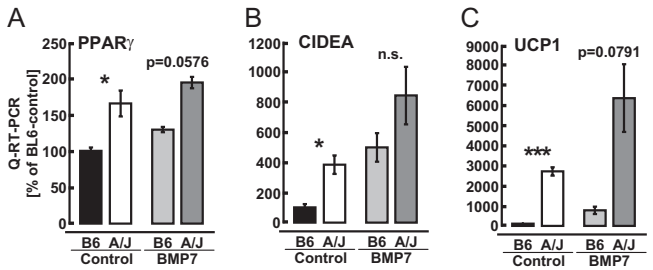

Fig. S8. Genetic background determines the propensity of ScaPCs isolated from muscle to undergo brown adipogenesis. (*A*–*C*) Quantitative RT-PCR analysis of (*A*) PPARγ, (*B*) CIDEA, and (*C*) UCP1 expression in muscle-derived ScaPCs, comparing the C57BL/6 mouse strain to the A/J strain. Bars indicate untreated cells from C57BL/6 (black), untreated from A/J mice as indicated (white), BMP7-pretreated from C57BL/6 (light gray), and BMP7-pretreated from A/J mice (dark gray). All data are presented as mean ± SEM (n = 3; *P < 0.05; **P < 0.01; ***P < 0.001).

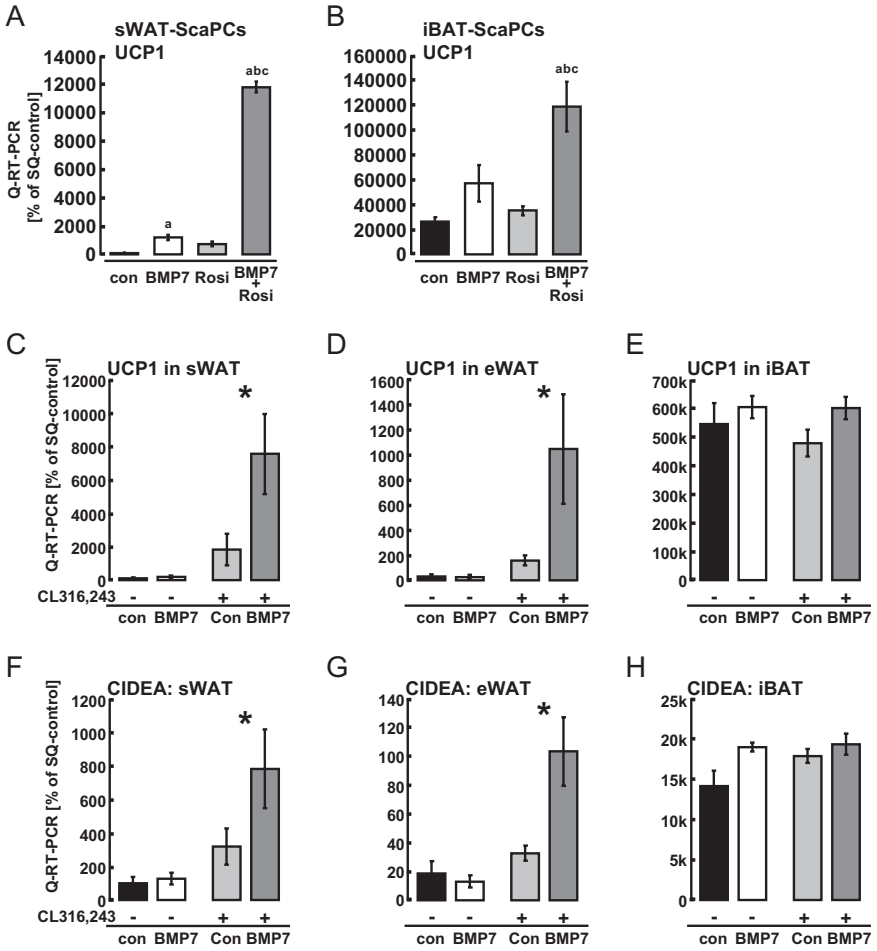

Fig. S9. BMP7 synergistically interacts with inducers of a brown-fat phenotype. Expression of UCP1 in ScaPCs derived from (*A*) sWAT and (*B*) iBAT. Cells were treated with vehicle (black bars), 3.3 nM BMP7 (white bars), 1 μM rosiglitazone (light gray bars), or a combination of BMP7 and rosiglitazone (dark gray bars). Data are presented as mean ± SEM (n = 3). "a" depicts statistically significant differences (P < 0.05) between control and the indicated condition; "b" depicts statistically significant (P < 0.05) differences between BMP7 and indicated condition; "c" depicts statistically significant (P < 0.05) differences between rosiglitazone and indicated condition. (*C*–*H*) Expression of UCP1 and CIDEA in (*C* and *F*) sWAT, (*D* and *G*) eWAT, and (*E* and *H*) iBAT following injection with BMP7 alone or in combination with CL316,243. Mice received injections of control (vehicle, black bars), BMP7 (white bars), control in combination with CL316,243 (light gray bars), and BMP7 in combination with Cl316,243 (dark gray bars). All data are presented as mean ± SEM (n = 5; *P < 0.05).

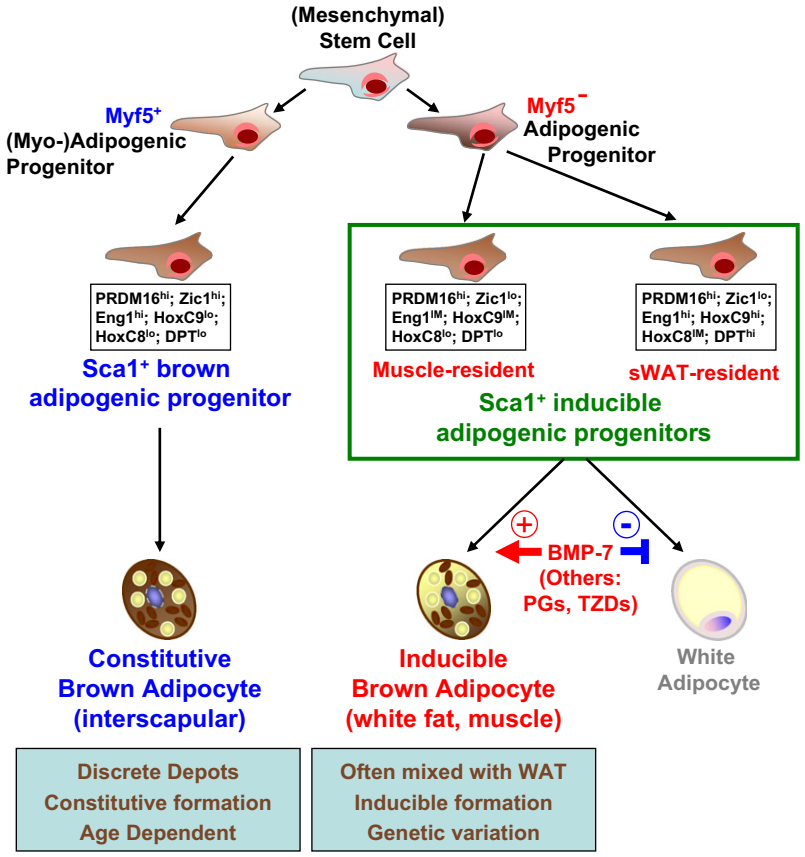

Fig. S10. Proposed model of different types of brown-fat progenitors giving rise to distinct brown adipocytes. Our data suggest the existence of three types of brown-fat progenitors: Whereas ScaPCs isolated from interscapular BAT serve as constitutively committed brown fat precursors, Sca-1^{+} progenitor cells from skeletal muscle and sWAT are highly inducible to become mature brown adipocytes by BMP7 stimulation. These precursors possess unique markers that allow the distinction of cellular origin. Other agents that may efficiently be used to induce brown adipogenesis include TZDs (thiazolidinediones) and PGs (prostaglandins).

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)