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Endothelial Differentiation in Multipotent Cells Derived from Mouse and Human White Mature Adipocytes

SUPPLEMENTAL DATA
Detailed Method Section
Mice and human samples

AP2-Cre$^{+/+}$;LacZ ROSA (R26R)$^{+/+}$ double transgenic mice that expressed ß-galactosidase (ß-Gal) under the control of the aP2 promoter were generated as previously described [1], using aP2-Cre+/+ (strain B6.Cg-Tg(Fabp4-cre) 1Rev/J) mice and LacZ ROSA26+/+ (R26R) (strain B6.129S4-Gt(ROSA)26Sortm1Sor) reporter mice obtained from the Jackson Laboratory. For collection of mouse adipose tissue for cell isolation, transgenic or wild type C57BL/6 mice were euthanized at 8-10 weeks of age by inhalation of Isoflurane (5-30%) and the adipose tissue was collected postmortem.

Ligation of the left anterior descending artery in wild type C57BL6/J male mice was performed as previously described [2]. After LAD ligation, the mouse was closely monitored. Once the heart rate stabilized (approximately 15 minutes), a cocktail of 500,000 DFAT cells in alpha-minimum essential medium (MEM) supplemented with 1% ES cell qualified fetal bovine serum (Invitrogen) and 1% Methylcellulose (Sigma-Aldrich) was injected into the infarct zone. Green Fluorescent Protein (GFP)-expressing DFAT cells used for injection were prepared from transgenic mice that expressed GFP under the control of the human ubiquitin C promoter (strain C57BL/6-Tg(UBC-GFP)30Scha/J) obtained from the Jackson Laboratory. The cells were previously unpassaged and used approximately 10 days after adipocyte isolation. The chest was closed after the cell injection. The mice were sacrificed 2, 3, or 6 weeks after the procedure, and the heart was fixed for histological analysis. The hearts were analyzed for the presence of cells that co-expressed GFP and EC lineage markers by immunostaining.

The animal studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996). Samples of fresh human subcutaneous lipoaspirate were used for this study, but we were blinded to the identities, all characteristics, and the medical histories of the human subjects.

Isolation of adipocytes and culture of DFAT cells

Lipid-filled mature adipocytes were prepared from 2 grams of mouse subcutaneous adipose tissue or human fresh lipoaspirate (within 2-4 hours of lipoaspiration) as previously described [3-5]. Adipocytes were washed three times in phosphate-buffered saline (PBS) before they were used for further analysis or culture. Adipocytes ($5x10^4$) were placed in 25-cm^2 culture flasks filled completely with Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (HyClone) and 0.5% of antibiotic-antimycotic solution (Mediatech), and cultured in ceiling cultures as previously described [4]. Similar to previous studies [4, 6], approximately 30-40% of the adipocytes adhered to the "ceiling" of the culture flask, released lipids droplets and took on a fibroblast-like morphology. Once the cells became firmly attached, conventional culture was applied [4]. The medium was changed in part every 3 days. As before, these cells were referred to as DFAT cells [4].

To enhance endothelial cell (EC) differentiation in DFAT cells, adipocytes ($1x10^5$ cell/well) or DFAT cells ($1x10^4$ cells/well) were seeded in 24-well plates. The adipocytes initially floated on the medium, then sank and attached to the bottom. Previous studies have shown that these cells are indistinguishable from cells initially grown in ceiling culture [7]. Bone morphogenetic protein-4 (BMP4) (50 ng/ml, R&D Systems) and BMP9 (10 ng/ml, R&D Systems) were added 3 days after the cells were seeded, and the treatment media were changed after 5 days. GW1929, a PPAR-γ agonist (100 ng/ml, Sigma-Aldrish) was used to modulate the effect of BMP-4.

Cell Culture and SiRNA Transfection

DFAT cells were seeded at 50-80% confluency, and transient transfections with siRNA were performed with Lipofectamine™2000 (Invitrogen) using 60 nM siRNA as previously described [8]. Briefly, the amount of siRNA was optimized as per the manufacturer's instructions. Three separate siRNAs (Silencer® predesigned siRNA, Ambion) and scrambled siRNA with the same nucleotide content were tested. When compared with unrelated control siRNA and scrambled siRNA, the specific ALK2 siRNAs resulted in an >85% decrease in mRNA and protein levels as previously shown by real-time PCR and immunoblotting, respectively [9]. The siRNA that provided the most efficient inhibition (90–95%) was used for experiments, and treatments were usually initiated 24 hours after the start of the transfection. Silencer® predesigned siRNAs were obtained for ALK2.

Tube Formation Assay

A collagen gel solution was prepared on ice by mixing the following solutions: collagen I, rat tail (BD Biosciences), 10 x F12 medium, and reconstitution buffer (HEPES buffer, pH 7.4, with 0.2 N sodium bicarbonate) in a ratio of 8:1:1 (volume). The prepared collagen gel was mixed with Matrigel™ (BD Biosciences) 1:1 (volume) with ice-cold pipettes. The DFAT cells were suspended in ice-cold gel solution at a concentration of $1x10^6$ cells/ml of total solution. Once the cells were suspended in the gel solution, they were immediately transferred into chamber slides and incubated in a humidified incubator (5% CO_2, 95% air) to allow the gel to solidify for 30 min at 37°C before adding culture medium. Cultures were maintained for 1-4 weeks, and the medium was changed every 2-3 days. At the end of the experiments, the gels were fixed overnight in 4% phosphate-buffered formalin, paraffin-embedded, and used for histological analysis.

ß-Galactosidase (ß-Gal) staining and quantification of ß-Gal positive cells

Cells were washed with PBS, fixed in 0.05% glutaraldehyde for 5 min at room temperature, washed again with PBS 4 times. ß-Gal (X-Gal) staining solution was prepared as follows: 250 μl of the 200mM $K_4Fe(CN)_6$, 250 μl of the 200mM $K_3Fe(CN)_6$, 20 μl of 1M $MgCl_2$, and 10 mg X-gal in 1 ml of dimethyl sulfoxide (DMSO) were combined, PBS was combined to a final volume of 10 ml, and pH was adjusted to 8.1-8.5. Cells were incubated with the staining solution for 4 hours at 37°C, washed 3 times with PBS, and analyzed under phase contrast microscopy. As previously shown by other investigators, it is essential to perform the ß-Gal staining at basic pH to avoid non-specific ß-Gal staining [10, 11]. All ß-Gal staining was performed at the basic pH, and was negative in control tissues. The percentage of ß-Gal positive cells was calculated after estimation of 10 different optical fields (10x magnification) in each culture well.

Flow Cytometric Analysis

The purity of the isolated adipocytes was assessed by fluorescence-activated cell sorting (FACS) using the lipophilic fluorescent dye Nile red (Sigma-Aldrich) as previously described [12, 13]. The adipocytes were washed with PBS, fixed in 2% formaldehyde for 10 min, washed again with PBS, and stained with Nile red at a final concentration of 1 μg/ml for 30 min. FACS emission was set at 575 nm. For characterization of the phenotype of DFAT cells, FACS analysis was performed when passaged for the first time using previously described methods [4]. Briefly, the cells were detached from the culture dish with 0.25% trypsin/EDTA, centrifuged at low speed, and stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Alexa Fluor 488 (AF-488)-conjugated antibodies against human CD34, CD31, CD45, CD90, CD133, CD14, CD140b, NG2, and CD146, and mouse CD34, CD31, CD45, CD90, CD133, CD11b, CD140b, NG2, and CD146, (all 1:200; BD Pharmingen and eBioscience). Nonspecific fluorochrome- and isotype-matched IgGs (BD Pharmingen) served as controls. Flow cytometer gates were set using unstained cells and the isotype-matched controls. Cells were gated by forward scatter (FSC) versus side scatter (SSC) to eliminate debris. A region was established to define positive PE/AF-488 fluorescence using a PE/AF-488-conjugated isotype-specific control. The number of cells stained positive for a given marker was determined by the percentage of cells present within a gate, which was established such that fewer than 2% of positive events represented nonspecific binding by the PE/AF-488-conjugated isotype-specific control. Minimums of 10,000 events were counted for each analysis. All FACS analyses were performed using a BD LSR II

flow cytometer (BD Biosciences). FACS files were exported and analyzed using BD Cellquest software v.3.3.

RNA analysis

Total RNA and cDNA were prepared as previously described [8]. Regular RT-PCR was performed for 35 cycles (15 seconds at 94°C, 30 seconds at 60°C, 1 minute at 72°C, followed by 10 minutes at 72°C) using Advantage Polymerase Mix (Clontech) in a GeneAmp PCR system, model 9700 (PerkinElmer). The primers used for RT-PCR are listed in Supplemental Tables II and III. The products were analyzed by 2% agarose gel electrophoresis. Real-time PCR was performed as previously described [8]. The primers and probe used for real-time PCR for mouse vascular endothelial growth factor (VEGF), CD31, fetal liver kinase (Flk)1, and VE-cadherin, and human VEGF, CD31, and VE-cadherin were pre-designed and obtained from Applied Biosystems (Foster City, CA) as part of Taqman® Gene Expression Assays. Already prepared cDNA from human embryonic cell line HFS-1 was used as control.

Immunohistochemistry and immunocytochemistry

Tissue sections were processed and stained as previously described in detail [14, 15]. For immunocytochemistry, cells in glass chamber slides were fixed in 4% paraformaldehyde for 10 min at room temperature, washed 3 times with PBS, permeabilized with 0.2% Triton X-100 for 20 minutes, and washed 2 times with PBS. Non-specific antibody binding sites were blocked by incubating the cells for 60 minutes in blocking buffer (1% bovine serum albumin (BSA), 10% goat serum in PBS). Primary antibodies were diluted 1:200 in antibody buffer (1% BSA in PBS), and cells were incubated for overnight at 4°C with primary antibodies. The next day, cells were washed 3 times with PBS. Alexa Fluor 488-conjugated (green fluorescence) or Alexa Fluor 594-conjugated (red fluorescence) goat anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR) were applied to the cells and incubated for 60 minutes at room temperature. The cells were washed 4 times in PBS, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). A DAPI stock solution was diluted to 300 nM in PBS, and 300 µl of the diluted solution was added to the cells, making certain that they were completely covered. The cells were incubated for 15 minutes and rinsed 2 times in PBS. Staining without primary antibodies served as controls. Images were acquired with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY). To eliminate the possibility of false colocalization caused by emission filter bleed through, only images showing signals that were clearly visible by eye through the microscope when using the appropriate filters for the respective antibodies were considered significant and included in the results. Primary antibodies included rabbit anti-ß-galactosidase, (Molecular Probes), hamster anti-mouse CD31 and CD34 (Millipore/Chemicon), rabbit anti-VE-cadherin, anti-VEGF (both from Santa Cruz Biotechnology), rabbit anti-von Willebrand Factor (vWF) and mouse anti-human CD31 (both from Dako, Carpinteria, CA), goat anti-alpha-smooth muscle cell actin (ASMA) (Abcam), and rat monoclonal anti-mouse F4/80 (AbD Serotec). Biotinylated Isolectin B4 was obtained from the Vector Laboratories, and Streptavidin-R-phycoerythrin conjugates from Invitrogen. Staining with AdipoRed™ (Lonza) was performed as per manufacturer's instructions before immunofluorescence.

Immunoblotting

Immunoblotting was performed as previously described [14, 16]. Equal amounts of cellular protein or culture medium were used. Blots were incubated with specific antibodies to CD31 or VE-cadherin (400 ng/ml; from Santa Cruz Biotechnology). ß-Actin (1:5000 dilution; Sigma-Aldrich) was used as loading control.

Statistical Analysis

Data were analyzed for statistical significance by two-way analysis of variance with post hoc Tukey's analysis. The analyses were performed using the GraphPad Instat® 3.0 software (GraphPad Software). P-values less than 0.05 were considered significant. All experiments were repeated a minimum of three times.

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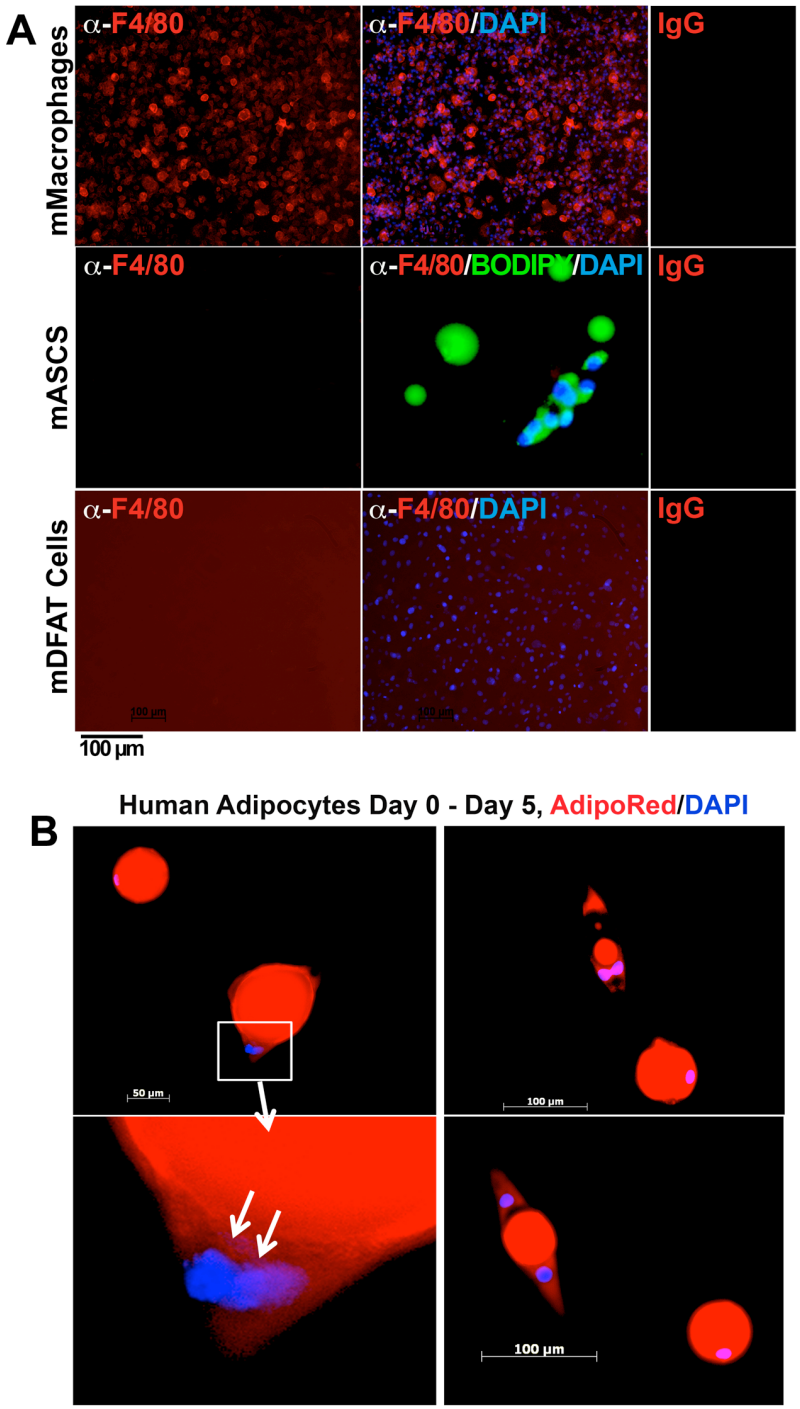
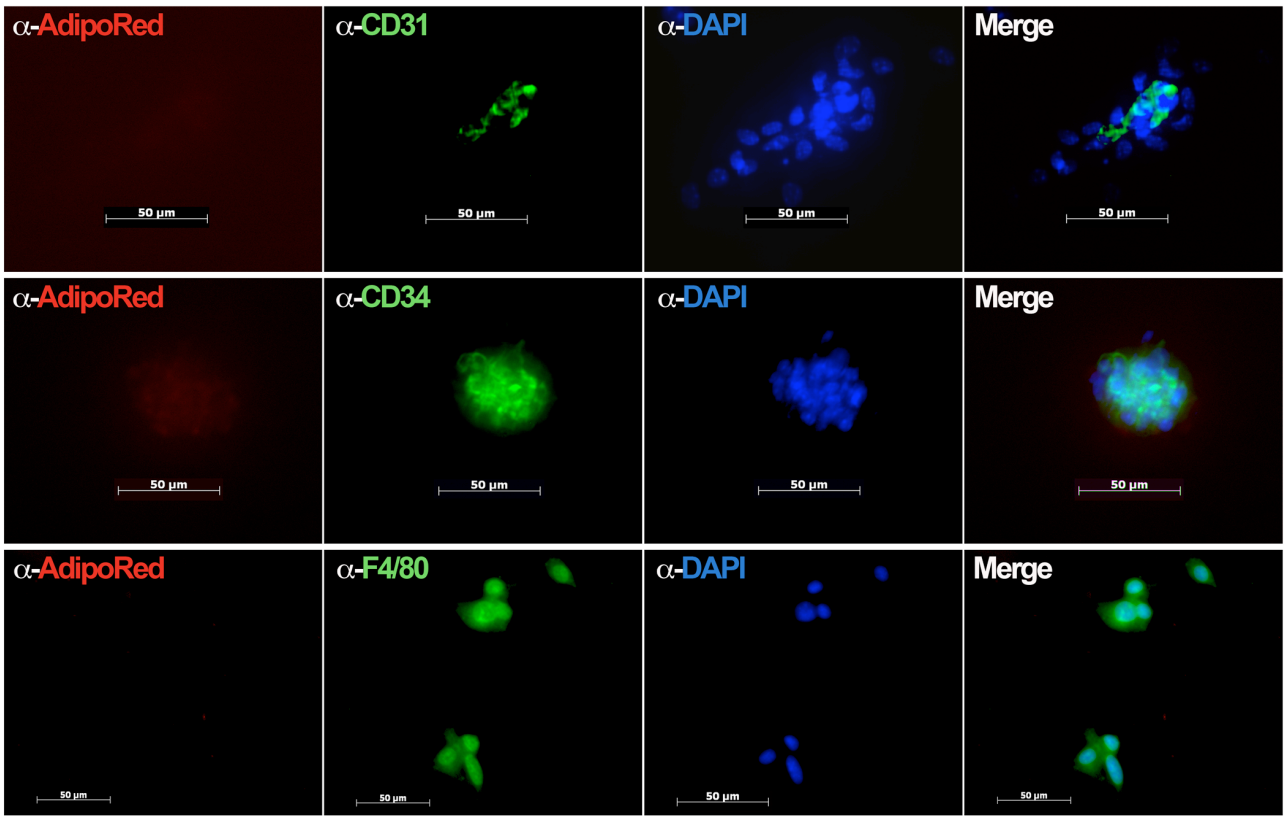


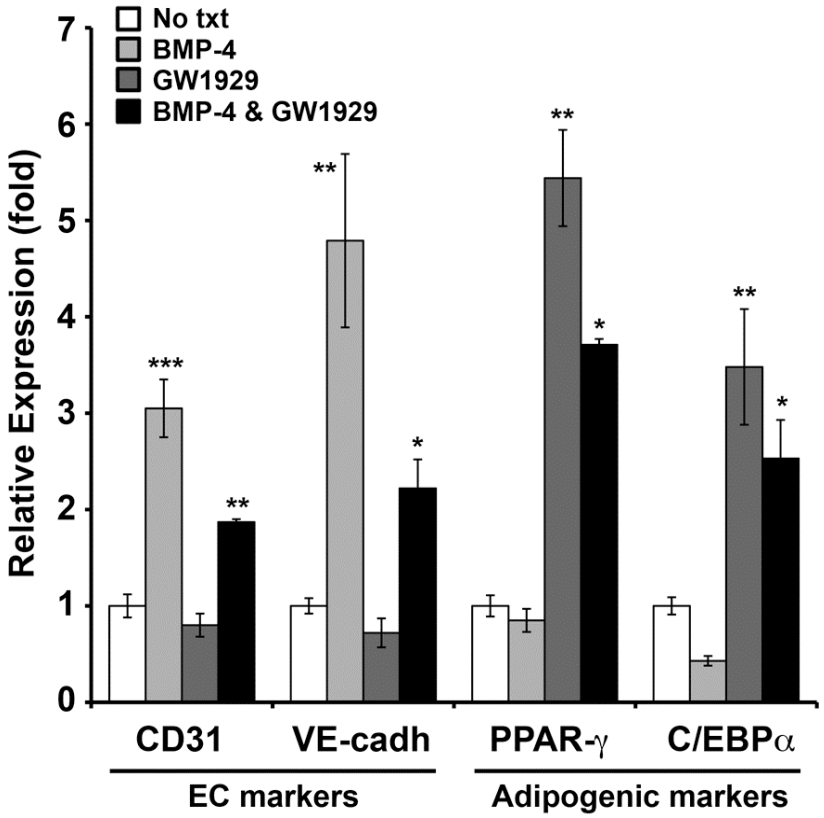
Supplemental Figure I
(A) Macrophage staining in mouse DFAT cells. Immunostaining for F4/80 (red), marker for mouse macrophages, in mFAT cells was negative; about 1,500-2,000 adipocytes were examined. Mouse macrophages were included as a positive control. DAPI (blue) was used to visualize nuclei.
(B) Bi-nucleated human adipocytes stained with AdipoRed and DAPI. The adipocytes appear to be in different stages of division.



Supplemental Figure II

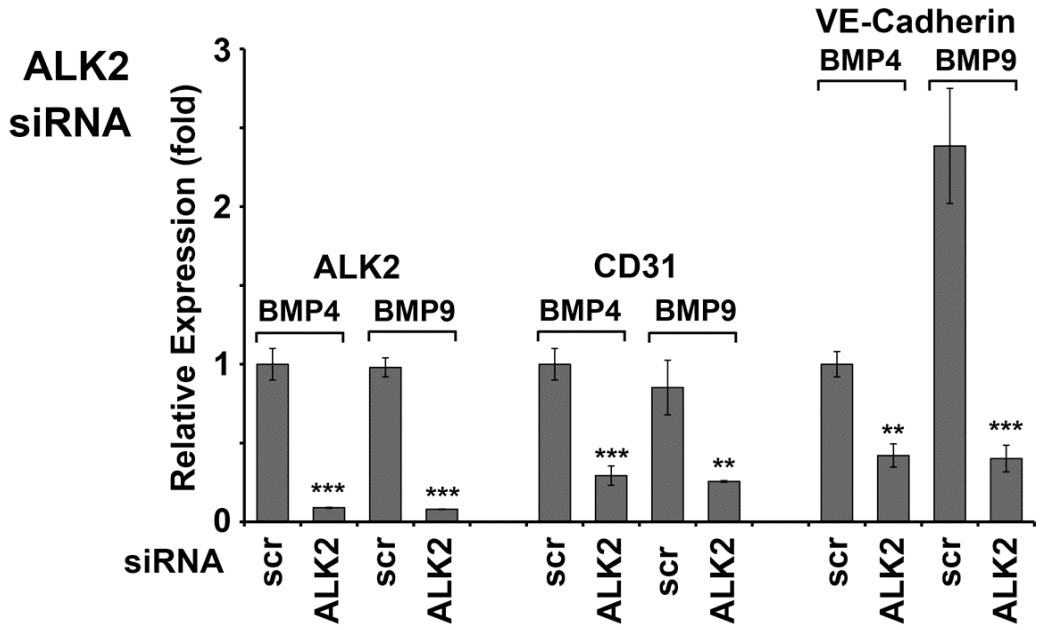
Endothelial cells and macrophages in mouse ASCs are AdipoRed-negative.

ECs and macrophages that were identified in mouse ASCs by immunostaining for CD31/34 (top and middle panels, green fluorescence) and F4/80 (bottom panels, green), respectively, did not stain with AdipoRed lipid stain (red). DAPI (blue) was used to visualize nuclei.



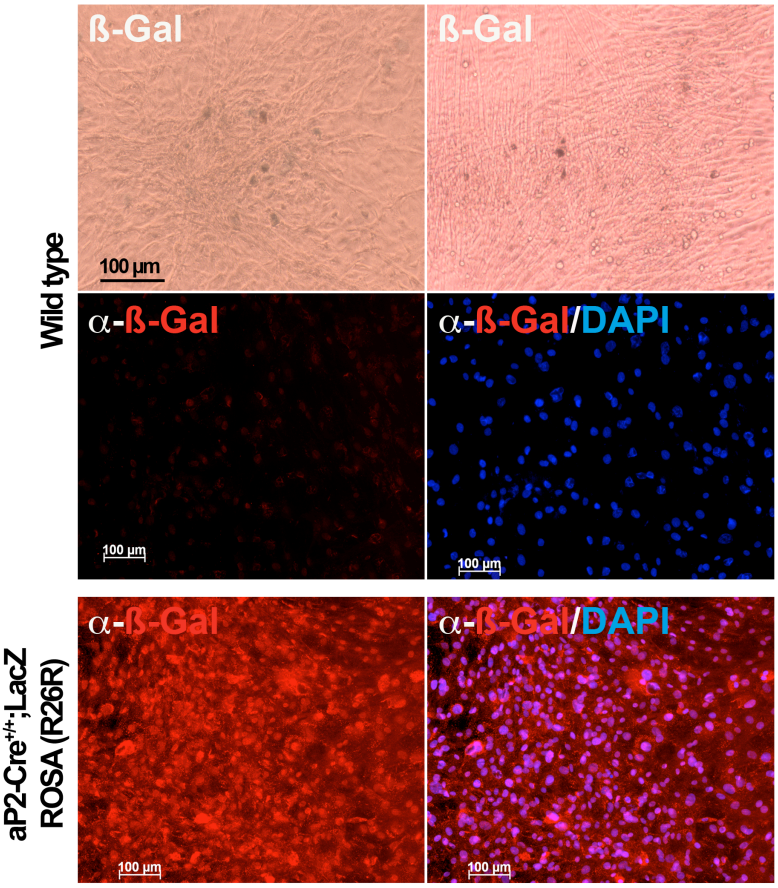
Supplemental Figure III

The PPAR-g agonist GW1929 modifies the effect of BMP-4 on EC and adipogenic differentiation in DFAT cells. Passaged DFAT cells were treated for 7 days with vehicle control, BMP-4 (50 ng/ml), GW1929 (100 ng/ml), or BMP-4 in combination with GW1929. Expression of EC markers (CD31 and VE-cadherin) and adipogenic markers (PPAR-γ and C/EBPα) was determined by real-time PCR. Asterisks indicate a statistically significant difference as compared to control treatment. *<0.05, **<0.01, ***<0.001 *Tukey's test*.



Supplemental Figure IV

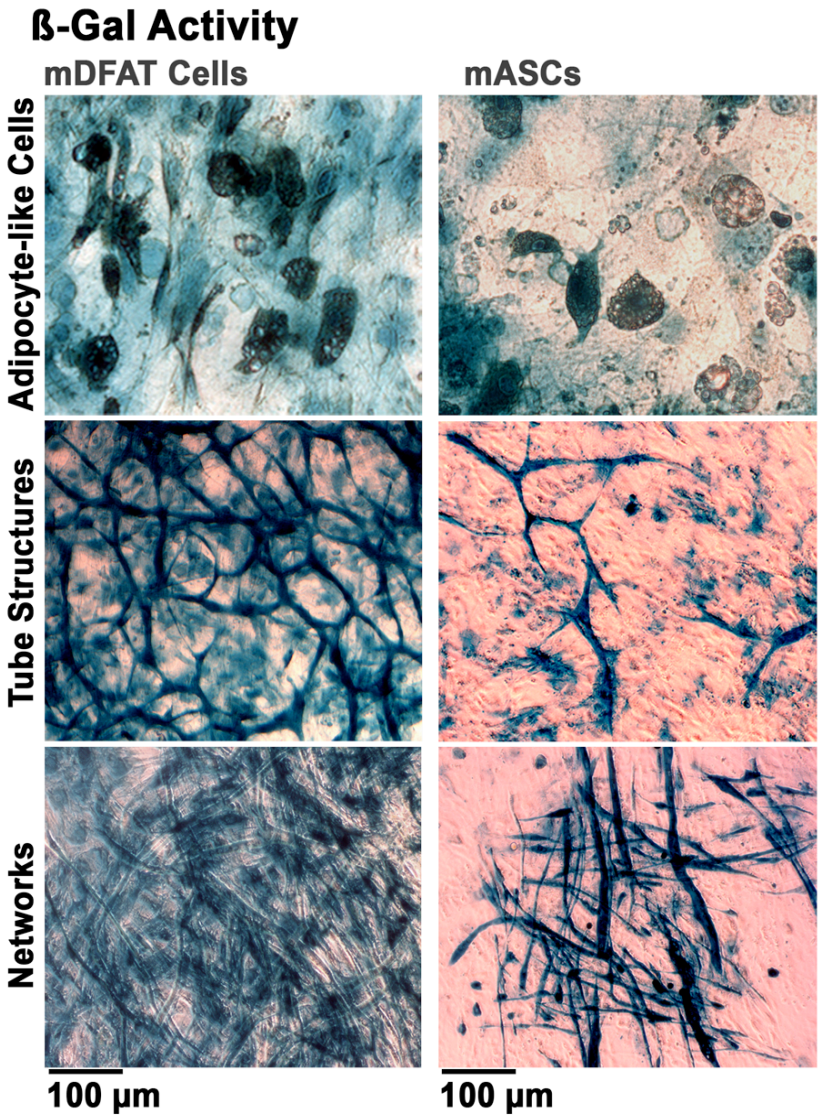
Effect of depletion of ALK2 on BMP-stimulated expression of endothelial lineage markers in DFAT cells. MDFAT cells were transfected with scrambled siRNA or siRNA to ALK2. BMP-4 or BMP-9 were added to the cells 24 hours after transfection, and expression of ALK2, CD31 and VE-cadherin was determined after 24 hours of treatment. Asterisks indicate a statistically significant difference between scrambled siRNA and ALK2 siRNA. **<0.01, ***<0.001 *Tukey's test*.



Supplemental Figure V

ß-Gal activity and immunostaining for ß-Gal in wild type cells.

ß-Gal activity and immunostaining (red) for ß-Gal in mDFAT cells from wild type mice (top 4 panels), and immunostaining for mDFAT cells from aP2-Cre$^{+/+}$;LacZ ROSA (R26R)$^{+/+}$ mice (bottom 2 panels). DAPI (blue) was used to visualize nuclei.



Supplemental Figure VI
Colonies formed in mouse DFAT cells and ASCs generated from aP2-Cre$^{+/+}$;LacZ ROSA (R26R)$^{+/+}$ double transgenic mice. ß-Gal staining in adipocyte-like colonies, and in cells participating in the formation of tubes and cardiomyocyte networks after 15 days of culture of unpassaged mDFAT cells and mASCs.

Supplemental Movie
The movie illustrates the proliferation of a single cell that was generated from a floating adipocyte and dropped to the bottom of the culture dish after about 24 hours.

Supplemental Table I.
Expression pattern of surface markers in human and mouse DFAT cells 5 days after adipocyte isolation

CD number	Alternative names	Adipocytes	DFAT Cells	Marker
CD34	L-selectin ligand	Negative	Negative	Endothelial progenitors
CD31	PECAM	Negative	Negative	Endothelial cells
CD45	LCA	Negative	Negative	Hematopoietic progenitors, leukocytes
CD90	Thy-1	Negative	++	Hematopoietic progenitors
CD133	Prominin 1	Negative	(+)	Neuroepithelial progenitors
CD14 (human) CD11b (mouse)	Mac-1	Negative	Negative	Macrophages
CD140b		Negative	Negative	Pericytes
NG2		Negative	Negative	Pericytes
CD146		Negative	Negative	Pericytes

Abbreviations:
CD, Cluster of Differentiation (Cluster of Designation); NG2, Neuron-glia antigen 2.

Supplemental Table II. Mouse Primer Sequences

Mouse Primers	Accession	Sequence (5′–3′)
CD31	NM_008816	ATTGGAGTGGTCATCGCCACCTTA
		AATCCAGGAATCGGCTGCTCTTCT
vWF	NM_011708	TCATCGCTCCAGCCACATTCCATA
		ACAGAAGCTAGTGTTCCCATCGCA
VE-cadherin	X83930	AACCATGACAACACCGCCAACATC
		TGAATCTCCAGCGCACTCTTGCTA
GAPDH	NM_008084	AACTTTGGCATTGTGGAAGGGCTC
		TGGAAGAGTGGGAGTTGCTGTTGA

Supplemental Table III. Human Primer Sequences

Human Primers	Accession	Sequence (5′–3′)
PPAR-γ	NM_138711	TCAAGACAACCTGCTACAAGCCCT
		AAGAAGGGAAATGTTGGCAGTGGC
CD34	NM_001773	AAGCCTAGCCTGTCACCTGGAAAT
		TGGCTTGCAACATCTTGCTCAGTG
CD31	NM_000442.4	CACAACAGACATGGCAACAAGGCT
		TCCTTCTGGATGGTGAAGTTGGCT
CD45	AY567999	TTGGCTTTGCCTTTCTGGACACAG
		AAGTGGAACACTGGGCATCTTTGC
CD90	NM_006288	TTCCAGAGCTGCTTCTGTCTGGTT
		TCCCTCACAAGGTAGGCCACAAAT
CD133	NM_001145847	TCTATGTGGTACAGCCGCGTGATT
		GCTGGTGCATTTCTCCACCACATT
CD14	NM_001174104	ACATAAACTGTCAGAGGCAGCCGA
		TCTTCATCGTCCAGCTCACAAGGT
CD140b	NM_002609	TTGTCCTCAATGTCTCCAGCACCT
		TCGGCATGGAATGGTGATCTCAGT
NG2	NM_001897	TTTCCGTGTCACAGCTCCACCATA
		ATCTACGGCCACGATACTGCCAAA
CD146	NM_006500	ACAAGACCAAGATCCACAGCGAGT
		ATGCACACAATCACAGCCACGATG
ASMA	NM_001141945	AAAGACAGCTACGTGGGTGACGAA
		ATAGCCACATACATGGCTGGGACA
VEGF	NM_001204384.1	TTTCTGCTGTCTTGGGTGCATTGG
		TCTGCATGGTGATGTTGGACTCCT
ß-actin	NM_001101	AGGCGGACTATGACTTAGTTGCGTTACACC
		AAGTCCTCGGCCACATTGTGAACTTTG