

A Protocol to Induce Brown and Beige Adipocyte Differentiation From Murine and Human Adipose-Derived SVF

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Abstract

Adipose cells vary functionally, with white adipocytes storing energy and brown/beige adipocytes generating heat. Mouse and human subcutaneous white adipose tissue (WAT)-derived stromal vascular fraction (SVF) provides mesenchymal stem cells (MSCs) that can be differentiated into thermogenic adipocytes using pharmacological cocktails. After six days of browning induction, these cells exhibited significant upregulation of thermogenic markers (*UCP1*, *Cidea*, *Dio2*, *PRDM16*) along with adipogenic genes (*PPAR γ* , *aP2*), showing enhanced thermogenic potential. This in vitro system offers a practical platform to study adipogenesis and thermogenic regulation.

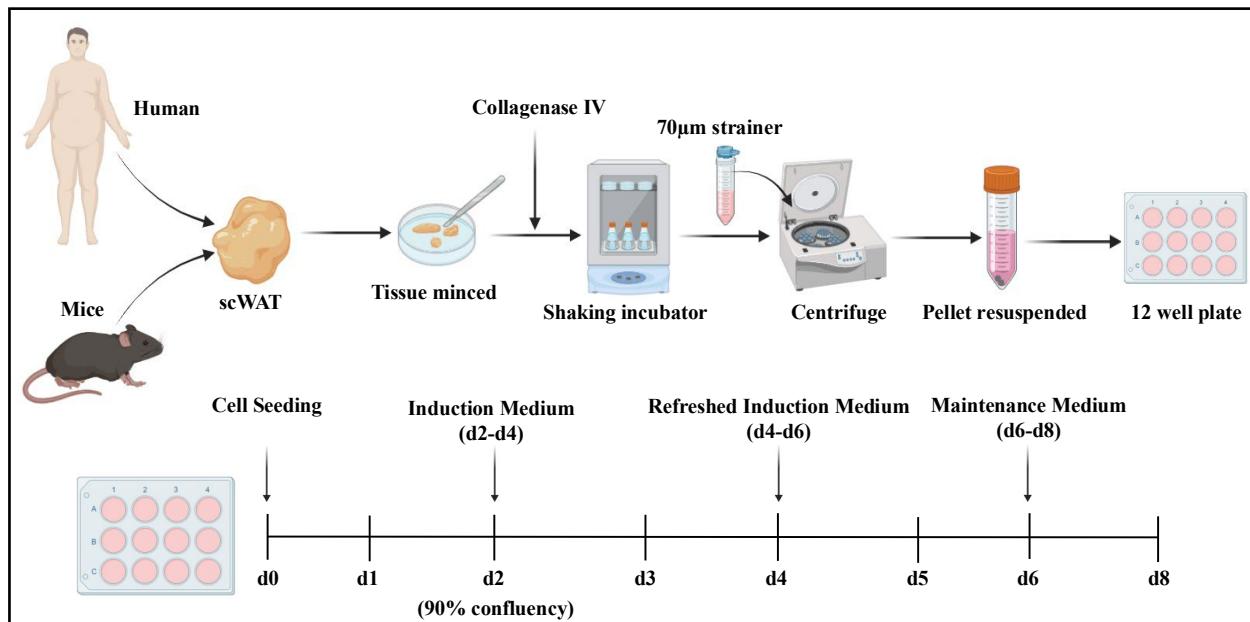
Key features

- The protocol aims to differentiate murine and human SVF from subcutaneous fat into brown/beige adipocytes for assays.
- This approach employs a straightforward methodology that effectively differentiates SVF cells using optimal chemicals, their concentrations, and durations.

Keywords: Adipocyte differentiation, Thermogenesis, SVF, Beige adipocytes, Brown adipocytes, MSCs

This protocol is used in: J Biol Chem (2020), DOI: 10.1074/jbc.RA119.012072

Graphical overview



Background

Obesity, as defined by WHO, is a chronic complex condition characterized by excessive adipose tissue that can adversely affect health [1]. Adipose tissue, often known as "fat," serves as a passive energy reservoir and is involved in various physiological functions, including the regulation of food consumption, energy balance, insulin production, immune response, and the maintenance of body temperature [2,3]. Mammals possess three different types of adipocytes—white, brown, and beige adipocytes—each located in specific anatomical regions and characterized by distinct gene expression profiles [4]. White adipose tissue (WAT) is extensively found in subcutaneous areas and surrounding internal organs. Its cellular structure is characterized by a prominent single lipid droplet, minimal mitochondria, and limited cytoplasm, serving primarily as an energy reservoir. Additionally, WAT functions as an endocrine organ, releasing adipokines like leptin, lipotropin, and TNF α , which play crucial roles in regulating energy metabolism and sustaining physiological balance [5]. Brown adipose tissue (BAT), on the other hand, has many mitochondria and multilocular lipid droplets and is heavily innervated and vascularized. Its major function is thermoregulation, which involves oxidizing fatty acids (FAs) to maintain body temperature [6]. Another form of adipose tissue, known as beige adipose, is found in WAT depots but exhibits brown-like characteristics including multilocular lipid droplets and high mitochondrial density [7,8]. Brown and beige adipocytes are often referred to as "thermogenic adipocytes." This process is facilitated by uncoupling protein 1 (UCP1) and is referred to as non-shivering thermogenesis [9,10].

Although beige adipocytes exhibit functional similarities to brown adipocytes, these two thermogenic cells differ in their developmental origins. In fact, beige adipocytes originate from the same progenitor cells as white adipocytes [11]. The process by which beige adipocytes are formed is commonly referred to as browning, typically occurring in subcutaneous fat deposits. Collectively, these findings indicate that both extrinsic and intrinsic cellular factors play a role in determining the fate of adipocyte differentiation. In this protocol, we used mouse and human stromal vascular fraction (SVF) isolated from fat depots to identify and evaluate methods for inducing differentiation into brown and beige adipocytes. Slight modifications were introduced in the earlier protocol [12] in our laboratory, where they have been replicated and refined to implement fundamental procedures with reduced toxicity and time interval. It includes the addition of optimized novel chemicals (1 nM T3 and 125 μM Indomethacine), decreased concentration of the earlier used compounds (20 nM insulin, 1 μM dexamethasone, and 0.5 μM IBMX), and shorter time duration for adipocyte differentiation.

Materials and reagents

Biological materials

1. Subcutaneous fat depots (scWAT) of mice
2. Subcutaneous fat depots (scWAT) of human

Reagents

1. Dulbecco's modified Eagle's medium (DMEM), high glucose (Corning, catalog number: 10-013-CV), store at 4 °C
2. Fetal bovine serum (FBS) (Thermo Fisher Scientific, catalog number: A5256801), store at -20 °C
3. Penicillin-streptomycin (Pen/Strep) (Thermo Fisher Scientific, catalog number: 15070063), store at -20 °C
4. Phosphate-buffered saline (PBS), 1× (Corning, catalog number: 21-040-CMX12)
5. Collagenase/dispase (Roche, catalog number: 10269638001)
6. TRIzol reagent (Fisher Scientific, catalog number: 15596018)
7. Chloroform (Sigma-Aldrich, catalog number: 472476)
8. Ultrapure DNase/RNase-free distilled water (nuclease-free water) (Fisher Scientific, catalog number: 10-977-023)
9. iScript™ cDNA Synthesis kit (Bio-Rad, catalog number: 1708890)
10. iQ™ SYBR Green Supermix (Bio-Rad, catalog number: 1708880)
11. Insulin (20 nM final concentration) (Sigma-Aldrich, catalog number: I1507), powder storage temperature -20 °C, store at 4 °C after dilution
12. Triiodothyronine (T3) (1 nM final concentration) (Sigma-Aldrich, catalog number: T2877), store at -20 °C
13. Indomethacin (125 µM final concentration) (Sigma-Aldrich, catalog number: I7378), store at -20 °C
14. Dexamethasone (1 µM final concentration) (Sigma-Aldrich, catalog number: D4902), store at -20 °C
15. Rosiglitazone (1 µM final concentration) (Sigma-Aldrich, catalog number: 557366-M), store at -20 °C
16. 3-Isobutyl-1-methylxanthine (IBMX) (0.5 µM final concentration) (Sigma-Aldrich, catalog number: I5879), store at -20 °C
17. Calcium chloride (Sigma-Aldrich, catalog number: C5670)
18. Isopropanol (Sigma-Aldrich, catalog number: 190764)

Solutions

1. Stock solutions: 0.5 mg/mL (87.2 µM) insulin, 1 mM T3, 100 mM Indomethacin, 10 mM Dexamethasone, 0.25 M IBMX, 10 mM Rosiglitazone, and 10 mM CaCl₂ (see Recipes)
2. Growth medium (see Recipes)
3. Induction medium (see Recipes)
4. Maintenance medium (see Recipes)
5. Reverse transcription mix (see Recipes)
6. qPCR mix (see Recipes)
7. Washing medium (see Recipes)

Recipes

1. Stock solutions

Reagent	Final concentration	Quantity or volume
Insulin	0.5 mg/mL (87.2 µM)	1 mg of insulin dissolved in 2 mL of distilled water (pH = 2, add 0.5 µL HCl 16N). Filter the stock solution through a 0.22 µm sterile filter.
T3	1 mM	1.3 mg of T3 dissolved in 2 mL of ethanol. <i>Note: T3 requires amber storage due to light sensitivity.</i>
Indomethacin	100 mM	71.558 mg of Indomethacin in 2 mL of ethanol. To help with solubilization, it may be heated to 75 °C for 1 min using a heating block.
Dexamethasone	10 mM	7.85 mg of Dexamethasone in 2 mL of ethanol. <i>Note: Dexamethasone requires amber storage due to light sensitivity.</i>
IBMX	0.25 M	55.56 mg of IBMX in 1 mL of ethanol. To help with solubilization, it may

		be heated to 75 °C for 1 min using a heating block.
Rosiglitazone	10 mM	7.15 mg of Rosiglitazone in 2 mL of ethanol. <i>Note: Rosiglitazone requires amber storage due to light sensitivity.</i>
CaCl ₂	10 mM	1.11 mg of CaCl ₂ (anhydrous) in 1 mL of sterile distilled water. Filter sterilize with 0.22 µm and store at 4 °C.

Note: Stocks are aliquoted and stored for 2–3 months at -20 °C, except for insulin (working vial), which needs to be stored at 4 °C. Avoid multiple freeze-thaw cycles to maintain the stability of the chemicals.

2. Growth medium

Reagent	Final concentration	Quantity or volume
DMEM, high glucose	90%	450 mL
FBS	10%	50 mL
Pen/Strep	1%	5 mL

3. Induction medium

Reagent	Final concentration	Quantity or volume
Growth medium		1 mL/well of 12-well plate
Insulin	20 nM	Diluted from stock in growth medium
T3	1 nM	Diluted from stock in growth medium
Indomethacin	125 µM	Diluted from stock in growth medium
Dexamethasone	1 µM	Diluted from stock in growth medium
IBMX	0.5 µM	Diluted from stock in growth medium
Rosiglitazone	1 µM	Diluted from stock in growth medium
Total	n/a	1 mL/well of 12-well plate

4. Maintenance medium

Reagent	Final concentration	Quantity or volume
Growth Medium		1 mL/well of 12-well plate
Insulin	20 nM	Diluted from stock in growth medium
T3	1 nM	Diluted from stock in growth medium
Total	n/a	1 mL/well of 12-well plate

5. Reverse transcription mix (per 1 sample)

Reagent	Volume
Nuclease-free water	Variable
iScript reaction mix (5×)	4 µL
iScript RT	1 µL
RNA (1 µg)	Variable
Total	20 µL

6. qPCR mix (per 1 sample)

Reagent	Volume
iQ™ SYBR Green Supermix	5 µL
Forward primer (final concentration 300–500 nM)	0.5 µL
Reverse primer (final concentration 300–500 nM)	0.5 µL
Nuclease-free water	2 µL
cDNA (100–500 ng)	2 µL
Total	10 µL

7. Washing medium

Reagent	Final concentration	Quantity or volume
PBS	1×	9.99 mL
Calcium (Ca ²⁺)	10 µM	10 µL of 10 mM CaCl ₂ stock solution
Total		10 mL

Laboratory supplies

1. Sterile forceps and scissors
2. 70 μ m cell strainer (BD Falcon, catalog number: 352350)
3. 12-well cell culture plate (Corning, catalog number: 12-565-321)
4. 0.22 μ m sterile filter (NalgeneTM, catalog number: 7262520)

Equipment

1. Heat block (Fisher Scientific, model: 88870001)
2. Tissue culture incubator (37 °C, 5% CO₂) (Eppendorf, New Brunswick, model: Galaxy 170R)
3. Water bath (MRC, model: SWBR27-2)
4. Centrifuge (Eppendorf, model: 5430R/refrigerated)
5. Tissue culture hood (Heal force: HF Safe-1200 LC Biosafety Cabinet, Class II, Type II)
6. Inverted microscope (Thermo Fisher Scientific, model: EVOS M3000)
7. Automated cell counter (Thermo Fisher Scientific, model: Countess 3)
8. PCR machine (Thermo Fisher Scientific, model: VeritiTM Thermal Cycler 96 well)
9. NanoDrop Lite Plus (Thermo Fisher Scientific, model: NDPLUSGL)
10. qPCR machine (Thermo Fisher Scientific, model: QuantStudio 3)
11. Shaking incubator (Helix)

Software and datasets

1. Graphpad Prism 9[®] software

Procedure

A. Isolation of stromal vascular fractions

1. Wash the dissected scWAT depots in 1× PBS supplemented with 1% Pen/Strep and transfer to another clean Petri dish with forceps. The carryover PBS helps to keep the tissue moist through the mincing process.
2. Mince the tissues with sterile scissors into small pieces until it is smooth. Insufficient mincing will result in larger tissue chunks, extended digestion, and ultimately reduced yield of precursors; prolonged mincing will cause unnecessary mechanical stress and may lower viability and/or adipogenic potential of isolated precursors.
3. Digest 1 g of tissue with 1 mg/mL type IV collagenase at 37 °C for 1 h in a shaking incubator (120 rpm). Stop digestion when the majority of the tissue chunks have disappeared and the solution appears cloudy. Usually, this step takes around 30–40 min.
4. Add 10 mL of washing medium to stop the enzymatic activity in the digestion mixture.
5. Pipette up and down to mix and filter this cell suspension in a 50 mL Falcon tube through a 70 μ m cell strainer to eliminate clumps of undigested cells.
6. Centrifuge the filtered suspension at 200 \times g for 10 min at room temperature.
7. The cells of the SVF pellet down. Carefully remove the supernatant and resuspend the pellet in growth medium.
8. Count the cells using an automated cell counter to ensure accurate and consistent results.

B. Seed cells

1. Day 0: Seed 1 \times 10⁵ SVF cells per well in a 12-well plate and culture in 1 mL of growth medium (see Recipes).
2. Change media every 2 days until confluent.

C. Brown/beige differentiation

1. (Day 2) Replace media with 1 mL of induction medium (see Recipes) and culture for 2 days.
2. (Day 4) Refresh with 1 mL of induction medium.
3. (Day 6) After 2 days, replace the media with 1 mL of maintenance medium (see Recipes). Replace maintenance medium after every 2 days.
4. (Day 8) Cells are fully differentiated and ready to use.

D. RNA extraction and cDNA synthesis

1. Discard media from the culture at day 8 of differentiation.
2. Wash cells twice with PBS.
3. Add 500 μ L per well of TRIzol and shake the 12-well plates with a plate mixer for 5 min.
4. Collect cell lysate from the wells into 1.5 mL tubes.
5. Add 100 μ L of chloroform and shake vigorously.
6. Centrifuge at 12,000 \times g for 15 min at 4 °C.
7. Transfer the top aqueous layer into new 1.5 mL tubes.
8. Add an equal amount of isopropanol (~250 μ L) and shake vigorously.
9. Incubate at 4 °C for 10 min.
10. Centrifuge at 12,000 \times g for 20 min at 4 °C.
11. Remove supernatant and wash with 75% cold ethanol.
12. Resuspend pellet and centrifuge at 10,000 \times g for 5 min at 4 °C.
13. Remove supernatant and air-dry the pellet.
14. Add 20 μ L of nuclease-free water into the pellet-containing tube.
15. Dry bath at 65 °C for 10 min.
16. Measure total RNA concentration with NanoDrop Lite plus spectrophotometer.
17. Mix RNA (1 μ g) with reverse transcription mix (see Recipes) in a 96-well plate and run reverse transcription with the PCR machine (Table 1).

Table 1. Reverse transcription setup

Step	Temperature	Time	Cycles
Incubation	42 °C	60 min	1
Termination	70 °C	5 min	1
Storage	-20 °C	hold	

E. qPCR

1. Dilute the reverse-transcribed cDNA solution (~500 ng), as appropriate for your downstream application, with nuclease-free water.
2. Mix the 2 μ L diluted cDNA solution with qPCR mix (see Recipes) in 96-well plates in triplicate ($n = 3$) for qPCR and run qPCR with the qPCR machine (Table 2).

Table 2. qPCR setup

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	15 s	40
Annealing	60 °C	30 s	40
Extension	72 °C	30 s	40

3. Calculate gene expression level according to the $\Delta\Delta Ct$ method.
4. Calculate the difference in Ct values (ΔCt) between the gene of interest and the housekeeping gene (*36B4*).
5. Calculate the difference between the ΔCt of the experimental samples and the ΔCt of the control sample (ethanol).

Data analysis

Here, we describe an in vitro brown/beige differentiation protocol using murine and human SVF. In addition to the cells described here, this protocol has also been applied to the murine preadipocyte cell line C3H10T1/2 [15]. The protocol typically results in 70%–90% of cells exhibiting cytoplasmic lipid droplets, although the proportion of differentiated cells and the size of the lipid droplets largely depend on factors such as the FBS lot and the type, passage, commitment, and confluence of the adipocytes. To illustrate the protocol's efficiency, we used subcutaneous white adipose tissue (scWAT) from both mice and humans and differentiated the SVF with a brown/beige differentiation cocktail. This cocktail induced the expression of thermogenic genes *UCP1*, *Cidea*, *Dio2*, and *PRDM16* in both murine and human SVF (Figure 1). It also upregulated pan-adipocyte markers *PPAR γ* and *aP2* (Figure 2). Since classic thermogenic marker *UCP1* was induced in brown-like differentiated adipocytes, we further determined their functionality by assessing the mitochondrial genes on day 8. The expression of key brown fat functionality markers such as *Elovl3*, *Mcad*, *Cpt1a*, *CytC*, and *Cox4 β* was significantly induced, confirming that these cells exhibit a highly functional thermogenic phenotype (Figure 3). The primer sequences used for RT-PCR (5' to 3') are summarized in Table S1.

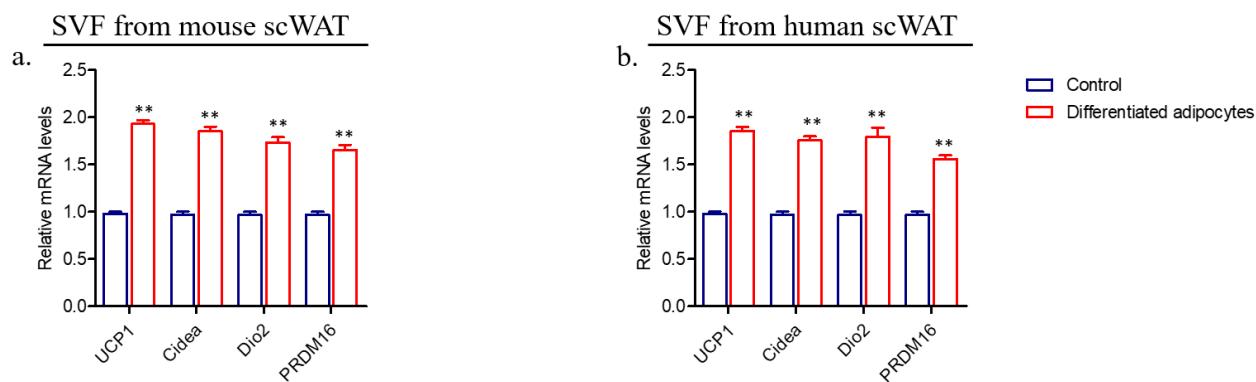


Figure 1. Gene expression analysis of brown/beige selective genes in control and differentiated adipocytes. Relative mRNA levels of *UCP1*, *Cidea*, *Dio2*, and *PRDM16* in brown-like differentiated adipocytes of stromal vascular fraction (SVF) derived from subcutaneous white adipose tissue (scWAT) of mouse (a) and human (b) ($n = 3$). *36B4* mRNA expression was used for normalization. Results are expressed as a mean \pm SD; different groups were compared via Student's t-test or by one-way ANOVA; *p-value < 0.05, **p-value < 0.005, ***p-value < 0.001. Primer sequences are available upon request.

Adipocytes differentiated using this protocol can be applied in multiple experimental settings. In our original research article where this protocol was employed [13–15], we performed gene expression analysis, oxygen consumption measurements, and fluorescence imaging microscopy using the differentiated adipocytes. SVF-derived adipocytes displayed brown/beige-like features, including induction of a thermogenic gene program, increased mitochondrial content, multilocular lipid accumulation (Figure 4), and elevated oxygen consumption rate with high uncoupled respiration. All data, along with information about data processing, statistical analyses, and details of replicates, are provided in the original research article [13].

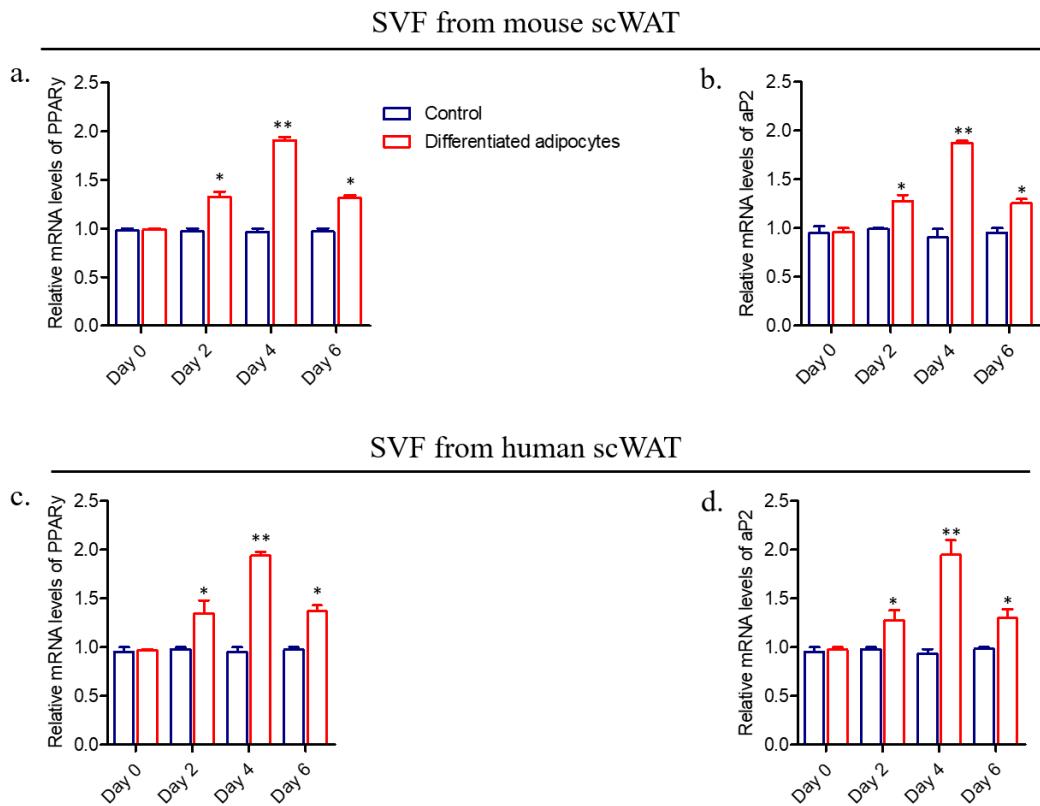


Figure 2. Expression of adipogenic markers in brown-like differentiated adipocytes. Relative mRNA levels of *PPARγ* (a, c) and *aP2* (b, d) in mouse and human subcutaneous white adipose tissue (scWAT)-derived stromal vascular fraction (SVF) differentiated into brown-like adipocytes ($n = 3$). *36B4* mRNA expression was used for normalization. Results are expressed as mean \pm SD; different groups were compared via Student's t-test or by one-way ANOVA; *p-value < 0.05, **p-value < 0.005, ***p-value < 0.001. Primer sequences are available upon request.

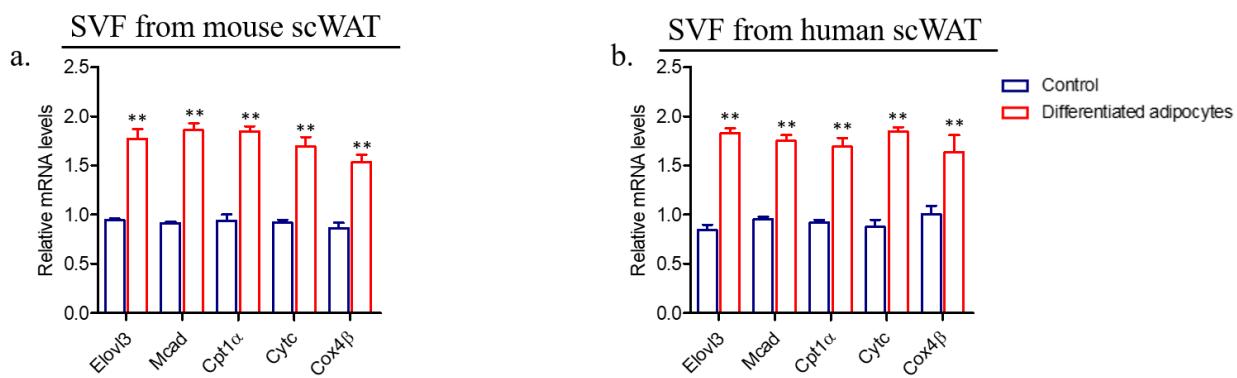


Figure 3. Expression of the key brown fat functionality markers in control and differentiated adipocytes. Relative mRNA levels of *Elov3*, *Mcad*, *Cpt1α*, *CytC*, and *Cox4β* in brown-like differentiated adipocytes of stromal vascular fraction (SVF) derived from subcutaneous white adipose tissue (scWAT) of mouse (a) and human (b) ($n = 3$). *36B4* mRNA expression was used for normalization. Results are expressed as mean \pm SD; different groups were compared via Student's t-test or by one-way ANOVA; *p-value < 0.05, **p-value < 0.005, ***p-value < 0.001. Primer sequences are available upon request.

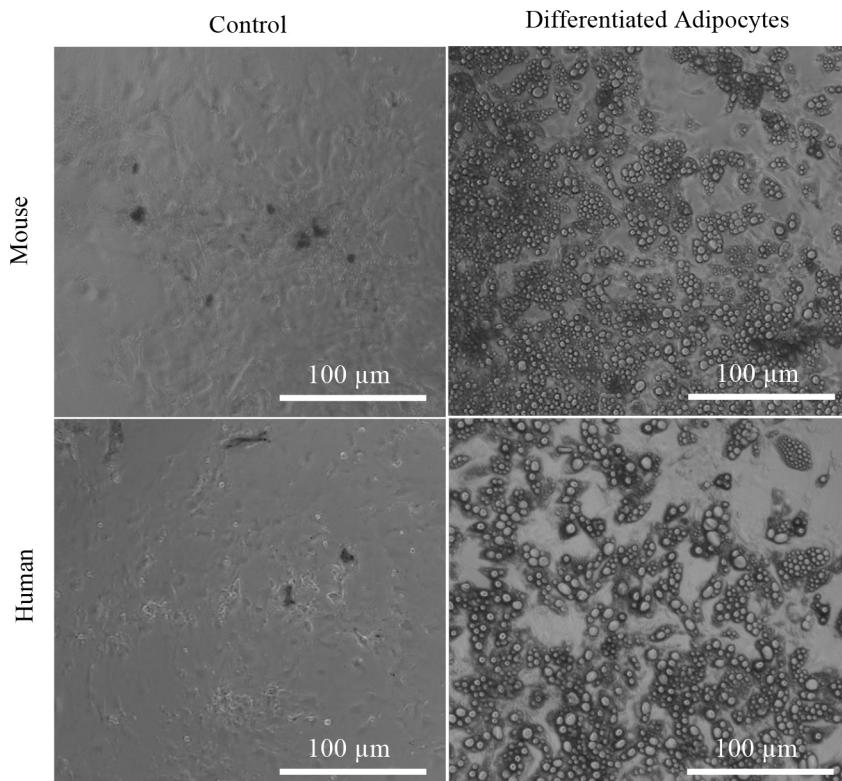


Figure 4. Brightfield microscopy images of brown-like adipocytes differentiated from subcutaneous white adipose tissue (scWAT) -derived stromal vascular fraction (SVF) of mouse and human (day 8). Ethanol was used as control. Magnification, 20×; scale bar represents 100 μm.

Validation of protocol

This protocol or parts of it has been used and validated in the following research article(s):

- Verma et al. [13]. The mRNA levels of heat shock factor 1 are regulated by thermogenic signals via the cAMP-dependent transcription factor ATF3. *Journal of Biological Chemistry*, 295(18), 5984–5994. DOI: 10.1074/jbc.RA119.012072 (Figure 1c).
- Perie et al. [14]. Transcriptional regulation of ZNF638 in thermogenic cells by the cAMP response element binding protein in male mice. *Journal of the Endocrine Society*, 3(12), 2326–2340 (Figure 1d).

General notes and troubleshooting

General notes

1. Ensure consistent timing for tissue digestion to avoid over- or under-digestion, which can compromise yield and cell surface marker integrity.
2. Be precise about the ingredients (e.g., buffer or media), quantities, and conditions established for your experiments.
3. Stocks should be aliquoted and stored for 2–3 months at -20 °C, except for insulin (working vial), which needs to be stored at 4 °C. Avoid multiple freeze-thaw cycles to maintain the stability of the chemicals.
4. Prewarm media to 37 °C for 20 min prior to the procedure.
5. SVF isolation and differentiation should be performed in a BSL-2 cabinet, RNA extraction should be performed on ice or in a cold room, and the TRIzol step should be performed in a fume hood.
6. If using this protocol in female mice, optimize digestion and gating parameters accordingly.

Troubleshooting

Problem 1: Low cell yields after digestion.

Possible causes: Incomplete digestion or poor tissue mincing and handling.

Solutions: Ensure uniform mincing of adipose tissue and verify collagenase activity. Extend digestion time slightly if needed.

Problem 2: Low differentiation efficiency (few lipid droplets).

Possible cause: Too low cell confluence at induction.

Solutions: Differentiate at 90% confluence. Use fresh, properly aliquoted induction cocktails (e.g., IBMX, dexamethasone, insulin, rosiglitazone, etc.).

Supplementary information

The following supporting information can be downloaded [here](#):

1. Table S1. Primer sequences

Acknowledgments

Authors' contribution: Conceptualization, N.V.; Investigation, R.R.Y., N.M., N.V.; Writing—Original Draft, N.V.; Writing—Review & Editing, N.V., R.R.Y., N.M.; Funding acquisition, N.V.; Supervision, N.V.

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Competing interests

The authors declare no competing interests.

Ethical considerations

All animal work was approved under IACUC protocol ID IA16-01767 by the NYU Grossman School of Medicine, Institutional Animal Care and Use Committee.

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