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Isolation and Differentiation of Primary White and Brown Preadipocytes from Newborn Mice

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TITLE:

Isolation and Differentiation of Primary White and Brown Preadipocytes from Newborn Mice

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KEYWORDS:

White adipocytes, brown adipocytes, mouse, primary culture, adipogenesis, obesity, diabetes, metabolic disease

SUMMARY:

This report describes a protocol for the simultaneous isolation of primary brown and white preadipocytes from newborn mice. Isolated cells can be grown in culture and induced to differentiate into fully mature white and brown adipocytes. The method enables genetic, molecular, and functional characterization of primary fat cells in culture.

ABSTRACT:

The understanding of the mechanisms underlying adipocyte differentiation and function has greatly benefited from the use of immortalized white preadipocyte cell lines. These cultured cell lines, however, have limitations. They do not fully capture the diverse functional spectrum of the heterogenous adipocyte populations that are now known to exist within white adipose depots. To provide a more physiologically relevant model to study the complexity of white adipose tissue, a protocol has been developed and optimized to enable simultaneous isolation of primary white and brown adipocyte progenitors from newborn mice, their rapid expansion in culture, and their differentiation in vitro into mature, fully functional adipocytes. The primary advantage of isolating primary cells from newborn, rather than adult mice, is that the adipose depots are actively developing and are, therefore, a rich source of proliferating preadipocytes. Primary preadipocytes isolated using this protocol differentiate rapidly upon reaching confluence and become fully mature in 4–5 days, a temporal window that accurately reflects the appearance of developed fat pads in newborn mice. Primary cultures prepared using this strategy can be expanded and studied with high reproducibility, making them suitable for genetic and phenotypic screens and enabling the study of the cell-autonomous adipocyte phenotypes of genetic mouse models. This protocol offers a simple, rapid, and inexpensive approach to study the complexity of adipose tissue in vitro.

INTRODUCTION:

Obesity results from a chronic imbalance between energy intake and energy expenditure. As obesity develops, white adipocytes undergo a massive expansion in cell size that results in hypoxia in the microenvironment, cell death, inflammation, and insulin resistance[1]. Dysfunctional, hypertrophied adipocytes cannot properly store excess lipids, which accumulate instead in other tissues where they dampen insulin action[2,3]. Agents that improve adipocyte function and restore normal lipid partitioning amongst tissues are predicted to be beneficial for the treatment of obesity-associated conditions characterized by insulin resistance such as type 2 diabetes. Phenotypic screens in adipocytes using immortalized cell lines, such as 3T3-L1, F442A, and 10T ½, have proven useful to identify genetic factors that regulate adipogenesis and to isolate pro-adipogenic molecules with anti-diabetic properties[4-7]. These cell lines, however, do not fully reflect the heterogeneity of cell types present in adipose depots, which includes white, brown, beige, and other adipocyte subtypes with unique characteristics, all of which contribute to systemic homeostasis[8-10]. Further, cultured cell lines often show a diminished response to external stimuli.

In contrast, cultures of primary adipocytes recapitulate more accurately the complexity of in vivo adipogenesis, and primary adipocytes show robust functional responses. Primary preadipocytes are typically isolated from the stromal vascular fraction of adipose depots of adult mice[11-14]. However, because the adipose depots of adult animals consist primarily of fully mature adipocytes that have a very slow turnover rate[15-17], this approach yields a limited quantity of preadipocytes with a low proliferation rate. Therefore, isolation of preadipocytes from newborn mice is preferable to obtain large quantities of rapidly growing cells that can be differentiated in vitro. Here, a protocol has been described, inspired by the initial work with primary brown adipocytes of Kahn et al.[18] to efficiently isolate both white and brown preadipocytes that can be expanded and differentiated in vitro into fully functional primary adipocytes (**Figure 1A**). The advantage of isolating primary cells from newborn, as opposed to adult mice, is that the adipose depots are rapidly growing and are thus a rich source of actively proliferating preadipocytes[17]. Cells isolated using this protocol have high proliferative capacity, enabling rapid scale-up of cultures. In addition, preadipocytes from newborn pups display higher differentiation potential than adult progenitors, which reduces well-to-well variability in the extent of differentiation and thus increases reproducibility.

PROTOCOL:

This protocol follows all IACUC guidelines of The Scripps Research Institute.

1. Collection and digestion of adipose depots (day 1)

1.1. Prepare two 1.5 mL tubes for each pup: one for brown adipose tissue (BAT) and one for white adipose tissue (WAT). Add 250 µL of phosphate-buffered saline (PBS) + 200 µL of 2x isolation buffer (123 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin-streptomycin, and 4% fatty acid-free bovine serum albumin) to each tube. Keep solutions sterile and on ice.

1.2. Place the pups into small chambers (e.g., one well of a 6-well plate), and set them on ice until they become hypothermic. Make sure that there is no direct contact between the pups and the ice. Prick a paw with a tip to assure loss of consciousness, and euthanize the pups by decapitation using sharp scissors.

NOTE: if working with different genotypes, prepare an additional tube to collect a tail biopsy (3 mm cut) for genotyping. If genotyping is performed immediately, keep the euthanized pups on ice until the dissection to collect adipose depots.

1.3. Calculate and weigh out the amount of collagenase needed to digest all the depots. Add 50 µL of 15 mg/mL collagenase type I in 2x isolation buffer to each tube. Do not resuspend the tissue in isolation buffer until all the tissues have been collected.

1.4. To collect subcutaneous WAT, cut the skin around the pup's abdomen (avoid peritoneal rupture), and gently pull the skin down below the legs. Without taking the skin, carefully collect the fat depot, which will appear as a clear (P1 or younger) or white (P2 and older), thin, elongated tissue attached on the inside or on top of the quadriceps (**Figure 1B**). Rinse the fat depot in PBS, and place it in one of the tubes containing 250 µL of PBS + 200 µL of 2x isolation buffer. Keep on ice.

NOTE: P0 and P1 mice give the best yield. In P0–1 pups, the subcutaneous WAT depot is nearly transparent. In P2 mice and older, the depot is easier to identify because it is already turning white, indicating the presence of fully mature, lipid-loaded, white adipocytes.

1.5. To collect interscapular BAT, pull the skin from the shoulder blades over the head. Lift the BAT—the deep red tissue between the shoulder blades—and carefully make incisions all around it to detach it from the body (**Figure 1B**). Check for consistency and color; rinse with PBS; place it in the other tube containing 250 µL of PBS + 200 µL of 2x isolation buffer; and keep on ice.

NOTE: When harvesting BAT from P2 mice and older, carefully remove the white adipose tissue surrounding the BAT. It consists of a thin, soft, white sheet of adipocytes located between the skin and the BAT, which is deeper between the shoulder blades.

1.6. Once all depots have been collected, gently mince each depot (4–6 times) using small scissors directly inside the tubes.

1.7. Resuspend collagenase type I in the appropriate volume of 2x isolation buffer to obtain a 10x stock solution at 15 mg/mL. Add 50 µL of 10x collagenase to each tube, keeping the tubes on ice until collagenase has been added to all the tubes.

1.8. Invert the tubes quickly to mix, and transfer to a temperature-controlled mixer. Incubate the samples for 30 min at 37 °C on a shaker (1,400 rpm frequency for effective sample mixing).

2. Plating preadipocytes (day 1)

2.1. After digesting the tissues, place the tubes back on ice.

NOTE: From this step onwards, all work is carried out in sterile conditions in a biosafety cabinet.

2.2. Strain the digested tissues through a 100 µm cell strainer into new 50 mL tubes. If working only with WT mice, or if genotypes are known, pool the relevant, dissociated BATs together; repeat this for the WATs. To maximize the cell yield, rinse each tube with 1 mL of isolation medium (Dulbecco's modified Eagle medium (DMEM) + 20% fetal bovine serum (FBS), 10 mM HEPES, 1% penicillin-streptomycin), and filter it through the cell strainer. If working with unknown genotypes, go to step 2.4

NOTE: FBS is an essential determinant of both preadipocyte proliferation and differentiation. Rigorously test different lots of FBS to ensure high performance and consistency between lots.

2.3. Dilute BAT and WAT suspensions to plate 2–3 wells of a 6-well plate for each BAT sample and 4–6 wells of a 6-well plate for each WAT sample. For instance, if 6 BATs and 6 WATs were pooled together, dilute the BAT cell suspension up to 24–36 mL and the WAT cell suspension up to 48–72 mL. Plate 2 mL of the cell suspensions per well.

2.4. For samples with unknown genotypes, keep each sample separate; place a 100 µm cell strainer on top of each well of a 6-well plate, and filter one sample per well. Rinse the tube with 1.5 mL of isolation medium, and pass it through the strainer, making up the final volume to 2 mL per well. Discard the cell strainer, place the lid on the plate, and transfer the plate to the incubator.

NOTE: The medium in the wells should look turbid 1–1.5 h after plating due to floating blood cells, cell debris, and lipids from lysed cells.

2.5. Aspirate the medium, and wash with 2 mL of DMEM without serum. Gently agitate the plate to detach blood cells from the bottom of the wells. After 3 washes, add 2 mL of fresh isolation medium, and transfer the cells to the incubator (37 °C, 5% CO_2).

NOTE: Check the cells under the microscope. Floating material (blood cells, cellular debris) should be minimal. Brown preadipocytes will appear as small non-translucent cells, whereas white preadipocytes will display a more elongated form. Both brown and white preadipocytes should be tightly attached to the plate.

3. Expansion of preadipocyte culture (day 2 to day 5)

3.1. On the next day, aspirate the medium, wash the cells with 2 mL of DMEM without serum, and add back 2 mL of the isolation medium.

3.2. Repeat step 3.1 once every 2 days until the cells reach 80–90% confluence.

NOTE: For primary brown preadipocytes, reaching 80–90% confluence can take 4–5 days. For primary white preadipocytes, it usually takes 2–3 days. Once the preadipocytes reach ~60% confluence, they can be efficiently infected with viral particles for knockdown or overexpression experiments. Infect preadipocytes with an appropriate viral load for up to 8 h. The use of cationic polymers to increase infection efficiency is discouraged, as it often results in toxicity and in a significant reduction of the adipogenic potential of infected preadipocytes.

3.3. To split the cells, coat new destination plates using a sterile solution of 0.1% w/v gelatin (dissolved in distilled water; do not use any heat). Use enough volume to cover the bottom of the well/dish. Incubate plates at 37 °C until the cells are ready to be seeded (at least 10 min).

NOTE: This step is optional. Coating of cell culture plates does not affect the yield or differentiation potential, but it substantially simplifies the maintenance and handling of differentiating adipocytes.

3.4. When cells reach sub-confluent density (85–95%), aspirate the medium, wash with PBS, and add trypsin for 3 min to detach the cells. Block trypsin activity by adding 2.5x trypsin volume of isolation medium. Pipette the cell suspension up and down to maximize cell recovery, and transfer into a new tube. Wash the wells with 1 mL of isolation medium, and add it to the first collection.

3.5. Centrifuge the cells for 5 min at 800–1200 × *g*, aspirate the supernatant, and resuspend the cells in 3–5 mL of the isolation medium. Count the cells, and dilute them to the desired final density.

NOTE: For instance, 50,000–80,000 cells/mL (2.5, 1, and 0.5 mL for 6-, 12- and 24-well plates, respectively) will result in fully confluent wells within 72–96 h.

3.6. Aspirate the coating solution in step 3.3, and wash off excess gelatin with PBS. Seed the cells, and return the plates to the incubator until fully confluent.

4. Differentiation of white and brown preadipocytes (days 7–12)

4.1. When preadipocytes become confluent, aspirate the medium, and replace it with differentiation medium consisting of 10% FBS in DMEM containing 170 nM insulin, 1 µM dexamethasone, and 0.5 mM 3-isobuthyl-1-methylxantine. If differentiating BAT preadipocytes, also add 1 nM triiodothyronine (T3). Mark the day on which adipogenic differentiation is induced as day 0 of differentiation.

NOTE: Both white and brown preadipocytes can spontaneously differentiate upon reaching confluence. However, to maximize adipocyte differentiation, the traditional pro-adipogenic cocktail described above (plus T3 for BAT preadipocytes) should be used.

4.2. After 48 h (day 2 of differentiation), observe small lipid droplets accumulating in the cells under the microscope using standard bright field. Refresh the medium with maintenance medium consisting of 10% FBS in DMEM and 170 nM insulin (plus 1 nM T3 for BAT).

NOTE: On day 4 or 5 of differentiation, the majority of cells will appear loaded with lipids. Terminally differentiating adipocytes can be kept in culture longer or used at this stage for experiments.

5. Re-plating for bioenergetics experiments

NOTE: If the intention is to perform bioenergetics studies in mature adipocytes in the 96-well format, the following steps need to be taken. Ideally, cells that have just fully differentiated (day 4 or 5) should be used. The procedure described below starts from 1 well of a 6-well plate.

5.1. Prior to trypsin digestion, prepare coating for 96-well plates. Add 50 µL of 0.1% gelatin to each well. Leave the plate in a tissue culture incubator until the cells are ready to be seeded.

5.2. On day 4 or 5 of differentiation, aspirate the medium, wash with 1 mL of PBS, and add 300 µL of 0.25% trypsin-ethylenediamine tetraacetic acid (for 1 well of a 6-well plate). Gently tilt the plate to ensure trypsin completely covers the bottom of the well; incubate for 2 min at room temperature. Block the action of trypsin with 700 µL of maintenance medium, pipette up and down to maximize cell recovery, and transfer the cell suspension into a new 1.5 mL tube.

5.3. Centrifuge the cells at 1200 × *g* for 5 min. Remove the supernatant, resuspend the pellet in 1 mL of maintenance medium, and count the cells.

NOTE: One well of a 6-well plate should yield approximately 1.5–1.8 million cells.

5.4. Dilute the cells to have a final concentration of 60,000 to 80,000 cells/mL for brown adipocytes and 100,000 to 120,000 cells/mL for white adipocytes. Plate 100 µL of the cell suspension per well in gelatin-coated 96-well plates.

NOTE: One well of a 6-well plate provides enough cells for up to two 96-well plates. For bioenergetics experiments, low-density plating (30–40% confluence) is needed to enable the accurate measurement of oxygen consumption and avoid oxygen depletion in the wells during the assay.

5.5. Allow the cells to adhere to the bottom of the plate for at least 48 h. If cells are kept in the plate for more than 48 h, refresh the medium after 2 days.

5.6. On the day of the assay, aspirate the medium, and replace it with assay medium. Incubate for 1 h at 37 °C in a non-CO_2-controlled incubator, and perform the assay as per the manufacturer's instructions.

REPRESENTATIVE RESULTS:

Section 1 of the protocol will yield a heterogeneous suspension of cells that are visible under a standard light microscope. Filtering of digested tissues with a cell strainer (section 2) will remove undigested tissue. However, some cellular debris, blood cells, and mature adipocytes will pass through (**Figure 1C**). Gentle washes 1 h after plating will remove non-relevant cells as preadipocytes attach rapidly to the bottom of the well (**Figure 1C**). In section 3, adipocyte precursors are expanded to obtain the number of cells required for the experimental plan. Although both white and brown preadipocytes isolated from newborn pups have high proliferative capacity, the yield of white preadipocytes is generally twice that of brown preadipocytes on a per-depot basis (**Figure 2A**). Therefore, if synchronized cultures are desired, the starting density of white preadipocytes must be calculated accordingly. Section 4 offers guidelines to obtain fully mature adipocytes.

At the end of differentiation, cells will appear loaded with lipid droplets and express classical markers of white and brown adipocytes, respectively (**Figure 2B**,**C**). Both white and brown adipocytes can be used for bioenergetics studies as described in section 5. A comparative analysis of oxygen consumption in a mitochondrial stress test of primary white and brown adipocytes under basal conditions, as well as in response to known stimulators of mitochondrial function (e.g., norepinephrine) is shown in **Figure 2D**. Upon isolation, it is also possible to differentiate primary white and brown adipocytes on the plates that will be directly used for bioenergetic experiments[19]. In this case, preadipocytes are isolated and plated as described in sections 1 and 2. When cells become confluent, they are induced to differentiate as described in section 4 until they reach terminal differentiation and are ready for bioenergetics analysis. This procedure is common for a 24-well plate format, but less so for 96-well plates.

FIGURE AND TABLE LEGENDS:

Figure 1: Collection and processing of fat pads. (**A**) Schematic representation of primary white (top) and brown (bottom) adipocyte isolation. (**B**) Subcutaneous white (top) and brown (bottom) adipose depots. In P0 mice, subcutaneous WAT is almost invisible, but becomes distinguishable on ~day 2 after birth. In contrast, BAT has a distinct dark color even at P0. In older pups, the BAT is surrounded by a thin superficial layer of WAT, which requires removal when the tissue is dissected. (**C**) Representative images of primary white and brown precursor cells after filtration through the 100 µm cell strainer, after the initial washes, and 24 h after isolation. Scale bars = 100 µm. Abbreviations: WAT = white adipose tissue; BAT = brown adipose tissue.

Figure 2: Differentiation of adipocyte progenitors. (**A**) Average number of subcutaneous WAT and BAT preadipocytes obtained per newborn (P0) pup. (**B**) Representative images of terminally differentiated white and brown adipocytes. For fluorescence imaging, cells were incubated with Nile Red and Hoechst 33342 to stain neutral lipids and nuclei, respectively. Scale bars = 100 µm. (**C**) Gene expression analysis of classical adipocyte markers, white-beige markers, and brown-specific markers in primary white and brown adipocytes differentiated for 6 days (n=3). For each gene, BAT expression is relative to WAT levels (set to 1). (**D**) OCR of white and brown adipocytes in a mitochondrial stress test and in response to norepinephrine (n=3). Brown adipocytes show

uncoupled respiration and a robust response to norepinephrine, whereas white adipocytes show little uncoupled respiration and no significant response to adrenergic stimulation. *p<0.05; **p<0.01, determined by two-tailed Student's *t*-test. Abbreviations: WAT = white adipose tissue; BAT = brown adipose tissue; OCR = oxygen consumption rate; Oligo = oligomycin; FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; RAA = rotenone, antimycin A; *PPARγ* = peroxisome proliferator-activated receptor gamma; *Acrp30* = adipocyte complement-related protein of 30 kDa; *Fabp4* = fatty acid-binding protein 4; *Glut4* = glucose transporter 4; *Cd36* = cluster of differentiation 36; *Retn* = resistin; *Slc27a1* = solute carrier family 27 member 1; *Ear2* = eosinophil-associated, ribonuclease A family 2; *Pgc1a* = PPARγ coactivator 1alpha; *Prdm16* = positive regulatory domain I-binding factor 1 (PRDI-BF1) and retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) homologous domain containing 16; *Eva1* = epithelial V-like antigen 1; *Cidea* = cell death-inducing DNA fragmentation factor-alpha-like effector A; *Ucp1* = uncoupling protein 1; *Dio2* = type 2 deiodinase.

DISCUSSION:

Adipose tissue is critical for systemic insulin sensitivity and glucose homeostasis[20]. Obesity-linked adipocyte dysfunction is tightly associated with the onset of type 2 diabetes. Therefore, greater understanding of the basic biology and physiology of adipose tissue may enable the design of new treatments for metabolic disorders. As a complement to direct functional and transcriptional analysis of mature adipocytes isolated from fat depots[21,22], cultured primary adipocytes have been shown to recapitulate many aspects of adipose tissue pathophysiology, including secretion of adipokines, resistance to insulin in response to pro-inflammatory stimuli, and induction of the thermogenic program[23-25]. Although previous protocols have described the isolation of adipocyte precursors from adult mice[11,12], this protocol provides a method for the efficient isolation of cells from newborn pups. This strategy yields a significantly larger population of white and brown adipocyte progenitors with higher differentiation potential, as postnatal adipose depots are still relatively undifferentiated compared to the adipose depots of adult mice[26]. Moreover, cells isolated using this method are already committed and yield fully functional differentiated adipocytes that express markers of mature cells and exhibit their unique physiological features, including lipid storage and thermogenic capacity.

Primary cells cannot be expanded indefinitely in vitro. In addition, primary preadipocytes start to lose their adipogenic potential after several passages in culture. This is likely because continual cell culture results in an intrinsic enrichment of less committed, more proliferative cells. Thus, one limitation of this protocol is the window of time during which preadipocytes can be used. Adipogenic potential is fully preserved if cells are induced to differentiate within 7–8 days from the time of isolation. Adipocyte progenitors are particularly resistant to enzymatic digestion, but it is nonetheless important to correctly time the collagenase treatment. Overdigestion of tissues may result in reduced cell survival and decreased ability of cells to adhere to the plate. Throughout the expansion phase, both white and brown preadipocytes are strongly adherent and can tolerate vigorous washes and frequent media exchange. However, the use of coated plates is recommended when preadipocytes are induced to differentiate. Mature, lipid-laden adipocytes have decreased surface adhesion and cell-cell interactions, resulting in a tendency to detach during differentiation unless gently handled. The most delicate step of the protocol is the

induction of differentiation. FBS, insulin, T3, and other drugs must be tested, and their final concentrations optimized, to obtain the highest extent of differentiation. A PPARγ agonist (e.g., rosiglitazone) can also be added to further stimulate adipogenesis.

It is important to note that the differentiation conditions can be adapted to the experimental needs. For instance, in screens with genetic or chemical libraries designed to identify proteins/compounds that enhance white and/or brown adipocyte differentiation, preadipocytes can be induced to differentiate using a minimal permissive medium that includes 10% FBS in DMEM and 170 nM insulin only. Assessment of each component of the differentiation cocktail is recommended to determine ideal assay windows for differentiation assays. T3 and dexamethasone are dispensable for primary white and brown adipocyte differentiation. These conditions will ensure a low rate of differentiation in control cells, thus increasing the window of the assay and maximizing the ability to detect pro-adipogenic factors. Cultures of primary brown and white adipocytes are a powerful tool to interrogate adipocyte cell-autonomous function in response to genetic manipulation and metabolic stresses to complement the study of brown and white adipose tissue in vivo. Hence, protocols for isolation and culture of primary preadipocytes are needed to enable reproducible, high-throughput investigations of adipocyte function in vitro. The strategy described here allows study of primary white and brown preadipocytes that can be differentiated into fully mature adipocytes and tested under a variety of experimental manipulations.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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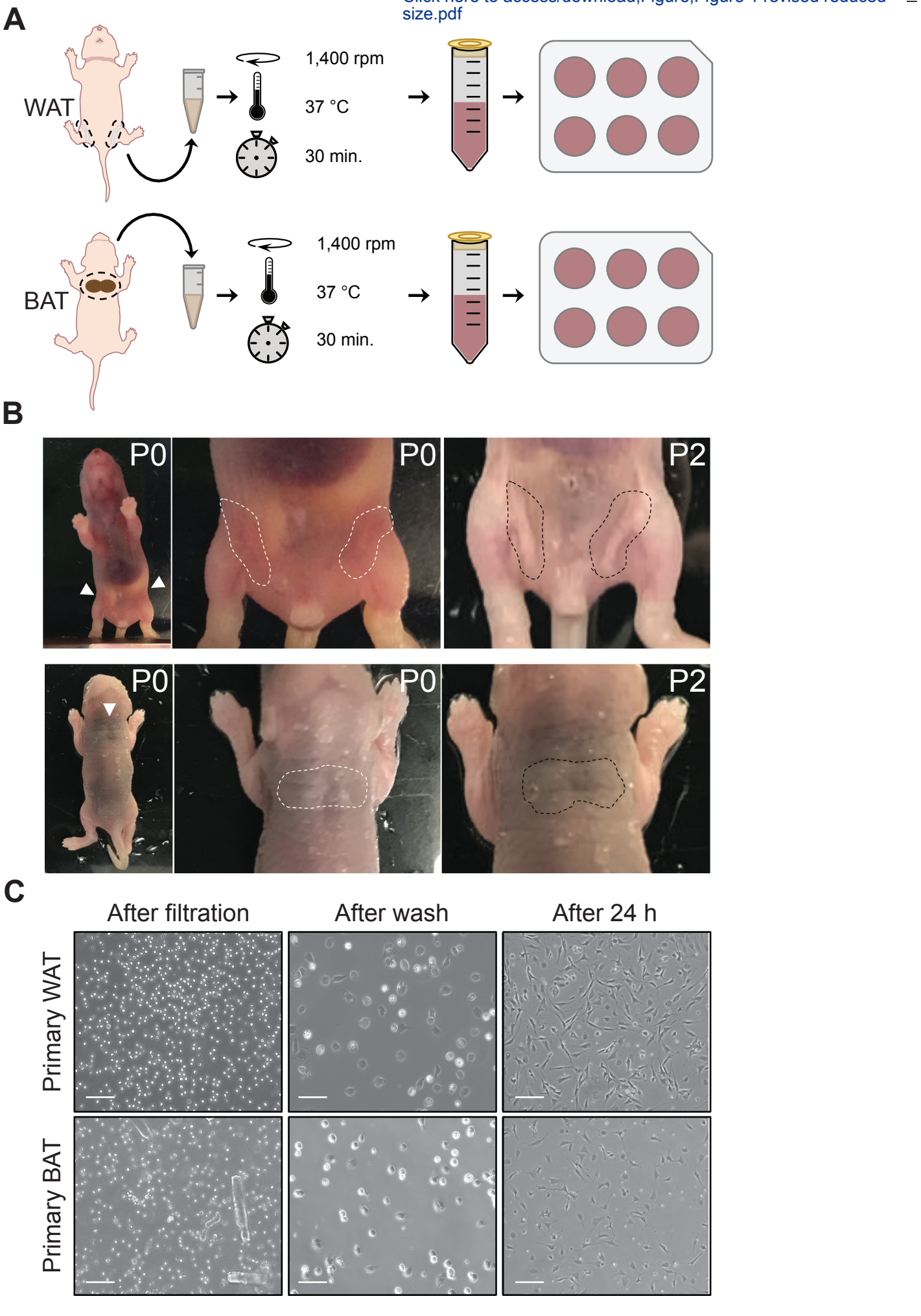
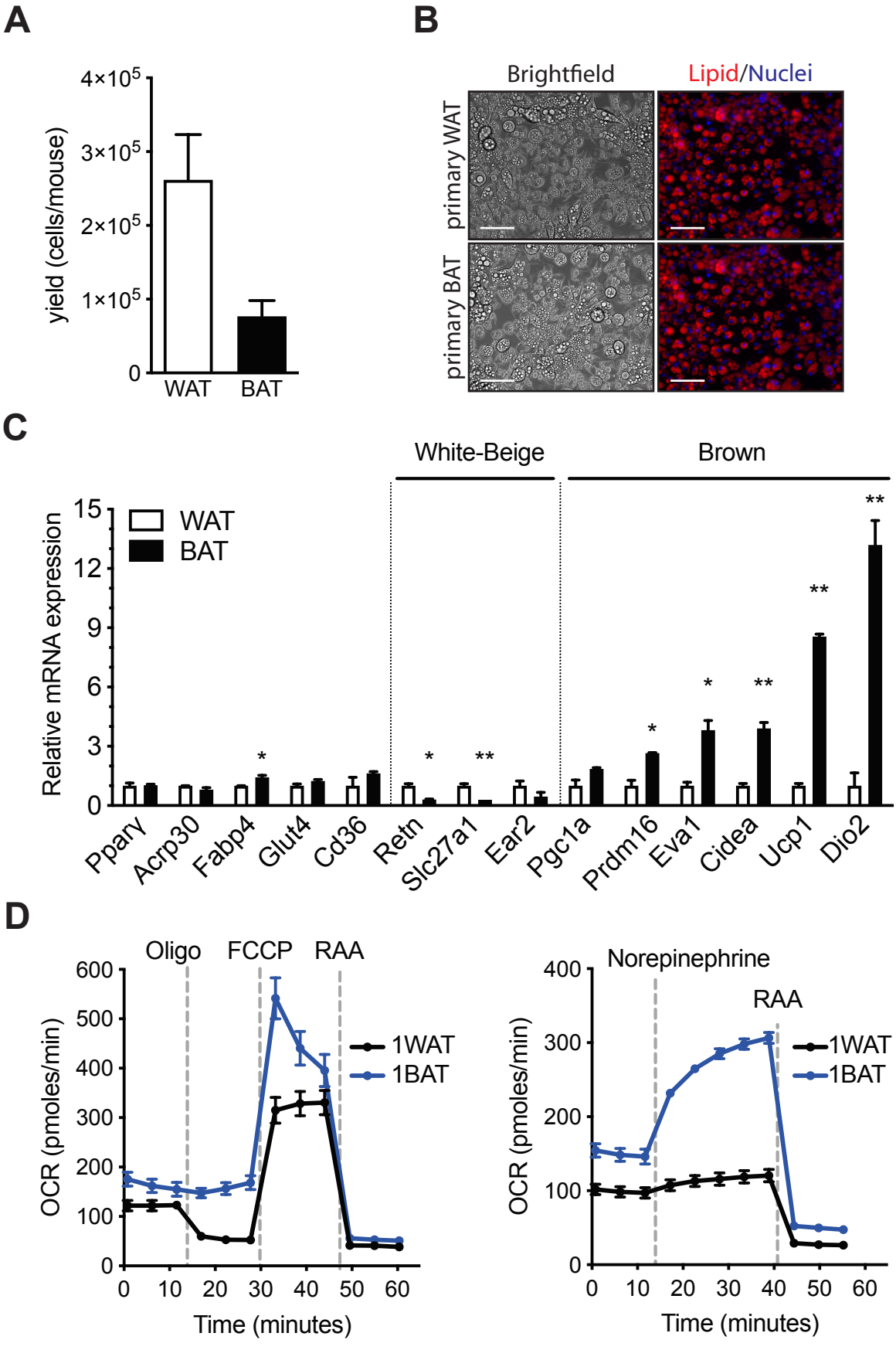




Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-Isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	I7018	
6-well plates	Corning	353046	
AdipoRed (Nile Red)	Lonza	PT-7009	
Antimycin A	Sigma-Aldrich	A8674	
BenchMark Fetal Bovine Serum	Gemini Bioproducts LLC	100-106	
$CaCl_2$	Sigma-Aldrich	C4901	
Cell strainer	Fisher Scientific	22363549	
Collagenase, Type 1	Worthington Biochemical Corp	LS004196	
ddH_2O	Sigma-Aldrich	6442	
Dexamethasone	Sigma-Aldrich	D4902	
DMEM	Sigma-Aldrich	D5030	For Bioenergetics studies
DMEM, High Glucose, Glutamax	Gibco	10569010	
DPBS, no calcium, no magnesium	Gibco	14190144	
Fatty Acid-Free BSA	Sigma-Aldrich	A8806	
FCCP	Sigma-Aldrich	C2920	
Gelatin	Sigma-Aldrich	G1890	
Glucose	Sigma-Aldrich	G7021	
HEPES	Sigma-Aldrich	H3375	
Hoechst 33342	Invitrogen	H1399	
Insulin	Sigma-Aldrich	I6634	
KCl	Sigma-Aldrich	P9333	
NaCl	Sigma-Aldrich	S7653	
Norepinephrine	Cayman Chemical	16673	
Oligomycin	Sigma-Aldrich	75351	
Pen/Strep	Gibco	15140122	
Rosiglitazone	Sigma-Aldrich	R2408	
Rotenone	Sigma-Aldrich	557368	
Seahorse XFe96 FluxPak	Agilent Technologies	102416-100	For Bioenergetics studies

Surgical forceps	ROBOZ Surgical Instrument Co	RS-5158
Surgical Scissors	ROBOZ Surgical Instrument Co	RS-5880
ThermoMixer	Eppendorf	T1317
triiodothyronine (T3)	Sigma-Aldrich	642511

RESPONSE TO REVIEWERS – JoVE62005

We thank reviewers for their interest in our work and for their careful and insightful comments. We have incorporated their suggestions. A point-by-point response to their specific comments follows.

Editorial comments:

We thank the editor for the careful review of our work. We believe we have addressed all the editorial comments. Specific to comment 13 (*Figure 1A: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). Please add abbreviations for WAT, BAT to the legend)*, please note that our protocol does not involve centrifugation at this step. The rpm refers to the shaking frequency of the temperature-controlled shaker used during the digestion of the adipose depots.

Reviewer #1:

This manuscript describes a protocol for the isolation and differentiation of primary white and brown adipocytes. The authors provide a clear description of procedures and of representative results. The introduction and discussion are nicely written. Overall terrific job.

We are glad that our protocol was well received. We thank the reviewer for his/her positive feedback.

Major Concerns:
None

Minor Concerns:
Another common way for bioenergetic analysis of primary adipocytes is to differentiate adipocytes directly on the Seahorse plates. It would be helpful for the authors to include discussion of this procedure.

We have modified the text and references to mention this valuable alternative to our protocol. We thank the reviewer for the suggestion.

It would be helpful if the authors could further discuss which components of the cocktail are strictly required, and which can be omitted (e.g., T3).

We agree with the reviewer that this is an important point. We have expanded the text to incorporate this aspect in the Discussion.

It could be helpful if the authors provide additional discussion that isolation and direct analysis of mature adipocytes from the fat pad is also becoming a more popular approach (see for example PMID 31270323, Pydi et al Nat Comm 2019).

We now address this issue in the Discussion and highlight the complementary nature of these two approaches.

Reviewer #2:

Galmozi et al. describe a protocol to isolate and differentiate iBAT and iWAT preadipocytes obtained from newborn mice. The authors claim that because no previous published protocols for newborn mice are available, their procedure is a novel contribution. Nonetheless, the extraction and differentiation of iBAT preadipocytes from newborn mice has recently described. Because of this background deficiency, it is unclear the comparative advantages of this protocol offers in terms of efficiency to obtain preadipocytes as well as to reach differentiated adipocytes.

We believe that the key advancement our work provides is the ability to easily obtain primary *white* adipocytes in large quantities. To our knowledge, this is the first protocol that enables isolation of white preadipocytes in an efficient manner at such scale. We agree with the reviewer that our protocol for isolation of primary iBAT adipocytes is not as novel. We simply included it because they can be simultaneously isolated while the primary white adipocytes are harvested.

Major Concerns:
Protocol:
1.0 Describe the euthanasia procedure with detail, emphasizing the precautions that were taken to prevent animal suffering. Clarify if this protocol was approved by the corresponding IACUC.

We now include a detailed description of the euthanasia procedure in a new section 1.2 in the revised protocol, and have added a statement to indicate that the described procedure was approved by our institutional IACUC.

2.2 The recommendation of specific reagent brands, particularly for FBS, must be backed up on experimental data. Please provide a supplemental table for the comparison between different FBS brands

We agree with the reviewer that it is perhaps not proper to suggest the use of specific FBS brands, and we have removed all commercial names from the protocol. However, we firmly believe that suitable FBS is a critical reagent for efficient adipocyte differentiation, and that the choice of a particular FBS lot needs to be carefully considered. Presenting a comparison of brands and currently available FBS lots would not be useful, as it is likely to become quickly outdated. Instead, we have modified the text to highlight the need, in our view, to test multiple lots of FBS to identify those that support the most robust level of preadipocyte proliferation and differentiation in the user's hands.

2.5.

- During the period of 1 - 1.5 hours after plating, how is the cell suspension preserved? In the incubator or at RT?

We apologize for having left this detail out. Cells are transferred to a 37°C tissue culture incubator. We now include this in the protocol.

- Because this suspension usually looks very turbid; how did you prevent preadipocytes loosing after washing the wells with DMEM without serum? In our experience, the use of ACK buffer in early steps is a good alternative to eliminate blood red cells to improve the optical properties of the suspension as well as to decrease the contamination of the first plated cells

Incubation with ACK buffer is an alternative to eliminate red blood cells, and we use ACK buffer when we isolate primary macrophages. However, incubation with ACK buffer needs to be timed quite precisely to avoid death of other cell types in addition to red blood cells. Preadipocytes stick to the plastic very efficiently, faster than any other cell type generated during tissue digestion. In our experience, washing the cells 1-1.5 hours after plating is an efficient procedure to separate preadipocytes that are already firmly attached to the plate from other cell types. This allows us to avoid the potential toxicity associated with ACK buffer use.

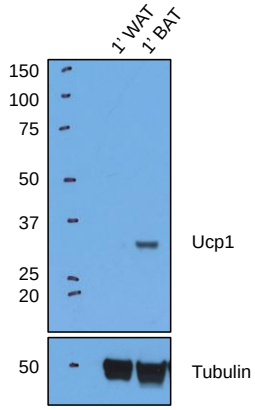
3.4 *What is the reason for coating the plates? Do you have evidence that this procedure increases the yield or improves differentiation efficiency? Again, this recomendation must be supported by empirical data showed in supplemental material*

Our protocol recommends coating plates only for preadipocyte differentiation. This is not a required step of the protocol, and this additional precaution does not affect either the yield or differentiation ability of the isolated preadipocytes. We recommend coating plates prior to differentiation simply because as the cells differentiate and become lipid-laden, repeated washes and media exchanges, as necessary over several days of culture, can cause differentiated cells to detach. The presence of an organic matrix, as opposed to uncoated plastic, reinforces cell attachment to the plate and minimizes potential cell loss. We have added a note in the protocol to clarify this point.

4.1 *In our experience, the lack of rosiglitazone determines no expression of UCP1 at all. Please justify the reason for not adding this compund in your protocol. Ideally, make a side by side comparision of the impact of rosiglitazone with a dose-response curve. Provide the raw data of the qPCRs for the gene expression studies showed in the "Results" section*

We respectfully disagree with the reviewer. Although the addition of rosiglitazone certainly boosts the extent of differentiation, direct activation of PPARγ using a synthetic agonist is not required at all for complete differentiation of primary white and brown adipocytes using our protocol. Brown adipocytes differentiated using our cocktail lacking

rosiglitazone robustly express UCP1, as evidenced not only by the Western blot we include below, but also by the fact that mitochondrial respiration in these cells is ~90% uncoupled vs. ~10% uncoupled in primary white adipocytes (**Figure 2d**, left panel). The rapid and sustained increase in oxygen consumption upon norepinephrine stimulation further supports the presence of high levels of UCP1 protein in these cells (**Figure 2d**, right panel). Given the robust level of UCP1 expression we see in our primary brown adipocytes differentiated without rosiglizatone, we see no need to provide a side-by-side comparison between cells differentiated with and without rosiglitazone, as the effects of this compound in brown adipocytes is an issue beyond the scope of this protocol. We urge the reviewer to try our protocol and change his/her FBS lot. Please see the protein blot below and similar data in Extended Figure 4J and 4K of our recent *Nature* publication (DOI: 10.1038/s41586-019-1774-2)



Results:

On page 7, lines 253-254 indicates that "Although both white and brown preadipocytes isolated from newborn pups have high proliferative capacity," How was cell proliferation was evaluated?

We assessed cell proliferation by counting cells every 24 hours over a period of 4 days.

Figure 1. Provide pictures of the animals with the skin removed. This is to provide representative images of the macroscopic aspect of both WAT and BAT in a newborn mouse for readers without previous anatomic experience.

We believe these are unnecessary, as these aspects will be shown in the video of our protocol.

Figure 2. 1) Indicate the day of differentiation corresponing to the showed data.

Data reported in figure 2 refer to primary white and brown adipocytes differentiated for 6 days. We now specify this in the figure legend.

2) To illustrate the degree of differentiation, it is better to express mRNA abundance as fold-change relative to day 0 for each cell type (undifferentiated preadipocytes).

We agree with the reviewer that a time course of gene expression throughout differentiation would be the best way to show the level of differentiation. However, the purpose of figure 2c is to highlight the relative expression of classical markers of fully mature white and brown adipocytes. The comparison the reviewer suggests would be biased by the basal, gene expression levels of white and brown precursors. The markers of fully mature adipocytes will be logs of magnitude higher than in undifferentiated precursors which we thought would be less helpful than a comparison of their relative expression in our mature white and brown adipocytes, to highlight the identity of the two cell populations we can isolate using this protocol.

3) Indicate the sample size and the statistical test used to compare the data

We have added the requested information to the figure legend.

Fig. 2b. Considering that this protocol aims to isolate and differentiate preadipoytes, why do not show representative fluorescence images of non-differentiated and early stages of brown and white differentiation. This will be an adequate way to demonstrate the absence of mature adipocytes in the starting material, before adding the adipogenic stimuli and show the evolution to fully mature adipocytes.

In figure 1c we show brightfield images of both white and brown preadipocytes. We believe this type of microscopy is more informative than fluorescence microscopy using Nile Red to stain lipid, for in preadipocytes the resulting images would only show the nuclear counterstain. As discussed in the protocol, lipid droplet appearance becomes visible within a few days after induction of differentiation under bright field microscopy. The ultimate goal of this protocol is to isolate precursor cells that can be differentiated *in vitro*. Therefore, we considered it appropriate to show images of freshly isolated cells in figure 1 and emphasize, in figure 2, the end product of our efforts – mature fully functional fat cells that behave as expected.

Fig. 2c. What is the reason to express mRNA levels of brown adipocytes relative to white adipocytes? Is this a correct way to normalize gene expression, taking into account that the adipogenic cocktail used was different?

The adipogenic cocktail for white and brown adipocytes is the same except for the addition of T3 in the BAT cocktail. We have chosen to show gene expression analysis relative to white adipocytes to highlight the differences in gene expression profiles between these two cell types. Specifically, gene expression in brown <u>is not</u> normalized to white adipocytes' gene expression but is presented <u>*relative*</u> to that in white adipocytes. Normalization is performed using a standard curve derived from the pool of all brown and white adipocyte samples. White and brown adipocyte samples were extracted and all qPCRs ran at the same time and normalized using the same standard curve. Therefore, we believe this way of presenting the data is more informative because it demonstrates that markers of brown vs. white adipocytes are specifically enriched in the appropriate cell types.

Discussion:

Please cite previously published protocols for preadipocyte isolation from new born mice.

We have modified the introduction to include the initial work of Dr. Kahn describing the isolation of primary brown preadipocytes from newborn mice. There are, however, no published protocols describing the isolation of white preadipocytes from newborn mice. That is the key advance that our novel method provides.

Discuss the critical differences between your protocol and those published and justify why your alternative could be a good option.

We have modified the text to highlight that our protocol, relative to those already published, allows us to simultaneously isolate a significantly larger amount of highly-proliferative white and brown preadipocytes with high differentiation potential.

Regarding the recomendation of coating the plates, provide the references supporting this asset. In our experience coating plates is not required for differentiation nor increase the number of differentiated cells

As stated in a prior response, coating plates is not a required step, and it does not increase the yield or differentiation potential of preadipocytes. It simply eases the work of the user during multi-day differentiation protocols, for it prevents the differentiating cells from detaching during washes and media changes. We include a note to this effect.