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TITLE:

Isolating Brown Adipocytes from Murine Interscapular Brown Adipose Tissue for Gene and Protein Expression Analysis

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SUMMARY:

This study describes a new method of isolating murine brown adipocytes for gene and protein expression analysis.

ABSTRACT:

Brown adipose tissue (BAT) is responsible for non-shivering thermogenesis in mammals, and brown adipocytes (BAs) are the functional units of BAT. BAs contain both multilocular lipid droplets and abundant mitochondria, and they express uncoupling protein 1 (UCP1). BAs are categorized into two sub-types based on their origin: embryo derived classical BAs (cBAs) and white adipocytes derived BAs. Due to their relatively low density, BAs cannot be isolated from BAT with traditional centrifugation method. In this study, a new method was developed to isolate BAs from mice for gene and protein expression analysis. In this protocol, interscapular BAT from adult mice was digested with Collagenase and Dispase solution, and the dissociated BAs were enriched with 6% iodixanol solution. Isolated BAs were then lysed with commercial reagent used for simultaneous isolation of RNA, DNA, and protein. After RNA isolation, the organic phase of the lysate was used for protein extraction. Our data showed that 6% iodixanol solution efficiently enriched BAs without interfering with follow-up gene and protein expression studies. Platelet-derived growth factor (PDGF) is a growth factor that regulates the growth and proliferation of mesenchymal cells. Compared to the brown adipose tissue, isolated BAs had significant higher expression of *Pdgfa*. In summary, this new method provides a platform for studying the biology of brown adipocytes at a single cell-type level.

INTRODUCTION

Both mice and humans have two types of adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT)[1]. WAT stores energy in the form of triglycerides in white adipocytes, and the brown adipocytes (BAs) of BAT dissipate chemical energy as heat[2]. Based on their developmental origin, BAs are further categorized into classical BAs (cBAs) that formed during embryo

development and white adipocytes derived BAs (beige/brite cells, converted from white adipocytes under stress conditions)[3]. BAs are multilocular and express the thermogenic protein uncoupling protein 1 (UCP1)[4]. Interscapular BAT (iBAT) depot is one of the primary cBAs depots in small mammals[5], whereas beige cells are dispersed within WAT[6].

Due to their nature of dissipating energy, BAs have received much attention as a therapeutic target for reducing obesity[7]. To exploit BAs for the purpose of treating obesity, it is essential to understand the molecular mechanisms that control BAs function, survival, and recruitment. Adipose tissues including BAT and WAT are heterogeneous. Except for adipocytes, adipose tissues contain many other cell types, such as endothelial cells, mesenchymal stem cells and macrophages[8]. Although genetic tools to specifically deplete candidate genes in mice BAs are available, such as UCP1::Cre line[9], techniques for purifying BAs from BAT or WAT are limited, making it hard to study BAs at a single-cell type level. Additionally, without obtaining pure BAs, the relationship between BAs and non-BAs will not be clearly delineated. For instance, platelet-derived growth factor receptor alpha (PDGFRα) has been used as a marker for undifferentiated mesenchymal cells, and it is expressed in the endothelial and interstitial cells of BAT. In cold stressed BAT, PDGFRα positive progenitor cells give rise to new BAs[10]. PDGFRα is activated by its ligand PDGF, a growth factor that regulates the growth and proliferation of mesenchymal cells[11]; however, it is unclear whether BAs influence the behavior of PDGFRα positive progenitor cells by secreting PDGF.

Recently, a BAs isolation protocol has been published, which is based on fluorescence-activated cell sorting (FACS)[12]. In this protocol, 3% bovine serum albumin (BSA) solution was used to separate BAs from non-BAs, and the enriched BAs were further purified by FACS. The application of this protocol is limited by the requirement of FACS process, which relies on both equipment and FACS operation experiences. In this study, a new protocol for isolating BAs from BAT was developed. The BAs isolated by this protocol can be directly used for gene and protein expression studies. Furthermore, data from this study suggest that BAs are a major PDGF resource.

PROTOCOL

All mice were maintained in pathogen-free conditions, and all procedures were approved by Masonic Medical research Institutional Animal Care and Use Committee (IACUC). UCP1::Cre[9] and Rosa $26^{tdTomato}$ mice lines[13] were reported previously. All mice were kept at room temperature with a 12 h light/dark cycle.

1. Preparing the Solutions and brown adipose tissue (BAT)

1.1. Prepare digestion solution and separation solution in 15 mL centrifuge tubes.

1.2. 10 mL of BAT Digestion solution: To 10 mL of sterile phosphate-buffered saline (PBS), add 3.5 mg/mL Dispase II, 1 mg/mL Collagenase II and 10 mM $CaCl_2$ to make BAT digestion solution.

1.3. 10 mL of 12% iodixanol solution (separation solution): Mix 1 mL of 10x PBS, 2 mL of 60% iodixanol, 0.01 mL of 1 M $MgCl_2$, 0.025 mL of 1 M KCl, 0.1 mL of 0.2 M ethylenediaminetetraacetic

acid (EDTA) and 6.865 mL of ddH_2O to obtain 12% iodixanol.

1.4. Euthanize one adult mouse with CO_2 overdose. Briefly, fill the mouse cage with 100% CO_2 at a displacement rate of 10-30% cage volume per min. 5 min later, confirm death by checking for the absence of visibly breathing.

1.5. Dissect the animal and collect interscapular BAT. Remove WAT and muscle layers under a stereo microscope.

1.6. To have sufficient digestion, cut each BAT lobe into ~ 3 mm^3 parts and place them into a clean 50 mL flask with a metal stir bar and 5 mL digestion solution. Before starting digestion, let the flask containing BAT and digestion buffer sit on ice for 1 h.

NOTE: In the following steps, around 80 mg BAT was used for brown adipocytes isolation.

2. BAs isolation procedure

2.1. Place the flask on a magnetic stirrer that is enclosed in an incubator. Set the stirring speed at 60 rpm and the temperature of the incubator at 35 °C, respectively. The digestion will last for around 30 min. If the BAT slices form clumps around the stirrer bar during digestion, use a 1 mL pipette tip to disrupt the aggregated tissues.

2.2. Place a 70 µm strainer filter on top of a clean 50 mL centrifuge tube. Pipette around 4 mL cell suspension through the strainer. Wash the strainer with 4 mL of 12% iodixanol solution. Pipette up and down to mix the cells and the iodixanol solution. Transfer the cell mixture into two clear 5 mL polystyrene test tubes.

NOTE: At the end of digestion, the digestion solution should be cloudy, indicating sufficient digestion. Use fresh digestion solution every time. Once prepared, the digestion solution should be used within 2 h.

2.3. Repeat step 2.2 and 2.3 once more if the digestion is not sufficient.

2.4. Leave the clear polystyrene tubes containing the separation solution and BAs on ice for 1 h. The BAs will form a layer on the top.

2.5. Take out 20 µL of the isolated BAs for microscope examination.

3. RNA and protein isolation from BAs

3.1. Pipette the BAs layer into two 1.7 mL microcentrifuge tubes for RNA and protein isolation. Carefully remove the excessive iodixanol solution without disrupting the BAs layer.

3.2. Add 1 mL commercial reagent (e.g., Trizol) to simultaneously isolate RNA, DNA, and protein

into the cell solution. Mix sufficiently to lyse the cells.

3.3. Add 200 µL of Chloroform to separate the phases.

3.4. Centrifuge the tube for 9,981 x *g* for 10 min at 4 °C.

3.5. After centrifugation, use the aqueous phase for RNA isolation. In this study, total RNA was used for reverse transcription, and quantitative RT-PCR was carried out with Real-Time PCR. Primer sequences are listed in **Table of Materials**.

3.6. Transfer 300 µL of the organic phase into a 2 mL microcentrifuge tube.

3.7. To the aqueous phase, add 2.5 volume 100% ethanol and vortex for 10 s.

3.8. Add 200 µL of 1-Bromo-3-chloropropane and vortex for 10 s.

3.9. Add 600 µL of double distilled water and vortex for 10 s.

3.10. Let the mixed solution stand for 10 min at room temperature.

3.11. Centrifuge at 9,981 x *g* for 10 min at 4 °C. At this step, the phases will be separated. The protein phase is localized in the middle layer.

3.12. Remove the top aqueous solution. Add 1 mL 100% ethanol into the remaining solution.

3.13. Centrifuge for 10 min, 9,981 x *g*, 4 °C. After centrifugation, the protein pellet will form. Discard the supernatant.

3.14. Wash the pellet with 1 mL 100% ethanol.

3.15. Centrifuge for 10 min, 9981 x *g*, 4 °C. Save the pellet and discard the supernatant.

3.16. Air-dry the pellet for 10 min at room temperature.

3.17. Measure the weight of the wet pellet. Add in 1% SDS solution at a ratio of 20 µL/mg pellet.

3.18. Dissolve the pellet by putting the tube in a heated shaker. Set the temperature at 55 °C, and the speed at 11 x *g*. It usually takes 5-10 min to completely dissolve the protein pellet.

NOTE: The concentration of the dissolved protein can be measured by BCA assay[14].

REPRESENTATIVE RESULTS

Preparation of interacapular BAT for brown adipocytes isolation

The brown adipocytes (BAs) isolation process is depicted in **Figure 1A**. The whole process, from

preparing BAT and digestion/separation solutions to obtaining isolated BAs will take around 4 h.

In adult mice, abundant BAT exists in the interscapular region. This interscapular BAT (iBAT) is covered by muscle layers and WAT (**Figure 1B**). Before starting the digestion procedure, the muscle layers and WAT need to be removed to yield out clean iBAT (**Figure 1C**). In a published BAs isolation protocol, minced BAT was used for BAs isolation[12]. In this study, digestion of 3 mm^3 size BAT (**Figure 1D**) yielded out more BAs than minced BAT.

Separation of BAs from non-BAs with 3% BSA solution

After iBAT digestion, dissociated BAs were mixed with non-BAs in the digestion product. Because BAs contain lipid droplets, their density is lower than non-BAs; however, the BAs density is not low enough to let them efficiently float to the top of a regular PBS solution. PBS containing 3% bovine serum albumin (BSA) has been used to separate BAs from non-BAs[12], which was successfully repeated in this study (**Figure 2A**).

Rosa $26^{tdTomato}$ is a reporter mouse line, which expresses strong tdTomato (tdTom) fluorescence protein following Cre-mediated recombination[13]. Ucp1::Cre transgenic mice express Cre recombinase in the BAs[9]. Ucp1::Cre mouse line was crossed with Rosa $26^{tdTomato}$ mice to genetically label BAs with tdTom (**Figure 2B**). For validating the BAs isolation procedure, iBAT from Ucp1::Cre;tdTom/+ mice was dissociated. Most of the cells enriched in the top layer of BSA solution were raspberry shape and contained multilocular lipid droplets. Furthermore, most of these raspberry shape cells were tdTom positive (**Figure 2C**), confirming that they were brown adipocytes.

Separation of BAs from non-BAs with 6% iodixanol solution

3% BSA separation solution enriched BAs. It was unclear whether these isolated BAs could be used for gene and protein expression analysis. RNA and protein were then extracted from the enriched BAs. RNA extraction was successfully performed according to the standard RNA isolation procedure. However, the standard Trizol protein isolation protocol, also known as Guanidinium thiocyanate-phenol-chloroform method (GTPC method), did not work well, which was tedious and had very low protein yield. Therefore, an improved protein isolation method was adopted to extract protein from Trizol-lysed BAs.

In this improved GTPC protocol, ethanol, bromo-chloropropane, and water was used for extracting protein from the organic phase[15]. After adding ethanol, bromo-chloropropane and water into the organic phase, and after centrifugation, protein pellet formed between the aqueous phase and the organic phase (**Figure 3A**). Protein pellet was then washed with 100% ethanol and dissolved in 1% SDS. This improved GTPC method was used to extract protein from iBAT and BSA solution-enriched BAs. Although the BAT protein pellet was easily dissolved in 1% SDS, major part of the BAs protein pellet was not soluble. Then the dissolved protein was examined with SDS-PAGE gel. As shown in a Coomassie blue stained SDS-PAGE gel (**Figure 3B**), a massive protein band around 60 kDa was present in the isolated BAs but not in the BAT samples. Because the molecular weight of BSA is 66 kDa, and abundant BSA exists in the BAs separation solution, this dominant protein band should be BSA. These data suggest that the BSA from the

BAs separation solution interferes with protein extraction.

Iodixanol is a nonionic and iso-osmotic gradient medium[16] that has been widely used for cell[17] and adeno-associated virus (AAV) purification[18]. To avoid BSA interference of protein expression studies, iodixanol was used to replace BSA in a new BAs separation solution. 3% BSA solution has a density of 1.03, which is similar with 6% iodixanol. In 6% iodixanol solution, BAs floated to the top in 30-60 min (**Figure 3C**). The BAs isolated with this solution showed out typical raspberry shape and contained multilocular lipid droplets (**Figure 3D**). Proteins extracted from these isolated BAs were nicely separated in SDS-PAGE gel (**Figure 3E**).

To verify whether the 6% iodixanol solution efficiently separated BAs from non-BAs, we genetically labeled the BAs with tdTom and examined the cells residing in the clear 6% iodixanol solution. After separating the BAs from non-BAs (step 2.4), the 6% iodixanol solution below the BAs layer was diluted 6 times with PBS and was then centrifuged at 600 x *g* for 5 min. After centrifugation, a small red cell pellet was formed on the bottom, which might be stromal vascular fraction cells. As shown in **Figure 3**, cells from the BAs layer were tdTom positive cells (**Figure 3F**); however, cells recovered from the pellet were tdTom negative (**Figure 3G**). Additionally, no obvious lipid droplets were visible in the tdTom negative cells. These data suggest that our new protocol can efficiently separate the BAs from the non-fat cells.

Together, these data demonstrate that isolating BAs with 3% BSA solution interferes with following-up biochemistry studies and suggest that 6% iodixanol solution is better than 3% BSA solution for isolating BAs.

Gene and protein expression analysis with isolated BAs.

For validating this new BAs isolation procedure on molecular level, the expression of three genes was compared between BAT and isolated BAs: *Ucp1*, *Pdgfa and Pdgfra.* In BAT, *Pdgfra* is expressed in endothelial cells and interstitial cells, and PDGFRα positive cells are putative progenitor cells[10]. The mRNA levels of *Ucp1 and Pdgfa* were both significantly higher in the isolated BAs than in the BAT (**Figure 4A,B**). On the contrary, the mRNA of *Pdgfra* was only detected in BAT (**Figure 4B**).

PPARγ is a transcriptional factor controlling adipose tissue development, UCP1 is a mitochondria protein, and PDGFRα is a membrane receptor protein. These three proteins represent proteins distributed in different cellular compartments. Western blots were performed to test whether protein extracted from Trizol-lysed BAs and BAT was suitable for protein expression analysis. UCP1 and PPARγ were detected in both BAs and BAT (**Figure 4C,D**), confirming that the total protein isolated from the Trizol-lysed BAs or BAT is suitable for western blot. Furthermore, consistent with the qRT-PCR results, UCP1 protein was enriched in BAs (**Figure 4C**); whereas PDGFRα was only detected in BAT but not in pure BAs (**Figure 4D**). In summary, these data demonstrate that our new BAs isolation method is efficient and suggest that BAs enriched by this method can be directly used for gene and protein expression studies.

FIGURE LEGENDS

Figure 1: Preparation of iBAT for brown adipocytes isolation. (**A**) Workflow of the brown adipocytes isolation procedure. (**B**) Ventral view of the interscapular tissue containing BAT, WAT and muscle layers. (**C**) Interscapular BAT (iBAT). Muscle layers and WAT adjacent to iBAT were removed. (**D**) A representative image of iBAT pieces used for brown adipocytes isolation. B-D, Scale bar= 5 mm.

Figure 2: Separation of brown adipocytes from digestion solution. (**A**) Images of dissociated brown adipocytes before and after separation. 3% BSA solution was used to separate brown adipocytes from non-brown adipocytes. Scale bar = 1 cm. (**B**) Schematic view of genetic labeling brown adipocytes with tdTomato fluorescence protein. (**C**) Images of isolated brown adipocytes. DIC, differential interference contrast. Scale bar = 50 μm.

Figure 3: Extraction of total protein from lysed brown adipocytes. (**A**) Separation of protein phase from the organic phase**.** (**B**) Coomassie staining of SDS-PAGE gel. Total protein was extracted from BAT or 3% BSA solution purified brown adipocytes. BSA protein band was indicated by an arrow. (**C**) Brown adipocytes layer formed on top of 6% iodixanol solution. Scale bar = 1 cm. (**D**) Brown adipocytes isolated with 6% iodixanol solution. Brown adipocytes were indicated by yellow arrows. Scale bar = 50 μm. (**E**) Coomassie staining of SDS-PAGE gel. Total protein was extracted from BAT or 6% iodixanol solution enriched BAs. (**F**) Images of tdTom labeled brown adipocytes. (**G**) Images of cells recovered from the iodixanol solution below the BAs layer. F and G, scale bar = 50 μm.

Figure 4: Gene and protein expression analysis of isolated brown adipocytes. Brown adipocytes isolated with the iodixanol method was used in these gene and protein expression studies. (**A,B**) qRT-PCR measurement of gene expression. mRNA levels were normalized to 36B4. N=3. Student t test, *, $P<0.01$; **, $P<0.01$. (**C**) Western blotting of PPARγ and UCP1. (**D**) Western blotting of PDGFRα. C and D, Ponceau S-stained membrane was used as loading control.

DISCUSSION:

In this study, a new method of isolating BAs for gene and protein expression analysis was developed.

In a published BAs isolation protocol, 3% BSA solution was used to enrich BAs[12]. Nevertheless, the enriched BAs achieved by this published protocol could not be directly used for protein expression analysis. This is because the concentrated BSA existing in the BAs solution interferes with following-up protein extraction. When the BAs enriched in the 3% BSA solution were treated with Trizol reagent, sticky protein aggregates would form, the majority of which was not soluble in the GTPC protein extraction process. Additionally, in the GTPC protein extraction product, most of the protein was BSA (**Figure 3B**). In this new protocol, 3% BSA separation solution was replaced with 6% iodixanol for purifying BAs. 6% iodixanol solution efficiently separated the BAs from non-BAs, and the isolated BAs had preserved morphology (**Figure 3D**). Superior to 3% BSA solution, 6% iodixanol solution did not interfere with protein extraction, and the extracted protein was suitable for western blot analysis (**Figure 4C,D**).

Adipose tissue contains large amount of lipids, and lipids contamination in extracted protein samples obstructs protein concentration measurement. Recently, a protocol to remove lipids from protein extractions has been published. In this protocol, a series of low temperature centrifugations are required, which is tedious and needs a large amount of starting materials[19]. In the current study, an improved GTPC method[15] was adopted to isolate protein from BAT. In this protocol, the BAT was first lysed with commercially available reagent which simultaneously isolate RNA, DNA, and protein, and then chloroform was added into the lysate. At this step, both protein and lipids were extracted into organic phase, which contained chloroform and phenol. After centrifugation and removing the RNA and DNA phase, the left organic phase was used for protein extraction. Different from the classical GTPC protocol, this improved GTPC protocol used ethanol, bromo-chloropropane and water to extract protein out of the organic phase. Our data showed that protein isolated with this improved GTPC method was compatible with bicinchoninic acid assay (BCA) based protein concentration measurement.

In this protocol, before the start of the enzyme dissociation process, BAT and digestion solution mixture was placed on ice for one hour. The purpose of this procedure is to reduce the cell metabolism and gene expression rate[20], as well as to let the digestion enzymes efficiently perfuse into the brown adipose tissue.

The current study aimed to develop a strait forward method to isolate brown adiopocytes from interscapular BAT for gene expression study; however, white adipocytes contamination may exist in the isolated BAs. The BAs were enriched based on their relatively low density. Because white adipocytes (WAs) also have low density, the purity of isolated BAs can potentially be affected by WAs. The interscapular BAT is sometimes attached by white adipose tissue, such as in old or high fat-diet treated mice. Although the white adipose tissue needs to be removed during the BAT preparation step, the white adipose tissue might not be completely removed. The current protocol cannot separate BAs from WAs based on cell density. Therefore, if very high purity is essential, the isolated BAs need to be sorted by FACS before being analyzed.

The interscapular BAT (iBAT) contains abundant classical BAs that derived from the *Myf5* cell lineage during embryonic development[21]. Here iBAT was used as an example for isolating BAs. Similar with the published protocol[12], our method can also be used for isolating beige cells from WAT. Nevertheless, to acquire pure beige cells from WAT, the beige cells need to be labeled with fluorescence protein and be enriched by FACS. Following our current protocol, the FACS enriched beige cells can be used for both gene and protein expression analysis, which will greatly improve the efficiency of biological materials utilization.

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DISCLOSURES:

None

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myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. *Development*. **135**, 1597-1604 (2008).

Figure 1

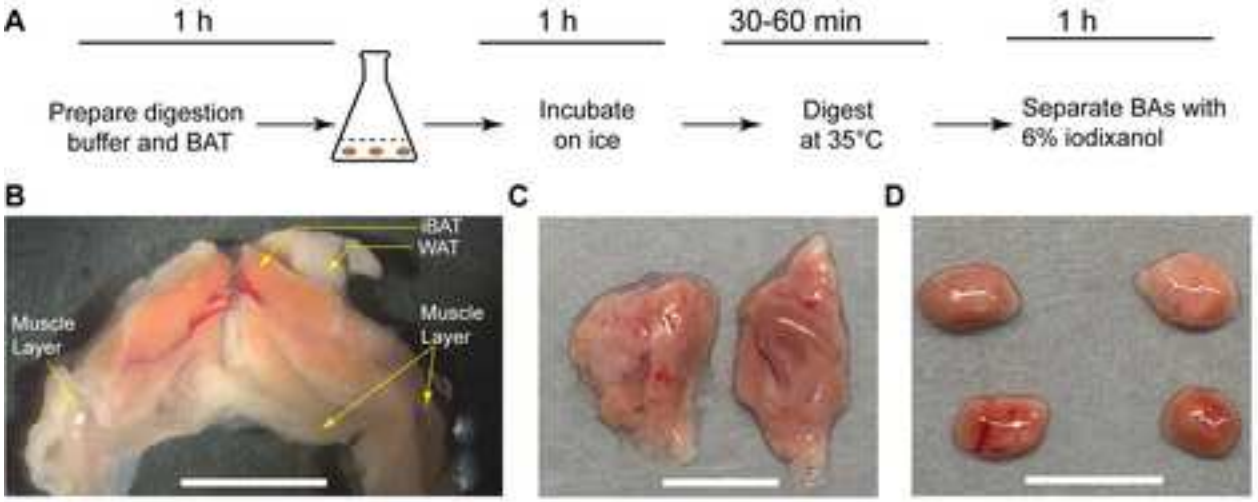

Figure 2

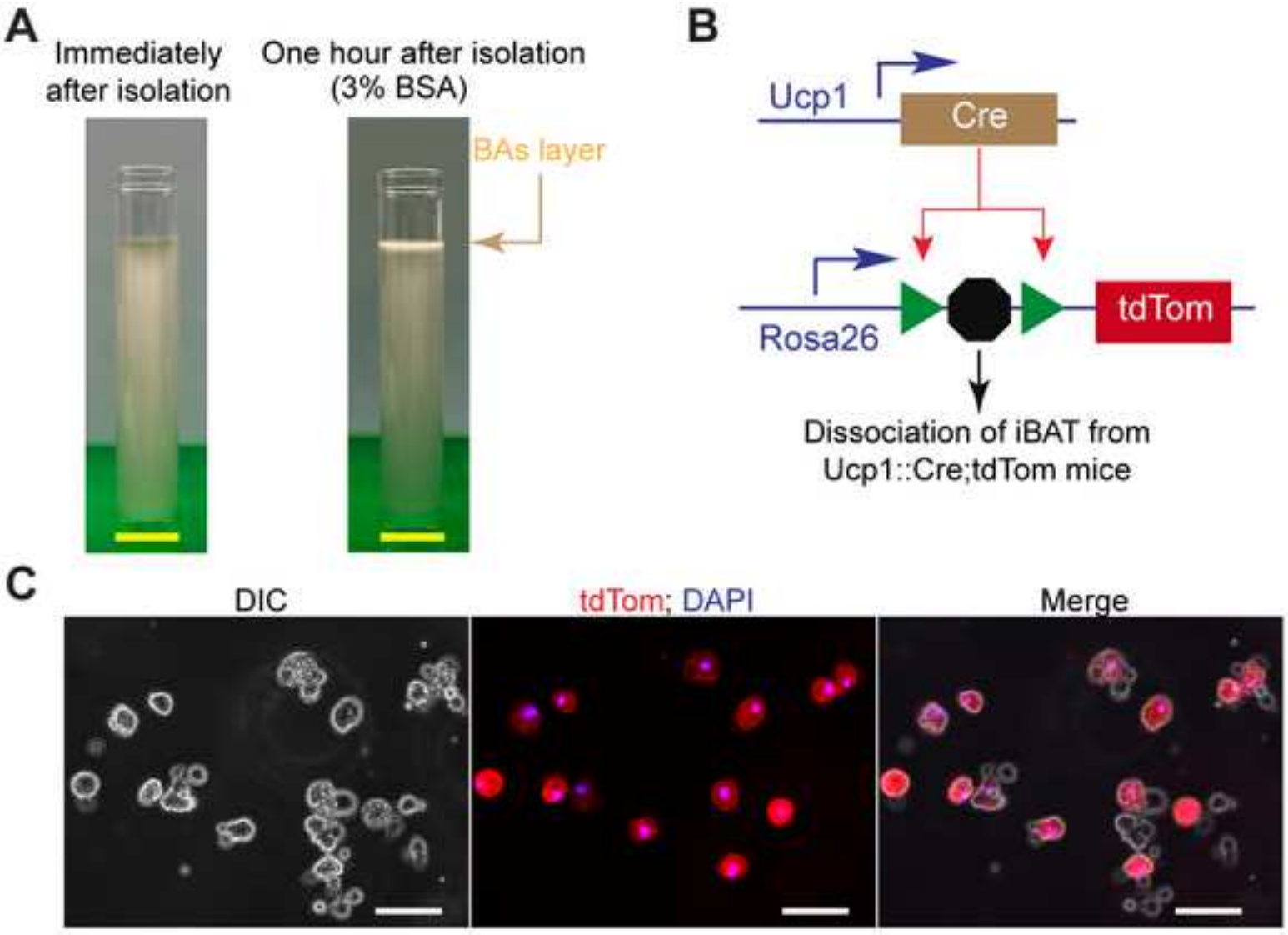

Figure 3

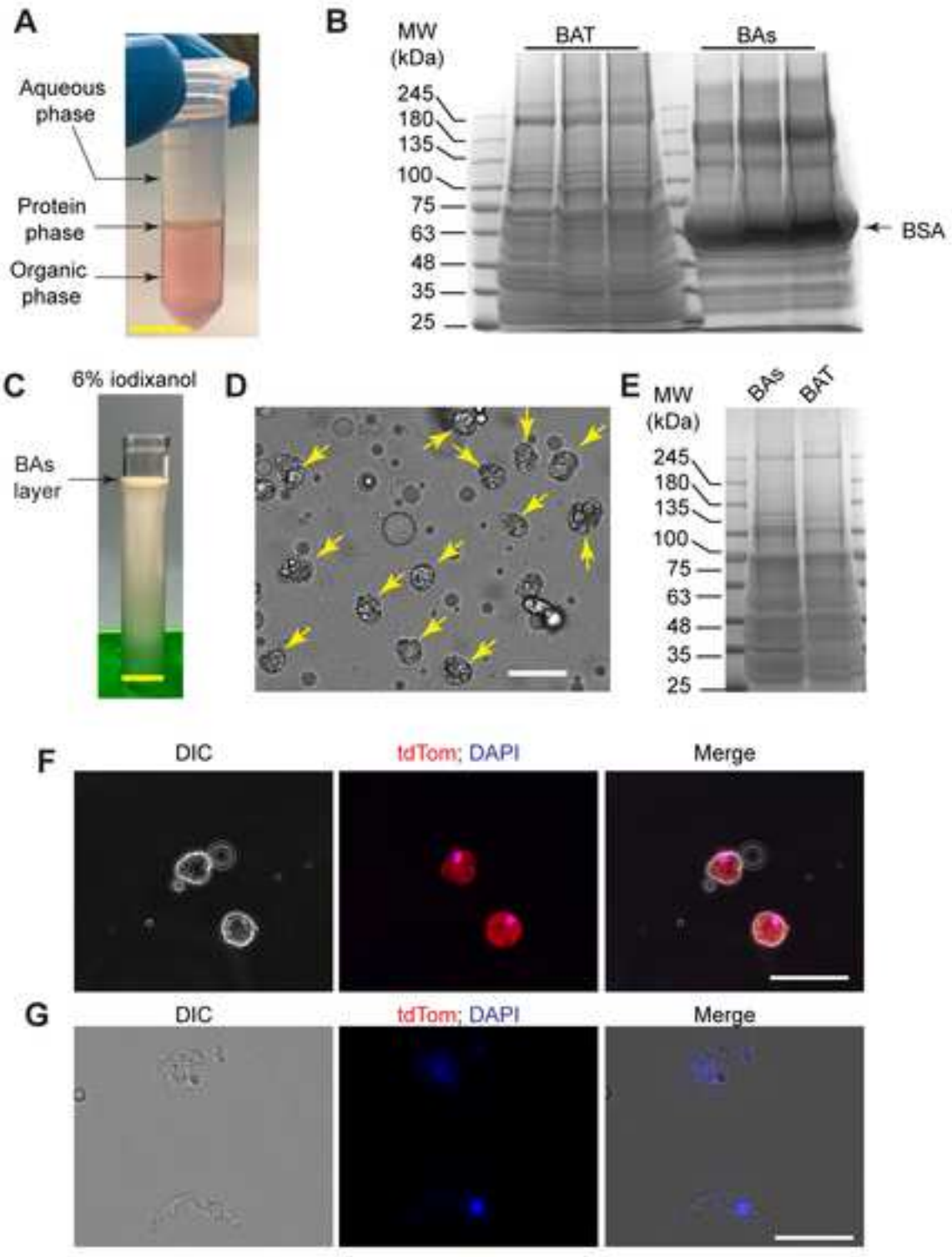

Figure 4

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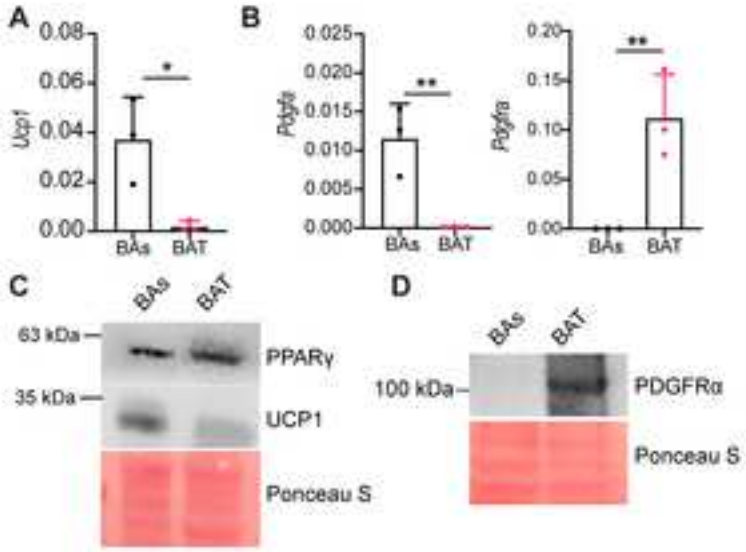

Table of Materials

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Material/Equipment	Company	Catalog number
Antibodies		
Antigen	**Company**	**Catalog**
PPARγ	LSBio	Ls-C368478
PDGFRa	Santa Cruz	sc-398206
UCP1	R&D system	IC6158P
Chemical and solutions		
Collagenase, Type II	Thermo Fisher Scientific	17101015
1-Bromo-3-chloropropane	Sigma-Aldrich	B62404
Bovine Serum Albumin (BSA)	Goldbio	A-421-10
Calcium chloride	Bio Basic	CT1330
Chloroform	IBI Scientific	IB05040
Dispase II, protease	Sigma-Aldrich	D5693
EDTA	Bio Basic	EB0107
Ethanol	IBI Scientific	IB15724
LiQuant Universal Green qPCR Master M	LifeSct	LS01131905Y
Magnesium Chloride Hexahydrate	Boston BioProducts	P-855
OneScrip Plus cDNA Synthesis SuperMix	ABM	G454
OptiPrep (Iodixanol)	Cosmo Bio USA	AXS-1114542
PBS (10x)	Caisson Labs	PBL07
PBS (1x)	Caisson Labs	PBL06
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23227
Potassium Chloride	Boston BioProducts	P-1435
SimplyBlue safe Stain	Invitrogen	LC6060
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	75746
Trizol reagent	Life technoologies	15596018
Primers		
Gene name (Species)	Forward	Reverse
Pdgfra (Mouse)	CTCAGCTGTCTCCTCACAgG	CAACGCATCTCAGAGAAAAGG
Pdgfa (Mouse)	TGTGCCCATTCGCAGGAAGAG	TTGGCCACCTTGACACTGCG
36B4(Mouse)	TGCTGAACATCTCCCCCTTCTC	TCTCCACAGACAATGCCAGGAC
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG

Equipment

Name	Company	Application
Keyence BZ-X700	Keyence	Imaging brown adipocytes
Magnetic stirrer	VWR	Dissociate BAT
QuantStudio 6 Flex Real-Time PCR Syste	Applied Biosystem	Quantitative PCR
The Odyssey Fc Imaging system	LI-COR	Western blot immaging

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
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2. Please provide an institutional email address for each author.

Response: Email addresses were provided.

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3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: personal pronouns were removed.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Falcon, OptiPrep, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
Response: Commercial language has been removed.

5. Line 73: Please elaborate on euthanasia. What % of CO2 was used? How long was the animal given CO2?
Response: Euthanize one adult mouse with CO_2 overdose. Briefly, 100% CO_2 was filled into the mouse cage a displacement rate of 10-30% cage volume per minute. 5 min later, death was confirmed by checking for the absence of visibly breathing.

6. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral, throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
Response: The time units have been corrected.

7. Please convert stirring speeds/ centrifugation speeds to centrifugal force (x g) instead of revolutions per minute (rpm) throughout the protocol.
Response: The centrifuge speeds have been converted into centrifugal force.

8. Line 106-109: Please mention how long is the vortex performed.
Resposne: the duriation of vortex has been provided.

9. Line 125: Please include the details of the BCA assay. A citation will suffice.
Response: A reference was provided.

10. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.
Response: Line space between protocol steps was provided. The protocol section for video was highlighted.

11. Please move the Figure Legends to the end of the Representative Results.

Response: Figure legends were moved to the end of the representative results.

12. Please include any limitations of the technique described in the protocol.
Response: One paragraph about BAs purity was added into the discussion section. Page 10, line 296 to 305.

13. Please do not use abbreviations for journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
Response: The reference format has been updated.

14. Figure 1: please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Examples: 5 h, 10 min.
Response: corrected.

15. Figure 2: Please include scale bars for all the images in the panel.
Response: scale bar provided.
16. Please sort the Table of Materials in alphabetical order.
Response: corrected.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this study, Lin and his colleagues developed a new method to isolate mouse BAs for gene and protein expression analysis. It is a topic of interest to researchers in metabolic areas. My detailed comments are as follows:

Major Concerns:
1. The rest of cells after removing the BAs layers should be validated by UCP1 IHC and staining of Lipophilic Dyes or tracing the UCP-1-Cre;tdTom BAs
Response: We thank the reviewer's suggestion. In this revised manuscript, we examined the cells exist in the solution below the BAs layer. The BAs were genetically labeled with tdTom, so that we can trace whether BAs were completely separated from the non-BA cells with our protocol. As shown in Figure 3 G, cells recovered from the clear 6% iodixanol solution were tdTom negative. Also, no obvious lipid droplets were visible in these cells. These data suggest that our new protocol can efficiently separate the BAs from the non-fat cells. On page 7, line 209 to line 217, we put a paragraph to describe these new results.

2. The thermogenic capacity of isolated BAs should be tested and compared between two methods.
Response: In this study, we developed a new protocol to isolate BAs for gene expression studies. The reviewer provided a good suggestion to test whether the BAs isolated with two different protocols have similar thermogenic capacity.
There are two methods to measure brown adipocytes thermogenic activity:
Seahorse XF Analyzer based respirometry analysis[1] and thermosensitive fluorescent dye based imaging analysis[2]. In these two methods, pre-adipocytes are first seeded on the plates and then differentiated into brown adipocytes. The primary BAs isolated with our current protocol are mature and lipid loaded cells. These low density BAs float in the cultue medium and cannot be seeded in culture plates/dishes, which makes these cells not suitable for either respirometry or imaging based thermogenic activity measurements. Therefore, due to the

current technical difficulties, we are unable to compare the thermogenic capacity of isolated BAs.

Minor Concerns:
It should be clear that this protocol is applicable to mouse brown adipocytes isolation, especially in the title and abstract.

Response:
We changed the title and revised the abstract to highlight that this protocol is for isolating mouse brown adipocytes.

Reviewer #2:
Manuscript Summary:
In this manuscript, Dr. Xu and colleagues describe a new method to isolate individual brown adipocytes from brown adipose depots. As the authors correctly indicated, the isolation of individual brown adipocytes is essential for the in-depth understanding of the functional variance and dynamics of this thermogenic cell type. The protocol in this manuscript is advantageous in that 1) it does not involve FACS sorting and 2) it is compatible with TRIZol-based protein purification from isolated brown adipocytes.

Major Concerns:
1. The purity is most important for an isolation protocol for individual cell types. The authors showed a DIC image of isolated BAs using the 6% iodixanol protocol (Fig. 3D) and qPCR and IB comparisons of BAs and non-BAs for some markers. However, these are not sufficient to quantify the purity of the isolation. I suggest that the authors isolate BAs from UCP1-TdTomato mice using the iodixanol protocol and quantify the purity of the isolated cells by FACS.
Response: We thank the reviewer for the suggestions of using FACS to analyse the BAs purity. Adipocytes are well known for their fragile nature. Large amount of debris or lipid particles are generated during the adipose tissue dissociation process, which makes the FACS analysis very challenging[3]. In our previous study, we labeled the brown adipocytes with tdTom, and isolated brown adipocytes from interscapular BAT. Consistent with published data[3], we found that most of the FACS events were tdTom negative[4]. Therefore, FACS will unlikely be a reliable method to quantify the purity of the purified BAs.

In the current study, we aimed to develop a strait forward method to isolate brown adiopocytes from interscapular BAT for gene expression study. The BAs were enriched based on their relatively low density. Because white adipocytes (WAs) also have low density, the purity of isolated BAs can potentially be affected by WAs but not other cell types. The interscapular BAT is sometimes attached by white adipose tissue, such as in old or high fat diet treated mice. Although we described that the white adipose tissue need to be removed during the BAT preparation step, the white adipose tissue might not be completely removed. Therefore, if very high purity is essential, the isolated BAs need to be sorted by FACS. We put a new paragraph in the discussion section (Page 10, lines 296 to 305) to adress this purity issue.

2. The current iodixanol protocol is based on cell density. It is well known that lipolysis during brown adipose activation is accompanied with reduced triglyceride storage in BAs, which presumably increases the density of brown adipocytes (under certain conditions). It would be

essential to confirm that the 6% iodixanol protocol non-selectively enriches all differentiated brown adipocytes (UCP1+) rather than only those lipids-laden brown adipocytes. As such, the cells underneath the upper BA layer should be examined carefully (e.g., after dilution of iodixanol and centrifugation) for UCP1+ cells.

Response: The reviewer raised an excellent question about the BAs density dynamics. As shown in our previous study[4], BAs at different developmental stages contain different amount of lipids. At postnatal day 1, BAs contain very limited amount of lipid droplets. Using this protocol, we isolated BAs from different age mice: postnatal day 1 (P1), 8 and 30. BAs from the BAT of P8 and P30 but not from P1 mice can be isolated with the current protocol. Additionally, BAs from P1 can be enriched by low speed centrifuge.

In this study, we isolated BAs from adult mice (6-8 weeks old) breed in a thermoneutral condition. In this revised manuscript, we examined the cells exist in the solution below the BAs layer. The BAs were genetically labeled with tdTom, so that we can trace whether BAs were completely separated from the non-BA cells with our protocol. As shown in Figure 3 G, cells recovered from the clear 6% iodixanol solution were tdTom negative. Also, no obvious lipid droplets were visible in these cells. On page 7, line 209 to line 217, we put a paragraph to describe these new results. These data suggest that our new protocol can efficiently separate the BAs from the non-fat cells.

Minor Concerns:

Step 2 "let the flask containing BAT and digestion buffer sit on ice for 1 hour". The purpose and importance of this 1-hour incubation at 4'C is not clear. The authors could provide more information on this.

Response: Cold treatment usually slows down enzymatic activity and gene expression[5]. We put the tissue on ice for 1 hour to reduce the BAs cell activity and to let the digestion enzymes efficiently perfuse into the brown adipose tissue. One page 10, we put a paragraph to explain the rationals (lines 292-295).

Step 3 "the temperature of the incubator at 35°C". Is 35°C a critical temperature for the digestion? Is 5% CO2 recommended? After all, 37°C incubators are commonly found in biomedical laboratories for mammalian and bacteria cell culture.

Response: For the enzyme digestion process, 37°C is a theoretically best digestion setting. However, in our hands, the incubator sometimes is overheated and the temperature can reach up to 38°C. At 35°C, the digestion works as well as at 37°C, and we do not need to worry about the overheating.

This digestion does not need to be done in a 5% CO2 incubator.

References

1. Mahdaviani, K., Benador, I. & Shirihai, O. Assessment of Brown Adipocyte Thermogenic Function by High-throughput Respirometry. *Bio Protoc* **5**, (2015).

2. Kriszt, R. et al. Optical visualisation of thermogenesis in stimulated single-cell brown adipocytes. *Scientific Report.* **7**, 1383 (2017).

3. Hagberg, C. E. et al. Flow Cytometry of Mouse and Human Adipocytes for the Analysis

of Browning and Cellular Heterogeneity. *Cell Report*. **24**, 2746-2756.e5 (2018).

4. Negron, S. G., Ercan-Sencicek, A. G., Freed, J., Walters, M. & Lin, Z. Both proliferation and lipogenesis of brown adipocytes contribute to postnatal brown adipose tissue growth in mice. *Scientific Report*. **10**, 20335 (2020).

5. Sonna, L. A., Fujita, J., Gaffin, S. L. & Lilly, C. M. Invited review: effects of heat and cold stress on mammalian gene expression. *Journal of applied physiology* **92**, 1725-1742 (2002).