Point-by-point responses

(Manuscript ID: JoVE62460 by Liu *et al*)

We would like to express our thanks to the reviewers' comments. We have revised our manuscript by including additional data and clarifications. Our point-by-point responses to all the comments by the editor and the reviewers are as follows.

Responses to Editor

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Reply: We have had the manuscript proofread by a native speaker so that there shall be minimal spelling or grammar issues in the current version.

2. Please revise the following lines to avoid previously published work: 128-130, 146, 148-150, 189-190, 233-234, 277-280.

Reply: These lines have been revised.

3. Please provide an institutional email address for each author.

Reply: Institutional email address for Dr. Liu has now been provided.

4. Please provide at least 6 keywords or phrases.

Reply: 6 keywords have now been provided.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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.

Reply: Commercial language within the manuscript has now been replaced by generic terms.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Reply: The said statement has been added before the protocol number.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Reply: The numbering of the Protocol has now been adjusted to conform to the JoVE Instructions.

8. Line 79-86: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Reply: We now split the protocol to numbered steps with each step contains less than 4 sentences.

9. Line 84: Please specify the dose of meloxicam used.

Reply: The dose of meloxicam has been provided in the manuscript.

10. Line 93-98: Please ensure that all the steps are numbered. We cannot have non-numbered paragraphs/steps/headings/subheadings.

Reply: OK.

11. Line 139: Please use standard abbreviations for time units preceded by a numeral. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Reply: We now checked throughout the manuscript to ensure standard abbreviations for time units are used.

12. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: Essential steps for the video have been highlighted.

13. Please title case and italicize journal titles and book titles. Do not use any abbreviations. 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. Please do not use the &-sign or the word "and" when listing authors.

Reply: The format of the references has been checked.

14. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Reply: The titles and legends were removed from the figures.

15. Figure 1B: Please include scale bars in all the images of the panel. Please include the details of magnification in the Figure Legends.

Reply: Scale bars are added in the images and the length of the scale bar is indicated in the legends.

Responses to Reviewer #1

Reviewers' Comments: The manuscript faces an interesting topic, though not commonly found in literature. Indeed, this argument can be deeply studied with FDG PET/CT, also in human settings.

Major Concerns:

1. It might be interesting arguing about a comparison between what you obtained in your study and what emerged from human studies, also using FDG PET/MRI. See "Andersson J, Lundström E, Engström M, Lubberink M, Ahlström H, Kullberg J. Estimating the cold-induced brown adipose tissue glucose uptake rate measured by 18F-FDG PET using infrared thermography and water-fat separated MRI. Sci Rep. 2019 Aug 26;9(1):12358." and "Lundström E, Ljungberg J, Andersson J, Manell H, Strand R, Forslund A, Bergsten P, Weghuber D, Mörwald K, Zsoldos F, Widhalm K, Meissnitzer M, Ahlström H, Kullberg J. Brown adipose tissue estimated with the magnetic resonance imaging fat fraction is associated with glucose metabolism in adolescents. Pediatr Obes. 2019 Sep;14(9):e12531." or "Lundström E, Strand R, Johansson L, Bergsten P, Ahlström H, Kullberg J. Magnetic resonance imaging cooling-reheating protocol indicates decreased fat fraction via lipid consumption in suspected brown adipose tissue. PLoS One. 2015 Apr 30;10(4):e0126705." for more details.

Reply: Thanks the reviewer for the constructive comment. In the revised discussion section, a paragraph discussing and comparing the findings between ours and those in human studies has been added. Briefly, 18-FGF/MRI based studies in humans mainly reveal functional BAT in the supraclavicular region.

2. What were the limits of your study?

Reply: The current study is mainly limited by the fact that our current protocol of cold challenge only causes a weak induction of glucose uptake in inguinal subcutaneous adipose tissue (iWAT). Therefore study has to be performed in mice with the removal of the interscapular BAT, in which glucose uptake and biogenesis of beige adipocytes were prominent. More efficient method to induce beige adipocytes in the iWAT and/or other adipose depots in normal mice are to be identified, which is beyond the scope of the current study. This limitation has now been added in the last paragraph of the discussion section.

Minor Concerns:

None.

Responses to reviewer #2

The reviewer's comment: The authors reported on a protocol for quantitative brown fat activation detection with 18F-FDG PET imaging by SUV calculation and simultaneous MR imaging for better anatomic delineation of iBAT and inguinal WAT in mice. 9 mice were distributed into 3 groups with 3 mice each, where one group was kept at thermoneutrality, one group at 6°C with removed iBAT, and one group at 6°C that only had a sham surgery. Higher SUV was found for the cold acclimated cohorts, especially for the cohort with removed iBAT.
The surgery procedure and image analysis steps including post-processing steps were described.
The text reads well, but I have some doubts whether really the up-to-date technique was described and whether the up-to-date literature was considered in both Introduction, study design and Discussion part.

Major Concerns:
1) It would be helpful to have a visualization in form of a flow chart the preparation steps you are describing, such as the mouse preparation procedure. Delineation of the anatomy on photographic images or sketches would complement the explanation of how surgery was done step by step.

Reply: Thank the reviewer for this constructive suggestion. A flow chart describing the preparation steps is provided as Figure 1A. For surgery procedures, video will be taken in which the anatomy of the mice will be clearly shown.
2) As the mouse positioning was described in the text, a schematic drawing of the PET/MR scanner including the mouse positioning would be desirable.

Reply: A schematic drawing including the PET/MR scanner and the mouse positioning is proved as Figure 1B. The detailed positioning of the mouse will also be demonstrated in the video.

3) Since MR was available - why not adding some quantitative scans such as chemical-shift-separated water-fat imaging for quantification of changes in fat fraction, or T1 mapping?

For a summary of potential MR parameters to look at, consider:

** DOI: 10.3389/fendo.2020.00421*

Reply: We thank the reviewer for suggesting the addition of the more advance MR imaging techniques for BAT quantification besides the FDG-PET scans proposed in this study. We feel that however inclusion of MR quantification scans is beyond the aim and scope of the current study, which is to investigate the glucose utilization on both BAT and WAT in vivo using FDG-PET. In addition, MR scans performed in this study serve as an anatomic reference to PET scans, where the excellent soft tissue contrast is sufficient to allow visualization and delineation of BAT and WAT,

which is not possible to achieve as reported previously using CT scans. Advance MR imaging techniques proposed by the reviewer certainly warrant thorough investigation and optimization on the preclinical scanner, and these kind of studies are currently underway in our laboratory which we hope to report the findings in due course.

4) In my opinion it is not feasible to do statistically significant tests at this low number of animals per cohort. Could you then also specify which test you used for calculating the p-values? This was missing in both the text and the figure caption. I think it would be enough showing the results for the mice measured, and leaving out the statistical component as this was not the intention of the paper anyhow, if I understood correctly.

Reply: We agree with the reviewer that the statistical tests were not feasible due to the low number of animals used. Thus we have now deleted the statistical component in Figure 2, as suggested by the reviewer.

Abstract:

5) Line 24-25: To my knowledge BAT is still in an early research stage and how to use it as a therapeutic target is still very unknown. Dangerous hyperthermia conditions have resulted from early tests.

Reply: Yes the reviewer is correct that BAT is potential but not yet new therapeutic targets for diseases. This statement is toned down.

Introduction:

6) Line 60: Here you say correctly, that it is a potential target for therapy of obesity, but only potential! Please adapt in the abstract.

Reply: Indeed claiming BAT as a potential target is more appropriate. We have rectified the overstatement in the abstract.

Discussion

7) Line 268-280: In contrary as stated by the authors, fasting status is important to control when examining BAT activation, as shown for instance in:
*Postprandial activation of BAT: * DOI: 10.1016/j.cell.2018.10.016*

Reply: We compared the FDG uptake in both fasting and fed mice and our results showed that glucose uptake in BAT was minimized after the mice were fasted overnight. This study (DOI: 10.1016/j.cell.2018.10.016) demonstrated that secretin, which is elevated after meal, mediates prandial thermogenesis in BAT, which consequentially induces satiation. Therefore this study is consistent with our notion that BAT is more activated in fed state of the mice.

8) Please read and consider following publications on PET/MR scanning of BAT in humans:

https://doi.org/10.1002/oby.22560

doi: 10.1002/mrm.26589

Reply: We do notice that there are several PET/MRI studies for assessment of BAT in humans. We have now discussed and compared the findings in humans and in mice by us in the discussion section. Briefly, 18-FGF/MRI based studies in humans mainly reveal functional BAT in the supraclavicular region.

9) Line 314-315: How would the authors suggest to suppress iBAT activity and how to selectively activate beige adipose tissue? Do the authors have any reference literature? And more importantly: WHY would that be of interest?

Reply: There are studies indicating that impairment on classical BAT induces beige cell biogenesis in iWAT, as described in the studies: (1) Brown Fat Paucity Due to Impaired BMP Signaling Induces Compensatory Browning of White Fat. Nature. 2013 Mar 21; 495(7441): 379–383. (2) Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. Cell Metab. 2014 Apr 1; 19(4): 593–604. Lin et al. reported that administration of GC-1, the thyroid hormone receptor beta (TR β) agonist, induces multilocular beige adipocytes in iWAT (Pharmacological Activation Of Thyroid Hormone Receptors Elicits A Functional Conversion Of White To Brown Fat. Cell Rep. 2015 Nov 24; 13(8): 1528–1537.) Several flavonoids have also been demonstrated as inducers of beige adipocytes, which is reviewed by Zhang et al. (Flavonoids as inducers of white adipose tissue browning and thermogenesis: signalling pathways and molecular triggers. Nutrition & Metabolism. 2019; 16: 6022).

Therefore, although in the current study, only a weak glucose uptake was induced in iWAT, the protocol described for measurement of glucose uptake and functionality in iWAT would be of interest for other researchers for evaluation of pharmacological reagents, or characterization of specific animal models.

10) Could you please indicate what kind of mice were used? special genetic sort?

Reply: We are using 8-week-old C57BL/6J mice. This information is now provided at the beginning of the protocol.

Minor Concerns:

Line 140: Please add reference to given formula.

Reply: A reference is added.

Line 155: … body temperature and the respiratory …

Reply: The missing word “and” has been added.

Line 154-155: please check wording/grammar of this sentence

Reply: This sentence is rectified as “Adjust the mouse bed position to include the whole mouse body, and to ensure the center FOV of MR is located in the center of mouse body.”.

Line 188: using the MR image for reference

Reply: The sentence has been rectified. Thank you.

Line 198-200: please add reference to equation

Reply: A reference is added.

Line 210: …, that are …

Reply: This typo has been corrected.

Line 215: are you here referring to in this case sham operated or iBATx?

Reply: Here we are referring to sham operated mice. We now further clarified this point in the description.