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August 12, 2020

Dear Dr. Nguyen,

We are pleased to submit our revised manuscript JoVE 61732 “Isolation, Proliferation and Differentiation of Rhesus Macaque Adipose-Derived Stem Cells” for publication in the Journal of Visualized Experiments. We appreciate the time and effort that the editor and the reviewers dedicated to providing feedback and are grateful for the critiques and have edited the manuscript according to the comments. We believe that our manuscript has been strengthened by the changes requested and we appreciate the opportunity for revision.

The details of the changes made are as follows. **The changes are highlighted in the manuscript. All page numbers, text lines and figures refer to the revised manuscript file with tracked changes.**

Editorial Comments:

COMMENT #1: Significant portions show overlap with previously published work. Please re-write lines 254-269, 334-338 to avoid this overlap.

We appreciate the comment, we have now rephrased the mentioned lines and have gone through the text to ensure that there is minimal overlap with published work.

COMMENT #2: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) 1) Some examples NOT in the imperative: 4.5. 2) Avoid long descriptions such as on lines 78-90.

We have rephrased the protocol section to be in the imperative tense. We have also edited the text to avoid long sentences and descriptions.

COMMENT #3: Include magnification for steps 2.2.9 and 3.2.3 and mention all settings for step 4.5.

We apologize for the oversight. We have now added the Magnification for microscope objectives in steps 2.2.9 (line 174) and 3.2.3 (line 220) and settings for step 4.5 (line 245).

COMMENT #4: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have restructured the discussion to describe the 5 major points as instructed.

COMMENT #5: Fig 1-2: Add scale bars.

We apologize for the oversight; we have now added scale bars to Figures 1 and 2.

COMMENT #6: Please spell out journal names.

We have edited the references to reflect the JoVE style of citations.

COMMENT #7: Please sort table of materials in alphabetical order.

We have now sorted the Table of Materials in alphabetical order.

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Comments from Peer-Reviewers:

Reviewer #1:

COMMENT #1: I recommend using English editing service because there are multiple grammatical errors found in the text.

We have thoroughly gone through the text and ensured that there are no grammatical or textual errors. We apologize for the typographical mistakes.

COMMENT #2: In my experience, the process human ADSCs isolation from visceral fat (collagenase digestion/centrifugation/separation of adipocytes) produces 1st SVF that after few days in culture in regular medium (DMEM/10% FCS/antibiotics) contains ~50% ADSCs and ~ 50% endothelial cells distinguished by their own morphology. The authors use 20% FCS, that it is ok, although most of the protocols indicate the use of 10%FCS.

Following collagenase digestion and separation of adipocytes and RBC lysis, the SVF fraction is plated in ASC media. The media is changed after the first 24 h, to remove most immune and other non-adherent cells and subsequently, media is changed every 48 h until the cells are about 80% confluent. We and others have shown using this protocol that most adherent cells have a fibroblast morphology and differentiate efficiently into adipocytes. Although we cannot unequivocally prove, the cultured SVF fraction is free of most of the contaminating cell types including RBCs, immune cells, and cells of endothelial lineage. We were using 10% FBS initially as suggested by most protocols but based on studies we have performed with primary macaque ADSCs, the cells proliferate and differentiate better in 20% FBS. Hence, for primary macaque ADSCs we recommend 20% FBS be used.

COMMENT #3: The preparation of collagenase digestion buffer (CB) does not require 1% BSA. If BSA is added to CB it is necessary adjust the PH to 7.4, because BSA make CB acidic. If 1% BSA is present in CB, I do not recommend to keep it at 4C for 4 weeks, because the possibility of bacterial infection, without BSA it is OK.

BSA is added in the collagenase digestion buffer to allow for separation of mature adipocytes during the digestion process. These mature adipocytes can then be used for downstream analysis. As mentioned in the protocol (lines 100-102), we recommend that CB be made fresh during the day of isolation and be used within 1 hour.

COMMENT #4: Besides dex, IBMX, adipogenic medium require the presence of insulin and PPARg agonist. CRITICAL

We and others have successfully differentiated both rhesus macaque and human adipose samples without insulin and PPARγ agonists in adipogenic medium (Ford, SM *et al.* 2018; Bunnell, BA *et al.* 2008; Gagliardi, C & Bunnell, BA 2011). In our laboratory, ADSCs are primarily used for metabolic studies involving assays for insulin-stimulated responses. To reduce the potential for insulin used in the differentiation cocktail to affect adipocyte metabolic capacity, and because the cocktail we use allows for optimal adipogenic differentiation, we recommend using the cocktail without insulin, especially for studies that will use differentiated adipocytes for downstream insulin-stimulated assays.

COMMENT #5: If cells are not adherent after 48 hs (line, 178) mean all cells are death, it is irrelevant to increase concentration of FBS in the ADSC growth medium.

We agree with the reviewer's comment. We have rephrased the sentence to read that after 24 hours at least 10-20 % of cells should be adherent and if there are no adherent cells, it indicates cell death due

to unforeseen issues during the isolation process or contamination of cell cultures (lines 183-187).

COMMENT #6: For 300,000 cells, I recommend to resuspend them in 5 cc culture medium and plate in 60 mm dish.

With primary ADSCs, one of the objectives is to have maximal number of cells for all proposed experiments. We have performed a number of optimization experiments for seeding densities and have found that if 300,000 cells are plated on a 100 mm culture dish, we can achieve ~100% confluency in 96 hours and it does not affect the gene expression or differentiation potential of ADSCs. Hence, we are fairly confident in recommending 300,000 cells in 100mm cell culture plates for expanding primary macaque ADSC cell lines.

COMMENT #7: Authors should describe concs of Oil Red O and BODIPY used, time of incubations, washes, etc.

We apologize for the oversight. The protocol is now updated to include all relevant information for Oil Red O and BODIPY staining.

COMMENT #8: I also recommend to characterize ADSCs using immunofluorescent label CD105.

We thank the reviewer for the suggestion. We use Oil Red O staining, BODIPY immunofluorescent staining and adipogenic gene expression to determine differentiation potential of ADSCs. CD105 is a good marker to confirm that the cells isolated are indeed adipose derived mesenchymal stem cells, although it is not routinely done in our laboratory. However, we have now added that CD105 can be used to confirm ADSCs in the Note section (lines 249-251).

Reviewer#2? (comments in PDF document)

COMMENT #1: plastic adherence is not a feature of multipotency

We have rephrased the sentence to describe the characteristics of stem cells and the adherence properties of ADSCs (lines 56-58).

COMMENT #2: how similar? please indicate the differences

We have rephrased the sentence to read “For the same amount of tissue used, we have observed that subcutaneous adipose tissue is more dense, tougher and yields less cells from digestion compared to omental adipose tissue ” (lines 79-81).

COMMENT #3: identification of lipid vesicles

We have now replaced “vesicles” with “droplets” where appropriate.

COMMENT #4: Please, explain the use of 5-PBS vs 2-PBS wash buffers

Because necropsy and collection of adipose tissue from macaques are performed in a non–sterile environment, we use higher concentrations of antibiotics to minimize risk of contamination. We have now the explained the use of 2 different concentrations of antibiotics in the Note section (lines 97-98).

COMMENT #5: Vendor, Country of origin, heat inactivated?

The details of FBS is provided in the Table of Materials. This is to adhere to the manuscript guidelines.

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COMMENT #6: Indicate the weight of adipose tissue

We generally use ~50 g of adipose tissue. We have now added this to the manuscript, line 119.

COMMENT #7: Mincing does not free cells

We apologize for the error. We have rephrased the sentence to indicate that mincing allows to increase the tissue surface area for more efficient and complete enzymatic digestion (lines 124-125).

COMMENT #8: Since the tube is sealed, there is no need to keep it in 5%CO2

We agree with the reviewer, enzymatic digestion need not be performed in 5% CO2. We have revised the step (2.1.7).

COMMENT #9: RT or 37

We have clarified the step and added that room temperature PBS is used.

COMMENT #10: "Rotate"

Step 3.1.2 indicates how to add Trypsin to the plate. Because only 2 ml of trypsin is added, the sentence was added to emphasize that the whole surface area should be covered. We have rephrased the sentence to read "Aspirate PBS and add 2 mL of 0.25 % trypsin-EDTA per 100-mm culture dish. Ensure the entire plate surface area is covered with trypsin."

COMMENT #11: Please replace Fig 1B with a sharper image

We apologize and have replaced Fig 1B.

COMMENT #12: Why does nuclei appear blue?? There is no DAPI being added to the staining buffer

We agree with the reviewer and we apologize, we have now removed the sentence.

COMMENT #13: Vendor #cat

The details of BODIPY is provided in the Table of Materials. As mentioned earlier, this is to adhere to the manuscript guidelines.

COMMENT #14: Indicate the wavelength and filter sets used for imaging

We have added the details used for imaging in the text (lines 304-306).

COMMENT #15: Typographical errors

We have addressed the specific comments by the reviewer and have thoroughly gone through the text and ensured that there are no grammatical or textual errors. We apologize for the typographical mistakes.

Reviewer #3:

COMMENT#1: As mentioned by authors on line 54 "Primary ADSCs can be isolated directly from white adipose tissue and stimulated to differentiate into adipocytes, cartilage or bone cells", I would suggest that both osteogenic and chondrogenic differentiation should be performed to ensure the multipotency of the isolated cells.

We agree with the reviewer that ADSCs are multipotent. However, our laboratory's primary focus is to elucidate how adipogenic metabolic capacity of adipocytes is influenced by external stressors. Based

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on our expertise, we have focused this manuscript on isolation, proliferation and adipogenic differentiation of ADSCs primarily for metabolic studies and assays. However, we have added in the introduction that ADSCs are multipotent and can be use in studies relating to regenerative medicine.

COMMENT #2: Figure 1B should be replaced by a higher quality image since it's impossible to distinguish the morphology of the cells.

We apologize and have replaced Fig 1B.

Thank you, in advance, for your time and consideration.

Sincerely,

Liz Simon
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