We appreciate the enthusiasm for our work and the specific feedback from the editors and reviewers to improve upon our work. We have listed our responses to comment in blue font below.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. We have carefully proof-read the manuscript for spelling and grammar issues.

2. Keywords: Please provide at least 6 keywords or phrases. The following keywords/phrases have been added to the title page of the manuscript: 1) human perivascular adipose tissue, 2) adipose progenitor cell, 3) mesenchymal stem cell, 4) chondrogenesis, 5) osteogenesis, 6) adipogenesis, 7) cell differentiation, 8) cardiovascular disease

3. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method-We have included this information in the first paragraph of the introduction.

b) The rationale behind the development and/or use of this technique We have included this information in the second paragraph of the introduction.

c) The advantages over alternative techniques with applicable references to previous studies We have is discussed this throughout the introduction/discussion.

d) A description of the context of the technique in the wider body of literature We have included this information throughout the discussion.

e) Information to help readers to determine whether the method is appropriate for their application We have included this information in the second paragraph of the discussion.

4. 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 and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: glutaMAX, Barnstead-Labline MAX, Peprotech, Accutase, Permount, etc. We have removed commercial language from materials in the main text and referenced appropriately in the table of materials.

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion. We have corrected the language to imperative tenses and avoided using “could be”, “should be” etc… We have also removed any comments about the protocol from the methods steps to the discussion.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below. We have added additional citations/detail to adequately describe the protocol.

7. 2.7: What is used to mince the tissue? We use forceps and dissecting scissors to finely mince the tissue prior to enzymatic digestion. This information has been added.

8. 2.19, 3.1, 5.1: Please specify the culturing conditions (e.g., temperature). Cells are cultured at 37°C and 5% CO2 , This information has been added to the methods e.g. line 169.

9. 4.13: Please provide some guidance on the appropriate number of replicates. We added this information in section 4.1.

10. 8.15: How to dehydrate? We added this information (line 254).

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Steps have been combined to approximately 3-4 sentences, drastically reducing the number of steps.

12. Please include single-line spaces between all paragraphs, headings, steps, etc. We made these formatting changes.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

16. Figures: Please include a space between numbers and their corresponding units of the scale bars (e.g., 1 cm, 100 µm, 200 µm). We corrected this.

17. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique. This is now discussed in the first paragraph of the discussion.

18. References: Please do not abbreviate journal titles.

19. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment. We have corrected this.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a clear and simple description of the methodological approach to harvest periaortic adipose tissue from human patients to derive perivascular adipose mesenchymal stromal cells. The methods describe both the preparation of the MSC population and their adipogenic, osteogenic and chondrogenic differentiation under standard inductive conditions. The novelty lies in the use of human peri-aortic adipose as the source of MSCs, which is relevant to studying perivascular fat and its progenitor cell content in human vascular diseases, including atherosclerosis. I have mainly minor comments/questions for the authors to address as listed below:

Major Concerns:

The first three sentences of the abstract and opening paragraph of the Introduction both focus on the important paracrine role of PVAT in signalling to the vasculature. The rest of the abstract and the main text itself do not dwell on this aspect of PVAT again. In my opinion, it would have been ideal if the authors had demonstrated or at the very least discussed the appropriate methodology to study the paracrine properties of PVAT, given the context with which they introduce the importance of PVAT in their manuscript. As they have not done this, they should consider rewording the opening of their Abstract to more closely reflect the subject of this manuscript - i.e. the study of perivascular adipose progenitor cells. We thank the reviewer for this suggesting and agree that our wording was off topic to the main focus of the paper. We have revised the wording in the abstract to be more focused on the perivascular adipose progenitor cells rather than paracrine properties of PVAT.

Minor Concerns:

Page 3, L65: change "aortic" to "aorta"-corrected

Page 4, L123: add "tube" after "conical"- corrected

Page 5, L146: change "drastic" to "marked"- corrected

2.18: Do the cells need to be cultured into CFU-F before reaching confluent stromal cultures? No, they are cultured to 100% confluence and immediately used for one of the three differentiation assays.

2.18: "confluency" is best replaced by "confluence"- corrected

What passage(s) of cells would you recommend using for the differentiation assays? We have included this information in the manuscript. The PVAT adipose progenitor cells require several rounds of replication to reach sufficient density and numbers for experimentation. We utilized cells in passage 5-7.

Is gelatin required for differentiation experiments? No

3.1 L164: Please provide a reference(s) for human BM MSC preparation we have included the appropriate reference in the manuscript. Nadri et al. “An efficient method for isolation of murine bone marrow mesenchymal stem cells” *The International Journal of Developmental Biology”.* 51: 723-729 (2007).

5.2: Why are the non-induced conditions also carried forward until D14 to serve as a more suitable control?- Yes that’s exactly correct. It also demonstrates that there is not spontaneous differentiation of control cells into chondrogenic/osteogenic/adipogenic lineages.

Page 10, L371: please add in "to" before "test their propensity"- corrected

Page 10, L370-2: A mixture of tenses is used in some parts of the discussion - e.g. fluctuating between "we study" and "we exhibited" - please use consistent tenses- corrected

Page 10, line 377: add "more" before "lineage committed than BM MSC"- corrected

Reviewer #2:

Generally speaking, it is a fair good work. I suggest major revision should be done for the further publication. Please pay more attention about the following lists:

(1) Is it possible to explain the difference of progenitor cells from human thoracic PVAT samples and other species? We appreciate this feedback and have made reference to this concern as a discussion point. Adipose progenitor cells are highly variable in cell surface protein expression, gene expression and differentiation capacity depending on their depot of origin. Given that so little is known regarding the phenotype of progenitor cells from human thoracic PVAT, and the high degree of heterogeneity of progenitor cells from other adipose depots, it is not possible at this time to make strong conclusions about the differences in the cells types.

(2) About the figures, the details of the statistical analysis from each lineage differentiation are better than image only. The readouts for this body of work were predominantly qualitative to demonstrate feasibility of the approach and techniques.

(3) Line 122, is it possible to define the size of the pieces, not only the weight. Because the shape of the small pieces influence the digestion for single cell suspension or tissue dissociation. We have highlighted additional details in section 2.2.

(4) Line 123, please define type of the DMEM, high or low glucose. Corrected.

(5) Line 138, for the cell strainer, do you need to squeeze or only rinse with solution? **We rinse the strainer and have emphasized this in section 2.4.**

(6) Line 151, please define the solution liquid for the FGF2. Is it culture medium? We have added this information in section 2.7.

(7) Line 156, the authors should provide more details of the confluency. Because in some cases 80-90% confluency are more conducive to cell growth for passaging. We have added several pieces of information regarding the confluency and passing the cells in the discussion.

(8) Line 196, "Incubate the micromass cultures for 2h", how to make sure the 10μL droplet would not dry out? Any moisturize process? We included in this step that the micromass droplet is in a plate containing PBS to remain humidified during formation (section 4.6).

(9) Line 257, please provide more details about the pre-process of the embedding. Substantial new detail has been provided regarding the dehydration, processing and embedding of the tissue/mass.

Reviewer #3:

Manuscript Summary:

Author describe protocol including three individual assays designed to induce perivascular adipose tissue derived cells to differentiate toward adipogenic, osteogenic, or chondrogenic lineages

Major Concerns:

Authors should provide alternative readout for the differentiation protocol. PCR of specific genes such as PLIN2, PPAR, hSox9, Acan, RUNX2, SPP1, Osteonectin may represent an alternative. We thank you for this suggestion and agree that analysis of osteogenic/chondrogenic gene expression in the differentiated cells is an important next step. Given that this is a methodology paper, we believe that getting into gene expression is beyond the scope of the research.

For chondrogenic differentiation authors should provide a protocol for Toluidine blue staining. We did Alcian blue for the purposes of showing chondrogenic lineage commitment in this report and have provided additional details regarding the staining procedure.

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