Author response to editorial and reviewers' comments

Editorial comments:

1. Please include spaces between numbers and units.
[Answer] We thank you for this comment. We checked and fixed it in the revised manuscript.

2. Please define abbreviations at first occurrence (ie, DMEM, etc.).
[Answer] Thank you for reminding us. According to the editorial comments, we define abbreviations at first occurrence.

3. Please use the Greek symbol Mu rather than a lowercase u to denote micro (see 1.3, 4.3.2).
[Answer] We thank you for this comment. According to the editorial comments, we changed it.

4. Please split 3.1 into two steps.
[Answer] Thank you for your comments. According to the editorial comments, we split 3.1 into two steps.

5. Materials table is missing. Please include all reagents and materials, including media components.
[Answer] We are grateful for this editorial comment. According to the editorial comments, we made the material section and attached a supplement table.

6. References – Please abbreviate all journal titles.
[Answer] We thank you for this comment. According to the editorial comments, we changed it.

7. Length exceeds 3 pg of material. Please highlight 2.75 pg (1 pg minimum) of continuous protocol for filming.
[Answer] Thank you for your comments. We highlighted it yellow (Line 146-330).

8. Grammar:
-Line 82 – "This novel methods"
-1.6 – "senescence cells"
-3.1 – "each gelatin coated culture dishes"
-4.3.3 – "45 cycles for 15 seconds at 95°C for 15 seconds"
[Answer] We thank you for this comment. We checked and fixed in the revised manuscript.

9. Additional detail is required:
-2.2 – How are cells counted?
[Answer] Thank you for your comments. We explained it in 2.3

-2.5 – How many cells are seeded? What medium is used? How long after changing medium in 2.4 are mESCs added to the culture?
[Answer] We checked and modified the protocol in the revised manuscript.

How are mESCs acquired?
[Answer] We acquired G4 mESCs line from Dr. Andras Nagy in Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, 25 Orde Street, Toronto, ON, M5T 3H7 Canada.

-2.6 – How are cells passaged?
[Answer] Thank you for your comments. We explained it in 3.1

-4.1.3 – Are cells fixed prior to PFA addition? This is currently how the step is written.
[Answer] Thank you for reminding us. We checked and fixed this in the revised manuscript.

10. Branding:
-Figure 1B – Opti-Mem
-4.3.1 – Trizol
-4.3.3 – "ABI 7500"
[Answer] Thank you for reminding us. We removed brand names in the revised manuscript.

11. Results: Figure 3 – What statistical test was used?
[Answer] Thank you for your comments. Statistical analyses were performed using GraphPad Prism 5, and significant differences between groups were identified by one-way ANOVA and Tukey's post-hoc test.

12. Discussion: Please discuss the significance of the method with respect to alternative techniques, and include independent citations. Please also discuss the limitations and any modifications/troubleshooting that can be performed.
[Answer] Thank you for your comments. We explained it in discussion section.

13. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
[Answer] We are grateful for the reviewer's comment. We added phrases and will send a copy of the modification/re-print permission for JoVE's record keeping purposes.

14. JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and

obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.
[Answer] We thank you for this comment. We added DOIs in the revised manuscript.

15. IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published
version.

Reviewer 1 Comments for the Author:

Manuscript Summary:
This manuscript is adequately written and logically organized, making it easy for readers to follow. I do not have any further comments for this manuscript. I just have one suggestion to the authors. I think that it would be more helpful for the readers if this protocol has a separate section for MATERIALS which describe some more additional details for the materials used in this protocol such as composition of reagents (culture media) and company information (vendor and batch number) etc.
[Answer] We appreciate the Reviewer's constructive suggestion very much. According to the editorial comments, we made a material section. However, commercial language (brand name) is restricted by editorial policy.

Reviewer 2 Comments for the Author:

Manuscript Summary:
This manuscript demonstrates an optimized method for the collection of mESCs-CM under serum- and feeder-free conditions, and for the characterization of mESCs-CM using senescence associated multiple readouts. This protocol provides a method to collect pure mESCs-specific secretory factors without serum and feeder contamination. The authors think that this technique facilitate therapeutic applications of mESCs-CM toward aging interventions, aging-associated diseases, and other regenerative medicine.

Major Concerns:
The reviewer's opinion is that mouse ESCs-condition medium does not contribute to any therapeutic applications for human patients. Furthermore, mESCs can easily culture on gelatin-coated dishes with conventional chemically defined medium with LIF (Leukocyte inhibitor factor). Therefore, it is quite easy to culture mESCs in serum- and feeder-free conditions. If this topics is applied to human ESCs, hESCs can be cultured on recombinant vitronectin-coated dishes in Essential 8 medium (chemical defined, serum-free, xeno-free culture medium). Therefore, the collection of hESCs-CM is quite easy under serum- and feeder-free conditions. The reviewer cannot find original and novel points of this manuscript.
[Answer] Thanks for the comment. We agree with the reviewer that mouse ESC conditioned medium would not contribute to therapeutic application for human aging or aging-associated

diseases. We rephrased these sentences in our revised version. We agree that serum- or feeder- or LIF (Leukemia Inhibitory Factor)-free culture conditions for mESCs are established technique. However, accumulated data have demonstrated that even though CMs were derived from same type of cells, their biological functions and molecular components are different, depending on their culture conditions, passage number of stem cell, culture medium and so on (PMID 25530971). In addition, there are just a few studies addressing a biological function of CM from murine iPS cell (not even ES cell), and their culture conditions were not clearly described and were very unlikely serum-free or feeder-free conditions (PMID: 23063297, 21835903). Further more, we were not able to find any publication where biological function of ESC-derived CM was tested or secreted molecules of CM were analyzed. Therefore, our work provides details of culture condition that enables us to collect and test the role of ESC-derived CM on cellular senescence, thereby readers in scientific community can easily repeat or modify this protocol for their own research purposes.

Minor Concerns:
1. The abbreviation should be defined when it appeared at the first time. "HDF" is appeared at line 115, but definition is Line 162.
[Answer] Thank you for reminding us. According to the comments, we define abbreviations at first occurrence.

2. DMEM, FBS should be written at the first time.
[Answer] Thank you for your comment. According to the editorial comments, we define abbreviations at first occurrence.

3. This manuscript is 1. Preparation of MEF, 2. Culture on mESCs on MEF, 3. Culture of mESCs on gelatin-coated dishes, 4. Collection of conditioned medium, 5. Characterization such as SA β-gal Assay, Cell Cycle assay, and qRT-PCR. These techniques are old and everyone knows these techniques. This manuscript for the textbook of mESC culture. It might be necessary to show how to culture human ESCs and iPSCs. However, there is no meanings to show mESC culture method. This is why JOVE does not upload this techniques until now.
[Answer] As we described above, main goal of our manuscript is not addressing mESC or hESC or hiPS culture condition or techniques, which are already established and are standardized.

Reviewer 3 Comments for the Author:

Manuscript Summary:
The manuscript describes a method for collection of conditioned medium (CM) from mouse embryonic stem cells (mESCs) cultured in the absence of feeder cells and using serum-free medium. The mESC-CM is shown to have an anti-senescence effect on human dermal fibroblast shown as decreased number of senescence associated beta-galactosidase positive staining, increased S and G2/M -phase cell populations by flowcytometry, and decreased senescence-associated gene expression levels.

Major Concerns:
Collection of conditioned medium from ESCs is quite standard and there is nothing particularly new or novel in the approach. The collection of CM should be standardized and a video describing the protocol would itself be useful for a large audience. However, I feel that there should be a deeper evaluation, and experimental, comparative data to show why this particular way of CM collection described in the manuscript is considered optimal. Many things such as cell lines, culture conditions, cell passage, cell numbers, matrix for feeder-free culture, CM collection time, and culture medium affect the factors that are secreted by the ESCs to the CM. These are not addressed or discussed in the manuscript. Currently there are many commercially available matrix-media combinations available for feeder independent culture of pluripotent stem cells that can be used for optimal, long-term culture in undifferentiated, pluripotent state. The gelatin + OPTI-MEM medium (?)combination for 24 hours in unlikely optimal for the mESC culture or at least this is not shown. The authors state that they provide an optimised method for CM collection but no optimisation is shown.

[Answer] We appreciate the Reviewer's constructive suggestion very much. We added some information about cell lines, culture conditions, cell passage, cell numbers, matrix for feeder-free culture, CM collection time, and culture medium in the revised manuscript. With regards to description about optimization process, we discussed briefly about how we selected this condition for collecting CM. Due to the space limitation, we are not able to provide all the optimization processes in our manuscript. Interestingly, in our ongoing experiment, we were able to perform high throughout screening on our CM, such as whole secretome analysis and micro RNA (miRNA) analysis (unpublished data). Although this protocol may not be a 'standard protocol' for mESC-CM collection, we believe that our method could be at least 'optimal' condition to show the anti-senescence effect of CM harvested from mESC in our study.

Also, I think it is very unlikely that mESC-CM is ever going to be used as therapeutic agent to treat patients. Parts like: "… facilitate therapeutic applications of mESCs-CM toward aging interventions, aging-associated diseases and other regenerative medicine" should be tuned down. An optimal method for CM collection from both human and mouse sourced pluripotent cells is useful mostly for proteomics based identification the (growth) factors that mediate the anti-senescence or other cellular responses. These factors could then be used as therapeutic agents.
[Answer] We do agree with Reviewer's comment and appreciate this valuable suggestion. We did tune down the sentence (last sentence of abstract and introduction). Currently, we try to establish in hESC system in our facility. Although we did not perform any experiments with conditioned media from human ESCs on cellular senescence process, CM from hESC-derived endothelial precursor cells has been shown to contain FGF-2, PDGF-AA, PDGF-AB/BB, and MCP-1 (PMID: 21235296), suggesting that hESC-CM might possess similar anti-senescence activity.

The data shown in the manuscript has been recently published in more detail by the authors in Yun-Ui Bae et al. 2016. Antisenescence effect of mouse embryonic stem cell conditioned

medium through a PDGF/FGF pathway. FASEB Journal vol. 30 no. 3 1276-1286. A more general approach to CM collection should be taken for a video publication for learning purposes.

[Answer] We thank you for this comment. We will try to coordinate with JOVE team to film more general approach as well.

Minor Concerns:
Abstract: References in the abstract? Last sentence should be tuned down.
[Answer] We thank you for this comment. We deleted references in the abstract and last sentence.

Introduction: Last paragraph is not realistic.
[Answer] Thank you for your constructive suggestion, we changed last paragraph of introduction.

Protocol: Reduced Serum Media? OPTI-MEM?
hDFs - abbreviation not introduced
[Answer] Thank you for reminding us. According to the editorial comments, we define abbreviations at first occurrence.

Cell numbers at each step?
[Answer] We thank you for this comment. We checked and modified protocol in the revised manuscript.

1.6) Are any of the chemicals hazardous?
[Answer] We are grateful for the reviewer's comment. DMF (dimethylformamide) is toxic. We added caution information in the revised manuscript.

2.2) Trypsinize?
[Answer] We thank you for this comment. We checked and modified protocol in the revised manuscript.

2.3) Medium?
[Answer] Thank you for your comments. We added exact medium name in 2.3

3.3) Collect "supernatant" after centrifugation?
[Answer] Thank you for your comments. We explained this in 3.4

4. The CM is not characterised. It is the effect of the CM to hDF that is shown.
[Answer] We thank you for this comment. We changed title of protocol 4.

4.1.1) Supplier of the fibroblasts?
[Answer] We are grateful for the reviewer's comment. We purchased human dermal fibroblasts (HDFs, NHDF-Ad-Der-Fibroblast) from Lonza. Please see Supplementary Table.

4.1.2) All medium should be changed the following day after seeding to discard cells that have not attached.
[Answer] Thank you for your comments. Although changing medium after seeding would be better for the overall experimental setting, we did not change medium as HDFs have excellent attachment abilities and we hardly found floating cells.

4.3.1) Which cells?
[Answer] Thank you for your comments. We explained this in 4.3.1

Results:
Why do mESCs show different morphology in the two culture conditions? Do they also secrete different factors? Are the mESCs alive/pluripotent/apoptotic?
[Answer] We are grateful for the reviewer's comments. Figure 1 (A) is normal mESCs culture condition and mESCs are pluripotent and alive. But Figure 1 (B) mESCs are in serum- and feeder- free as well as LIF-free condition for 24 hours. Due to this reason, as described previously (PMID 17546008), their morphologies are different from Figure 1 (A) and they are still healthy cells. Also, as LIF was not included in Figure 1 (B) condition, mESCs may not be fully pluripotent cells. However, we were able to detect pluripotency-specific micro RNA (miRNA) 290-295 cluster (PMID 18692474, 19363475) from our CM, indicating that these cells are still pluripotent cells and they produce and secret miRNA 290-295 cluster to CM.

Senescent hDFs? Was senescence induced?
[Answer] Thank you for your comments. We induced senescence of HDF by multiple passaging (replicative senescence model, PMID; 13905658, 23590226).

Figure Legend 3. Characterisation of anti-ageing affect of mESC-CM to hDFs.
What is Y in the 3C? Cells trated with?
[Answer] We thank you for this comment. We used Y (=non-senescent cell, young cell) with no treatment for positive control. We explained in the Figure legends section.

Use standard deviation instead of SEM. Statistical test?
[Answer] Thank you for your comments. We changed our data using standard deviation instead of SEM. Statistical analyses were performed using GraphPad Prism 5 and significant differences between groups were identified by one-way ANOVA and Tukey's post-hoc test.