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Collection of serum- and feeder-free mouse embryonic stem cell conditioned media for a cell-free approach

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Abstract:	The capacity of ESCs and induced pluripotent stem cells (iPSCs) to generate various cell types has opened new avenues in the field of regenerative medicine. However, despite their benefits, the tumorigenic potential of ESCs and iPSCs has long been a barrier for clinical applications. Interestingly, it has been shown that ESCs produce several soluble factors that can promote tissue regeneration and delay cellular aging, suggesting that ESCs and iPSCs can also be utilized as a cell-free intervention method. Therefore, the method for harvesting mESCs-CM with minimal contamination of serum components (FBS) and feeder cells (MEFs) has been highly demanded. Here, the present study 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 will provide a method to collect pure mESCs-specific secretory factors without serum and feeder contamination.
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Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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Dear Teena Metha,

We would like to submit our revised manuscript entitled 'Collection of serum- and feeder- free mouse embryonic stem cell conditioned media for cell-free approach' into Journal of Visualized Experiments (JoVE). In this revised manuscript, we address all the comments/suggestions from editorial and reviewers.

Please see below for some information about our manuscript.

Title	Collection of serum- and feeder- free mouse embryonic stem cell conditioned media for cell-free approach
Author contributions	Y.-U.B: designed and performed experiments, demonstrate experimental procedures, wrote manuscript. H.-K.S. & J.-R.K: designed project and experiments, wrote manuscript
The name of editor	Dr. Teena Mehta
Reviewer	Suggested: 1) Dr. Goo Jang, Associate professor, Seoul National Univ. snujang@snu.ac.kr. 2) Kiwon Ban, Assistant professor, City University of Hong Kong, Ban.KW@cityu.edu.hk

Although embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) hold promise for future cell-based therapy in the regenerative medicine, there are still multiple roadblocks for therapeutic application. Interestingly ESCs or iPSCs are known to produce multiple soluble factors that might have beneficial contribution to tissue regeneration and aging process.

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To explore biological function of soluble factors from ESCs, we optimized protocol for the collection of mouse ESCs conditioned medium (mESC-CM). Our protocol provides a method to collect mESC-specific secretory factors without contamination from serum and feeder cell layer, which will enable full characterization of mESCs-derived factors for regenerative and anti-aging medicine.

Thank you for your positive consideration.

Sincerely yours,

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TITLE:
Collection of serum- and feeder-free mouse embryonic stem cell-conditioned medium for a cell-free approach

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Cellular senescence, Conditioned media (CM), Embryonic stem cells (ESCs), Cell-free approach

SHORT ABSTRACT:
This protocol provides a method for the collection of mouse embryonic stem cell (mESC)-conditioned medium (mESC-CM) derived from serum (fetal bovine serum, FBS)- and feeder (mouse embryonic fibroblasts, MEFs)-free conditions for a cell-free approach. It may be applicable for the treatment of aging and aging-associated diseases.

LONG ABSTRACT:
The capacity of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to generate various cell types has opened new avenues in the field of regenerative medicine. However, despite their benefits, the tumorigenic potential of ESCs and iPSCs has long been a barrier for clinical applications. Interestingly, it has been shown that ESCs produce several

soluble factors that can promote tissue regeneration and delay cellular aging, suggesting that ESCs and iPSCs can also be utilized as a cell-free intervention method. Therefore, the method for harvesting mouse embryonic stem cell (mESC)-conditioned medium (mESC-CM) with minimal contamination of serum components (fetal bovine serum, FBS) and feeder cells (mouse embryonic fibroblasts, MEFs) has been highly demanded. Here, the present study demonstrates an optimized method for the collection of mESC-CM under serum- and feeder-free conditions and for the characterization of mESC-CM using senescence-associated multiple readouts. This protocol will provide a method to collect pure mESC-specific secretory factors without serum and feeder contamination.

INTRODUCTION:

The goal of this protocol is to collect mouse embryonic stem cell (mESC)-conditioned medium (mESC-CM) from serum- and feeder-free culture conditions and to characterize its biological functions.

In general, embryonic stem cells (ESCs) have great potential for regenerative medicine and cell therapy due to their pluripotency and capacity for self-renewal[1-3]. However, the direct transplantation of stem cells has several limitations, such as immune rejection and tumor formation[4,5]. Therefore, a cell-free approach may provide an alternate therapeutic strategy for regenerative medicine and aging interventions[6,7].

Senescence is viewed as a cellular counterpart to the aging of tissues and organs, characterized by a permanent state of growth arrest, altered cell physiology, and behaviors. Aging is the main risk factor for prevalent diseases including cancer, cardiovascular disease, type 2 diabetes, and neurodegeneration[8]. One of the obvious characteristics of aging is the decline in the regenerative potential of tissues, which is caused by stem cell aging and exhaustion[9]. Many significant studies have shown pharmacological molecules, such as rapamycin[9], resveratrol[10], and metformin[11], and blood-borne systemic factors, namely GDF11[12], that have the ability to consistently delay aging and extend life span.

In the present study, mESC-CM has been harvested without serum (fetal bovine serum, FBS) and feeder (mouse embryonic fibroblasts, MEFs) layers to exclude the contamination of serum factors and secretory factors from MEFs. These conditions allowed for a serum- and feeder-free CM that consequently enabled the accurate identification of mESC-specific secretory factors.

This proposed protocol is highly efficient, relatively cost effective, and easy to operate. This technique provides insights into the characterization of mESC-derived soluble factors that can mediate an anti-senescence effect, which may be used for the development of a safe and potentially advantageous cell-free therapeutic approach toward interventions for aging-associated diseases and other regenerative treatments.

PROTOCOL:

NOTE: A schematic of the serum- and feeder-free CM collection protocol is shown in Figure 1.

1. Materials (Preparation of MEFs, Medium, Plates, and Solutions)

1.1) Prepare 500 mL of medium to culture the MEFs. Supplement Dulbecco's Modified Eagle;s

Medium (DMEM) with 10% FBS (ESC quality), 50 units/mL penicillin, and 50 mg/mL streptomycin.

1.2) Isolate MEFs from embryos following an established routine protocol[13] and maintain them in MEF medium.

1.3) Prepare 500 mL of medium to culture the mESCs. DMEM is supplemented with 15% FBS and 2 mM L-glutamine, 100 μM non-essential amino acids (NEAA), 100 μM β-mercaptoethanol, 100 units/mL leukemia inhibitory factor (LIF), 50 units/mL penicillin, and 50 mg/mL streptomycin.

1.4) Prepare the gelatinized plates (5 gelatinized plates / 1 mESC plate) by coating 10-cm cell culture dishes with 5 mL of 0.1% gelatin solution. Incubate for at least 10 min at room temperature.

1.5) Prepare 500 mL of Reduced Serum Medium for a serum-free condition of mESCs. Supplement Reduced Serum Media with 1.2 g of sodium bicarbonate (pH 7.0). Filter through a 0.2-μm bottle-top filter.

1.6) Prepare the senescence-associated β-galactosidase (SA β-gal) staining solution for the detection of senescent cells: 1 mg/mL X-gal (dissolved in dimethylformamide, DMF), 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$[14].
CAUTION! Hazardous! DMF is a toxic and corrosive solution. Wear personal protective clothing (*e.g.*, nitrile or latex gloves, a lab coat, and goggles) when handling solution. Use a fume hood.

1.7) Prepare 500 mL of medium to culture human dermal fibroblasts (HDFs, NHDF-Ad-Der-Fibroblast). Supplement DMEM with 10% FBS and 100 units/mL penicillin and 100 mg/mL streptomycin.

2. Culture of Mouse Embryonic Stem Cells (Figure 1A and 2A)
NOTE: Carry out all steps in a cell culture biological safety hood.

2.1) Treat the MEFs with 20 mL of MEF medium containing 10 μg/mL of mytomycin C in a 15-cm cell culture dish. Incubate for 2 h at 37 °C and 5% CO_2.

2.2) Aspirate the medium from MEFs. Wash the cells with PBS three times. Add 3 mL of trypsin-EDTA (TE, 1x) and incubate for 3 min at 37 °C and 5% CO_2. After 3 min, neutralize the TE with 6 mL of MEF medium and centrifuge for 3 min at 300 x g.

2.3) Resuspend in 5 mL of MEF medium. Determine the cell number in the resulting cell suspension using trypan blue staining and a hemocytometer. Plate inactivated MEFs (feeder) at a density of $2x10^6$ cells per 10-cm cell culture dish in MEF medium. Incubate for 24 h at 37 °C and 5% CO_2.

2.4) Replace the MEF medium with mESC medium 24 h after plating the feeder (on the following day).

2.5) Plate the mESCs (G4 F1 hybrid ES cell) at a density of $2x10^6$ cells on the feeder with mESC medium. Incubate for 48 h at 37 °C and 5% CO_2.
NOTE: The anti-aging effect of mESC-CM is likely stronger when lower passage number mESCs are used[15-17]. We acquired a G4 mESC line from Dr. Andras Nagy in Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, 25 Orde Street, Toronto, ON, M5T 3H7 Canada.

2.6) Keep the cells at a relatively high density and passage at a 70-80% sub-confluent state. Daily replace the medium with fresh mESC medium.

3. Collection of Serum- and Feeder-Free Conditioned Medium (Figure 1B and 2B)
NOTE: Carry out all steps in a cell culture biological safety hood.

3.1) Rinse the mESC plate with 5 mL of PBS. Add 1 mL of TE (2.5x) and incubate for 3 min at 37 °C and 5% CO_2. After 3 min, neutralize the TE with 2 mL of mESC medium and centrifuge for 3 min at 300 x g.

3.2) Resuspend in 5 mL of mESC medium and plate 1 mL into each gelatin-coated culture dish (5 gelatinized plates / 1 mESC plate) in mESC medium. Culture at 37 °C and 5% CO_2 until 80-85% confluency is reached.

3.3) Wash mESCs with sufficient PBS to cover the cells (8 mL per 10-cm plate) for 10 min per wash, for a total of three washes. Incubate in Reduced Serum Medium for 24 h at 37 °C and 5% CO_2.
NOTE: The washing step is important to prevent FBS contamination. It is important to follow the incubation time[18,19].

3.4) Collect mESC-CM into a 50-mL conical tube and centrifuge for 20 min at 2,500 x g. Collect the supernatant solution (CM) after the centrifugation. Filter through a 0.2-µm bottle-top filter.

4. Effects of Mouse Embryonic Stem Cell-Conditioned Medium (mESC-CM)
NOTE: The effects of mESC-CM were validated by several methods, such as SA β-gal assay, cell cycle analysis, and qRT-PCR.

4.1) SA β-gal assay (Figure 3A)
4.1.1) Seed HDFs at a density of $2x10^4$ cells per well in 6-well plates in HDF medium. Incubate overnight at 37 °C and 5% CO_2.

4.1.2) Following an overnight incubation, discard half of the HDF medium and add mESC-CM and control medium. Incubate for 72 h at 37 °C and 5% CO_2. Control medium is derived from serum-free medium (Reduced Serum Media) in a gelatin-coated dish in the absence of mESCs.

4.1.3) Wash the cells with sufficient PBS to cover the cells (2 mL per 6-well plate) for 30 s per

wash, for a total of two washes. Add 3.7% paraformaldehyde (PFA) for fixation. Incubate for 5 min at room temperature.
CAUTION: Hazardous! Paraformaldehyde is a toxic and corrosive solution. Wear personal protective clothing (*e.g.*, nitrile or latex gloves, a lab coat, and goggles) when handling the solution. Use a fume hood.

4.1.4) Aspirate the fixation solution. Wash the fixed cells with PBS twice, as described in step 4.1.3.

4.1.5) Add the SA β-gal staining solution (1-2 mL per well in a 6-well plate). Incubate for 17.5 h at 37 °C.
NOTE: It is not to be incubated in a CO_2 incubator.

4.1.6) Aspirate the SA β-gal staining solution and wash the cells with PBS twice, as described in step 4.1.3.

4.1.7) Add the Eosin solution for counter-staining. Incubate for 5 min at room temperature. Wash the cells with PBS twice, as described in step 4.1.3.

4.1.8) Image cells at 100x magnification using a light microscope and capture images using an attached digital camera for subsequent analysis.
NOTE: The total number of cells can be counted in a blind manner and the percentage of SA β-gal positive blue cells can be calculated.

4.2) Cell Cycle Analysis (Figure 3B)
4.2.1) Seed HDFs at a density of $8x10^4$ cells per well in a 6-cm cell culture dish in HDF medium. Incubate overnight at 37 °C and 5% CO_2.

4.2.2) Following an overnight incubation, discard half of the HDF medium and add mESC-CM and control medium. Incubate for 24 h at 37 °C and 5% CO_2.

4.2.3) Trypsinize the cells, as described step in 3.1, and centrifuge for 5 min at 300 x g. Wash the cells with cold PBS solution (PBS with 0.5 mM $CaCl_2$ and 2% FBS, 1 mL per 1.5-mL tube) twice and centrifuge for 3 min at 2,500 x g. Resuspend in 100 µL of cold PBS solution.

4.2.4) Fix the cells by dropping 200 µL of cold ethanol while vortexing. Store at 4 °C for at least 1 h.

4.2.5) Wash the cells with cold PBS solution twice, as described in step 4.2.3.

4.2.6) Resuspend the cells in 250 µL of sodium citrate buffer (1.12%, pH 8.5) containing 50 µg/mL RNAse. Incubate for 30 min at 37 °C.

4.2.7) Add 250 µL of sodium citrate buffer containing 50 µg/mL propidium iodide. Incubate for 20 min at room temperature.

4.2.8) Measure the 10,000 cells in each sample using flow cytometry[20].

4.3 qRT-PCR (Figure 3C)

4.3.1) Seed HDFs and add mESC-CM, as described in steps 4.1.1 and 4.1.2.

4.3.2) Isolate total RNA from the HDFs using an RNA extraction kit according to the manufacturer's protocol. Quantify the extracted total RNA using a spectrophotometer[21].

4.3.3) Synthesize cDNA by adding 1 µg of the total RNA to a 20 µL of reaction mixture containing oligo (dT) primers and M-MLV reverse transcriptase, according to the manufacturer's protocol[20].

4.3.4) Measure the amplification of the cDNA with a real-time PCR machine and use Green PCR master mix and specific gene primers (Supplement Table 1). Normalize the data with GAPDH expression. Use the following PCR protocol: initial denaturation for 10 min at 95 °C; 45 cycles for 15 s at 95 °C, 20 s at 55 °C, and 35 s at 72 °C; and the melting curve stage for 15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C, and 5 s at 60 °C[15].

REPRESENTATIVE RESULTS:

Originally, mESCs are maintained on an MEF feeder in mESC medium with FBS and other supplements (Figure 1A and 2A). CM was collected from mESCs in Reduced Serum Media without a feeder layer, FBS, or other supplements (Figure 1B and 2B). This culture condition allows us to collect mESC-specific conditioned medium without potential contamination by the factors from the feeder, FBS, or other supplements. The control medium was collected under the same culture conditions, without mESCs.

mESCs show different morphologies between the two culture media: i) normal mESC culture conditions (Figure 2A) and ii) serum- and feeder-free culture conditions (Figure 2B). The mESC colonies grew on an MEF layer and demonstrated an oval and shiny appearance under the normal mESC culture conditions (Figure 2A). On the contrary, the mESCs in the serum- and feeder-free culture conditions showed a flattened and irregular morphology (Figure 2B).

The functional characterization of mESC-CM was achieved by senescence-associated methods, such as SA β-gal assay (Figure 3A), cell cycle analysis (Figure 3B), and qPCR (Figure 3C). Treatment of senescent HDFs with mESC-CM decreased the number of positive SA β-gal-positive cells, which is an indicator of cellular senescence (Figure 3A). Cell cycle analysis revealed that mESC-CM treatment dramatically increased the number of cells in the S and G_2/M phase, whereas it reduced the number of cells in the G_0/G_1 phase (Figure 3B). In addition, mESC-CM treatment decreased the senescence-associated gene expression levels (namely, p53, p21, and p16) and the senescence-associated secretory phenotype (SASP) expression levels (IL-6).

FIGURE LEGENDS:

Figure 1: Preparation and optimization of mESC-CM. Experimental strategy for the preparation and optimization of serum-free and feeder-free CM. (A) Normal mESC culture condition and (B) serum- and feeder-free mESC-CM culture condition. C = control medium

without FBS and MEF; CM = conditioned medium without FBS and MEF. Modified with permission from FASEB J (Reference 15).

Figure 2: Bright field images of mESCs. mESCs under (A) normal conditions and (B) serum- and feeder-free conditions. Yellow arrows indicate feeder cell (MEFs) in normal mESC culture conditions. Scale bars, 100 μm.

Figure 3: Characterization of the anti-aging effect of mESC-CM. (A) SA β-gal activity staining and the percentage of SA β-gal-positive cells. (B) Cell cycle analysis by flow cytometry. (C) Expression levels of senescence-associated gene expression levels (p53, p21, and p16) and senescence-associated secretory phenotype (SASP) expression levels (IL-6) by qRT-PCR. Values are the mean ± SD. Figures are representative of three independent experiments. Statistically-significant differences between groups were identified by one-way ANOVA and Tukey's post-hoc test.*$p<0.05$, **$p<0.01$ Y = non-senescent cells; S = senescent cells; C = control medium without FBS and MEF; CM = conditioned medium without FBS and MEF. Scale bars, 10 μm. Modified with permission from FASEB J (Reference 15).

DISCUSSION:

For the successful collection of serum- and feeder-free mESC-CM, the following suggestions should be taken into consideration. The most critical factor is using early passage mESCs for the collection of mESC-CM. Previously, it has been shown that early passage mESC-CM has better anti-aging effects compared to late passage mESCs. The passage number of mESCs has been reported to affect their developmental potential[16] and pluripotency[17].

While additional research is needed to analyze the specific factors of the mESC secretome, which induce anti-senescence effects, we can currently conclude that mESC-CM is sufficient to decrease senescence at the cellular level.

The identification of mESC-specific secretory factors that revert senescent cells back to young cells will be critical for future studies. For high-quality analyses on the secretory molecules, such as antibody array[15] and secretome analysis, the washing step during the medium collection process (step 3) is critical. If the washing step is not properly conducted, the secretory molecules will be contaminated by serum (FBS) components[18,19].

The serum- and feeder-free incubation time (24 h) is very important in the medium collection process (step 3), as the longer incubation time (over 24 h) may increase the possibility of cell autolysis or apoptosis by starvation under the serum- and feeder- depleted conditions[18,19]. The normal ESC culture condition requires a feeder layer for long-term culturing of undifferentiated cells, as the feeder secretes a large number of molecules[22]. The gelatin-coated plate prevents the possibility of contamination from the feeder cells.

The mESC-CM, harvested from serum- and feeder-free culture conditions, has an anti-senescence ability in senescent HDFs. Anti-senescence effects of mESC-CM have been demonstrated by senescence-associated multiple readouts, such as SA β-gal activity; an enhanced proliferative potential (cell cycle analysis); and reduced p53, p21, p16, and IL-6 gene expression levels (Figure 3A-C).

When human primary cells are treated with mESC-CM, xeno-contamination would be a critical issue for clinical application. Therefore, an investigation of the secretory factors from human ESCs or iPSCs would be an important future study for the clinical application of CM derived from human origins. The convergence of a cell-free approach based on a stem cells and an anti-senescence study is expected to expand the current understanding of senescence-associated diseases, resulting in greater insight into improvements on therapeutic approaches.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

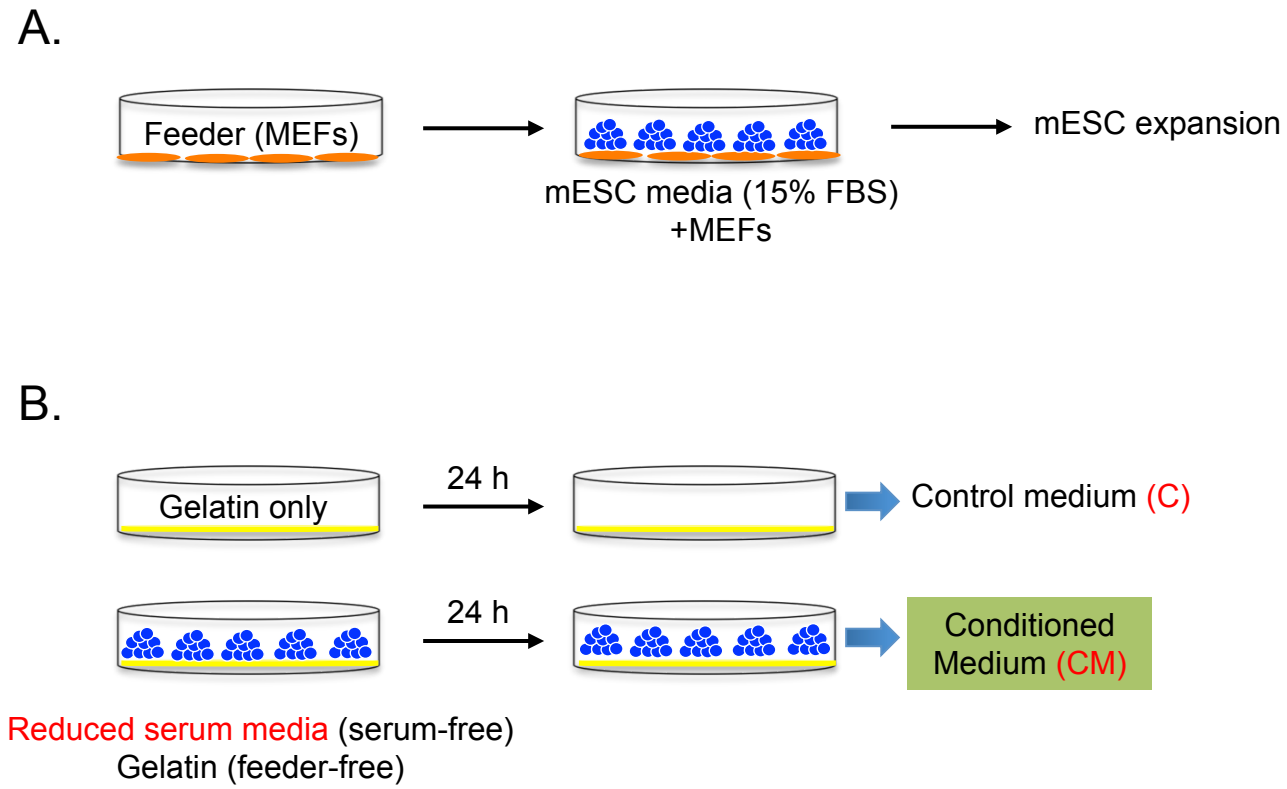
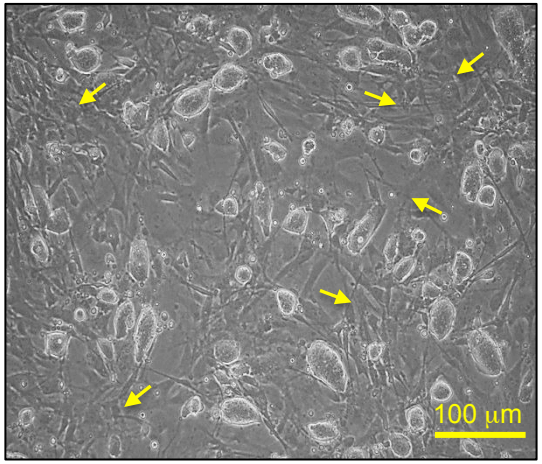


Figure 2

A.



B.

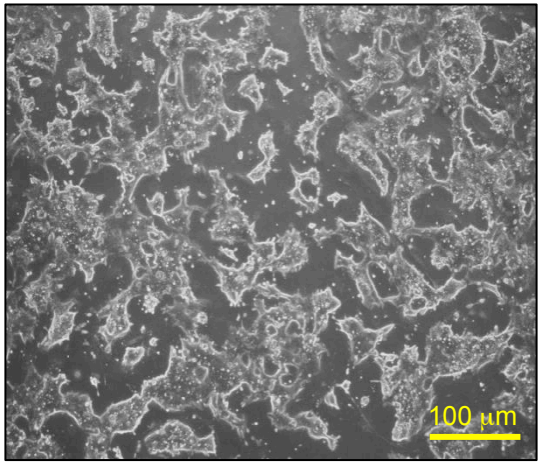
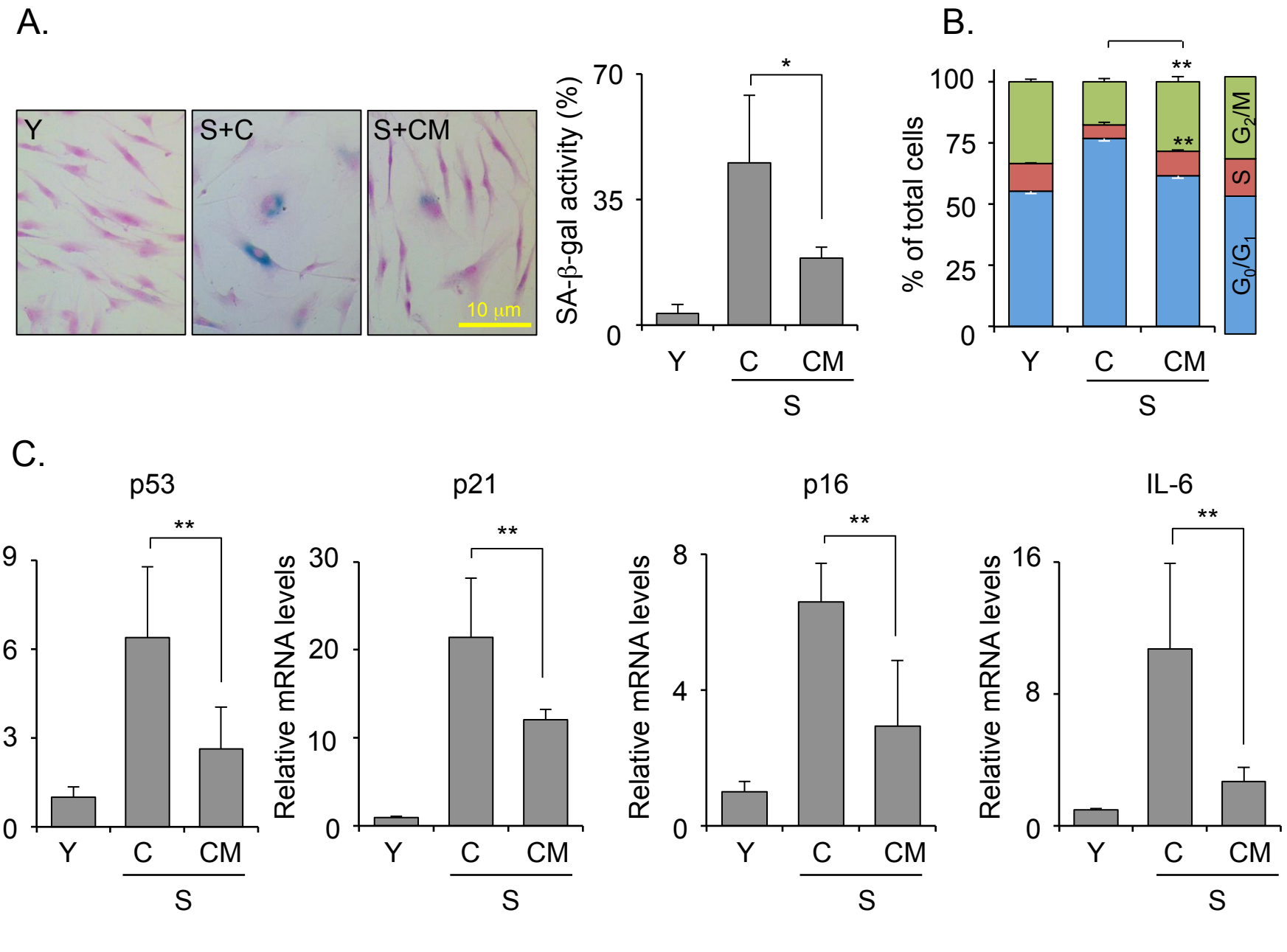


Figure 3


A.
Y
S+C
S+CM
10 μm
SA-β-gal activity (%)
70
35
0
*
Y
C
CM
S
B.
% of total cells
100
75
50
25
0
**
**
G2/M
S
G0/G1
Y
C
CM
S
C.
p53
p21
p16
IL-6
Relative mRNA levels
**
9
6
3
0
30
20
10
8
4
16
Y
C
CM
S

Supplementary Table 1. Primer sequences

Gene	Primer sequence
p53	forward- CTGTCCCCGGACGATATTGA reverse- CTGGCATTCTGGGAGCTTCA
p21	forward- GGCAGACCAGCATGACAGATT reverse- ATTAGGGCTTCCTCTTGGAGAAG
P16	forward-CCCTCGTGCTGATGCTACTG reverse-CCATCATCATGACCTGGTCTTCT
IL-6	forward-TGAGGGCTCTTCGGCAAAT reverse-CCAGAAGAAGGAATGCCCATT
GAPDH	forward- CGACCACTTTGTCAAGCTCA reverse- AGGGGTCTACATGGCAACTG

Supplementary Table 2. Materials

Name	Company	Catalog Number	Comments
DMEM	Invitrogen	#11960-044	
FBS	Invitrogen	#30044333	20%, ES cell quality
Penicillin and streptomycin	Invitrogen	#15140	50units/ml penicillin and 50mg/ml strepto -mycin.
L-glutamine	Invitrogen	#25030	2mM
Nonessential amino acids (NEAA)	Invitrogen	#11140	100uM
β-mercaptoethanol	Sigma	#M3148	100uM
Leukemia inhibitory factor	Millipore	#ESG1107	100units/ml
OPTI-MEM	Invitrogen	#22600	

X-gal	Sigma	#B4252	1mg/ml
Paraformaldehyde (PFA)	Sigma	P6148	3.7%
Dimethylformamide (DMF)	Sigma	#D4551	
Potassium ferricyanide	Aldrich	#455946	5mM
potassium ferrocyanide	Aldrich	#455989	5mM
NaCl	Sigma	#S7653	150mM
$MgCl_2$	Sigma	#M2393	2mM
Mytomycin C	Sigma	#M4287	10ug/ml
Propidium iodide	Sigma	#P4170	50ug/ml
TRIzol	Ambion	#15596018	
M-MLV reverse transcript-tase	Promega	#M170B	
Power SYBR Green PCR master mix	Applied Biosystems	#4367659	
HDFs, NHDF-Ad-Der-Fibroblast	LONZA	#CC-2511	
Bottle top filter,	Corning	#430513	0.2μm

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CORRESPONDING AUTHOR:

Name: Hoon-ki Sung

Department: Physiology and Experimental Medicine,

Institution: The Hospital for sick Children Research Institute.

Article Title: Collection of serum- and feeder- free mouse embryonic stem cell conditioned media for cell-free approach

Signature:  Date: May 24, 2016

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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.

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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
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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.