**TITLE:**

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

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**SHORT ABSTRACT:**

This protocol provides a method for the collection of mouse embryonic stem cells (mESCs) conditioned media (mESCs-CM) derived from serum- and feeder- 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 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 and iPSCs also produce several soluble factors that can promote tissue regeneration and delay cellular aging [1-3](#_ENREF_1), suggesting that ESCs and iPSCs can be also utilized as a cell-free intervention method. Therefore, we tried to identify an anti-senescence or therapeutic molecules from mESCs conditioned medium with minimal contamination of serum components (fetal bovine serum, FBS) and feeder cells (mouse embryonic fibroblasts, MEFs). Here, we present an optimized method for the collection of mESCs-CM under serum- and feeder- free condition and for the characterization of mESCs-CM using senescence associated multiple readouts. Our protocol will provide a method to collect pure and mESCs-specific secretory factors without serum and feeder layer contamination. Therefore, this technique will enable full characterization of mESCs-derived factors, and facilitate therapeutic applications of mESCs-CM toward aging intervention, aging-associated diseases and other regenerative medicine.

**INTRODUCTION:**

The goal of this protocol is to collect mESCs-CM from serum- and feeder- free culture conditions, and characterize its biological functions.

In general, embryonic stem cells have great potential for regenerative medicine and cell therapy, due to their pluripotency and capacity for self-renewal[4](#_ENREF_4). However, direct transplantation of stem cells has several limitations, such as immune rejection and tumor formation[5](#_ENREF_5),[6](#_ENREF_6). Therefore, a cell-free approach may represent an alternative therapeutic strategies[7](#_ENREF_7),[8](#_ENREF_8).

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[9](#_ENREF_9). One of the obvious characteristics of aging is the decline in the regenerative potential of tissues, which is caused by stem cell exhaustion[10](#_ENREF_10). Many significant studies have shown pharmacological molecules, such as rapamycin[10](#_ENREF_10" \o "Harrison, 2009 #18), resveratrol[11](#_ENREF_11), and metformin[12](#_ENREF_12), and blood-borne systemic factors, namely, GDF11[13](#_ENREF_13), that have the ability to consistently delay aging and extend life span.

We harvested mESCs-CM maintained without FBS and a feeder layer to exclude contamination of serum factors and secretory factors from mouse embryonic fibroblasts (MEFs). These conditions allowed for a feeder- and serum- free CM that consequently enabled the accurate identification of mESCs-specific secretory factors.

This protocol will facilitate novel cell-free therapy for aging intervention and regenerative medicine. Our novel methods will overcome the major limitation of stem cell therapy, and will help to identify the specific mESCs secretory factors. Therefore, these techniques will be beneficial when applied to potential future treatments for aging, aging-associated diseases and regenerative medicine.

**PROTOCOL:**

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

**1. Preparation of MEFs, Medium, Plates and Solutions**

1. Prepare 500ml of medium to culture mouse embryonic fibroblasts (MEFs): DMEM medium (Invitrogen/#11960-044) is supplemented with 10% FBS (ES cell quality, Invitrogen/#30044333) and 50units/ml penicillin and 50mg/ml streptomycin (Invitrogen/#15140).

2. Isolate MEFs from embryos following an established routine protocol[14](#_ENREF_14), and maintain in MEFs medium.

3. Prepare 500ml of medium to culture mouse embryonic stem cells (mESCs): DMEM medium is supplemented with 15% FBS and 2mM L-glutamine (Invitrogen/#25030), 100uM nonessential amino acids (NEAA) (Invitrogen/#11140), 100uM β-mercaptoethanol (Sigma/#M3148), 100units/ml leukemia inhibitory factor (Millipore/#ESG1107), 50units/ml penicillin and 50mg/ml streptomycin.

4. Prepare the necessary number of gelatinized plates by coating 10 cm cell culture dishes with 5ml of 0.1% gelatin solution for at least 10 minutes at room temperature.

5. Prepare 500ml of OPTI-MEM (Invitrogen/#22600) for a serum-free condition of mESCs: OPTI-MEM is supplemented with 1.2g sodium bicarbonate (pH 7.0). Immediately process by membrane filtration (0.2μm bottle top filter, Corning/#430513).

6. Prepare the senescence associated β-galactosidase (SA β-gal) staining solution for detection of senescence cells: 1mg/ml X-gal (Sigma/#B4252) (dissolved in dimethylformamide, DMF, Sigma/#D4551), 40mM citric acid/sodium phosphate buffer (pH 6.0), 5mM potassium ferricyanide (Aldrich/#455946), 5mM potassium ferrocyanide (Aldrich/#455989), 150mM NaCl (Sigma/#S7653), and 2mM MgCl2 (Sigma/#M2393)[15](#_ENREF_15).

**2. Culture of Mouse Embryonic Stem Cells (Figure 1 A, 2A)**

All steps are to be carried out in a cell culture biological safety hood.

1. Treat MEFs with 20ml MEFs medium containing 10ug/ml of mytomycin C (Sigma/#M4287) in a 15 cm cell culture dish. Incubate for 2 hours at 37℃, 5% CO2.

2. Aspirate the medium from MEFs. Wash the cells with PBS three times.

3. Plate inactivated MEFs (feeder) at a density of 2X106 cells per 10 cm cell culture dish. Incubate the cells for 24 hours at 37℃, 5% CO2.

4. Replace the MEFs medium with mESCs medium 24 hours after plating the feeder (on the following day).

5. Culture the ESCs on the feeder at 37℃, 5% CO2.

NOTE: Anti-aging effect of mESCs-CM is likely stronger when lower passage number mESCs were used[16-18](#_ENREF_16).

6. Change mESCs medium daily. Keep the cells at a relatively high density, and passage when a 70-80% sub-confluent state is reached.

**3. Collection of Serum- and Feeder- Free Conditioned Medium (Figure 1B, 2B)**

All steps are to be carried out in a cell culture biological safety hood.

1. Transfer mESCs on the feeder to gelatin coated culture dishes in mESCs medium. Culture at 37℃, 5% CO2 until 80-85% confluency is reached.

2. Wash mESCs with PBS for 10 minutes, three times. Incubate in OPTI-MEM for 24 hours at 37℃, 5% CO2.

NOTE: The washing step is important for excluding FBS contamination.

NOTE: It is important to keep the incubation time[19](#_ENREF_19),[20](#_ENREF_20).

3. Collect mESCs-CM and centrifuge for 20 minutes at 5000rpm. Filter it through a 0.2μm bottle top filter.

**4. Characterization of Mouse Embryonic Stem Cell Conditioned Medium (mESCs-CM)**

NOTE: Characterize mESCs-CM using several methods, such as SA β-gal Assay, Cell Cycle analysis and qRT-PCR.

A) SA β-gal Assay (Figure 3A)

1. Growing human dermal fibroblasts (HDFs, NHDF-Ad-Der-Fibroblast LONZA/#CC-2511) at 2X104 cells per well in 6-well plates.

2. Following overnight incubation, discard half of the HDFs medium, add mESCs-CM, and control medium. Incubate for 72 hours at 37℃, 5% CO2. Control medium is derived from serum-free medium (OPTI-MEM) in gelatin-coated dish, in the absence of ESCs.

3. Wash the cells with PBS, twice. Add 3.7% paraformaldehyde (PFA) for fixation. Incubate for 5 minutes at room temperature.

4. Remove the fixation solution. Wash the fixed cells with PBS, twice.

5. Add the SA β-gal staining solution (1-2ml per well in a 6-well plate). Incubate for 17.5 hours at 37℃.

NOTE: Not to be incubated in a CO2 incubator.

6. After the incubation, wash the cells with PBS, twice.

7. Add the Eosin solution for counter staining for 5 minutes. Wash the cells with PBS, twice.

8. Collect images using a microscope. The total number of cells was counted in a blind manner and the percentage of blue cells was calculated.

B) Cell Cycle Analysis (Figure 3B)

1. Wash the cells with cold PBS solution (0.5mM CaCl2 and 2% FBS), two times. Resuspend in 100ul PBS solution.

2. Fix by dropping 200ul of cold ethanol while vortexing. Store at 4℃ for at least 1 hour.

3. Wash the cells with cold PBS solution, two times.

4. Resuspend the cells in 250ul of sodium citrate buffer containing 50ug/ml RNAse in (1.12%, pH 8.5). Incubate for 30 minutes at 37℃.

5. Add 250ul of sodium citrate buffer containing 50ug/ml propidium iodide (Sigma/#P4170). Incubate for 20 minutes at room temperature.

6. Measure the 10,000 cells in each sample using flow cytometry.

C) qRT-PCR (Figure 3C)

1. Isolate total RNA from the cells using TRIzol (according to the manufacturer’s protocol, Ambion/#15596018). Quantify the extracted total RNA using a NanoDrop spectrophotometer.

2. Synthesize cDNA by adding 1ug of the total RNA to a 20ul reaction mixture, containing oligo (dT) primers and M-MLV reverse transcriptase (according to the manufacturer’s protocol, Promega/#M170B).

3. Measure the amplification of the cDNA with an ABI 7500 Real-Time PCR system, using Power SYBR Green PCR master mix (Applied Biosystems/#4367659) and specific gene primers (Supplement Table 1). Normalize the data with GAPDH expression.

**REPRESENTATIVE RESULTS:**

The overall scheme of the mESCs-CM collection procedure is presented in Figure 1.

Originally, mESCs are maintained on MEF feeder in mESCs medium with FBS and other supplements (Figure 1A, 2A). CM was collected from mESCs in OPTI-MEM without a feeder layer, FBS or other supplements (Figure 1B, 2B). The control media was collected under the same conditions without mESCs.

mESCs show different morphologies between the two culture media: i) normal mESCs culture condition, and ii) serum-free and feeder-free culture condition. The mESC colonies under the normal culture condition had an oval and shiny appearance (Figure 2A). On the contrary, the mESCs in the serum-free and feeder-free culture condition showed flattened single cells with a uniform morphology (Figure 2B).

The characterization of mESCs-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 mESCs-CM decreased SA β-gal positive cell numbers, which is an indicator of cellular senescence (Figure 3A). Cell cycle analysis revealed that mESCs-CM treatment dramatically increased the S and G2/M phase cell population, whereas it reduced the G0/G1 phase (Figure 3B). In addition, mESCs-CM treatment decreased the senescence-associated gene expression levels (namely, p53, p21 and p16) and senescence-associated secretory phenotype (SASP) expression levels, such as IL-6.

**Figure 1:** Preparation and optimization of mESC-CM. Experimental strategy for preparation and optimization of serum-free and feeder-free CM. (A) Normal mESCs culture condition and (B) Serum-free and feeder-free mESCs-CM culture condition.

**Figure 2:** Bright field images of mESCs under (A) normal condition and (B) serum- and feeder-free condition. Scale bars, 100㎛.

**Figure 3:** Characterization of the anti-aging effect of mESCs-CM. (A) SA β-gal assay, (B) Cell cycle analysis and (C) qRT-PCR. Y=non-senescent cells; S=senescent cells; C=control medium; CM=conditioned medium

**DISCUSSION:**

For the successful collection of serum- and feeder- free mESCs-CM, the following suggestions should be taken into consideration. The most critical factor is using early passage mESCs for the collection of mESCs-CM. Previously, our paper showed that early passage mESCs-CM have better anti-aging effects, compared to late passage mESCs. Passage number has been reported to affect their developmental potential[17](#_ENREF_17) and pluripotency[18](#_ENREF_18" \o "Li, 2007 #44).

Identification of mESCs specific-secretory factors that revert senescent cells back to young cells will be critical for the future study. For further secretory molecule analysis, such as antibody array and secretome analysis, the washing step during the media collection process (Protocol 3) is essential. If washing is not properly conducted, the secretory molecules will be contaminated by serum (FBS) components[19](#_ENREF_19),[20](#_ENREF_20).

In addition, serum- and feeder- free incubation times are also important during the media collection process (Protocol 3). The 24 hour incubation increased the possibility of cell autolysis, which was induced by starvation under the feeder- and serum- depleted condition[19](#_ENREF_19),[20](#_ENREF_20).

The normal ESCs culture condition requires a feeder layer for long-term culturing of undifferentiated cells, as the feeder secretes a large number of molecules[21](#_ENREF_21). The gelatin-coated plate prevents the possibility of contamination from the feeder cells.

After the collection of conditioned medium, we demonstrated that the mESCs-CM has anti-senescence ability in the senescent HDFs. We showed a decrease in SA β-gal activity, an enhanced proliferative potential (cell cycle analysis), and reduced p53, p21, p16 and IL-6 expression levels (Figure. 3A-C).

The convergence of a cell-free approach based on stem cell and anti-senescence study is expected to expand our understanding of senescence-associated diseases, resulting in greater insight into improvements for therapeutic approaches.

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**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES**

1 Conboy, I. M., Yousef, H. & Conboy, M. J. Embryonic anti-aging niche. *Aging* **3**, 555-563 (2011).

2 Lavasani, M. *et al.* Muscle-derived stem/progenitor cell dysfunction limits healthspan and lifespan in a murine progeria model. *Nature communications* **3**, 608, doi:10.1038/ncomms1611 (2012).

3 Woo, D. H. *et al.* Direct and indirect contribution of human embryonic stem cell-derived hepatocyte-like cells to liver repair in mice. *Gastroenterology* **142**, 602-611, doi:10.1053/j.gastro.2011.11.030 (2012).

4 Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science (New York, N.Y.)* **282**, 1145-1147 (1998).

5 Lee, A. S., Tang, C., Rao, M. S., Weissman, I. L. & Wu, J. C. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. *Nature medicine* **19**, 998-1004, doi:10.1038/nm.3267 (2013).

6 Moon, S. H. *et al.* A system for treating ischemic disease using human embryonic stem cell-derived endothelial cells without direct incorporation. *Biomaterials* **32**, 6445-6455, doi:10.1016/j.biomaterials.2011.05.026 (2011).

7 Tongers, J., Roncalli, J. G. & Losordo, D. W. Therapeutic angiogenesis for critical limb ischemia: microvascular therapies coming of age. *Circulation* **118**, 9-16, doi:10.1161/circulationaha.108.784371 (2008).

8 Lazarous, D. F. *et al.* Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial. *Journal of the American College of Cardiology* **36**, 1239-1244 (2000).

9 Adams, P. D. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Molecular cell* **36**, 2-14, doi:10.1016/j.molcel.2009.09.021 (2009).

10 Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392-395, doi:10.1038/nature08221 (2009).

11 Baur, J. A., Ungvari, Z., Minor, R. K., Le Couteur, D. G. & de Cabo, R. Are sirtuins viable targets for improving healthspan and lifespan? *Nature reviews. Drug discovery* **11**, 443-461, doi:10.1038/nrd3738 (2012).

12 Martin-Montalvo, A. *et al.* Metformin improves healthspan and lifespan in mice. *Nature communications* **4**, 2192, doi:10.1038/ncomms3192 (2013).

13 Loffredo, F. S. *et al.* Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy. *Cell* **153**, 828-839, doi:10.1016/j.cell.2013.04.015 (2013).

14 Jozefczuk, J., Drews, K. & Adjaye, J. Preparation of mouse embryonic fibroblast cells suitable for culturing human embryonic and induced pluripotent stem cells. *Journal of visualized experiments : JoVE*, doi:10.3791/3854 (2012).

15 Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J. & Toussaint, O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. *Nature protocols* **4**, 1798-1806, doi:10.1038/nprot.2009.191 (2009).

16 Bae, Y. U., Choi, J. H., Nagy, A., Sung, H. K. & Kim, J. R. Antisenescence effect of mouse embryonic stem cell conditioned medium through a PDGF/FGF pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **30**, 1276-1286, doi:10.1096/fj.15-278846 (2016).

17 Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J. C. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 8424-8428 (1993).

18 Li, X. Y. *et al.* Passage number affects the pluripotency of mouse embryonic stem cells as judged by tetraploid embryo aggregation. *Cell and tissue research* **327**, 607-614, doi:10.1007/s00441-006-0354-6 (2007).

19 Mbeunkui, F., Fodstad, O. & Pannell, L. K. Secretory protein enrichment and analysis: an optimized approach applied on cancer cell lines using 2D LC-MS/MS. *Journal of proteome research* **5**, 899-906, doi:10.1021/pr050375p (2006).

20 Makridakis, M. & Vlahou, A. Secretome proteomics for discovery of cancer biomarkers. *Journal of proteomics* **73**, 2291-2305, doi:10.1016/j.jprot.2010.07.001 (2010).

21 Eiselleova, L. *et al.* Comparative study of mouse and human feeder cells for human embryonic stem cells. *The International journal of developmental biology* **52**, 353-363, doi:10.1387/ijdb.082590le (2008).