Video Article

Isolation and Enrichment of Rat Mesenchymal Stem Cells (MSCs) and Separation of Single-colony Derived MSCs

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URL: https://www.jove.com/video/1852
DOI: doi:10.3791/1852

Keywords: Cellular Biology, Issue 37, mesenchymal stem cells, magnetic cell sorting, flow cytometry, cloning cylinder

Date Published: 3/22/2010

Citation: Zhang, L., Chan, C. Isolation and Enrichment of Rat Mesenchymal Stem Cells (MSCs) and Separation of Single-colony Derived MSCs. *J. Vis. Exp.* (37), e1852, doi:10.3791/1852 (2010).

Abstract

MSCs are a population of adult stem cells that is a promising source for therapeutic applications. These cells can be isolated from the bone marrow and can be easily separated from the hematopoietic stem cells (HSCs) due to their plastic adherence. This protocol describes how to isolate MSCs from rat femurs and tibias. The isolated cells were further enriched against two MSCs surface markers CD54 and CD90 by magnetic cell sorting. Expression of surface markers CD54 and CD90 were then confirmed by flow cytometry analysis. HSC marker CD45 was also included to check if the sorted MSCs were depleted of HSCs. MSCs are naturally quite heterogeneous. There are subpopulations of cells that have different shapes, proliferation and differentiation abilities. These subpopulations all express the known MSCs markers and no unique marker has yet been identified for the different subpopulations. Therefore, an alternative approach to separate out the different subpopulations is using cloning cylinders to separate out single-colony derived cells. The cells derived from the single-colonies can then be cultured and evaluated separately.

Video Link

The video component of this article can be found at https://www.jove.com/video/1852/

Protocol

1. Isolation of Rat MSCs

Mesenchymal stem cells were isolated from 6-8 week old Sprague-Dawley female rat as previously described [1, 2]. Isolated MSCs can adhere to plastic surface and easily expand during in vitro culture.

1. The animal was put into an anaesthesia chamber and anaesthetized for around five minutes. During the anaesthesia, observe the rate of blinking, breathing and motor activity. Remove the animal from the chamber immediately after it stops motor activity and the blinking rate became infrequent.
2. Lay the animal down on an operation station and kill the animal by cervical dislocation. Cut off the femurs and tibias from the back limbs, remove the skin and muscles.
3. Put the dissected femurs and tibias in 70% isopropanol for a few seconds and transfer to 1X D-PBS.
4. In a biosafety cabinet, the femurs and tibias were transferred to a 10cm dish containing DMEM. Each bone was then held with tweezers and the two ends were cut open with a scissor. Attach a 22G needle to a 3ml syringe and fill it with DMEM, then flush the marrow into a 50ml tube by inserting the needle to one open end of the bone. Repeat 2~3 times for each bone. When all the marrows were obtained, resuspend the cells and pass the cell suspension through a 70μm cell strainer to remove the bone debris and blood aggregates.
5. Spin down cells at 200g, 4°C for 5 minutes and remove the supernatant by aspiration. Resuspend cells in 25ml MSC medium (DMEM containing 10% FBS and 1% Pen-Strep). 10ml cell suspension was seeded into each 10cm culture dish for a total of two dishes. Keep the culture dishes in a 37°C and 5% CO_2 incubator for 1~2 weeks. Medium was changed every 2~3 days.

2. Enrichment of Rat MSCs

A number of surface proteins have been used to enrich MSCs, including CD54, CD90, CD73, CD105 and CD271 [3-5]. In our study, we used CD54 and CD90 as markers to enrich MSCs by magnetic cell sorting.

1. When cells reached around 80% confluency, aspirate the medium and add 4~5ml trypsin-EDTA to each dish. Put the dishes back to the incubator and incubate for around 5 minutes to allow cell detachment. Once cells were detached, add equal amount of culture medium to inactivate trypsin. Collect cell suspension into a 15ml tube and spin cells down at 200g, 4°C for 5 minutes.
2. The next steps describe how to enrich MSCs by two surface markers CD54 and CD90 according to the manual for cell separation using BD IMagnet. Cell pellet was resuspended in cell staining buffer (3% heat inactivated FBS in 1X D-PBS) at 20 million cells/ml. Biotinylated CD54 antibody (0.25μg per million cells) and biotinylated CD90 antibody (0.15μg per million cells) was added and mixed gently with the cell

suspension. After incubation on ice for 15 minutes, labeled cells were washed with an excess volume of 1X BD IMag buffer. The labeled cells were spun down at 200g, 4°C for 5 minutes.

3. Vortex the BD IMag streptavidin particles thoroughly, add 40µl particles for every 10 million cells. Mix the particle with the labeled cells thoroughly and incubate the cells at 6~12°C for 30 minutes. This allows the streptavidin particles to bind to the biotinylated anti-CD54 and anti-CD90, which is bound to the surface proteins CD54 and CD90 respectively.
4. During the incubation time, label a round-bottom test tube to collect the positive fraction. After incubation, bring the labeling volume to 20 million cells/ml with 1X BD IMag buffer and transfer the labeled cells to the positive-fraction collection tube. Place the positive-fraction tube onto the BD IMagnet and let it stay for 6 minutes, then remove the supernatant with a glass Pasteur pipette with the positive-fraction tube still on the BD IMagnet.
5. Remove the positive-fraction tube from the BD IMagnet and place it on ice. Add 1ml ice cold 1X BD IMag buffer and resuspend cells by gentle mixing. Place the tube back onto the BD IMagnet and let it stay for 2~4 minutes. Remove the supernatant with a new glass Pasteur pipette. Repeat the washing as described in 2.5 one more time.
6. Remove the tube from the BD IMagnet and resuspend cells in culture medium, seed one 75cm^2 flask for maintaining the cells and a 10cm dish for flow cytometry.

3. Verification of Surface Marker Expression by Flow Cytometry

Flow cytometry analysis was performed to verify the cells we obtained express CD54 and CD90. The HSC marker CD45 was used to confirm that the MSCs were depleted of HSCs.

1. When cells become ~80% confluent, trypsinize cells with trypsin-EDTA and collect cells into a 15ml tube. Centrifuge at 200g, 4°C for 5 minutes to collect cells.
2. Aspirate the supernatant and wash cells with 1X D-PBS once. Resuspend cells in cell staining buffer (1X D-PBS containing 2% FBS and 0.05% sodium azide) to a final concentration of 5~10 million cells/ml and keep cells on ice. Aliquot 100µl cell suspension into the six labeled tubes (1. cells only; 2. isotype control IgG2a; 3. Isotype control IgG1; 4. CD45; 5. CD54; 6. CD90).
3. Add isotype controls and primary antibodies at appropriate concentrations (IgG2a and IgG1: 20µl per million cells; CD45: 0.5µg per million cells; CD54: 0.25µg per million cells; CD90: 0.15µg per million cells) and incubate at 4°C for 30 minutes.
4. Wash cells with 1X D-PBS twice and then resuspend cells in 100µl cell staining buffer. Add SA-PE (streptavidin- phycoerythrin, use 0.15µg per million cells) and incubate at 4°C for 30 minutes in dark.
5. Wash labeled cells with 1X D-PBS twice. Resuspend cells in 400µl cell staining buffer and transfer to a falcon tube for flow cytometry analysis.

4. Separation of Single-colony Derived MSCs

MSCs is a heterogeneous population composed of different subpopulations with different cell shape, growth rate as well as differentiation ability [6]. However, all the subpopulations express the known MSC markers and therefore it is not feasible to use markers to separate out these subpopulations. Therefore, we applied cloning cylinders to separate out the different subpopulations, which are colonies formed by single cells.

1. Plate cells at about 50~100 cells per 10cm dish. Incubate in a 37°C and 5% CO_2 incubator for 1~2 weeks. During this period, examine well isolated colonies with an inverted microscope. Once the colonies have reached big enough size (better more than 100 cells in each colony), mark the colonies with a sharpie at the bottom of the dish. When pick the colony to be marked, make sure there are no surrounding colonies near the picked one.
2. Aspirate medium and wash the dish once with 1X D-PBS. Pick up a sterile cloning cylinder with a sterile forceps and gently place it around the marked colony. Repeat this until all the marked colonies has a cloning cylinder placed over. The picked colonies should be far away from each other such that every cloning cylinder only contains one colony.
3. Add 100µl trypsin-EDTA to each cloning cylinder and put the dish back to the incubator for ~5 minutes. After 5 minutes, check the cells under the microscope to see whether they are rounding up. When the cells have lifted up, add equal amount of culture medium to inactivate trypsin. Mix the cell suspension with a 200µl micropipettor and transfer the cell suspension to a 60mm dish containing 3ml prewarmed culture medium. Label the dishes properly and put them back into the incubator. Tracking morphological change over the next few days.

5. Representative Results

According to the protocol described in the part for rat MSCs isolation, plastic adherent MSCs should be visible the next day after plating. As cells continue to proliferate, the confluent cells should look like the cells shown in Figure 1A. When cells reach ~80% confluency (Figure 1B), subculturing can be carried out. During subculture, trypsin-EDTA was used to detach cells and lifted cells are small and round as shown in Figure 1C.

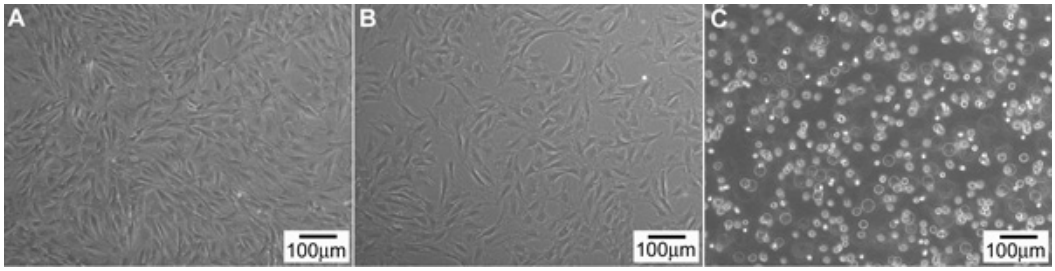

Figure 1. Phase contrast images of rat MSCs. (A) Confluent MSCs. The majority of cells are spindle-like or star-like. (B) MSCs around 80% confluency. (C) Lifted cells after trypsinization are small and round.

Once MSCs are enriched by magnetic cell sorting, flow cytometry analysis is performed to verify the surface marker expressions. If the enrichment is good, the cells should show positive staining against MSC markers CD54 and CD90 but negative against the HSC marker CD45 (Figure 2). Isotype controls IgG2a and IgG1 are used as negative controls.

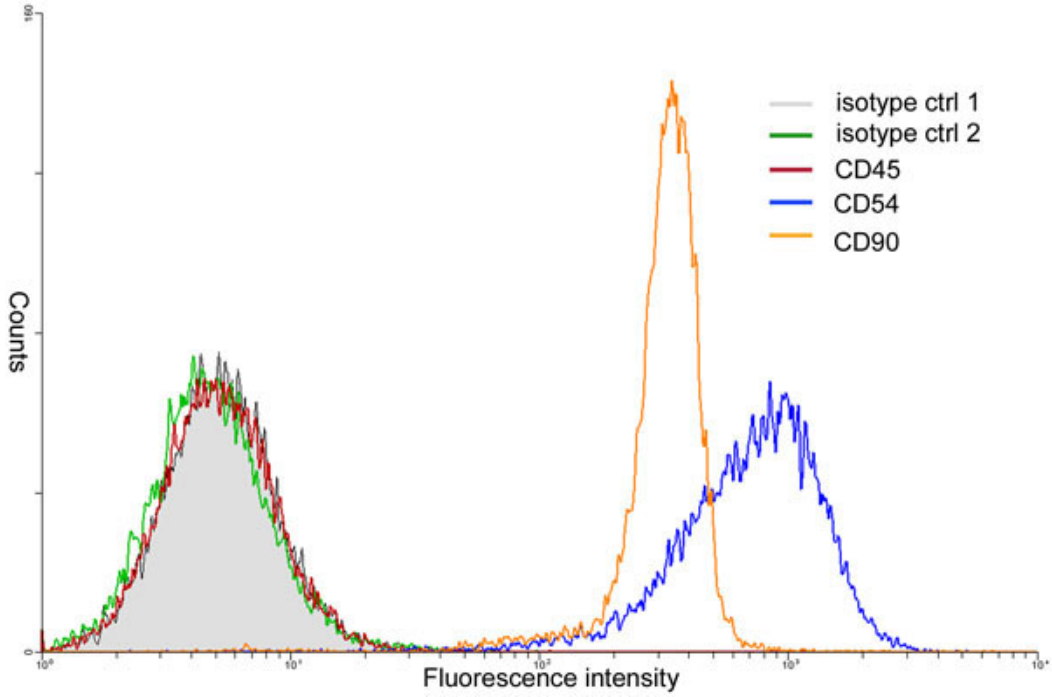

Figure 2. Flow cytometry analysis of MSCs for surface markers. MSCs were labeled with antibodies against IgG2a (isotype control 1), IgG1 (isotype control 2), CD45, CD54 and CD90. MSCs expressed CD54 and CD90 but not CD45.

When cells are seeded at proper clonal density, colonies should rise from single cells. Figure 3A represents a colony formed by a single cell. Cloning cylinders can then be used to separate the colonies and cells derived from the colonies can be cultured separately. Figure 3B and 3C represents cells derived from two individual colonies. The cells derived from colony 1 are spindle like (Figure 3B) whereas the cells derived from colony 2 are round (Figure 3C).

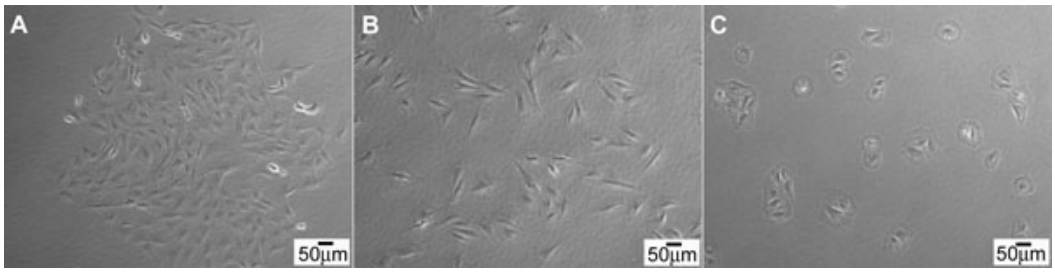

Figure 3. Colony formation by MSCs and single-colony derived cells. MSCs cultured at clonal density form individual colonies. These colonies can be separated by cloning cylinders and cells from different colonies can be cultured separately. (A) A representative colony formed by MSCs when plated at clonal density. (B) Spindle-like cells derived from one colony. (C) Round cells derived from another colony.

Discussion

This protocol describes how to isolate and enrich MSCs. A method to separate the single-colony derived cells is also incorporated. There are several steps that are important for a successful isolation, enrichment and colony separation. While doing cell isolation from rat, it is recommended to filter through a cell strainer or a sterile nylon mesh of similar size to get rid of the blood clots and bone debris. After plating the cells overnight, many dead cells will be floating in the medium and the dead cells are removed by replacing with fresh medium which should help the growth of the attached cells.

The magnetic cell sorting in this protocol describes how to perform a positive selection, and similar procedures can be used to perform a negative selection. The amount of antibodies to be added may differ and optimization is required to achieve better sorting. This is also true for labeling the cells for flow cytometry analysis. If not running flow cytometry analysis for the labeled samples immediately, samples can be fixed

with 2% formaldehyde and run later. However, long-term storage is not recommended since this tends to increase the auto-fluorescence and sacrifice sample quality.

The key part for the colony separation is seeding at the right cell density (which should be optimized experimentally) and locating single clones that are not surrounded by other clones. If there are other clones nearby, the cloning cylinder may encompass the nearby clones and the cells obtained will no longer be from one clone. When placing the cloning cylinder over the clone, also be careful not to slide it over the dish surface as this will cause the silicon grease at the bottom of the cloning cylinder to cover the cells and prevent the trypsin from reaching the cells to detach them.

Acknowledgements

The work was supported in part by National Institute of Health (R01GM079688, R21RR024439, and R21GM075838), National Science Foundation (CBET 0941055), and the MSU Foundation.

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