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Isolation of Murine Adipose Tissue-derived Mesenchymal Stromal Cells (mASCs) and the Analysis of Their Proliferation *in vitro*

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[Abstract] Mesenchymal stromal cells (MSCs) are non-hematopoietic, perivascular cells which support hematopoiesis and are thought to participate in tissue repair *in vivo*. MSCs can be isolated from various tissues and organs and are defined *in vitro* as plastic adherent cells expressing CD73, CD90, CD105 (human MSCs) or CD29, CD44, sca-1 (murine MSCs) which can differentiate into osteoblasts, adipocytes, chondroblasts and myocytes. MSCs possess potent immunomodulatory and trophic capacities *in vitro* and *in vivo* and have thus emerged as a promising treatment of inflammatory/autoimmune diseases. The use of MSCs for human disease relies on the injection of a large number of cells and much effort has been focused on acquiring MSCs with high proliferative capacity. Thus, establishing simple and accurate protocols for measuring MSC proliferation is of importance for both basic and applied research. The current protocol provides details on how to isolate and measure the proliferation of murine MSCs derived from inguinal and/or intraabdominal adipose tissue (mASCs) using the xCELLigence system and CellTiter-Blue reagent (Carrillo-Galvez *et al.*, 2015; Anderson *et al.*, 2013). The protocols described below can also be easily translated to human MSCs.

Materials and Reagents

1. Scalpel blades No. 24 (VWR International, catalog number: 233-5487)
2. Cell strainers (100 µm and 70 µm) (BD Biosciences, catalog numbers: 352360 and 352350)

Note: Currently, it is "Corning , catalog numbers: 352360 and 352350".

3. Black 96-well plate (Greiner Bio-One GmbH, catalog number: 655076)
4. 16-well E-plate (E-plate 16) (ACEA BIO, catalog number: 06465382001)
5. Tissue culture treated Falcon 96-well plates (BD Biosciences, catalog number: 353072)

Note: Currently, it is "Corning, catalog number: 353072".

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6. Nunc™ Cell Culture Treated EasYFlasks™ (Thermo Fisher Scientific, catalog number: 156499)
7. BALB/c (or any strain of choice) 8-12 weeks old male or female mice (Charles River Laboratories)
8. Hank's balanced salt solution (HBSS) w/Calcium w/Magnesium (Biowest, catalog number: L0612-500)
9. Collagenase Type I (Sigma-Aldrich, catalog number: C0130-100MG)
10. MesenCult™ Proliferation Kit with MesenPure™ (Mouse) (STEMCELL Technologies, catalog number: 05512)
11. Penicillin-Streptomycin 10,000 U/ml (Life Technologies, Gibco, catalog number: 15140-122)

Note: Currently, it is "Thermo Fisher Scientific, Gibco™ catalog number: 15140-122".

12. Stable Glutamine 200 mM (Biowest, catalog number: X0551-100)
13. DPBS w/o Calcium w/o Magnesium (Biowest, catalog number: L0615-500)
14. TrypLE™ Express Enzyme (1x) (Life Technologies, catalog number: 12604021)

Note: Currently, it is "Thermo Fisher Scientific, Gibco™ catalog number: 12604021".

15. Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, catalog number: 10309433)
16. Trypan blue (Sigma-Aldrich, catalog number: T8154-20 ml)
17. CellTiter-Blue reagent (Promega Corporation, catalog number: G8081)
18. Collagenase Type I (see Recipes)
19. Complete MesenCult medium (see Recipes)

Equipment

1. Sterile stainless steel scissors and forceps
2. Neubauer cell counting chamber (VWR International, catalog number: 631-1111)
3.
4. Cell culture incubator (THERMO, model: HEPA class 100)
5. Phase contrast microscope (OLYMPUS, model: CKX41)
6. Centrifuge for cells (Eppendorf, model: 5810R)
7. xCELLigence RTCA station and control unit (lap top) with the RTCA Software (ACEA BIO/ROCHE)
8. Fluorometro [Glomax multidetection system (Promega Corporation) or equivalent]

Procedure

A. Isolation of mASCs

Mesenchymal stromal cells are isolated from inguinal (subcutaneous) and gonadal (intraabdominal fat deposits attached to the epididymis/testes in male mice and ovaries/uterus in female mice) fat pads.

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1. Sacrifice the mice by cervical dislocation and aseptically remove fat tissue and collect it in a 50 ml tube containing 10 ml of ice cold HBSS (Note 1).
2. Wash the fat tissue twice in 10 ml HBSS containing 2% (v/v) of penicillin/streptomycin at room temperature (RT) and transfer the fat to a Petri dish. Remove as much liquid as possible and weigh the fat. Cut the fat tissue using sterile scissors or scalpel blades into small pieces (≤ 3 mm^3).
3. Resuspend the minced fat tissue in 2.5 ml HBSS containing 2 mg/ml collagenase Type I (equivalent to 1-5 FALGPA units and ≥ 625 collagen digestion units (CDU)/g of fat tissue) and transfer the mixture to a 15 ml tube (< 5 ml) or 50 ml tube (≥ 5 ml). Place the tube in a 37 °C water bath for 30 min; gently vortex tube for 10 sec every 5 min.
4. Add 10 ml of HBSS to the digested fat solution and filter it using 100 µm cell strainers (Note 2).
5. Spin down the released cells (300 *x g*, 7 min at 4 °C), aspirate the supernatant (Note 3) and resuspend the pellet in 10 ml HBSS.
6. Filter the cell suspension using a 70 µm cell strainer and pellet the cells by centrifugation (300 *x g*, 7 min at 4 °C).
7. Resuspend the pellet in 2 ml complete Mesencult medium and count cells using Trypan blue. We generally obtain 0.25 ± 0.03 g fat/mouse (mean ± SEM, n = 6) and 1.9 ± 0.4 x 10^6 cells/g fat (mean ± SEM, n = 11).
8. Plate the released cells in tissue culture-treated Nunc EasYFlasks at 20-30,000 cells/cm^2 in complete Mesencult medium.
9. After 24 h, change the medium in order to remove non-attached cells and debris.

B. Cell culture

Culture the adherent cells using complete Mesencult at 37 °C, 5% CO_2, 21% O_2, 90% relative humidity (RH) in 75 cm^2 (T75) Nunc EasYFlask at 10,000 cells/cm^2.

1. When cultures reach 80-90% confluency, wash the cells twice with 10 ml PBS.
2. Add 2 ml TrypLE/T75 flask and incubate at 37 °C for 5 min. Tap firmly on the side of the bottle in order to detach the cells and then add 8 ml of PBS containing 2% FBS.
3. Resuspend the cells gently until obtaining a homogenous cell suspension and spin down the cells at 300 *x g* for 5 min.
4. Count the cells as described below and seed the cells at 10,000 cells/cm^2 and expand the culture until it reaches 80-90% confluency. mASCs can be used at passage 3-7 (Note 4).
5. The phenotype of passage 3 mASCs from Balb/c mice, as measured by flow cytometry, is $CD11b^-$ (0.6 ± 0.2%), $CD31^-$ (0%), $CD45^-$ (0.03 ± 0.06%), $sca\text{-}1^+$ (80.3 ± 14.5%), $CD29^+$ (99.9 ± 0.1%) and $CD44^+$ (99.8 ± 0.2%). The expression levels are shown as % of the population expressing the marker relative to the corresponding isotype control staining (mean ± standard deviation, n = 3). The expression range can

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vary between mouse strains. Differentiation of murine MSCs into osteoblasts, adipocytes and chondroblasts (Anderson *et al.*, 2013; Peister *et al.*, 2004) can be performed using commercial differentiation kits from *e.g.,* Stem Cell Technologies (http://www.stemcell.com) and Lonza (http://www.lonza.com).

C. Proliferation (Note 5)

1. Preparing the mASCs (passage 3-7) for the proliferation assays.
   a. Harvest the cells as described above.
   b. Resuspend the cell pellet gently but thoroughly in 2 ml Mesencult/confluent T75 flask to obtain a cell concentration between 5 x 10^5 and 1 x 10^6 cells/ml.
   c. Dilute the cell suspension 1:1 in Trypan blue (20 µl cell suspension + 20 µl Trypan blue).
   d. Count. Mix well and add 10 µl to a Neubauer counting chamber and count the four corner squares (Note 6).
   e. Prepare a working solution of 10,000 cells/ml in complete Mesencult for measuring cell proliferation using the xCELLigence or 6,600 cells/ml for measuring cell proliferation using the CellTiter-Blue reagent and keep the tube on ice (Note 7).
2. xCELLigence

   The xCELLigence system can be used to measure cell proliferation in real time and uses specific cell culture plates in which the well bottoms are covered with electrodes (Figure 1). Attachment of cells to the electrodes increases their impedance which is displayed as an increase in cell index. The cell index gives information about cell number, viability and morphology in each well. The xCELLigence RTCA station should be placed in a cell incubator set at 37 °C, 21% O_2, 5% CO_2, 90% RH.

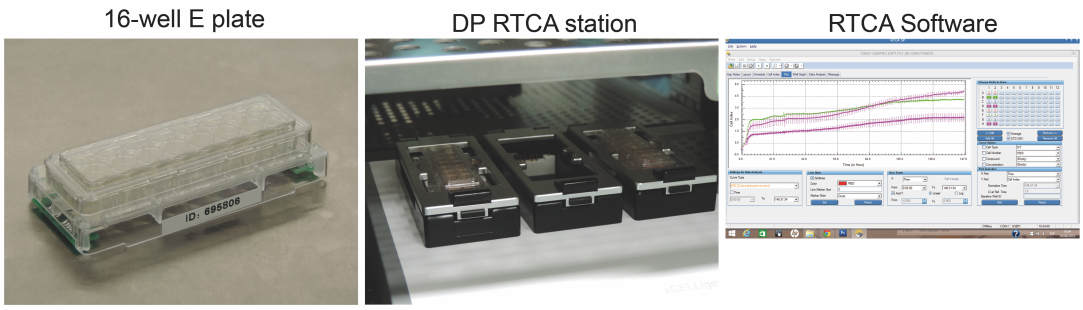

Figure 1. Components of the xCELLigence system. The left panel shows a 16-well E-plate used for measuring proliferation of adherent cells. The middle panel shows the DP RTCA station with its three integrated stations for E-plates. The right panel gives an example of how the RTCA Software visualizes cell proliferation in real time by plotting cell index vs. time.

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a. In a sterile hood, add 100 µl/well of complete Mesencult in a 16-well E-plate, put on the lid and place it on the xCELLigence RTCA station. Check the connections and the cell index readings to make sure that the 16-well E-plate is correctly installed. Leave the 16-well E-plate on the xCELLigence RTCA station for 30-45 min and then measure the background impedance in the absence of cells.

b. Remove the E-plate from the xCELLigence RTCA station and place it in a sterile hood. Vortex the tube containing 10,000 mASCs/ml to resuspend the cells and carefully add 100 µl of cell suspension (prepared in step C1e)/well in duplicates being careful not to touch the bottom of the wells with the tips. The final volume/well should now be 200 µl.

c. Leave the mASCs in the xCELLigence RTCA station in the cell incubator for 7 days, reading the impedance every hour (Note 8).

d. Plot the cell index versus time in culture (see Figure 2 below).

3. CellTiter-Blue

This method is based on the conversion of resazurin to the fluorescent product resorufin by metabolically active cells. The fluorescence signal obtained is proportional to the number of viable cells in the culture.

a. Add 150 µl of cell suspension/well in a flat-bottomed 96-well tissue culture plate (each well will contain approximately 1,000 cells) in triplicates. Make 1 plate/time point. Add culture medium alone in triplicates to each plate to serve as background control. Place the plates in a cell incubator set at 37 °C, 5% CO_2, 21% O_2, 90% RH.

b. Read the plates on day 1, 3 and 5-6. Add 30 µl of CellTiter-Blue reagent/well, tap the sides of the plate gently and incubate the plate for another 4 h.

c. Transfer 100 µl from each well to a black 96-well plate and read fluorescence in a fluorometro (a Glomax multidetection system or equivalent) at $560_{Ex}/590_{Em}$.

d. Subtract the average background fluorescence from sample fluorescence and plot fluorescence (proliferation) versus time (see Figure 2 below).

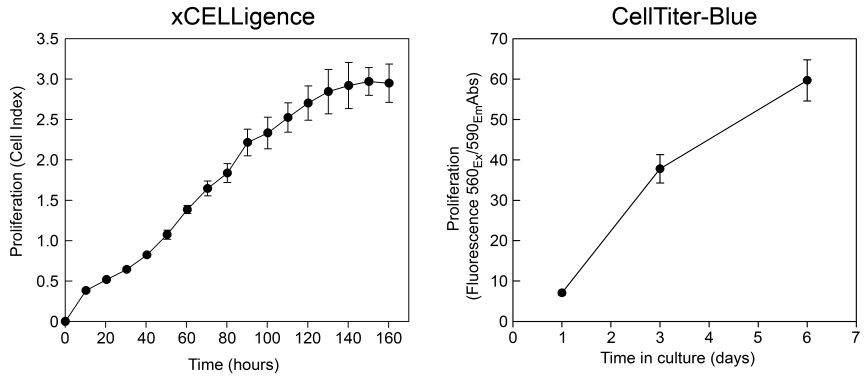

Figure 2. Representative data showing the proliferation of mASCs using the xCELLigence system (left panel) and the CellTiter-Blue reagent (right panel)

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Notes

1. Thoroughly spray the abdomen of the sacrificed mouse with 70% ethanol and secure the animal, ventral side up, on a polystyrene support using medical syringe needles. Use one set of sterile scissors/forceps to carefully make a midline incision, being careful not to damage the peritoneum. Separate the skin from the peritoneum and secure the skin flaps with syringe needles. When acquiring the inguinal fat pads care should be taken to remove the inguinal lymph nodes that are embedded into the fat at the Y-shaped junction of blood vessels. Using a new set of sterile scissors/forceps, collect both inguinal fat pads and transfer them to a 50 ml tube containing 10 ml HBSS and keep on ice. Proceed to cut open the peritoneum, taking care not to damage the intestines. Collect the gonadal fat pads attached to the testes and ovaries in male and female mice, respectively. We pool the fat from both locations when isolating mASCs. However, depot-specific differences in MSC phenotype have been reported and depending on your scientific aims you may have to isolate mASCs from one location or the other.
2. The solution can be viscous and it might be necessary to use several cell strainers.
3. The pellet in the following steps is loose and care should be taken when removing the supernatants.
4. We seed the mASCs at a concentration of 10,000 cells/cm^2 which allows us to passage the cells every 3-4 days. Under these culturing conditions we have been able to measure the proliferation of mASC from passage 3-7 using the xCELLigence system or CellTiter-Blue reagent. However, it should be noted that the size of mASCs increases with increasing passage number whereas their proliferative capacity decreases. It is therefore best to use mASC cell lines with the same passage number when comparing their proliferative capacity with the above methods.
5. We provide two protocols for measuring mASC proliferation. The CellTiter-Blue reagent is easy to use and feasible in most laboratories. However, some studies have shown that resazurin can affect cell viability upon prolonged exposure and inclusion of drugs that increase mitochondrial number/activity can give false results. The xCELLigence system requires the purchase of the RTCA station/software and the cost of specialized E-plates. However, the xCELLigence system enables the label-free and non-invasive measurement of proliferation in real-time. We generally use both methods in parallel when studying mASC proliferation *in vitro*. To obtain more information about the CellTiter-Blue reagent and xCELLigence system, please read the manufacturer's instructions.
6. We prefer to count in average 20-50 cells/corner square in order to obtain reliable cell concentrations. Calculate the cell concentration using the following formula: (total number of cells in the four corner squares/4) x 2 (dilution factor) x 10,000 = no. cells/ml.

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7. We found that the addition of 1,000 mASCs/well (3,125 cells/cm^2) allows the monitoring of mASC proliferation for 5-7 days before the culture reaches confluence. This cell number gives reproducible growth curve which includes an initial 24-48 h lag phase followed by a proliferative phase according to Figure 1.
8. We usually do not add fresh media to the E-plates during the experiment. However, it is possible to pause the experiment, remove the E-plates, add another 100 µl medium/well and restart the impedance measurements.

Recipes

1. Collagenase Type I
   The collagenase Type I (100 mg) is dissolved in 10 ml of HBSS to a concentration of 10 mg/ml
   Aliquots of 0.5 ml are stored at -20 °C
2. Complete MesenCult medium
   Thaw MesenCult™ stem cell stimulatory supplements (mouse), stable glutamine, penicillin/streptomycin at 4 °C and mix:
   400 ml of MesenCult MSC basal medium (mouse)
   100 ml MesenCult stem cell stimulatory supplements (mouse)
   5 ml stable glutamine (final concentration: 2 mM)
   5 ml penicillin/streptomycin (final concentration: 100 U/ml)
   0.5 ml Mesenpure (optional)
   Store the complete MesenCult at 4 °C and use it within one month

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