Published in final edited form as:

Curr Protoc Stem Cell Biol. ; 28: Unit-2B.6.. doi:10.1002/9780470151808.sc02b06s28.

Preparation of anti-inflammatory mesenchymal stem/precursor cells (MSCs) through sphere formation using hanging drop culture technique

Thomas J. Bartosh¹ and Joni H. Ylostalo²

Institute for Regenerative Medicine, Texas A&M Health Science Center College of Medicine at Scott & White, 5701 Airport Rd., Module C, Temple, TX 76502

Abstract

Herein, we describe a protocol for preparation of pre-activated anti-inflammatory human mesenchymal stem/precursor cells (MSCs) in 3D culture without addition of exogenous chemicals or gene transfer approaches. MSCs are an easily procurable source of multipotent adult stem cells with therapeutic potential largely attributed to their paracrine regulation of inflammation and immunity. However, the culture conditions to prepare the ideal MSCs for cell therapy remain elusive. Furthermore, reported lag time for activation in experimental models have prompted investigations to pre-activate the cells prior to their administration. In this protocol, standard 2D culture expanded MSCs are activated by aggregation into 3D spheres using hanging drop cultures. MSC activation is evaluated by real-time PCR and/or ELISA for anti-inflammatory factors (TSG-6, STC-1, PGE2), and by a functional assay using lipopolysaccharide-stimulated macrophage cultures. Furthermore, we elucidate methods to prepare MSC sphere conditioned medium, intact spheres, and suspension of single cells from spheres for experimental and clinical applications.

Keywords

MSC; sphere; anti-inflammatory; macrophage; activation; spheroid; LPS

INTRODUCTION

This unit describes a proficient method to generate anti-inflammatory human mesenchymal stem/stromal cells (MSCs) using hanging drop cultures. Also, basic assays to test the potency and properties of the activated MSCs will be described. Three dimensional (3D) culture techniques, such as hanging drop cultures, have been used in studies to mimic more closely the microenvironments and cellular interactions occurring *in vivo* (Achilli et al., 2012; Page et al., 2013). These cell-to-cell and cell-to-matrix interactions direct the behavior and properties of the cells, making 3D cultures an efficient way of activating cells. Recent studies have demonstrated that MSCs in 3D cultures have properties that could enhance their

Correspondence to: Joni H. Ylostalo.

¹Phone: 254-771-6845, Fax: 254-771-6839, bartosh@medicine.tamhsc.edu

²Phone: 254-771-6820, Fax: 254-771-6839, ylostalo@medicine.tamhsc.edu

therapeutic potential (Qihao et al., 2007; Potapova et al., 2008a; Potapova et al., 2008b; Xie et al., 2009; Frith et al., 2009; Wang et al., 2009a; Wang et al., 2009b; Bartosh et al., 2010; Saleh and Genever., 2011; Jing and Jian-Xiong., 2011; Ylostalo et al., 2012; Baraniak and McDevitt., 2012). The protocols presented below are detailed descriptions of those we published previously (Bartosh et al., 2010; Ylostalo et al., 2012).

Hanging drop cultures permit cells to aggregate and form a sphere in the apex of the drop. The size of the sphere is easily controlled by the volume of the drop or the concentration of the cell suspension. This unit begins with the standard culture of human MSCs on adherent dishes in 2D, followed by the hanging drop culture of the cells in 3D, harvest of spheres and individual cells from the hanging drop cultures, preparation of conditioned medium from transfer cultures, and real-time PCR, ELISA, and macrophage assays to evaluate the activated MSCs.

NOTE: The procedures involving cell cultures are to be performed in a Class II biological safety cabinet and everything entering the hood must be sprayed with a 70% alcohol solution.

NOTE: Aseptic techniques must be maintained when handling live cells.

NOTE: All cell cultures are performed in a 37°C, 5% CO₂ humidified incubator.

BASIC PROTOCOL 1: HANGING DROP CULTURE TECHNIQUE FOR THE DEVELOPMENT OF MSC SPHERES

The protocol reported in this section has been designed to generate homogenous 3D micro-tissue aggregates or 'spheres' from MSC cultures. The methods described have been adapted from conventional hanging-droplet protocols (Achilli et al., 2012; Page et al., 2013) but herein are tailored to enhance the therapeutic potential of MSCs (Bartosh et al., 2010; Ylostalo et al., 2012).

To obtain the cells for 3D cultures MSCs, isolated from human bone marrow aspirates, are first propagated as standard plastic-adherent 2D cultures (Support Protocol 1). When the expanded cells reach 70-80% confluence and are of sufficient quantity, 3D cultures are initiated. Using hanging drop technique, MSC spheres are generated from a defined number of cells resulting in uniform size with reproducible anti-inflammatory characteristics.

Materials

Cell culture dish, treated, 150 mm × 25 mm

Lab marker

Culture expanded and harvested bone marrow MSCs in CCM (Support Protocol 1)

Complete culture medium (CCM, Reagents and Solutions)

1000-μl pipette with sterile tips

Motorized pipettor with 5-ml, 10-ml, 25-ml, and 50-ml serological sterile pipets

Sterile reagent reservoir

Phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride, pH 7.4

100- μ l multichannel pipette with sterile tips

Humidified cell culture incubator set to 37°C and 5% CO₂

1. Obtain a sufficient number of 150 mm cell culture dishes and label the lids.

It is important to label the dishes prior to initiation of hanging drop cultures as dish handling can disrupt the hanging drops.

2. Use the culture expanded and harvested bone marrow MSCs prepared in Support Protocol 1 for hanging drop cultures (Figure 1A, B).

Each 150 mm culture dish of passage 1 or 2 MSCs yields approximately 1 – 1.5×10^6 cells, sufficient to produce 40–60 spheres.

3. For hanging drops consisting of 25,000 cells per drop, dilute the MSC cell suspension to 714 cells/ μ l with CCM and transfer the suspension to a sterile reagent reservoir.

Cell concentration can be adjusted to generate spheres of different sizes.

4. Remove the lid of a 150 mm dish and add 20 ml PBS (room temperature) to the base.

Keep the lid off of the plate to prevent accumulation of condensation.

5. With the underside of the lid facing upward, use a multi-channel pipette to dispense 35 μ l droplets in even rows onto the lid (Figure 1C, D).

Approximately 15 rows of 8, or 120 droplets (3×10^6 cells), can be comfortably transferred onto a 150 mm culture dish lid.

6. In one continuous motion, flip the lid so the apex of the droplets is facing downward and carefully position the lid on the base of the culture dish previously filled with PBS (Figure 1E).

Proper speed and directionality are critical parameters for inverting a lid with droplets as large as 35 μ l. For best results, simultaneously move the lid in an upward motion while turning it over. Prior to preparing hanging drops, practice pipetting droplets of CCM (without cells) and flipping the plate. The surface tension of the CCM without cells is slightly less, therefore, preparing hanging drops with cells will be easier after practice with CCM.

7. Keeping the dish level, transfer it to a humidified incubator set to 37°C and 5% CO₂ (Figure 2A).

8. Repeat steps 4–7 for each additional plate.

It is important to keep plates in the incubator as a single layer. Stacking the plates can interfere with air flow and prevent the spheres from assembling properly.

9. Incubate the cells in hanging drops for 3 days at 37°C in the incubator to generate MSC spheres.

For optimal results, make certain that the incubator is level and the cultures are uninterrupted. Opening the incubator door for even a short period of time can result in condensation build-up on the lid around the drops or disruption of the droplets.

SUPPORT PROTOCOL 1: RECOVERY OF FROZEN MSCs AND EXPANSION AS 2D ADHERENT CULTURES

This protocol describes the culture conditions necessary to obtain a sufficient quantity of human bone marrow MSCs for 3D cultures while limiting passage number (Bartosh et al., 2010). The protocol starts with MSC recovery from a frozen vial and describes the basic techniques of MSC harvest and re-plating for expansion in 2D adherent cultures.

Materials

Complete culture medium (CCM, Reagents and Solutions)

Cell culture dish, treated, 150 mm × 25 mm

Humidified cell culture incubator set to 37°C and 5% CO₂

Liquid nitrogen tank for cell storage

A frozen vial containing approximately 10⁶ passage 1 or 2 bone marrow MSCs (Center for the Preparation and Distribution of Adult Stem Cells, Texas A&M Health Science Center, Institute for Regenerative Medicine)

Water bath fixed to 37°C

Motorized pipettor with 5-ml, 10-ml, 25-ml, and 50-ml serological sterile pipets

Vacuum aspirator

Phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride, pH 7.4

0.25% Trypsin-EDTA (1x)

Upright microscope with a 10x objective

50-ml sterile conical tubes

Centrifuge with swinging bucket rotor and adaptors for 50-ml conical tubes

20-μl, 100-μl, 200-μl, and 1000-μl pipette with sterile tips

1.5-ml sterile microcentrifuge tubes

Hemocytometer

Trypan blue

MSC recovery from a frozen vial

- 1** Prepare a recovery plate for MSC cultures by placing 30 ml CCM into a 150 mm cell culture dish and incubate 30 min at 37°C in a humidified incubator containing 5% CO₂.

Equilibrating the medium in the incubator ensures better recovery of the cells since the medium is warm and pH correct before the addition of the cells.
- 2** Remove frozen cryovial of approximately 10⁶ MSC in passage 1 or 2 from liquid nitrogen tank and incubate 2 min at 37°C water bath.

Monitor thawing process carefully and longer/shorter incubations might be needed depending on the thawing speed. Gentle swirling of the tube in the water bath will aid in the thawing process. Make sure the tube does not get immersed into the water as this will increase the contamination risk.
- 3** Transfer contents of the cryovial to the culture dish using motorized pipettor and 5-ml serological pipet.

Cryovial can be washed couple times with the CCM from the plate to ensure transfer of all the cells from the tube.
- 4** Place the dish overnight in the incubator (Figure 1A).

Do not exceed over 24 h as the DMSO from the frozen cell stock can become toxic for the cells.
- 5** Wash the dish twice with 15–20 ml PBS and add 3 ml pre-warmed 0.25% trypsin/EDTA (1x).

Make sure all the PBS is removed from the dish before addition of trypsin solution.
- 6** Incubate 3–4 min at 37°C incubator and examine the detachment efficiency under a microscope.

Detachment of cells should be regularly monitored to minimize cell damage caused by over-exposure to trypsin. To facilitate detachment, the side of the dish can be gently tapped during the trypsinization process. Exact time required for complete cell detachment is dependent on cell confluency and the potency of the trypsin/EDTA.
- 7** Add 6 ml of pre-warmed CCM to the dish and transfer contents to a 50-ml conical tube.

Serum in CCM will neutralize trypsin.
- 8** Wash the plate with 15–20 ml PBS and transfer to same 50-ml tube to maximize cell recovery.

- 9 Centrifuge the cells for 10 min at 450 x g, room temperature.
- 10 Aspirate the supernatant and resuspend cell pellet in up to 1 ml CCM.
- 11 Count viable cells with hemocytometer.

Follow the hemocytometer instructions. We generally make 2–3 separate dilutions of the cell solution and count each dilution twice to get a more accurate cell count. Viable cells will have a round morphology with light coloring and typically count for over 90% of the cells. Dead/dying cells are often black or odd shaped. Viability dye trypan blue can also be used to aid in counting viable cells (dead/dying cells will stain blue).

MSC expansion

- 12 Seed an appropriate number of 150 mm culture dishes at 100 cells/cm² in 30 ml of pre-warmed CCM.

Distribute the cells evenly across the surface of the plate by carefully moving the plate in a pattern reflecting the number '8'.
- 13 Incubate the culture dishes 7 days in the incubator with medium changes on days 3 and 6.

To change the medium, aspirate old CCM from the dishes and carefully add 30 ml of the new CCM for each dish.
- 14 Examine the 2D culture-expanded MSCs by microscopy to verify that the cells are 70–80% confluent and ready-to-use (Figure 1B).

It is vital to periodically monitor confluency of the cultures. Under the conditions described, the cells should be 70–80% confluent on day 7. Culture time should be adjusted to prevent deleterious results that can occur from using cells that are over-confluent.
- 15 Follow the instructions for steps 5–9 in this protocol to harvest the cells from the dishes.

After adding trypsin/EDTA to 4 dishes, take another set of 4 dishes and wash and trypsinize them. Now the first set of 4 dishes should be ready for harvest while the second set of 4 are in the incubator. This allows the harvest of 8 dishes quickly. Another set of 8 dishes can then be harvested while the first set of 8 are in the centrifuge.
- 16 Discard supernatant and combine the cell pellets from all tubes into a single tube using up to 50 ml CCM.

Since the cells are cultured at a high density in spheres, second wash in CCM is important to remove residual trypsin/EDTA from the cells.
- 17 Centrifuge the cells 10 min at 450 x g, room temperature.
- 18 Aspirate supernatant, and resuspend pellet in CCM for cell counts as in step 11.

BASIC PROTOCOL 2: HARVEST OF MSC SPHERES AND PREPARATION OF SINGLE CELLS FROM SPHERES

This protocol describes the harvest of MSC spheres from hanging drop cultures for further potency assays (Bartosh et al., 2010; Ylostalo et al., 2012). The method to prepare activated single cells from spheres is also described in this protocol (Bartosh et al., 2010; Ylostalo et al., 2012). For many downstream applications intact spheres are the first choice of activated cells, but for some applications sphere-derived cells might be more appropriate. For example, flow cytometry, cell sorting applications and intravenous delivery of pre-activated cells would require single cell suspension whereas immunofluorescence microscopy and delivery of cells into peritoneum would permit use of whole spheres.

Materials

Hanging drop culture dish with 3 day MSC spheres (Basic Protocol 1)
Cell lifter
20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with sterile tips
15-ml and 50-ml sterile conical tubes
Centrifuge with swinging bucket rotor and adaptors for 15-ml and 50-ml conical tubes
Phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride, pH 7.4
Vacuum aspirator
Freezer that can reach temperatures below -70°C
0.25% Trypsin-EDTA (1x)
Water bath fixed to 37°C
Motorized pipettor with 5-ml, 10-ml, 25-ml, and 50-ml serological sterile pipets
40 μ m cutoff cell strainer
Complete culture medium (CCM, Reagents and Solutions)
Fetal bovine serum (FBS)
Hemocytometer
Trypan blue
Upright microscope with a 10x objective

Harvest of MSC spheres

- 1 Lift the lid off a 150 mm dish with day 3 hanging drop cultures of MSCs (Basic Protocol 1) and invert to position the droplets facing upward.

It is imperative to not let the plate sit at room temperature for long periods of time after removing from the incubator as the temperature

change can cause deposition of condensation on the lid and alter experimental results.

- 2 Angle the lid to approximately 10–20 degrees and push the droplets containing the MSC spheres, to the plate edge using a cell lifter (Figure 2B, C).

Although the spheres are sturdy tissue-like structures that do not dissociate by mechanical means alone, care should be taken to avoid mechanically altering the properties of the cells. The spheres do not adhere to the dish lid and therefore can easily be forced to the edge.

- 3 Transfer the spheres and medium into a 15-ml conical tube with a 1000- μ l pipette (Figure 2E, F).

The spheres can be tacky therefore it is recommended to use the same pipette tip throughout sphere collection. Moreover, it is not recommended to collect in a larger serological pipet to avoid loss of spheres resulting from adherence to the wall of the pipet. Use a 50-ml conical tube to harvest spheres and medium from multiple dishes.

- 4 Centrifuge 5 min at 450 x g, room temperature.

Centrifugation of the spheres is not recommended when using the spheres for applications where sphere morphology is critical such as immunofluorescence microscopy. Since the spheres are denser than the medium, they will settle to the bottom of the tube in 2–3 minutes.

- 5 Wash the spheres with PBS and centrifuge 5 min at 450 x g, room temperature.

Dissociation of spheres to single cell suspension

- 6 To obtain single cells from spheres, wash the spheres with PBS and centrifuge 5 min at 450 x g, room temperature.

- 7 Aspirate the supernatant and add 2–3 ml 0.25% trypsin/EDTA (1x) to the pelleted spheres.

Do not mix the solution at the start of the incubation as the spheres are tacky and can become lodged on the wall of the pipette tip. Complete removal of PBS is important particularly when dissociating small numbers of spheres in a low volume of trypsin/EDTA. Fresh trypsin/EDTA prewarmed to 37°C just prior to use is recommended to ensure efficient dissociation.

- 8 Use the following guideline to determine the amount of trypsin/EDTA for different numbers of spheres:

<u>No. of spheres</u>	<u>Trypsin/EDTA volume</u>
30	1–2 ml
120	3 ml

<u>No. of spheres</u>	<u>Trypsin/EDTA volume</u>
500	10–15 ml
1000	20 ml

- 9 Incubate 3 min at 37°C then mix the solution by pipetting 5–6 times using a 1000- μ l pipette.
For larger volumes, use a 10-ml serological pipet to ensure proper mixing.
- 10 Incubate 3 min at 37°C.
- 11 Repeat sample mixing and carefully inspect tube for the presence of aggregates. If no visible clumps are observed, inactivate trypsin by addition of CCM at 2 times the volume of enzyme solution used.
If aggregates remain, incubate additional 3 min at 37°C, mix, and inactivate trypsin. If large sphere numbers and trypsin are used, inactivate trypsin by addition of FBS at volume of the trypsin/EDTA used.
- 12 Pass the cell suspension through a 40 μ m strainer to remove undigested material and any small aggregates that might be remaining.
- 13 Centrifuge 10 min at 450 x g, room temperature to pellet the cells.
Sphere-derived cells are very small (approximately ¼ volume of standard MSCs) and therefore the cell pellet is often not visible particularly following the dissociation of a small number of spheres.
- 14 Aspirate supernatant and wash cells with PBS.
- 15 Centrifuge 10 min at 450 x g, room temperature and aspirate the supernatant.
- 16 Suspend the cell pellet into appropriate volume of CCM and count the viable sphere-derived cells with a hemocytometer.
Generally, recovery of sphere-derived cells is approximately 30–50% of the input number of MSCs suspended in the initial drops. Use of trypan blue can aid in determining the number of viable cells.

BASIC PROTOCOL 3: PREPARATION OF MSC SPHERE CONDITIONED MEDIUM USING TRANSFER CULTURES

The types of paracrine factors secreted by MSCs provide valuable information regarding cell activation and potential therapeutic benefit (Prockop and Oh., 2012). Here we describe a method to prepare conditioned medium from MSC spheres to evaluate the secretome of MSCs pre-activated by sphere formation in hanging drop cultures (Bartosh et al., 2010; Ylostalo et al., 2012).

For the assay, MSCs (sphere or adherent monolayer) are collected then transferred into a multi-well plate containing medium. We have successfully transferred sphere MSCs to

CCM, macrophage medium, and medium with reduced serum, and measured secreted factors after 2 h, 6 h, and 24 h.

Materials

Adherent monolayer MSCs (Support Protocol 1)
MSC spheres and single cells from spheres (Basic Protocol 2)
Motorized pipettor with 10-ml serological sterile pipets
15-ml sterile conical tubes
CCM (Reagents and Solutions)
Centrifuge with swinging bucket rotor and adaptors for 15-ml conical tubes
Vacuum aspirator
Hemocytometer
20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with sterile tips
6-well cell culture dish, treated
Humidified cell culture incubator set to 37°C and 5% CO₂
1.5-ml sterile microcentrifuge tubes
Microcentrifuge with rotor for 1.5-ml tubes and capability for 10,000 x g speed
0.22 μ m filter
Freezer that can reach temperatures below –70°C

Preparation of cell suspension

- 1 Transfer a sufficient number of adherent monolayer MSCs (Support Protocol 1), MSC spheres (Basic Protocol 2), or single cells from MSC spheres (Basic Protocol 2) to a 15-ml conical tube.
- 2 Add 12 ml CCM and centrifuge 5 min at 450 x g, room temperature.

All the steps below can also be performed with reduced serum medium (e.g. 2% or 5% FBS) or other medium formulations if downstream applications are serum sensitive or require specific medium. However, not all medium is compatible with MSC growth and viability and should be tested prior to use.
- 3 Aspirate supernatant and resuspend cells in CCM.
- 4 Obtain cell counts using hemocytometer or estimate the counts based on input number of cells into hanging drop cultures.
- 5 Adjust cell concentration to 100,000 cells/ml with CCM.

- 6 Transfer 2 ml of the cell suspension or 8 spheres (25,000 cells/sphere) to 6-well plate in triplicate. Place medium alone in a well to be used as a reagent control in downstream assays.

The amount of cells described here has been used routinely for detection of PGE2 in the conditioned medium. Cell concentration and growth area can be modified for detection of soluble factors that are less abundant.

Transfer of spheres to non-adherent dishes has also been performed for collection of sphere-conditioned medium.

- 7 Incubate up to 24 h in a humidified incubator set to 37°C and 5% CO₂ (Figure 3A–D).

It is normal, particularly in cultures of sphere-derived cells, to observe numerous cell fragments and microparticles as a fraction of the cells in spheres undergo apoptosis/necrosis.

Collection of the MSC conditioned medium

- 8 Collect the medium conditioned by the cells and place into a 15-ml conical tube.
- 9 Centrifuge 5 min at 500 x g, room temperature.
- 10 Transfer the MSC-conditioned medium to 1.5-ml sterile tubes and centrifuge 10 min at 10,000 x g, room temperature to further clarify the sample.

If large volume of MSC-conditioned medium is prepared, the conditioned medium can be filtered through a 0.22 µm filter to remove cell debris and fragments instead of the high speed centrifugation.

- 11 Transfer supernatant to sterile 1.5-ml tubes, aliquot, and store at –80°C.

MSC conditioned medium can be stored for several months. Avoid repeated freeze-thaw cycles.

BASIC PROTOCOL 4: GENE EXPRESSION ANALYSIS OF ANTI-INFLAMMATORY MARKERS

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product, usually a protein. Gene expression studies with real-time polymerase chain reaction (PCR) are most commonly used to detect changes, either increases or decreases, in the expression of a gene. Real-time PCR measures PCR amplification in real time permitting the determination of the starting concentration of a nucleic acid using fluorescent reporter molecules. This protocol describes expression analysis of 3 anti-inflammatory protein coding genes in MSC spheres and adherent monolayers. The protocol starts with a total RNA isolation method followed by quantification of the RNA, reverse transcription (RT), and real-time PCR method for COX-2, TSG-6, and STC-1 genes using GAPDH as a housekeeping gene.

Materials

Adherent monolayer MSCs (Support Protocol 1)

MSC spheres (Basic Protocol 2)

10- μ l, 20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with RNase and DNase-free sterile tips

RNeasy Mini Kit (Qiagen, cat. no. 74104)

B-mercaptoethanol

1.5-ml RNase-free sterile microcentrifuge tubes

Freezer that can reach temperatures below -70°C

Vortex

QIAshredder (Qiagen, cat. no. 79654)

Microcentrifuge with rotor for 2 ml tubes and capability for over 15,000 x g speed

200 proof ethanol

RNase and DNase-free water

RNase-Free DNase Set (Qiagen, cat. no. 79254)

Microcentrifuge for spinning down solutions

Microvolume spectrophotometer (e.g., NanoDrop)

High Capacity cDNA Reverse Transcription Kit (Life Technologies, cat. no. 4368814)

0.2-ml RNase and DNase-free sterile PCR tubes or strips with caps

Cold block

Thermal cycler

Freezer that can reach temperatures below -20°C

GAPDH TaqMan® Gene Expression Assay FAM (Life Technologies, Hs02758991_g1)

PTGS2 (COX-2) TaqMan® Gene Expression Assay FAM (Life Technologies, Hs00153133_m1)

TNFAIP6 (TSG-6) TaqMan® Gene Expression Assay FAM (Life Technologies, Hs01113602_m1)

STC1 (STC-1) TaqMan® Gene Expression Assay FAM (Life Technologies, Hs00174970_m1)

TaqMan® Fast Universal PCR Master Mix (2x), No AmpErase UNG (Life Technologies, cat. no. 4352042)

96-well PCR plates (compatible with real-time PCR system; e.g., Life Technologies)

PCR hood

Optical Adhesive Film with plate sealer tool

Centrifuge with plate adaptor

Real-time PCR system capable of detecting FAM and ROX reporter dyes (e.g., ABI 7900HT, Life Technologies)

Total RNA isolation from monolayer and sphere MSCs

- 1 Use the RNeasy Mini Kit with Qias shredder columns and RNase-Free DNase Set to isolate total RNA without DNA contamination from adherent monolayer MSCs (Support Protocol 1) and MSC spheres (Basic Protocol 2) previously washed with PBS.
- 2 Lyse at least 100,000 monolayer MSCs and 20 spheres each prepared from 25,000 cells (but no more than 10^7 cells) in separate conical tubes with 350 μ l of RLT buffer (supplemented with 1:100 of β -mercaptoethanol) mixing thoroughly.

Make sure no excessive PBS remains in the tube before adding the RLT buffer to ensure thorough cell lysis.
- 3 Transfer the cell lysate into a 1.5-ml RNase-free sterile tube and store for at least couple hours at -80°C to ensure complete sphere lysis.
- 4 Thaw cell lysate on ice and vortex briefly and follow the manufacturer's instruction for RNA isolation.

Vortexing will ensure thorough lysing of the spheres.
- 5 Proceed to RNA quantification or store samples at -80°C .

RNA quantification

- 6 Thaw samples on ice.
- 7 Measure the concentration of total RNA using microvolume spectrophotometer.

The concentration of RNA is measured spectrophotometrically at 260 nm in which absorbance of 1 corresponds to RNA concentration of 40 ng/ μ l. The ratio of the absorbances at 260 nm and 280 nm provides an estimate of the purity of RNA in regards to protein contamination. For pure RNA this ratio is typically 1.9–2.1.

cDNA synthesis

- 8 Use the High Capacity cDNA Reverse Transcription Kit according to manufacturer's directions to generate enough cDNA for real-time PCR.

Use 1 μ g of RNA. If the concentration of the RNA is less than 100 ng/ml, lower amounts of RNA may be used, however, the same amount should be used for each sample in the study to ensure accurate comparisons. Also, total sample volume can be increased up to 30 μ l, but equal amount of RT-PCR master mix must be added.
- 9 Proceed to real-time PCR or store the cDNA at -20°C

Real-time PCR

- 10 Thaw the samples and TaqMan® Gene Expression Assays on ice, vortex them and briefly spin them down.

At least one target and one endogenous control (GAPDH) Gene Expression Assay is needed.

- 11 Prepare the reaction mix in triplicate according to manufacturer's instructions for TaqMan® Gene Expression Assays and Fast Universal PCR Master Mix.
- 12 Follow the guidelines for the real-time PCR instrument for generating the experiment document, plate layout, and run parameters.
- 13 Analyze the data according to the instructions for the real-time PCR instrument.

The instrument will automatically define the baseline signal level and threshold to permit determination of the C_T values for each well. Use the C_T method to calculate relative changes in gene expression using GAPDH as the endogenous control and monolayer MSCs as a baseline. This will result in relative change of each gene between monolayer and sphere MSCs.

BASIC PROTOCOL 5: QUANTIFICATION OF PGE2 SECRETED BY MSC SPHERES

The following protocol describes the quantification of PGE2 in the MSC sphere-conditioned medium. PGE2 is an important immunomodulatory molecule that, among other functions, can convert pro-inflammatory M1 macrophages into more anti-inflammatory M2 macrophages (Nemeth et al., 2009; Ylostalo et al., 2012; Prockop and Oh., 2012). The amount of PGE2 in sphere-conditioned medium is directly proportional to the anti-inflammatory effect of the sphere-conditioned medium on lipopolysaccharide (LPS)-stimulated mouse macrophages and thus is an important measure of the level of activation of MSCs (Ylostalo et al., 2012).

Materials

MSC monolayer and sphere-conditioned medium (Basic Protocol 3)

Vortex

Prostaglandin E2 Parameter Assay Kit (R&D Systems, cat. no. KGE004B)

Deionized water

10- μ l, 20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with sterile tips

1.5-ml sterile microcentrifuge tubes

100- μ l, 200- μ l multichannel pipette

Horizontal orbital microplate shaker capable for 500 rpm speed

Multichannel vacuum aspirator

Microplate reader capable of measuring absorbances at 450 nm and 540 nm

Computer software that can generate a four parameter logistic curve fit

1. Use the Prostaglandin E2 Parameter Assay Kit to measure the concentration of PGE2 in the MSC monolayer and sphere-conditioned medium collected in Basic Protocol 3.
2. Thaw samples on ice and vortex after thawed.
3. Reconstitute the PGE2 standard with 1 ml deionized water or thaw a previously frozen aliquot of the standard on ice.

The stock PGE2 standard concentration is 25,000 pg/ml.

4. Prepare the PGE2 standards by diluting into calibrator diluent (RD5-56) according to the following protocol:

A	280 µl stock	420 µl RD5-56	10,000 pg/ml
B	350 µl A	350 µl RD5-56	5,000 pg/ml
C	350 µl B	350 µl RD5-56	2,500 pg/ml
D	350 µl C	350 µl RD5-56	1,250 pg/ml
E	350 µl D	350 µl RD5-56	625 pg/ml
F	350 µl E	350 µl RD5-56	313 pg/ml
G	350 µl F	350 µl RD5-56	156 pg/ml
H	350 µl G	350 µl RD5-56	78 pg/ml

Mix each solution well by pipetting before transfer to the next tube. Use new tip for each transfer.

5. Dilute the sphere-conditioned medium samples 1:50 and monolayer-conditioned medium samples 1:5 in 350 µl RD5-56 in 1.5-ml microcentrifuge tubes.
6. Design the layout of the plate to include samples, standards, zero standard, and non-specific binding well, all in duplicate.
7. Remove excess microplate strips from the plate frame and return them to the foil pouch with the desiccant and run the assay according to manufacturer's instructions.
8. Determine the optical density of each well within 30 min of addition of the stop solution using a microplate reader set to 450 nm and 540 nm.
9. Subtract the 540 nm readings from the 450 nm readings.

This will correct for optical imperfections in the plate.

10. Subtract the average signal of the non-specific binding wells of the signal from all other wells.

This will correct for the non-specific signal from the reagents.

11. Calculate the signals for each sample and standard by averaging the duplicate values.
12. Create a standard curve using a computer software that can generate a four parameter logistic curve fit and use it to determine the PGE2 concentrations for each sample.

Alternatively, you can create the standard curve by plotting optical density on linear y-axis and PGE2 concentration on logarithmic x-axis and drawing the best fit curve. You can then read the values from the standard curve manually.

13. Multiply the values obtained from the standard curve with the dilution factor to acquire the final PGE2 concentrations.

BASIC PROTOCOL 6: FUNCTIONAL MEASUREMENT OF THE ANTI-INFLAMMATORY PROPERTIES OF MSC SPHERES USING MACROPHAGE CULTURES

Macrophages are important immune effector cells with well-documented roles in inflammatory response and in tissue repair (Martinez et al., 2009; Murray and Wynn., 2011; Ortega-Gomez et al., 2013). The assay described here was developed as a valuable tool to measure the ability for MSC spheres to modify cytokine production of stimulated macrophages. It is based on our recent findings that MSC spheres can reduce secretion of pro-inflammatory cytokines (such as TNF α) by stimulated macrophages while enhancing the secretion of anti-inflammatory cytokines (such as IL10) (Ylostalo et al., 2012).

This protocol describes a macrophage assay, in which pro-inflammatory and anti-inflammatory cytokine secretion, by lipopolysaccharide-stimulated mouse macrophages incubated with MSC conditioned medium, is monitored.

Materials

12-well cell culture dish, treated

Macrophage medium (Reagents and Solutions)

Water bath set at 37°C

10- μ l, 20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with sterile tips

MSC-conditioned medium from adherent monolayer, sphere, and sphere-derived cell cultures (Basic Protocol 3)

CCM (Reagents and Solutions)

J774 mouse macrophages (Support Protocol 2)

15-ml and 50-ml sterile conical tubes

0.1 mg/ml lipopolysaccharide (LPS) solution

Vortex

Motorized pipettor with 10-ml serological sterile pipets
Humidified cell culture incubator set to 37°C and 5% CO₂
Upright microscope with a 10x objective
1.5-ml sterile microcentrifuge tubes
Microcentrifuge with rotor for 1.5-ml tubes and capability for 500 x g speed
Freezer that can reach temperatures below –70°C
Vortex
Mouse TNFα Quantikine ELISA kit (R&D Systems, cat. no. MTA00B)
Mouse IL10 Quantikine ELISA kit (R&D Systems, cat. no. M1000).

Assay plate preparation

- 1 Determine the number of samples that will be tested and obtain a sufficient number of 12-well plates for the assay.

Each sample should be tested in triplicate.
- 2 Add 480 µl of pre-warmed macrophage medium to each well.
- 3 Transfer 20 µl of each MSC-conditioned medium (Basic Protocol 3) or CCM (vehicle control) into a well in triplicate. Also, add 20 µl macrophage medium into a well in triplicate for the unstimulated macrophage control (Figure 4A).

The volume in each well is now 500 µl and each sample is being tested in triplicate.
- 4 Carefully tap the side of the plate minimum of 7 times to mix the sample and distribute the medium across the entire surface of the wells.

It is important that the medium is appropriately distributed to avoid macrophage clumping in later steps.

Macrophage stimulation and treatment

- 5 Suspend J774 mouse macrophages (Support Protocol 2) to a concentration of 200,000 cells/ml in macrophage medium in a 50-ml conical tube.
- 6 Transfer 500 µl of the cell suspension to the 3 wells containing 500 µl macrophage medium only (unstimulated macrophage control).

To evenly distribute the macrophages in the wells, gently rock the plate 3 times. Avoid circular movement of the plate to prevent the cells from settling in the center of the well.
- 7 In a separate 15-ml tube, add 1.1 µl LPS (from 0.1 mg/ml working solution) for every sample well that will be assayed in the presence of LPS-stimulated macrophages.

This volume includes a 10 % excess. For example, for an assay with 12 sample wells, 13.2 μ l of LPS is required. It is important to vortex LPS solution well prior to pipetting.

- 8** To the tube containing LPS, add 550 μ l of the macrophage suspension for every sample well to be tested in the presence of stimulated macrophages.

This volume includes a 10% excess. For example, for an assay with 12 sample wells, approximately 6.6 ml macrophage suspension is required.

- 9** Mix the solution immediately using a 10-ml serological pipet and incubate 5–10 min at room temperature.

LPS must be evenly distributed very quickly. Careful mixing will minimize bubble or foam formation that could alter cell and LPS concentration.

- 10** Mix the cell suspension and transfer 500 μ l to wells containing MSC-conditioned medium or CCM. Distribute the macrophages by rocking the plate 3 times.

For best results and to prevent settling of the cells, mix the cell suspension regularly, after addition of macrophages to 5 or 6 wells. Each well now contains 100,000 macrophages in 100 ng/ml LPS with 1:50 dilution of MSC conditioned medium in total volume of 1 ml.

- 11** Incubate 16–18 h in a humidified cell culture incubator set to 37°C and 5% CO₂.

- 12** After 16–18 h, examine macrophage morphology to ensure the cells were properly activated (Figure 4B, C).

LPS-stimulated macrophages will appear larger, flatter, and more granular than the unstimulated cells.

- 13** Collect the medium conditioned by the treated macrophages into 1.5-ml tubes and centrifuge 5 min at 500 x g, room temperature to remove residual cells.

- 14** Transfer supernatant to new 1.5 ml tubes, aliquot, and store samples at –80°C for ELISAs.

Assays for macrophage cytokine production

- 15** Thaw samples on ice and vortex before use.

- 16** Dilute the macrophage conditioned medium samples 1:3 for mouse TNF α quantification by commercial ELISA kit. No dilution is needed for determination of mouse IL10 by commercial ELISA kit.

Follow the manufacturer's instructions for appropriate diluents and procedures.

ALTERNATIVE PROTOCOL 1: MEASUREMENT OF THE ANTI-INFLAMMATORY EFFECTS OF MSC SPHERES AND SPHERE-DERIVED CELLS USING A TRANSWELL MACROPHAGE SYSTEM

This protocol describes an alternative approach to measure the effects of sphere MSCs on inflammatory macrophage cultures (Bartosh et al., 2010; Ylostalo et al., 2012). The use of a transwell system here permits crosstalk of soluble factors between cell types.

For the assay MSCs, seeded in 6-well dishes are overlaid with a permeable transwell membrane containing stimulated macrophages. Here, use of the cells instead of conditioned medium allows more thorough assessment of the functional effects of the sphere-derived MSCs. However, this protocol is more time consuming and requires multiple investigators working simultaneously.

Materials

Costar 6-well tissue culture plates with 24 mm permeable transwell supports, pore size 0.4 μm

Sterile forceps

Macrophage medium (Reagents and Solutions)

Water bath set at 37°C

10- μl , 20- μl , 100- μl , 200- μl , 1000- μl pipette with sterile tips

Adherent monolayer MSCs (Support Protocol 1)

MSC spheres and single cells from spheres (Basic Protocol 2)

CCM (Reagents and Solutions)

Upright microscope with a 10x objective

J774 mouse macrophages (Support Protocol 2)

15-ml and 50-ml sterile conical tubes

0.1 mg/ml lipopolysaccharide (LPS) working solution

Vortex

Motorized pipettor with 10-ml serological sterile pipets

Humidified cell culture incubator set to 37°C and 5% CO_2

1.5-ml sterile microcentrifuge tubes

Microcentrifuge with rotor for 1.5-ml tubes and capability for 500 x g speed

Freezer that can reach temperatures below -70°C

Mouse TNF α Quantikine ELISA kit (R&D Systems, cat. no. MTA00B)

Mouse IL10 Quantikine ELISA kit (R&D Systems, cat. no. M1000).

MSC seeding

- 1 Obtain a sufficient number of 6-well plates containing 24 mm transwell® permeable inserts.
It is recommended that samples be assayed in triplicate.
- 2 Using sterile forceps, temporarily remove the inserts.
Keep the inserts in a biosafety cabinet to maintain sterility.
- 3 Add 2.4 ml macrophage medium to each well.
- 4 Suspend the adherent monolayer (Support Protocol 1), spheres (Basic Protocol 2), and sphere-derived MSCs (Basic Protocol 2) in CCM to a final concentration of 2×10^6 cells/ml.
The concentration of MSCs can be adjusted to change the MSC to macrophage ratio. We have routinely used an MSC to macrophage ratio of 1:1, 1:2, and 1:8.
- 5 Transfer 100 µl of the MSC cell suspension or CCM alone in to a well in triplicate. Immediately distribute the cells across the surface by moving the plate in a pattern resembling the number '8'.
Transfer 100 µl macrophage medium (without cells) to 3 wells to serve as the unstimulated macrophage control.
- 6 Incubate 15 min at room temperature.
This will allow the cells to settle to the bottom of the plate.

Preparation of transwell supports

- 7 Examine the plates with a microscope to ensure that most cells have adhered to the bottom.
- 8 Position the transwell inserts above the cells into the appropriate wells.
- 9 Overlay the transwell with 500 µl macrophage medium and distribute the solution completely across the surface.

Macrophage stimulation and co-culture

- 10 Suspend J774 macrophages (Support Protocol 2) to a concentration of 400,000 cells/ml in macrophage medium in a 50-ml conical tube.
- 11 Transfer 1 ml of the macrophage cell suspension to the top of the insert of the 3 wells void of MSCs or CCM (unstimulated macrophage control).
Distribute the macrophages across the transwell by rocking the plate 2–3 times.
- 12 In a separate 50-ml conical tube, add 4.4 µl LPS (from 0.1 mg/ml working solution) for every sample well that will be assayed in the presence of LPS-stimulated macrophages.

This volume includes a 10% excess. For example, an assay with 12 sample wells will require 52.8 μ l of LPS.

- 13 To the tube containing LPS, add 1.1 ml of the macrophage suspension for every sample well to be assayed. Mix immediately to disperse the LPS.
- 14 Incubate 5–10 min at room temperature.
- 15 Mix the cell suspension and transfer 1 ml to the upper part of the transwell insert above wells containing MSCs or CCM.

Distribute the macrophages evenly across the transwell by rocking the plate 2–3 times. Each well now contains 400,000 macrophages, 200,000 MSCs, and 100 ng/ml LPS in 4 ml of macrophage medium.

- 16 Incubate 16–18 h in a humidified cell culture incubator set to 37°C and 5% CO₂.
- 17 Collect the medium (1.5 ml) on top of the transwell and transfer into 1.5 ml-tubes.
- 18 Centrifuge 5 min at 500 x g, room temperature.
- 19 Transfer supernatant to new 1.5 ml-tubes, aliquot, and store at –80°C for ELISAs (Basic Protocol 6).

SUPPORT PROTOCOL 2: J774 MACROPHAGE CULTURE IN PETRI DISHES

This protocol describes the culture of mouse macrophages (J774) as adherent monolayers on non-tissue culture treated dishes (Ylostalo et al., 2012). The use of petri dishes permits harvest without enzyme solutions or cell scrapers.

Materials

Liquid nitrogen tank for cell storage

A frozen vial containing approximately 10⁶ J774 mouse macrophages

Water bath set at 37°C

15-ml and 50-ml sterile conical tubes

Pre-warmed macrophage medium (Reagents and Solutions)

Motorized pipettor with 10-ml serological sterile pipets

Centrifuge with swinging bucket rotor and adaptors for 15-ml and 50-ml conical tubes

Vacuum aspirator

150 mm × 15 mm petri dishes

Humidified cell culture incubator set to 37°C and 5% CO₂

Upright microscope with a 10x objective

10- μ l, 20- μ l, 100- μ l, 200- μ l, 1000- μ l pipette with sterile tips

1.5-ml sterile microcentrifuge tubes

Hemocytometer

1. Remove frozen cryovial of approximately 10^6 J774 mouse macrophages from liquid nitrogen tank and incubate 2 min at 37°C water bath.

To reduce the possibility of contamination, keep the vial O-ring and cap dry. More or less than 2 min might be required for complete thaw. Monitor carefully.
2. Transfer the contents of the cryovial to a 15-ml conical tube.
3. Add 9 ml of macrophage medium drop by drop and centrifuge 5 min at 200 x g, room temperature.
4. Aspirate the supernatant and carefully resuspend the cell pellet in 1 ml macrophage medium.
5. Transfer the cells to a 150 mm petri dish containing 30 ml macrophage medium and incubate 2–3 days in a humidified incubator set to 37°C and 5% CO₂.
6. Change the medium and check the confluency of the culture with a microscope.

Adherence of the macrophages onto the bacterial dish takes a considerable amount of time. Therefore, leave the cells undisturbed for 2–3 days. Even after this time period, it is common to see a small percentage of viable cells in the culture remain in suspension. These cells are typically removed by aspiration during medium change and thus do not affect the assay.
7. Incubate the macrophage culture in the incubator until it reaches 70–80% confluency.

Change the medium every 2–3 days.
8. To harvest the cells, aspirate the old culture medium, and add 10 ml fresh medium.
9. Remove the cells from the plate by spraying (with the pipettor) the culture medium onto the cells throughout the plate multiple times.

Do not use cell scrapers or enzymatic solutions, such as trypsin, for cell harvest, as these can promote cell death and non-specifically activate the cells. Avoid bubbles during the cell lifting and monitor the cell removal efficiency by tilting the plate towards light. Cell-free areas will appear clear.
10. Transfer the cells to a 50-ml conical tube and centrifuge 5 min at 200 x g, room temperature.
11. Aspirate the supernatant and suspend the cells in macrophage medium.
12. Count the cells using a hemocytometer.

REAGENTS AND SOLUTIONS

Complete culture medium (CCM)

Minimal Essential Medium alpha (α MEM, Life Technologies, cat. no. 12561) containing:

17% Fetal Bovine Serum-Premium Select (Atlanta Biologicals, cat. no. S11550), selected for optimal growth and differentiation of human MSCs

2 mM L-glutamine from 200 mM stock (Life Technologies, cat. no. 25030)

1x Penicillin-Streptomycin from 100x stock (Life Technologies, cat. no. 15140)

Sterile filter and store up to 1 month at 4°C

Macrophage medium

Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™-I (Life Technologies, cat. no. 10569) containing:

10% Fetal Bovine Serum-Premium Select (Atlanta Biologicals, cat. no. S11550)

1x Penicillin-Streptomycin from 100x stock (Life Technologies, cat. no. 15140)

Sterile filter and store up to 1 month at 4°C

COMMENTARY

Background Information

Stem cells residing in the bone marrow referred to as multipotent stromal cells, mesenchymal stem cells, or simply as MSCs, have drawn much interest recently as potential therapeutic adult stem cells (Prockop et al., 2010; Prockop and Oh., 2012; Ranganath et al., 2012; Keating., 2012). In addition to bone marrow, MSCs can also be isolated relatively easily from numerous other tissue sources, such as adipose tissue and umbilical cord (Keating., 2012; Prockop and Oh., 2012). Other factors that make MSCs suitable for therapy are the ease of expansion in adherent cultures and their apparent lack of tumorigenicity (Prockop et al., 2010; Prockop and Oh., 2012). Promising results in pre-clinical animal models and in human clinical trials have paved way for research in furthering the mechanistic understanding of the observed results and developing methods to prepare the best possible MSCs for therapy. Important part of the therapeutic effect of MSCs is their ability to get activated *in vivo* to secrete and express many anti-inflammatory, anti-cancer, and other potentially therapeutic molecules as engraftment and differentiation do not appear to be major contributing factors for therapeutic effects of MSCs (Prockop et al., 2010; Prockop and Oh., 2012; Ranganath et al., 2012; Keating., 2012). In addition, retention of infused MSCs *in vivo* is limited (Prockop et al., 2010; Prockop and Oh., 2012). Therefore, large numbers of cells are required for clinical applications to ensure that adequate amounts of beneficial molecules are secreted and expressed. One way to reduce the amount of cells required for therapy would be to pre-activate the cells to express the desired molecules before injection into patients.

Culture of cells in three dimension (3D) mimics cell-to-cell and cell-to-extracellular matrix interactions occurring *in vivo* better than traditional 2D adherent cultures in which cell-to-plastic interactions prevail (Achilli et al., 2012; Page et al., 2013). Closer contacts between cells in 3D permit activation of many signaling pathways more readily supporting the basis for normal cell function. Various methods have been developed to culture cells in 3D including pellet cultures, spinner cultures, non-adherent dishes, rotating wall vessels, cell sheets, micro-fluidics, and hanging drop cultures (Achilli et al., 2012; Page et al., 2013). When MSCs are cultured in conditions that do not permit adherence to tissue culture plastic the cells aggregate to form multicellular spheroids or spheres (Potapova et al., 2008a; Arufe et al., 2009; Frith et al., 2009; Wang et al., 2009a; Wang et al., 2009b; Bartosh et al., 2010; Saleh et al., 2011; Saleh and Genever., 2011; Jing and Jian-Xiong., 2011; Ylostalo et al., 2012; Baraniak and McDevitt., 2012). In most cases spheres of various sizes are formed but when MSCs are cultured in small volumes in non-adherent U- or V-shaped wells or in hanging drops, a single sphere is formed. Several studies have suggested enhanced therapeutic potential of MSC spheres over standard MSCs from 2D cultures making 3D cultures an important part of obtaining pre-activated MSCs for pre-clinical and clinical studies (Qihao et al., 2007; Potapova et al., 2008a; Potapova et al., 2008b; Xie et al., 2009; Frith et al., 2009; Wang et al., 2009a; Bartosh et al., 2010; Saleh and Genever., 2011; Ranganath et al., 2012; Ylostalo et al., 2012; Suzuki et al., 2012).

Hanging drop cultures of MSCs have been used to generate spheres with some advantages over other methods for sphere generation (Potapova et al., 2008a; Potapova et al., 2008b; Bartosh et al., 2010; Ylostalo et al., 2012). First, the size of the sphere is easily manipulated by changing the size of the drop or the cell concentration. Second, no specialized and expensive equipment is required for experimental and small animal model scale work as large amounts of spheres can be formed easily using multichannel pipettors and harvested conveniently by just scraping the culture dish lid and collecting the spheres with a pipette for centrifugation. If large scale production (i.e. for human clinical trial) of uniform size spheres is required, several automated instruments exist that can pipette accurately tens of drops per second while multiple parameters such as drop volume and cell concentration can be easily manipulated (Faulkner-Jones et al., 2013).

In hanging drop cultures, MSCs quickly aggregate and then the aggregates coalesce into a single central sphere in the apex of the drop (Bartosh et al., 2010; Ylostalo et al., 2012). Further compaction of the sphere occurs during the following days resulting in a sphere composed of small round cells embedded in matrix (Bartosh et al., 2010). During compaction, some of the MSCs undergo apoptosis that is a key driving force for the activation of the anti-inflammatory secretome in MSCs (Bartosh et al., 2010; Ylostalo et al., 2012). The activated MSCs can be used as intact spheres for *in vitro* studies or injected into cavities, such as peritoneum or joint spaces. Alternatively, the spheres can be dissociated into single cell suspension consisting of very small activated MSCs for intravenous injections.

Critical Parameters and troubleshooting

Hanging drop cultures of MSCs are mainly influenced by factors such as uninterrupted culture, proper airflow in the incubator and ensuring the same temperature of the reagents and plastics used. Maintaining these critical parameters will ensure proper aggregation and compaction of the cells into a single sphere by the end of the 3 day culture resulting in activated MSC. Most common problems and solutions encountered during the hanging drop cultures of MSCs and the potency assays can be found in Table 1.

For initiation of the hanging drop cultures MSCs should be of high viability and suspended in relatively high concentrations. Careful mixing of the cell suspension before pipetting the drops ensures formation of uniform sized spheres. Also, care must be taken when pipetting to maintain the surface tension within the drop to allow easier rotation of the plate for upside-down culture. During harvesting, when obtaining single cell suspension of sphere cells, careful mixing of the sphere/trypsin suspension approximately every 3 minutes, while monitoring the process, will result in good yields and viability.

For the assays of the anti-inflammatory MSCs, quality of the isolated RNA is of great importance in real-time PCR. For success of ELISA, samples must be stored in freezer, and freeze-thaw cycles should be minimized. For the functional assays, response of macrophages is dependent on the age of the cultures, thus low passage cells should be used when testing the potency of spheres or conditioned medium.

Anticipated Results

This method of generating anti-inflammatory MSCs using hanging drop culture is expected to generate a sphere of approximately 400–500 μm in diameter in each drop when making 35 μl drops of cells suspended at 714 cells/ μl . Approximately 30–50% of the initial amount of cells are routinely recovered from MSC spheres dissociated with trypsin/EDTA after 3 day culture in hanging drops. The viability of sphere-derived cells is approximately 80–90%. Incubating cells for longer than 3 days in hanging drops will decrease the cell viability and recovery by dissociation as cells have used most of the nutrients from the medium and medium will become acidic (Bartosh et al., 2010). Large increases in gene expression of anti-inflammatory molecules are detected in MSCs after 3 day hanging drop culture with TSG-6 expression increasing over 500-fold, while STC-1 expression increases generally over 10-fold and COX2 over 100-fold. The concentration of PGE2 after transfer culture is usually approximately 20–100 ng/ml.

In the macrophage assay, when the mouse macrophages are cultured at 100,000 cells/ml in macrophage medium and stimulated with 100 ng/ml of LPS, the expected concentration of TNF α after 18–24 hours is approximately 800–1200 pg/ml, whereas IL10 concentration is approximately 10–30 pg/ml. When the stimulated macrophages are incubated with 1:50 dilution of medium conditioned by transferred sphere or sphere-derived cells, the TNF α concentration drops to 200–400 pg/ml and IL10 concentration increases to 60–120 pg/ml.

Time Considerations

Expansion of MSCs from a frozen vial at a low density is usually done in 7 days which includes 2 medium changes (day 3 and day 6). Harvesting the MSC plates takes approximately 2 hours and generating the hanging drop cultures 1 hour. After 3 days of hanging drop culture, the spheres and conditioned medium can be harvested in approximately 1 hour. To generate sphere-derived cells, dissociation procedure takes approximately 1 hour. RNA isolation from spheres can be done in 2 hours followed by quantification of the RNA in 1 hour and cDNA generation in 4 hours. Setting up the real-time PCR takes approximately 1 hour per plate and the PCR run takes approximately 1 hour including basic data analysis. PGE2 ELISA can be done in 5 hours. Macrophage assay set up takes usually 2 hours and harvest after 18–24 hours another 2 hours. TNF α and IL10 ELISAs can be run in 6 hours. Extra time will be required for detailed data analysis of real-time PCR and ELISA data.

Acknowledgments

Supported in part by NIH grant P40RR17447 and CPRIT grant RP110553-P1.

LITERATURE CITED

- Achilli TM, Meyer J, Morgan JR. Advances in the formation, use and understanding of multi-cellular spheroids. *Expert Opin Biol Ther.* 2012; 12:1347–1360. [PubMed: 22784238]
- Arufe MC, De la Fuente A, Fuentes-Boquete I, De Toro FJ, Blanco FJ. Differentiation of synovial CD-105(+) human mesenchymal stem cells into chondrocyte-like cells through spheroid formation. *J Cell Biochem.* 2009; 108:145–155. [PubMed: 19544399]
- Baraniak PR, McDevitt TC. Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. *Cell Tissue Res.* 2012; 347:701–711. [PubMed: 21833761]
- Bartosh TJ, Ylostalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, Lee RH, Choi H, Prockop DJ. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc Natl Acad Sci U S A.* 2010; 107:13724–9. [PubMed: 20643923]
- Faulkner-Jones A, Greenhough S, King JA, Gardner J, Courtney A, Shu W. Development of a valve-based cell printer for the formation of human embryonic stem cell spheroid aggregates. *Biofabrication.* 2013; 5 015013-5082/5/1/015013. Epub 2013 Feb 4.
- Frith JE, Thomson B, Genever PG. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods.* 2009; 16:735–49. [PubMed: 19811095]
- Jing Y, Jian-Xiong Y. 3-D spheroid culture of bone marrow mesenchymal stem cell of rhesus monkey with improved multi-differentiation potential to epithelial progenitors and neuron in vitro. *Clin Experiment Ophthalmol.* 2011; 39:808–819. [PubMed: 22050567]
- Keating A. Mesenchymal stromal cells: new directions. *Cell Stem Cell.* 2012; 10:709–716. [PubMed: 22704511]
- Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol.* 2009; 27:451–483. [PubMed: 19105661]
- Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol.* 2011; 11:723–737. [PubMed: 21997792]
- Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med.* 2009; 15:42–9. [PubMed: 19098906]

- Ortega-Gomez A, Perretti M, Soehnlein O. Resolution of inflammation: an integrated view. *EMBO Mol Med*. 2013
- Page H, Flood P, Reynaud EG. Three-dimensional tissue cultures: current trends and beyond. *Cell Tissue Res*. 2013; 352:123–131. [PubMed: 22729488]
- Potapova IA, Brink PR, Cohen IS, Doronin SV. Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells. *J Biol Chem*. 2008a; 283:13100–7. [PubMed: 18334485]
- Potapova IA, Doronin SV, Kelly DJ, Rosen AB, Schuldt AJ, Lu Z, Kochupura PV, Robinson RB, Rosen MR, Brink PR, Gaudette GR, Cohen IS. Enhanced recovery of mechanical function in the canine heart by seeding an extracellular matrix patch with mesenchymal stem cells committed to a cardiac lineage. *Am J Physiol Heart Circ Physiol*. 2008b; 295:H2257–63. [PubMed: 18835924]
- Prockop DJ, Kota DJ, Bazhanov N, Reger RL. Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). *J Cell Mol Med*. 2010; 14:2190–2199. [PubMed: 20716123]
- Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Ther*. 2012; 20:14–20. [PubMed: 22008910]
- Qihao Z, Xigu C, Guanghui C, Weiwei Z. Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells. *DNA Cell Biol*. 2007; 26:497–503. [PubMed: 17630854]
- Ranganath SH, Levy O, Inamdar MS, Karp JM. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell*. 2012; 10:244–258. [PubMed: 22385653]
- Saleh FA, Genever PG. Turning round: multipotent stromal cells, a three-dimensional revolution? *Cytotherapy*. 2011; 13:903–912. [PubMed: 21696237]
- Saleh FA, Whyte M, Genever PG. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *Eur Cell Mater*. 2011; 22:242–57. discussion 257. [PubMed: 22012735]
- Suzuki S, Muneta T, Tsuji K, Ichinose S, Makino H, Umezawa A, Sekiya I. Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. *Arthritis Res Ther*. 2012; 14:R136. [PubMed: 22676383]
- Wang CC, Chen CH, Hwang SM, Lin WW, Huang CH, Lee WY, Chang Y, Sung HW. Spherically symmetric mesenchymal stromal cell bodies inherent with endogenous extracellular matrices for cellular cardiomyoplasty. *Stem Cells*. 2009a; 27:724–32. [PubMed: 19259939]
- Wang W, Itaka K, Ohba S, Nishiyama N, Chung UI, Yamasaki Y, Kataoka K. 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials*. 2009b; 30:2705–15. [PubMed: 19215979]
- Xie QP, Huang H, Xu B, Dong X, Gao SL, Zhang B, Wu YL. Human bone marrow mesenchymal stem cells differentiate into insulin-producing cells upon microenvironmental manipulation in vitro. *Differentiation*. 2009; 77:483–491. [PubMed: 19505629]
- Ylostalo JH, Bartosh TJ, Coble K, Prockop DJ. Human Mesenchymal Stem/Stromal Cells Cultured as Spheroids are Self-activated to Produce Prostaglandin E2 that Directs Stimulated Macrophages into an Anti-inflammatory Phenotype. *Stem Cells*. 2012; 30:2283–2296. [PubMed: 22865689]

Figure 1. Preparation of hanging drop cultures with human MSCs

Phase-contrast microscopy of (A) passage 1 MSCs recovered from frozen cryovial and (B) ready-to-use MSC cultures seeded at 100 cells/cm² and expanded for 7 days. To generate hanging drop cultures, (C, D) pipette 35- μ l droplets of CCM containing MSCs onto the underside of a lid to a 150-mm or equivalent culture dish, then (E) flip the lid back to its original position on top of the dish containing PBS.

Figure 2. Harvesting spheres from hanging drop cultures

(A) Hanging drops containing MSCs cultured for 3 days are shown as single layers in a 37°C incubator. (B) With the underside of the dish lid facing upward and slightly angled, use a cell lifter to force the droplets containing spheres to the edge. (C) Spheres assembled from 25,000 cells can be easily visualized during the collection process (arrows). (D) Magnified view of a single sphere from the dish. Scale bar, 100 μ m. (E, F) Images of approximately 500–1,000 spheres transferred to a conical tube. The majority of spheres descend rapidly to the bottom of tube. For optimal yields during PBS wash steps, centrifugation is important.

Figure 3. Analysis of sphere transfer cultures

Representative images of (A) monolayer-derived MSCs, (B) intact spheres, and (C) sphere-derived cells 24 h after transfer into tissue culture treated 6-well plates. Sphere-derived MSCs are considerably smaller than MSCs from monolayer cultures. (D) Spheres transferred for 24 h to non-adherent plates remain in suspension and tend to fuse together. Scale bar, 100 μm .

Figure 4. Macrophage assay

(A) Plate set up for macrophage assay using MSC-conditioned medium. To prepare the 12-well assay plate, macrophage medium (M ϕ medium) and CCM (vehicle control) or conditioned medium (CM) from spheres (Sphere CM) or adherent monolayer cultures (2D MSC CM) are first added to the wells as shown. Then, 500 μ l of macrophage suspension is added to each well. Unstimulated macrophages are transferred to the wells shown in blue (M ϕ control) while lipopolysaccharide-stimulated macrophages are transferred into the wells shown in red. The final volume per well is 1.0 ml. For simplicity, wells containing CM from sphere-derived cells have been omitted. Representative images of unstimulated (B) and stimulated (C) macrophages after 16–18 h culture.

Table 1

Troubleshooting guide for common problems faced during MSC hanging drop culture and macrophage assay.

Problem	Possible cause	Solution
Poor sphere formation	Surface tension was lost	Dispense the cells carefully to maintain the surface tension
	Drops were smeared during plate flipping	Flip the plate in one continuous motion while raising your hands to ensure equal speed throughout the plate lid
Poor yield/viability of cells dissociated from spheres	Plate or the incubator was bumped during culture	Make sure the culture stays uninterrupted
	Poor sphere harvest	Harvest all the spheres carefully
	Loss of spheres/cells during centrifugation	Check the centrifuge speed and aspirate the supernatant carefully while leaving the cells in small amount of liquid
	Incomplete dissociation	Make sure the spheres are mixed every 3 minutes and are completely dissociated (no visible aggregates) before stopping
	Too long in trypsin/EDTA	Shorten the trypsin/EDTA incubation time and monitor carefully
Poor yield of macrophages	Incomplete harvest of cells from the plate	Harvest all the cells from the plate by dispersing liquid forcefully onto the culture
Poor response of the macrophage cultures to LPS	Cells harvested with too much mechanical force	Harvest the cells with limited number of forceful dispersions
	Old culture of cell	Use earlier passage macrophages
	LPS is ineffective	Check the LPS stock date and endotoxin level and remake the solution
	Reagents contain endotoxins	Check the endotoxin levels of the reagents and prepare new reagents