TECHNICAL MANUAL
VERSION 1.2.0

Enumeration and Expansion of Mouse Mesenchymal Progenitor Cells Using MesenCult®

STEMCELL TECHNOLOGIES

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1.0 Introduction

Isolation and characterization of mouse mesenchymal stem cells

The bone marrow stroma was originally thought to function mainly as a structural framework for hematopoietic stem and progenitor cells in the bone marrow. It has now been established that the stroma consists of a heterogeneous population of cells including endothelial cells, fibroblasts, adipocytes, and osteogenic cells, a subset of which exerts both positive and negative regulatory effects on the proliferation and differentiation of hematopoietic cells.[1,2] The adherent stromal cell population is also believed to contain other non-hematopoietic cells that are capable of both self-renewal and differentiation into bone, cartilage, muscle, tendon, and fat.[3-5] These primitive progenitor cells are often referred to as mesenchymal stem cells (MSCs) and are distinct from hematopoietic stem cells. Characterization of mesenchymal stem cells has included morphological as well as cytochemical characterization of the cultured cells.

The colony-forming unit - fibroblast (CFU-F) assay is used by many investigators as a functional method to quantify mesenchymal stem and progenitor cells. Enrichment of mouse CFU-F has been best described by Short and Simmons who identified the compact bone (femur and tibia) itself as a richer source of these progenitor cells than the marrow plug within it.[6] Using a number of physical and enzymatic treatments of the bone to generate a single cell suspension, followed by depletion of cells expressing the following lineage (Lin) antigens: CD3, CD4, CD5, CD8, CD11b, and GR1, Short *et al.* significantly enriched for CFU-F. Further cell sorting experiments using flow cytometry identified the stromal (mesenchymal) mouse precursor as $Lin^-CD45^-CD31^-SCA1^+$.[7]

Mouse mesenchymal progenitor cells are more difficult to isolate and culture from bone marrow (BM) than their human equivalents. One factor contributing to the difficulties in isolating relatively homogenous mesenchymal progenitor cells is their extremely low frequency. The progenitor cell frequency in mouse BM (as quantified by the CFU-F assay) has been estimated to be only 0.001 - 0.0001% (1 in 100,000 - 1 in 1,000,000).[8] The extreme rarity of the mesenchymal progenitor cells in mouse bone marrow is further complicated by frequent contamination with hematopoietic cells which persist in culture after several passages.[8,9] Furthermore, lack of specific markers unique to mouse mesenchymal stem cells has made it difficult to either characterize or positively select specific functional cell populations. Many studies have focused on describing the characteristics of culture-expanded mesenchymal cells.[10-12]

The effect of oxygen concentration on MSC culture

Mouse mesenchymal stem cells have been shown to be highly sensitive to atmospheric oxygen tension as culturing cells under hypoxic conditions facilitates optimal clonogenicity and cell proliferation (Brenton Short, unpublished data). Many other cell types have also been shown to have enhanced proliferation when cultured with lowered atmospheric oxygen tension, including rat central nervous system (CNS) stem cells, adult mouse skeletal muscle satellite cells, human $CD34^+$ bone marrow progenitor stem cells and rat mesenchymal progenitor cells.[13-17] It has been suggested that the increased proliferation of rat bone marrow-derived mesenchymal progenitor cells that occurs at 5% oxygen is due to increased expression of hypoxia inducible factor (HIF), which in turn upregulates genes involved in metabolism, cell proliferation and survival.[18] Low oxygen conditions appears to be a critical factor in the expansion of mouse mesenchymal cells in culture.

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2.0 Materials

2.1 Product Description

MesenCult® products for mouse mesenchymal stem cell research include:

PRODUCT DESCRIPTION	VOLUME	CATALOG #
MesenCult® MSC Basal Medium (Mouse)	400 mL	05501
Mesenchymal Stem Cell Stimulatory Supplements (Mouse)	100 mL	05502
MesenCult® Proliferation Kit (Mouse), containing Catalog #05501 and 05502	500 mL	05511

2.2 Storage Conditions

MesenCult® MSC Basal Medium (Mouse) should be stored at 2 - 8°C and is stable until expiry date as indicated on the label.

Mesenchymal Stem Cell Stimulatory Supplements (Mouse) should be stored at -20°C and are stable until expiry date as indicated on the label. Storage at 2 - 8°C is not recommended. Mesenchymal Stem Cell Stimulatory Supplements (Mouse) can be thawed, aliquoted into smaller volumes and refrozen to facilitate the preparation of small volumes of Complete MesenCult® Medium (Mouse). Do not freeze-thaw more than twice. Do not thaw at a temperature greater than 37°C; thawing at 2 - 8°C is recommended.

Once the Mesenchymal Stem Cell Stimulatory Supplements (Mouse) have been added to the MesenCult® MSC Basal Medium (Mouse), store at 2 - 8°C for up to 1 month. Long-term storage at 2 - 8°C is not recommended. Refer to Section 3.0 for preparation of Complete MesenCult® Medium (Mouse).

2.3 Equipment and Supplies Required

- Biohazard safety cabinet certified for level II handling of biological materials (e.g. Canadian Cabinets)
- 37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO_2 in air (e.g. Forma 3326)
- Inverted microscope
- Routine light microscope for hemacytometer cell counts
- Hemacytometer
- 6-well tissue culture-treated plates (Falcon Catalog #353502 or Corning Catalog #3506)
  Cells can also be grown in tissue culture-treated 35 mm dishes (Corning Catalog #430165) or T-25 cm^2 flasks (Falcon Catalog #353109). Always use tissue culture-treated plates, dishes or flasks for mesenchymal cell culture.
- 1 mL and 10 mL sterile pipettes
- Pipette-aid (e.g. Drummond Scientific)
- 21g or 23g needles (BD Catalog #305167 and #305145)
- 3 mL syringes
- 70 µm cell strainer (Falcon Catalog #352350)
- 50 mL conical polypropylene tubes (Fisher Catalog #05-539-8)
- 14 mL conical polypropylene tubes (Falcon Catalog #352001)
- Sterile scissors and forceps
- Scalpel
- Mortar and pestle and 100 mm petri dish (if using Bone Crushing procedure)
- Hypoxia Chamber (Catalog #27310)

2.4 Additional Reagents Required

- Alcohol wipes
- 70% Isopropanol
- PBS with 2% Fetal Bovine Serum (Catalog #07905)
- IMDM with 2% Fetal Bovine Serum (Catalog #07700)
- Trypsin-EDTA (Catalog #07901)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Trypan Blue (Catalog #07050)
- 0.25% Type I Collagenase in PBS with 20% FBS (Catalog #07902)
- Dispase (5mg/mL; Catalog #07913)
- FBS (Catalog #06902 or quality cell culture-tested equivalent)

The following materials are required to stain CFU-F colonies:

- PBS (Catalog #37350)
- Methanol ACS (BDH Catalog #ACS531)
- Giemsa Staining Solution (EMD Chemicals Catalog #R03055)
- Distilled water

3.0 Preparation of Complete MesenCult® Medium (Mouse)

Mesenchymal Stem Cell Stimulatory Supplements (Mouse) should be thawed overnight under refrigeration (at 2 - 8°C) or for several hours at room temperature (15 - 25°C) prior to usage.

To prepare Complete MesenCult® Medium (Mouse), thaw one bottle of Mesenchymal Stem Cell Stimulatory Supplements (Mouse) as described. Once thawed, mix well and add the entire volume to one bottle of MesenCult® MSC Basal Medium (Mouse) (1/5 dilution). Thoroughly mix Complete MesenCult® Medium (Mouse).

Complete MesenCult® Medium (Mouse) should be prepared in volumes that can be used within 1 month (refer to Section 2.2 for storage information). If less than 500 mL will be required, smaller volumes can be prepared. Prepare Complete MesenCult® Medium (Mouse) by diluting Mesenchymal Stem Cell Stimulatory Supplements (Mouse) 1/5 with MesenCult® MSC Basal Medium (Mouse). For example, prepare 100 mL of Complete MesenCult® Medium (Mouse) by adding 20 mL of Mesenchymal Stem Cell Stimulatory Supplements (Mouse) to 80 mL of MesenCult® MSC Basal Medium (Mouse).

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4.0 Mouse Colony-Forming Unit - Fibroblast (CFU-F) Assay

4.1 Isolation of Cells for CFU-F Assay

4.1.1 Isolation of Cells from Marrow Plug: Marrow Flushing

Note: Mice should be sacrificed using procedures approved by your institution.

1. After sacrificing the mouse, wet the pelt thoroughly with 70% isopropanol, then clip and peel it back to expose the hind limbs. Using sterile sharp scissors (to avoid splitting the bone), cut the knee joint in the center and remove ligaments and excess tissue.
2. Remove the femur and tibia by severing them from the mouse at the hip and ankle, respectively. Remove muscle, ligaments and excess tissue from bone.
3. Trim the ends of the bones to expose the interior of the marrow shaft.
4. Collect the marrow cells in 1 - 2 mL of IMDM with 2% FBS (Catalog #07700). Flush the marrow from the femoral shaft using a 21g needle attached to a 3 mL syringe. A smaller needle (23g) may be required to flush marrow from the tibia. Use the same medium to flush bones from 1 - 3 animals.
5. Make a single cell suspension by gently drawing medium and cells up and down several times using the same needle and syringe.
6. Keep the cells on ice. For most applications, it is not necessary to purify or wash the cells.
7. Remove a small aliquot of cells and dilute 1/50 to 1/100 in 3% Acetic Acid with Methylene Blue (Catalog #07060). Count nucleated cells using a hemacytometer.

   Expected cell recovery: $1.0 - 2.0 \times 10^7$ cells per femur
   6.0×10^6 cells per tibia
   $3.0 - 5.0 \times 10^7$ cells per mouse (2 femurs and 2 tibias)

4.1.2 Isolation of Cells From Compact Bones

A cell population derived from compact bone tissue in the adult mouse has been reported to be highly enriched for CFU-F. In the adult mouse, the CFU-F frequency in the compact bone is significantly higher than the marrow plug,[6] indicating the site of the major reservoir of CFU-F is the surrounding bone tissue rather than the marrow itself. To isolate cells by crushing compact bones (femur and tibia), the following procedure is recommended:

1. Clean a mortar and pestle with 70% isopropanol. Remove the isopropanol from the mortar and pestle, and allow to air dry in a sterile biohazard safety cabinet for 30 minutes. Rinse mortar and pestle with sterile phosphate buffered saline (PBS) just prior to use.
2. Wipe the instruments (scissors and forceps) with an alcohol wipe and air dry. Ensure instruments are completely dry as residual alcohol may reduce marrow cell viability.
3. After sacrificing the mouse, wet the pelt thoroughly with 70% isopropanol, then clip and peel it back to expose the hind limbs. Using sterile sharp scissors (to avoid splitting of the bone), cut the knee joint in the center and remove ligaments and excess tissue.
4. Remove the femur and tibia by severing them from the mouse at the hip and ankle, respectively.
5. Using a scalpel, scrape bones thoroughly to remove muscle, and cut to remove epiphyses. Ensure that the bones are cleaned thoroughly, with no remaining muscle tissue attached.
6. Place clean bones in the mortar containing 10 mL PBS with 2% FBS (Catalog #07905) and 1 mM EDTA. The solution of PBS with 2% FBS and 1 mM EDTA is now referred to as 'Buffer'.

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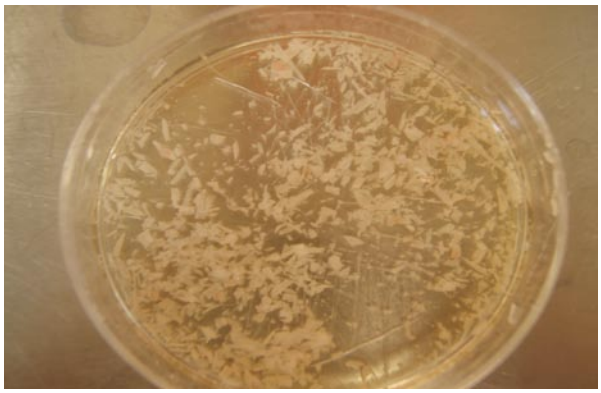
7. Crush bones with pestle, using only enough force to crack open the bones. Agitate gently to free bone marrow (BM) from bone fragments and pipette Buffer off. Buffer containing BM can be filtered through a 70 µm cell strainer (Falcon Catalog #352350) and used for other applications (for example, perform the CFU-F assay as recommended in point 7 of Section 4.1.1 or for expansion as recommended in Section 4.2.1).
8. Add 10 mL fresh Buffer and repeat agitation and removal of BM. Repeat wash step an additional 4 times (for a total of 6 washes) or until the majority of the BM has been removed (bone fragments will turn white in color).

   A loss of cell viability and excess debris will be generated when bones are harshly ground. It is important to only use gentle pressure to crack open the bones. Bone fragments must only be crushed to the point of removal of bone marrow. Once bone marrow is removed, fragments will be white in color.
9. Transfer the bone fragments to a 100 mm dish. Add 2 mL of 0.25% Collagenase Type 1 in PBS containing 20% FBS (Catalog #07902). Ensure bones are completely covered in solution. Let sit for 3 - 5 minutes.

   This step softens the bone allowing it to be chopped more easily.

10. Using a scalpel, chop the remaining bone fragments into fine pieces (1 - 2 mm fragments).

    Proper bone fragmentation is required to release a sufficient number of cells for cell separation.



11. Transfer the bone fragments and collagenase solution to a 50 mL polypropylene tube and add further 0.25% Collagenase Type I with 20% FBS (Catalog #07902) to a final volume of 2 mL per mouse used, or a minimum of 10 mL.
12. Seal lid with Parafilm® and place tube in a shaking 37°C waterbath at maximum speed for 45 minutes. If using a bacterial culture shaker, set speed to ~200 rpm.

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13. After 45 minutes, remove the tube from the shaker and add Buffer (refer to Step 6) to a final volume of 30 mL. Collect supernatant and filter through a 70 µm cell strainer (Falcon Catalog #352350). Wash bone fragments by mixing with an additional 10 mL of Buffer and allowing fragments to settle for 3 - 4 minutes. Filter the wash through the 70 µm strainer, combining with the previously collected cells (for a final volume of 40 mL).
14. Centrifuge at 300 x *g* (~1200 rpm) for 10 minutes at room temperature (15 - 25°C) with the brake on. Remove supernatant and resuspend cell pellet in ~100 - 250 µL medium (note: small particles and debris may be visible in the cell suspension):
    a. For cell culture, resuspend cells in Complete MesenCult® Medium (Mouse). Refer to Sections 4.2.2. and 5.2 for more information.
    b. For cell separation experiments, resuspend cells in the medium recommended by the desired cell separation protocol (e.g. the EasySep® Mouse Mesenchymal Progenitor Cell Enriched Kit (Catalog #19771) requires that cells be resuspended in PBS with 2% FBS).
15. Place cells on ice until ready for use.
16. Remove a small aliquot of cells and dilute 1/50 to 1/100 in 3% Acetic Acid with Methylene Blue (Catalog #07060). Count nucleated cells using a hemacytometer.
17. Expected cell recovery: 1.5 - 3.5 x 10^6 cells per mouse (2 femurs and 2 tibias). If cell yield is > 5 x 10^6 cells/mouse, this is an indication that the marrow was not sufficiently depleted.

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4.2 Mouse CFU-F Assay Procedure

4.2.1 CFU-F Assay for Cells Isolated from Bone Marrow

To ensure sufficient colony numbers to accurately assess progenitor cell content of mouse bone marrow and/or compact bone, mouse CFU-F assays should be initiated at 2 different cell concentrations in a 6-well dish:

- 5.0 x 10^5 cells/well
- 1.0 x 10^6 cells/well

1. Prepare Complete MesenCult® Medium (Mouse) as described in Section 3.0.
2. Perform a nucleated cell count using 3% Acetic Acid with Methylene Blue (Catalog #07060). Dilute cells with Complete MesenCult® Medium (Mouse) to obtain a stock cell concentration of 1.0 x 10^7 cells/mL.
3. For duplicate assays in 6-well plates (Falcon Catalog #353502 or Corning Catalog #3506), prepare tubes of the cell suspension as follows:
   - Prepare to plate 5.0 x 10^5 cells/well by adding 100 µL of cells at 1.0 x 10^7 cells/mL (prepared in Step 2) to 3.9 mL Complete MesenCult® Medium (Mouse), to obtain 4.0 mL of cells at 2.5 x 10^5 cells/mL.
   - Prepare to plate 1.0 x 10^6 cells/well by adding 200 µL of cells at 1.0 x 10^7 cells/mL (prepared in Step 2) to 3.8 mL of Complete MesenCult® Medium (Mouse), to obtain 4.0 mL of cells at 5.0 x 10^5 cells/mL.
4. Gently vortex the tubes (medium setting for 5 seconds) and then plate 2 mL of each dilution into each of 2 wells of a 6-well plate (Falcon Catalog #353502 or Corning Catalog #3506). Each dilution should be plated in duplicate (2 mL/well, 2 wells/dilution).

   Cells must be plated in tissue culture-treated plates.
5. Culture cells for 10 days at 37°C in 5% CO_2. A medium change may not be required, but if the medium becomes yellow/orange in color, perform a half-medium change by gently removing approximately half the medium from the culture dish without disturbing the cells, and replacing with an equal volume of Complete MesenCult® Medium (Mouse) prewarmed to 37°C.

Table 1: CFU-F-derived colony size and CFU-F frequency in 5% and 20% oxygen from bone marrow

CFU-F/10^6 CELLS		CFU-F SIZE (mm)	
Mean ± SD; n = 3		Average diameter ± SD; n = 3 (Range)	
5% Oxygen	20% Oxygen	5% Oxygen	20% Oxygen
11.72 ± 3.53	3.05 ± 1.86	1.83 ± 0.64 (1 - 3.5)	1.63 ± 0.43 (1 - 2.5)

4.2.2 CFU-F Assay for Cells Isolated from Compact Bone

1. Mouse CFU-F assays performed using cells obtained from compact bone should be initiated at 3 different cell concentrations, to ensure sufficient colony numbers to accurately assess progenitor cell content. Plate cells in 2 mL of Complete MesenCult® Medium (Mouse) in a 6-well plate as follows:

   a. For cells from compact bone prepared according to the protocol described in Section 4.1.2 and grown in a hypoxic environment (5% O_2, 10% CO_2, 85% N_2 in a Hypoxia Chamber, Catalog #27310) plate 1,000, 5,000 and 10,000 cells/cm^2 in duplicate (e.g. plate 10,000, 50,000 and 100,000 cells/well).

   b. For cells from compact bone prepared according to the protocol described in Section 4.1.2 and grown in a normal environment (20% O_2, 5% CO_2) plate at 10,000, 20,000, and 40,000 cells/cm^2 in duplicate (e.g. plate 100,000, 200,000 and 400,000 cells/well).

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c. For enriched compact bone cells (e.g. enriched for mesenchymal progenitor cells using the EasySep® Mesenchymal Progenitor Enrichment Kit, Catalog #19771) grown in a hypoxic environment (5% O_2, 10% CO_2, 85% N_2 in a Hypoxia Chamber, Catalog #27310) plate 50 - 250 cells/cm^2 in duplicate (e.g. plate 500 - 2,500 cells/well)

d. For enriched compact bone cells (e.g. enriched for mesenchymal progenitor cells using the EasySep® Mesenchymal Progenitor Enrichment Kit, Catalog #19771) grown in a normal environment (20% O_2, 5% CO_2) plate at 200 - 1,000 cells per cm^2 in duplicate (e.g. plate 2,000 - 10,000 cells/well).

2. Culture cells for 13 days. No medium change is required.

   Cells from compact bone grown in the CFU-F assay occasionally undergo spontaneous differentiation to form adipocytic or chondrogenic cells. For additional information, please contact STEMCELL Technologies' Technical Support (techsupport@stemcell.com).

4.2.3 CFU-F Assay for Cells Isolated from Fetal Liver

1. Prepare Complete MesenCult® Medium (Mouse) as described in Section 3.0.
2. Prepare mouse fetal liver cells according to your usual laboratory procedures, or refer to the Technical Manual for Mouse Colony-Forming Cell Assays Using MethoCult® (Catalog #28405, Section 4.3.4).
3. Dilute mouse fetal liver cells to 4.0 x 10^6 cells/mL (stock concentration).
4. To set up duplicate cultures at 2.0 x 10^5 cells/well, add 100 µL of cells at 4.0 x 10^6 cells/mL to 3.9 mL of Complete MesenCult® Medium (Mouse) to obtain 4.0 mL of cells at 1.0 x 10^5 cells/mL.
5. Gently vortex the tube (medium setting for 5 seconds) and then plate 2 mL into each of 2 wells of a 6-well plate (Falcon Catalog #353502 or Corning Catalog #3506).

   Cells must be plated in tissue culture-treated plates.

6. Culture cells for 10 days at 37°C in 5% CO_2. A medium change may not be required, but if the medium becomes yellow/orange in color, perform a half-medium change by gently removing approximately half the medium from the culture dish without disturbing the cells, and replacing with an equal volume of Complete MesenCult® Medium (Mouse) prewarmed to 37°C.

4.3 Staining and Enumeration of Mouse CFU-F-Derived Colonies

1. After 10 - 13 days in culture, remove medium from the culture plate by decanting into a waste container containing bleach. The adherent colonies will remain attached to the plate. Gently wash each well twice with PBS (Catalog #37350) to ensure that residual FBS is removed. Allow to air dry for 5 minutes.
2. Add enough methanol to cover each well and incubate at room temperature (15 - 25°C) for 5 minutes. Decant methanol and allow to air dry for 5 minutes.
3. Add enough Giemsa Staining Solution (EMD Chemicals Catalog #R03055) to completely cover the well and incubate at room temperature (15 - 25°C) for 5 minutes.
4. Remove Giemsa Staining Solution. Wash gently with water to remove non-bound stain. Rinse until water remains clear.
5. Discard the water and allow to air dry. Count colonies microscopically. Examples of CFU-F-derived colonies are provided in Sections 4.4 and 4.5. CFU-F-derived colonies from compact bone (bone crushing) may contain debris.

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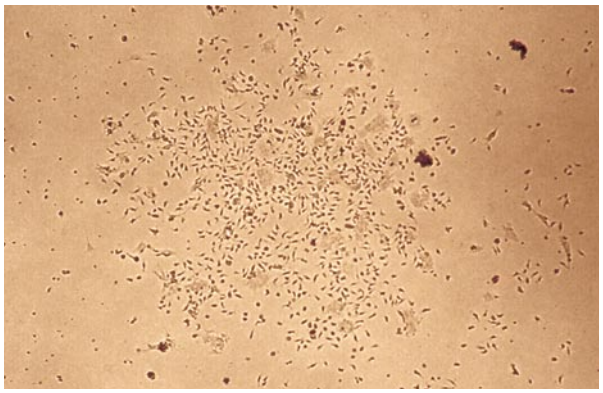
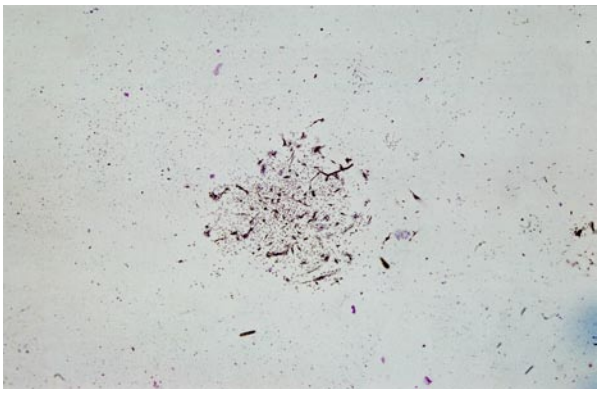
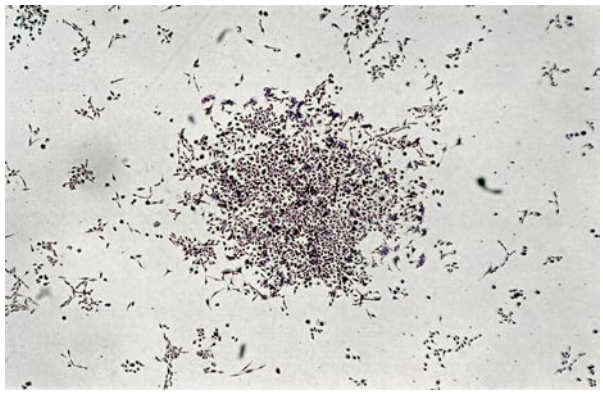
4.4 Examples of Mouse CFU-F-Derived Colonies from Marrow Plug Cultured with Complete MesenCult® Medium (Mouse)



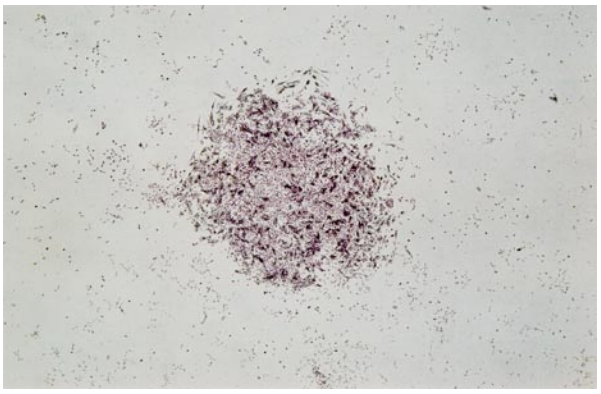
Colony before staining.



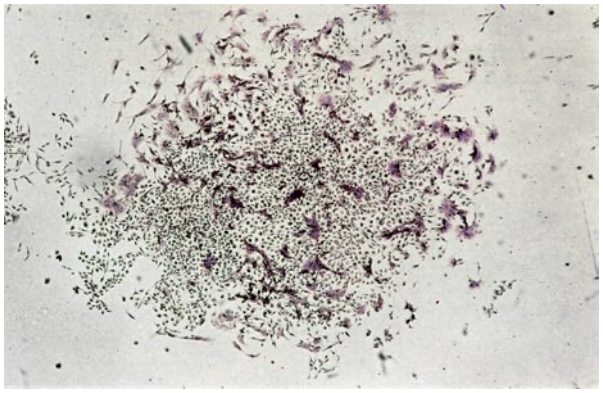
Small colony stained with Giemsa.



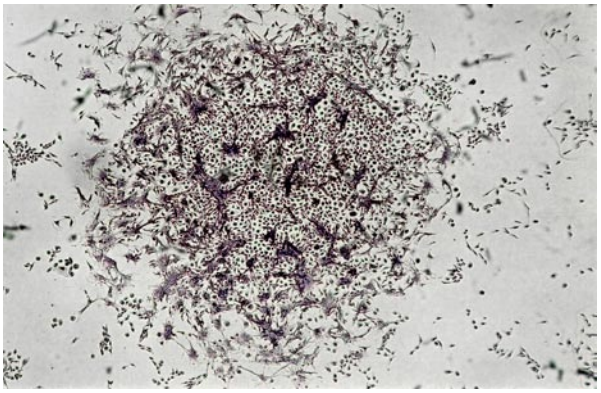
Medium colony stained with Giemsa.



Medium colony stained with Giemsa.



Large colony stained with Giemsa.



Large colony stained with Giemsa.

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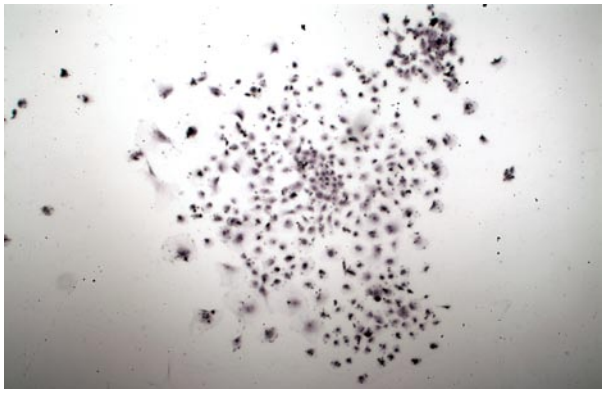
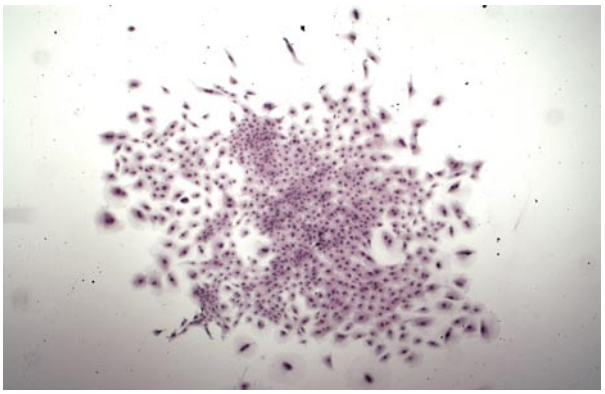
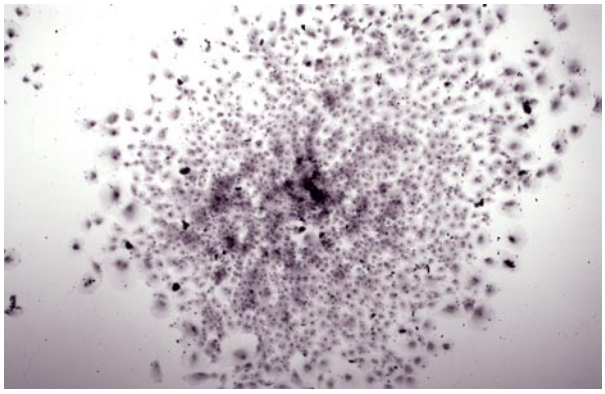
4.5 Examples of Mouse CFU-F-Derived Colonies from Compact Bone Cultured with Complete MesenCult® Medium (Mouse)



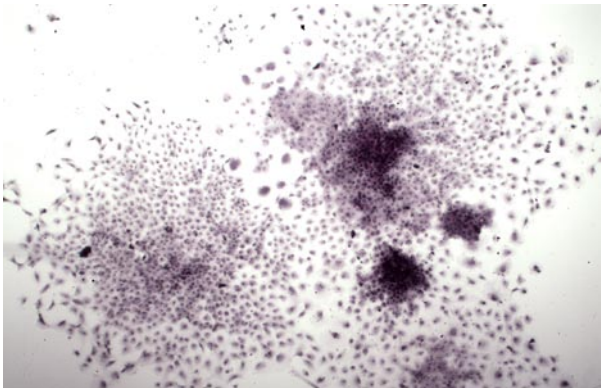
Small colony stained with Giemsa.



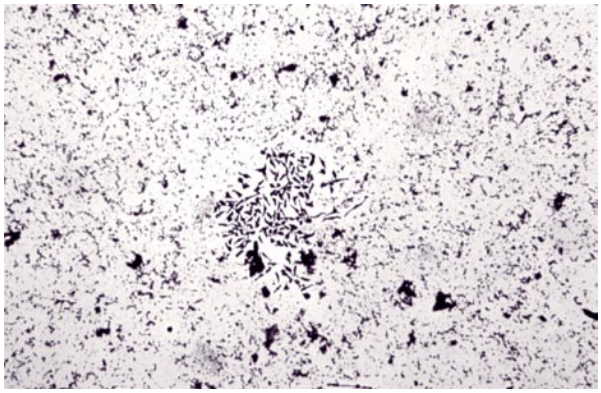
Medium colony stained with Giemsa.



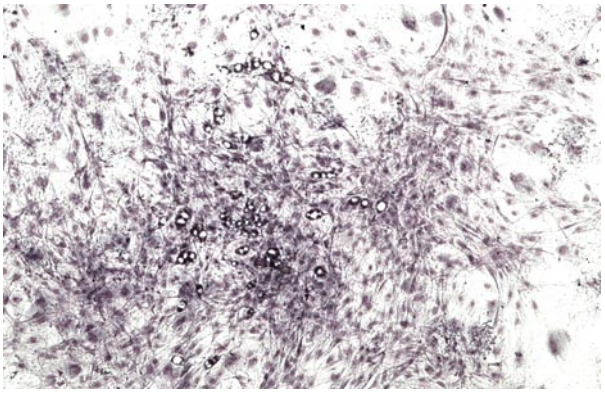
Large colony stained with Giemsa.



Two medium colonies stained with Giemsa. Note that the morphology can be different between colonies.



Debris from bone crushing can interfere with colony identification as the cells are difficult to distinguish from background.



Spontaneous differentiation of compact bone-derived mesenchymal cells into adipocytes can occur at a low frequency.

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5.0 Expansion of Mouse Mesenchymal Stem Cells

5.1 Expansion Protocol for Cells Isolated from Bone Marrow or Fetal Liver

1. Harvest mouse bone marrow cells as described in Section 4.1.1 or obtain mouse fetal liver cells.
2. Plate unprocessed bone marrow or fetal liver cells as follows:
   - 7.5 - 10 x 10^6 cells in 2 mL Complete MesenCult® Medium (Mouse) in 1 well of a 6-well plate (Falcon Catalog #353502 or Corning Catalog #3506) or one 35 mm dish (Corning Catalog #430165)
   - 2.5 x 10^7 cells in 10 mL Complete MesenCult® Medium (Mouse) in a T-25 cm^2 flask (Falcon Catalog #353109).

   Cells must be plated using tissue culture-treated plates, dishes or flasks.
3. Grow cells at 37°C in 5% CO_2 for 1 - 4 weeks until an adherent layer has formed. At weekly intervals, half-medium changes of Complete MesenCult® Medium (Mouse) are recommended. To perform a half-medium change, gently remove approximately half of the medium from the culture dish without disturbing the cells, and replace with an equal volume of Complete MesenCult® Medium (Mouse) prewarmed to 37°C.
4. Observe mesenchymal cells under the microscope at least once a week to determine confluency. When the cells have reached ~80% confluency, they are ready to be passaged.

   80% confluency may be reached in 7 - 28 days.
5. Once cells are 80% confluent, remove MesenCult® medium by decanting or aspirating. The adherent mesenchymal cells will remain attached. Wash the bottom of the flask with PBS (Catalog #37350) to remove residual FBS. Remove the PBS.
6. Add enough Trypsin-EDTA (Catalog #07901) to cover cells and incubate at 37°C for 7 minutes (e.g. add 1 mL of Trypsin to 1 well of a 6-well plate or 35 mm dish, or 4 mL to a T-25 cm^2 flask). Observe under the microscope to ensure that the mesenchymal cells have detached. Add FBS (Catalog #06902 or quality cell culture-tested equivalent) to a final concentration of 20% (v/v) to neutralize the action of the Trypsin (e.g. add 250 µL to 1 well of a 6-well plate or 35 mm dish, or 1 mL to a T-25 cm^2 flask). If any cells are still attached, tap the flask on the surface of the bench/hood to detach cells.
7. Collect trypsinized cells into a 14 mL tube, top up with Complete MesenCult® Medium (Mouse) to 10 mL, and centrifuge the cells at 300 x *g* (~1200 rpm) for 8 minutes at room temperature (15 - 25°C) with the brake on. Remove supernatant and resuspend pelleted cells in Complete MesenCult® Medium (Mouse).
8. The cells can now be divided into new tissue culture-treated dishes or plates. A recommended dilution is 1/3 (e.g. 1 well of a 6-well plate or a single 35 mm dish containing 80% confluent mesenchymal cells can be passaged into 3 wells of a 6-well plate, 3 x 35 mm dishes, or a single T-25 cm^2 flask). Cells can be cultured under normal oxygen tension or under hypoxic conditions.

Table 2: Average number of days between passaging

	RANGE OF DAYS BETWEEN PASSAGING	AVERAGE # DAYS BETWEEN PASSAGING FROM P0 TO P6 (Mean ± SD)	AVERAGE # DAYS TO REACH P6 FROM P0*
5% Oxygen	1 - 5	3.3 ± 1.5	20 days
20% Oxygen	8 - 21	10.6 ± 5.5	70 days

*Cells were passaged at 80% confluency

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5.2 Expansion Protocol for Cells Isolated from Compact Bone

1. Harvest mouse compact bone cells as described in Section 4.1.2.
2. Plate cells in Complete MesenCult® Medium (Mouse) as follows:
   - For cells from compact bone prepared according to the protocol described in Section 4.1.2, plate 2 - 5 x 10^5 cells per well of a 6-well plate in a volume of 2 mL or plate 0.6 - 1.2 x 10^6 cells in a T-25 cm^2 flask in a volume of 10 mL.
   - For enriched compact bone cells (e.g. enriched for mesenchymal progenitor cells using the EasySep® Mesenchymal Progenitor Enrichment Kit, Catalog #19771) plate 1 - 5 x 10^4 cells per well of a 6-well plate in a volume of 2 mL or plate 0.4 - 1.2 x 10^5 cells in a T-25 cm^2 flask in a volume of 10 mL.
3. Grow cells at 37°C in a Hypoxia Chamber (Catalog #27310) at an atmosphere of 5% O_2, 10% CO_2, 85% N_2 for 8 - 14 days until an adherent cell layer has formed. After 8 days, if the color of the medium has become orange, a half-medium change can be performed.
4. Observe mesenchymal cells under the microscope after 7 days to determine confluency. Once the cells have reached 80% confluency, they are ready to passaged.

   80% confluency may be reached between 7 - 16 days
5. Passage according to the protocol provided in Section 5.1, Steps 5 - 8.

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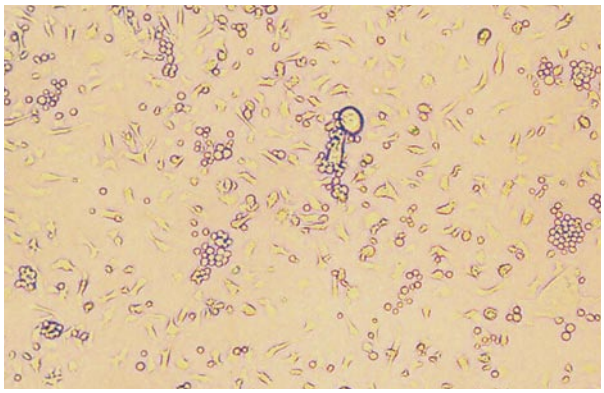
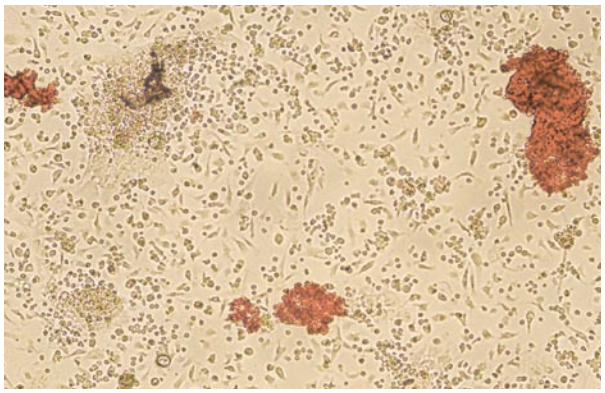
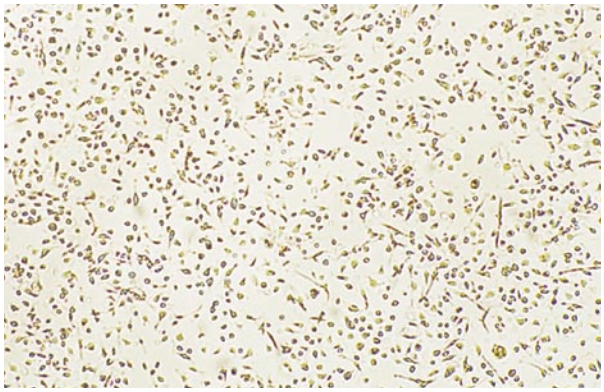
5.3 Examples of Culture-Expanded Mouse Mesenchymal Stem Cells



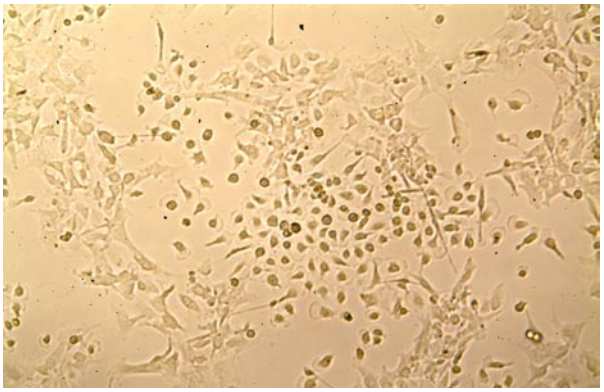
Passage 0 cells at 60% confluency. Cells were obtained by marrow flushing. Bright round cells are contaminating hematopoietic cells.



Passage 0 cells at 70% confluency. Cells were obtained by marrow flushing. Red aggregates are sometimes observed in cultures obtained from marrow flushing.

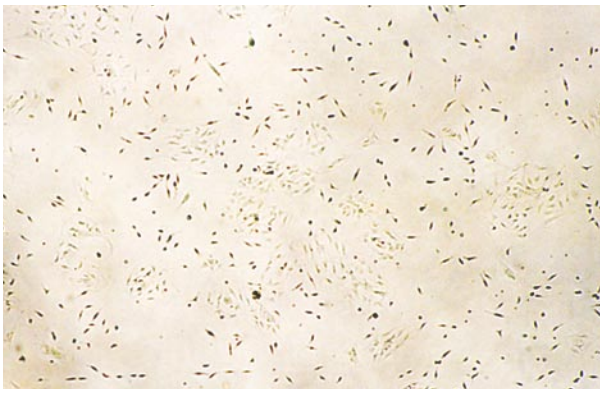


Passage 1 cells at 80% confluency. Cells were obtained by marrow flushing.



Passage 2 cells at 40% confluency. Cells were obtained by bone crushing.

Passage 2 cells. Notice that the confluency is uneven. The cells in the center of the frame are at 40% confluency but the edges are only at 20% confluency.



Passage 3 cells at 50% confluency. Cells were obtained by marrow flushing. The short and dark cells are contaminating macrophages.

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6.0 Helpful Hints

- Mesenchymal Stem Cell Stimulatory Supplements (Mouse) should be thawed overnight under refrigeration (at 2 - 8°C). If this is not possible, thaw supplements in a 37°C water bath.

  Do not thaw the supplement in a 56°C water bath!

- Once prepared, Complete MesenCult® Medium (Mouse) is stable at 2 - 8°C for 1 month. If the entire 500 mL volume of the complete medium exceeds your monthly requirements, it is possible to aliquot the supplements and store these at -20°C. Therefore, smaller volumes of complete medium can be prepared, ensuring that the supplements represent one-fifth of the total volume (i.e. 10 mL of supplements to 40 mL of basal medium).
- Cell counts should be performed in 3% Acetic Acid with Methylene Blue (Catalog #07060) to obtain an accurate nucleated cell count.
- Dilution and suspension of cells in Complete MesenCult® Medium (Mouse) is recommended prior to plating in tissue culture-treated plates, dishes or flasks to ensure equal distribution of colonies on culture surface.
- Tissue culture-treated plates, dishes or flasks must be used to support the proliferation of CFU-F and expansion of mesenchymal cells.
- Variability between samples is to be expected. As with human cells, one would expect variability in the number of CFU-F in mouse bone marrow and compact bones. A number of factors may contribute to this variability, including the age of the mouse and any treatments the mouse received prior to cell harvest. We have tested different wild type mouse strains and have not seen significant differences between strains (C57BL/J6, BALB/c, and N/M mice).

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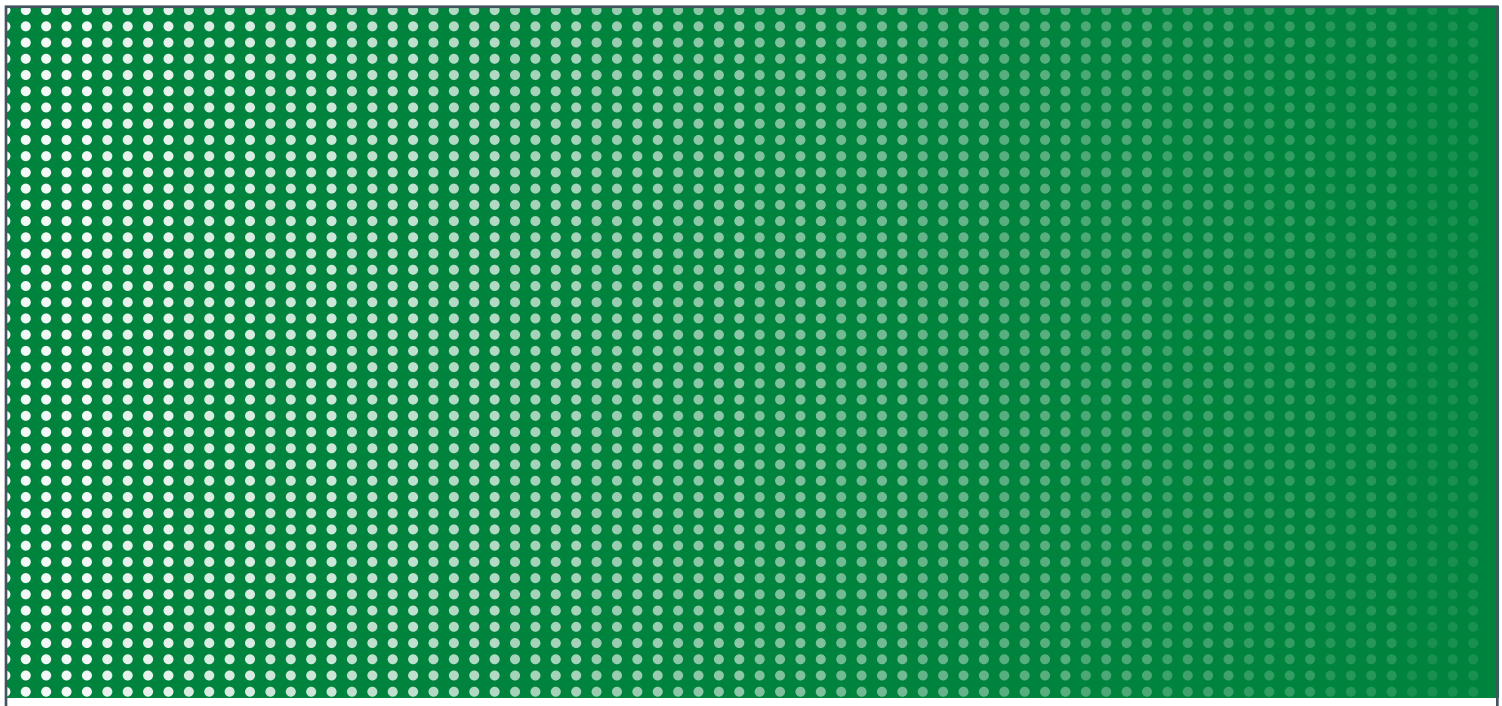

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