Video Article

Isolation, Culture, and Differentiation of Bone Marrow Stromal Cells and Osteoclast Progenitors from Mice

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URL: https://www.jove.com/video/56750
DOI: doi:10.3791/56750

Keywords: Developmental Biology, Issue 131, Mesenchymal, isolation, differentiation, osteoblast, adipocytes, osteoclasts

Date Published: 1/6/2018

Citation: Maridas, D.E., Rendina-Ruedy, E., Le, P.T., Rosen, C.J. Isolation, Culture, and Differentiation of Bone Marrow Stromal Cells and Osteoclast Progenitors from Mice. *J. Vis. Exp.* (131), e56750, doi:10.3791/56750 (2018).

Abstract

Bone marrow stromal cells (BMSCs) constitute a cell population routinely used as a representation of mesenchymal stem cells *in vitro*. They reside within the bone marrow cavity alongside hematopoietic stem cells (HSCs), which can give rise to red blood cells, immune progenitors, and osteoclasts. Thus, extractions of cell populations from the bone marrow results in a very heterogeneous mix of various cell populations, which can present challenges in experimental design and confound data interpretation. Several isolation and culture techniques have been developed in laboratories in order to obtain more or less homogeneous populations of BMSCs and HSCs *invitro*. Here, we present two methods for isolation of BMSCs and HSCs from mouse long bones: one method that yields a mixed population of BMSCs and HSCs and one method that attempts to separate the two cell populations based on adherence. Both methods provide cells suitable for osteogenic and adipogenic differentiation experiments as well as functional assays.

Video Link

The video component of this article can be found at https://www.jove.com/video/56750/

Introduction

Primary murine BMSCs are commonly used as an *in vitro* model of mesenchymal stem cells since their discovery in the early 1980s[1]. Indeed, cultures of plastic-adherent cells flushed from the bone marrow cavity of long bones maintain the capacity to be differentiated into osteoblasts, osteoclasts, chondrocytes, or adipocytes in many studies[2,3,4,5]. However, the bone marrow is a unique tissue composed of many different cell populations including, but not limited to, BMSCs, HSCs, endothelial, and immune cells. Thus, isolation and culture techniques can yield cell populations with different homogeneity. Using such techniques to test the differentiation potential from cells can be challenging. For example, when comparing cells from mice with different genotypes, starting with a mixed cell population limits the interpretation of the data. Conversely, obtaining homogenous populations of BMSCs and HSCs can be technically difficult and may not be as representative of an *ex vivo* model.

In our laboratory, we are primarily interested in the utilization of BMSCs due to their potential to be differentiated into osteoblasts, osteoclasts, and adipocytes. Here, we present techniques of BMSCs and HSCs isolation and culture used to assess osteoblastogenesis or adipogenesis *in vitro*, as well as cultures of HSCs to differentiate into osteoclasts. One method uses a mixed population of Bone Marrow Cells (BMCs) containing BMSCs and HSCs directly suitable for adipogenesis, osteoblastogenesis, and osteoclastogenesis (called Total BMCs). This method is a closer *ex vivo* representation of the heterogeneity found amongst the cells of the bone marrow microenvironment. Another method separates adherent from non-adherent cells in an attempt to culture "purer" populations of BMSCs and HSCs (called Adherent BMSCs). The later method allows the cell culture experiments to start with a more accurate number of BMSCs or HSCs and reduces the potential of complex indirect effects of other cell populations that remain in the culture. Both methods have been previously published and used to address different research questions[6,7,8,9].

Protocol

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Maine Medical Center Research Institute.

1. Collection Tubes Preparation

1. Make the BMSC culture media by supplementing Minimum Essential Medium α (MEM α) with 10% Fetal Bovine Serum and 1% of Penicillin/ Streptomycin.
2. Place 100 µL of BMSC culture media in 1.5 mL microcentrifuge tubes. 3 bones will usually fit in one single tube so prepare accordingly.
3. Cut the ends of 200 µL pipette tips enough so that they fit in the centrifuge tubes. Insert the tips inside the tubes and ensure that the lids can close before placing the tubes on ice.
   NOTE: Alternatively, 0.75 mL microcentrifuge tubes with the ends cut can be inserted into the 1.5 mL microcentrifuge tube.

2. Harvest of Bones from Mice

1. Euthanize the animals using CO_2 followed by cervical dislocation and spray them with 70% ethanol.
   NOTE: Ensure that animals from the same sex and preferably the same age are used. We routinely use animals aged 7 to 8 weeks. We recommend pooling cells from at least 3 animals per experimental groups to yield a better representative and reproducible cell population.
2. **Place the euthanized mouse on its back on a dissecting board. Use forceps to create a tent of skin on the abdomen. Make a small incision (around 1 cm) using sterile dissecting scissors. Peel back the skin towards the limbs and the feet from the cut in order to expose the lower abdomen and the legs.**
   1. Cut below the ankle joint to remove the foot. Cut along the iliac crest at the hip to separate the femur head from the hipbone. While the mouse is still on its back, make an incision in the middle of the hipbone to separate the two iliac bones.
3. Using lint-free wipes, carefully remove the muscle from the femurs, tibias, and iliac bones.
   NOTE: Cutting as much of the tendons and muscle from the bone as possible makes the cleaning faster and easier with the lint-free wipes (*e.g.*, kimwipes). Use care when manipulating the bones as they tend to break easily and their fragility may be increased with certain genotypes.
4. Once all the samples have been dissected, place them in PBS on ice and move to a sterile culture hood for the remaining steps of isolation.
   NOTE: Working aseptically as much as possible will reduce the potential for contamination in the cultures.
5. Make small cuts (approximately 1 to 2 mm) at both the proximal and distal ends of the bones before placing them in the sectioned tips within the centrifuge tubes.
   Note: Ensure to cut a sufficient amount so that marrow can be centrifuged out; however, care must be taken to avoid cutting too much as cell populations tend to vary based on proximity within the marrow space.
6. Centrifuge at 10,000 x g for 15 s at room temperature. Ensure that all the marrow is flushed and pelleted at the bottom of the 1.5 mL tube while the bones are inside the inserts (either 200 µL pipet tip or 0.75 mL microcentrifuge tube). Once all the marrow is collected, remove the inserts with the bones from the collection tubes.
   NOTE: If all the marrow is flushed, the bones will appear white. However, if marrow remains in certain bones, try cutting ends again and centrifuge.

3. Cell Suspension

1. Using a 25 G needle, pull the cell pellets up and down slowly to break up clumps and combine all the samples into a single 15 mL conical tube. Add 10 mL of BMSC Culture Media per 500 µL of samples.
2. Place a 70 µm filter on top of a 50 mL conical tube and pass the cell suspension through this filter in order to remove possible bone fragments.

4. Plating and Culture of Mixed Populations of Bone Marrow Cells (Total BMCs)

NOTE: Cells are cultured at 37 °C in an incubator with 5% CO_2.

1. Calculate the number of cells and plate them at 1.0 x 10^6 cells/cm^2 in culture media.Allow 72 h of mixed culture for cells to attach.
   NOTE: We recommend diluting the cell suspension 20x in culture media and diluting again in trypan blue before counting the cells using a hemocytometer. For 7-week-old male C57Bl6/J mice, we routinely yield a cell number of around 50 x 10^6 cells but age, sex, and strain can affect cell number greatly.
   NOTE: A mix population of cells should attach at the bottom of the culture well.
2. After 72 h, remove the media and replace it with fresh media. Continue changing the media every other day and check for confluency. When the cells reach 80-100% confluency, they are ready for differentiation.
   NOTE: Because this culture is mixed with both BMSCs and HSCs, it can be directly used for osteoblastogenesis, adipogenesis as well as osteoclastogenesis.

5. Plating and Culture of the Split Population of BMSCs (Adherent BMSCs)

NOTE: Cells are cultured at 37 °C in an incubator with 5% CO_2.

1. Plate all the cells collected from the femurs, tibias, and iliac bones of one mouse into a 10 cm culture dish (55 cm^2 of culture area).
   NOTE: When pooling 2-3 C57BL/6J mice, we typically plate this 'total' bone marrow population in a 150 cm^2 flask.
2. After 48 h of mixed culture, remove the culture media (containing the non-adherent HSCs). If osteoclastogenesis experiments are to be performed, set this population of non-adherent cells aside (see section 7), if not, aspirate and discard these cells.
3. Wash the plate/flask with PBS carefully as to not disturb the attached cells.
4. Remove PBS and lift the cells by adding 0.25% trypsin and incubating at 37 °C for 1-3 min. Quench the trypsin by adding cell media to the cell suspension. At this point, BMSCs, which are now lifted, can be counted (as previously done in step 4.1) and plated for future experiments.
5. Calculate the number of cells needed for plating. Centrifuge the required volume of cell suspension at 1,000 x g for 5 min at room temperature.
   Note: Cells are typically plated based on end-point experiments. For example, if protein/RNA is to be isolated we typically plate at a higher density (~1.0-2.0 x 10^5 cells/well in 6-well plate).
6. Remove the media and resuspend the cell pellet in the volume of culture media needed for plating. Plate the cells in culture media according to the experiments and allow them to attach for a few days. When the BMSCs reach confluency, proceed to perform differentiation.

6. Differentiation of BMSCs

1. **Osteoblasts**
    1. Make osteoblast differentiation media by adding 800 µL of 1 M β-glycerol phosphate in PBS and 200 µL of 0.5 M ascorbic acid in PBS to 99 mL of BMSC culture media (see step 1.1 for the composition of the BMSC culture media).
        1. Once BMSCs cultures (either total BMCs from step 4.3 or adherent BMSCs from step 5.6) are confluent, differentiate them into osteoblasts by switching the culture media to osteoblast differentiation media.
    2. Replace the differentiation media every other day. Cells start producing white nodules of mineralization. Visualize them with the naked eye on the bottom of the well; this process can occur within a few days to 3 weeks.
        1. In order to assess osteoblastogenesis, perform alkaline phosphatase staining and/or Von Kossa staining on the wells as described in section 8.
        NOTE: Change the media carefully by tilting the plates at a slight angle in order to avoid disruption of the cell monolayer.

2. **Adipocytes**
    1. Make adipocyte base media by mixing together 90 mL of Dulbecco Modified Eagle Medium (DMEM) High Glucose, 10 mL of Fetal Bovine Serum, 1 mL of Penicillin/Streptomycin, 100 µL 20 mM Rosiglitazone and 100 µL of 2 mM Insulin. If needed, store this media at 4 °C for the week during the differentiation experiment.
    2. Make adipocyte induction media by adding 200 µL of 62.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 25 µL of 1 mM Dexamethasone to 25 mL of Adipocyte Base Media.
    3. Once BMSCs cultures (either total BMCs from step 4.3 or adherent BMSCs from step 5.6) are confluent, change the media to adipocyte induction media. Consider this Day 0 of differentiation.
    4. After 48 h, remove the media and refresh the culture with adipocyte induction media.
    5. On Day 4, switch to adipocyte base media.
    NOTE: Lipid droplets can start appearing as early as Day 2 or Day 4.
    6. On Day 7, observe that cells have accumulated maximum lipid droplets and display a phenotype similar to a mature adipocyte. Do not culture past this point, as it may result cell death/detachment.

7. Differentiation of HSCs into Osteoclasts

1. Make Osteoclast Differentiation Media by adding 50 µL of 25 µg/mL of Receptor Activator of NFKB Ligand (RANKL) and 15 µL of 25 µg/mL of macrophage Colony Stimulating Factor (mCSF) to 25 mL of BMSC Culture Media.
2. Once the total BMCs (from step 4.3) or the non-adherent cells (from step 5.2) are attached to the well, culture media can be switched to Osteoclast Differentiation Media. Replenish differentiation media every other day.
3. Observe the osteoclast cultures every day under a microscope at 10X magnification. Observe that osteoclasts fuse and form multinucleated cells, typically around Day 5-7 of differentiation.

8. Staining

1. **Osteoblasts**
NOTE: For the Alkaline Phosphatase (AP) staining, we often use an AP staining kit (see **Table of Materials**) according to the guidelines.
    1. Perform AP staining by following kit manufacturer's guidelines.
        1. Aspirate media and rinse cells with PBS. Fix the cells with 4% Paraformaldehyde (PFA) in PBS for 12 min at room temperature.
        2. In the meantime, make the AP solution using the reagents from the AP staining kit by adding 500 µL Sodium Nitrite to 500 µL FRV-Alkaline solution and mixing gently; let stand for 2 min. Add 22.5 mL dH_2O and 500 µL Naphthol AS-BI Alkaline. Mix gently. Caution: PFA is toxic and must be handled with care.
        3. Once the cells are fixed, rinse with PBS and add AP solution to each well (1 to 2 mL per well). Cover with aluminum foil and let stain for 30 min at room temperature protected from light. Aspirate the solution, wash with distilled water, and allow the plate to dry.
    2. Acquire images of stained colonies using a camera in order to observe alkaline phosphatase positive cells.
    3. Alternatively perform Von Kossa instead of alkaline phosphatase staining. For this, wash wells thoroughly with distilled water as any remaining PBS will precipitate with silver nitrate.
        1. Fix the cells with 4% PFA in PBS for 12 min at room temperature. Then, add enough 5% silver nitrate solution to cover the well and expose to strong light for 1 h at room temperature.
    4. Remove the silver nitrate solution and rinse each well with distilled water. Add 5% sodium thiosulfate solution to the cells and let stand for 3 min.
    5. Aspirate the sodium thiosulfate and wash with distilled water. Let the plate dry and observe the mineral deposit stained in dark brown/black with naked eye.

2. **Adipocytes**
    1. For Oil Red O (ORO) staining, fix the cells using 10% Neutral Buffered Formalin for 1 h at room temperature.
    2. In the meantime, make the ORO Stock Solution by dissolving 0.035 g in 10 mL of isopropanol. This is sufficient for a 6-well plate. Mix and pass the solution using one filter paper (pore size 11 µm).
    3. Make the ORO Working Solution by diluting 9 mL of ORO stock solution with 6 mL of distilled water. Leave the solution on a rocker until ready for use.

4. Prepare 60% isopropanol in distilled water (make enough to cover each well).
5. Once the cells are fixed, remove the fixative and wash once with distilled water. Replace the water with the 60% isopropanol solution for 1 min.
   NOTE: Add liquid slowly while tipping the plate; adipocytes dislodge very easily.
6. Remove the isopropanol and add enough ORO Working Solution to cover all the cells. Incubate for 15 min on a low-speed rocker at room temperature. Remove ORO and wash twice with distilled water.
   NOTE: At this point, ORO-stained lipid droplets can be visualized under the microscope.
7. To quantify ORO, destain the ORO by adding 1 mL of 100% isopropanol to each well and by rocking slowly for 10 min.
8. In the meantime, prepare a dilution series to create a standard curve based on the following 8 standards using ORO Working Solution (2,100 µg/mL) and isopropanol as a diluent: 500, 350, 300, 250, 150, 100, 75, and 0 µg/mL.
9. Load 200 µL of the standards and the destained samples in triplicates on a plate and read the absorbance at 490 nm.
   1. Determine concentration of ORO for each sample based on the standard curve.
      Note: We recommend normalizing the concentration ORO to cell number. Cell number can be quantified by crystal violet staining or DNA quantity.

3. **Osteoclasts**
   NOTE: When ready, we suggest staining osteoclasts for Tartrate-Resistant Acid Phosphatase (TRAP) using the TRAP staining kit (see **Table of Materials**).
   1. For TRAP staining, fix cells for 30 min at room temperature with 2.5% glutaraldehyde in PBS. In the meantime, make the TRAP solution by mixing together 50 µL of Fast Garnets Base Solution, 50 µL of Sodium Nitrite, 4.55 mL of distilled water, 50 µL of Napthol AS-Biphosphate, 200 µL of Acetate solution and 100 µL Tartrate solution.
   2. Aspirate off fixative, and rinse the cells with PBS twice before adding TRAP solution for 1 h at 37 °C.
   3. Wash with distilled water before counting osteoclasts under a microscope (magnification 10X). The $TRAP^+$ cells appear purple with 3 or more nuclei.

Representative Results

Overview of the Cell Cultures
The two culture techniques allow the assessment of differentiation on a mixed population of BMCs comprised of BMSCs and HSCs (Total BMCs, **Figure 1A**) or a population of BMSCs split from HSCs (Adherent BMSCs, **Figure 1B**). 48 hr after the cells from the marrow are isolated and plated (**Figure 1C**), they rapidly attach to the bottom of the plastic culture plate and tend to form a monolayer. Once the cells reach confluence (**Figure 1D**), they can be used for differentiation assays.

BMSCs Differentiated into Osteoblasts
Confluent cultures of BMSCs can be differentiated into osteoblasts using an osteogenic media composed of ascorbic acid and β-glycerol phosphate (**Figure 2A**, B). After a few days of differentiation, osteoblasts start expressing alkaline phosphatase (**Figure 2C**). Further differentiation will trigger osteoid matrix production and ultimately the mineralization of this matrix (**Figure 2D**). Interestingly, only a proportion of the cells will differentiate into osteoblasts *in vitro*. Indeed, staining with crystal violet (**Figure 2E**) reveals that not all cells express alkaline phosphatase or mineralize the plate.

BMSCs Differentiated into Adipocytes
Adipogenesis can be tested on cultures of mixed BMCs as well as on the more homogenous cell population. As observed with osteoblast differentiation, whether the mixed populations (**Figure 3A**) or the split populations (**Figure 3B**) are used, only a proportion of the cells are adipogenic. Adipocytes appear round with multiple lipid vacuoles that can be stained in red with ORO (**Figure 3C**). In our experience, *in vitro* adipocytes always appear multi-vacuolated unlike *in vivo* white adipocytes. Differences in genotype or culture conditions might increase adipogenesis to a point where adipocytes detach and float in the culture plate. To resolve this, the adipogenesis experiment can be stopped at an earlier time point.

HSCs Differentiated into Osteoclasts
Osteoclastogenesis can be induced in the cultures by supplementing the media with mCSF and RANKL. HSCs start to fuse rapidly to form multinucleated cells that stain positive for TRAP (**Figure 4A**). Those osteoclasts are capable of resorbing mineral matrix *in vitro* and thus can be used to assess osteoclastic activity (**Figure 4B**).

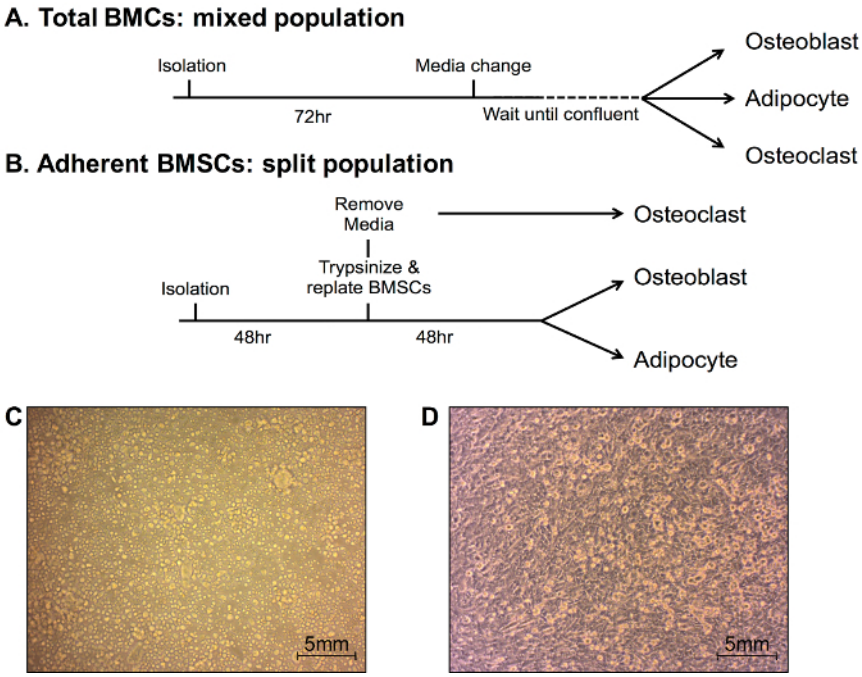

Figure 1: Schematic timeline of the protocols for the culture of BMSCs and HSCs. Different culture techniques to yield Total BMCs composed of a mixed population of cells (**A**) or Adherent BMSCs (**B**). Representative image of BMSCs culture isolated from C57B6/J mice 48 hr after their plating (**C**) and after they are split from HSCs (**D**). Please click here to view a larger version of this figure.

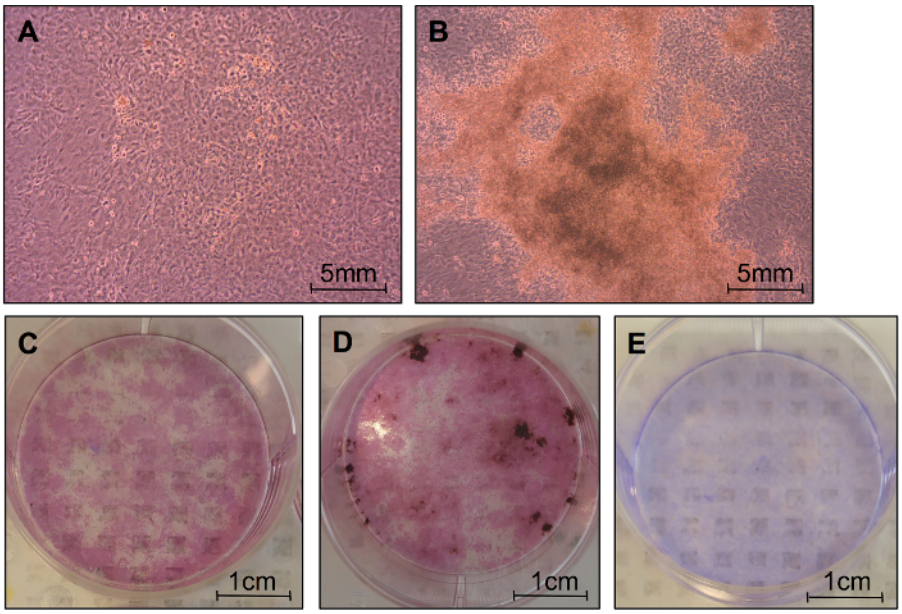

Figure 2: Images of wells of BMSCs cultures differentiated into osteoblasts. BMSCs before (**A**) and after (**B**) being placed in osteogenic media. Osteoblasts can be stained for alkaline phosphatase expression in pink (**C**) and for mineral deposit in brown (**D**). Crystal violet can be used in order to represent cell number after differentiation (**E**). Please click here to view a larger version of this figure.

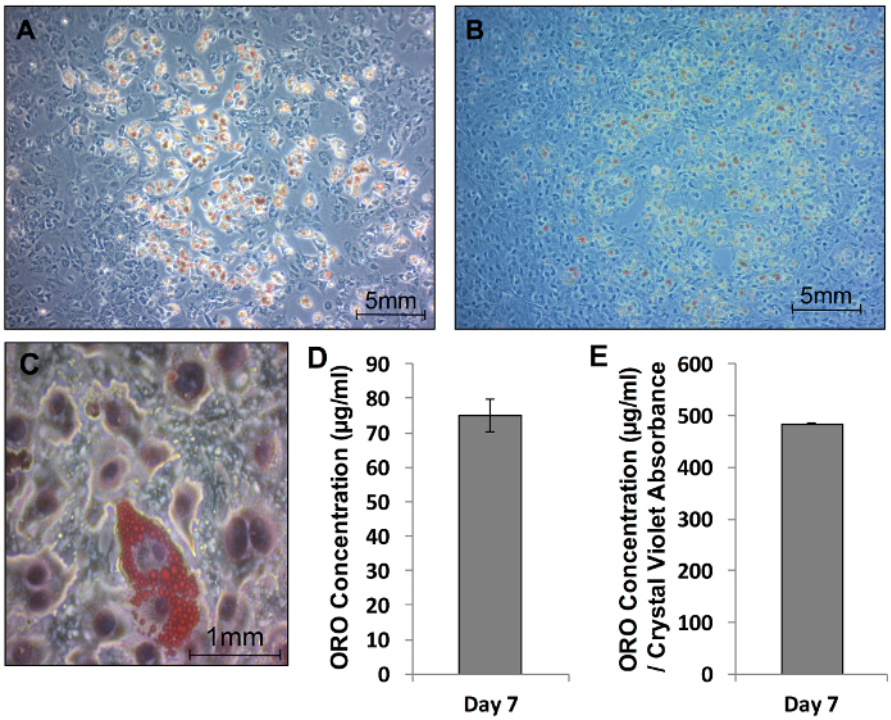

Figure 3: Representative images of the cultures of BMSCs differentiated into adipocytes. ORO-stained adipocytes obtained from a mixed population of BMSCs (**A**) and a split culture (**B**) after 7 days in adipogenic media. Image of an adipocyte stained with ORO (**C**). Quantification of ORO after 7 days in adipogenic media before (**D**) and after (**E**) normalization to crystal violet absorbance. Data are presented as means with error bars representing standard error of the mean. Please click here to view a larger version of this figure.

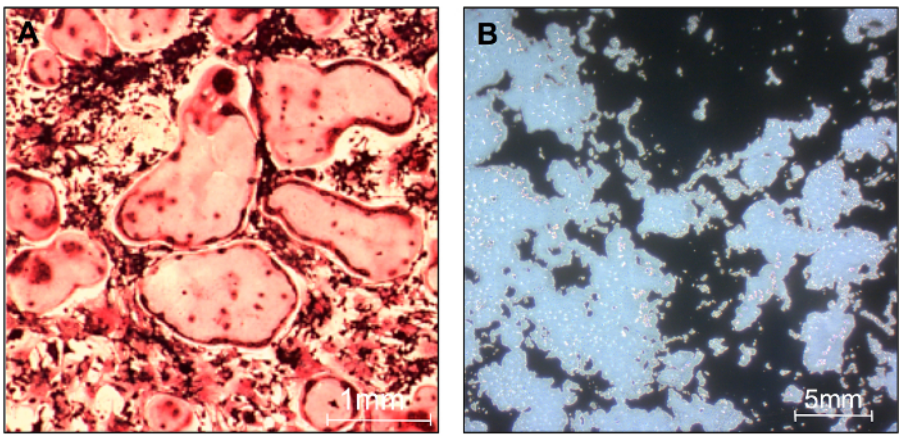

Figure 4: HSCs cultures differentiated into osteoclasts. TRAP staining of osteoclasts appearing as large purple-pink multinucleated cells (**A**). Mineralized surface stained in black by Von Kossa with resorption pits left by osteoclasts differentiated from HSCs after 7 days (**B**). Please click here to view a larger version of this figure.

Discussion

In this article, two methods of culture of BMSCs are presented with their advantages and limitations. Isolating cells coming from the bone marrow is a relatively effortless process. However, obtaining a cell population representative of the mesenchymal stem cells or osteoclastic progenitors can be challenging due to the diverse cellular environment of the marrow cavity.

Culturing the entirety of the bone marrow contents provides a close representation of the *in vivo* microenvironment. Yet, isolating cells from different groups of mice (genotypes, treatments, ages, *etc.*) can result in a very different number of BMSCs or HSCs at the inception of the *in vitro* experiment. Therefore, separating BMSCs and HSCs and re-plating them allow a better control over the cell number at the start of the differentiation experiment. However, reducing the heterogeneity of the cell culture might affect the outcomes of the BMSCs or HSCs as both secrete factors that influence mesenchymal and osteoclastic differentiation. It is important to consider those limitations when designing the isolation and the culture of the BMSCs and HSCs.

The multitude of cell populations present in the bone marrow can introduce challenges in the interpretation of the data. In addition to HSCs and BMSCs, the bone marrow microenvironment also contains differentiated immune cells, red blood cells, endothelial cells,*etc.* Those populations may remain in the adherent or non-adherent fraction and thus can affect the experimental outcomes. Sorting BMSCs can be an option in order to achieve a "purer" cell population. Indeed, BMSCs are thought to express CD34 while HSCs do not[10]. However, it was also suggested that the lack of CD34 on the surface of BMSCs may be the result of culture and a study has shown that a non-adherent subset of $CD34^+$ BMSCs could differentiate into osteoblast, adipocytes and chondrocytes[11,12]. Therefore, when considering sorting to isolate BMSCs, an array of well-defined markers should be designed beforehand. Other obstacles to cell sorting are timing and cost. Indeed, adding a sorting step could increase the time of the isolation protocol considerably as well as a significant increase in cost. In summary, sorting cells present technical challenges that may reduce the yield and quality of BMSCs.

Disclosures

The authors disclose that they have no financial conflict of interests.

Acknowledgements

This work was supported by the National Institutes of Health (R01 AR061164-01A1).

References

1. Friedenstein, A. J., Latzinik, N. W., Grosheva, A. G. **&** Gorskaya, U. F. Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porous sponges. *Exp Hematol.* **10** (2), 217-227 (1982).
2. Chen, T. L. Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. *Bone.* **35** (1), 83-95 (2004).
3. Kokabu, S. *et al.* BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b. *Mol Endocrinol.* **26** (1), 87-94 (2012).
4. Ghali, O. *et al.* Dexamethasone in osteogenic medium strongly induces adipocyte differentiation of mouse bone marrow stromal cells and increases osteoblast differentiation. *BMC Cell Biol.* **16** 9 (2015).
5. Kretlow, J. D. *et al.* Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. *BMC Cell Biol.* **9** 60 (2008).
6. DeMambro, V. E. *et al.* Igfbp2 Deletion in Ovariectomized Mice Enhances Energy Expenditure but Accelerates Bone Loss. *Endocrinology.* **156** (11), 4129-4140 (2015).
7. Maridas, D. E. *et al.* IGFBP-4 regulates adult skeletal growth in a sex-specific manner. *J Endocrinol.* **233** (1), 131-144 (2017).
8. Nadri, S. *et al.* An efficient method for isolation of murine bone marrow mesenchymal stem cells. *Int J Dev Biol.* **51** (8), 723-729 (2007).
9. Sreejit, P., Dilip, K. B. **&** Verma, R. S. Generation of mesenchymal stem cell lines from murine bone marrow. *Cell Tissue Res.* **350** (1), 55-68 (2012).
10. Abdallah, B. M. *et al.* CD34 defines an osteoprogenitor cell population in mouse bone marrow stromal cells. *Stem Cell Res.* **15** (3), 449-458 (2015).
11. Akiyama, K. *et al.* Characterization of bone marrow derived mesenchymal stem cells in suspension. *Stem Cell Res Ther.* **3** (5), 40 (2012).
12. Lin, C. S., Ning, H., Lin, G. **&** Lue, T. F. Is CD34 truly a negative marker for mesenchymal stromal cells? *Cytotherapy.* **14** (10), 1159-1163 (2012).

