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In vitro Osteoclastogenesis Assays Using Primary Mouse Bone Marrow Cells

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[Abstract] Osteoclasts are a group of bone-absorbing cells to degenerate bone matrix and play pivotal roles in bone growth and homeostasis. The unbalanced induction of osteoclast differentiation (osteoclastogenesis) in pathological conditions, such as osteoporosis, arthritis and skeleton metastasis of cancer, causes great pain, bone fracture, hypercalcemia or even death to patients. *In vitro* osteoclastogenesis analysis is useful to better understand osteoclast formation in physiological and pathological conditions. Here we summarized an easy-to-follow osteoclastogenesis protocol, which is suitable to evaluate the effect of different factors (cytokines, small molecular chemicals and conditioned medium from cell culture) on osteoclast differentiation using primary murine bone marrow cells.
Keywords: Osteoclastogenesis, Osteoclast, Primary bone marrow, TRAP staining, Cell culture

[Background] The skeleton is maintained by successive and well-controlled absorbance and formation of bone mass during lifetime. In the bone cavity, each of these two activities is carried out by a specialized cell type: the bone-forming osteoblasts and bone-degrading osteoclasts. Osteoblasts and osteoclasts are derived from bone-resident mesenchymal cells and hematopoietic lineage progenitor cells, respectively. The differentiation of hematopoietic myeloid progenitor cells into mature osteoclasts are majorly controlled by receptor activator of nuclear factor-κB ligand (RANKL, encoded by *TNFSF11*) and macrophage colony-stimulating factor (M-CSF, encoded by *CSF1*) derived from osteoblasts and its progenitor cells (Suda *et al.*, 1999). Unlike other cells, osteoclasts differentiate through fusion of a certain number of progenitor cells (Boyle *et al.*, 2003). Thus, a key histological feature of mature osteoclasts is their multiple nuclei. After maturation, osteoclasts are capable of bone resorption by producing an acidified microenvironment to dissolve bone mass mainly composed of calcium phosphate, along with proteases to degrade extracellular matrix (Boyle *et al.*, 2003). The dissolved bone matrix releases sequestered growth factors utilized by osteoblasts to expand their population (Kassem and Bianco, 2015). This cross-talk between osteoblasts and osteoclasts ensures coordinate bone-forming and -degenerating activity, which is dysregulated in a plethora of diseases, including osteoporosis, arthritis and bone metastasis of cancers (Rodan and Martin, 2000; Raisz, 2005; Gupta and Massague, 2006). Based on previous literature (Lu *et al.*, 2009; Wang *et al.*, 2014; Zhuang *et al.*, 2017), here we describe a step-by-step protocol for an *in vitro* osteoclastogenesis assay using primary murine bone marrow cells that allows studying the effect of a broad range of factors/conditions (such as cytokines and conditioned medium) on osteoclast differentiation.

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Materials and Reagents

1. Pipet tips (Autoclaved, any brand)
2. 29 gauge syringe (BD, catalog number: 328421)
3. 40 µm nylon mesh cell strainer (Corning, catalog number: 352340)
4. Minisart® NML syringe filter, 0.2 µm (Sartorius, catalog number: 17597-K)
5. 14 mm round coverslips (any brand suitable for cell culture)
6. 24 well plate and 100 mm Petri dish (Thermo Fisher Scientific, Nunc™, catalog numbers: 142475 and 172931)
7. 15 ml conical tubes (Corning, catalog number: 352196)
8. Glass slides (OMANO, catalog number: OMSK-50PL)
9. Cell culture Petri dishes and multi-well plate (Thermo Fisher Science, catalog numbers: 174888, 150350, 142485)
10. 1.5 ml Eppendorf tubes (Fisher Scientific, catalog number: 05-408-129)
11. Mouse aged between 4-7 weeks (any eligible provider, mouse strain/genetic background should be consistent with other assays)
12. α-MEM (Thermo Fisher Scientific, catalog number: A1049001)
13. Fetal bovine serum (Thermo Fisher Scientific, catalog number: 10099141)
14. Recombinant murine M-CSF (PeproTech, catalog number: 315-02)
15. Recombinant murine RANKL (PeproTech, catalog number: 315-11)
16. Red blood cell lysing buffer (BD, catalog number: 555899)
17. Albumin, bovine serum, fraction V (Merck, catalog number: 12659)
18. Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich, catalog number: 387A)
19. Acetone (Fisher Scientific, catalog number: A18-1)
20. 37% formaldehyde (Fisher Scientific, catalog number: BP531-500)
21. Neutral Balsam (Sangon Biotech, catalog number: E675007)
22. Sodium chloride (NaCl) (Fisher Scientific, catalog number: BP358)
23. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P5405)
24. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S5136)
25. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
26. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: H9892)
27. Culture medium (see Recipes)
28. Phosphate buffered saline (see Recipes)
29. Murine rRANKL and rM-CSF solution (see Recipes)

Equipment

1. Pipette (Eppendorf, sterilize surface by 70% ethanol)
2. Hemacytometer (OMANO, catalog number: OMSK-HEMA)

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3. Dissecting tweezers and scissors (Autoclaved)
   Scissors (Fisher Scientific, catalog number: 08-940)
   Tweezers (Fisher Scientific, catalog number: 12-460-612)
   Manufacturer: Integra LifeSciences, Integra® Miltex®, catalog number: MH18782.
   Tweezers (Fisher Scientific, catalog number: 12-460-611)
   Manufacturer: Integra LifeSciences, Integra® Miltex®, catalog number: MH18780.
4. Cell culture incubator (any brand)
5. Centrifuge (Thermo Fisher Scientific, model: Hearus Labofuge 400 R)
6. Balance
7. Inverted microscope (Nikon Instruments, model: Eclipse Ti-S)
8. Water bath (any brand which can reach 37 °C)

Procedure

Note: For the animal experiment included in the protocol, please refer to specific regulations in your institute. In our study, all animal studies were conducted according to the guidelines for the care and use of laboratory animals and were approved by the Institutional Biomedical Research Ethics Committee.

A. **Day 0:** Extraction of bone marrow cells

   Note: The volumes of medium or buffer in the procedures are for one mouse. Adjust the volume if handling multiple mice.

   1. Sacrifice a mouse aged between 4-7 weeks by cervical dislocation and quickly sterilize the body surface with 70% ethanol.
   2. Immediately dissect out the hinder limbs of the mouse by cutting at the ankle and the upper end of the thigh with scissors. Remove skin and muscle to reveal bones. Dislocate the femur and tibia by cutting at the knee with scissors. Rinse bones twice with pre-cooled PBS to remove excessive muscle tissues (Figure 1A).
   3. Remove both ends of each piece of bone (Figure 1B) and flush the bone marrow cells out with a 29 gauge syringe. Use 0.5 ml α-MEM per bone and temporarily collect cell solution in a 6 cm Petri dish (Figure 1C).
      Note: If bone marrow cells do not flow out easily, stir within the bone cavity with the syringe needle briefly to mobilize cells.
   4. Pipet the resultant cell solution gently and filter it through a 40 μm mesh cell strainer (Figure 1D) into a 15 ml conical tube. This step is to remove unwanted cell clusters and bone matrix. Collect cells via centrifugation at 400 *x g* for 5 min at 4 °C and discard supernatant.
   5. Resuspend cell pellets in 200 μl α-MEM and add 2 ml diluted red blood cell lysing buffer. Gently mix the solution by inverting the tube a couple of times and then leave at room temperature for 5-10 min until the solution becomes transparent red (Figure 1E).
      Note: Prepare the red blood cell lysing buffer just before use. Red blood cell lysing buffer is

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supplied as 10x stock. Dilute it with deionized water to the working concentration.

6. Collect cells by centrifugation at 400 *x g* for 5 min at 4 °C. Cell pellets should be almost white at this stage. If not, repeat Steps A5 and A6.
7. Discard supernatant and resuspend cells in 10 ml culture medium and culture in a 10 cm Petri dish in a 37 °C, 5% CO_2 cell culture incubator overnight.

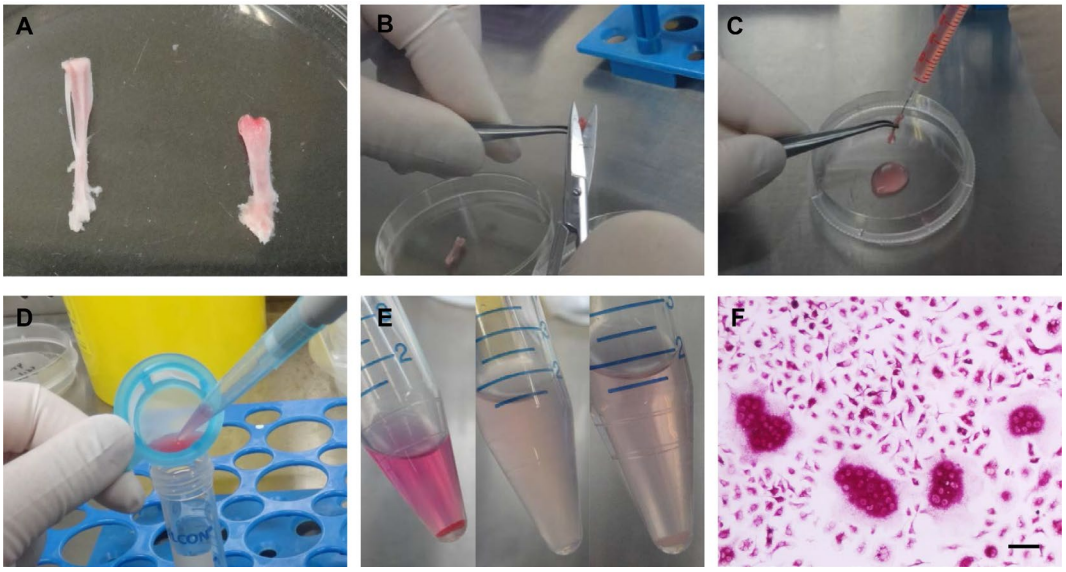

Figure 1. Overview of osteoclastogenesis assay. A. Dissected femur and tibia; B. Removal of bone ends; C. Flushing of bone marrow cells; D. Filtering through a cell strainer; E. Primary bone marrow cells before (left), during (middle) and after (right) red blood cell lysis; F. Representative image of mature osteoclasts. Scale bar = 50 μm.

B. **Day 1:** Osteoclastogenesis

1. Collect suspended myeloid precursor cells in a 15 ml conical tube and centrifuge at 400 *x g* for 5 min at 4 °C.
2. Resuspend cell pellets in 1 ml culture medium and count cell number by hemacytometer.
   Note: Usually 1-2 x 10^7 cells can be obtained with one mouse at this step.
3. Place sterilized coverslips into the central 8 wells of 24 well plates.
   Note: Usually four replicates are required for each setting.
4. Dilute cell solution with culture medium or culture medium/conditioned medium mixture to the concentration of 1.6-2.0 x 10^6/ml. Aliquot 2 ml cell solution for each setting (four replicates). Add M-CSF (final concentration, 25 ng/ml) and RANKL (final concentration, 50 ng/ml) stock to their final concentration.
   Note: The final concentration of rRANKL may vary and please refer to Notes section for guidance.
   If specific cytokines or chemicals are of interest, please also add them into the cell aliquots. Gently but thoroughly pipet the cell solution and transfer 500 μl mixtures to each of the four replicates.

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C. **Day 4:** Refresh medium with the same medium recipe as Day 1

D. **Day 7:** TRAP staining for mature osteoclasts

1. Conduct staining procedures of coverslips following the manufacturer's protocol (Sigma). Here is the Sigma protocol with our modification:
   Note: The whole fixation and staining procedures can be conducted in the same multi-well plate of osteoclast differentiation.
   a. Prepare Fixative Solution by combining 25 ml Citrate solution (provided by the staining kit), 65 ml acetone and 8 ml 37% formaldehyde. Place Fixative Solution in glass bottles and cap tightly. Fixative solution is stable up to 2 months at 2-8 °C and discard if evaporation is observed.
   b. Pre-warm sufficient deionized water (at least 10 ml for each well) to 37 °C for the whole assay, including washes before and after staining.
   c. Bring Fixative Solution to room temperature (18-26 °C). Aspirate culture medium and rinse once with PBS. Fix cells on coverslips by adding 500 µl Fixative Solution/well for 30 sec. Rinse thoroughly in deionized water: Do not allow slides to dry.
   d. To a 1.5 ml Eppendorf tube add 0.5 ml Fast Garnet GBC Base Solution and 0.5 ml Sodium Nitrite Solution. Mix by gentle inversion for 30 sec. Let it stand for 2 min.
   e. Prepare staining solution in the "Beaker B" column (45 ml deionized water pre-warmed to 37 °C, 1.0 ml diazotized Fast Garnet GBC solution from Step D1d, 0.5 ml Naphthol AS-BI Phosphate solution, 2.0 ml Acetate Solution and 1.0 ml Tartrate Solution).
      Note: All solutions except deionized water are provided by the staining kit.
   f. Add at least 500 µl staining solution to each well. Place multi-well plates in a 37 °C incubator for 1 h and protect plates from light.
   g. Aspirate staining solution and rinse several times with deionized water. Carefully take cover slips out and air dry them.
2. Mount of air-dried coverslips
   a. Take a clean glass slide and place one drop of 20 µl neutral balsam onto the slide.
   b. Carefully hold coverslips on the edge, facing the surface with cells toward glass slide, and slowly mount to avoid air bubbles.
   c. Count results within one week after mounting. Mature osteoclasts are TRAP-positive cells with ≥ 3 nuclei and diameter ≥ 50 µm (Figure 1F).

   Note: We usually count TRAP-positive multi-nuclei cells of a whole coverslip to collect data. It is not suggested to collect data by taking random snapshots since it is very difficult to pre-determine how many snapshots are sufficient to estimate the number of osteoclasts.

Notes

1. The administration of antibiotics may cause unsuccessful induction of osteoclasts and should

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be avoided. Please use sterile equipment to handle mouse tissue and cells. The conical tubes, syringes, Petri dishes, multi-well plates and cell strainers mentioned in the Materials and Reagents section are all sterilized. Other tools, such as tips, dissecting tweezers, scissors, and coverslips can be autoclaved. Equipment which cannot be autoclaved, such as pipettes, can be sterilized by 70% ethanol spraying. After the equipment has been properly handled, the risk of contamination should be quite low.

2. The quality of FBS may strongly affect the differentiation of osteoclasts. Also, the rRANKL final concentration can vary from 0 to 100 ng/ml due to the different quality of FBS, and 50 ng/ml should be an appropriate point to start with. Users need to adjust the concentration of rRANKL since too much rRANKL can mask the modulation caused by the specific reagents which users are interested in. Users can test a series of concentrations ranging from 0 to 100 ng/ml in pilot trials. The appropriate concentration should 1) allow users to observe mature osteoclasts with the right morphology and 2) not saturate osteoclastogenesis, which means increased rRANKL administration can still induce more osteoclasts.

Recipes

1. Culture medium
   α-MEM with 20% heat-inactivated FBS
   Note: FBS can be heat-inactivated by incubation at 56 °C for 30 min.
2. Phosphate buffered saline
   1 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   Diluted in 800 ml deionized water and adjust to pH 7.4 with HCl
   Add deionized water to the final volume of 1 L and autoclave the solution
3. Murine rRANKL and rM-CSF solution
   a. Dissolve 0.5 g BSA in 10 ml sterilized PBS and filter through a 0.2 μm filter
   b. Reconstitute lyophilized protein powder in PBS containing 0.5% BSA solution to the final concentration of 0.1 mg/ml and gently agitate to ensure it is fully dissolved
   c. Aliquot cytokine solution and store at -80 °C

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Competing interests

Authors declare no conflict of interest or competing interests.

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