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LABORATORY METHODS

Generation and culture of osteoclasts

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Osteoclasts are highly specialized cells of haematopoietic lineage that are uniquely responsible for bone resorption. In the past, osteoclasts were isolated as mature cells from chicken long bones, or were generated using osteoblasts or stromal cells to induce osteoclast formation in total bone marrow from mice or rabbits. The Copernican revolution in osteoclast biology began with the identification of macrophage-colony stimulating factor (M-CSF) and receptor activator NFκB-ligand (RANKL) as the key regulators of osteoclast formation, fusion and function. The availability of recombinant human and mouse M-CSF and RANKL has enabled researchers to reliably generate osteoclasts from primary monocyte/macrophage cells as well as from cell lines such as RAW 264.7. This article summarizes the most commonly used procedures for the isolation, generation and characterization of human, rodent and chicken osteoclasts *in vitro.* Lists of further reading and recommendations are included to facilitate a successful application by the reader.

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Introduction

Osteoclasts are one of the most distinct and specialized cell types found in the body. They are large, multinucleated, motile cells of haematopoietic lineage formed through the fusion of multiple monocyte or macrophage precursors.[1] Their survival, proliferation and differentiation from haematopoietic stem cells to functional osteoclasts is driven by two essential cytokines, macrophage-colony stimulating factor (M-CSF), secreted by osteoblasts, and receptor activator of NFκB ligand (RANKL), found as a soluble factor or as a membrane-bound cytokine expressed on osteoblasts, osteocytes, dendritic cells, mature T cells and haematopoietic precursors.[2–7]

The main function of osteoclasts is bone resorption: the degradation of both the hydroxyapatite mineral component of bone and the organic bone matrix. At the site of resorption, mature osteoclasts become polarized and attach to the bone surface through actin-rich podosomes. These form a circular structure, the actin ring, isolating the bone matrix from the extracellular space in the 'sealing zone'. Within this 'zone' the complex folded membranes of the ruffled border serve as a secretory site for the protons, chloride ions and various enzymes required to degrade the organic and inorganic portions of the bone matrix.[8,9] Degraded resorption products are removed from the 'sealing zone' by endocytosis and transcytosis and are released from the basolateral membrane into the extracellular fluid away from the site of resorption.[10]

The ability to culture and study osteoclasts *in vitro* is therefore a key basic skill for any scientist wishing to study bone metabolism, the mechanisms of bone disease or any novel skeletal therapeutic agent.

In this technical review we have provided an overview of the different techniques used to generate, differentiate and analyse osteoclasts *in vitro*. This has been done by combining previously published protocols with those optimized in our laboratories, with the aim of giving a straightforward and comprehensive handbook.

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Materials and Methods

Historical osteoclast cultures

In the past, the only way to study osteoclasts *in vitro* was to extract them as mature cells from neonatal animals by fragmentation of their bones.[11–14] Subsequently, osteoclasts were generated by co-culturing osteoclast precursors with osteoblasts under osteoclastogenic conditions.[15–17] While the isolation of osteoclast precursors is still required for modern primary osteoclast *in vitro* techniques, in general these historical methods are now rarely used. However, for certain investigations these may still be the most appropriate culture to use and knowing them helps in understanding how modern methods were derived.

Isolation of mature osteoclasts

Isolation of rabbit osteoclasts. Mature rabbit osteoclasts can be isolated from the long bones (tibia and femur) of a 2–4-day-old rabbit. The limbs are carefully dissected out, the skin removed and the limbs transferred into a tube containing ice-cold phosphate-buffered saline (PBS). Using a scalpel/forceps, remove the soft tissues and articular cartilage and place the bones into fresh ice-cold PBS. In a Petri dish, finely mince all the isolated bones into small pieces in a small volume of culture medium that has not been supplemented with fetal calf serum (FCS). To do this, first cut the bones lengthwise, remove the marrow and then chop transversely as finely as possible. Avoid using FCS and perform this stage of the procedure as quickly as possible to prevent mature osteoclasts from adhering to the bottom of the dish. Transfer the medium containing the minced bones into a 50 ml conical tube and vigorously vortex for three 10-second bursts and then allow the bone fragments to settle for 3 min. Transfer the supernatant to a fresh centrifuge tube and add the required volume of culture medium supplemented with 10% FCS. Plate the cell suspension into Petri dishes and culture overnight under standard cell culture conditions. The next day remove the medium and gently wash the cell monolayer with sterile PBS to eliminate contaminating stromal and non-adherent cells. Add fresh culture medium and maintain under standard cell culture conditions[18] (**Figure 1a**).

Isolation of chicken osteoclast. Mature chicken osteoclasts can be isolated from female White Leghorn hens (*Gallus domesticus*) that have been kept for 7 days on a low-calcium diet.[19] The low-calcium diet enhances osteoclastogenesis and bone resorption, causing osteoclasts to not only cover the trabecular bone but also to be present in the bone marrow completely detached from the bone surface. It is interesting to note that chicken osteoclasts do not undergo apoptosis when detached from the bone and this is why this technique allows the isolation of mature osteoclasts.

After killing, femurs and tibias are collected, cleaned of soft tissue and placed in ice-cold PBS. The bones are then fragmented with sterile surgical blades and sharpened needles used to gently scrape the cells from the bone into fresh PBS. This suspension is transferred to a 15 ml tube and left to rest for a few minutes to allow the bone fragments to settle. When the fragments reach the bottom of the tube, the supernatant containing the cells is collected, transferred to a new 15 ml tube and centrifuged (250 *g* for 5 min). A fresh 15 ml tube is half filled with 10% albumin in PBS (or to increase cell viability PBS supplemented with 75% FBS) and the resuspended cells gently layered on top. The tubes are left for 45 min to allow sedimentation of the cells by gravity. The supernatant is again collected and centrifuged (250 *g* for 5 minutes). The sedimentation/centrifugation cycle is repeated 3–4 times in order to obtain an enriched population of osteoclasts (90–95%). Resuspend the final cell pellet in culture medium and plate 1×10^5 cells per 35 mm Petri dish. On average 1–1.5 $\times$ 10^6 osteoclasts can be isolated from one chicken. After 24 h, replace half of the medium with fresh medium, and then after a further 24 h remove the medium gently and completely and replaced with fresh medium. On the third rinse the culture vigorously to remove non-adherent cells. The culture medium should be changed every other day[20,21] (**Figure 1b**).

Isolation of rat osteoclasts. Mature rat osteoclasts can be mechanically isolated from long bones of new-born rats (2–4 days). Long bones of new-born rats are isolated, cleared of soft tissues using sterile surgical blades and placed in ice-cold PBS. Bone cells are then isolated by finely chopping the bone using a scalpel blade, using osteoclast isolation medium buffered with HEPES.

The suspension and the small bone fragments are collected with a pipette and placed in a tube, where larger bone fragments are allowed to settle for 10 s. The cells are then dropped onto slices of devitalized bone, and placed onto plastic Petri dishes or glass coverslips according to the analysis needed. Osteoclasts are then allowed to seed and attach to the support (bone, plastic or glass) for 30 min, at 37 °C, at 5% CO_2. The support is then washed in medium containing heat-inactivated fetal bovine serum (10% v/v) and placed in separate wells containing 2 ml of the same medium and cultured at 37 °C, 5% CO_2, for up to 18 h.[22]

Isolation of osteoclast precursors

Isolation of mouse osteoclast precursors. To obtain osteoclast precursors from new-born mice, seven to nine 1-day-old mice are killed and placed in 90% alcohol. The hindlimbs and forelimbs are harvested and transferred into a sterile Petri dish on ice. Here, the soft tissues are carefully removed from the limbs with a sterile scalpel and the clean bones are placed in a new 35 mm Petri dish filled with 2 ml of culture medium that had been stored for 30 min in an incubator (5% CO_2, 37 °C) to allow it to reach the correct pH and temperature. The bones are finely chopped using sterile surgical blades, and the bone marrow mechanically isolated from the bone fragments by pipetting the medium repeatedly using a sterile plastic Pasteur pipette. This step is repeated five times using fresh medium each time and the five fractions of bone marrow cells (containing both haematopoietic and stromal cells) are collected and combined in a 15 ml tube. The bone marrow cell suspension is centrifuged at 300 *g* for 3 min, the supernatant is discarded and the pellet is resuspended in 1 ml of culture medium[23] (**Figure 2a**).

To isolate adult mouse osteoclast precursors, total bone marrow is isolated from the long bones (tibia and femur) of 8–12-week-old mice. First, scissors are used to cut the hindlimbs from the dead mice while keeping the long bones intact. The paws and skin are removed from the limbs and placed in PBS for transport. The limbs are transferred to a sterile Petri dish and a scalpel is used to cut through the knee joint, separating the femur and the tibia. The remaining soft tissue is scraped from the bones and the epiphyses is cut off to expose the bone marrow. The culture medium is used to flush out the bone

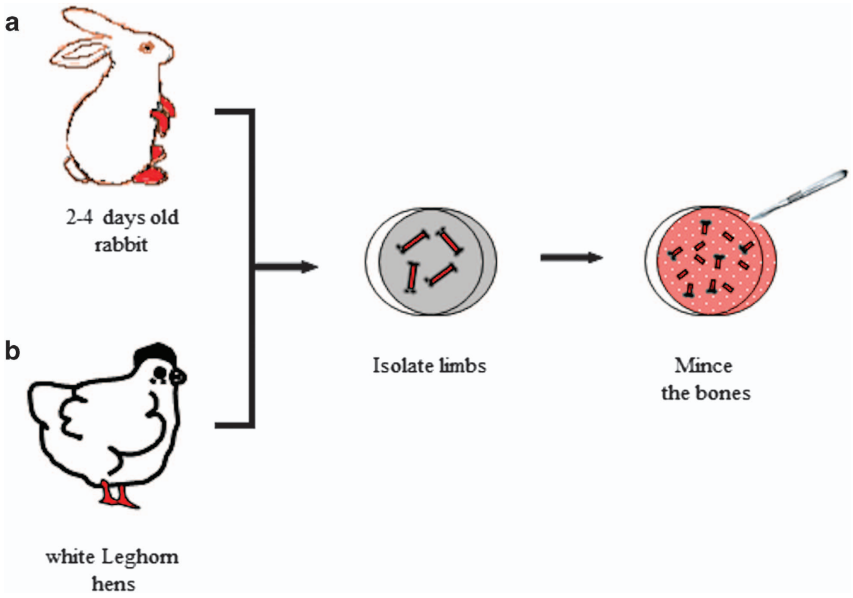

Figure 1 Schematic illustration of isolation of mature osteoclasts from 2–4-day-old rabbits (**a**) and white Leghorn hens (**b**). See text under Isolation of mature osteoclasts in Materials and methods section for more details.

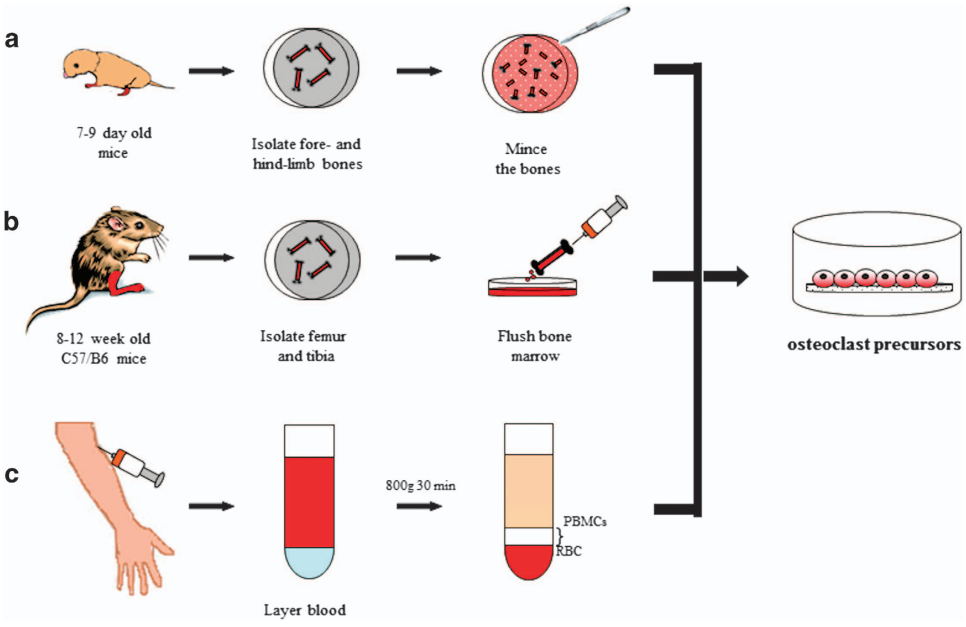

Figure 2 Schematic illustration of isolation of osteoclast precursors from new-born mice (**a**) and adult mice (**b**) peripheral blood (**c**). See text under Isolation of osteoclast precursors in Materials and methods section for more details.

marrow from each bone into a fresh sterile 60 mm Petri dish using a 5 ml syringe with a 25-gauge (G) needle. A single cell suspension is obtained by passing the flushed bone marrow through needles of decreasing size (19G→21→25G) and transferring it into a conical 15 ml tube. The cell suspension is then centrifuged at 300 *g* for 3 min, the supernatant is discarded and the pellet is resuspended in 1 ml of culture medium (**Figure 2b**).

Isolation of human osteoclast precursors. To generate human osteoclasts, density centrifugation is used to isolate human peripheral blood mononuclear cells (PBMCs) from samples of fresh peripheral/venous blood or from buffy coat. These samples must have been collected into anticoagulant vessels (e.g. EDTA or heparin coated). First, dilute 10 ml of the blood (or buffy coat) sample with 10 ml of warm, sterile PBS. Then take a conical 50 ml tube and pipet in 10 ml of Lymphoprep mixture (Ficoll-Paque may also be used and is equally potent for isolating PBMCs[24]). Slowly add the 20 ml of diluted blood sample onto the Lymphoprep using the side of the tube. The blood should form a layer on top of the Lymphoprep. Centrifuge the 50 ml tube at 800 *g* for 30 min with the brake off. This will separate the solution into three layers, of which the small middle layer contains the PBMCs. Carefully collect this layer and

transfer it into a clean 50 ml tube. Add PBS until there is a total volume of 20 ml and resuspend the cells to wash them. Centrifuge the tubes at 300 *g* for 3 min and discard the supernatant to remove any traces of Lymphoprep (additional washes may be done if necessary). Resuspend the cell pellet in culture medium (1 ml or more depending on the volume of the pellet) and estimate the cell number with a haemocytometer. The cells can be put straight into culture or aliquoted and stored in liquid nitrogen for use later[25] (**Figure 2c**).

Osteoclast generating co-cultures. Mouse osteoclasts can be generated from total bone marrow or by co-culturing previously isolated osteoblasts and bone marrow cells together. Both protocols use 1,25 Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and prostaglandin E_2 (PGE_2) to promote the expression of RANKL and M-CSF by osteoblasts, leading to osteoclast formation.

Total bone marrow is isolated as previously described and the cell suspension centrifuged at 300 g for 3 min to collect the cells. Resuspend the pellet at a final concentration of 2×10^6 cells ml^{-1} in culture medium supplemented with 10^{-8} M $1,25(OH)_2D_3$. The medium should be changed the following day and every 3 days after that. The first osteoclasts will appear between 5 and 7 days after seeding and a good number should be present between days 7 and 10.

Using the method described above it is also possible to generate rabbit and human osteoclast-like cells using whole bone marrow isolated from 2–4 days-old rabbits or human adult volunteer donors respectively.

To generate osteoclasts using the bone marrow-osteoblast co-culture system, primary mouse calvarial osteoblasts are isolated from the calvarial bones of 2-day-old mice by sequential collagenase digestion.[26] Maintain the osteoblast culture under standard cell culture conditions until it reaches confluence (~3 days). Once confluent, treat the osteoblasts with trypsin for 3 min at 37 °C in order to detach the cells; then add culture medium and transfer the cells into a fresh 15 ml tube. Centrifuge at 300 g for 3 min, discard the supernatant and resuspend the osteoblast pellet in 1 ml of culture medium. Estimate osteoblast numbers using a haemocytometer and plate them into 96-well plates at 8×10^3 cells per well in 150 μl of culture medium supplemented with 10 nM $1,25(OH)_2D_3$ and 1 μM PGE_2. The next day (day 4), isolate the bone marrow cells as previously described. Remove 100 μl of culture medium from the 96-well plates and add the bone marrow cells at 1.5-2 × 10^5 cells per well in 100 μl culture medium supplemented with 10 nM $1,25(OH)_2D_3$ and 1 μM PGE_2. Incubate the culture under standard conditions and refresh the medium every other day until multinucleated osteoclasts are formed. The osteoblast layer can easily become detached if it is disturbed, and therefore care should be taken during medium changes. This can be advantageous at the end of the culture. The corner of the osteoblast layer can be detached by pipetting PBS onto the wall of the well, and the whole layer then removed by washing with further PBS. The purified osteoclasts will remain and can now be more easily analysed. The first osteoclasts will appear on day 7 or 8 and a large number should be present between days 9 and 11[27] (**Figure 3**).

Current osteoclast culture methods

M-CSF-RANKL-generated osteoclasts. The generation and study of osteoclasts was forever changed by the identification of M-CSF and RANKL as the essential cytokines responsible for the differentiation of haematopoietic stem cells into mature and functional osteoclasts.[28–31] M-CSF is secreted by osteoblasts and binds to the membrane receptor c-Fms expressed on early osteoclast precursors.[31–33] This interaction induces the expression of receptor activator of nuclear factor κB (RANK). RANKL is found as a soluble factor or as a membrane-bound cytokine expressed on osteoblasts, osteocytes, dendritic cells, mature T cells and haematopoietic precursors.[34–37] The binding of RANKL to RANK on the surface of osteoclast precursors initiates their differentiation, and fusion into osteoclasts, and enhances osteoclast survival and activity.[38]

Recombinant M-CSF and RANKL are now widely available, allowing researchers to easily generate large numbers of osteoclasts *in vitro* and study their formation and activity in the absence of other cell types.

A variety of cell populations can be driven to differentiate into multinucleated bone resorptive osteoclasts. This includes directly treating adult mouse bone marrow cells or mouse/human PBMCs with M-CSF (human or mouse 25–50 ng ml^{-1}) and RANKL (human or mouse 10–120 ng ml^{-1}). However, the number of osteoclast precursors present in these samples can be highly variable. It is therefore desirable to normalize the number of osteoclast precursors by using mouse/human M-CSF to expand the macrophage population prior to the addition of RANKL. Alternatively, the RAW 264.7 murine macrophage cell line[38] can be used as an abundant source of RANKL-sensitive osteoclast precursors. Regardless of the source of the precursors, a failure to generate osteoclasts is most often due to inactive M-CSF or RANKL. It is therefore recommended that every batch of cytokines be tested to determinate the optimal concentration (dose–response curve) before any experimental cultures are undertaken. In addition, the serum used during a culture greatly affects the number of osteoclasts that will form. Several batches of FCS should be tested and the batch giving the best result reserved for osteoclast cultures.

Osteoclast precursors. Isolate the desired osteoclast precursors into a sterile 100 mm Petri dish and culture in medium supplemented with 25–50 ng ml^{-1} of M-CSF. After 48–72 h the attached cells will be confluent M-CSF-dependent macrophages. Wash the monolayer with 5 ml ice-cold PBS and use a cell scraper to gently scrape the adherent M-CSF-dependent macrophages from the Petri dish. Collect the cells in culture medium and transfer them to a conical 15 ml tube. Centrifuge the tube at 300 g for 3 min, discard the supernatant and resuspend the pellet in 1 ml of culture medium. Use a haemocytometer to estimate the number of M-CSF-dependent macrophages and then seed them in 96-well plates at 12×10^3 cells per well in 150 μl culture medium supplemented with 25–50 ng ml^{-1} M-CSF. Allow the M-CSF-dependent macrophages to attach overnight and then replace the medium with culture medium supplemented with M-CSF (25–50 ng ml^{-1}) and RANKL (10–120 ng ml^{-1}). Fifty per cent of this medium should be refreshed every 48 h. Osteoclasts will begin to form after 72–96 h (**Figure 4**).

RAW 264.7 cells. Rather than using primary cells, osteoclasts can be generated from some immortalized cell lines, of which the most commonly used is the murine macrophage cell line

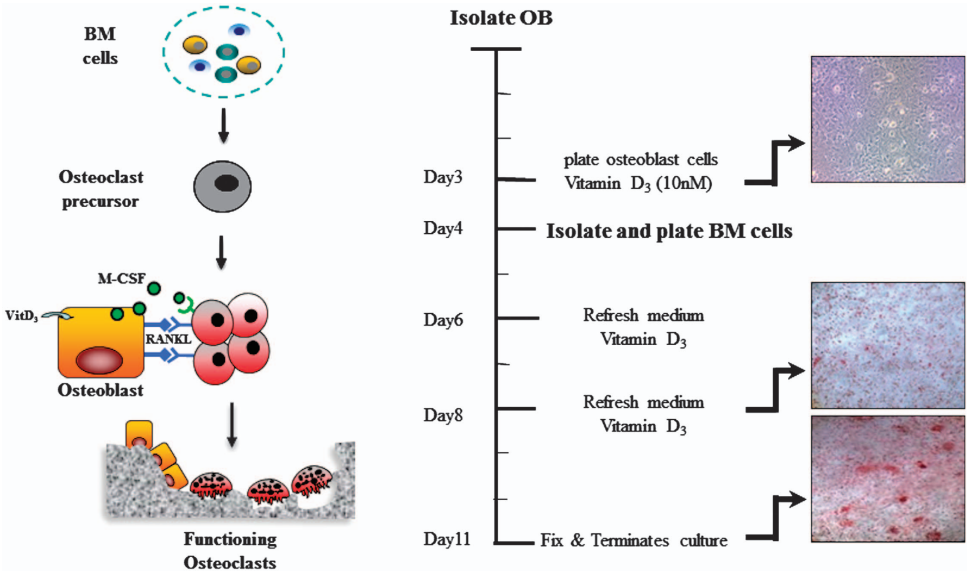

Figure 3 Timetable of bone marrow–osteoblast co-culture. See text under Osteoclast generating co-cultures in Materials and methods section for more details. This figure was adapted and modified from A.I. Idris unpublished data.

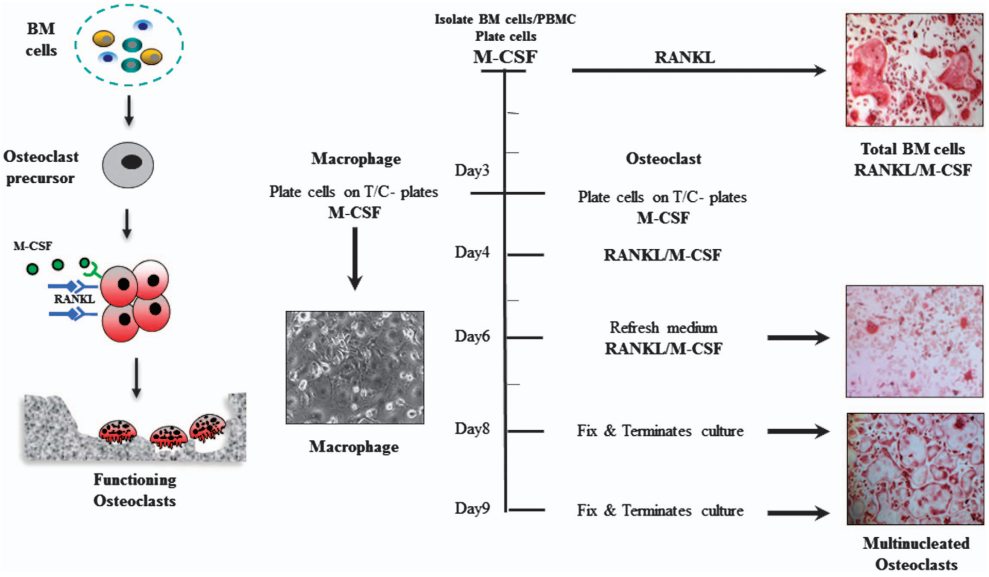

Figure 4 Timetable of M-CSF/RANKL-generated osteoclasts. See text under M-CSF-RANKL-generated osteoclasts in Materials and methods section for more details. This figure was adapted and modified from A.I. Idris unpublished data.

RAW 264.7.[39] Using a cell line rather than primary cells confers several advantages, including wide availability, removing the need to cull a live animal, and ease of transfection. However, by their nature, cell lines may not react in the same way as primary cells and should be used only in preliminary studies or in parallel to primary investigations.

Cultures of RAW 264.7 cells can be maintained indefinitely in complete medium by scraping and sub-culturing the cells 1:3 whenever confluence is reached. To differentiate the cells into osteoclasts, cells are plated into 24-well plates at a concentration of 1–4 $\times 10^4$ cells in 500 μl of complete medium supplemented with 20–100 ng ml^{-1} RANKL. As RAW 264.7 cells express both M-CSF and its receptor c-fms, no treatment with M-CSF is necessary. On day 3 the medium should be replaced with 500 μl of fresh complete medium supplemented with 20–100 ng ml^{-1} RANKL. Osteoclasts will first appear around day 4 and will be plentiful by day 5 or 6. The number of osteoclasts that form varies considerably with both the number of cells seeded and the passage number of the RAW 264.7 cells used. Early and late passages tend to produce low numbers of osteoclasts with optimal results achieved using cells from passages 4–18. RAW 264.7 cells can be grown and differentiated on plastic, glass or dentine depending on the experimental purpose and can be analysed using the same techniques as primary osteoclasts.

Induced pluripotent stem cells. Recently, Grigoriadis *et al.*[40] described an exciting new method for studying osteoclasts.

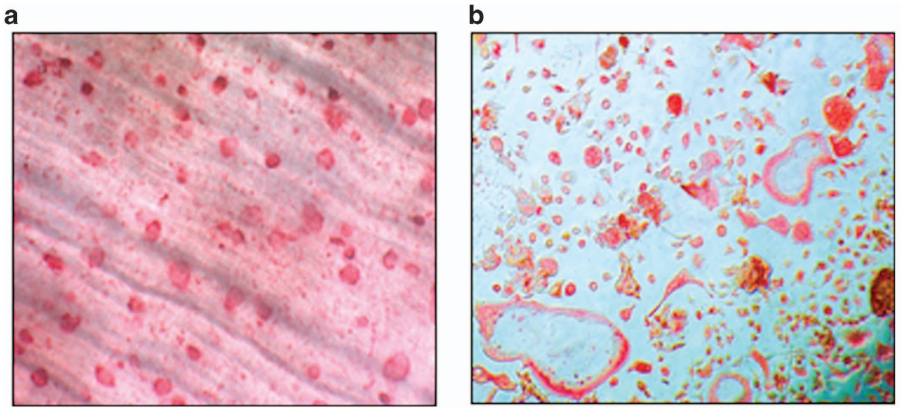

Figure 5 TRAcP-positive osteoclasts on dentin slices (**a**) and osteo assay surface multiple-well plates (**b**).

Using cocktails of growth factors and cytokines, they guided the differentiation of a reprogrammed human iPS cell line into osteoclasts. This method may possibly provide a reliable and renewable source of primary-like human osteoclasts and could allow the direct study of osteoclasts from patients with genetic disorders and the manipulation of these osteoclasts in novel genetic therapies.

Specialized embryonic cell culture techniques are required for this method and are best described in Grigoriadis *et al.*[40] In brief, the cells are differentiated over four stages: mesoderm induction, haematopoietic specification, haematopoietic expansion and maturation, and differentiation into osteoclasts. First, the formation of embryoid bodies with a mesoderm streak is induced by the treatment of 10–20 cell human iPS cell aggregates with BMP-4 and bFGF for 4 days in serum-free media. These embryoid bodies are then harvested and haematopoietic specification promoted by culturing with VEGF, bFGF, IL-6, IL-3, IL-11 and stem cell factor. On day 8 serum is added and treatment is changed to VEGF, EPO, TPO, IL-6, IL-3, IL-11 and stem cell factor. This promotes haematopoietic cell maturation and myeloid expansion. On days 18–22, the embryoid body is dissociated and the cells cultured on dentine in the presence of M-CSF and RANKL. Osteoclasts form after a further 10–14 days of culture and can be analysed using standard techniques.

Characterization of osteoclast cultures

The following assays are commonly used to characterize osteoclasts.

Staining for tartrate-resistant acid phosphatase. Multinucleated osteoclasts are characterized by high expression of the enzyme tartrate-resistant acidic phosphatase as previously described in Coxon *et al.*,[18] and Itzstein and van't Hof.[27] This can easily be stained for and visualized. To do this, upon termination of an experiment, fix and stain the culture as follows:

Fixation:

1. Carefully rinse the culture with PBS (do not scratch the bottom of the well).
2. Fix cells with 4% formaldehyde in PBS for 10 min at room temperature.
3. Rinse twice with PBS and either stain immediately or store the plates at 4 °C.

Staining: Prepare the following solutions (see Supplemented materials). Greater detail of the components is given in supplementary materials:

Solution A:

- 150 μl of Napthol-AS-BI-phosphate solution
- 750 μl of Veronal buffer
- 900 μl of Acetate buffer
- 900 μl of Acetate buffer with 100 nM Sodium Tartrate

Solution B (do not mix vigorously):

- 120 μl of Pararosanilin
- 120 μl of 4% Sodium Nitrate

Mix solutions A and B in a clean glass beaker and filter through a 0.45 μm filter. Add 50–100 μl per well (for a 96-well plate) of the filtered staining solution to the fixed cells and incubate at 37 °C. The time needed to obtain a good stain can vary between 30 and 60 min, and therefore the cells should be carefully monitored to avoid overstaining. When the desired level of stain has appeared, rinse with PBS and then add 200 μl of 70% ethanol to each well. The plates should now be wrapped in cling film and stored at 4 °C. Tartrate-resistant acidic phosphatase-positive cells (bright red) containing 3 or more nuclei are osteoclasts (**Figure 5**). The number, size and number of nuclei of osteoclasts can be analysed by counting and image analysis. Alternatively, cells can be stained using a leucocyte acid phosphatase staining kit (Sigma 387A), or acid phosphatase activity can be directly measured with the acid phosphatase assay kit (Sigma CS0740).

Detection of F-actin ring. The formation of a ring of intracellular actin filaments in the sealing zone of an osteoclast is widely used as an indicator of correct polarization and resorptive activity. To study actin rings, osteoclasts can be grown on a glass slide or in an optic bottom flask, although for physiologically accurate rings osteoclasts should be grown on dentine. At the end of the culture, cells are fixed, permeabilized in 0.5% Triton X100 in PBS for 5 min at room temperature and then incubated with 0.5 $\mu g\,ml^{-1}$ FITC- or TRITC-labelled Phalloidin for 30 min. The cells are then rinsed with PBS and F-actin rings can be visualized using fluorescence or confocal microscopy. High magnification can show the presence of

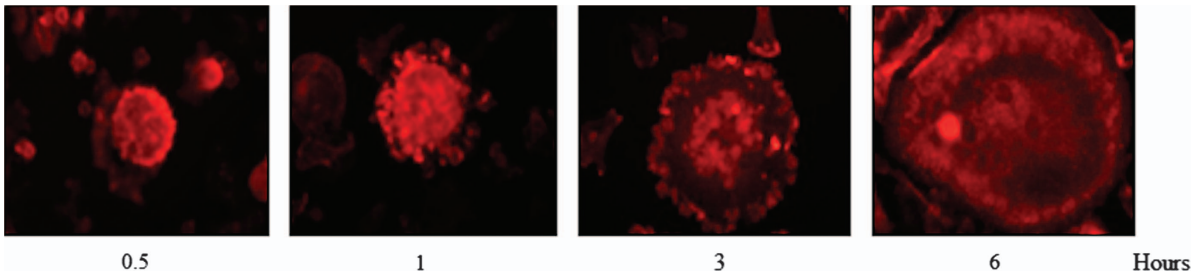

Figure 6 F-actin ring generation (TRITC-labelled Phalloidin staining).

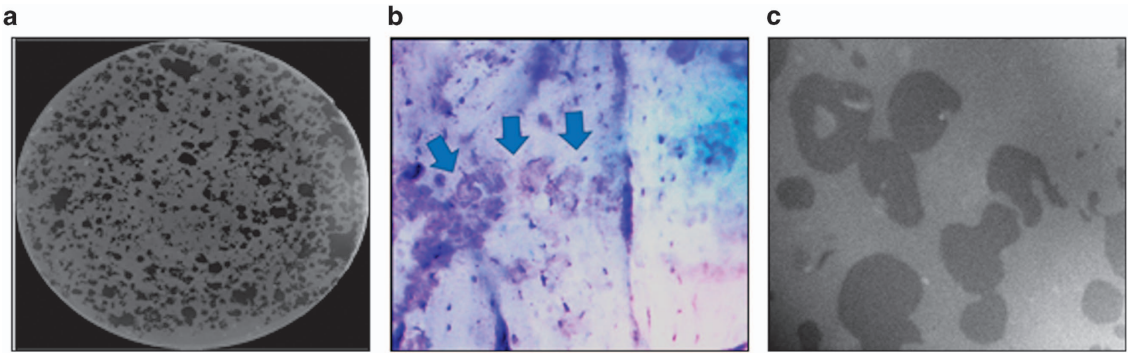

Figure 7 Resorption pits on dentin slices (**a**), bone slice (**b**) and osteo assay surface multiple-well plates (**c**).

podosomes. These are highly specialized sub-cellular components, composed of actin microfilaments, actin-binding proteins, adhesion proteins, adapter proteins, signalling proteins, tyrosine kinases and integrin receptors. During osteoclast adhesion and polarization, podosomes will appear as randomly dispersed dots. They then cluster and move to the peripheral area of the cell where they form a podosome belt or actin ring (**Figure 6**). When the podosome belt is formed, osteoclasts can start the bone resorption process. The analysis of the actin ring can be very informative about the status of an osteoclast culture. The most common measure is the percentage of mature, multinucleated osteoclasts with a complete actin ring. This quantification provides the number of osteoclasts that have the morphological characteristic of a resorbing cell and should be always calculated when a deep characterization of an osteoclast culture is required.

Resorption-pit assay. The activity of multinucleated osteoclasts can be assessed using the resorption-pit assay. To activate the resorption process, a low pH is required as osteoclasts are 'activated' at a pH of $\sim$6.9, whereas resorption is drastically reduced at pH above 7.2. Culture medium can be alkalinized with NaOH or acidified with concentrated HCl to allow resorption to occur in a controlled manner.[41,42] Mature osteoclasts, obtained using the methods described above, are removed from the dish using trypsin-EDTA solution. Cells are then plated on dentine or bone slices and allowed to resorb bone for 48 h. Pits can be analysed as follows:

(1) For dentine slices: Following tartrate-resistant acidic phosphatase staining, the surface of the slices are vigorously cleaned with tissue paper to remove the adherent cells. Resorption pits can then be visualized using a reflected light microscope as they will appear dark when compared with the unresorbed areas. The resorbed area can be quantified using ImageJ or custom image analysis software.[27] The results are expressed as number of pits per osteoclast or area resorbed per osteoclast. It is important to normalize the resorbed pits/area by the number of osteoclasts to allow comparison between different cultures (**Figure 7a**).
(2) For bone slices: The slices are vigorously cleaned with a cotton swab to remove cells and then stained with 1% toluidine blue in order to reveal the resorbed areas. Resorption exposes collagen fibres in the bone and it is these that are toluidine blue stained. The pit area can be quantified using a number of software programs as above, or the Pit index can be computed. The Pit index was the first quantification method accepted for the analysis of pits.[43] In brief, the resorption pits are divided into three visual categories according to their diameter: small, $<10\,\mu m$; medium, 10–30 μm; and large, $>30\,\mu m$. Each pit category is then given a score by multiplying the numbers of pits found by a different factor according to their dimensions: for small pits, 0.3; for medium pits, 1; and for large pits, 3. The sum of the three scores is the pit index. Osteoclast differentiation, morphology and viability can be studied using parallel bone slices (**Figure 7b**).

Osteoclast activity can also be directly detected by generating and culturing osteoclasts on osteo assay surface multiple-well plates. Following tartrate-resistant acidic phosphatase staining, the plates are incubated with a solution of 50% bleach for 10 min to remove the adherent osteoclasts. They are rinsed four times with PBS and then air-dried to visualize the surface properly. Resorption pits are visualized by light microscopy and the total area resorbed can be quantified using ImageJ software (**Figure 7c**).

Materials

All reagents and materials used for collecting the samples, isolating the cells and culturing the cells should be sterile.

- Phosphate-buffered saline, pH 7.2 (PBS).
- Isolation and culture medium: minimal essential medium (αMEM or DMEM)) supplemented with 10% fetal calf serum (FCS), 5% L-Glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (complete medium). A volume of 100 mM sodium Pyruvate and Hepes 1 M pH 7.2 can be added to the medium.
- Human/mouse recombinant RANKL at a working concentration of 10–120 ng ml^{-1}.
- Human/mouse recombinant M-CSF at a working concentration of 25–50 ng ml^{-1}.
- Lymphoprep or Ficoll-Paque.
- A 96-well plate, a 12-well plate or Petri dishes. Falcon tissue culture plate (produces the best results).
- Sterile scissors, tweezers, forceps and scalpels.
- Sterile syringes and needles (19, 21 and 25G).
- 4% Paraphormaldehyde.
- 1,25 Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$).
- Prostaglandin E2 (PGE_2).
- Trypsin 1%.

Supplemented materials

The stock solutions for TRAcP stain are prepared in glass containers as follows and kept at 4 °C.

- Veronal buffer: dissolve 1.17 g sodium acetate anhydrous and 2.94 g Veronal (Sodium 5,5-diethylbarbiturate) in 100 ml of distilled water.
- Acetate buffer: dissolve 0.82 g sodium acetate anhydrous in 100 ml of distilled water and adjusting the pH at 5.2 with glacial acetic acid.
- Pararosanilin solution: add 1 g of Pararosanilin powder to 20 ml of distilled water and add 5 ml of concentrated HCl. Carefully heat the solution to ~60 °C in water bath and stir until dissolved. Allow the solution to cool and filter.
- Naphthol-AS-BI-phosphate solution: Dissolve 10 mg ml^{-1} of Naphthol-AS-BI-phosphate in Dimethylformamide. This solution should be prepared fresh before use, though it is stable at 4 °C for up to 2 weeks.

Discussion

Obtaining a good osteoclast culture is an essential skill for a bone biologist, and is required for almost all high-level studies on bone physiology and pathology. Over the years many different protocols have been set up and tested, and hence it is now possible to select an appropriate protocol for any desired experiment or analysis.

We hope that this technical review will aid researchers in identifying the most appropriate protocol for their osteoclast studies. In fact, choosing the appropriate cellular model is essential to properly validate a hypothesis. For example, in a study investigating the effect of a drug treatment on osteoclasts, M-CSF and RANKL osteoclast cultures would be a good model for investigating direct effects on osteoclast formation. However, this culture would not account for any indirect effects of the treatment mediated by osteoblasts or stromal cells. If those were to be investigated, an osteoblast–osteoclast co-culture could be used. This culture would then give information about the overall consequences to osteoclast formation, but would not differentiate between direct and indirect effects. Moreover, although both of these methods are useful for determining how a treatment moderates osteoclast formation, they would be less informative if an effect on osteoclast function needed to be determined. In this case, mature osteoclasts grown on dentine should be subjected to the treatment. These can be from M-CSF and RANKL cultures or co-cultures, but in some cases it may be most appropriate to directly isolate the mature cells using the techniques described in the historical section.

Recent findings have revealed new and unexpected roles for the bone and bone cells. These results show how bone interacts with other areas of study, including the immune, endocrine and reproductive systems, and bring scientists from these disciplines into the bone field. We hope that this protocol outline will help new and old bone students shed light into the functions of osteoclasts.

Recommended Further Reading

Bone Research Protocols:

Orriss IR and Arnett TR. Rodent osteoclast cultures. *Methods Mol Biol* 2012;**816**:103–117.

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Conflict of Interest

The authors declare no conflict of interest.

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