Chapter 2

Osteoblast Isolation from Murine Calvaria and Long Bones

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Abstract

This chapter describes the isolation of primary mouse osteoblasts from adult mouse calvaria and long bones, as well as the process of isolation of bone cells from neonatal mouse calvaria. Osteoblasts from adult mouse bone are obtained as outgrowth from collagenase-treated bone pieces. Isolation of osteoblasts from neonatal calvaria is achieved by sequential enzymatic digestion of the bone matrix. Because of differences in origin and isolation method, each of the primary bone cell cultures described will have their own characteristics.

Key words: Osteoblast, Osteocyte, Calvaria, Long bone, Cell culture, Mouse

1. Introduction

When conducting in vitro research on bone, often a choice has to be made between the use of bone organ cultures or bone cell cultures. When using cell culture the choice is between primary cells or cell lines. The advantage of cell lines over freshly isolated cells lies in the immediate availability of large numbers of cells, the homogeneity of the cell cultures, and the expected stability of the phenotype. In the long run, however, many cell lines appear unstable to some extent, and subclones of cell lines tend to develop in different laboratories. In addition, the clonal selection generally favors rapidly growing cells, but these might not express all typical features of cells from a certain tissue, and the resulting cell line may thus not be entirely representative. In addition, although silencing RNA techniques are rapidly evolving, enabling the silencing of specific molecular targets in cell lines, transfection efficiency, and duration of silencing are still hard to control. An obvious advantage of using primary cells is that cells can be isolated from genetically

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modified animals, expressing a stable phenotype. This means that in certain experiments, the use of primary bone cells is preferred above the use of cell lines.

Peck and coworkers initiated the use of primary bone cell cultures in 1964 (1). They isolated cells from frontal and parietal bones of fetal and neonatal rat calvaria by collagenase digestion of the uncalcified bone matrix. The isolated cells were viable, proliferated during culture, and exhibited high activity of the osteoblast marker alkaline phosphatase (ALP). The real nature of the cells, however, especially the amount of contamination with connective tissue fibroblasts, could not be unambiguously defined (1). Wong and Con (1974) tried to isolate a better defined and more homogeneous cell population by removing the outer layers of the periosteum with successive collagenase treatments (2). While this method led to cell cultures that were more osteoblastic in nature, these were still not completely free from other cell types, such as osteoclast precursors (3). Other investigators have tried to improve the osteoblastic character of the isolated bone cell populations by removing the fibroblastic outer periosteum before using enzymatic digestion to isolate the cells from the calvarium (4, 5). This method resulted in two cell populations, one of which was still osteogenic after prolonged culture time (osteoblastic cells), and another one which was not osteogenic after prolonged culture (periosteal fibroblasts) (6). Nowadays, a broad range of methods is available for obtaining well-defined osteoblast-like cells in vitro (also see Chapters 1 and 3), which are widely used as tools in bone biology (7–9).

This chapter describes the isolation of primary mouse bone cells from adult mouse calvaria and long bones, as well as the process of isolation of bone cells from neonatal mouse calvaria. Due to their difference in origin and method of isolation, one might expect that each of the primary bone cell cultures described will have their own characteristics. For instance, it is likely that neonatal cell cultures contain more immature rapidly growing cells than cultures from adult bone. Indeed, it has been shown that neonatal cells show a higher basal release of nitric oxide and a higher response to 1,25-dihydroxyvitamin D_3 treatment than bone cells obtained from adult bone (10). Thus, for in vitro studies investigating the cellular behavior of adult bone, it seems advisable to use cells from adult bone fragments to best reproduce the inherent cellular properties of the adult tissue.

There are also profound differences between bones from the skull and the axial and appendicular skeleton, with regard to matrix composition (11), osteoclast functionality (12), and osteocyte morphology (13). Therefore, one might expect that differences exist between cell cultures derived from calvaria and long bones, and care should be taken which cells are used for in vitro research. We found no difference between cultured osteoblasts from adult mouse calvaria or adult mouse long bones with regard to the nitric

oxide response to mechanical loading (10), suggesting that either cell culture can be used for in vitro mechanotransduction experiments. Interestingly, it has been reported that the differential mechanosensitivity of bones from C57BL/6J and C3H/HeJ mice in vivo is also reflected in the in vitro mechanical loading response of osteoblasts derived from these mouse strains (14). All these factors should be taken into consideration when planning experiments with murine osteoblasts.

2. Materials

2.1. Tissues

Cells are obtained from the long bones and the calvaria of adult (age 9 weeks or older) mice, or from the calvaria from neonatal mouse pups (age 3–4 days).

2.2. Instruments

All of the following materials are supposed to be sterile.

1. Polystyrene plate and needles for fixing the mice.
2. Scalpels (no. 10 and 11), scissors, tweezers, and curved forceps.
3. 5 ml and 10 ml syringes, 27G½ needles and 0.2 µm disposable filter units (Schleicher & Schuell GmbH, Dassel, Germany).
4. 25 and 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark), 94/16 mm cellstar petri dishes (Greiner), and 145/20 mm cellstar (large) petri dishes.
5. 100×16 mm (10 ml) conical base test tubes with screw cap (Bibby Sterilin ltd, Staffordshire, UK) and conical base, 15 and 25 ml polypropylene centrifuge tubes (Greiner).

2.3. Media and Solutions

1. Sterile phosphate-buffered saline (PBS), pH 7.4.
2. Dulbecco's modified Eagle's medium, 1 g/l glucose + L-glutamine + pyruvate (DMEM).
3. Complete culture medium (cCM):DMEM supplemented with 100 U/ml penicillin, 50 µg/ml streptomycin sulfate, 50 µg/ml gentamycin, 1.25 µg/ml fungizone, 100 µg/ml ascorbate, and 10% FBS (e.g., Hyclone, Logan, UT, USA; see Note 1). Make fresh before each medium change and filter sterilize.
4. Collagenase II solution. 2 mg collagenase II (260 U/mg; Worthington, Lakewood, NJ, USA) per ml DMEM. Make fresh and filter sterilize.
5. Trypsin solution. 0.25% trypsin 1:250 (Gibco) plus 0.1% EDTA in PBS, filter sterilize.

6. Digestion solution: Add 1 ml trypsin solution plus 3.2 mg collagenase II (Worthington) to 4 ml PBS. Make up fresh just before use.
7. Stock solution of collagenase type I: 10 mg/ml collagenase type I in Hank's balanced salt solution (HBSS). Filter sterilize and freeze aliquots for single use at –20°C.
8. Collagenase I work solution: Dilute stock solution of collagenase type I in HBSS to 1 mg/ml just before use.
9. 4 μM EDTA in PBS. Filter sterilize and store at 4°C.

3. Methods

Normal techniques for working under sterile conditions (use of sterile media and instruments and working in a flow cabinet) should be adhered to.

3.1. Isolation and Culture of Primary Bone Cells from Adult Mouse Long Bones

1. Euthanise one or two adult mice.
2. Fixate the mouse in a supine position on a polystyrene plate or in a large petri dish, and rinse the abdomen and extremities using a small amount of 70% ethanol.
3. Make a single incision through the skin, starting at the top of the sternum and ending a few millimeters above the genitals, using a number 10 scalpel. Make a second incision starting from the top of the first incision and ending at the wrist of the upper left extremity. Repeat this procedure with the other paws. Carefully remove the skin from the abdomen with a blade.
4. Change your blade for a sterile number 11 scalpel. Carefully remove all the muscles from one of the long bones (femur, tibia and fibula, or humerus, radius and ulna), and thoroughly scrape the bone with a scalpel until it is clean (see Note 2). Excise the long bone and place it in a petri dish with PBS. Continue until all the bones are excised.
5. When all the long bones have been removed, cut off the epiphyses.
6. Thoroughly flush out the bone marrow with PBS, using a 5 ml syringe and a 27 gauge needle.
7. Cut the clean diaphyses into little pieces of approximately 1–2 mm^2 using scissors.
8. The bone pieces are washed several times with PBS, and incubated in 4 ml collagenase II solution at 37°C in a shaking water bath in order to remove all remaining soft tissue and adherent cells.

9. Vigorously shake the collagenase II solution containing the bone pieces, by hand, every 30 min.
10. After 2 h, rinse the bone pieces three times with cCM, shaking the solution containing the pieces for a few seconds during every wash step.
11. Transfer the bone pieces to 25 cm^2 flasks, containing 5 ml cCM, at a density of about 20–30 fragments per flask. Replace culture medium three times per week. After each medium replacement, make sure that the bone chips are evenly distributed over the bottom of the culture flask, by gently swirling the culture flask.
12. Adult mouse bone cells will start to migrate from the bone chips after 3–5 days. On average the cells growing from the bone fragments will be ready for use after 11–15 days. Do not allow the cells to become over-confluent around the bone chips.
13. To obtain more cells, cCM is removed from the flask, cells, and bone pieces are gently washed three times with PBS, and incubated in 1 ml trypsin solution at 37°C for 10 min. Swirl and tap the flask on a level surface a few times during the 10 min incubation period. Carefully check whether the cells are released from the tissue culture plastic using a microscope. Remove the trypsin solution containing the cells using a small pipette. Leave the bone pieces in the flask, and discard.
14. The cells are plated at $2.5–5 \times 10^3$ cells per cm^2 in T25 or T75 flasks in cCM.
15. Medium is changed three times per week, and after approximately 7–10 days cells will reach subconfluency, upon which they can be used for experiments (see Note 3). The average number of cells thus obtained is between 4×10^6 and 6×10^6 cells.

3.2. Isolation and Culture of Primary Bone Cells from Adult Mouse Calvaria

1. Euthanise two adult mice and fixate them on a polystyrene plate, or in a large petri dish.
2. Clean the head using 70% ethanol, and make a cut through the skin at the base of the skull, using scissors.
3. Make an incision starting at the nose bridge, and ending at the base of the skull. Remove the skin from the top of the head.
4. Use scissors to cut through the bone at the base of the neck. Cut the calvaria loose while holding the head with curved forceps placed in the orbita (see Fig. 1).
5. Transfer the calvaria to a petri dish with PBS and remove the soft tissues using tweezers or by scraping with a knife (see Note 2).
6. Remove the sutures using scissors, and chop the remaining bone into small fragments of approximately 1–2 mm^2.

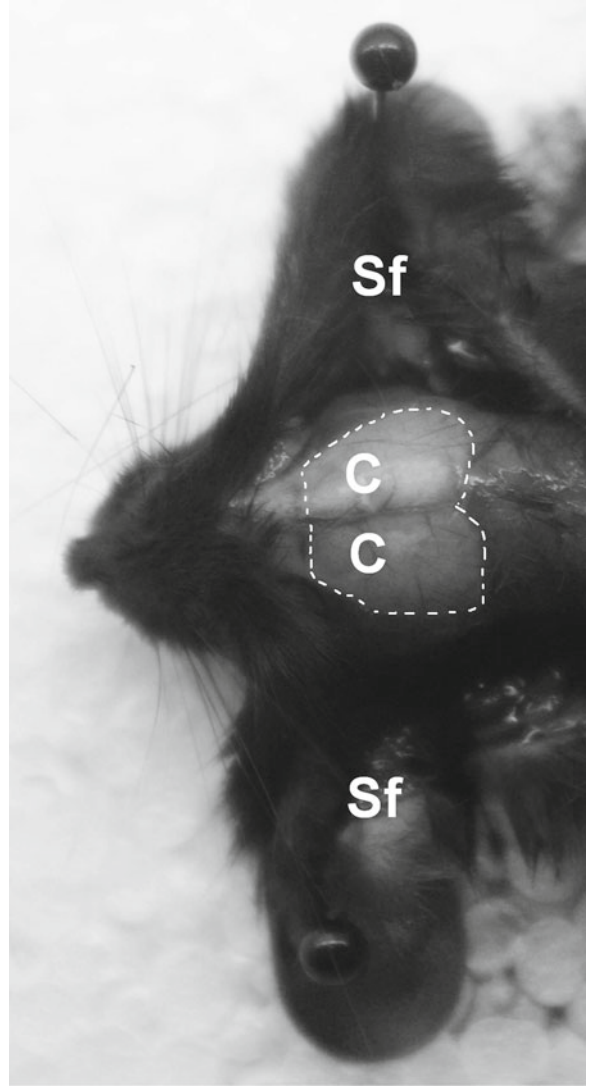

Fig. 1. Mouse calvaria. Make a cut through the skin at the base of the skull, using scissors. For adult tissue, make an incision starting at the nose bridge, ending at the base of the skull, and remove the skin from the top of the head. Use scissors to cut away the skin from the top of the neonatal mouse heads. Dissect calvaria as indicated by the dotted line and remove as much soft tissue as possible. Do not include the area near the neck, since this will result in heavy fibroblast contamination of the cultures. *Sf* skin flap, *C* calvaria.

7. Incubate the fragments for 30 min in 4 ml collagenase II solution at 37°C in a shaking waterbath.
8. Discard the collagenase II solution and replace with fresh collagenase II solution. Incubate another 30 min and then replace the collagenase II solution for trypsin solution.
9. Incubate in trypsin for 30 min. Replace by 4 ml collagenase II solution for the fourth and final incubation step of 30 min.

10. Rinse the bone pieces three times with cCM and transfer the bone pieces to 25 cm^2 flasks, containing 5 ml cCM, at a density of about 20–30 fragments per flask.
11. Medium is changed three times per week. After each medium replacement, make sure that the bone chips are evenly distributed over the bottom of the culture flask, by gently swirling the culture flask. Adult mouse bone cells will start to migrate from the bone chips after 3–5 days.
12. On average the cells growing from the bone fragments will reach subconfluency after 11–15 days, upon which the cells are incubated with 1 ml trypsin solution at 37°C for 10 min. Swirl and tap the flask on a level surface a few times during the 10 min incubation period. Remove the trypsin solution containing the cells using a small pipet. Leave the bone pieces in the flask and discard.
13. The cells are plated at 2.5 to 5×10^3 cells per cm^2 in T25 or T75 flasks in cCM.
14. Medium is changed three times per week, and after approximately 7–10 days cells will reach subconfluency, upon which they can be used for experiments. The average number of cells thus obtained lies between 4×10^6 and 6×10^6 cells.

3.3. Isolation and Culture of Bone Cells from Neonatal Mouse Calvaria

1. Euthanise 20–30 neonatal mice pups (2 nests) by means of decapitation, or inhalation of halothane, and place the heads in a petri dish with PBS (see Note 4).
2. Grab a head in the nape of the neck, and cut the skin away using scissors.
3. Hold the head with curved forceps placed through the orbita, cut the calvaria loose along the edge, and place it in a petri dish with PBS (see Fig. 1).
4. Pin the calvaria down with tweezers and cut away the edges and sutures with a small scalpel. Transfer the calvaria halves to a 25 ml tube with PBS and wash twice with PBS.
5. Incubate the calvaria in 4 ml digestion solution at 37°C in shaking water bath. After 10 min shake the calvaria by hand for a few seconds.
6. Incubate for a total of 20 min, and then transfer the supernatant containing cells to a 10 ml tube. Add 700 µl FBS to the cell suspension to inhibit trypsin activity.
7. Wash the calvaria with 3 ml DMEM (without FBS!), shake well, and add the supernatant to the tube containing the cell suspension. This is population number 1.
8. Add new digestion solution to the calvaria, and repeat the previous three steps to obtain population number 2. During the 20 min that the calvaria have to incubate in the water bath,

centrifuge cell population number 1 at 300×*g* for 5 min. Discard supernatant, resuspend cell pellet in 1 ml cCM, and add to 17 ml cCM. Pipette in a 6-well plate at 3 ml cell suspension per well.

9. Repeat the entire procedure for a total of four times to obtain population 1–4.
10. Culture medium is changed 1 day after isolation of the bone cells.
11. Within approximately 5 days cells will reach subconfluency, upon which they are trypsinized by incubation with 200 µl trypsin solution per well, at 37°C for 10 min.
12. To enhance the number of cells available for experiments, population 1 and 2, resembling osteoblast progenitor cells, are usually pooled, as well as population 3 and 4. This latter pooled cell population is enriched with cells exhibiting biochemical characteristics of differentiated osteoblasts, such as high ALP activity and osteopontin expression. Both pooled and unpooled populations can be directly used for experiments.
13. The number of cells obtained using this method varies between 6×10^6 and 10×10^6 cells.

3.4. Isolation and Culture of Bone Cells from Neonatal Mouse Calvaria (Alternative)

Although we prefer the use of collagenase type II, other groups have reported use of crude collagenase type I, which is cheaper and intrinsically contains trypsin as a contaminant. Therefore, an alternative protocol for isolation of osteoblasts from neonatal mouse calvaria, which uses alternate collagenase I and EDTA incubations to remove as much mineralized matrix as possible, and increases cellular yield, is given below:

1. Dissect calvaria as described in Subheading 3.3 steps 1–3 and collect them in 3 ml HBSS in a 25 ml centrifuge tube in a shaking waterbath at 37°C.
2. Incubate calvaria in 3 ml collagenase I work solution for 10 min in a shaking waterbath at 37°C.
3. Replace the collagenase I work solution (fraction 1) with fresh solution and discard fraction 1.
4. Incubate for 30 min in collagenase I work solution. Collect the solution containing cells (fraction 2) and place in a conical centrifuge tube. Wash calvaria in 7 ml of PBS and add wash to fraction 2.
5. Add EDTA solution to the calvaria and incubate for 10 min at 37°C. Collect EDTA solution (fraction 3). Wash calvaria in 7 ml HBSS and add wash to fraction 3.
6. Add collagenase I work solution to the calvaria and incubate for 30 min at 37°C. Collect the solution containing cells (fraction 4)

and place in a conical centrifuge tube. Wash in HBSS and add wash to fraction 4.

7. Further fractions, presumably containing more mature osteocyte-like cells, can be collected by repeating steps 5 and 6, but cell yields will be increasingly lower.
8. Centrifuge all fractions immediately after collection (250 × *g* for 5 min) and resuspend pellets in cCM (see Subheading 2.3.3).
9. Plate out pooled or single fractions in 75 cm^2 culture flasks using cells derived from 2 to 3 animals/flask.
10. Cultures will be confluent in 3–4 days. To minimize contamination by other adherent cell types, replace medium once cells have adhered (2–3 h after plating). Use the more differentiated fractions (3 and 4) for osteoclast cocultures (see Chapter 12, this volume).

4. Notes

1. Addition of serum to the medium is necessary for the survival and stimulation of proliferation of the primary mouse bone cells. However, "serum" is not a constant and homogeneous product, and the growth rate of primary bone cells can vary considerably between several batches of serum. It is therefore recommended to test several batches of serum on their cell proliferative ability and continue to use the one that produces the best results.
2. Sometimes, the primary bone cultures can contain fibroblasts, which grow faster than the bone cells and can quickly overgrow the primary bone cell cultures. If this problem occurs, care should be taken to remove all soft tissues by scraping the bones with a knife before starting the collagenase treatment. Also make sure the collagenase is not expired, and that the collagenase solution is made fresh every time.
3. Primary bone cell cultures are not 100% pure and may contain some fibroblasts and other cell types that are not from the osteoblast lineage. The osteoblastic phenotype of the primary mouse bone cell cultures can be determined by stimulating the cells with 1,25dihydroxyvitamin D_3, which should lead to enhanced ALP activity (11). The exact nature of the bone cells that are isolated from adult long bones and adult calvaria has not been fully determined. Since the cell isolation protocols involve mechanically removing the soft tissues first and then all adhering cells by means of incubation with collagenase, the cells that are isolated from the bones might represent osteocytes that reverted to proliferation after several days of exposure to serum.

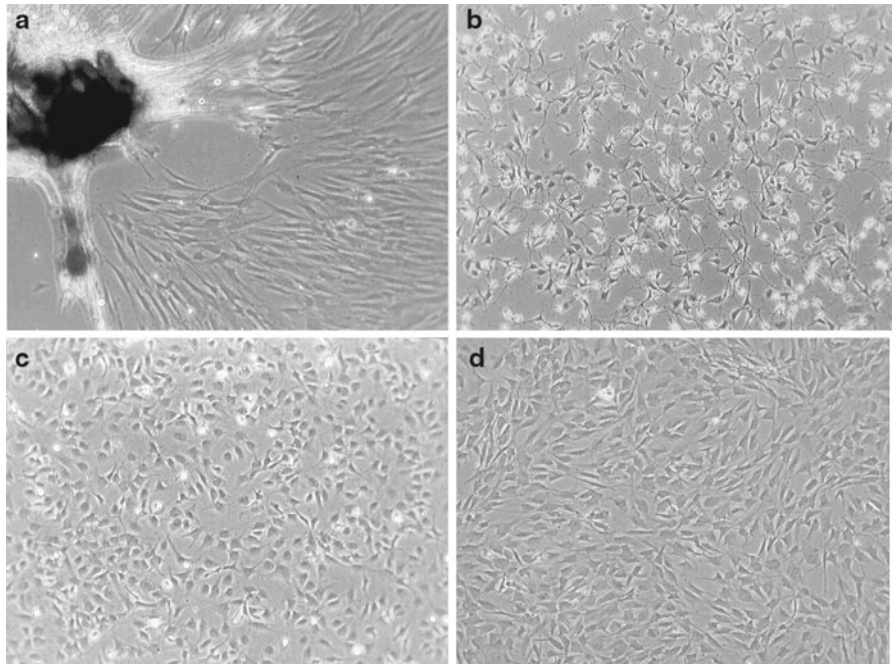

Fig. 2. Phase contrast microscopy of primary mouse bone cell cultures. (**a**) Adult mouse bone cells growing out of the bone chips, day 6 of culture. (**b**) Subconfluent layer of adult mouse bone cells, first passage. (**c**) Neonatal mouse calvarial cells, population number 1 and 2, day 2 of culture. (**d**) Neonatal mouse calvarial cells, population number 3 and 4, day 2 of culture. Note the oblong trapezoid shaped morphology of the osteoblastsl.

The microscopical appearance of the isolated bone cells is mostly osteoblastic (Fig. 2), but mRNA expression of several markers for (pre)osteocytes, such as MEPE, Phex, and DMP1 can be detected by means of PCR. Absence of staining for von Willebrand factor (factor VIII) shows that the bone cell cultures do not contain endothelial cells.

4. Smaller numbers of calvaria can be used successfully, leading to a proportionately lower yield of osteoblasts.

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