Generation of rodent and human osteoblasts

Sarah E B Taylor, Mittal Shah & Isabel R Orriss

Print

Abstract

This paper describes the isolation, culture and staining of primary osteoblasts from neonatal rodents and human samples. The calvaria and long-bone assays allow direct measurement of bone matrix deposition and mineralisation, as well as producing osteoblasts at defined stages of differentiation for molecular and histological analysis. Culture of human osteoblasts enables cell function to be investigated in targeted patient groups. The described methods will provide a step-by-step guide of what to expect at each stage of the culture and highlight the varied tissue culture conditions required to successfully grow osteoblasts from different sources. A special focus of this paper is the methods used for analysis of bone mineralisation and how to ensure that nonspecific mineral deposition or staining is not quantified.

Subject terms: Cell biology

Introduction

Osteoblasts, the bone-forming cells, are derived from mesenchymal stem cells. *In vitro* culture of osteoblasts constitutes a central part of research into the regulation of bone formation. Many approaches have been developed to study osteoblasts including primary cell cultures, bone organ cultures and immortalised osteoblast-like cell lines (for example, MC3T3-E1, MG-63 and SaOS-2). Together, these techniques have provided abundant information about the processes regulating osteoblast proliferation, differentiation, survival and function.

Several techniques for obtaining primary osteoblasts have now been described.[1, 2, 3] These methods use cells isolated from different skeletal locations (for example, long bones, calvaria) and animal models (for example, human, rat and mouse). The *in vitro* culture of calvarial osteoblasts from neonatal rats is an important and widely used technique for studying the regulation of osteoblast function. This method provides high numbers of cells and is relatively easy to do, making it ideal for experimental

purposes. Osteoblasts isolated from long bones are less frequently used but also represent an important source of cells.[4] Although the mechanisms of bone formation differ between the calvaria (intramembranous ossification) and the long bones (endochondral ossification) recent work has shown that osteoblasts isolated from these different anatomical locations behave in a similar manner *in vitro*.[4]

The calvarial and long-bone osteoblast bone-formation assays have several advantages, that make them good models for studying the cellular responses to exogenous stimuli. First, they allow the key function of osteoblasts, namely bone formation, to be quantified.[1] Second, it enables the processes of bone matrix deposition and mineralisation to be studied independently.[5] Finally, osteoblasts can be studied at clearly identified stages of differentiation from immature, proliferating cells through to the mature bone-forming osteoblasts.

These methods also allow osteoblast activity to be studied in an environment that is relatively free from the influence of other cell types normally found in bone such as endothelial and haematopoietic cells. Furthermore, it allows the extracellular environment (for example pH and pO_2) to be tightly controlled and if required, manipulated, in a manner which is not possible *in vivo* or using bone organ cultures.[6, 7]

Human osteoblasts, from several anatomical sources, have also been widely used to study osteoblast physiology.[2, 8, 9, 10, 11] Human cells obtained from explant bone tissues consist of a heterogeneous cell population that predominantly consists of early osteoblasts. *In vitro* these cells can be differentiated into mature, bone-forming osteoblasts by the addition of glucocorticoids, vitamin D and bone morphogenetic proteins.[8, 9, 10, 11] Although slower growing compared with neonatal rodent cells, the use of human osteoblasts allows cell function to be investigated in patients suffering from age-related bone diseases.

Here we describe methods for the isolation of rat calvarial and long-bone osteoblasts from the same animal. We also describe how these methods should be adapted to successfully culture mouse osteoblasts, which appear to require more nutritional support *in vitro*. Finally, we describe how to culture primary human osteoblasts from bone samples.

Materials and methods

Materials

All solutions, instruments and tissue culture plastics must be sterile. Tissue culture reagents were obtained from Life Technologies (Paisley, UK) and, unless stated, all other reagents from Sigma-Aldrich (Poole, UK).

- Animals: Cells are obtained from the calvaria and long bones of neonatal rats (2–3 days) and mice (2–5 days). One neonatal rat will produce 7×10^6–10^7 and 5×10^6 cells from the calvaria and long bones, respectively. One mouse calvaria will typically yield 3–5 $\times 10^6$ cells.

- Human specimens: Cells are obtained from the trabecular region of the humeral head from

patients undergoing total shoulder replacements. The amount of trabecular specimen received will vary depending on surgeries, but a specimen of ~5mm^3 will produce 1–2 × 10^6 cells. Other anatomical sources of bone can also be used.

- Phosphate-buffered saline (PBS): For storing tissues and washing cells. The addition of 100 U per ml penicillin, 100 μg per ml streptomycin and 0.25 μg per ml amphotericin (known as antibiotic/antimitotic or AB/AM) is recommended for human cells (PBS+AB/AM).

- Alpha-modified essential medium (αMEM): Add 10% foetal calf serum, 2 mM L-glutamine and AB/AM. Rat and human cells can also be cultured in Dulbecco's modified Essential medium (DMEM), supplemented as αMEM (Note 1). This supplemented medium is usually stable for ~4 weeks.

- Osteogenesis αMEM (osMEM) or DMEM (osDMEM): To αMEM or DMEM add 50 μg per ml ascorbate, 2 mM β-glycerophosphate (Note 2) and 10 nM dexamethasone (not mouse cells, Note 3). Always make fresh on day of use.

- Sodium hydroxide (NaOH): 6 M NaOH to alter the pH of the culture medium if required (Note 4).

- Trypsin-EDTA: 0.25% (*w/v*) trypsin with 1 mM EDTA.

- Accutase: with PBS containing 0.5 mM EDTA and 3 mg per l phenol red for human cell disassociation (Note 5).

- Collagenase: 0.2% (*w/v*) collagenase solution (Type II collagenase from *Clostridium histolyticum*, made up in Hank's balanced salt solution).

- Fixative: 2.5% glutaraldehyde in dH$_2$O, 70% ethanol or 4% paraformaldehyde in PBS (pH 7.4).

- Alizarin red stain for mineralised bone nodules: 1% (*w/v*) alizarin red in dH$_2$O pH 5.5.

- Alkaline phosphatase (tissue nonspecific alkaline phosphatase (TNAP)) staining: Required components—naphthol AS-MX-phosphate, dimethylformamide and fast blue b salt.

- TNAP activity: SensoLyte pNPP TNAP assay kit (Anaspec, Fremont, CA, USA).

- Sirius red stain for deposited collagen: Sircol dye reagent (Biocolor, County Antrim, UK).

- Masson's trichrome collagen staining: required components—Bouin's fixative, Weigert's haematoxylin solution, 0.5% acid fushcin solution with 5% acetic acid, phosphomolybdic acid solution, 2% methyl blue solution with 2.5% acetic acid and 1% acetic acid solution.

- Tissue culture plastics: Large petri dishes (100 mm), 5 ml flat-bottomed tubes, 15 ml and 50 ml centrifuge tubes, 25 and 75 cm^2 tissue culture flasks and 6-, 12- or 24-well tissue culture plates (Note 6).

- Dissection tools: Scalpels and blades (no.20), rongeurs, bone cutters, tweezers and scissors.

Methods

Isolation of primary osteoblasts from neonatal rodent calvaria

- Euthanise 2–6 neonatal rats (2–3 days) or mice (2–5 days) and sterilise with 70% ethanol. Remove the head using large scissors; retain the body for long-bone isolation.

- Remove the skin and brain tissue from the skull using a scalpel and tweezers.

- Cut away the jaw and carefully scrape off any excess tissue and cartilage from around the calvaria.

- Cut the calvaria in half and place in a flat-bottomed 5-ml tube; wash with PBS.

- Repeat steps 2–5 as required.

- Incubate in 0.25% trypsin (500 µl per calvaria) for 10 min at 37 °C. Remove and discard the trypsin solution; wash in αMEM or DMEM.

- Incubate in 0.2% collagenase solution (600 µl per calvaria) for 30 min at 37 °C.

- Remove the collagenase digest, discard and replace with fresh solution for 60 min at 37 °C.

- Transfer the final digest to a 15-ml centrifuge tube. Wash any remaining calvaria with αMEM or DMEM (5 ml) and add the solution to the final digest.

- Spin at 1500 g for 5 min. Discard the supernatant and resuspend the cells in αMEM or DMEM (1 ml per calvaria).

- Rat cells: Add 20 ml of αMEM or DMEM and 1 ml cell suspension per 75 cm^2 flask (1 calvaria per flask).

- Mouse cells: Pool the cell suspensions; add 17 ml of αMEM and 3 ml cell suspension per 75 cm^2 flask (3 calvaria per flask). If the calvaria need to be kept separate (for example, different genotypes) expand cells in a 25 cm^2 flask (7 ml of αMEM plus 1 ml cell suspension).

- Incubate the flask at 37 °C/5% CO_2 until the cells become confluent (~3 days and 5–6 days for rat and mouse cells, respectively).

Isolation of primary osteoblasts from the long bones of neonatal rats

- Remove the limbs from the body by cutting with sharp scissors taking care to preserve as much of the limb as possible.

- Using a scalpel cut off the paws and cut the limb in half (at the joint).

- Remove the skin and scrape away the soft tissue. Cut off the epiphyses and place the bone fragments into a flat-bottomed 5 ml tube. Wash and vortex in PBS to remove any residual soft tissue.

- Incubate the bone fragments in trypsin (1 ml per animal) for 10 min at 37 °C. Remove and discard trypsin solution; wash in DMEM.

- Incubate in collagenase solution (1 ml) for 30 min at 37 °C.

- Remove the collagenase digest, discard and replace with fresh solution for 60 min at 37 °C.

- Keep the final digest and transfer to a 15-ml centrifuge tube. Wash remaining bone fragments with DMEM (5 ml), add the solution to the final digest.

- Spin at 1500 g for 5 min. Discard the supernatant and resuspend the cells in DMEM (1 ml). The initial cell suspension may be slightly more heterogeneous compared with a calvarial bone isolate but behaves similarly on culture in osDMEM.[4]

- Add 20 ml of sDMEM to a 75-cm^2 flasks; add cell suspension (1 ml per flask).

- Incubate the flask at 37 °C/5% CO_2, until the cells become confluent (~3–4 days).

Isolation of primary osteoblasts from human shoulder humeral head trabecular bone

- Patients who are undergoing total shoulder replacements for osteoarthritis can provide a source of bone for explant cultures (consult local Ethical Committee for guidelines on seeking ethical approval and patient consent). This tissue is remote from the shoulder joint and free from contaminating soft tissue. Transfer tissue into a sterile container with PBS+AB/AM and transport to laboratory with minimal delay (Note 7).

- Remove soft connective tissue and coagulated marrow from the outer surfaces of the bone by scraping.

- Place tissue in sterile petri dish containing 5–10 ml of PBS+AB/AM. Using rongeurs, bone cutters and scissors carefully cut the bone into 2–4 mm fragments.

- Carefully decant the PBS+AB/AM and transfer the fragments into a 50-ml universal container with 10–15 ml PBS+AB/AM. Vortex for 3 × 5 s; allow bone fragments to settle for 30 s. Carefully decant off the supernatant to remove haematopoietic marrow, marrow fat and dislodged cells. Add additional 10–15 ml of PBS+AB/AM and vortex as before. Repeat this process at least six times or until no remaining haematopoietic marrow and marrow fat is visible and the bone fragments have assumed a white ivory-like appearance (Note 8).

- Incubate the fragments in 1% trypsin for 10 min at 37 °C. Remove and discard trypsin solution; wash in DMEM, then PBS.

- Incubate in 0.2% collagenase solution (enough to cover the fragments) for 30 min at 37 °C.

- Remove the collagenase digest, discard and wash with DMEM.

- Culture these fragments as explants at a density of 0.2–0.5 g tissue per 100 mm diameter in a petri dish or a 75-cm^3 flask in 10 ml of DMEM at 37 °C/5% CO_2.

- Leave the cultures undisturbed for 7 days to ensure the outgrowing cells adhere to the tissue culture plastic. The first cells will be observed after 7–10 days of culture; cells will be confluent after 6–8 weeks. Medium should be exchanged every 7 days.

Osteoblast bone mineralisation assay. This protocol is used for rodent and human osteoblasts.

- Once the cells are confluent remove the media and wash with PBS. Add 1 ml per flask trypsin-EDTA (rodent) or 2 ml per flask Accutase (human) and incubate for 10–15 min at 37 °C.

- Add αMEM (10 ml) to inactivate the trypsin; transfer the cell suspension to a 15 ml centrifuge tube.

- Spin at 1500 *g* for 5 min. Discard the supernatant and resuspend the cells in αMEM or DMEM (1 ml per flask).

- Perform a cell count using a haemocytometer; seed the cells in tissue culture trays (Note 6) in osMEM or osDMEM. Human osteoblasts should be seeded in DMEM to allow cell growth; once confluent, medium can be replaced with osDMEM (Note 3). This is day 1 of culture.

- Exchange half the medium every 2–3 days.

- Rodent cultures will be fully confluent by day 4; the overall culture duration will depend on the cells used (Note 9). Rat calvarial osteoblasts will begin to form discreet bone nodules from ~day 10 with extensive mineralised nodules present by day 14 (Figure 1). Mouse osteoblasts are significantly slower with bone nodules not evident until ~21 days (Figure 1). Rat long-bone osteoblasts will display collagen deposition from day 10, giving rise to dense, and organised trabecular-shaped networks by day 20 (Figure 2). Collagen deposition and mineralisation can be visualised in detail by electron microscopy (Figure 3).

- Human osteoblasts from younger patients (<40 years old) will begin to form bone nodules from day 21 giving rise to dense, organised trabecular-shaped networks by day 36. Human osteoblasts from aged diseased individuals (>65 years old) will form bone nodules from day 36 giving rise to organised trabecular-shaped networks by day 56. It is important to note that depending on disease state and age/sex/race of the patient, time taken for *in vitro* bone formation may vary (Figure 4, Note 10).

- The pH should be monitored throughout and maintained at ~pH 7.4 by adding 6 M NaOH if

required (Note 5).

- TNAP activity, collagen production, cell number and gene expression can be measured at clearly defined stages of osteoblast differentiation (Figures 1, 2 and 5, Note 9).

Figure 1
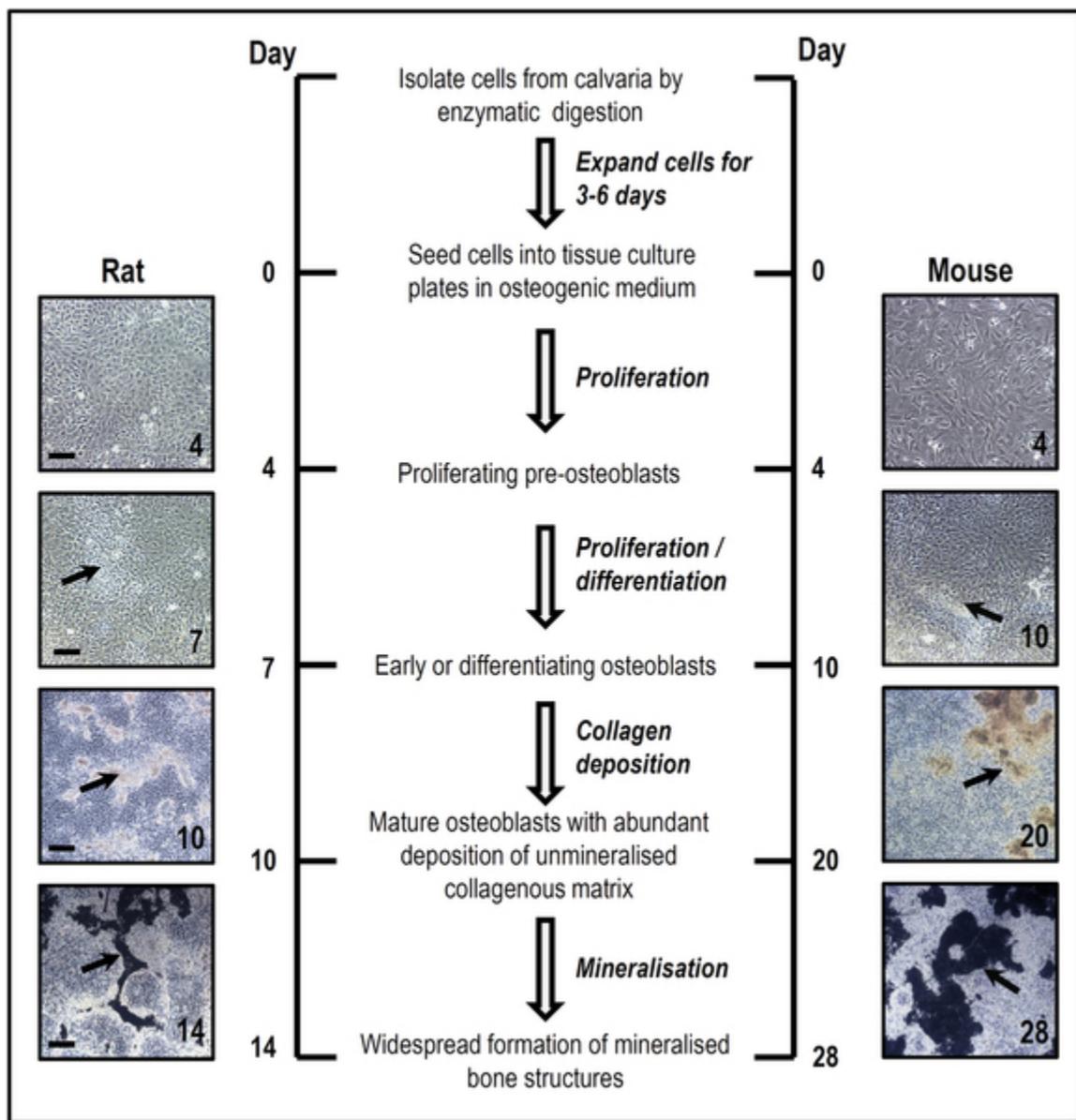
Calvarial osteoblast cultures. Rat calvarial osteoblast cultures are ~14 days in duration when grown in DMEM. Representative images of unstained cell layers show that by day 4 of culture, a confluent monolayer of preosteoblasts is evident. At day 7, the cells are more compacted and organic matrix is starting to be deposited (as shown by the arrow). By day 10, there is abundant deposition of unmineralised collagenous matrix, and after 14 days there is widespread formation of mineralised bone structures. Mouse osteoblasts take ~28 days to form mineralised matrix nodules. Representative images of mouse cells show the defined stages of differentiation at 4, 10, 20 and 28 days of culture. Scale bars: day 4/7=50 µm, days 10/14=500 µm.

Figure 2
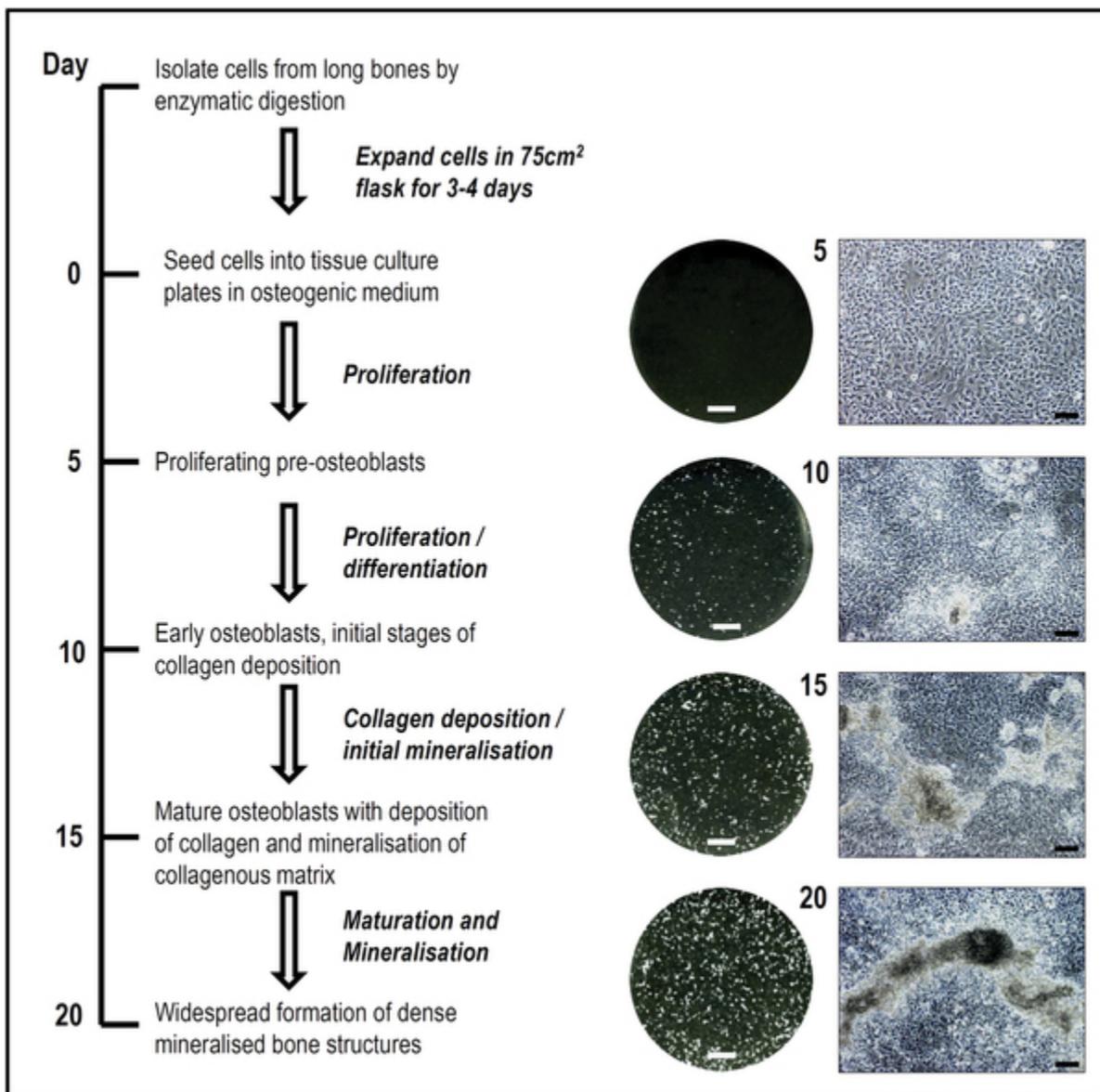
Long-bone osteoblast cultures. Rat long-bone osteoblast cultures are ~15–20 days in duration when grown in DMEM. Representative images of unstained cell layers and bone formation show that by day 5 of culture, a confluent monolayer of preosteoblasts is evident. At day 10, the cells are more compacted and the initial stages of collagen matrix deposition are evident. The collagen matrix is much denser by day 15 and mineralisation of collagenous structures can be seen. By day 20, there is abundant deposition of mineralised bone structures. Scale bars: whole-well scan=50 mm, phase contrast images=100 μm.

Figure 3
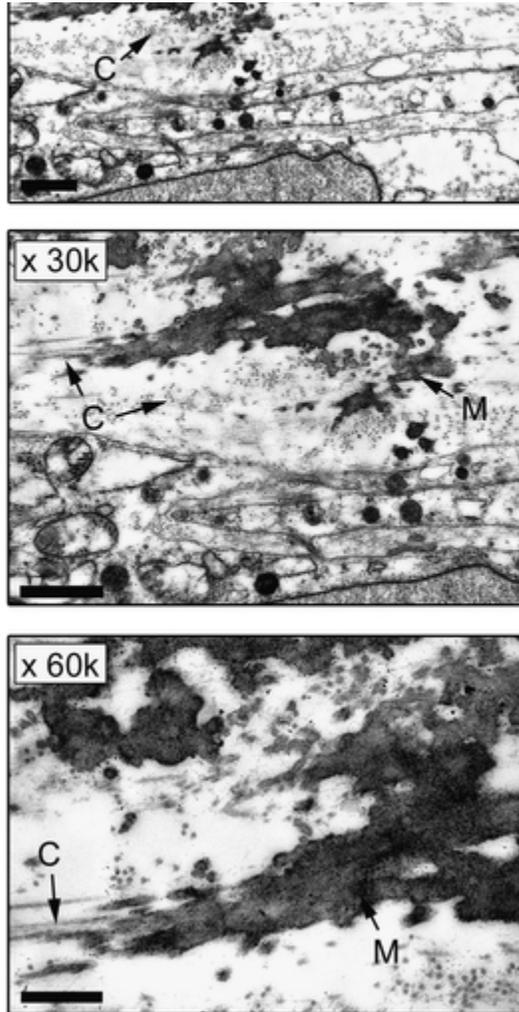
TEM images of the extracellular matrix produced by long-bone osteoblasts. Long-bone osteoblasts were grown in osDMEM for 28 days and then viewed by TEM. **C**=collagen fibres, which can be seen longitudinally and in transverse section (where they appear as dots). **M**=specific mineralisation of the collagenous extracellular matrix. Magnification at × 20 k and × 30 k scale bar=1 µm, × 60 k scale bar=500 nm.

Figure 4
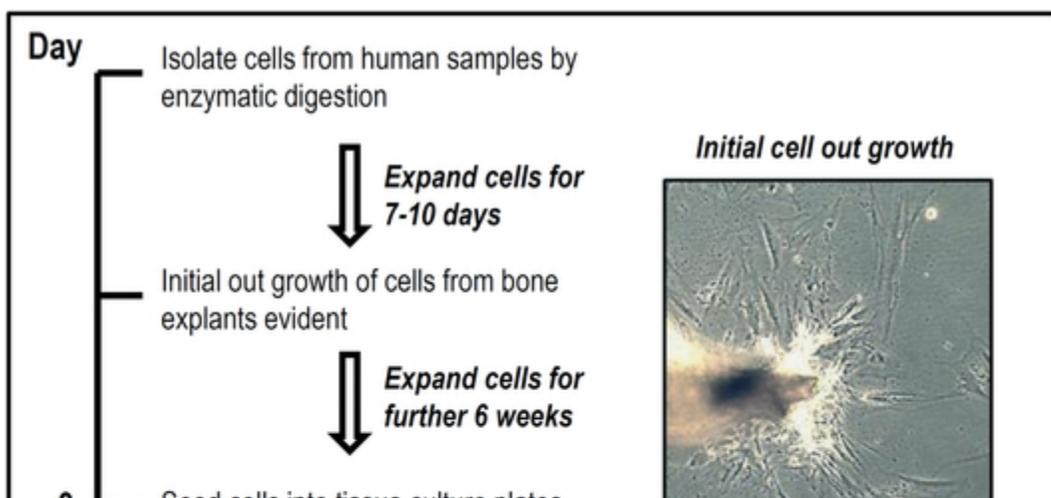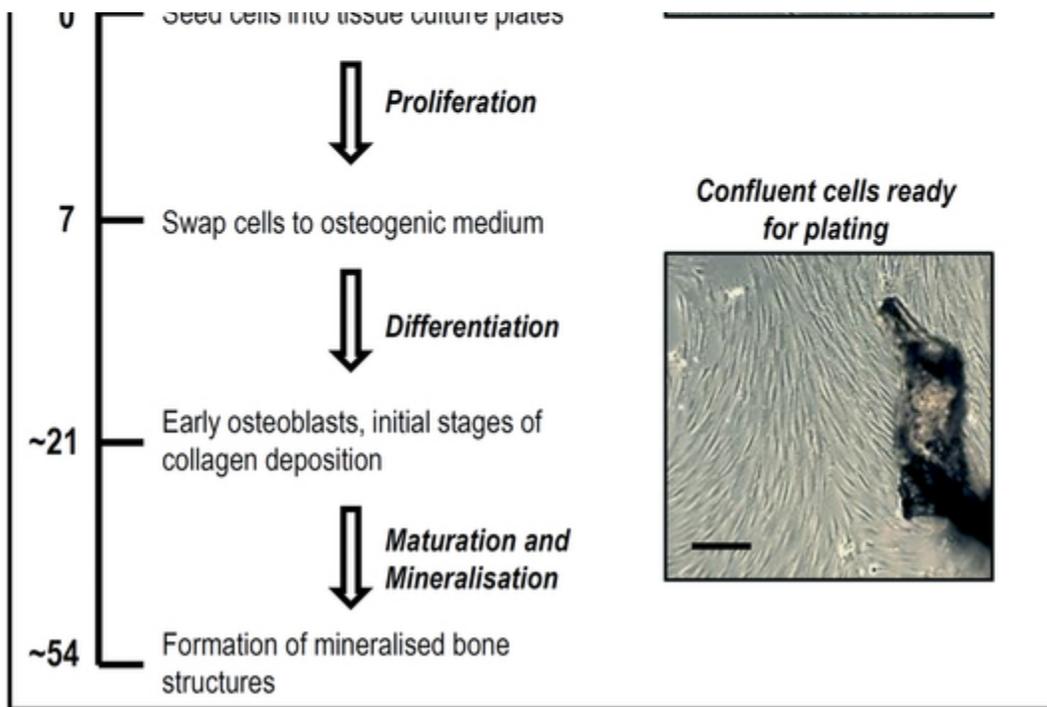
Human humeral head trabecular bone explant cultures. Human osteoblasts take ~6–8 weeks to reach subconfluence when cultured in DMEM by the explant method. Representative images show unstained cell layers from explant bone at initial out growth and confluent monolayers of human humeral head trabecular osteoblasts before subculture. Following subculture mineralised bone structures are usually evident after ~54 days. Scale bar=50 μm.

Figure 5
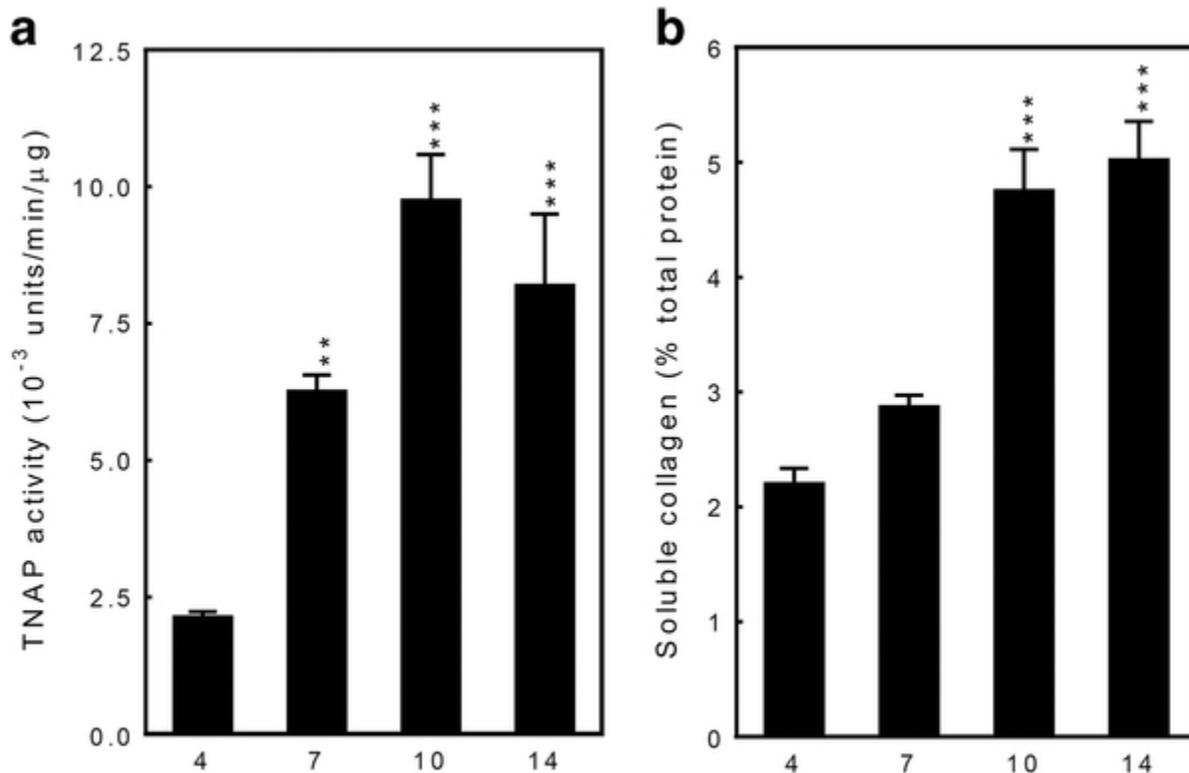
TNAP activity and soluble collagen levels in rat osteoblast cultures. TNAP activity and soluble collagen levels were measured at regular intervals throughout the osteoblast culture period. (**a**) TNAP activity was 2.5-fold and ⩽fourfold higher in differentiating and mature osteoblasts, respectively, compared with proliferating, preosteoblasts. (**b**) Soluble collagen levels were increased up to twofold in mature osteoblasts. Values are mean±s.e.m.; **$P<0.01$, ***$P<0.001$.

Fixation and staining

- On termination of the experiment, carefully wash the cell layers with PBS. Transfer to appropriate fixative:

  - – Alizarin red: 2.5% glutaraldehyde for 5 min

  - – Sirius red: 70% ethanol for 1 h

  - – Masson's trichrome: Bouin's fixative overnight

  - – TNAP: 4% paraformaldehyde (pH 7.4) for 20 min at 4 °C

- Wash twice with PBS and leave to dry.

- Once fully dry, scan unstained plates at 2000 dots cm^2 on a high-resolution flat-bed scanner (Note 11) for analysis of mineralised nodule formation.

- Stain as required (Figures 5, 6, 7, 8):

  - – Three times 70% ethanol washes, leave to dry. Alizarin red for 5 min followed by three times 50% ethanol washes.

  - – Sirius red for 1 h; wash with dH$_2$O and allow to dry.

  - – Masson's trichrome collagen stain: Following fixation wash the cell layers with running water until the yellow colour disappears. Stain with Weigert's haematoxylin for 10 min; wash with tap water. Stain with 0.5% acid fuchsin solution for 5 min and rinse with dH$_2$O. Treat with phosphomolybdic acid solution for 5 min before staining with 2% methyl blue for 5 min. Rinse with dH$_2$O, followed by 1% acetic acid for 2 min. Dehydrate the cell layers by several washes with 50, 70 and 100% alcohol. Nuclei should appear black, cytoplasm red and collagen fibres blue.

  - – TNAP stain: In glass vials, dissolve 1 mg per ml napthol AS-MX-phosphate in dimethylformamide and 1 mg per ml of fast blue-BB salt in 0.1 M Tris HCl pH 9.2. Combine

both solutions and mix well; filter through a 0.45 µM syringe filter. Cover cells evenly (0.2 ml per well in a 12-well tray) and incubate the plates for 25 min at 37 °C in the dark. Remove stain solution and wash the monolayers twice in dH$_2$O.

Figure 6

TNAP activity and mineralisation in human trabecular osteoblasts from normal and osteoarthritic patients. The level of mineralised nodule formation increases with culture time in human osteoblasts from (**a**) normal (30 years old) and (**b**) osteoarthritic patients (86 years old). (**c**) Representative whole-well scans of alizarin red stained human osteoblasts at different stages of culture. Human cells appear to be unable to form the discrete trabecular bone structures characteristic of neonatal rodent osteoblasts; instead, the mineralisation is more widespread. Human osteoblasts from (**d**) normal and (**e**) osteoarthritic patients display TNAP activity throughout the culture period. (**f**) Representative images showing increased levels of TNAP staining with osteoblast differentiation. Values are mean±s.e.m.; **$P<0.01$, ***$P<0.001$. Scale bar=50 mm.

Figure 7

Different staining methods in primary calvarial osteoblast cultures. Images show 14-day rat and 28-day mouse osteoblast cultures either left unstained, or stained with TNAP, alizarin red or sirius red. The individual bone structures formed by rodent osteoblasts displayed marked differences in appearance. Rat osteoblasts typically produced smaller bone nodules that had a 'trabecular-shaped' appearance, whereas in mouse osteoblast cultures the bone structures were larger but fewer in numbers. TNAP staining was closely associated with the mineralised matrix in rat osteoblast cultures but more widespread in mouse osteoblasts. Sirius red staining shows the presence of collagen fibres in these cultures. Scale bars=50 mm (whole-well scans) and 500 µm (phase contrast microscopy images).

Figure 8

Different staining methods in primary long-bone osteoblast cultures. Images show 20-day rat long-bone osteoblast cultures either left unstained, or stained with alizarin red or Masson's trichrome. The rat long-bone osteoblasts produce 'trabecular-shaped' bony structures that display positive staining for calcium and collagen deposition. Scale bars=50 mm (whole-well scans) and 500 μm (phase contrast microscopy images).

Figure 6

TNAP activity and mineralisation in human trabecular osteoblasts from normal and osteoarthritic patients. The level of mineralised nodule formation increases with culture time in human osteoblasts from (**a**) normal (30 years old) and (**b**) osteoarthritic patients (86 years old). (**c**) Representative whole-well scans of alizarin red stained human osteoblasts at different stages of culture. Human cells appear to be unable to form the discrete trabecular bone structures characteristic of neonatal rodent osteoblasts; instead, the mineralisation is more

widespread. Human osteoblasts from (**d**) normal and (**e**) osteoarthritic patients display TNAP activity throughout the culture period. (**f**) Representative images showing increased levels of TNAP staining with osteoblast differentiation. Values are mean±s.e.m.; **$P<0.01$, ***$P<0.001$. Scale bar=50 mm.

Figure 7

Different staining methods in primary calvarial osteoblast cultures. Images show 14-day rat and 28-day mouse osteoblast cultures either left unstained, or stained with TNAP, alizarin red or sirius red. The individual bone structures formed by rodent osteoblasts displayed marked differences in appearance. Rat osteoblasts typically produced smaller bone nodules that had a 'trabecular-shaped' appearance, whereas in mouse osteoblast cultures the bone structures were larger but fewer in numbers. TNAP staining was closely associated with the mineralised matrix in rat osteoblast cultures but more widespread in mouse osteoblasts. Sirius red staining shows the presence of collagen fibres in these cultures. Scale bars=50 mm (whole-well scans) and 500 µm (phase contrast microscopy images).

Figure 8

Different staining methods in primary long-bone osteoblast cultures. Images show 20-day rat long-bone osteoblast cultures either left unstained, or stained with alizarin red or Masson's trichrome. The rat long-bone osteoblasts produce 'trabecular-shaped' bony structures that display positive staining for calcium and collagen deposition. Scale bars=50 mm (whole-well scans) and 500 μm (phase contrast microscopy images).

Quantitation of mineralised nodule formation

- Several programs are available, such as Adobe Photoshop and Image J (http://rsbweb.nih.gov/ij/), which can be used to determine the number and surface area of mineralised bone nodules.

- Using these programs, binary images of each individual well can be subjected to automated analysis, using constant threshold and minimum particle levels (Figure 9).

Figure 9
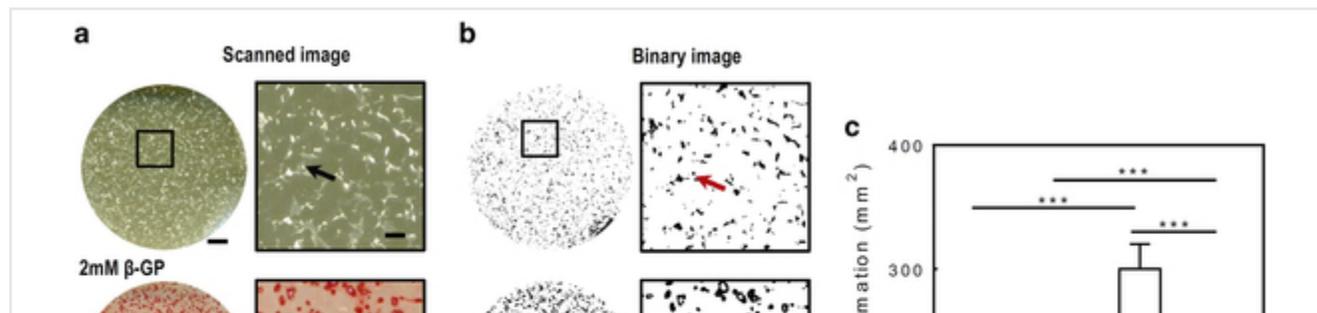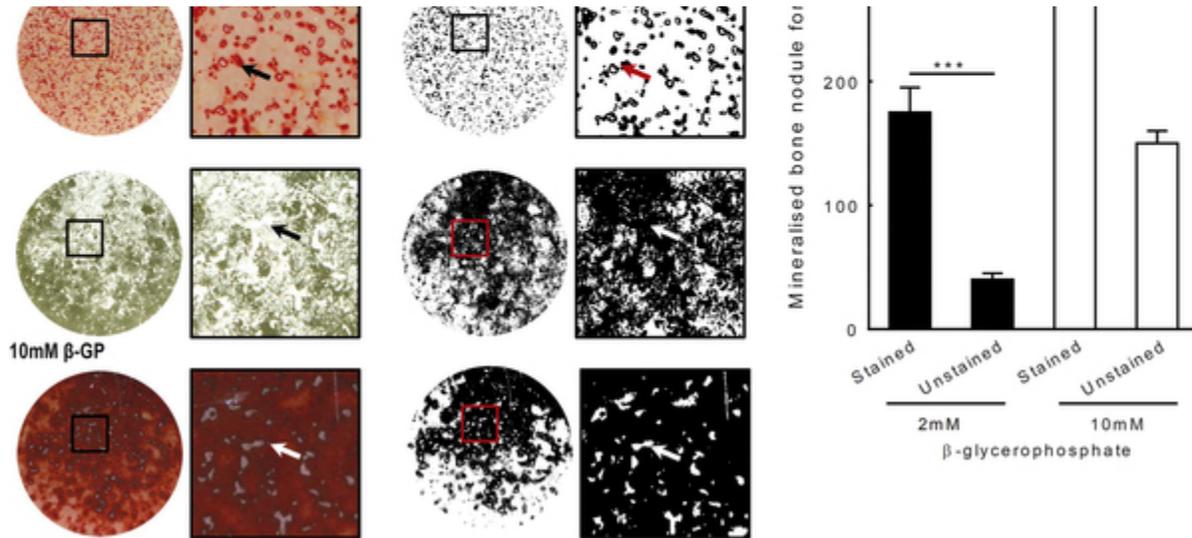
Quantification of bone mineralisation by image analysis. The representative images in (**a**) are of the same cell layers before and after alizarin red staining; the stained images are examples of cultures that have been inadequately washed, leading to bleeding of the alizarin red into the surrounding cells (shown by the arrows). (**b**) Binary images of the same cell layers highlight the differences in the level of bone mineral detected between stained and unstained plates. (**c**) Comparison of stained and unstained plates by automated analysis shows that the level of bone formation is ⩽fourfold higher in the stained plates. Culture with excess β-glycerophosphate (10 mM) amplifies this problem due to the widespread nonspecific deposition of mineral. Scale bars=50 mm (whole-well scans) and 5 mm (nodule images). Values are mean±s.e.m.; ***$P<0.001$.

Notes

1. Tissue culture medium. For successful osteoblast cultures it is important to use the correct tissue culture medium. Mouse osteoblasts appear to require more nutritional support *in vitro* and to get significant mineralisation should be grown in αMEM.[12] The formulation of αMEM is designed to closely approximate the protein composition of cells[13] and it contains higher concentrations of amino acids and nucleotides compared with other tissue culture mediums (such as DMEM). Rat osteoblasts will successfully mineralise in both αMEM and DMEM; however, cultures will be 2–3 days shorter when grown in the former. The timelines in Figures 1 and 2 are based on mouse and rat osteoblasts cultured in osMEM and osDMEM, respectively.

2. β-Glycerophosphate. Bone mineralisation requires a constant supply of Ca^{2+} and orthophosphate (P_i). The main source of Pi in bone is TNAP, which hydrolyses a range of phosphate-containing substrates including ATP, pyrophosphate and β-glycerophosphate. To form mineralised bone nodules *in vitro* an exogenous source of phosphate is required; most commonly used is β-glycerophosphate (2–10 mM). Previous studies have shown in cultures of rat osteoblasts, 2 mM β-glycerophosphate reproducibly forms abundant, discrete nodules with a characteristic 'trabecular-shaped' morphology[1, 5] (Figure 6). However, culture with 5–10 mM β-glycerophosphate reduces cell viability and causes widespread, non-specific mineral deposition that differs from true-bone formation[1, 5] (Figure 9). Mouse osteoblasts

can tolerate a higher concentration of β-glycerophosphate (up to 5 mM) before non-specific mineralisation occurs.[12] Thus, when investigating osteoblast function it is critical that the appropriate, low concentration of β-glycerophosphate is used. This ensures that any effects on bone formation/mineralisation are due to differences in osteoblast activity rather than an excess level of P_i in the culture medium.

3. Dexamethasone. The ability of dexamethasone to promote osteoblast differentiation in rat cells was first identified in the 1980s [14] and it is now routinely added to these cultures in the nanomolar range.[1] In contrast, many studies have shown that dexamethasone inhibits osteogenic differentiation in murine cells.[12, 15, 16, 17, 18, 19] Using the above protocols, we have recently shown that 10 nM dexamethasone is required for differentiation in rat osteoblast cultures but slows differentiation in mouse cells.[12] Thus, we recommend that dexamethasone is only added to osteogenic medium when growing rat cells.

Because of the slow growth of human cells, dexamethasone should only be added from confluence; however, ascorbate can be added to facilitate cell growth.

4. The importance of pH. Extracellular pH is an important factor in the regulation of bone mineralisation.[6] Acidosis inhibits matrix mineralisation by reducing TNAP expression and activity, while increasing mineral solubility.[6] The metabolic activity of osteoblasts will cause progressive medium acidification especially in mature, bone-forming cultures when cell numbers are high. To ensure that pH does not influence mineralisation, it may be required to add OH^- ions (as 6 M NaOH) to the culture medium to ensure a pH of ~7.4.

5. Accutase. This is a gentler alternative to trypsin-EDTA for cell dissociation. It reduces the level of nonviable cells and does not require serum inhibition.

6. Osteoblast seeding density and tissue culture plates. Rat and human osteoblasts can be cultured in several different well plate formats. Typical seeding densities per well are 2.5×10^4 (24-well), 5×10^4 (12-well) and 10^5 (6-well). Mouse osteoblasts will only form abundant mineralised bone nodules in 6-well trays (10^5 cells per well). Seeding cells in 24- or 12-well trays will result in significant monolayer peeling before mineralisation.[12]

7. Human tissue storage and transport. To avoid degradation of viable cells it is important to place the bone samples in explants as soon as possible. Store specimens before explant for not >24 h at 4 °C in PBS (+AB/AM to the risk of reduce post-operative culture contamination) or serum-free DMEM.

8. Removal of marrow and fat from human bone. Samples from diseased and aged bone can contain high amounts of marrow and fat. This must be removed to avoid any adipocyte or stromal cell contamination.

9. Stages of differentiation in osteoblast cultures. These methods allow rodent osteoblasts to be studied at clearly identified stages of differentiation such as proliferating pre-osteoblasts, early or differentiating osteoblasts, mature osteoblasts and mature, mineralising osteoblasts (Figures 1, 2 and 5).

10. Young vs old cells. Human osteoblasts (particularly from older individuals) tend to grow and differentiate slower compared with neonatal rodent osteoblasts. Cells from young animals have considerable growth potential and thus will expand rapidly *in vitro*. In contrast, cells from adult animals (including human donors) may have progressed too far towards their 'Hayflick limit' (the number of times a cell population will divide until cell division is arrested[20]) to be capable of rapid expansion and differentiation.

11. Analysis of mineralised nodule formation. A commonly used method for quantifying bone formation is image analysis of alizarin red stained osteoblast cell layers. However, this technique has limitations and, if staining is performed incorrectly, can give inaccurate results. For example, when cell layers are not stained sufficiently the alizarin red will not reach the centre of the bone nodules leaving an unstained white region. Conversely, if the cells are not washed properly following staining the alizarin red can 'bleed' into the region immediately surrounding the bone structure. Figure 9a shows the same cultures before and after alizarin red staining. The corresponding binary images shown in Figure 9b highlight the obvious differences in the level of bone mineral detected. Comparison of stained and unstained plates by automated analysis shows that the level of bone formation is ≤fourfold higher in the stained plates (Figure 9c). Performing analysis of bone formation on unstained plates removes this imprecision and provides a more reliable representation of the actual level of mineralisation. Culture with excess β-glycerophosphate (10 mM) amplifies this problem due to the widespread nonspecific deposition of mineral (Figure 9).

Bone formation can also be quantified by eluting the alizarin red with acidic solutions and measuring the optical density by spectrophotometry. This process will dissolve all the stain into solution, whether it is associated with a discrete bone nodule or is nonspecific staining, and therefore can also give inaccurate results.

Discussion

The methods described here provide a robust *in vitro* model for studying the regulation of osteoblast proliferation, differentiation and function. However, these protocols do have some limitations that need to be taken into account when planning and interpreting experiments. First, the use of high levels of β-glycerophosphate will result in nonspecific, dystrophic mineralisation, which is not true-bone formation. Second, as the osteoblasts are grown in monolayers they cannot form the three-dimensional (3D) cell-to-cell interactions, which occur *in vivo.* Third, although rodent osteoblasts are readily available, the supply of human osteoblasts is limited and often restricted to cells from older patients undergoing surgery. Finally, once in culture, human osteoblasts are often very slow growing and

therefore it takes a significant amount of time to obtain experimental data.

There are some newer approaches/techniques that can be used to address some of these issues. The development of 3D culture systems has provided a novel method for growing osteoblasts (in isolation or cocultured with other cell types) in an environment that more accurately represents the *in vivo* situation.[21, 22] There have also been several studies that have described the isolation of human osteoblast precursors from peripheral or cord blood.[23, 24, 25] At present, work using these methods is limited; however, they do represent a potentially important source of human osteoblasts.

Recommended future reading

There are many other papers that cover different methods for isolating and culturing osteoblasts. The following publications are recommended for further information about rodent and human osteoblasts:

Orriss IR, Hajjawi MOR, Huesa C, MacRae VE, Arnett TR. Optimisation of the differing conditions required for bone formation *in vitro* by primary osteoblasts from mice and rats. *Int J Mol Med* 2014;**34**:1201–1208.

Dillon JP, Waring-Green VJ, Taylor AM, Wilson PJ, Birch M, Gartland A *et al.* Primary human osteoblast cultures. *Methods Mol Biol* 2012;**816**:3–18.

Detailed methodological information about the 3D coculture of osteoblasts with other cell types is provided by:

Unger RE, Halstenberg S, Sartoris A, Kirkpatrick CJ. Human endothelial and osteoblast cocultures on 3D biomaterials. *Methods Mol Biol* 2011;**695**:229–241.

References

1. Orriss IR, Taylor SE, Arnett TR. Rat osteoblast cultures. *Methods Mol Biol* 2012;**816**:31–41.

2. Dillon JP, Waring-Green VJ, Taylor AM, Wilson PJ, Birch M, Gartland A *et al.* Primary human osteoblast cultures. *Methods Mol Biol* 2012;**816**:3–18.

3. Bakker AD, Klein-Nulend J. Osteoblast isolation from murine calvaria and long bones. *Methods Mol Biol* 2012;**816**:19–29.

4. Taylor SEB, Key ML, Lander M, Orriss IR, Patel JJ, Arnett TR. A novel method for the isolation and culture of rat long bone osteoblasts. *Bone* 2009;**44**:S318–S319.

5. Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G *et al.* Extracellular nucleotides block bone mineralization *in vitro:* evidence for dual inhibitory mechanisms involving both P2Y$_2$ receptors and pyrophosphate. *Endocrinology* 2007;**148**:4208–4216.

6. Brandao-Burch A, Utting JC, Orriss IR, Arnett TR. Acidosis inhibits bone formation by osteoblasts *in vitro* by preventing mineralization. *Calcif Tissue Int* 2005;**77**:167–174.

7. Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J, Arnett TR. Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. *Exp Cell Res* 2006;**312**:1693–1702.

8. Siggelkow H, Rebenstorff K, Kurre W, Niedhart C, Engel I, Schulz H *et al.* Development of the osteoblast phenotype in primary human osteoblasts in culture: comparison with rat calvarial cells in osteoblast differentiation. *J Cell Biochem* 1999;**75**:22–35.

9. Beresford JN, Graves SE, Smoothy CA. Formation of mineralized nodules by bone derived cells *in vitro*: a model of bone formation? *Am J Med Genet* 1993;**45**:163–178.

10. Beresford JN, Gallagher JA, Poser JW, Russell RG. Production of osteocalcin by human bone cells *in vitro*. Effects of 1,25(OH)2D3, 24,25(OH)2D3, parathyroid hormone, and glucocorticoids. *Metab Bone Dis Relat Res* 1984;**5**:229–234.

11. Couchourel D, Aubry I, Delalandre A, Lavigne M, Martel-Pelletier J, Pelletier JP *et al.* Altered mineralization of human osteoarthritic osteoblasts is attributable to abnormal type I collagen production. *Arthritis Rheum* 2009;**60**:1438–1450.

12. Orriss IR, Hajjawi MOR, Huesa C, MacRae VE, Arnett TR. Optimisation of the differing conditions required for bone formation *in vitro* by primary osteoblasts from mice and rats. *Int J Mol Med* 2014;**34**:1201–1208.

13. Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science* 1955;**122**:501–514.

14. Bellows CG, Aubin JE, Heersche JN, Antosz ME. Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcif Tissue Int* 1986;**38**:143–154.

15. Chen TL, Cone CM, Feldman D. Glucocorticoid modulation of cell proliferation in cultured osteoblast-like bone cells: differences between rat and mouse. *Endocrinology* 1983;**112**:1739–1745.

16. Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS *et al.* Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J Cell Biochem* 1997;**66**:1–8.

17. Li H, Qian W, Weng X, Wu Z, Li H, Zhuang Q *et al.* Glucocorticoid receptor and sequential P53 activation by dexamethasone mediates apoptosis and cell cycle arrest of osteoblastic MC3T3-E1 cells. *PLoS ONE* 2012;**7**:e37030.

18. Li J, Zhang N, Huang X, Xu J, Fernandes JC, Dai K *et al.* Dexamethasone shifts bone marrow stromal cells from osteoblasts to adipocytes by C/EBPalpha promoter methylation. *Cell Death Dis* 2013;**4**:e832.

19. Esmail MY, Sun L, Yu L, Xu H, Shi L, Zhang J. Effects of PEMF and glucocorticoids on proliferation and differentiation of osteoblasts. *Electromagn Biol Med* 2012;**31**:375–381.

20. Hayflick L. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965;**37**:614–636.

21. Unger RE, Halstenberg S, Sartoris A, Kirkpatrick CJ. Human endothelial and osteoblast co-cultures on 3D biomaterials. *Methods Mol Biol* 2011;**695**:229–241.

22. Jones GL, Walton R, Czernuszka J, Griffiths SL, El Haj AJ, Cartmell SH. Primary human osteoblast culture on 3D porous collagen-hydroxyapatite scaffolds. *J Biomed Mater Res A* 2010;**94**:1244–1250.

23. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. *N Engl J Med* 2005;**352**:1959–1966.

24. Rosada C, Justesen J, Melsvik D, Ebbesen P, Kassem M. The human umbilical cord blood: a potential source for osteoblast progenitor cells. *Calcif Tissue Int* 2003;**72**:135–142.

25. Liu G, Li Y, Sun J, Zhou H, Zhang W, Cui L *et al. In vitro* and *in vivo* evaluation of osteogenesis of human umbilical cord blood-derived mesenchymal stem cells on partially demineralized bone matrix. *Tissue Eng Part A* 2010;**16**:971–982.

Download references

Acknowledgments

Author information

Affiliations

Department of Orthopaedic Surgery, Stanford University School of Medicine, Stanford, CA, USA

Sarah E B Taylor

Department of Surgery and Cancer, Imperial College London, London, UK

Mittal Shah

Department of Comparative Biomedical Sciences, Royal Veterinary College, London, UK

Mittal Shah & Isabel R Orriss

Conflict of Interest

The authors declare no conflict of interest.

Corresponding author

Correspondence to: Isabel R Orriss