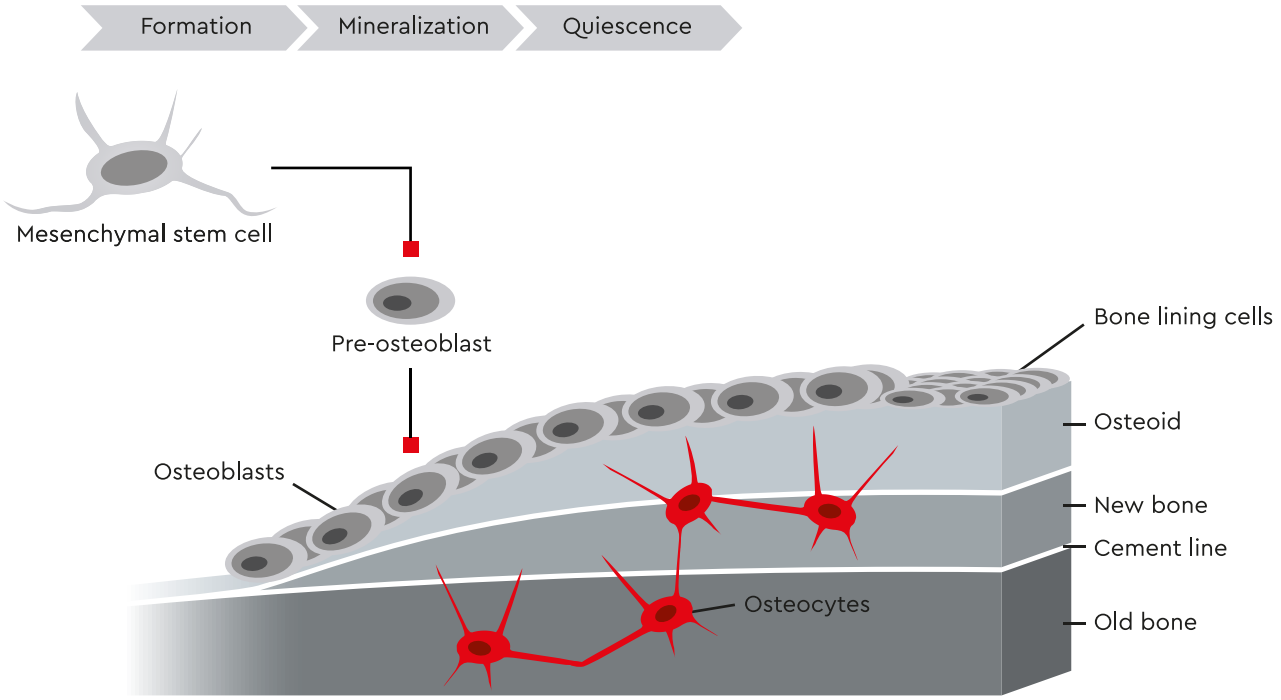
Osteoblast differentiation and mineralization

Application note

Background

Osteoblasts (HOB) are specialized fibroblasts that secrete and mineralize the bone matrix. They develop from mesenchymal precursors. The mineralized extracellular matrix is mainly composed of type I collagen and smaller but significant amounts of osteocalcin (OC), matrix gla protein, osteopontin (OPN), bone sialoprotein (BSP), BMPs, TGF-β, and the in-organic mineral hydroxylapatite. Osteoblast differentiation *in vitro* and *in vivo* can be characterized in three stages: (a) cell proliferation, (b) matrix maturation, and (c) matrix mineralization [1]. *In vitro*, matrix maturation and mineralization are usually enhanced by growing the cells to complete confluency and by adding specific osteogenic factors [2].

(a) During proliferation, several extra-cellular matrix proteins (procollagen I, TGF-β, and fibronectin) can be detected. The matrix maturation phase (b) is characterized by maximal expression of alkaline phosphatase (AP). Finally, at the beginning of matrix mineralization (c), genes for proteins such as OC, BSP, and OPN are expressed and once mineralization is completed, calcium deposition can be visualized using adequate staining methods. Analysis of bone cell-specific markers like AP, OC, and collagen type I or detection of functional mineralization is frequently used to characterize osteoblasts *in vitro* [2]. The mineralization process of osteoblasts in *in vitro* culture has also been used as a model for testing the effects of drug treatments and mechanical loading on bone cell differentiation and bone formation [3, 4].



Protocol for the detection of osteoblast proliferation and mineralization

I. Detection of alkaline phosphatase*

Materials

- Saccomanno Fixation Solution (Morphisto, Cat. No. 13881.00250)
- BCIP/NBT tablets (Sigma Aldrich, SigmaFastTM BCIP-NBT)
- Dulbecco's phosphate-buffered saline (PBS) without Ca^{++}/Mg^{++} (C-40232)

Important: Do not let the cells dry for longer than 30 sec. throughout the entire staining procedure!

Proliferating osteoblasts show alkaline phosphatase (AP) activity, which is greatly enhanced during *in vitro* bone formation. AP activity is therefore a feasible marker for HOB. AP can easily be detected using BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium) as a substrate, which stains cells blue-violet when AP is present. Alternatively, assess AP activity using commercially available AP staining solutions/kits according to the manufacturer's instructions.

* AP activity is not limited to osteoblasts. Therefore a second confirmation, e.g. direct staining of extracellular calcium deposits (mineralization), may be necessary.

1

Prepare solutions and reagents

Obtain Saccomanno Fixation Solution (Morphisto). Dissolve one BCIP/NBT tablet (Sigma Aldrich) in 10 ml distilled water to prepare the substrate solution. Store in the dark and use within two hours.
Add 0.05% Tween 20 to PBS without Ca^{++}/Mg^{++} (C-40232) to prepare the washing buffer.

2

Wash the cells

Remove the cells from the incubator and carefully aspirate the medium. Carefully wash the cells with PBS.

Note: Do not disrupt the cell monolayer.

3

Fixation of the cells

Carefully aspirate the PBS and add enough Saccomanno Fixation Solution to cover the cellular monolayer. After 60–90 seconds gently aspirate the fixation solution and wash the cells with washing buffer.

Note: Longer fixation will lead to irreversible inactivation of AP.

4

Stain the cells

Carefully aspirate the washing buffer and add enough BCIP/NBT substrate solution to cover the cellular monolayer. Incubate at room temperature in the dark for 5–10 minutes. Check staining progress every 2–3 minutes.

5

Wash the cells

Carefully aspirate the substrate solution and wash the cell monolayer with washing buffer. Carefully aspirate the washing buffer and add PBS.

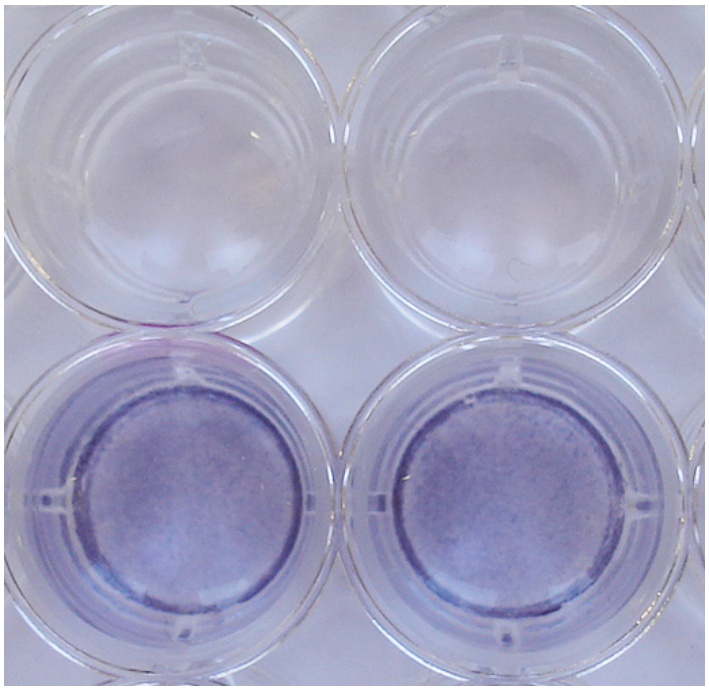


Fig. 1: Alkaline phosphatase detection. *HUVEC (AP negative, upper row) are colorless or faintly bluish, whereas osteoblasts (AP positive, lower row) are dark blue-violet. The higher the AP activity, the more intense the color.*

6

Analyze the cells

Evaluate staining results. Refer to Fig. 1 for an example of AP detection.

II. Osteoblast mineralization

Materials

- Human Osteoblasts (C-12720)
- Osteoblast Growth Medium (C-27001)
- Osteoblast Mineralization Medium (C-27020)
- Collagen I coated 24-well tissue culture plate (Corning, Cat. No. 354408)

Use aseptic techniques and a laminar flow bench.

1

Seed osteoblasts (HOB) on collagen precoated culture plates

Plate 3×10^4 HOB (C-12720) per well on a collagen I coated 24-well tissue culture plate (Corning). Work in duplicate. Use Osteoblast Growth Medium (C-27001) for one well as a negative control and Osteoblast Mineralization Medium (C-27020) for the other well.

2

Differentiation culture of induced osteoblasts

Incubate the cells for 17–21 days. Change the medium every third day. Be careful not to disturb the cell monolayer.

Proceed with the protocol in the following section **Detection of calcium deposits (mineralization)**.

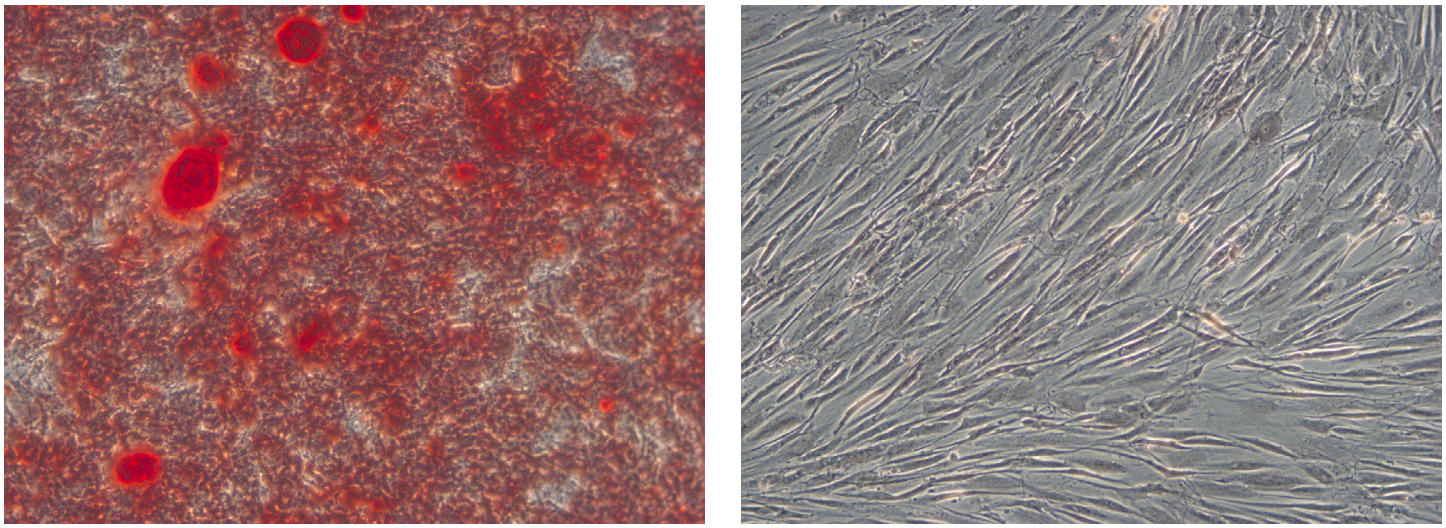


Fig. 2: Microscopic appearance of osteoblasts after mineralization in vitro. Left: *Mineralized osteoblasts in Osteoblast Mineralization Medium (C-27020) show vast extracellular calcium deposits, stained in bright orange-red.* ***Right:*** *The negative control in Osteoblast Growth Medium (C-27001) is slightly reddish.*

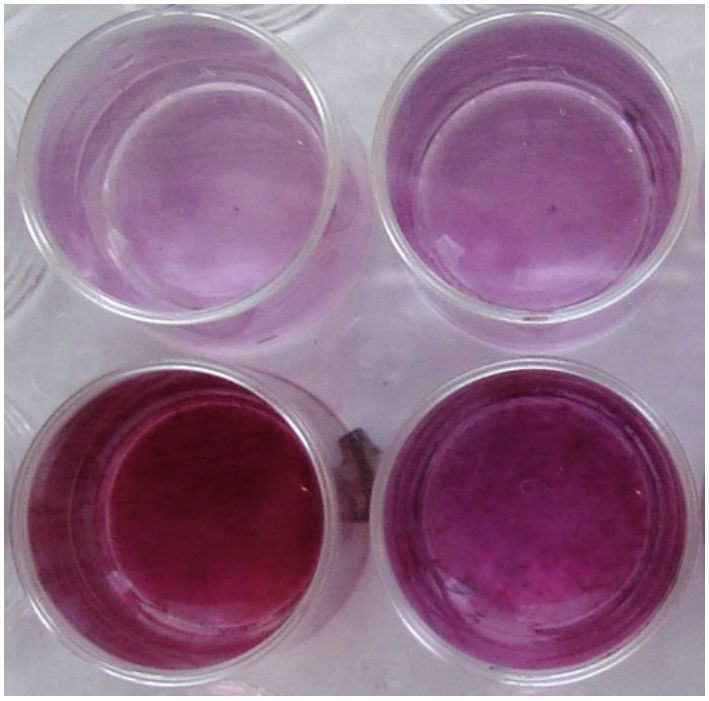


Fig. 3: Macroscopic appearance of HOB after mineralization in vitro. *The negative control in Osteoblast Growth Medium (upper row) is slightly reddish, whereas the mineralized osteoblasts in Osteoblast Mineralization Medium (C-27020) show vast extracellular calcium deposits, stained in bright orange-red (lower row).*

III. Detection of calcium deposits (Mineralization)

Materials

- Saccomanno Fixation Solution (Morphisto, Cat. No. 13881.00250)
- Alzarin Red S
- Dulbecco's phosphate-buffered saline (PBS) without Ca^{++}/Mg^{++} (C-40232)

Important: Do not let the cells dry for longer than 30 sec. throughout the entire staining procedure!

Osteoblasts can be induced to produce vast extracellular calcium deposits in vitro. This process is called mineralization. Calcium deposits are an indication of successful in vitro bone formation and can specifically be stained bright orange-red using Alizarin Red S.

1

Prepare solutions and buffers

Use Saccomanno Fixation Solution (Morphisto). To prepare the Alizarin Red S staining solution dissolve 2 g Alizarin Red S in 90 ml distilled water, mix and adjust the pH to 4.1–4.3 with hydrochloric acid, as necessary. Then, bring up to a final volume of 100 ml with distilled water and filter the dark-brown solution. Store in the dark at 2–8 °C.

Note: The correct pH of the solution is critical. Check pH (at ambient temperature) if the solution is more than one month old.

2

Wash the cells

Remove the cells from the incubator and carefully aspirate the medium. Carefully wash the cells with Dulbecco's PBS without Ca^{++}/Mg^{++} (C-40232).

Note: Do not disrupt the cell monolayer.

3

Fixation of the cells

Carefully aspirate the PBS and add enough Saccomanno Fixation Solution to cover the cellular monolayer. After at least 60 minutes gently aspirate the fixation solution and wash the cells with distilled water.

4

Stain the cells

Immediately before use, pass the required amount of Alizarin Red S staining solution through a 0.22 µm syringe filter equipped with a PES-membrane.
Carefully aspirate the distilled water and add enough filtered Alizarin Red S staining solution to cover the cellular monolayer. Incubate at room temperature in the dark for 45 minutes.

5

Wash the cells

Carefully aspirate the Alizarin Red S staining solution and wash the cell monolayer four times with 1 ml distilled water. Carefully aspirate the distilled water and add PBS.

6

Analyze the cells

Analyze the sample immediately, as the dye may bleed upon prolonged storage without embedding. Undifferentiated osteoblasts (without extracellular calcium deposits) are slightly reddish, whereas mineralized osteoblasts (with extracellular calcium deposits) are bright orange-red. Refer to Fig. 2 and 3 for an example of osteoblast mineralization.

Related products

Media	Size	Catalog number
Human Osteoblasts (HOB)	500,000 cryopreserved cells 500,000 proliferating cells	C-12720 C-12760
Osteoblast Growth Medium (Ready-to-use)	500 ml	C-27001
Osteoblast Mineralization Medium (Ready-to-use)	100 ml	C-27020
DetachKit	30 ml 125 ml	C-41200 C-41210
Cryo-SFM Plus	30 ml 125 ml	C-29920 C-29922
Cryo-SFM Plus, phenol red-free	30 ml 125 ml	C-29930 C-29932
Dulbecco's PBS, without Ca^{++}/Mg^{++}	500 ml	C-40232
HOB Pellet	> 1 million cells per pellet	C-14071

References

1. Stein GS and Lian JB. Molecular mechanisms mediating developmental and hormone-regulated expression of genes in osteoblasts: an integrated relationship of cell growth and differentiation. In: Noda M, editor. Cellular and molecular biology of bone. Tokyo: Academic Press. p 47–95, 1993.
2. Kasperk C. et al. Human bone cell phenotypes differ depending on their skeletal site of origin J Clin Endocrinol Metab. Aug;80(8): 2511–7, 1995.
3. Kostenuik, P.J. et al. Skeletal unloading inhibits the in vitro proliferation and differentiation of rat osteoprogenitor cells. Am. J. Physiol. 273, E1133, 1997.
4. Kostenuik, P.J. et al. Skeletal unloading causes resistance of osteoprogenitor cells to parathyroid hormone and to insulin-like growth factor-I. J. Bone Miner. Res. 14, 21, 1999.

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