

Osteoblast Differentiation and Mineralization

Application Note

Background

Osteoblasts 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 inorganic 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].

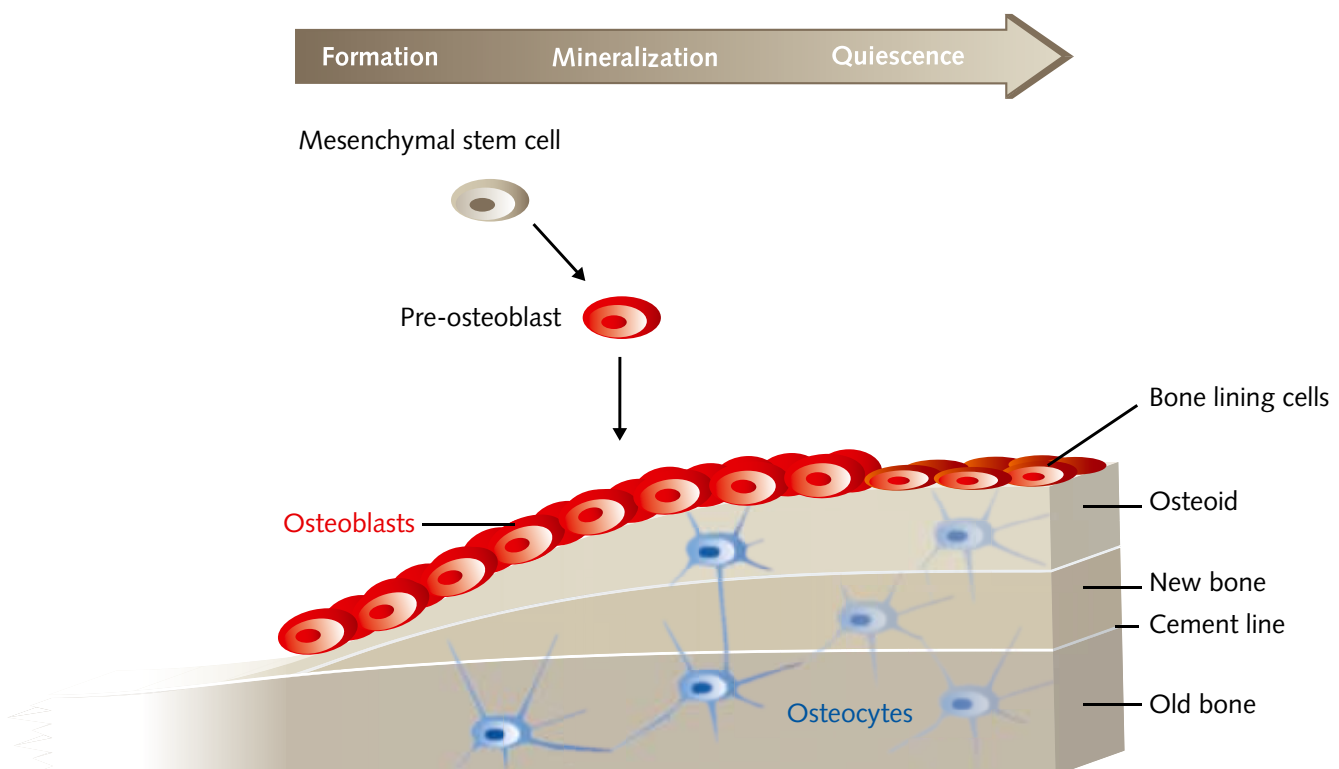
(a) During proliferation, several extracellular matrix proteins (procollagen I, TGF- β , and fibronectin) can be detected. The matrix maturation phase (b) is characterized by maximal expression of alkaline phosphatase (AP).

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].



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

Detection of Alkaline Phosphatase*

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 as a substrate, which stains cells blue-violet when AP is present.

1. Prepare solutions and buffers

Dissolve one BCIP/NBT tablet (SigmaFast™ BCIP-NBT; Sigma Aldrich) in 10 ml distilled water to prepare the substrate solution. Store in the dark and use within 2 hours.

Add 0.05% Tween 20 to Dulbecco's PBS, w/o Ca⁺⁺/ Mg⁺⁺ (Cat. No. C-40232) to prepare the Washing Buffer.

2. Wash the cells

Take 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 transfer the tissue culture dish to a fume hood. Add enough neutral buffered formalin (10%) to cover the cellular monolayer. After 60 sec. carefully aspirate the formalin 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 min. Check staining progress every 2-3 min.

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.

6. Analyze the cells

Evaluate staining results.

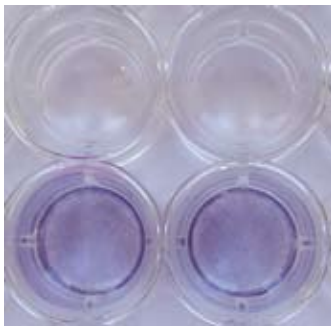


Fig. 2: HUVEC (AP negative, upper row) are colorless or faintly bluish, whereas osteoblasts (AP positive, lower row) are dark blue-violet. The higher the AP activity, the more intense the color.

Please follow the recommended safety precautions for the chemicals used in this procedure!

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

*Detection
of Alkaline
Phosphatase*

Use aseptic techniques and a laminar flow bench.

Osteoblast Mineralization

1. Seed Osteoblasts (HOB)

Plate 6×10^4 HOB per well of a 24-well tissue culture plate (3.15×10^4 cells/cm²) using HOB Growth Medium (Cat. No. C-27001). Work in duplicate.

2. Grow Osteoblasts

Important: Let the cells reach $\geq 100\%$ confluency (24 - 72 hours).

3. Induce Osteoblasts

Induce one of the duplicate samples with Osteoblast Mineralization Medium (Cat. No. C-27020). Use HOB Growth Medium for the remaining well as a negative control.

4. Differentiation culture of induced Osteoblasts

Incubate for 21 days to complete the mineralization process.

Change Medium every third day. Be careful not to disturb the cell monolayer.

Osteoblast Mineralization

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

Detection of Calcium Deposits (Mineralization)

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

Dissolve 2 g Alizarin Red S (C. I. 58005) in 100 ml distilled water, mix, and adjust pH to 4.1 - 4.3 with 0.1% NH_4OH to prepare the Alizarin Red S staining solution. Filter the dark-brown solution and store it in the dark.

Note: The correct pH of the solution is critical. Check pH, if the solution is older than 1 month.

2. Wash the cells

Take the cells from the incubator and carefully aspirate the medium. Carefully wash the cells with Dulbecco's PBS, w/o Ca^{++} / Mg^{++} (Cat. No. C-40232).

Note: Do not disrupt the cell monolayer!

3. Fixation of the cells

Carefully aspirate the PBS and transfer the flask to a fume hood. Add enough neutral buffered formalin (10%) to cover the cellular monolayer. After at least 30 min. carefully aspirate the formalin and wash the cells with distilled water.

4. Stain the cells

Carefully aspirate the distilled water and add enough Alizarin Red S staining solution to cover the cellular monolayer. Incubate at room temperature in the dark for 45 min.

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 Washing Buffer and add PBS.

6. Analyze the cells

Undifferentiated HOB (without extracellular calcium deposits) are slightly reddish, whereas mineralized osteoblasts (with extracellular calcium deposits) are bright orange-red.

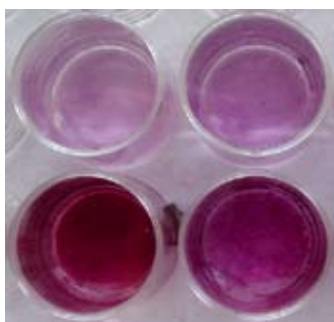


Fig. 1: HOB after mineralization in vitro. The negative control in HOB Growth Medium (upper row) is slightly reddish, whereas the mineralized osteoblasts in HOB Mineralization Medium show vast extra-cellular calcium deposits, stained in bright orange-red (lower row).

Please follow the recommended safety precautions for the chemicals used in this procedure!

*Detection
of Calcium
Deposits*

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.

Related Products

Product	Size	Catalog Number
Human Osteoblasts (HOB)	500,000 cryopreserved cells	C-12720
	500,000 proliferating cells	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	C-41200
	125 ml	C-41210
	250 ml	C-41220
Cryo-SFM	30 ml	C-29910
	125 ml	C-29912
Dulbecco's PBS, w/o Ca ⁺⁺ / Mg ⁺⁺	500 ml	C-40232
HOB Pellet	> 1 million cells per pellet	C-14071

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