Osteoblast Differentiation and Mineralization

**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].

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**Diagram:**

- **Formation**
  - Mesenchymal stem cell
- **Mineralization**
  - Pre-osteoblast
  - Osteoblasts
  - Bone lining cells
- **Quiescence**
  - Osteoid
  - New bone
  - Cement line
  - Old bone

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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.

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

1. Seed Osteoblasts (HOB)
   Plate 6 x 10^4 HOB per well of a 24-well tissue culture plate (3.15 x 10^4 cells/cm^2) using HOB Growth Medium (Cat. No. C-27001). Work in duplicate.

2. Grow Osteoblasts
   **Important:** Let the cells reach ≥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.
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 HCL or NH₄OH 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!
References


Related Products

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