Utility of Recombinant Hedgehog Proteins and RANKL to Generate Bone Cell Models *In Vitro*

Hortensia Soto, Juan Moyron-Quiroz, Dan Kim, Mohar Chattopadhyay, Miguel Tam, Xifeng Yang, Dzung Nguyen, and Jie Zhou
BioLegend, San Diego, CA

Abstract

Bone remodeling is a lifelong process that begins during the embryonic stage and continues after birth. The two main bone cells involved in bone remodeling are osteoblast (bone formation) and osteoclast (bone resorption). The strict balance between bone formation and bone resorption is maintained by several cytokines and growth factors, including RANKL, the Hedgehog family of proteins (SHH, DHH, and IHH), and M-CSF secreted by bone cells, as well as systemic hormones. Any imbalance in bone remodeling might result in bone related diseases. Osteoblast differentiation was first studied using C3H10T1/2 mouse mesenchymal cells. Recombinant SHH, DHH, and IHH were able to induce osteoblast differentiation of C3H10T1/2 cells. Bone morphogenetic protein 9 (BMP-9) enhanced the differentiation of C3H10T1/2 cells induced by Hedgehogs. Osteoclasts are multinuclear cells that are derived from hematopoietic precursors of the monocyte/macrophage macro lineage, and their differentiation depends on osteoblast-produced RANKL and M-CSF. In order to study osteoclast differentiation and the role of different cytokines in bone homeostasis, murine bone marrow derived macrophages (BMDM) were obtained and treated with mouse GM-CSF for 7 and 14 days. Flow cytometric analysis of cellular markers and functional assays confirmed the identity of macrophages. Subsequently, BMDM were differentiated to osteoclasts with mouse M-CSF and RANKL. The multinucleated cells were identified by cell morphology and functional TRAP detection. Similar results were obtained with RAW264.7 cells, a mouse macrophage cell line. Our results confirm that BMDM, osteogenic, and osteoblastic cell lines are good models to study cytokines involved in cell differentiation and bone remodeling.

Introduction

- Bone homeostasis is highly regulated.
- Bone cells can be divided in two main groups: Osteoblast and osteoclast lineages
- Osteoblast lineage (bone forming axis) includes MSCs, pre-osteoblasts, mature osteoblasts, bone-lining cells, and osteocytes.
- Osteoclast lineage (bone resorption) comprises macrophages, osteoclasts, and multinucleated giant cells.
- The Hedgehog family includes Indian hedgehog (IHH), Sonic hedgehog (SHH) and Desert hedgehog (DHH), regulates cell proliferation , cell fate, cell renewal and tissue repair, and induces osteoblast differentiation.
- Endoglin (CD105) is a type III receptor (co-receptor) that belongs to the TGFβ superfamily, and plays key roles in hematopoiesis, angiogenesis, cardiovascular development, and vascular remodeling.
- M-CSF and RANKL are essential cytokines for survival, expansion, and differentiation of osteoclasts.

Results

Osteoblast Differentiation

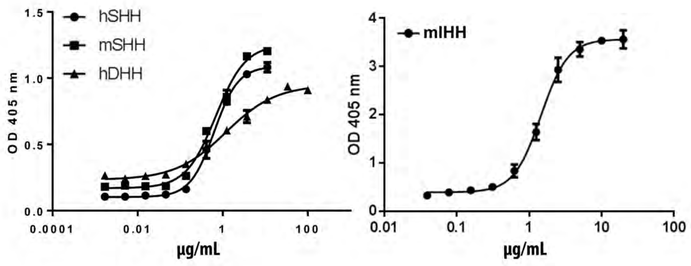

Fig 1. Osteoblast differentiation. Mouse mesenchymal stem cells C3H10T1/2 were used to induce osteoblast differentiation. C3H10T1/2 stem cells were grown to confluence and treated with BMP9 (20 ng/mL, Cat. No. 553102) and different concentrations of recombinant human or mouse SHH, human DHH, or mouse IHH (Cat. No. 753502, 754602, 759802, and 758602 respectively). Alkaline phosphatase was measured as a marker for osteoblast differentiation using p-nitrophenyl phosphate as a substrate.

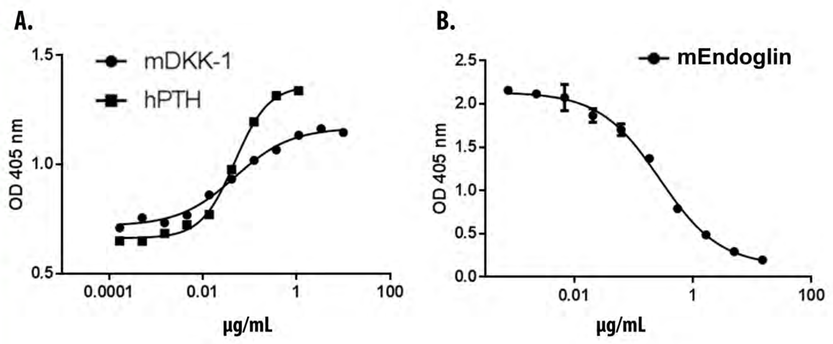

Fig 2. A. Osteoblast differentiation. Murine MC3T3-E1 preosteoblasts were grown in alpha MEM and differentiated to osteoblasts once they reached approximately 80 – 90 % confluency. The cells were treated with BMP9 (20 ng/mL) and different concentrations of recombinant mouse DKK1 and recombinant human PTH (Cat. No. 759602 and 753202 respectively). Alkaline phosphatase was measured as a marker for osteoblast differentiation using p-nitrophenyl phosphate. B. Mouse endoglin inhibits the activity of BMP9. Different concentrations of mouse endoglin (Cat. No. 719702) were pre-incubated with BMP9 and subsequently the cells were treated with the endoglin/BMP9 complex.

BMDM Characterization

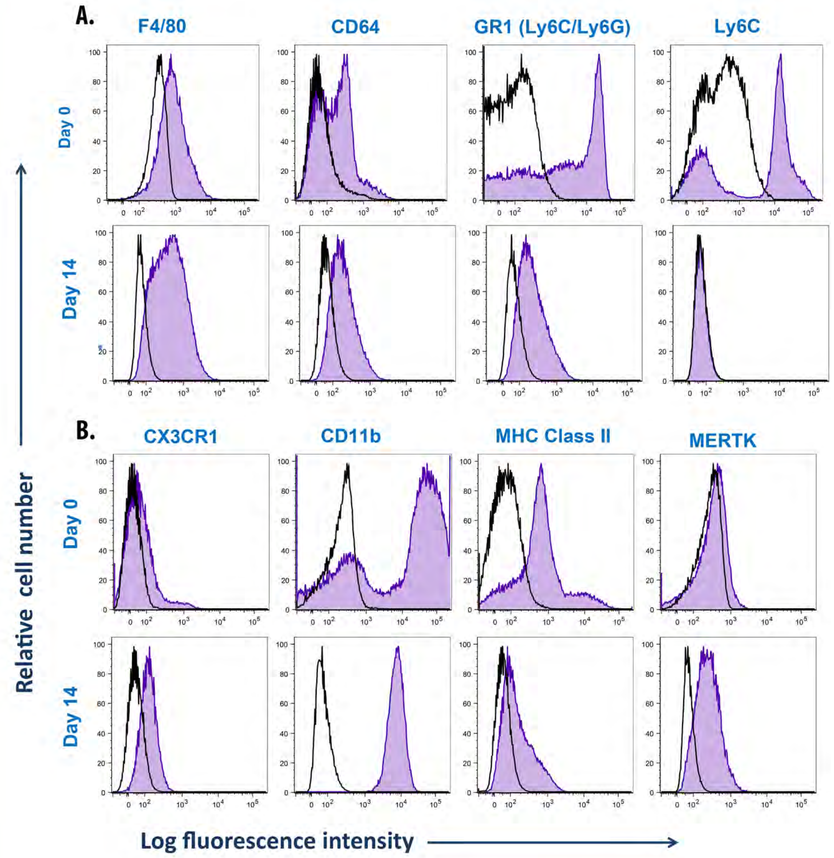

Fig 3. Bone Marrow Derived Macrophages (BMDM) FACS analysis. BM was obtained from Balb/C mice and differentiated to macrophages with GM-CSF (Troupling V *et al.*, *J Vis Exp* 81: e50966). GM-CSF was used initially at 20 ng/mL for three days and subsequently at 5 ng/mL. The derived macrophages were stained at day 14 with macrophage cell markers (filled histogram), or matched isotype control antibodies (open histograms). A. Anti mouse F4/80 (clone BM8), CD64 (X54-5/7.1), Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5), and Ly6C (clone HK1.4). B. Anti mouse CX3CR1 (clone SA011F11), CD11b (clone M1/70), MHC class II (clone 39-10-8), and MERTK (clone 2B10C42).

Phagocytic Activity

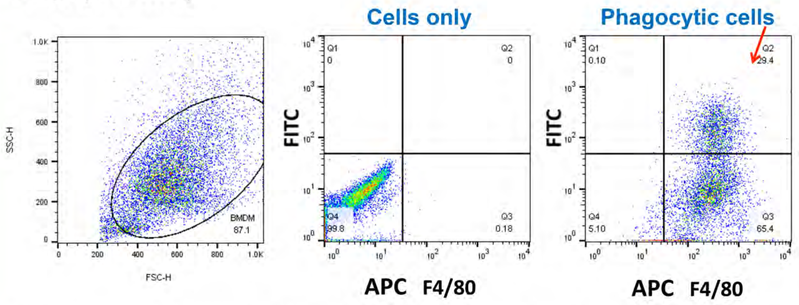

Fig 4. Functional assay for BMDM. Macrophages derived after 14 days showed phagocytic activity. This activity was tested using latex beads-IgG-FITC. Macrophages and beads were incubated for 3.5h at 37°C; subsequently, the cells were stained with APC-F4/80.

Osteoclast Differentiation

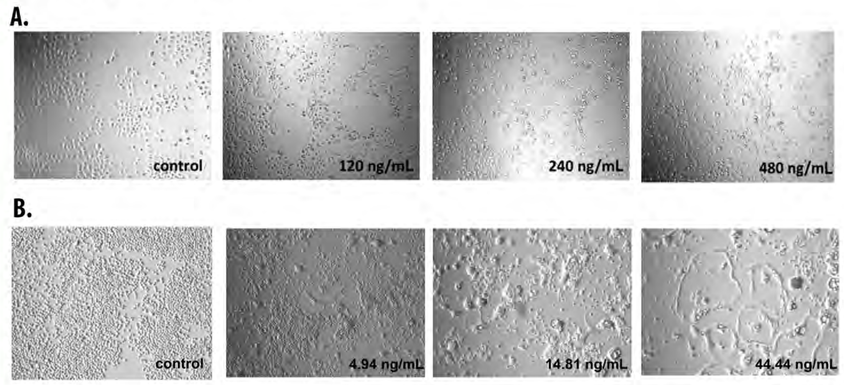

Fig 5. A. Osteoclast differentiation of BMDM. Macrophages derived after 14 days were treated with recombinant mouse M-CSF (25 ng/mL, Cat. No. 576402) for three days in MEM alpha medium with 10% FBS. Subsequently, the cells were treated with different concentration of recombinant mouse RANKL (Cat. No. 577102) in MEM alpha medium with 10% FBS for eight days. B. Osteoclast differentiation of RAW 264.7 macrophage cell line. RAW 264.7 cells were treated with different concentrations of recombinant mouse RANKL in MEM alpha medium with 10% FBS for four days. The osteoclast formation was detected with the presence of multinucleated cells and tartrate resistant acid phosphatase (TRAP) activity (see below).

TRAP Activity

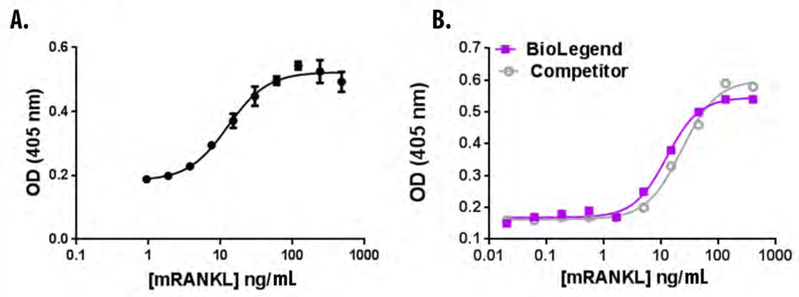

Fig 6. Tartrate resistant acid phosphatase activity (TRAP). A. TRAP activity was measured in the osteoclasts differentiated from BMDM (described in figure 5). TRAP activity was quantified in the cell layer (Ghayor C, *et al. J Biol Chem* 286:24458, 2011). B. TRAP activity detected in osteoclasts differentiated from RAW 264.7 macrophage cell line with recombinant mouse RANKL.

Conclusions

Mouse mesenchymal stem cells C3H10T1/2 and murine MC3T3-E1 preosteoblasts have been used to characterize the osteoblast differentiation using the Hedgehog family members.

BMDM is a good model to study M-CSF and RANKL in osteoclast differentiation and bone remodeling.