High-throughput tool for discovery of bone regulating factors

Parul Sharma, Keith R. Solomon, and Peter V. Hauschka
Childrens Hospital Boston, Boston, MA, USA

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Pathological changes in bone are a major medical, dental, and sports health care problem. They can arise from genetic factors or be induced by aging, trauma, environment, or drug interactions. Bone turnover is a complex, highly regulated remodeling process. Mesenchymally derived cells that make bone (osteoblasts) and hematopoietically derived cells that resorb bone (osteoclasts) are the two major cell types responsible for normal continuous turnover of the skeleton. These cell types arise from distinct precursor lineages in the bone marrow, and their complex differentiation is regulated by multiple signaling pathways. This regulation involves protein ligand interactions with cell surface receptors, including RANKL-RANK, BMP-BMP receptors, and Wnt-LRP5 (1–4). Some other proteins known to regulate bone turnover include Akt (5), fos (6), src (7), Dkk1, and SOST (8). Dysregulation in any of these genes can potentially lead to bone disorders characterized by increased (osteopetrosis) or decreased (osteopenia, osteoporosis) bone mass. For LRP5, the transducer of Wnt signaling expressed on osteoblasts, either osteoporosis or osteopetrosis may result, depending on the site of LRP5 gene mutation (4,9). A multiplex high-throughput assay for osteogenesis would be most helpful, as bone modeling is a multifactorial process, involving a variety of cell types and factors. Such an assay could analyze the effects of drugs and other agents on osteoblast differentiation and could screen limited amounts of different bone marrow samples for their osteogenic ability.

Osteogenic activity is proportional to the number of osteoprogenitor cells, and each of these cells is potentially capable of forming a colony of osteoblasts in vitro; these colonies are termed osteoblastic colony forming units (CFU-Os). Research in bone biology capitalizes on the highly elevated expression of alkaline phosphatase and tartarate-resistant acid phosphatase (TRAP) as markers for differentiated osteoblasts and osteoclasts, respectively. Catalytic activities of these enzymes can withstand simple histological fixation processes. Here we report the development of a 96-well alkaline phosphatase high-throughput assay that is compatible with multiparameter analysis for studying the osteoblast differentiation process. Alkaline phosphatase has been critically reviewed and evaluated as a true marker for differentiated osteoblasts (10–12). The alkaline phosphatase enzyme assay described here is a modification of the assays earlier described by Huggins et al. (13,14) and Sabokbar et al. (15).

Most studies assessing osteoblast differentiation have used either primary cultures of osteoblasts, expanded from rodent calvarial collagenase digests (16,17), or bone marrow stromal cells, flushed from mouse long bones (18–21). These methods involve selective culture of adherent cells, variable culture time, and multiple subcultivations, all of which are likely to affect the outcome of drug/agent studies. To avoid these confounding variables, we chose to validate our novel high-throughput assay on unexpanded bone marrow-derived osteoblasts. To optimize the assay conditions for these naïve primary cells, the assay well size was reduced to a 96-well format so that the cultures would reflect the initial osteogenic properties of the bone marrow. On day 0, femurs were freshly excised from C3H or BL/6 mice sacrificed by CO_2 inhalation, and marrow was flushed with serum-free α minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (final concentration of 100 U/mL for each antibiotic; both from Sigma, St. Louis, MO, USA). Cells were passed through a 70 µm strainer and pelleted at

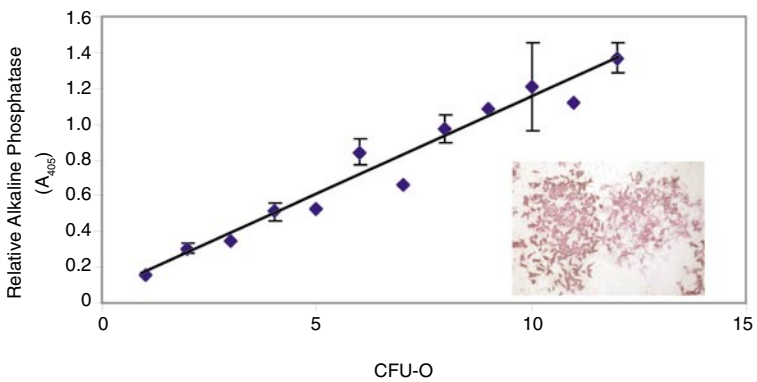

Figure 1. Correlation between the number of osteoblastic colony forming units (CFU-Os) and total alkaline phosphatase level. Alkaline phosphatase levels were measured in fixed wells from bone marrow cultures by quantitating A_{405} of the hydrolyzed soluble substrate p-nitrophenyl phosphate. There is an excellent correlation [Pearson correlation, $R = 0.98$, using GraphPad InStat program (GraphPad Software, San Diego, CA, USA), $P < 0.0001$) between A_{405} and microscopically counted alkaline phosphatase-stained positive colonies, CFU-Os. Variability is contributed by the intensity of alkaline phosphatase expression and size of the CFU-Os (figure inset). To avoid colony overlap, only wells containing 12 or fewer colonies were counted. The inset shows two differentiated CFU-Os in bone marrow cultures stained histochemically with the 86-R leukocyte alkaline phosphatase kit. Even within each colony unit, the individual cells may have varying levels of alkaline phosphatase. Data are graphed as means ± SEM, showing values for CFU-Os (x-axis) and A_{405} (y-axis).

4°C for 15 min. They were resuspended in α-MEM containing 10% fetal bovine serum (FBS), counted, plated at 5×10^4 cells/well of a Corning® Costar® 96-well tissue culture plate (Corning, Acton, MA, USA), and grown overnight at 37°C. On day 1, the medium was replaced by fresh media with and without various differentiation factors. Fresh medium and differentiation factors were again added on day 4. Since osteoblast expression of alkaline phosphatase was detectable after 4 days (22), we took advantage of this relatively early expression and stopped the assay on day 5 (for faster turnaround).

The cells were fixed for 1 min at 23°C with 27 mM citrate buffer, pH 3.6, containing 12 mM NaCl, 3% paraformaldehyde, and 66% acetone (Sigma) and quickly rinsed twice with deionized water. An alkaline phosphatase substrate that produces a yellow colored product, p-nitrophenyl phosphate (pNPP; Sigma), was added to the wells. Cells were incubated for 10 min at 23°C in the dark, and absorbance was measured at 405 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader.

To establish a correlation between the A_{405} values and the microscopically counted CFU-Os, we counted the colonies in 80 wells of a typical experiment. Histochemical staining of alkaline phosphatase positive colonies in the same wells was then done using the 86-R leukocyte alkaline phosphatase kit (Sigma). Figure 1 shows the excellent correlation coefficient ($R = 0.98$) of colony number (x-axis) and A_{405} (y-axis) for the paired data set. Variability in the readings results from differences in the intensity of alkaline phosphatase expression and sizes of the CFU-Os (see Figure 1, inset).

For secondary multiplex analysis, the same plate was rinsed in deionized water and processed further for detection of additional markers using standard staining techniques [e.g., TRAP, a marker for osteoclast differentiation; 4′-6-diamidino-2-phenylindole (DAPI) for counting nuclei] and imunohistochemical detection of markers of osteogenesis and cell lineage (e.g., osteocalcin, a late marker of osteogenesis; or desmin or smooth muscle actin, pericyte markers) (Figure 2). Plates processed in this sequence would be compatible with automated techniques for quantifying colony size, shape, cell fluorescence, and other parameters.

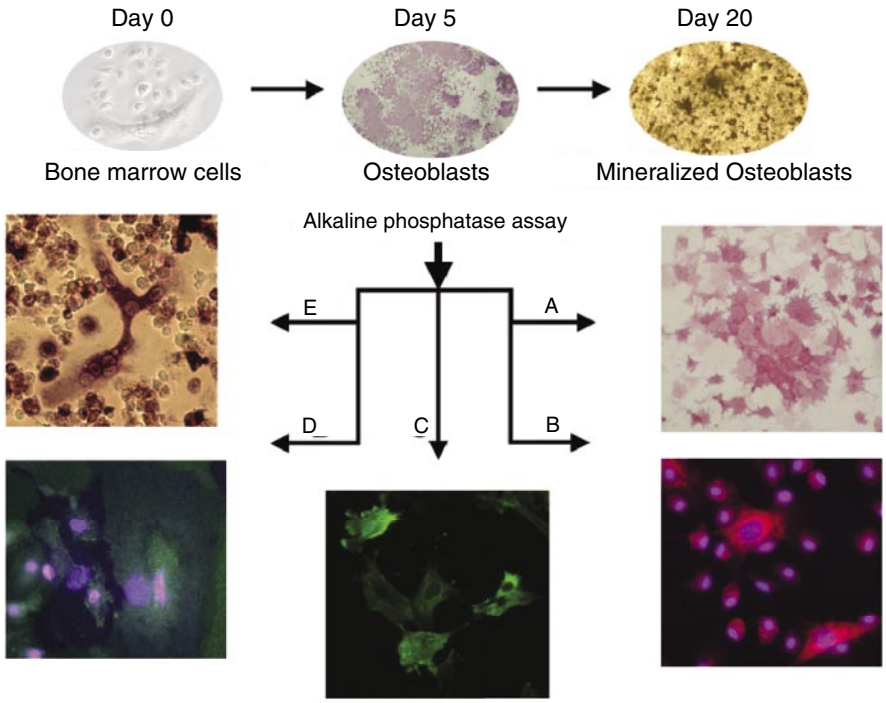

Figure 2. Secondary multiplex analysis. (Top panel) Osteoblast differentiation. Bone marrow cells treated with mineralizing medium (50 µg/mL ascorbic acid, 10 mM β-glycerol phosphate) have detectable alkaline phosphatase levels by day 5 [pink, alkaline phosphatase-stained osteoblastic colony forming units (CFU-Os)]. On continued treatment in mineralizing conditions, numerous mineralized nodules form by day 20 (black, von Kossa stain). (Bottom panel) Multiplex detection of differentiation markers for osteoblasts and other cell types in bone marrow cultures. Measurement of CFU-Os by alkaline phosphatase using p-nitrophenyl phosphate (pNPP) was followed in the same wells by (A) histochemical staining for alkaline phosphatase activity to reveal CFU-Os (pink); or (B) fluorescent immunostaining for osteocalcin, a late osteoblastic differentiation marker (red, goat antimouse osteocalcin BT-432; Biomedical Technologies, Stoughton, MA, USA), secondary antibody A-11057 (Molecular Probes™ Invitrogen), and nuclei [blue, 4′-6-diamidino-2-phenylindole (DAPI)]; or (C) immunostaining for smooth muscle actin, a marker of vascular smooth muscle cells (green, mouse anti-smooth muscle actin antibody M0851; DakoCytomation, Hamburg, Germany), secondary antibody A-11059 (Molecular Probes Invitrogen); or (D) immunostaining for desmin, typically a pericyte marker (green, mouse anti-desmin antibody M0760; DakoCytomation), secondary antibody A-11059, and nuclei (blue, DAPI), showing a desmin-negative alkaline phosphatase-positive osteoblast [black in fluorescein isothiocyanate (FITC) channel due to previous staining as in panel A]; or (E) histochemical staining for tartarate-resistant acid phosphatase (TRAP) activity (Sigma), an osteoclast marker (red-brown). Image acquisition utilized both an upright microscope (alkaline phosphatase, von Kossa, and TRAP staining) and a Nikon Eclipse TE2000-U inverted fluorescence microscope (all immunofluorescent staining; Nikon, Tokyo, Japan).

Our sequential assay design, optimized for cultures of primary osteoblasts and CFU-Os also works well with homogenous murine osteoblast cell lines. For instance, after taking account of the higher level of basal alkaline phosphatase activity of the immature murine MC3T3-E1 osteoblastic cell line, we found that the clonal colony assay was well correlated with total alkaline phosphatase activity (data not shown). Importantly, the procedure preserves most antigenic epitopes, allowing sequential measurement with fluorescent- and peroxidase-labeled secondary antibodies, thus permitting multiple readouts from the same sample. Antibody binding to various antigens (osteocalcin, desmin, etc.), staining of TRAP enzyme, fluorescein isothiocyanate (FITC)-phalloidin labeling of cytoskeletal structure, and DAPI labeling of nuclei were unaffected by the initial alkaline phosphatase assay.

Preservation and localization of osteogenic biomarkers is essential for identifying their roles in bone formation. This simple fix-and-measure procedure for quantitation of CFU-Os is compatible with a wide variety of downstream analyses using immunohistochemical reagents and high-throughput liquid handling instruments. In summary, this robust and versatile assay should permit co-measurement of the rapidly expanding number of pathways and new proteins that have been implicated in early osteoblast differentiation, skeletal growth, and maintenance of bone mass.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Peter V. Hauschka, Department of Orthopedic Surgery, Childrens Hospital Boston, Enders-1007, 320 Longwood Ave, Boston, MA 02115, USA. e-mail: peter.hauschka@childrens.harvard.edu