CELL-BASED OSTEOGENIC ASSAYS FOR DRUG DISCOVERY AND METASTATIC DISEASES

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Abstract

A cell-based assay for human osteogenesis is described. The assay is based on the differentiation of human MSCs into osteoblasts. The assay demonstrates differentiation induced early activation of alkaline phosphatase (ALP) and increased expression of Runx-2, and osteopontin (OPN) at 3 to 6 days followed by suppression of Runx-2 and OPN gene expression while osteocalcin (OC) gene expression was not elevated until 24 days of continuous exposure to differentiation agents. Differentiating agents included dexamethazone (10 nM & 100 nM) Lithium (0.5 mM & 1 mM) for Wnt pathway activation together with 200 ng/ml parathyroid hormone (PTH). Cytokine analysis of conditioned media showed differentiation induced secretion of GM-CSF, a well-known marker of osteogenesis. Also, differentiation induced secretion of MMP-2, TGF-β1 and TNF-α. This may reflect auto-regulation of this cell-based assay system. Smart Flare® probes were used to demonstrate live cell expression of β-actin. This MSC-based assay system is suitable for high throughput screening of specific molecular targets including Runx-2 and controlling microRNAs, especially since its expression is a key fate determinant in osteoblast differentiation from MSCs. Other molecular targets may also be analyzed using high throughput readout using this MSC-based assay.

Introduction

Osteoporosis is a growing global health problem now affecting 200 million women globally. This incidence is age related: 1/10 at 60, 1/5 at 70, 2/5 at 80 and 2/3 at 90 years of age. Furthermore, men are also at risk for osteoporotic fractures and for men over 50, the risk of osteoporotic fracture is greater than the risk of prostate cancer (International Osteoporosis Foundation). Bone growth involves the coordinated action of bone genesis and resorption. Osteoblasts generate new bone while osteoclasts are primarily responsible for bone resorption. Traditional treatments of osteoporosis involve blockage of resorption, e.g., by bisphosphonates. More recently, anabolic agents have been approved for treatment of osteoporosis and these activate osteogenesis resulting in new bone growth as therapy for osteogenesis, osteogenesis imperfect and other conditions associated with low bone density. However, current anabolic agents have problematic side effects including sarcoma and limited usage periods, driving the

development of new and improved osteoporosis treatments. Here we describe a human MSC-based assay that replicates osteoblast differentiation in a live-cell, *in-vitro* format, suitable for high-throughput readout of specific molecular events for use in development and QC of novel osteoporosis drugs and natural substances. This assay may also be extended for use in cell-based assays of bone metastatic disease.

Materials and Methods

Cell Culture

Cryopreserved cord blood-derived mesenchymal stem cells (Vitro Biopharma Cat. No. SC00A1) were initially expanded in T-25 tissue culture flasks (BD Falcon, Cat. No. 353108) using MSC-GRO low serum, complete medium (Vitro Biopharma Cat. No. SC00B1) for cell culture. Cells were harvested using Accutase (Innovative Cell Technologies Inc., Cat. No. AT-104) for 15 minutes and placed into a 15mL conical tube. Cells were collected via centrifugation (450 x G) for 7 minute. Cell supernatant was aspirated off and cells were resuspended in 1mL PBS and counted on a Beckerman-Coulter Z2 particle counter (range 10 μm-30 μm).

Osteogenic Differentiation

Cells were plated at 5,000/cm^2 in a Falcon BD tissue culture 6-well plates and maintained in MSC-Gro™ proprietary osteogenic differentiating low serum, complete media (Vitro Biopharma, Cat. No. SC00B11) in a reduced O_2 environment (1% O_2, 5% CO_2, 90% N_2) at 37°C in a humidified chamber. Differentiation was induced by exposure to 10nM dexamethasone, 100nM dexamethasone, 0.5mM lithium, 1mM lithium, and 200 ng/mL PTH. Cultures were maintained for a period of 30 days. Conditioned media and lysed cells were collected at day 3, 6, 12, 18, and 24 from each dose. Phase contrast images were obtained using an Olympus CKX41 microscope at 10x.

RNA extraction and expression of Osteogenic-specific genes

Cells were harvested and lysed at day 3, 6, 12, 18, and 24 of each dose using Accutase (Innovative Cell Technologies Inc., Cat. No. AT-104) for 15 minutes and placed into a 15mL conical tube. Cells were collected via centrifugation (450 x G) for 7 minute. RNA was extracted from the cells at each time period using RNeasy Mini Kit (Qiagen Cat. No. 74124) and quantified on a spectrophotometer measuring absorbance at 260 nm. RT-PCR was performed using OneStep RT-PCR Kit (Qiagen Cat. No. 210210) according to the manufacture's procedure. Runx-2, osteopontin, and osteocalcin expression was determined at each time period. Gene expression was estimated by positive amplification of each dose at each time point and graphed as percent gene expression over time using Origin 8.1.

Alkaline Phosphatase Analysis

Lysed cells were used to determine the concentration of alkaline phosphatase versus inhibited alkaline phosphatase (ALP). ALP was inhibited with 60 μM Tetramisole for 5 minutes at 37°C. 10 μL of each sample +/- inhibition was added to a black 96-well plate (ThermoScientific, Cat. No. 165305). 90uL of 4-MUP (Sigma, Cat. No. M3168) was added to all wells and incubated at

room temperature for 30 minutes in the dark. Alkaline phosphatase measurements were taken on a Modulus Microplate Reader (Ex= 365nm, Em= 410-460nm). Statistical analysis was performed using the Student's t-test.

Cytokine Secretion Analysis

Conditioned media was analyzed using a bone metabolism array (Ray Biotech Cat. No. QAH-BMA-1000) and read on a laser scanner (Molecular Devices, GenePix Pro 4000B) to measure cytokine secretion according to the manufacturer's suggested procedures.

Smart Flare Gene Expression Analysis

Uptake control, beta actin and beta-catenin Smart Flares were purchased from EMD-Millipore (Catalog numbers: SF-114, SF-145, SF-738). These were prepared according to the manufacturer's instructions and added to cell culture media at 100pM concentration. Following overnight incubation, fluorescent signals were visualized using the Cytation 3 imaging system (BioTek).

Results and Discussion

Cell Differentiation and Select Gene Expression

The typical gene expression profile of Runx-2, OPN & OC is shown in Figure 1. There were some minor differences between the differentiation conditions, e.g., 100 nM dexamethasone induced Runx-2 expression at 3 days while the other differentiation conditions did not. In general each differentiation agent produced similar changes in expression of these three genes while no expression was observed in the absence of differentiating agents. MSCs displayed typical fibroid morphology during growth and exhibited differentiated morphology, e.g, clusters of superficial differentiating osteoblasts, when exposed to differentiating conditions.

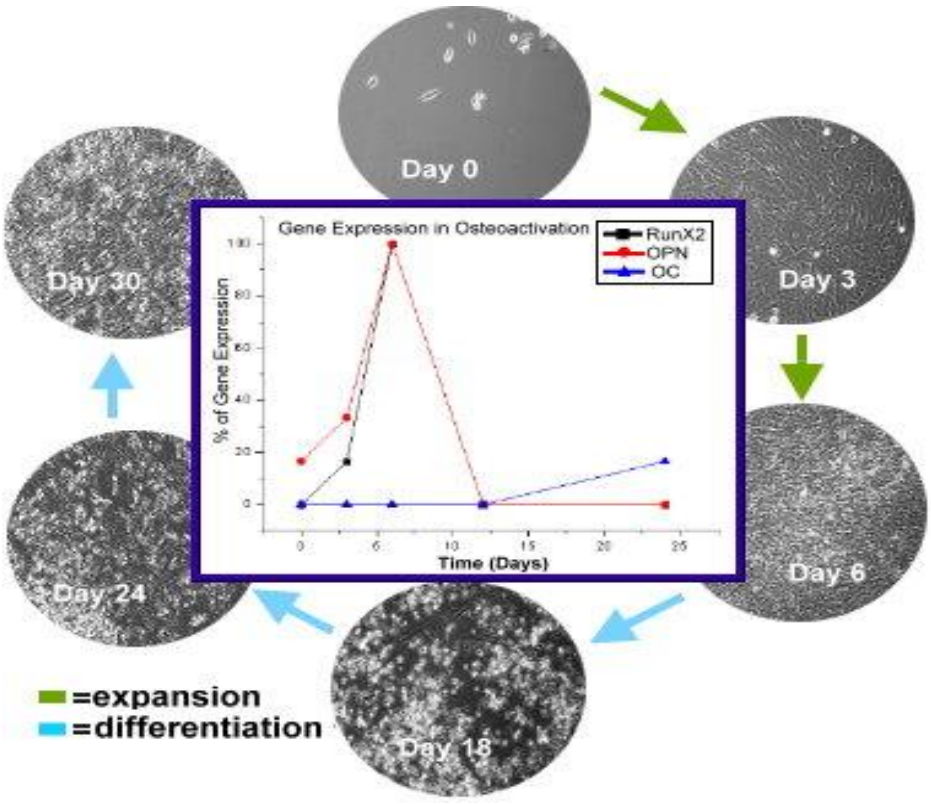


Figure 1: The images of osteogenic expansion and differentiation using phase-contrast microscopy. The graph depicts the percent of gene expression as a function of time. At day 12, Runx-2 and OPN were not expressed. Osteocalcin is expressed following 24 days of culture.

Alkaline Phosphatase Expression

The bar graph below shows the ALP activity in MSCs in control conditions or exposed to differentiation agents (gray bars) for three days. We also determined the effect of the ALP inhibitor, Tetramisole, and these results are shown as red bars (+/- SD of 6 determinations). Since some activity was inhibited by tetramisole, it is likely to reflect ALP.

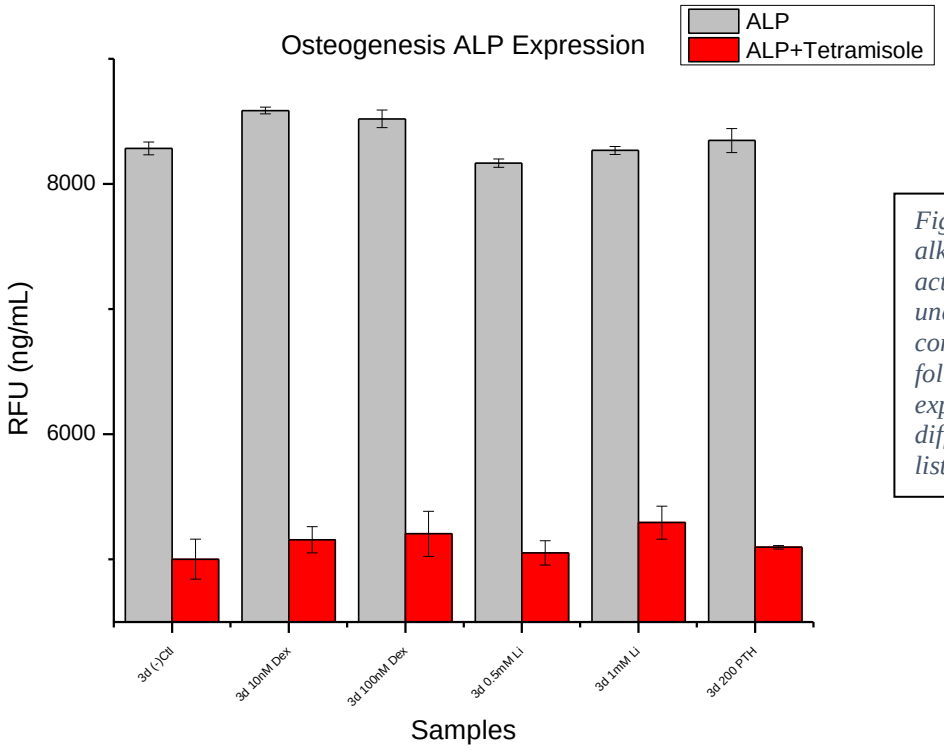


Figure 1: shows the alkaline phosphatase activity in MSCs under control conditions and following 3days exposure to the differentiating agents listed.

The results indicate substantial ALP activity in undifferentiated MSCs, possibly indicating expanded differentiation capacity of this cell line since ALP activity is associated with pluripotency. Also, early stages of differentiation induced small increases in ALP activity due to differentiation induced by 10 nM and 100 nM dexamethasone ($p<0.001$ for 10 nM dexamethasone and $p< 0.01$ for 100nM dexamethasone), while only small, non-significant, increases were seen with 1 mM Li and 200 ng/ml PTH. Thus, although ALP activity was increased by osteogenic differentiation, the increases were small at this stage of differentiation. Much larger increases in ALP were apparent after 22 days exposure to 100 ng/mL and 200 ng/mL PTH (data not shown). Increases in ALP are an early biomarker of osteogenesis together with the increased expression of Runx-2 and OPN.

Cytokine Secretion Analysis

The content of select cytokines in conditioned media exposed to control or differentiation conditions for 24 days was measured using antibody microarrays and the results are shown in Figure 3, below.

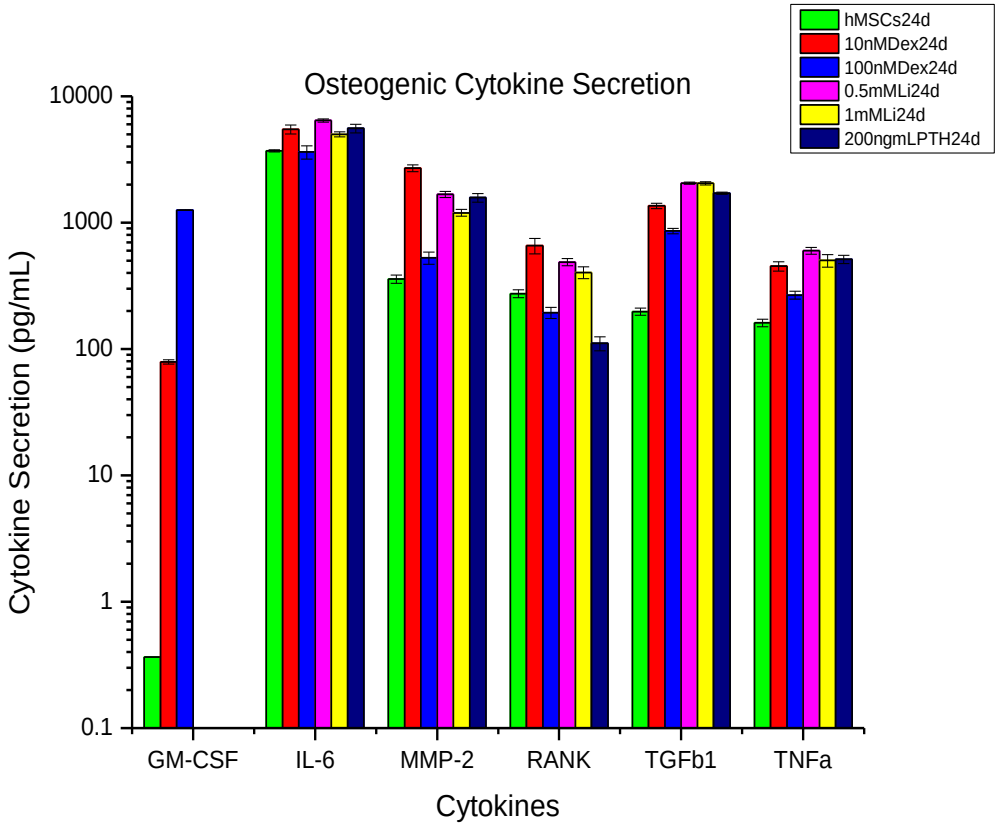


Figure 2: The graph shows cytokine secretion during osteogenesis. The concentration of cytokines is shown under control conditions and during continuous exposure to differentiation agents for 24 days.

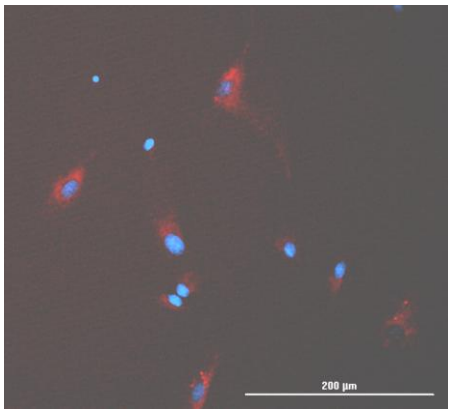
The secretion of GM-CSF, a well-known marker of osteogenesis (Chaudhory, LR, et al., Endocrinol. 130: 2528-2534, 1992) was substantially elevated by osteoblast differentiation induced by 10 nM dexamethasone (>200-fold) and further stimulated by 100 nM dexamthasone (> 2500-fold). While the secretion of IL-6 or RANK did not show consistent differentiation induced changes, MMP-2, TGF-β1 and TNF-α showed increased secretion correlated with osteoblast differentiation. There was a reduction in secretion of these cytokines at 100nM dexamethasone, possibly due to excessive dosage. These results provide additional evidence that the cell based assay system described here replicates osteogenesis and also provides insights into regulation of this process and potential novel sites of intervention in the treatment of osteoporosis.

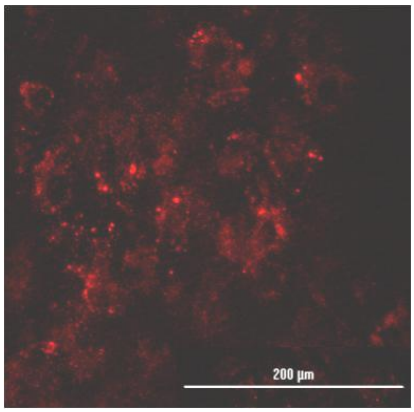
Thus, the increased secretion of TGF-β1 & TNF-α may reflect endogenous regulation of osteoblast differentiation since both TNF-α and TGF-β1 block osteoblast differentiation and TNF-α is also known to activate osteoclastogenesis (Loots, CG, et. al., Bone 50: 663-669, 2012 & Osta, et. al., Front Immunol 5:48, 2014). Also, MMP-2, is a proteolytic enzyme that breaks down bone effectively inducing bone resorption (Quan, J., et.a.l., Pathology 44: 221-227, 2012). Thus, differentiation induced secretion of regulatory molecules provides a cell-based assay

system to assess blockage of the osteogenesis inhibitors TGF-β1, TNF-α and MMP-2 as potential treatments or adjuvants to osteoporosis treatments. Also, MMP-2 may be involved with the invasive process of the metastasis of cancer to bone.

Live-Cell RNA Expression

The figures below show the expression of beta-actin in live cell cultures of MSCs without differentiation. The image shown on the left indicates nuclei (blue-DAPI) and β-actin expression-red (Cy3). On the right, a higher density culture is shown of MSCs without nuclear stain. These results show that the cell-based assay described here may be used for live cell determination of gene expression (RNA probes) or regulation of gene expression through use of specific microRNA Smart Flare® probes.





Conclusions:

We describe a human MSC-based assay system that replicates osteogenesis. This was indicted by sequential appearance of increased alkaline phosphatase activity, early expression of Runx-2 at day 3-6 that slightly proceeded OPN expression. Both Runx-2 & OPN expression were then suppressed while osteocalcin expression required 24-days continuous exposure to differentiation agents including dexamethasone, lithium and PTH. Osteogenesis was accompanied by multi-fold increases in GM-CSF secretion during exposure to dexamethasone. Interestingly, this system also appeared to exhibit auto-regulation by secretion of osteogenic inhibitory agents TGF-β1 and TNF-α. The latter cytokine also stimulates osteoclastogenesis further suggesting auto-regulation. In addition, MMP-2 secretion was potentiated during osteoblast differentiation. Since MMP-2 may dissolve bone, its secretion may be an additional auto-regulatory process of osteogenesis. This system is suitable for high-throughput screening of several events in bone generation and homeostasis including specific gene expression analysis and intervention.