# OSTEOCYTE INTERACTION WITH OSTEOBLASTS AND RESPONSE TO INTERMITTENT HYDROSTATIC PRESSURE LOADING IN A 3D TRABECULAR BONE EXPLANT CULTURE MODEL

**Erica Takai, Robert L. Mauck[+], Clark T. Hung[+], X. Edward Guo**

Bone Bioengineering Laboratory [+]Cellular Engineering Laboratory

Department of Biomedical Engineering
Columbia University
New York, NY, 10027

## INTRODUCTION

The long-term goal of this study is to develop an *in vitro* culture system that maintains the 3D extracellular matrix environment of live osteocytes, with the ability to selectively seed osteoblasts and/or osteoclasts. This system may potentially be used for the study of mechano-signal transduction between different cells that populate bone as well as in the development of new bone tissue engineered constructs using live trabecular bone scaffolds.

Despite the unique spatial distribution of osteocytes in bone tissue and their possible role in mechanotransduction, there has been limited characterization of osteocyte response to mechanical stimuli in their native three-dimensional (3D) environment [1, 2, 3]. Osteocytes have also been hypothesized to play an important role in the coordination of osteoclast and osteoblast activity in bone adaptation to mechanical stimuli [4], yet there are few studies demonstrating the direct influence of osteocyte-osteoblast interaction on osteocyte function *in vitro*. Moreover, although the peak hydrostatic pressures in bone pores are thought to be as high as 2.2MPa [5], there are no studies that have examined the effect of hydrostatic pressure on osteocyte function *in situ*. The specific aims of the current study are to examine the effect of 1) the presence of seeded osteoblasts (experiment I), and 2) dynamic hydrostatic pressure loading (experiment II) on osteocyte viability.

## MATERIALS AND METHODS

Cylindrical trabecular bone cores (Ø=5mm, h=4mm) were harvested from the epiphysis of tarsal bones of 3-month-old calves using a diamond tipped coring tool (Starlite Industries, PA) and an Isomet low-speed saw (Buehler, IL) under sterile conditions. The cores were cleaned of bone marrow and most lining cells with sterile PBS using an Interplak water pik (Conair Corp., CT). Some cores were seeded the next day with osteoblasts harvested from tarsal trabecular chips using a custom-made cell seeder, resulting in an initial seeding density of approximately $5x10^5$ cells/core. The cores were divided into four groups: 1) live cores with and 2) without osteoblasts, for experiment I; 3) live cores with 4) and without hydrostatic pressure loading for experiment II. All cores were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

In experiment II, intermittent hydrostatic pressure loading was applied using a custom-feedback controlled pressure chamber [6] (Fig. 1). The cores were placed in sealed sterile plastic bags with 7 ml of supplemented α-MEM, then placed in the pressure chamber. Control samples were similarly sealed in plastic bags, but were not subjected to pressure loading. Loaded cores were subjected to 3MPa (~500psi) hydrostatic pressure loading at 0.33Hz with a triangular waveform for 1 hour per day, for 5 out of an 8-day culture period. All experiments were performed at 37°C.

The cores from experiment I were harvested on days 2 (n=1), 7, and 11 (n=2), and cores from experiment II on days 1, 5, and 8 (n=4). At each time point, each core was cut vertically in the center to produce a rectangular cross section. One half of the cut cores were stained using a live/dead cell viability stain (Molecular Probes, OR) consisting of calcein-AM (live) and ethidium homodimer-1 (dead), and 16 images were taken with a Fluoview confocal microscope system (Olympus, NY) at 10μm increments. The remainder of the cut specimens were fixed in neutral buffered formalin, decalcified in a buffered formic acid solution for 2 days, paraffin embedded, and sectioned at 10μm thickness. All sections were stained with hematoxylin and eosin (H&E; Sigma, MO).

To determine osteocyte viability, osteocytes with intact nuclei were counted in a 400μm by 4,000μm area in the center of each H&E stained section and normalized to the bone area in that region, using a bone histomorphometry system (Osteometrics, GA). Three sections from each specimen were analyzed. To determine statistical significance, ANOVA with Fisher's post-hoc analysis was performed (Systat, IL).

## RESULTS

Experiment I: Osteocyte viability assessed from H&E stained sections of both seeded and unseeded cores were found to decrease over the culture period, with a significant ($p \leq 0.02$) decrease in

osteocyte viability between day 2 and day 7 or 11 (Fig. 2). The number of viable osteocytes was significantly (p=0.01) smaller in seeded cores compared to unseeded cores at day 11. Live/dead staining showed that at day 2 many osteocytes were alive for both seeded and unseeded cores, with a decrease in number of viable cells with time (Fig. 2, 3). Similarly, by day 11 there were more live osteocytes in the unseeded cores compared to seeded cores in live/dead stained cores (Fig. 3).

Experiment II: Consistent with experiment I, viability of the osteocytes were found to decrease over the culture period in both loaded and unloaded cores, with a significant ($p \leq 0.001$) decrease in osteocyte viability between day 1 and day 5 or 8 (Fig. 4). However, hydrostatic pressure loading prevented the decrease in viability between day 5 and day 8 seen in the unloaded group (p=0.003), with a statistically higher number of viable osteocytes in the loaded cores on day 8 (p=0.01). Live-dead staining showed similar trends with more live osteocytes in loaded cores at day 8 (Fig. 5).

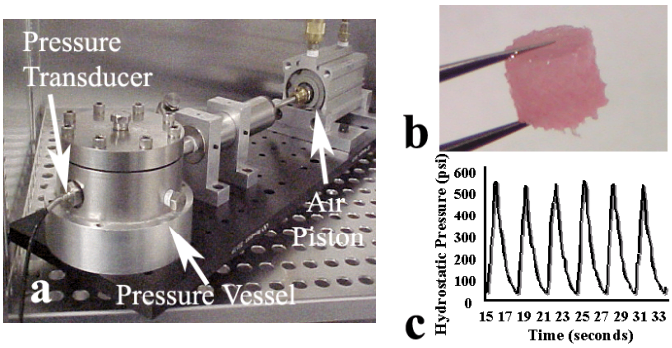


**Figure 1.** a) Custom hydrostatic pressure chamber; b) Trabecular bone core; c) Hydrostatic pressure loading profile.

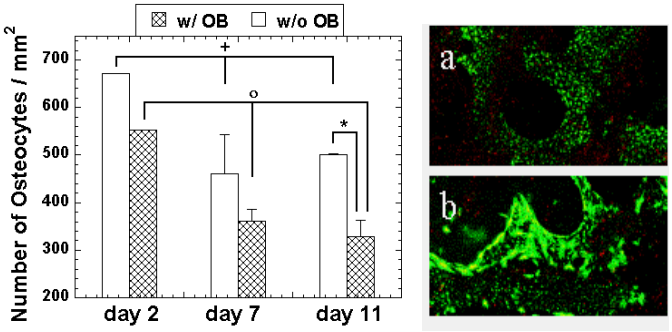


**Figure 2**. Experiment I: number of osteocytes/mm$^2$ in the presence and absence of osteoblasts (left), *p=0.01; +o $p \leq 0.02$. Live (green)/dead (red) staining at day 2 of a) unseeded and b) seeded cores (right). 100x

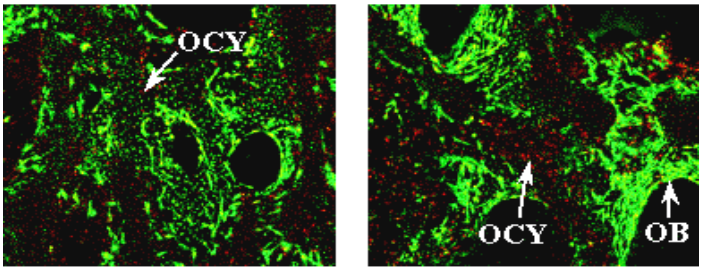


**Figure 3**. Live (green)/dead (red) stain at day 11 unseeded (left), and seeded (right). Osteocytes=OCY; Osteoblasts=OB. 100x

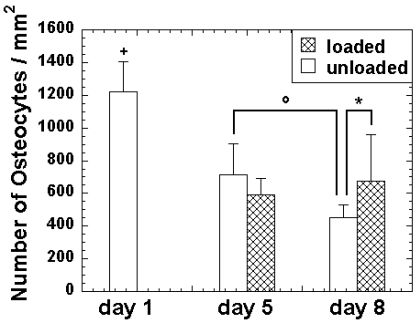


**Figure 4**. Experiment II: number of osteocytes/mm$^2$ ±hydrostatic pressure loading; +p≤0.001 with all others; op=0.003; *p=0.01.

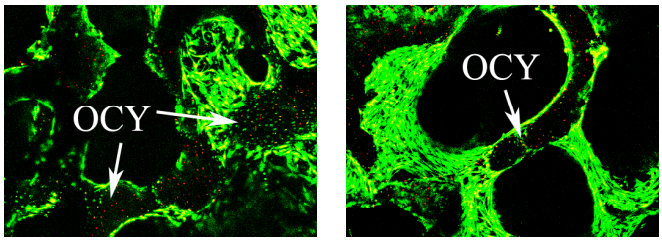


**Figure 5**. Live (green)/dead (red) stain at day 8 loaded (left), and unloaded (right). Osteocytes=OCY; 100x

## DISCUSSION

Although viability of osteocytes in bone explant cultures has been assessed for short culture periods (<1 week) [1], viability at longer time periods have not been assessed. Furthermore, to our knowledge, the decrease in osteocyte viability in the presence of osteoblasts has never been reported. In the first part of this study, both live/dead staining and H&E staining show that in the absence of mechanical stimuli, osteocyte viability is significantly decreased in the presence of osteoblasts by day 11. This finding may be attributed to nutrient limitations that arise with the addition of osteoblasts, or osteocyte-osteoblast interactions.

Interestingly, application of intermittent hydrostatic pressure enhanced cell viability over culture time. The mechanism underlying this phenomenon is unclear in light of the general thinking that application of hydrostatic pressure to an incompressible system (media and cells) would not induce cell/tissue deformation that would give rise to fluid flow and nutrient transport. Although most cells populating the trabecular surface are initially washed away (Fig. 2a), some persist and proliferate over time (Fig. 3, 5). It is possible that the re-populated surface cells are also affected by the pressure loading and in turn influence the osteocytes. A combined study, which examines the effects of hydrostatic pressure loading in the presence and absence of seeded osteoblasts, may further clarify this question. Our findings may have implications on the finding that intermittent compressive loading of rat metatarsals also increases osteocyte viability compared to static controls during a 5-day culture period [2].


## ACKNOWLEDGEMENTS

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