INTRODUCTION:
Bone adaptation, maintenance, and repair are dependent on local and systemic bio-regulators and their interaction with physical forces such as fluid shear, biaxial stretch, and hydrostatic pressure. In vitro, these types of loads have been shown to differentially affect gene expression by osteoblast lineage cells. For example, in osteoblasts strained at 1% [1] or exposed to static fluid shear, activation of the transcription factor NF-κB was observed; while oscillatory fluid flow reduced TNF-a mediated NF-κB activation [2]. NF-κB, along with the transcription factors serum response factor (SRF) and c-fos, are involved in many cell signaling pathways; including responses to proliferative, apoptotic, and mechanical stress cues. The purpose of this study was to examine the activation and expression of these early transcription factors in response to cytokine exposure, different mechanical loading regimes, and the possible interactions between these stimuli.

METHODS:
Cell cultures: Murine preosteoblasts (MC3T3-E1) were seeded at a density of 30,000 cells/cm² on 35mm dishes or Bioflex™ 6-well plates [Flexcell International] coated with fibronectin. Experiments were performed after 18 hours of serum starvation. All experiments were replicated.

Cytokines: Cells were exposed to 0.5ng/ml IL-1β (Roche) or 30ng/ml FGF-2 (Scios) to activate the NF-κB and SRF/c-fos pathways.

Hydrostatic Load: Cells on 35mm dishes were enclosed in a hydrostatic pressure chamber compressed to either 1 or 10psi via a pneumatic control system operated using a custom LabVIEW program. Pressures were applied at 0.05Hz (10s on, 10s off).

Substrate Stretch: The Bioflex plate wells were sealed in a specially designed clamp, and distended via a pneumatic control system to induce experimentally verified surface strains. Strains were applied at 0.05Hz (10s on, 10s off) up to 2h.

Immunocytochemistry: Cells were fixed, permeabilized, blocked, and incubated with anti-NF-κB p65 antibody followed by a rhodamine-conjugated secondary antibody. Images were recorded and analyzed using the BioQuant Imaging System. Optical densities were recorded in the nuclei and cytoplasm of >40 cells in 5 images per group.

Western blotting: Lysates were collected in RIPA Buffer and 20μg protein was analyzed by Western Blot for IBa, NF-κB p65, SRF, and c-fos. Signal was detected by chemiluminescence (Bio-Rad ChemiDoc).

RESULTS:
Substrate stretch stimulates SRF and c-fos:
In various cell types, including osteoblasts, c-fos is induced by strain. SRF is an early response gene responsible for c-fos upregulation via the serum response element in the c-fos promoter. Results demonstrated a transient induction of c-fos and SRF within 30 minutes of strain application over 8% (Fig 1), similar to an exposure to FGF-2. No activation of c-fos was observed at 1 or 10psi cyclic hydrostatic pressure. These results strongly demonstrate differential gene expression responses for different load regimes.

Figure 1: SRF & c-fos induction by 8% Strain

Substrate stretch represses IL-1β induced NF-κB activation:
NF-κB is typically sequestered in the cytoplasm by its inhibitory molecule IκBa. Upon stimulation, IκBa is degraded and NF-κB translocates to the nucleus. NF-κB activation was not observed in cells subjected to stretch. However, the stimulation of NF-κB by IL-1β was reduced. Immunofluorescence of NF-κB p65 subunit revealed a qualitative decrease in nuclear to cytoplasmic staining in activated cells at 30 and 45 minutes (Fig 2). To explore the mechanism of this load effect, we examined IκBa degradation via Western blot. IL-1β treatment resulted in temporal IκBa degradation, which was reduced by application of 8% cyclic substrate stretch (Fig 3). NF-κB levels remained consistent for all groups. No reduction in IκBa degradation was observed at 1 or 10psi cyclic hydrostatic pressure.

Figure 2: IL-1β induced NF-κB p65 activation is reduced in stretched cells

DISCUSSION:
We have demonstrated that mechanical load can directly activate transcription factors and also modulate their activation by cytokines. These results are the first demonstration of SRF induction by strain in osteoblasts. SRF induction could explain mechano-regulation of c-fos, however it did not temporally precede it.

No NF-κB activation was observed in biaxially strained MC3T3 preosteoblasts, in contrast to a previous study using a more mature ROS17/2.8 osteosarcoma cell line [1]. When NF-κB was hormonally activated, however, biaxial strain suppressed this response. The mechanism of this suppression was via decreased degradation of IκBa. These results are consistent with a prior study that exposed UMR106 osteosarcoma cells to oscillatory fluid flow [2]. It is likely that there is a component of fluid shear imparted to cells in our system.

Although it has been shown that MC3T3 cells can respond to hydrostatic pressure with increases in PGE2 and decreases in alkaline phosphatase [3], all of the load responses in this study were seen with cyclic biaxial strain. This supports the hypothesis that the specific local three-dimensional stress state substantially influences the biological response.

REFERENCES:

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