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Original Article

Hylonome

In vitro and *in vivo* study on osteocyte-specific mechanical signaling pathways

W. Yang[1], I. Kalajzic[2], Y. Lu[3], D. Guo[3], M.A. Harris[1], J. Gluhak-Heinrich[4], L.F. Bonewald[3], J.Q. Feng[3], D.W. Rowe[2], S.E. Harris[3]

[1]Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, TX, [2]Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, Conn, [3]Dept. of Oral Biology, University of Missouri at Kansas City, Kansas City, MO, [4]Department of Orthodontics, Dental School, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

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Introduction

Mechanical loading of bone results in various osteogenic stimuli, including new bone formation as well as repair. In this process, osteocytes which are derived from osteoblasts are critical for communicating and sending signals to other bone cells through gap junctions with their dendritic processes to initiate bone remodeling[1-2]. It is speculated that the history of weight bearing affects long-term cellular memory in the bone cell and osteocyte network that modulates the cellular response to a wide variety of stimuli[3]. However, few markers for osteocytes are recognized and the gene expression patterns and pathways of osteocytes responding to mechanical loading are not well defined.

To understand the mechanism of how cellular information is transmitted by mechanical loading in bone to the genome, we investigated load responsive gene expression patterns using the 2T3 osteoblast-osteocyte differentiation cell model[4], the MLO-Y4 osteocyte-like cell model[5] and the mouse ulna loading model[6].

DMP1 cis-regulatory regions

Dentin matrix protein 1 (DMP1) is selectively expressed in osteocytes and is activated in response to mechanical strain[7,8]. Using Northern analysis of the 2T3 osteoblast-osteocyte model and *in situ* analysis of loaded mouse ulna, we found that DMP1 expression increased 20-fold, selectively in regions of mineralizing matrix *in vitro*.

The transcriptional activity of three cis-regulatory regions, -9624 to +1996 (10kb), –7892 to +4439 (8kb) and –2433 to +4439 (2.5kb) of the DMP1 gene, driving GFP, were first examined *in vitro* in 2T3 cells to investigate the major cis-regulatory regions that are specific for osteocytes and for response to mechanical strain. The three cis-regulatory regions were selected based on DNA sequence conservation in non-coding regions of the human and mouse DMP1 genes using "*rvista*"[9]. For the three cis-regulatory regions, GFP expression increased dramatically and locally in areas of mineralized matrix. Many GFP positive cells were dendritic. Mineralizing cultures containing osteocytes with the 8kb DMP1 cis-regulatory construct showed 7-fold increases in GFP in response to fluid flow shear stress and Northern analysis showed that endogenous DMP1 was also increased.

Transgenic mice with the 8kb DMP1-GFP construct were generated that showed selective expression in osteocytes. The right ulnae of the transgenic mice were loaded for 1 bout of 60 cycles (2 Hz; peak force of 2.4 N) and the left ulna served as a control. After 24hrs, GFP expression was shown to increase in the loaded ulna. These mice can now be used to isolate and study osteocyte function in any genetic background.

Microarrays

To globally explore the osteocyte-specific mechanical signaling pathways, mouse 5k oligonucleotide microarrays were used to obtain gene expression profiles from *in vitro* (triplicate) and *in vivo* (5 ulnae) experiments.

First, 2T3 cells at low density and confluency were com-

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Corresponding author: Stephen E. Harris, PhD., Department of Periodontics, Dental School, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, MSC 7894, San Antonio, TX 78229-3900
E-mail: harris@uthscsa.edu

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pared to MLO-Y4 cells to identify genes that may be specific to osteocytes compared to osteoblasts. Then, to understand the osteocyte specific mechanical loading genomic regulatory network, the early osteoblast and osteocyte models were subjected to 0.4 Pascals of fluid shear stress for 2 hrs followed by analysis at 2 and 24 hrs after stopping flow. The time of 2 hrs after loading was chosen to identify short-term responsive genes; 24 hrs after loading for long-term responsive genes. In the mouse ulna loading model, the right ulna was loaded for 1 bout of 60 cycles (2 Hz; peak force of 2.4 N) and the left ulna served as a control.

After hybridization experiments, gene expression intensities were quantified and statistically analyzed. Northern analysis was performed to validate key gene expression patterns. We then performed several clustering algorithms based on pattern similarity to organize clusters of expression patterns.

Functional classification was used to evaluate the biological features of the *in vivo* and *in vitro* data. In the osteocyte model, osteopontin was increased, a gene that is well-known to be highly responsive to loading as well as CD44 and gp38/E11. CD44 which interacts with GP38/E11, osteopontin and most likely DMP1, may be associated with migration, generation and maintenance of dendritic processes[10,11]. These results suggest that major changes are occurring in the actin cytoskeleton and in osteocyte interaction and communication with the extracellular matrix. Ptger2 (Prostaglandin receptor) was increased at the early time point[12]. The voltage-dependent L-type calcium channel alpha 1s (Cacna1s) was also increased after mechanical loading, which may be linked to DMP1 export from the cell to the matrix[13]. Several other correlations were observed between the *in vitro* cell systems and the *in vivo* loading model. From these diverse data sets, we are building gene expression and pathway maps using GenMapp[14] and PathwayAssist[15] to be available to the scientific community through an established website.

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