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Bone Generation through Mechanical Loading

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Abstract*—**Bones are dynamic and responsive organs, they regulate their strength and mass according to the loads which they are subjected. Because, the Wnt/β-catenin pathway has profound effects on the regulation of bone mass, ***we hypothesized that mechanical loading of bone cells stimulates Wnt/β-catenin signaling, which results in the generation of new bone mass. Mechanical loading triggers the secretion of the Wnt molecule, which after binding to transmembrane proteins, causes GSK-3β (Glycogen synthase kinase 3 beta) to cease the phosphorylation of β-catenin. β-catenin accumulation in the cytoplasm, followed by its transport into the nucleus, binding to transcription factors (TCF/LEF) that initiate transcription of genes related to bone formation.

To test this hypothesis, we used TOPGAL (Tcf Optimal Promoter β-galactosidase) mice in an experiment in which cyclic loads were applied to the forearm. TOPGAL mice are reporters for cells effected by the Wnt/β-catenin signaling pathway. TOPGAL mice are genetically engineered mice in which transcriptional activation of β-catenin, results in the production of an enzyme, β-galactosidase. The presence of this enzyme allows us to localize transcriptional activation of β-catenin to individual cells, thereby, allowing us to quantify the effects that mechanical loading has on the Wnt/β-catenin pathway and new bone formation. The ulnae of loaded TOPGAL mice were excised and transverse slices along different parts of the ulnar shaft were assayed for the presence of β-galactosidase.

Our results indicate that loading increases β-catenin transcriptional activity in regions where this pathway is already primed (i.e. where basal activity is already higher) in a load magnitude dependent manner. Further experiments are needed to determine the temporal and spatial activation of this signaling in relation to bone formation.

***Keywords*—**Bone Resorption and Formation, Mechanical Loading of Bone, Wnt Signaling Pathway & β-catenin.

I. Introduction

THE mechanical loading that bones receive during daily activity are one of the key determinants of the extent of bone formation. The skeleton responds to physical exertion to accommodate an individual's activity levels, and increases bone mass accordingly. This physiological response is hypothesized to be the reaction of osteocytes being subjected to membrane shear stresses or mechanical stretch. We further hypothesize that a key biochemical/molecular pathway, i.e. the Wnt/β-catenin signaling pathway, is activated by application of load.

The Wnt/β-catenin pathway clearly plays a powerful role in the regulation of bone mass and new bone formation [1]. The Wnt/β-catenin pathway consists of a cascading series of biomolecules that eventually leads to the Wnt molecule bind to the transmembrane proteins, LRP5 and Frizzled. Binding of the Wnt molecule to transmembrane proteins, results in activation of the Wnt/LRP5/Frizzled complex, which through a series of other biochemical pathways initiates the kinases of GSK-3β, resulting in its inactivation. Inactivation of GSK-3β, results in accumulation of cytoplasmic β-catenin. Increased β-catenin in the cytoplasm, results in enhanced transport to the nucleus where it binds to the transcription factors TCF/LEF, and initiates the transcription of genes related to bone formation. This signaling pathway is known to be extremely important in initiating bone formation [2, 3], but its response to mechanical loading is not known. The activation of the Wnt/β-catenin pathway within a particular cell can be determined with the use of transgenic TOPGAL mice.

β-catenin is present in the cytoplasm under normal circumstances as part of the N-cadherin complex [4] and is also present in the Axin/APC/GSK-3β degradation complex [5]. However the presence of β-catenin in the cytoplasm is short lived, and cytoplasmic β-catenin concentrations are governed by phosphorylation by GSK-3β. As stated above, the binding of the Wnt signaling molecule to LRP5 transmembrane protein and the proceeding bio-molecular reactions cause the in activation of GSK-3β [6].

To test our hypothesis, that mechanical loading of bone cells initiates a cascading series of events in the Wnt/β-catenin pathway, which initiates new bone formation, we used transgenic mice. The transgenic mice, termed the TOPGAL reporter model, are reporters for cells that are effected by the Wnt/β-catenin pathway [7]. As β-catenin binds to the transcription factors, TCF/LEF, and the genes for bone generation are transcribed, an additional gene inserted into the TOPGAL mouse genome is also expressed. The transcription of the additional gene causes the secretion of β – galactosidase to the cytoplasm. After appropriate staining protocol, β – galactosidase becomes visible under light microscopy as a blue-green precipitate within cells. This staining can be used to determine activation of β-catenin at an individual cell level. To evaluate our hypothesis, we tested for the presence of β-galactosidase in the loaded forearm after conducting loading in the TOPGAL mouse model. We further correlate the presence of β-galactosidase to mechanical stress magnitudes determined using computational finite element analysis.

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II. Methods

To illustrate the effects that mechanical loading has on activation of the Wnt Signaling Pathway, mechanical loading was applied to the forearms of TOPGAL mice. All experiments were conducted in 4-month old female TOPGAL mice on a C56BL6 background, with IACUC approval. Only the right forearm was loaded, and the left forearm serves as contralateral controls. The forearm was loaded such that a peak strain of 4500 με stress was induced, as measured using strain gages. Loading was applied for 100 cycles, using a sinusoidal waveform and a frequency of 2Hz. The mice were sacrificed at 24 hrs (n =2) after being subjected to mechanical loading.

The transgenic TOPGAL mice were used to determine the response of osteocytes to external mechanical stimuli. Three different sections were taken from each of the two bone specimens. The figure shows sections that were obtained 3-4mm, 0, and 3-4mm proximal to the ulna mid-shaft. The percentage of cells expressing β-galactosidase, blue-green staining, were then counted in each section. Comparison of the number of blue specks between loaded bone specimens and control (unloaded) specimens indicates the effects that loading has on the Wnt Signaling Pathway and new bone formation.

We further correlate the presence of β-galactosidase to mechanical stress magnitudes determined using computational finite element analysis. This was performed to begin the construction of a three-dimensional model of where Wnt/β-catenin signaling was most prevalent. The 3-D model of Wnt/β-catenin signaling presence was then correlated against a finite element analysis of stress and strains on the ulnae under load.

Finite Element Analysis (FEA) of mouse ulna to obtain axial strain magnitudes: To relate the sites of initial activation of Wnt signaling to sites experiencing the highest strain magnitudes, we have begun to conduct finite element analysis of the ulna (Fig. 1). We have measured axial strains using strain gages in the mouse ulna and developed a finite element model. Strain gage measurements were conducted for 2 cases: intact forearm and excised ulnae. This enabled us to determine the magnitudes of loads being borne by the ulna in the intact forearm. Strains measured using strain gages were also used to verify and validate the finite element model. Briefly, finite element analysis (FEA) was performed on a model built from micro-CT scans of the ulna. FEA predicted strains were within 10% of strain gage measured strains at two locations, which were: (i) on a medial site, 9mm distal to the proximal end of the ulna (proximal is the side where the olecranon process is present), and (ii) 5mm distal to the proximal end on the lateral surface. Appropriate boundary conditions were applied to the ulna such that the contribution of the radius towards load-sharing was accounted for.

Within individual sections, strains obtained from FEA were correlated with pre-existing levels of Wnt/β-catenin signaling. The results from this correlation can be found in Figure 3 of the Results section. This further confirms our hypothesis that the Wnt/β-catenin pathway is triggered in response to high mechanical workloads.

III. Results

The β-galactosidase staining of control and loaded specimens are presented in Figure 1. Three sections were taken at different positions along the ulna length. It can be seen that the loaded specimens have a higher percentage of cells expressing Wnt/β-catenin pathway, this indicates that induced loads caused the molecular changes and that the bone is adapting. Results indicate that there is a strong correlation between the percentage of cells expressing β-galactosidase in a particular region with and without loading.

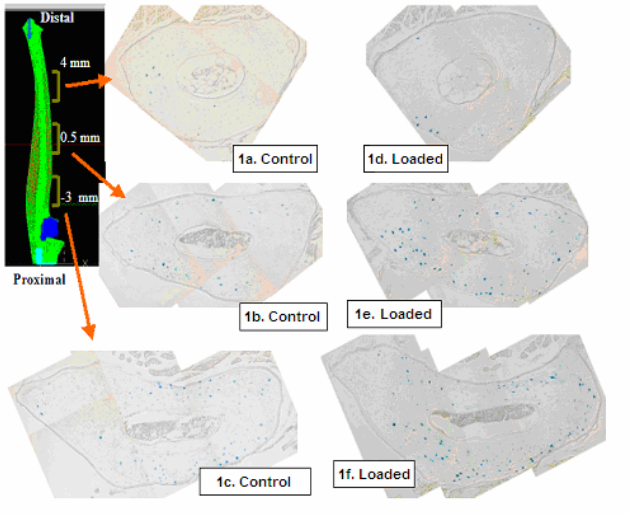
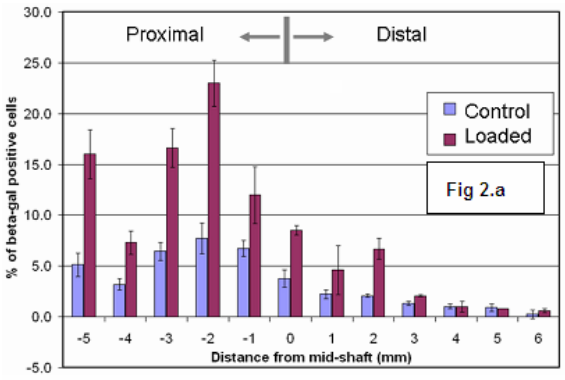
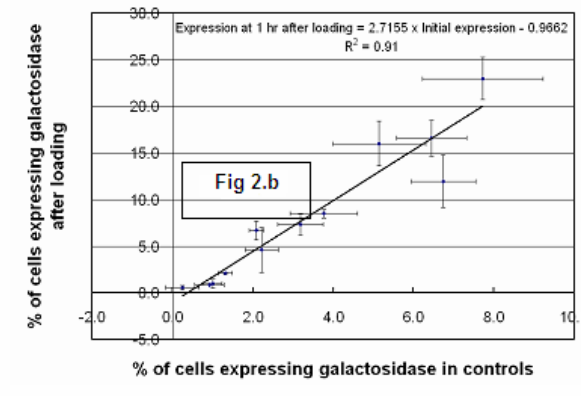


Fig. 1. β-gal enzyme expression (blue) in control (Fig. 1a, 1b and 1c) and loaded (Fig. 1d, 1e and 1f) bone in TOPGAL mice, at 24 hrs after *in vivo* loading. Representative transverse cross-sections of the ulna were obtained 4, 0.5 and -3 mm distal to the ulna mid-shaft. In both control and loaded bones, β-gal expression is observed mostly in osteocytes. In control bone, there is a low basal level of expression of β-gal in osteocytes. In loaded bone, the numbers of cells exhibiting β-gal and the intensity of staining are both increased, particularly in sections obtained 0.5 and -3 mm distal to the ulna mid-shaft. In the loaded bone, some staining is also seen in cells close to the periosteal surface (suggesting that the signaling is propagating to the bone surfaces).



(a)



(b)

Fig. 2 Figure 2.a depicts the percentage of osteocytes that were positive for β-galactosidase for control and loaded TOPGAL ulnae. The percentages are calculated for different distances distal and proximal along the ulna shaft. Firgure 2.b plots the percentage of cells expressing galactosidase after loading versus the percentage of cells expressing galactosidase in controls.

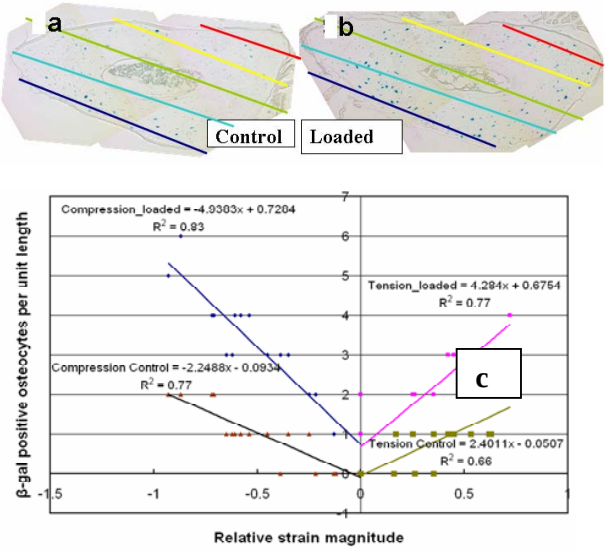


Fig. 3 On the transverse bone cross-sections obtained from the ulna mid-shaft, we superimpose lines that demarcate regions having equal axial strain values as determined from finite element analysis. The number of osteocytes expressing β-galactosidase per unit length is plotted against the relative strain magnitude (strain magnitude normalized by the highest strains in the bone which are different for control and loaded animals). Results demonstrate that in control bone, there is a relationship between strain magnitudes and β-galactosidase expression (and therefore Wnt/β-catenin activity). In loaded bones, the number of β-gal positive osteocytes is increased in a load dependent manner. These increases occurred in regions experiencing both tensile and compressive axial strains.

IV. Discussion

The objective of this paper was to test whether mechanical loading activates Wnt/β-catenin signaling. In this study, we found that higher loads caused more osteocytes to express the Wnt/β-catenin signaling, and that the highest rates of expression were in regions were the bone incurred higher levels of stress. Additionally it was determined that regions where basal levels of Wnt/β-catenin signaling were high demonstrated further increases in expression upon stimulation with mechanical load.

Our results demonstrate that mechanical loading causes the initiation of the Wnt/β-catenin signaling pathway. How the increases in mechanical stress mediated increases in Wnt/β-catenin signaling results in isolating the region where bone formation occurs, needs to be further investigated.

In conclusion, using the forearm compression loading model in the TOPGAL transgenic mice, we have demonstrated that a potent signaling pathway (Wnt/ β-catenin signaling) is activated.

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