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Transcriptional profiling of cortical versus cancellous bone from mechanically-loaded murine tibiae reveals differential gene expression

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ABSTRACT

Mechanical loading is an anabolic stimulus that increases bone mass, and thus a promising method to counteract osteoporosis-related bone loss. The mechanism of this anabolism remains unclear, and needs to be established for both cortical and cancellous envelopes individually. We hypothesized that cortical and cancellous bone display different gene expression profiles at baseline and in response to mechanical loading. To test this hypothesis, the left tibiae of 10-week-old female C57Bl/6 mice were subjected to one session of axial tibial compression (9 N, 1200 cycles, 4 Hz triangle waveform) and euthanized 3 and 24 h following loading. The right limb served as the contralateral control. We performed RNA-seq on marrow-free metaphyseal samples from the cortical shell and the cancellous core to determine differential gene expression at baseline (control limb) and in response to load. Differential expression was verified with qPCR. Cortical and cancellous bone exhibited distinctly different transcriptional profiles basally and in response to mechanical loading. More genes were differentially expressed with loading at 24 h with more genes downregulated at 24 h than at 3 h in both tissues. Enhanced Wnt signaling dominated the response in cortical bone at 3 and 24 h, but in cancellous bone only at 3 h. In cancellous bone at 24 h many muscle-related genes were downregulated. These findings reveal key differences between cortical and cancellous genetic regulation in response to mechanical loading. Future studies at different time points and multiple loading sessions will add to our knowledge of cortical and cancellous mechanotransduction with the potential to identify new targets for mouse genetic knockout studies and drugs to treat osteoporosis.

1. Introduction

Osteoporosis is characterized by bone loss that often leads to fracture of corticocancellous sites such as the hip, spine, and wrist [1,2]. The majority of current drug treatments are anti-catabolic and decrease the resorption of bone by osteoclasts. In contrast, parathyroid hormone (PTH) is the only FDA-approved anabolic drug proven to increase bone formation [3]. A promising route to discover other anabolic drug targets arises from the fact that bone is mechanosensitive.

Mechanical loading of the skeleton by exercise in humans increases bone mineral density [4–7]. In mouse models in vivo compression of the tibia allows for the application of controlled loads and differentially increases bone mass in cortical (Ct) and cancellous (Cn) sites [8–10]. The molecular mechanisms behind this anabolic response to mechanical loading need to be determined and compared between cancellous and cortical bone. This knowledge may enhance the development of drug therapies to increase bone formation in osteoporotic patients.

Gene expression following in vivo tibial compression in mice has been examined with qPCR and microarray of cortical or homogenized cortical and cancellous bone [11–14]. Wnt/β catenin signaling [11,12] and estrogen receptor alpha signaling [11,14] are involved in the anabolic loading response. Limitations of previous work include the combined examination of cortical and cancellous bone and the biased determination of pathways and genes of interest. Moreover, the presence of large numbers of contaminating bone marrow cells is likely to skew further any findings. RNA sequencing has become the standard method to examine total gene transcription at any point in time. Unlike qPCR or microarray techniques RNA sequencing is unbiased and does not examine a predetermined set of genes. Furthermore, RNA-seq requires a relatively small amount of input material (>10–100 ng), and thus is ideal for examining small tissue samples such as fractionated murine bone.

We recently published a method to examine gene expression in cortical and cancellous bone and demonstrated with qPCR that centrifugation removed marrow more efficiently and increased the expression of bone-related genes [15]. Expanding on this method, we now ask how

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transcriptional profiles differ in cortical and cancellous bone at baseline and in response to mechanical loading. To date, use of RNA-seq to study murine bone has been limited and only focused on the cortical diaphysis, homogenized metaphysis or whole bones [16–20]. Transcriptional profiling with RNA-seq provides an opportunity to determine gene expression in cortical and cancellous tissues separately and to examine the molecular mechanisms responsible for mechanical loading-related anabolism. We hypothesized that basal gene expression would differ in cortical and cancellous bone and that the transcriptional response to mechanical loading would differ between the two tissues. To test this hypothesis, we applied compressive loading to the mouse tibia and performed RNA-seq in cortical and cancellous bone separately at two time points.

2. Materials and methods

2.1. Animals

Fourteen 10-week-old female C57Bl/6 mice were subjected to a single session of in vivo mechanical loading of the left tibia (9 N max load, 1200 cycles, 4 Hz, triangle waveform) with the right tibia as contralateral control [9,10,21]. Three and twenty-four hours after a single loading session mice were euthanized (n = 7/group), and their tibiae rapidly dissected for RNA isolation. The epiphysis, all soft tissues, periosteum, fibula, and the distal end were removed. Tibiae were centrifuged for 20 s at 16,100 *g* at room temperature in microcentrifuge tubes, as previously described [15] and then cut approximately 5 mm distal to the growth plate to isolate the metaphysis. The cancellous core of the metaphysis was separated from the cortical shell with a 1 mm biopsy punch (Miltex, Integra LifeSciences Corp, Plainsboro, NJ). The IACUC of Cornell University approved all animal procedures.

2.2. RNA isolation

RNA isolation was performed using Trizol (Life Technologies, Carlsbad, CA) and RNeasy Mini kit (Qiagen, Germantown, MD) as described previously [13]. Briefly, individual cancellous and cortical samples were pulverized in liquid nitrogen-cooled flasks (Mikro-dismembrator S, Sartorius Stedim Biotech, Bohemia, NY, USA). Following pulverization, Trizol was added to the flasks and the powdered bone/Trizol mix was incubated at room temperature for 45 min. 300 μL of chloroform was added to samples, vortexed for 15 s, and decanted into phase lock gel tubes (PLG, heavy, 5 Prime, Gaithersburg, MD). Samples were centrifuged for 15 min at 4 °C and 11,500 rpm, to separate the nucleic acid phase (~600 μL), which was removed and added to an equal volume of 70% ethanol. This mixture was applied to purification columns (RNeasy Mini kit, Qiagen) following the manufacturer's instructions, including a DNase digestion (RNase free DNase kit, Qiagen). A final volume of 30 μL of RNA was eluted.

RNA purity and quantity were tested using a spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE) and RNA Quality Number (RQN) using a fragment analyzer (Advanced Analytical Technologies, Inc., Ames, IA). The mean (±SD) concentration of cortical samples was 92 (±40) ng/μL, and for cancellous samples was 30 (±17) ng/μL. The 260/280 ratio was within an acceptable range (1.8–2.1) for all samples. Cancellous samples were of higher quality than their cortical counterparts with a mean (±SD) RQN of 7.4 (±0.9) and 5.3 (±0.8), respectively.

2.3. RNA-seq library preparation and analysis

Total RNA from 8 animals (n = 32 samples; 4 samples/animal from cortical and cancellous tissue of two tibiae) was processed to create RNA-seq libraries (Illumina TruSeq RNA Sample Preparation Kit v2, San Diego, CA) following the manufacturer's instructions. mRNA was purified with polyA+ magnetic beads, chemically fragmented (120–200 bp fragments), reverse transcribed using random hexamers, and ligated to bar-coded adapters. The resulting cDNA fragments were amplified to provide sufficient material for subsequent analysis. Finally, primer-dimers were removed by washing with AMPure XP beads (Agencourt AMPure XP, Beckman Coulter, Brea, CA). Final libraries were tested using a fragment analyzer to determine the distribution of fragment sizes. The thirty-two individually bar-coded libraries were normalized, pooled (maximum of 12 libraries/lane), and sequenced (100-bp single-end reads, Illumina HiSeq 2000).

Sequences were aligned to the mouse genome (mm10) using TopHat followed by transcript assembly and gene counts with Cuffdiff [22]. On average, 77% of the 18 million reads/library were mapped to the mouse genome, 95% uniquely. Differential expression was determined with a paired design in edgeR [23]. The paired design consisted of an additive linear model with blocking for which the blocking factor was "tissue" when baseline cortical and cancellous tissue from the same bone were compared, and the blocking factor was "animal" when the loaded limb was compared to the control contralateral limb in the same animal. Genes with low expression were filtered from each library by removing genes with counts per million less than one in at least four samples. Finally, differentially expressed genes were determined by fitting gene-wise generalized linear models and performing likelihood ratio tests. Genes were differentially expressed based on fold-change (FC) cut-off and 5% false discovery rate (FDR) cut-off. A more stringent fold change cut-off was applied for normal developmental changes (Ct to Cn baseline, >2-fold) than loading induced changes (>1.5-fold).

For the basal samples only, we identified contaminants from our RNA-seq data based on genes determined to be highly expressed in muscle, bone marrow, or blood compared to tibial bone by Ayturk et al. [16]. For the loaded samples all gene changes are reported including genes considered contaminants in the basal analysis.

Lists of differentially expressed genes were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID, from the National Institute of Allergy and Infectious Diseases (NIAID), NIH — http://david.abcc.ncifcrf.gov/). Gene ontology (GO) for biological processes and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were determined for each group using the functional annotation tool ($p < 0.05$) [24,25].

2.4. Gene expression verification with qPCR

Using qPCR, we verified differential gene expression obtained from RNA-seq for 12 genes that were different between the two tissues or with loading: *Gpr50*, *Grem1*, *Ostn*, *Ptgs2*, *Ptn*, *Sost*, *Timp1*, *Tnfrsf11b*, *Wnt1*, *Wnt10b*, *Wnt16*, and *Wnt7b* (primer sequences in Supplementary Table S1). RNA from the remaining animals (n = 3/per group) was reverse transcribed to cDNA following the manufacturer's instructions (High Capacity cDNA Reverse Transcription kit, Life Technologies) and brought to 5 ng/μL with RNase-free water. A final volume of 20 μL containing 2X SYBR Green (Perfecta SYBR Green Fastmix, with ROX, Quanta Biosciences, Gaithersburg, MD) was assayed by triplicate qPCR using 39 cycles of denaturing (95 °C, 5 s) and annealing/elongation (60 °C, 30 s) (CFX96 Real-Time PCR System, BioRad, Hercules, CA). Quantification cycle (Cq) was determined for each gene and compared to the reference gene *Gapdh*. All groups are presented as fold change with loading or tissue type ($2^{-\Delta\Delta Cq}$). The $\log_2$ fold-change of RNA-seq versus qPCR was graphed to determine quality of correlation; a slope of one would define perfect agreement. The strength of the relationship was quantified by Pearson correlation.

3. Results

3.1. Control cortical and cancellous bone exhibit different transcriptional profiles

We first compared the transcription profiles of cortical and cancellous bone from control limbs (>2-fold). Cortical and cancellous bone