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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 (9N, 1200 cycles, 4Hz triangle waveform) and euthanized 3 and 24 hours 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 hours with more genes downregulated at 24 hours than at 3 hours in both tissues. Enhanced Wnt signaling dominated the response in cortical bone at 3 and 24 hours, but in cancellous bone only at 3 hours.

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In cancellous bone at 24 hours 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.

Keywords

osteoporosis; animal models; mechanical loading; transcriptome; cancellous bone; cortical bone

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 (>10-100ng), 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 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 (9N max load, 1200 cycles, 4Hz, 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 20s at 16,100g 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 1mm 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 (Mikrodismembrator 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 minutes. 300µL of chloroform was added to samples, vortexed for 15s, and decanted into phase lock gel tubes (PLG, heavy, 5 Prime, Gaithersburg, MD). Samples were centrifuged for 15 minutes at 4C and 11,500 rpm, to separate the nucleic acid phase (~600µL), 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 30µL 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 ($\pm$ SD) concentration of cortical samples was 92 ($\pm$ 40) ng/µL, and for cancellous samples was 30 ($\pm$ 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 ($\pm$ SD) RQN of 7.4 ($\pm$ 0.9) and 5.3 ($\pm$ 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 (120-200bp 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 RNAseq 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 5ng/μL with RNase-free water. A final volume of 20μL containing 2X SYBR Green (Perfecta SYBR Green Fastmix, with ROX, Quanta Biosciences, Gaithersburg, MD) was assayed by triplicate qPCR using 39 cycles of denaturing (95C, 5s) and annealing/elongation (60C, 30s) (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^{-Cq}). The log₂ 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 samples from the contralateral control (non-loaded) limbs were analyzed 3 hours and 24 hours after a single loading dose. A total of 178 genes were differentially expressed three hours after a single loading dose, with 110 genes more highly expressed in control cortical bone and 68 genes more highly expressed in control cancellous bone (Figure 1A, Supplementary table S2). Genes known to be expressed in mesenchymal-derived bone cells included *ostn* (osteocrin; 29-fold, Ct>Cn), *wnt16* (wingless-related MMTV integration site 16; 12-fold, Ct>Cn), *wnt7b* (wingless-related MMTV integration site 7b; 7-fold, Ct>Cn), and *sost* (sclerostin; 4-fold, Ct>Cn).

Three hundred and twenty six genes were differentially expressed 24 hours after a single loading dose, with 287 genes more highly expressed in cortical bone and 39 genes more highly expressed in cancellous bone (Figure 1A, Supplementary table S3). Ninety-three differentially expressed genes were shared at the two time points (77 Ct>Cn, 16 Cn>Ct) and included *ostn* (52-fold, 24h), *wnt16* (43-fold, 24h), *wnt7b* (4-fold, 24h), and *sost* (4-fold, 24h) (Figure 1B).

When cortical vs cancellous gene expression was examined in control tissue, five differentially-expressed genes from the 3-hour group and 84 differentially-expressed genes from the 24-hour group were previously shown to be more highly expressed in skeletal muscle than bone and thus designated as “contaminants” by Ayturk et al. [16]. When we examined these genes in our data, all “contaminating” genes that were more highly expressed in cortical bone (n=83) were genes previously associated with muscle. Of those Ayturk-designated contaminant genes that were more highly expressed in cancellous bone in our data (n=4), one was from marrow, two from blood, and only one from muscle. Because the previously-identified contaminant genes in cortical bone were all from muscle and the cancellous genes from marrow and blood, we excluded these genes from the pathway analysis of the control tissue.

Gene ontology (GO) was determined separately for genes that were more highly expressed in cancellous and cortical bone. All genes from both time points were included, with the exception of the contaminating genes identified by Ayturk et al. The extracellular matrix-

receptor interaction pathway was enriched in both cancellous and cortical bone, while melanogenesis was enriched in cortical bone only and complement and coagulation cascades were enriched in cancellous bone only (Figure 1C). In cancellous bone, the top biological processes were regulation of body fluid levels, coagulation, and blood coagulation while in cortical bone cell adhesion, biological adhesion, and skeletal system development were the identified processes (Figure 1D).

3.2 The mechanical loading response at 3h is driven by Wnt signaling in cortical and cancellous bone

Three hours after a single loading session 43 genes were differentially expressed >1.5-fold in cortical bone and 18 in cancellous bone. A majority of genes were upregulated by loading in both cortical and cancellous bone (Figure 2A). Eleven genes were shared and included *Wnt1* (wingless-related MMTV integration site 1; 6.2-fold Ct, 4.1-fold Cn), *Wnt7b* (3.2-fold Ct, 7.8-fold Cn), *Timp1* (tissue inhibitor of metalloproteinase 1; 1.6-fold Ct, 1.9-fold Cn), *Ptgs2* (prostaglandin-endoperoxide synthase 2; 4.3-fold Ct, 3.9-fold Cn), and *Tnfrsf11b* (tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin); 2.2-fold Ct, 1.9-fold Cn) (Figure 2B). Enriched signaling pathways included Wnt signaling in both cortical and cancellous bone and hedgehog signaling in cortical bone only (Figure 2C). Enriched biological processes included Wnt receptor signaling in cancellous and cortical bone, biomineral formation in cortical bone, and developmental maturation in cancellous bone (Figure 2D). Complete lists of differentially expressed genes with mechanical loading can be found in supplementary table S4 (cortical bone) and S5 (cancellous bone).

3.3 The mechanical loading response at 24h includes changes in Wnt signaling in cortical bone and muscle-related genes in cancellous bone

Twenty-four hours after a single loading session 58 genes were differentially expressed in cortical and 32 differentially expressed genes in cancellous bone (>1.5-fold). More genes were upregulated by loading in cortical bone (60%), while more genes were downregulated in cancellous bone (66%) (Figure 3A). Both tissue types shared twelve genes, including *Ptn* (pleiotrophin; 2.7-fold Ct, 3.1-fold Cn), *Vcan* (versican; 1.8-fold Ct, 1.6-fold Cn), and *Tubb3* (tubulin beta 3; 2.5-fold Ct, 1.8-fold Cn) (Figure 3B). The only enriched signaling pathway in cortical bone was melanogenesis, while the only enriched pathway in cancellous bone was tight junction (Figure 3C). Biological processes included response to organic substance and response to toxin in cortical and muscle contraction and muscle system process in cancellous (Figure 3D). Notably, many highly downregulated muscle-related genes were identified in cancellous bone including *Myh4* (myosin, heavy polypeptide 4, skeletal muscle; -59.5-fold), *Tnnc2* (troponin C2, fast; -41.6-fold), *Smpx* (small muscle protein, X-linked; -32.3-fold), *Tnnt3* (troponin, T3, skeletal, fast; -36.6-fold), *Mylk2* (myosin, light polypeptide kinase 2, skeletal muscle; -22.4-fold), *Myoz1* (myozenin 1; -23.4-fold), *Myom2* (myomesin 2; -21.5-fold), *Actn3* (actinin alpha 3; -14.7-fold), *Myf1* (myosin, light polypeptide 1; -13.8-fold), *Casq1* (calsequestrin 1; -12.4-fold), *Mybpc2* (myosin binding protein C, fast-type; -12.1-fold), *Myh2* (myosin, heavy polypeptide 2, skeletal muscle, adult; -12.2-fold), *Myf6* (myosin light chain, phosphorylatable, fast skeletal muscle; -12.9-fold), and *Tnni2* (troponin I, skeletal, fast 2; -7.7-fold). Complete

lists of differentially expressed genes can be found in supplementary table S6 (cortical) and S7 (cancellous).

3.4 Temporal comparison of differentially expressed genes with mechanical loading

The gene expression response within cortical bone was grossly different at 3 and 24 hours following a single loading dose (Figure 4A). More genes were upregulated at 3 hours and to a greater magnitude than at 24 hours, while at 24 hours more genes were highly downregulated than at 3 hours. Four genes were differentially expressed in cortical bone at both 3 and 24 hours following a single loading session: *Wnt1*, *Wnt10b*, *Ptgs2*, and *Mt2* (metallothionein 2; upregulated at 3h, downregulated at 24h).

In cancellous bone the upregulated genes at 3 hours and 24 hours had similar magnitudes of fold-change, but more genes were downregulated with higher magnitude fold-change at 24 hours (Figure 4B). Only *Timp1* was upregulated both 3h and 24h following a single loading dose in cancellous bone (1.9-fold 3h, 1.7-fold 24h).

Finally, more genes were differentially expressed following loading at 24 hours in both cortical and cancellous bone. At both 3 and 24 hours more genes were differentially expressed in cortical than cancellous bone. The magnitude of the fold change in upregulated genes at 3h in cortical bone was larger than in cancellous bone, while at 24h the magnitude of the fold change in downregulated genes was larger in cancellous bone than in cortical bone.

3.5 RNA-seq verification with qPCR

We found strong agreement between qPCR and RNA-seq for the twelve genes tested. When all differentially expressed genes were included (not only significant changes), the slope of the linear fit between RNA-seq and qPCR was close to one and the Pearson correlation was 0.89 (Figure 5).

4. DISCUSSION

We used RNA-seq to compare gene expression in cortical and cancellous bone of control and loaded limbs following a single bout of axial tibial compression in 10-week-old female mice. Cortical and cancellous bone expressed different genes basally and in response to in vivo mechanical loading at two time points. At baseline, many genes were differentially regulated between cortical and cancellous bone, the majority of which were more highly expressed in cortical bone. Genes expressed by osteoblasts and those mediating Wnt signaling dominated the response three hours after a single loading session. Cortical expression twenty-four hours after a single loading session was still enriched for upregulated bone-related and Wnt signaling genes, while the cancellous response involved downregulation of many muscle-related genes. More genes were differentially expressed at the twenty-four hour time point in both cortical and cancellous bone than three hours after loading.

The differential gene expression in baseline cortical and cancellous bone identified here is not surprising considering differences in growth and aging in these bone sites. Cortical and

cancellous bone arise through different growth processes and have different age-related bone mass accrual and subsequent loss. For both tissues, hypertrophic chondrocytes can become osteoblasts and osteocytes that contribute to cancellous bone, the endosteum and mature bone [26, 27]. Cortical bone mass increases during development, stabilizes at skeletal maturity, and decreases during aging [28, 29]. Cancellous bone mass also increases initially during growth but decreases immediately after reaching peak bone mass, without a stabilization phase [30]. These differences in development and age-related changes between cancellous and cortical bone suggest different regulatory mechanisms driven by the differential transcription demonstrated in our study. Furthermore, we confirmed that differences in gene expression did not reflect different proportions of bone cells in cortical and cancellous bone by determining the number of osteoblasts per bone surface and the number of osteocytes per bone area. These measures were not different in cortical and cancellous samples from six animals (data not shown).

More genes were expressed differentially twenty-four hours after a single loading session in control bone, implying that mechanical loading is not a purely local response in terms of gene transcription. Dynamic loading of the tibia causes adaptation in the loaded bone only and not in adjacent, contra-lateral or remote sites when measured at the tissue level with microcomputed tomography [31]. Future studies should examine transcription in cortical and cancellous bone of naïve (not externally loaded) animals to determine if loading causes systemic changes in gene expression.

Wnt signaling is vital for skeletal development and maintenance [32]. Recently, genetic variants in *Wnt16* were linked to cortical bone thickness, bone mineral density and fracture risk in humans [33]. Mice lacking *Wnt16* have 27% thinner femoral cortices, bone strength reductions of 43%-61% and develop spontaneous fractures without changes in cancellous mass or architecture [33, 34]. *Wnt16* is osteoblast-derived and inhibits osteoclastogenesis by decreasing osteoclast progenitors and increasing osteoblast expression of osteoprotegerin [34]. In the current study *Wnt16* was 12- to 43-fold more highly expressed in cortical bone, in agreement with the cortical bone phenotype seen in humans and mice. *Sost*, a Wnt signaling inhibitor, was also more highly expressed in cortical bone (4-fold); however, a clear cortical-only bone phenotype is not seen in humans or rodents. *Sost* gene loss-of-function in humans causes Sclerosteosis and van Buchem's diseases, characterized by drastically increased bone mass [35, 36]. Furthermore, sclerostin antibody increased bone mineral density by 3-12% at the lumbar spine, total hip and femoral neck after one year of treatment in a phase II clinical trial [37]. In addition, *Sost* deficient mice have high bone mass in both cortical and cancellous sites [38], and Sclerostin antibody increased bone formation in both cortical and cancellous bone in rats [39, 40] and cynomolgus monkeys [41].

Signaling pathways and genes regulating mechanoresponsiveness have been determined largely from studies of cortical bone, and the few studies that examine cancellous bone response have conflicting results. Osteocytes in the cancellous bone of the mouse vertebrae were examined six hours after a single loading dose. 287 genes were upregulated including *Wnt5a*, *Aspn*, and *Igfl*, while 52 genes were downregulated including *Spock2* and *Col19a1* [42]. Notably *Sost* did not change, while we saw a 2-fold downregulation of *Sost* 3 hours

following loading in cancellous bone. Furthermore, our two studies shared few genes that were differentially expressed due to mechanical stimulation. This disparity may reflect the different time points examined following loading, different bones examined, or their use of collagenase digestion, which affects gene expression [16]. Three days of hindlimb unloading in rats decreased *Sost* expression in metaphyseal cancellous bone, had no effect on metaphyseal cortical bone, and caused upregulation in the cortical diaphysis [43]. Differences in cortical and cancellous expression and the lack of studies of mechanotransduction in cancellous bone highlight the need for site-specific determination of loading-induced changes in transcription.

Canonical Wnt/ β -catenin signaling is a major regulator of bone's adaptive response to mechanical loading. In vivo tibial loading increased diaphyseal expression of Wnt pathway and Wnt/ β -catenin target genes including *Wnt10b*, *Sfrp1*, *Fzd2*, *Wisp2*, *Wnt2b*, and *Crebbp* [11, 44] and decreased expression of the Wnt signaling inhibitor *Sost* [14]. We found increased expression of *Wnt1*, *Wnt7b* and *Wnt10b* 3 hours after a single loading session in cortical bone, while *Crebbp* (CREB binding protein) and *Lrp5* (low density lipoprotein receptor-related protein 5) were modestly decreased. *Wnt1* and *Wnt10b* remained upregulated at 24 hours in cortical bone. *Wnt1* and *Wnt7b* were also upregulated in cancellous bone at the earlier time point, while the Wnt inhibitors *Sost* and *Dkk1* (dickkopf homolog 1) were downregulated. Furthermore, β -catenin activation by mechanical loading requires prostaglandin [45]. We saw loading-related upregulation of *Ptgs2* (prostaglandin-endoperoxide synthase 2, aka COX-2) in cortical bone at 3 and 24 hours, and in cancellous bone at 3h only. Our data confirm the previously determined importance of Wnt signaling in the cortical response to mechanical loading and identifies the role of Wnt signaling in the early loading response in cancellous bone.

Other skeletal genes were differentially expressed following mechanical loading. Tissue inhibitor of metalloproteinase (*Timp1*) was upregulated by loading in cortical bone at 3h (1.6-fold), and in cancellous bone at 3h (1.9-fold) and 24h (1.7-fold), in agreement with findings from homogenized tibial bone subjected to mechanical loading [14, 46]. Pleiotrophin (*Ptn*) was increased by 4.3-fold twenty-four hours after four sessions of 4-point bending of the mouse tibia [46], and was also upregulated in our study at 24 hours in both cortical (2.6-fold) and cancellous bone (3.1-fold). Osteoprotegerin (*Tnfrsf11b*) is well known to inhibit osteoclast differentiation [47] and was increased by loading at 3 hours in cortical (2.2-fold) and cancellous (1.9-fold) bone.

Muscle genes were the largest group of downregulated genes twenty-four hours following a single loading session in cancellous bone. A single loading session of the rat forearm (cortical site) also caused downregulation of muscle genes, seven of which were also identified in our study (*Ampd1*, *Smpx*, *Tnnt3*, *Mylk2*, *Actn3*, *Myhpf*, and *Tnni2*) [48]. In our experiment these gene changes are unlikely to reflect muscle contamination because muscle was carefully removed from the whole bone surface, and the cancellous core does not interface with muscle. Furthermore, calvarial-derived osteocytes express genes related to muscle function, development and differentiation, and to a greater extent than osteoblasts [49] as does young healthy articular cartilage [50]. Established expression of muscle-related genes in musculoskeletal tissues other than muscle suggests that their classification was

based upon the first tissue in which they were identified or the tissue in which they are most abundant, but may not be muscle-specific. The exact cellular population in our samples has not been determined, but embedded osteocytes are a likely source; our centrifugation protocol removes marrow and non-adherent osteoblasts and osteoclasts. The role of muscle-related genes in bone biology and mechanotransduction is not well understood, and elucidation of their functions in bone cells and the response to mechanical loading requires further study.

Despite the removal of contaminating genes determined by Ayturk et al., pathways and biological processes were identified in cancellous bone that are related to coagulation. These results may reflect the presence of blood or serum within the tibial samples.

The current study is limited in its examination of the response to loading at the transcriptional level in young mice at two time points following a single loading session. Protein levels resulting from the transcriptional changes need to be confirmed, localized to the different cells, and ideally related to the strain field, to more accurately determine spatial and temporal responses to mechanical loading. The selection of appropriate time points for these studies will be critical to the experimental design. Future work could include adult mice, earlier time points to capture changes in transcription factors, later time points to examine matrix- and mineralization-related gene expression, and multiple loading sessions.

The ability to examine cortical and cancellous bone separately and compare their baseline transcription and their response to mechanical loading is unique. Many gene knockout studies have demonstrated differential phenotypes in cortical and cancellous bone; some notable examples are the primary effect in cancellous bone of absence of estrogen receptor alpha in bone cells [51-55], the previously mentioned cortical phenotype in mice lacking Wnt16 [33, 34], and the decrease in cortical but not cancellous bone volume when BMP2 is deleted from early-stage osteoblasts [56]. With in vivo compression of the mouse tibia, cancellous bone is often more responsive to mechanical loading than cortical bone [21, 57]; however, gene knockouts can modify this responsiveness in a site-specific manner [55, 58]. Future studies utilizing our methods to determine the baseline transcription and response to loading in cortical and cancellous bone in genetically modified mice will help unravel the complex molecular mechanisms responsible for in vivo bone homeostasis and mechanotransduction.

4.1 Conclusions

In summary, we used RNA-seq to demonstrate differences in cortical and cancellous bone transcription separately at baseline and at two time points following a single loading session. The differences in transcription at baseline highlight the need to examine these bone tissue envelopes separately, particularly when cortical to cancellous phenotypic differences are observed. Furthermore, we discovered tissue-envelope-specific and time-dependent differences in the response to mechanical loading. In particular, further investigation into the large downregulation of muscle-related genes in cancellous bone is necessary. Next generation sequencing is a powerful tool to study bone transcription and identify future targets for treatment of osteoporosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Transcription was examined in cortical and cancellous bone separately.
- Cortical and cancellous bone have differential gene expression basally and in response to loading.
- Enhanced Wnt signaling defined the early response to loading in cortical and cancellous bone.
- Wnt upregulation persisted at a later time point in cortical, while muscle-related genes were downregulated in cancellous.

Figure 1. Control cortical and cancellous bone exhibit distinctly different transcriptional profiles
 (A) Venn diagram showing the number of differentially expressed genes between control cortical and cancellous bone at 3 (left) and 24 (right) hours (FDR<5%, FC>2). Arrows (%) indicate proportion of genes that were more highly expression in cortical (green) or cancellous (red) bone. A majority of genes were more highly expressed in cortical bone. B) Top 10 genes (by 3h FC) out of 93 total differentially expressed in cortical versus cancellous bone that were shared at both time points. All genes shown are more highly expressed in cortical bone. DAVID analysis determined C) significantly enriched pathways and D) biological processes in cortical and cancellous bone (p<0.05).

Figure 2. Differential expression of Wnt signaling genes 3h following a single loading session in cortical and cancellous bone

(A) Venn diagram showing the number of genes significantly altered by mechanical loading at 3 hours in cortical (left) and cancellous (right) bone (FDR<5%, FC>1.5). Arrows (%) indicate proportion of genes that were up- (green) or down- (red) regulated. A majority of genes were upregulated by loading. B) Genes differentially expressed due to mechanical loading common to both cortical and cancellous bone. DAVID analysis determined C) significantly enriched pathways and D) biological processes in cortical and cancellous bone following loading (p<0.05).

Figure 3. Wnt signaling changes in cortical bone and muscle-related gene changes in cancellous bone 24h following a single loading session

(A) Venn diagram showing the number of genes significantly altered by mechanical loading at 24 hours in cortical (left) and cancellous (right) bone (FDR<5%, FC>1.5). Arrows (%) indicate proportion of genes that were up- (green) or down- (red) regulated. A majority of genes were upregulated by loading in cortical bone, while in cancellous bone, a majority of genes were downregulated. B) Genes differentially expressed due to mechanical loading common to both cortical and cancellous bone. Negative fold change values indicate downregulation in response to loading. DAVID analysis determined C) significantly enriched pathways and D) biological processes in cortical and cancellous bone following loading (p<0.05).

Figure 4. More genes were differentially expressed in cortical bone and at 24h

Ranked $\log_2$ fold change histograms of differentially expressed genes in A) cortical and B) cancellous bone at 3h (grey bars) and 24h (black bars) after a single loading session. The y-axis was aligned with the inflection between up- and down-regulated genes at 24h to assist in visualization of trends in differentially expressed genes between time points and tissue types.

Figure 5. qPCR validated 12 differentially expressed genes

qPCR was performed for 12 genes identified as differentially expressed with RNA-seq. Log₂ fold change from RNA-seq vs qPCR. Grey dashed lines indicate 2-fold up- and down-regulation, black dashed line is the identity line (1:1 relationship), and the solid line is the linear regression.