Journal of Cellular Biochemistry 96:1049–1060 (2005)

Global Gene Expression Analysis in the Bones Reveals Involvement of Several Novel Genes and Pathways in Mediating an Anabolic Response of Mechanical Loading in Mice

Weirong Xing,[1] David Baylink,[1,2] Chandrasekhar Kesavan,[1] Yan Hu,[1] Susanna Kapoor,[1] Robert B. Chadwick,[1] and Subburaman Mohan[1,2,3,4]*

[1]Musculoskeletal Disease Center, JL Pettis Memorial Veterans Administration Medical Center, Loma Linda, California 92357
[2]Department of Medicine, Loma Linda University, Loma Linda, California
[3]Department of Biochemistry, Loma Linda University, Loma Linda, California
[4]Department of Physiology, Loma Linda University, Loma Linda, California

Abstract To identify the genes and signal pathways responsible for mechanical loading-induced bone formation, we evaluated differential gene expression on a global basis in the tibias of C57BL/6J (B6) mice after four days of four-point bending. We applied mechanical loads to the right tibias of the B6 mice at 9 N, 2 Hz for 36 cycles per day, with the left tibias used as unloaded controls. RNA from the tibias was harvested 24 h after last stimulation and subjected to microarray. Of the 20,280 transcripts hybridized to the array, 346 were differentially expressed in the loaded bones compared to the controls. The validity of the microarray data was established with the increased expression of bone-related genes such as pleiotrophin, osteoglycin, and legumain upon four-point bending and confirmation of increased expression of selected genes by real-time PCR. The list of differentially expressed genes includes genes involved in cell growth, differentiation, adhesion, proteolysis, as well as signaling molecules of receptors for growth factors, integrin, Ephrin B2, endothelin, and adhesion G protein coupled receptor. Pathway analyses suggested that 28 out of the 346 genes exhibited a direct biological association. Among the biological network, fibronectin and pleitrophin function as important signaling molecules in regulating periosteal bone formation and resorption in response to four-point bending. Furthermore, some expressed sequence tags (ESTs) with no prior known function have been identified as potential mediators of mechanotransduction signaling pathways. Further studies on these previously unknown genes will improve our understanding of the molecular pathways and mechanisms involved in bone's response to mechanical stress. J. Cell. Biochem. 96: 1049–1060, 2005.

Key words: osteogenesis; microarray; mechanical loading; bone; gene expression

Abbreviations used: AHR, aryl-hydrocarbon receptor; α5β3 integrin, alpha5beta3 integrin; ANXA2, annexin A2; AP-1, activating protein 1; β2M, β-2 microglobulin; COL18A1, Collagen type XVIII alpha 1; Csrp2, cystein and glycine-rich protein 2; CTSD, cathepsin D; EGF, epidermal growth factor; EGFR, EGF receptor; Emp1, epithelial membrane protein 1; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; Ephb2, ephrin receptor B2; EPS8, EGFR pathway substrate 8; ERK, extracellular signal-regulated kinases; EST, expressed sequence tag; FCGR1A, Fc receptor IgG high affinity I; FGF7, fibroblast growth factor 7; FGFR1, FGF receptor 1; FN, fibronictin receptor; HIF-1A, hypoxia-induced factor1alpha; IER3, immediate early response gene 3; IGF, insulin-like growth factor; IGFBP5, IGF binding protein 5; IGFR, IGF receptor; Itm2a, integral membrane protein 2; Lgmn, legumain; LRR, leucine-rich repeats; MAF, musculoaponeurotic fibrosarcoma oncogene; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; Npdc1, neural proliferation, differentiation and control gene 1; Ogn, osteoglycin; PCR, polymerase chain reaction; PDGF, platelet derived growth factor; PDGFA, PDGF alpha; PDGFRA, PDGFR alpha; PDGFRB, PDGF receptor beta; Ptn, pleiotrophin; QTL, quantitative trait loci; RGDS, Ral GDP dissociation stimulator; TEP1, telomerase associated protein 1; TIM, tissue inhibitor of metalloprotainase; TNC, Tenascin C; VEGF, vascular endothelial growth factor.

Grant sponsor: U.S. Army Medical Research Acquisition Activity; Grant number: DAMD17-01-1-0744.

*Correspondence to: Dr. Subburaman Mohan, Musculoskeletal Disease Center, Jerry L. Pettis Memorial Veterans Medical Center, Loma Linda, CA 92357.
E-mail: Subburaman.Mohan@med.va.gov

Received 11 July 2005; Accepted 12 July 2005
DOI 10.1002/jcb.20606

It is well established that bone adapts to mechanical loading by adjusting its density, shape, and strength during periods of growth and daily physical activities. Under normal conditions, such as exercise, mechanical loading stimulates bone formation, whereas overloading or unloading may result in unbalanced bone resorption and reduced growth rate [Frost, 1992; Sibonga et al., 2000]. However, the skeletal response to mechanical loading is widely varied in the normal human population [Devine et al., 2004; Rittweger et al., 2005]. For example, some postmenopausal women with severe osteoporosis display a poor anabolic response to mechanical stimuli loaded on their bones, while other patients respond normally to the same degree of mechanical loading [Preisinger et al., 1995; Yamazaki et al., 2004]. A similar variation in anabolic response to mechanical loading has also been observed in some animal models, such as the C57BL/6 (B6) and C3H/HeJ (C3H) mouse strains [Akhter et al., 1998; Pedersen et al., 1999]. We and others have found B6 mice to be more responsive to skeletal loading compared to C3H mice [Akhter et al., 1998; Kodama et al., 1999, 2000; Robling and Turner, 2002]. In addition, congenic mice of B6.C3H-4T containing a segment of mouse chromosome 4 from the C3H strain in the B6 genetic background were found to be more susceptible to mechanical loading than the B6 mice [Robling et al., 2003]. Together, these studies have confirmed that differential anabolic responses to mechanical loading are, in large part, genetically determined. Therefore, studies to identify the genes and signal transduction pathways involved in bone's adaptive response to mechanical loading are important for the future development of diagnostic markers and/or therapeutic targets for osteoporosis.

In the past few years, studies using in vitro culture systems have identified a number of genes susceptible to mechanical force. It has been found that these genes are involved in a number of signaling pathways, including calcium-regulated PI3K-Akt and protein kinase C [Danciu et al., 2003; Pines et al., 2003], growth factor activated extracellular signal-regulated kinases (ERK), prostaglandin synthesis [Kapur et al., 2003], and integrin pathway [Weyts et al., 2002]. While the data generated from these in vitro studies have provided important information, one major limitation has been that most of the data obtained were from homogenous osteoblast cells in cell culture systems lacking the vital communications of multiple cell types such as osteocytes and osteoblasts. This is a constraint because bone osteocytes can receive and transmit changes in mechanical forces to other cell types involved in bone remodeling [Ehrlich et al., 2002; Noble et al., 2003; Yang et al., 2005]. In addition, multipotent progenitor cells can also respond to mechanical signals to differentiate toward osteoblast lineage [Estes et al., 2004]. Accordingly, there has been a considerable need for an in vivo analysis of gene expression patterns of mechanically loaded bones compared to unloaded bones that would address these considerations.

Several approaches have been used to identify candidate genes that contribute to phenotypic variation in inbred strains of mice, including quantitative trait loci (QTL), polymerase chain reaction (PCR) differential display, cDNA microarray, and high-throughput mutation screening of target region [Doerge, 2002]. Of these techniques, microarray holds a great deal of promise for identifying the genes in question because of its ability to simultaneously characterize the expression levels of many thousands of genes associated with various biological functions and processes using very small amounts of RNA. This technology has been applied to identify mechanically induced genes in osteoblast cells exposed to fluid shear stress and has revealed multiple molecular pathways regulating osteogenic gene expression [Kapur et al., 2003]. Therefore, in the present study, we used oligonucleotide microarrays containing over 20,000 probes and evaluated differential gene expression on a global basis in the tibias of female B6 mice after four days of four-point bending. We hypothesized that mechanical activation of one or more sensitive signaling pathways would contribute to the robust increase in new bone formation in response to four-point bending in the female B6 mice.

MATERIALS AND METHODS

Animals and Materials

Ten-week-old C57BL/6J female mice were obtained from the Jackson Laboratory and housed at the Jerry L. Pettis Memorial VA Medical Center Animal Research Facility (Loma Linda, CA) under standard approved laboratory conditions with controlled illumina-

tion (14 h light, 10 h dark), temperature (22°C) and unrestricted food and water. Studies were performed with the approval of the Animal Ethics Committee of the Jerry L Pettis Memorial VA Medical Center. Mouse development oligonucleotide microarray (22 K) slides were purchased from Agilent Technologies, Inc (Mountain View, CA). All probes on each slide were 60 base-pairs in length with sense orientation designed from the National Institute on Aging/National Institute of Health cDNA mouse clone set [Carter et al., 2003]. The designed microarray slides contain a single set of 22,575 spots, of which, 20,371 are target genes. In addition, the slides also include a total of 1,075 control spots (144 negative controls, 18 corner mark negative controls, 196 spike-in probes, 215 staggered start probes, 10 corner mark positive controls and 492 positive control grid). The remaining 1,129 spots are blanks. The Cyanine 5-CTP and Cyanine 3-CTP were obtained from PerkinElmer Life Science (Boston, MA).

Mechanical Loading In Vivo and RNA Extraction

Mice were externally loaded in vivo in a four-point bending device, as described previously [Akhter et al., 1998; Wang et al., 2000]. This model has been used extensively in a number of laboratories for studies on mechanical loading [Torrance et al., 1994; Cullen et al., 2001; Robling and Turner, 2002]. In previous studies, we found that 2 weeks of four-point bending at 9 N, 2 Hz for 36 cycles per day increased total volumetric bone mineral density by 15% in B6 mice [Kesava et al., 2004]. In this study, we used 4 days of loading to minimize the number of genes that change in response to loading-induced bone remodeling. Briefly, the right tibias of the mice were loaded for 4 days at 9 N, 2 Hz for 36 cycles per day, and the left tibias of the same mice were used as unloaded controls. Twenty-four hours after last stimulation, the mice were sacrificed and the corresponding tibias were removed. The bones were dissected free of soft tissue, flushed with PBS to remove the bone marrow cells, and stored in a solution of RNAlater (Ambion, Inc. Austin, TX) at −20°C. To optimally evaluate mechanosensitive genes, only the region of bone that was subjected to four-point bending was used for RNA extraction. We pooled RNA from five loaded or unloaded bones to obtain sufficient RNA for microarray analysis and subsequent real-time PCR work. To generate five pairs of samples, a total of 25 mice were divided into five groups with five mice each. Total RNA was extracted using Trizol (Invitrogen Corporation, Carlsbad, CA), and further cleaned up through RNeasy mini spin columns (Qiagen, Valencia, CA). RNA concentration and integrity were analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Microarray Design and Hybridization

Probe labeling was performed according to the manufacturer's instructions using Agilent low RNA input fluorescent linear amplification kits (Agilent Technologies, Inc.). Briefly, an aliquot of 2 μg of total RNA was reverse transcribed using a primer containing oligo (dT) and a T7 RNA polymerase promoter. After synthesis of the first and second strands of cDNA, the product was amplified in an in vitro transcription reaction in order to generate enough cRNA labeled targets in the presence of cyanine 3- or cyanine 5-labeled CTP. The dye-labeled cRNA was then purified through RNeasy mini spin columns to remove free nucleotides. The cRNA concentration and dye incorporation were measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, NE). Hybridization was carried out according to the instructions provided in Agilent oligonucleotide microarray kit (Agilent Technologies, Inc.). Two micrograms of fragmented cyanine 3-labled cRNA of unloaded reference sample was mixed with equal amounts of cyanine 5-labeled cRNA of loaded experimental sample, and the mixture was hybridized to a 22 K mouse development oligonucleotide microarray for 17 h at 60°C at 7 rpm. After hybridization, the slides were dried using a nitrogen-filled air gun, and subsequently scanned using GSI Scanarray 4000 (GSI Lumonics, Inc., Moorpark, CA). The images were analyzed using the ImaGene 5.6 software (Biodiscovery, Inc., El Segundo, CA). The ImaGene software flagged spots with intensities lower than that of the background or spots with aberrant shapes.

Normalization and Analysis of Microarray Data

Expression analysis of the microarray data from five slides was performed using the GeneSpring 6.2 (Silicon Genetics, Redwood City, CA). Local background-subtracted median signal intensities were used as intensity measures,

with the data normalized using per spot and per chip intensity/dependent LOWESS normalization [Workman et al., 2002]. Transcripts that passed with flag values "present" in all five replicates and a raw signal greater than the background were targeted for further analyses. In order to minimize the false-positive/negative error rate in our high-density oligonucleotide microarray, we chose a combination of fold change and significant difference to restrict a small gene list [Costigan et al., 2002; Schmalbach et al., 2004]. The transcripts were first scaled to an expression level of 1.5-fold change. The filtered genes (e.g. $\geq$1.5-fold) were then further analyzed utilizing a one-sample Student's *t*-test with "Benjamini and Hochberg" Multiple Testing Correction [Hochberg and Benjamini, 1990]. Differentially-expressed genes in the loaded bones were defined as those whose normalized average data had a difference of 1.5-fold change or greater with P value <0.01, compared with the unloaded reference samples.

Identification of Signaling Pathways

We analyzed the gene list obtained from our microarray analysis using the PathwayAssist software (Stratagene, La Jolla, CA) to identify any specific signaling pathways, gene regulation networks, and protein interaction maps. The PathwayAssist program uses a natural language processor to retrieve information from databases such as PubMed in order to provide direct biological associations.

Real-Time PCR

We used reverse transcriptase polymerase chain reaction (RT-PCR) for selected genes to confirm changes in transcription observed in our microarray analysis. Total RNA (2 μg) was reverse-transcribed into cDNA by using a oligo$(dT)_{12-18}$ primer and SuperScript II RNase^{TM} H^- Reverse Transcriptase (Invitrogen). Real-time PCR was carried out in a 96 well plate using a 7700 ABI prism sequence detection system (Applied Biosystems, Foster City, CA). The PCR contained 100 ng of template cDNA, 1× SYBR GREEN master mix (Qiagen) and 100 nM of specific forward and reverse primers in 25 μl volume per reaction. Primers for the housekeeping gene, β-2 microglobulin (β2M), were used to normalize the expression data for each gene. The thermal cycling conditions for real-time PCR were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Sequences of the primers were: pleiotrophin (Ptn) (forward 5-gaaaatttgcagctgccttc, reverse 5′-ttcaaggcggtattgaggtc); osteoglycin (Ogn) (forward 5′-tgcaacaggcaattctgaag, reverse 5′-tcctt-ggcagtcagcttttt); legumain (Lgmn) (forward 5′-acctgggtgactggtacagc, revese 5′-gattccttcacgtc-gttggt); immediate early response gene 3 (IER3) (forward 5′-tctggtcccgagattttcac, reverse 5′-ctccgaggtcaggttcaaag); β2M (forward 5′-cgagcccaagaccgtctact; reverse 5′-gctatttctttctgcgtgcat); P37nb (forward 5′-aggaggcgttcatttacacg, reverse 5′-gggtttgtatgggaaacacg); neural proliferation, differentiation and control gene 1 (Npdc1) (forward 5′-taggcttcagcgagagatcc, reverse 5′-atggtcaaacagtgggttgc).

RESULTS

Genome-Wide Expression Profiles of Mechanically-Loaded Bones Versus Unloaded Bones

We examined global gene expression profiles in five different RNA pools from loaded and corresponding unloaded bones using an oligonucleotide array consisting of 20,371 target genes. We found that a total of 20,280 transcripts were hybridized to the array. After an initial filtering of the data, we arrived at an informative data set for further analysis consisting of 19,882 genes which had both passed with flag value "present" in all five replicates and a raw signal greater than the background. Comparison of the gene expression profiles of the loaded and unloaded bones revealed 346 differentially expressed sequences that differed by 1.5-fold or greater with a significance of $P < 0.01$ after "Benjamini and Hochberg" multiple testing correction. Table I shows a list of differentially regulated genes whose expression levels were either up- or down-regulated at least two-fold and organized in different biological categories. The complete list of 346 differentially regulated genes is provided in the Supplementary Data posted on this Journal's Website.

To determine the validity of our microarray data, we performed various permutation analyses, which include comparisons of two loaded samples versus three loaded samples, two unloaded samples versus three unloaded samples, and a pool of two loaded plus two unloaded versus three loaded and three unloaded samples. In addition, we also performed sample reproducibility analysis by comparing two

TABLE I. Functional Categories of Selected Genes Differentially Expressed in Mechanically Loaded Bones

Gene	Access number	Description	Change	*P* value	Location
Cell growth/differentiation					
Ptn	AK011346	Pleiotrophin	4.29	0.0006	6
Ogn	AK014259	Osteoglycin	2.47	0.0040	13
Itm2a	NM_008409	Integral membrane protein 2A	2.85	0.0031	X
Emp1	BC034257	Epithelial membrane protein 1	2.56	0.0015	6
Lepre1	NM_019783	Leprecan 1	2.46	0.0012	4
Tgfbi	XM_122567	Transforming growth factor, beta induced, 68 kDa	2.26	0.0026	13
Pdgfrl	NM_026840	Platelet-derived growth factor receptor-like	2.18	0.0026	13
Morf4l2	NM_019768	Mortality factor 4 like 2	2.04	0.0057	X
Csrp2	NM_007792	Cysteine and glycine-rich protein 2	4.12	0.0013	10 D1
Akr1b7	AK002705	Aldo-keto reductase family 1, member B7	4.72	0.0018	6
Ephb2	BM231330	Eph receptor B2	2.54	0.0048	4 D3
Npdc1	NM_008721	Neural proliferation, differentiation and control gene 1	2.24	0.0093	2 A3
Cell adhesion					
Col5a1	NM_015734	Procollagen, type V, alpha 1	1.97	0.0011	2
Col6a3	BC005491	Procollagen, type VI, alpha 3	3.36	0.0030	1
Col8a1	NM_007739	Procollagen, type VIII, alpha 1	2.56	0.0018	16 C1
Col14a1	XM_127997	Procollagen, type XIV, alpha 1	2.37	0.0026	15
Col18a1	BC008227	Procollagen, type XVIII, alpha 1	3.40	0.0020	10
PCDH19	XM_033173	Protocadherin 19	2.28	0.0092	X
Lamb1-1	XM_126863	Laminin B1 subunit 1	2.69	0.0011	12
Nid1	NM_010917	Nidogen 1	1.99	0.0042	13
Nrp	AK011144	Neuropilin (an alternate receptor for VEGF-A)	2.13	0.0028	8
ESTs	NM_172399	A930038C07Rik (Fibronectin, type III domain)	2.06	0.0019	6
Fn	XM_129845	Fibronectin receptor	2.58	0.0012	1
Matn2	NM_016762	Mus musculus matrilin 2	2.76	0.0012	15
Loxl3	NM_013586	Lysyl oxidase-like 3	2.14	0.0021	6
Lox	NM_010728	Lysyl oxidase (hydroxylysine residues in collagens)	3.25	0.0042	18
Cell death					
Gas1	NM_008086	Growth arrest specific 1	2.26	0.0015	13
Bok	BC030069	Bcl-2-related ovarian killer protein	2.48	0.0015	1 D
C1qtnf6	NM_028331	C1q and tumor necrosis factor related protein 6	2.11	0.0015	15 E1
Proteolysis					
Mest	NM_008590	Mesoderm specific transcript	3.05	0.0011	6
Lgmn	NM_011175	Legumain	3.25	0.0030	12 E
Timp1	NM_011593	Tissue inhibitor of metalloproteinase 1	2.46	0.0032	X
Pcolce	NM_008788	Procollagen C-proteinase enhancer protein	2.15	0.0086	5
Serpinb6	NM_009254	Serine (or cysteine) proteinase inhibitor, clade B, member 6a	1.97	0.0039	13
EST	NM_028072	2010004N24Rik (heparin-degrading endosulfatases)	1.95	0.0040	2
Signal transduction					
Semcap3	NM_018884	SemaF cytoplasmic domain associated protein 3	1.98	0.0023	6
Pdgfrb	NM_008809	Platelet derived growth factor receptor, beta polypeptide	1.97	0.0082	18
EPS8	BC030010	Epidermal growth factor receptor pathway substrate 8	1.98	0.0043	6
Gpr124	NM_054044	Adhesion G protein-coupled receptor 124	1.98	0.0015	8
Entpd2	NM_009849	Ectonucleoside triphosphate diphosphohydrolase 2	2.01	0.0029	2 A3
Scgn10	AK003659	Superiorcervical ganglia, neural specific 10 (stathmin-like 2)	2.06	0.0023	3
Dcamkl1	BE824672	Double cortin and calcium/calmodulin-dependent protein kinase-like 1	3.80	0.0038	3
Ania4	NM_021584	Activity and neurotransmitter-induced early gene, similar to CaM-Kinase	2.87	0.0025	Y
Ednrb	AE014177	Endothelin receptor type B	2.91	0.0063	14

(*Continued*)

TABLE I. (*Continued*)

Gene	Access number	Description	Change	*P* value	Location
Nek6	NM_021606	NIMA (never in mitosis gene a)-related expressed kinase 6	2.14	0.0014	2
Calu	NM_007594	Calumenin (Calmodulin and related proteins, cacium binding)	2.05	0.0100	6
Rcn	NM_009037	Reticulocalbin (Calmodulin and related proteins, cacium binding)	2.31	0.0037	2
D7Ertd671e	XM_133470	DNA segment, Chr 7,Ca2+-binding protein	2.02	0.0023	7
Fstl	NM_008047	Follistatin-like	3.10	0.0018	16
Tax1bp3	NM_029564	Tax1 (human T-cell leukemia virus type I) binding protein 3 (PDZ domain)	1.97	0.0018	11
Il13ra1	NM_133990	Interleukin 13 receptor, alpha 1	2.42	0.0060	X
Ccr5	XM_135269	Similar to C-C chemokine receptor 5, (rhodopsin family)	2.35	0.0015	9 F4
Igsf10	AK018323	Immunoglobulin superfamily, member 10	2.20	0.0020	3D
Transcription regulation					
Gsc	NM_010351	Goosecoid	2.30	0.0048	12
Ankrd1	BC037138	Ankyrin repeat domain 1 (cardiac muscle)	2.26	0.0099	19 C2
Gli5	BC021517	GLI-Kruppel family member GLI5		0.0094	16
Maged1	NM_019791	Melanoma antigen, family D, 1	2.33	0.0012	X
Mkrn1	NM_018810	Makorin, ring finger protein, 1	0.45	0.0008	6 B1
Heat shock proteins					
Hsp25	NM_013560	Heat shock protein, 25 kDa	2.15	0.0062	5
Serpinh1	NM_009825	Serine (or cysteine) proteinase inhibitor, clade H, member 1 (Hsp47)	2.72	0.0023	7 E1
Cytoskeleton movement					
Tnnt2	NM_011619	Troponin T2, cardiac	3.03	0.0006	1
Cald1	BC015839	Caldesmon 1	2.11	0.0040	7q33
ESTs	NM_023716	2410129E14Rik (tubulin, beta)	2.75	0.0026	13
Tuba1	NM_011653	Tubulin, alpha 1	2.40	0.0006	15
Acta2	NM_007392	Actin, alpha 2, smooth muscle, aorta	1.97	0.0015	19
S100a10	NM_009112	S100 calcium binding protein A10 (calpactin)	2.00	0.0050	3
ESTs	NM_026473	2310057H16Rik (tubulin)	4.69	0.0014	18 E1
Transport					
AI173274	NM_134090	*Mus musculus* expressed sequence AI173274, mRN	2.33	0.0008	15 E1
Sec23a	AY082671	SEC23A	2.31	0.0039	12
P4hb	XM_126743	PDI, Thbp, ERp59; protein disulfide isomerase;	2.29	0.0017	11 80.0 cM
Slc35f5	NM_028787	Solute carrier family 35, member F5	2.11	0.0057	1
Pr1	BC024613	Protein distantly related to the gamma subunit family	1.97	0.0100	1
Other					
Scr59	BC023432	Spermatogenesis associated, serine-rich 2	2.03	0.0065	15 F1
Ppfibp1	BC035209	PTPRF interacting protein, binding protein 1	2.18	0.0045	6 G3
Fkbp9	NM_012056	FK506 binding protein 9	2.15	0.0015	6
Scd2	NM_009128	Stearoyl-Coenzyme A desaturase 2	2.56	0.0025	19
P37nb	XM_131917	Mus musculus 37 kDa leucine-rich repeat (LRR) protein	3.67	0.0040	5
Fer-1-like 3	XM_148914	RIKEN cDNA 2310051D19 gene, similar to myoferlin isoform b	2.64	0.0039	19
Spon2	NM_133903	Spondin 2, extracellular matrix protein	2.46	0.0012	5
Fkbp11	NM_024169	FK506 binding protein 11 (protein turnover, chaperones)	1.98	0.0080	15
Ms4a6d	NM_026835	Membrane-spanning 4-domains, subfamily A, member 6D	1.96	0.0026	19
Rhced	AF057524	Rhesus blood group CE and D	0.49	0.0052	4
ESTs	BG071710	Mus musculus cDNA clone H3102C09 3′, mRNA sequence	0.48	0.0060	9
ESTs	BG071952	Mus musculus cDNA clone H3105A09 3′, mRNA sequence	0.43	0.0060	?
ESTs	BG074055	Mus musculus cDNA clone H3130C11 3′, mRNA sequence	0.47	0.0047	7
Unknown	BG072471	Mus musculus cDNA clone H3110H06 3′, mRNA sequence	0.49	0.0045	3

Functional categories of selected genes that show expression changes of two-fold (log value) or greater with *P* value ≤ 0.01 in loaded bones compared to unloaded bones. The genes with fold changes less than 0.5 are identified as down-regulated genes. GenBank accession numbers, chromosomal locations and statistical significance are also listed.

normalized samples to three other normalized samples in a two-color system. These analyses revealed that the genes identified in this study cannot be explained on the basis of false discovery rate.

Of the 346 differentially expressed genes/expressed sequence tags (ESTs) that we have identified, 304 were up-regulated, and 42 were down-regulated. A total of 157 (~46%) of these differentially-regulated genes encode proteins with no characterized function (Fig. 1). Interestingly, proteins encoded by some of the genes/ESTs contain functional motifs, such as fibronectin domain, heparin-degrading endosulfatases, tubulin, leucine rich repeats (LRR) and von Willebrand factor type C domain. The remaining 189 known genes encode proteins that could be functionally characterized into 12 categories as assessed by Gene Ontology (GO), including cell growth, differentiation, adhesion, cell cycle, cell death, proteolysis, signaling molecules, transcription regulation, heat shock proteins, cytoskeleton movement, and transport (Fig. 1). The majority of differentially expressed genes belonged to the signaling molecule category and included growth factor receptors, G-protein coupled receptors, integrin receptors, Ca++ dependent receptor, tyrosine/serine/threonine kinases, intracellular, and STAT cascade signaling molecules. The second, third, and fourth largest number of genes were found in cell growth, transport, and cell adhesion, respectively. In addition, we also observed increased expression of a number of genes in categories such as transcriptional regulation,

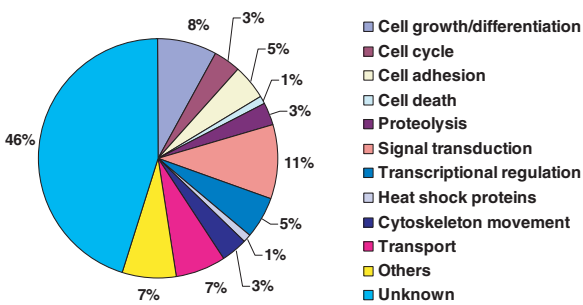

Fig. 1. Biological functions of the 346 genes differentially regulated between the loaded and unloaded bones. Other selections represent genes involved in protein modification, chaperoning pathways, extracellular matrix biogenesis, hemostasis, metabolism and development. A total of 157 genes/ESTs are yet to be characterized. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteolysis, cell death, and heat shock proteins in the loaded bones compared to unloaded bones.

Notably, mechanical loading stimulated a large number of genes involved in cell growth, proliferation, and differentiation (Table I). These genes included growth factors (Ptn, Ogn), receptor tyrosine protein (Ephb2), and oncogenes (leprecan1, Itm2a, Emp1, Csrp2 and Npdc1). In addition to the aforementioned genes, some transcription factors such as goosecoid and signaling molecules of growth factor receptor pathways, were also identified as promoters of cell growth.

Validation of Microarray Data

To confirm the expression data from our oligonucleotide microarray studies, we selected five genes differentially expressed between the loaded and unloaded bones and one unchanged gene for quantitative RT-PCR analysis on five pairs of samples. Three of these genes (Ptn, Ogn, and Lgmn) have been shown to be involved in the regulation of bone remodeling [Madisen et al., 1990; Choi et al., 1999; Yang et al., 2003]. IER3 has been shown to be an immediate early response gene that regulates cell growth and apoptosis in response to stress [Wu, 2003]. P37nb is a member of a leucine-rich repeat protein family with a conserved role in regulation of proliferation, morphology and dynamics of the cytoskeleton, cell adhesion, and tissue development [Segev et al., 2004]. Npdc1 is known to be expressed in the brain with an expression that can be coordinated with the regulation of cellular proliferation and differentiation [Evrard et al., 2004]. Based on the above information, we considered the six genes as potential candidates for mechanical signaling pathway. We therefore chose these genes for confirmation by real-time PCR. Consistent with the microarray data, Ptn, Ogn, Lgmn, P37nb, and Npdc1 were found by real-time PCR to be expressed at significantly higher levels in the loaded bones. The expression of IER3 was no different between the loaded and unloaded bones, as expected based on our microarray data (Fig. 2).

Identification of Mechanosensitive Signaling Pathways Involving in Anabolic Response

To identify potential signaling pathways associated with the skeletal anabolic response to mechanical loading, we analyzed our microarray expression data using PathwayAssist

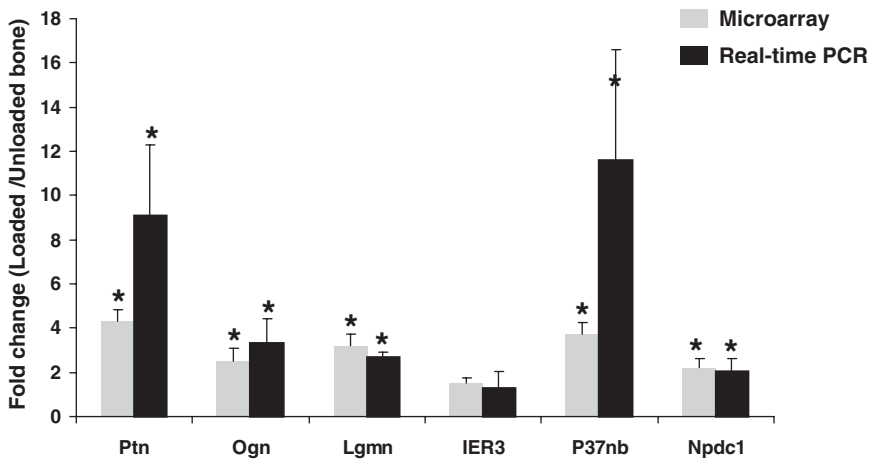

Fig. 2. Comparison of fold changes in expression for selected genes by microarray and real-time PCR. The results of real-time PCR were normalized to the expression of β-2 microglobulin (β2M) in each sample, and expressed as fold change of the loaded sample over the expression level of the unloaded sample. The data shown are means ± SD from five replicates. Ptn: pleiotrophin; Ogn: osteoglycin; Lgmn: legumain; IER3: immediate early response gene 3; Npdc1: neural proliferation, differentiation and control gene 1. * represents $P < 0.01$ for loaded bones vs. unloaded bones.

[Nikitin et al., 2003]. This software comes with a built-in natural language processing module MedScan and a comprehensive database containing more than 150,000 events of regulation, interaction and modification between proteins and cell processes obtained from PubMed which allows it to generate a biological association network (BAN) of known protein–protein interactions. By importing microarray expression data into the BAN, co-expressed genes associated with specific signaling pathways can be identified. In order to characterize signaling pathways involved in response to mechanical loading, we imported 346 differentially expressed genes into the PathwayAssist, and found that 28 of the 346 genes exhibited a direct biological association (Fig. 3). This network identified the following genes as coordinately being regulated in response to four-point bending: JUNB, MAF, TIMP1/2, MMP2, α5β3 integrin, FN, TNC, Col18A1, IGFBP-5, EGFR, EPS8, ENPP1, FGF7, FGFR1, PDGFA, PDGFRA/B,

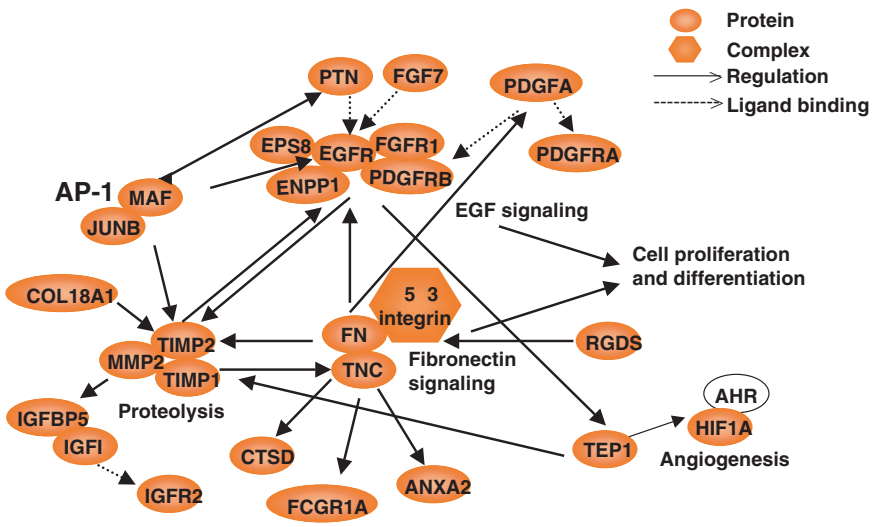

Fig. 3. Schematic representation of direct biological association of differentially expressed genes involved in bone remodeling. Analysis of a direct biological association of differentially expressed genes is performed using PathwayAssist 2.53 software (Stratagene, La Jolla, CA). Biologically linked proteins indicated by nodes and biological processes are shown in the diagram. An open node represents a gene that is down-regulated in the loaded bones compared to unloaded controls. Solid nodes are genes that are up-regulated in the loaded bones compared to unloaded controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PTN, TEP1, CTSD, FCGR1, HIF1A, AHR, ANXA2 and RGDS. Many of these genes represent transcription factors, growth factors and growth factor receptors which have been implicated in regulating the formation and/or activity of bone cells. Some of the genes associated with the network, such as TNC, FN, TIMP1, TIMP2, MMP2, JUNB, and PDGFA, have been also shown to respond to mechanical stress in bone cells in vitro [Granet et al., 2002; Carvalho et al., 2004; Hatori et al., 2004; Ponik and Pavalko, 2004]. In addition, it has been shown that the Ptn gene, a ligand for the EGF receptor, is induced in bladder smooth muscle cells in response to mechanical strain [Park et al., 1998]. Thus, a number of genes we have identified in our four-point bending studies in bone are the same genes that have been identified as mechanosensitive genes in other cell types. Among the 28 genes in the network, fibronectin and Ptn are key signaling molecules that can activate integrin and EGF signaling pathways.

DISCUSSION

The present study is a global microarray analysis of the mouse genome to identify differentially expressed genes in the tibias of female mice after four days of mechanical stimulation in vivo. We chose four-point bending as a loading regimen and B6 as our mouse model based on the findings that 2 weeks of four-point bending with this model caused a dramatic 15% increase in total volumetric bone mineral density, and a 40% increase in bone size [Kesavan et al., 2004]. We chose 4 days of consecutive loading based on the rationale that it leads to activation of important signaling pathways associated with rapid bone remodeling [Raab-Cullen et al., 1994b; Tanaka et al., 2003]. Consistent with the robust bone anabolic response, we identified 346 genes that were differentially expressed in the loaded bones with a 1.5 fold change or greater ($P < 0.01$). Some of these genes, such as TIMP1, heat shock proteins, fibronectin, and neuropilin, have been previously implicated in bone's response to mechanical force and in mediating bone remodeling [Park et al., 1999; Swartz et al., 2001; Shay-Salit et al., 2002]. The identification of previously identified mechanosensitive genes and confirmation of microarray data by real-time PCR provided validation of our microarray results.

Previous in vivo studies of mechanical loading on rat tibias using a four point bending device revealed dynamic changes of gene expression in periosteal bone cells [Raab-Cullen et al., 1994a,b]. In those studies, it was found that transiently stimulated expression of AP-1 proteins within 2 h after loading led to increased osteoblast cell proliferation [Raab-Cullen et al., 1994b]. However, the expression of alkaline phosphatase, osteopontin, and osteocalcin, which are typically produced by mature osteoblasts, was reduced [Raab-Cullen et al., 1994b]. The transcripts of growth factors such as transforming growth factor β (TGFβ) and IGF-I were increased to peak levels after 4 h of mechanical loading [Raab-Cullen et al., 1994b]. These studies suggested that acute periosteal response to external mechanical loading was associated with a change in expression of growth factors known to regulate osteoblast cell proliferation. In the present study, we found a moderate induction of AP-1 proteins (e.g. JunB and Maf), IGF-I, IGFBP-5, Ptn, EGF/FGF receptors, and other genes related to cell cycle after 4-day mechanical loading (Supplementary Data). It should be noted that all of the immediate response genes may not have been identified because the RNA used in this study was extracted 24 h after the last mechanical load. In addition, our global microarray analysis also revealed that a large number of highly expressed genes which have not been previously implicated in mechanical signaling pathways, such as Ogn, Itm2a, Emp1, leprecan 1, and Npdc1, could act as potential mediators of mechanical stress to promote cell growth.

Our microarray analysis identified 157 previously uncharacterized or unknown genes/ESTs, some containing interesting functional motifs. For example, Csrp2, located on mouse chromosome 10, has a zinc-binding domain present in Lin-11, Isl-1, and Mec-3 (LIM-domain) that can bind protein partners via tyrosine-containing motifs. These proteins have been implicated as key regulators of developmental pathways and it has been proposed that they also regulate cell proliferation and differentiation of vascular smooth muscle cells in response to injuries [Jain et al., 1996, 1998]. We also observed a novel EST (P37nb) with leucine-rich repeats (LRR), a known functional domain present in a number of proteins with diverse functions and cellular locations [Strausberg et al., 2002]. In addition, one recent study found

evidence that one of the LRR proteins, CMF608, a mechanical strain-induced bone-specific protein, is involved in promoting osteochondroprogenitor proliferation [Segev et al., 2004]. Similarly, osteoadherin is another small molecule with LRR that can promote integrin ($\alpha5\beta3$)-mediated cell binding in bone tissue [Sommarin et al., 1998]. Our findings suggest that further studies are needed to evaluate whether these ESTs with important functional domains are involved in regulating bone cell proliferation/activity in response to mechanical loading.

In this study, we have confirmed the existence of a direct biological network consisting of the EGF receptor, fibronectin signaling, and proteolysis that are typically involved in bone formation and bone resorption [Marie et al., 1990; Anderson et al., 2004]. Our findings are supported by the fact that expression of PTN, which interacts with the EGF receptor (ErbB1/4), is also stimulated by mechanical stretch in bladder smooth muscle cells [Park et al., 1999]. In addition, it has been reported that overexpression of the PTN protein in osteoblast cells resulted in an increase in cell proliferation and periosteal bone formation [Imai et al., 1998; Tare et al., 2002]. Therefore, the issue of whether or not the elevated expression of PTN and fibronectin are major extracellular mediators of mechanical stress that activate the EGF receptor and integrin signaling pathways that are essential for bone anabolic response in response to mechanical loading requires further study.

Our study also found that several signaling molecules, including adhesion G-protein-coupled receptor 124, chemokine receptor and Eph receptor B2, were modulated by mechanical loading. Of these molecules, it has been previously established that the Eph receptor is localized within the QTL region of mouse chromosome 4 which is believed to contain mechnosensitive gene(s) [Robling et al., 2003]. In addition, the Eph receptors and their membrane-anchored ephrin ligands are important in regulating cell-cell interactions and communications [Adams et al., 2001]. Targeted disruption of the Eph receptor ligand ephrinB1 in mice has also been reported to cause abnormal cartilage segmentation and the formation of additional skeletal elements, suggesting that ephrinB1 signaling is required for normal morphogenesis of skeletal elements [Compagni et al., 2003]. Our study provides, for the first time, evidence for increased Eph receptor B2 expression in bone cells in response to mechanical loading and establishes the groundwork for further examination of the Eph receptor signaling pathway's role in regulating bone formation.

Our experimental design involved a few waves of mechanical stimulation prior to evaluation of gene expression changes by microarray. Thus, it is possible that some of the genes altered after four days of four-point bending may be bone remodeling-related rather than mechanical responsive genes, and therefore the genes that are directly activated by mechanical stimulation cannot be discriminated. Our analysis also involved using RNA from loading region of bone that included multiple cell types (e.g. osteoblasts, stromal cells, ostocytes, and osteoclasts). Thus, we cannot conclude which cell types are contributing to changes in gene expression. Further studies are needed to address this issue.

In conclusion, we have examined the in vivo effect of mechanical loading on differentially expressed genes in the whole genome, and identified a number of novel genes/ESTs and pathways that may play important roles in mediating the skeletal anabolic response to mechanical force. Future studies on these unknown genes and signal molecules will provide a better understanding of the molecular pathways involved in mediating the skeleton's anabolic response to mechanical stress.

ACKNOWLEDGMENTS

The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014, is the awarding and administering acquisition office. The information contained in this publication does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. All work was performed in facilities provided by the Department of Veterans Affairs. We would like to thank Mr. Sean Belcher for his editorial assistance.

REFERENCES

Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R. 2001. The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell 104:57–69.

Akhter MP, Cullen DM, Pedersen EA, Kimmel DB, Recker RR. 1998. Bone response to in vivo mechanical loading in two breeds of mice. Calcif Tissue Int 63:442–449.

Anderson HD, Wang F, Gardner DG. 2004. Role of the epidermal growth factor receptor in signaling strain-dependent activation of the brain natriuretic peptide gene. J Biol Chem 279:9287–9297.

Carter MG, Hamatani T, Sharov AA, Carmack CE, Qian Y, Aiba K, Ko NT, Dudekula DB, Brzoska PM, Hwang SS, Ko MS. 2003. In situ-synthesized novel microarray optimized for mouse stem cell and early developmental expression profiling. Genome Res 13:1011–1021.

Carvalho RS, Einhorn TA, Lehmann W, Edgar C, Al-Yamani A, Apazidis A, Pacicca D, Clemens TL, Gerstenfeld LC. 2004. The role of angiogenesis in a murine tibial model of distraction osteogenesis. Bone 34:849–861.

Choi SJ, Reddy SV, Devlin RD, Menaa C, Chung H, Boyce BF, Roodman GD. 1999. Identification of human asparaginyl endopeptidase (legumain) as an inhibitor of osteoclast formation and bone resorption. J Biol Chem 274:27747–27753.

Compagni A, Logan M, Klein R, Adams RH. 2003. Control of skeletal patterning by ephrinB1–EphB interactions. Dev Cell 5:217–230.

Costigan M, Befort K, Karchewski L, Griffin RS, D'Urso D, Allchorne A, Sitarski J, Mannion JW, Pratt RE, Woolf CJ. 2002. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci 3:16.

Cullen DM, Smith RT, Akhter MP. 2001. Bone-loading response varies with strain magnitude and cycle number. J Appl Physiol 91:1971–1976.

Danciu TE, Adam RM, Naruse K, Freeman MR, Hauschka PV. 2003. Calcium regulates the PI3K-Akt pathway in stretched osteoblasts. FEBS Lett 536:193–197.

Devine A, Dhaliwal SS, Dick IM, Bollerslev J, Prince RL. 2004. Physical activity and calcium consumption are important determinants of lower limb bone mass in older women. J Bone Miner Res 19:1634–1639.

Doerge RW. 2002. Mapping and analysis of quantitative trait loci in experimental populations. Nat Rev Genet 3:43–52.

Ehrlich PJ, Noble BS, Jessop HL, Stevens HY, Mosley JR, Lanyon LE. 2002. The effect of in vivo mechanical loading on estrogen receptor alpha expression in rat ulnar osteocytes. J Bone Miner Res 17:1646–1655.

Estes BT, Gimble JM, Guilak F. 2004. Mechanical signals as regulators of stem cell fate. Curr Top Dev Biol 60:91–126.

Evrard C, Caron S, Rouget P. 2004. Functional analysis of the NPDC-1 gene. Gene 343:153–163.

Frost HM. 1992. Perspectives: Bone's mechanical usage windows. Bone Miner 19:257–271.

Granet C, Vico AG, Alexandre C, Lafage-Proust MH. 2002. MAP and src kinases control the induction of AP-1 members in response to changes in mechanical environment in osteoblastic cells. Cell Signal 14:679–688.

Hatori K, Sasano Y, Takahashi I, Kamakura S, Kagayama M, Sasaki K. 2004. Osteoblasts and osteocytes express MMP2 and −8 and TIMP1, −2, and −3 along with extracellular matrix molecules during appositional bone formation. Anat Rec A Discov Mol Cell Evol Biol 277:262–271.

Hochberg Y, Benjamini Y. 1990. More powerful procedures for multiple significance testing. Stat Med 9:811–818.

Imai S, Kaksonen M, Raulo E, Kinnunen T, Fages C, Meng X, Lakso M, Rauvala H. 1998. Osteoblast recruitment and bone formation enhanced by cell matrix-associated heparin-binding growth-associated molecule (HB-GAM). J Cell Biol 143:1113–1128.

Jain MK, Fujita KP, Hsieh CM, Endege WO, Sibinga NE, Yet SF, Kashiki S, Lee WS, Perrella MA, Haber E, Lee ME. 1996. Molecular cloning and characterization of SmLIM, a developmentally regulated LIM protein preferentially expressed in aortic smooth muscle cells. J Biol Chem 271:10194–10199.

Jain MK, Kashiki S, Hsieh CM, Layne MD, Yet SF, Sibinga NE, Chin MT, Feinberg MW, Woo I, Maas RL, Haber E, Lee ME. 1998. Embryonic expression suggests an important role for CRP2/SmLIM in the developing cardiovascular system. Circ Res 83:980–985.

Kapur S, Baylink DJ, Lau KH. 2003. Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways. Bone 32:241–251.

Kesava CB, Baylink DJ, Wergedal JE, Mohan S. 2004. Inbred mouse strains exhibit variations in the skeletal adaptive response to 4-point bending: Evidence for involvement of different genetic mechanisms. J Bone Miner Res 19:S396.

Kodama Y, Dimai HP, Wergedal J, Sheng M, Malpe R, Kutilek S, Beamer W, Donahue LR, Rosen C, Baylink DJ, Farley J. 1999. Cortical tibial bone volume in two strains of mice: Effects of sciatic neurectomy and genetic regulation of bone response to mechanical loading. Bone 25:183–190.

Kodama Y, Umemura Y, Nagasawa S, Beamer WG, Donahue LR, Rosen CR, Baylink DJ, Farley JR. 2000. Exercise and mechanical loading increase periosteal bone formation and whole bone strength in C57BL/6J mice but not in C3H/Hej mice. Calcif Tissue Int 66:298–306.

Madisen L, Neubauer M, Plowman G, Rosen D, Segarini P, Dasch J, Thompson A, Ziman J, Bentz H, Purchio AF. 1990. Molecular cloning of a novel bone-forming compound: Osteoinductive factor. DNA Cell Biol 9:303–309.

Marie PJ, Hott M, Perheentupa J. 1990. Effects of epidermal growth factor on bone formation and resorption in vivo. Am J Physiol 258:E275–E281.

Nikitin A, Egorov S, Daraselia N, Mazo I. 2003. Pathway studio—the analysis and navigation of molecular networks. Bioinformatics 19:2155–2157.

Noble BS, Peet N, Stevens HY, Brabbs A, Mosley JR, Reilly GC, Reeve J, Skerry TM, Lanyon LE. 2003. Mechanical loading: Biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. Am J Physiol Cell Physiol 284:C934–C943.

Park JM, Borer JG, Freeman MR, Peters CA. 1998. Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. Am J Physiol 275:C1247–C1254.

Park JM, Adam RM, Peters CA, Guthrie PD, Sun Z, Klagsbrun M, Freeman MR. 1999. AP-1 mediates stretch-induced expression of HB-EGF in bladder smooth muscle cells. Am J Physiol 277:C294–C301.

Pedersen EA, Akhter MP, Cullen DM, Kimmel DB, Recker RR. 1999. Bone response to in vivo mechanical loading in C3H/HeJ mice. Calcif Tissue Int 65:41–46.

Pines A, Romanello M, Cesaratto L, Damante G, Moro L, D'Andrea P, Tell G. 2003. Extracellular ATP stimulates the early growth response protein 1 (Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line. Biochem J 373:815–824.

Ponik SM, Pavalko FM. 2004. Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE2 release in MC3T3-E1 osteoblasts. J Appl Physiol 97:135–142.

Preisinger E, Alacamlioglu Y, Pils K, Saradeth T, Schneider B. 1995. Therapeutic exercise in the prevention of bone loss. A controlled trial with women after menopause. Am J Phys Med Rehabil 74:120–123.

Raab-Cullen DM, Akhter MP, Kimmel DB, Recker RR. 1994a. Periosteal bone formation stimulated by externally induced bending strains. J Bone Miner Res 9:1143–1152.

Raab-Cullen DM, Thiede MA, Petersen DN, Kimmel DB, Recker RR. 1994b. Mechanical loading stimulates rapid changes in periosteal gene expression. Calcif Tissue Int 55:473–478.

Rittweger J, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P, Felsenberg D. 2005. Muscle atrophy and bone loss after 90 days' bed rest and the effects of flywheel resistive exercise and pamidronate: Results from the LTBR study. Bone.

Robling AG, Turner CH. 2002. Mechanotransduction in bone: Genetic effects on mechanosensitivity in mice. Bone 31:562–569.

Robling AG, Li J, Shultz KL, Beamer WG, Turner CH. 2003. Evidence for a skeletal mechanosensitivity gene on mouse chromosome 4. Faseb J 17:324–326.

Schmalbach CE, Chepeha DB, Giordano TJ, Rubin MA, Teknos TN, Bradford CR, Wolf GT, Kuick R, Misek DE, Trask DK, Hanash S. 2004. Molecular profiling and the identification of genes associated with metastatic oral cavity/pharynx squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 130:295–302.

Segev O, Samach A, Faerman A, Kalinski H, Beiman M, Gelfand A, Turam H, Boguslavsky S, Moshayov A, Gottlieb H, Kazanov E, Nevo Z, Robinson D, Skaliter R, Einat P, Binderman I, Feinstein E. 2004. CMF608-a novel mechanical strain-induced bone-specific protein expressed in early osteochondroprogenitor cells. Bone 34:246–260.

Shay-Salit A, Shushy M, Wolfovitz E, Yahav H, Breviario F, Dejana E, Resnick N. 2002. VEGF receptor 2 and the adherens junction as a mechanical transducer in vascular endothelial cells. Proc Natl Acad Sci USA 99:9462–9467.

Sibonga JD, Zhang M, Evans GL, Westerlind KC, Cavolina JM, Morey-Holton E, Turner RT. 2000. Effects of spaceflight and simulated weightlessness on longitudinal bone growth. Bone 27:535–540.

Sommarin Y, Wendel M, Shen Z, Hellman U, Heinegard D. 1998. Osteoadherin, a cell-binding keratan sulfate proteoglycan in bone, belongs to the family of leucine-rich repeat proteins of the extracellular matrix. J Biol Chem 273:16723–16729.

Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, Jordan H, Moore T, Max SI, Wang J, Hsieh F, Diatchenko L, Marusina K, Farmer AA, Rubin GM, Hong L, Stapleton M, Soares MB, Bonaldo MF, Casavant TL, Scheetz TE, Brownstein MJ, Usdin TB, Toshiyuki S, Carninci P, Prange C, Raha SS, Loquellano NA, Peters GJ, Abramson RD, Mullahy SJ, Bosak SA, McEwan PJ, McKernan KJ, Malek JA, Gunaratne PH, Richards S, Worley KC, Hale S, Garcia AM, Gay LJ, Hulyk SW, Villalon DK, Muzny DM, Sodergren EJ, Lu X, Gibbs RA, Fahey J, Helton E, Ketteman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Young AC, Shevchenko Y, Bouffard GG, Blakesley RW, Touchman JW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Krzywinski MI, Skalska U, Smailus DE, Schnerch A, Schein JE, Jones SJ, Marra MA. 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci USA 99:16899–16903.

Swartz MA, Tschumperlin DJ, Kamm RD, Drazen JM. 2001. Mechanical stress is communicated between different cell types to elicit matrix remodeling. Proc Natl Acad Sci USA 98:6180–6185.

Tanaka SM, Alam IM, Turner CH. 2003. Stochastic resonance in osteogenic response to mechanical loading. Faseb J 17:313–314.

Tare RS, Oreffo RO, Clarke NM, Roach HI. 2002. Pleiotrophin/osteoblast-stimulating factor 1: dissecting its diverse functions in bone formation. J Bone Miner Res 17:2009–2020.

Torrance AG, Mosley JR, Suswillo RF, Lanyon LE. 1994. Noninvasive loading of the rat ulna in vivo induces a strain-related modeling response uncomplicated by trauma or periostal pressure. Calcif Tissue Int 54:241–247.

Wang Y, Fraefel C, Protasi F, Moore RA, Fessenden JD, Pessah IN, DiFrancesco A, Breakefield X, Allen PD. 2000. HSV-1 amplicon vectors are a highly efficient gene delivery system for skeletal muscle myoblasts and myotubes. Am J Physiol Cell Physiol 278:C619–C626.

Weyts FA, Li YS, van Leeuwen J, Weinans H, Chien S. 2002. ERK activation and alpha v beta 3 integrin signaling through Shc recruitment in response to mechanical stimulation in human osteoblasts. J Cell Biochem 87:85–92.

Workman C, Jensen LJ, Jarmer H, Berka R, Gautier L, Nielser HB, Saxild HH, Nielsen C, Brunak S, Knudsen S. 2002. A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol 3:research0048.

Wu MX. 2003. Roles of the stress-induced gene IEX-1 in regulation of cell death and oncogenesis. Apoptosis 8:11–18.

Yamazaki S, Ichimura S, Iwamoto J, Takeda T, Toyama Y. 2004. Effect of walking exercise on bone metabolism in postmenopausal women with osteopenia/osteoporosis. J Bone Miner Metab 22:500–508.

Yang X, Tare RS, Partridge KA, Roach HI, Clarke NM, Howdle SM, Shakesheff KM, Oreffo RO. 2003. Induction of human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation by osteoblast stimulating factor-1/pleiotrophin: Osteoconductive biomimetic scaffolds for tissue engineering. J Bone Miner Res 18:47–57.

Yang W, Lu Y, Kalajzic I, Guo D, Harris MA, Gluhak-Heinrich J, Kotha S, Bonewald LF, Feng JQ, Rowe DW, Turner CH, Robling AG, Harris SE. 2005. Dentin matrix protein 1 gene *cis*-regulation: Use in osteocytes to characterize local responses to mechanical loading in vitro and In vivo. J Biol Chem.