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Mechanical Modulation of Molecular Signals Which Regulate Anabolic and Catabolic Activity in Bone Tissue

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Abstract Identifying the molecular mechanisms that regulate bone's adaptive response to alterations in load bearing may potentiate the discovery of interventions to curb osteoporosis. Adult female mice (BALB/cByJ) were subjected to catabolic (disuse) and anabolic (45 Hz, 0.3*g* vibration for 10 min/day) signals, and changes in the mRNA levels of thirteen genes were compared to altered indices of bone formation. Age-matched mice served as controls. Following 4 days of disuse, significant ($P = 0.05$) decreases in mRNA levels were measured for several genes, including collagen type I (−55%), osteonectin (−44%), osterix (−36%), and MMP-2 (−36%) all of which, after 21 days, had normalized to control levels. In contrast, expression of several genes in the vibrated group, which failed to show significant changes at 4 days, demonstrated significant increases after 21 days, including inducible nitric oxide synthase (iNOS) (39%, $P = 0.07$), MMP-2 (54%), and receptor activator of the nuclear factor kB ligand (RANKL) (32%). Correlations of gene expression patterns across experimental conditions and time points allowed the functional clustering of responsive genes into two distinct groups. Each cluster's specific regulatory role (formation vs. resorption) was reinforced by the 60% suppression of formation rates caused by disuse, and the 55% increase in formation rates stimulated by mechanical signals ($P < 0.05$). These data confirm the complexity of the bone remodeling process, both in terms of the number of genes involved, their interaction and coordination of resorptive and formative activity, and the temporal sensitivity of the processes. More detailed spatial and temporal correlations between altered mRNA levels and tissue plasticity may further delineate the molecules responsible for the control of bone mass and morphology. J. Cell. Biochem. 94: 982–994, 2005.

Key words: bone adaptation; mechanical loading; disuse; gene expression; osteoporosis

Functional load bearing has a strong influence on bone remodeling, as evidenced by its direct impact on osteoblastic and osteoclastic activity, and ultimately, by alterations of bone mass and morphology. Decreases in functional loading, which occur under conditions, such as spaceflight or bedrest, suppresses osteoblast activity while elevating osteoclastic activity [Turner, 2000], resulting in a net loss of bone [Alexandre et al., 1988; Judex et al., 2004]. Conversely, specific increases in load bearing, as in the form of exercise, can stimulate osteoblastic activity while suppressing osteoclast recruitment and activity [Judex and Zernicke, 2000; Tajima et al., 2000], resulting in net improvements in both bone quantity and quality [Rubin et al., 2002; Judex et al., 2003].

In vivo studies, which have investigated the influence of mechanical loading on bone plasticity, have focused on identifying those specific mechanical parameters responsible for regulating the response. By design, many of these

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studies have precluded a rigorous characterization of the molecular mechanisms which control bone's adaptive processes, thus hampering a full understanding of the etiology of osteoporosis, and limiting a systematic examination of potential drug targets involved in regulating bone disease. In vitro models of bone adaptation have been used to demonstrate that a number of diverse molecules are related to mechanically mediated changes in bone formation and resorption—ranging from immediate early genes such as c-fos to the most abundant protein in bone, collagen type I [Nomura and Takano-Yamamoto, 2000; Rubin et al., 2000; Genever and Skerry, 2001; Jiang et al., 2002; Hatton et al., 2003; Peake and el Haj, 2003; Saunders et al., 2003]. Critical putative functions of genes involved in mechano-reception and transduction have been derived from these studies. Ultimately, however, removal of cells from their matrix may mask critical interdependent interactions between osteoblasts, osteoclasts, osteocytes, and other cells [Parfitt, 1995], and emphasizes the benefits of using integrative in vivo models to more fully characterize the molecules, which ultimately control bone remodeling activity, and more rigorously consider the complexity of the bone remodeling response [Pavlin and Gluhak-Heinrich, 2001; Hadjiargyrou et al., 2002; Karsenty, 2003].

We have recently demonstrated that extremely small magnitude forces, induced noninvasively to the skeleton as whole body vibrations, can be perceived as osteogenic [Rubin et al., 2001a]. In the proximal tibia of adult BALB/cByJ mice, for example, 10 min per day of a 45 Hz, 0.3g acceleration (1g = acceleration on Earth, or 9.8 m/s^2) is anabolic to trabecular bone [Judex et al., 2002], while disuse is catabolic and may also suppress bone formation [Judex et al., 2002]. The goal of the study reported here was to relate these mechanically mediated changes in bone formation rates (BFR) to the expression of a broad set of genes anticipated to play a role in regulating bone adaptation. The thirteen genes considered all have critical, but not necessarily unique, tasks in (mechanically induced) bone formation (Cbfa1 [Ontiveros and McCabe, 2003], osterix [Nakashima et al., 2002], BMP-2 [Sato et al., 1999], IGF-1 [Kawata and Mikuni-Takagaki, 1998], MMP-2 [Blumenfeld et al., 2002], collagen type I [Moalli et al., 2000], integrin β3 [Weyts et al., 2002], osteonectin [Pioletti et al., 2003]) and bone resorption (RANKL [Rubin et al., 2003], iNOS [Watanuki et al., 2002], osteopontin [You et al., 2001], MMP-9 [Rantakokko et al., 1999], cathepsin K [Rantakokko et al., 1999]). It was hypothesized that alterations in load bearing (increase or decrease) will stimulate differential responses in the activity of these formation and resorption gene "families," including their temporal expression patterns.

MATERIALS AND METHODS

Experimental Design

All procedures were reviewed and approved by the University's Animal Care and Use Committee, and met or exceeded all AALAC guidelines. Sixteen-week-old female BALB/cByJ mice were randomly distributed into one of three groups: control, disuse, and mechanical stimulation (n = 15, each). Mice were housed individually in standard cages (28 × 17 × 13 cm) and had access to rodent chow (autoclaved diet NIH-31 with 6% fat, 18% protein, Ca:P 1:1, vitamin, and mineral fortified) ad libitum. The catabolic stimulus was achieved by functional disuse, initiated by applying (24 h/day) a hindlimb unloading apparatus (tail suspension), originally developed for the rat [Morey-Holton and Globus, 2002], and modified for use in the mouse [Judex et al., 2002]. The mechanically mediated anabolic signal was achieved by placing them on a platform, which oscillated vertically at 45 Hz, 0.3g, for 10 min/day, 5 days/week [Judex et al., 2002]. Six mice from each group were sacrificed after 4 days, and nine mice from each group sacrificed after 21 days. To facilitate the accurate measurement of bone formation, mice in the 21-day group were injected with calcein (i.p.,10 mg/kg) on day 10 and 19. Upon sacrifice, left and right tibiae were harvested, cleaned of all surrounding soft tissue, and preserved in 70% EtOH (right tibia; histomorphometry), or immediately snap-frozen in liquid nitrogen and stored in −80°C (left tibia; gene expression).

Histomorphometry

Processing of the mouse tibia for histomorphometry has been described previously [Judex et al., 2002]. Briefly, the proximal and diaphyseal region of the right tibia of each mouse was embedded in methyl-methacrylate (Fisher Scientific, NJ). Following polymerization, a 7-μm-thick undecalcified frontal section was cut

with a sledge microtome (Reichert Jung, Germany) from the proximal tibia and a 40-μm transverse section was cut from the middiaphysis with a diamond wire saw (Well Diamond Wire Saws, GA). For each tibia, mididaphyseal cortical (Ct.Ar) and metaphyseal trabecular bone area (Tb.Ar) were measured, and BFR were determined with bone surface (BS) as referent. Further, mineralizing surface (MS/BS), an indicator for the presence of osteoblasts on surfaces and mineral apposition rates (MAR), an indicator of how fast osteoblasts are producing new bone, were measured. All analyses were performed with the operator blinded to the identity of the mouse from which the section had been harvested. Bone sections that failed to display double labels anywhere within the entire tibial section, indicating that one of the fluorescent label injections was not absorbed, were removed from the dynamic bone analyses (applicable to one specimen from the control group). To compare changes in gene expression (based on RNA extracted from the entire tibia) to altered static and dynamic histomorphometric bone indices at the tissue level, all formation rates and bone areas were averaged between trabecular and cortical bone.

RNA Extraction

Upon storage in -80°C, individual left tibiae (including bone marrow and cartilage) were crushed into a fine powder using a liquid nitrogen cooled mortar and pestle, and total RNA was isolated using standard protocols [Reno et al., 1997; Majima et al., 2000]. TRIzol reagent (Life Technologies, MD) was added to the bone powder and further homogenized. Phases were separated with chloroform under centrifugation (12,000*g*). One volume of ethanol was added to the aqueous phase and total RNA was isolated using the RNeasy Total RNA isolation kit (Qiagen, CA) as directed by the manufacturer. Any potential DNA contamination was removed with RNase free DNase (Qiagen). A cyto-fluorometer and a fluorescent dye (Ribo Green, Molecular Probes, OR) were used to quantify the amount of total RNA extracted from the tibia. The integrity of all RNA samples was assessed by agarose gels and RT-PCR amplification curves, leading to the exclusion of three bones from each of the 21-day groups. Consideration of a relatively large number of genes across three different experimental groups (control, vibrated, disuse) and two time points (4 and 21 days) necessitated the pooling of RNA samples [Kendziorski et al., 2003] for each group at a given time point (two pools of $n = 3$). Experimental error was minimized by running all samples (pools) in triplicates, and the average of the three values was used for further analyses.

Quantification of Gene Expression

The thirteen candidate genes monitored in this study were selected based on their proposed critical roles in regulating bone-remodeling activity. Although most, if not all, of the chosen genes almost certainly have multiple functions, the 13 candidate genes were categorized into those critical to: (1) formative processes and (2) resorptive processes. Of course, the pool of possible candidate genes implicated in bone formation and resorption is enormous [Karsenty, 2003; Teitelbaum and Ross, 2003], and an entirely different set of candidate genes could have been used to characterize changes in the mechanically mediated expression levels of formative and resorptive genes as a function of time. Thus, many genes essential to the control of bone's response to mechanical perturbations were inherently not considered. Similarly, the number of possible time points, especially for the screening of immediate early genes, is essentially infinite, and the 4 and 21 days time points were chosen as indicators of relatively early and intermediate processes occurring at the tissue level [Pead et al., 1988].

Genes characterized as essential to bone formation included: core binding factor alpha-1 (Cbfa1), osterix, insulin growth factor-1 (IGF-1), bone morphogenetic protein-2 (BMP-2), integrin β3, collagen type I (α1), metalloproteinase-2 (MMP-2 or gelatinase A), and osteonectin. Genes related to bone resorption included: receptor activator of the nuclear factor kB ligand (RANKL), inducible nitric oxide synthase (iNOS or NOS2), cathepsin K, metalloproteinase-9 (MMP-9 or gelatinase B), and osteopontin. The expression levels of these 13 candidate genes were quantified using a real-time PCR cycler (LightCycler, Roche, IN) relative to the expression levels of a housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH).

Mouse specific primers for the coding region of each gene were designed and verified through sequencing. A "one-step" real-time RT-PCR kit (Qiagen, CA) provided the chemistry for the

quantification process (e.g., reverse transcriptase, buffer, nucleotides, SYBR Green). Annealing temperature and the amount of RNA template (~1–10 ng) were optimized for each primer pair. The cycling conditions for each reaction consisted of reverse transcription at 50°C for 20 min, activation at 95°C for 15 min, and amplification for 40 cycles. Each amplification cycle included denaturation at 94°C for 15 s, annealing at temperatures specified in Table I for 20 s, and extension at 72°C for 12 s. Upon amplification, a melting curve analysis (95°C for 5 s, 55°C for 15 s, and 95°C for 0 s) was performed to check for single amplicons. Both the gene of interest and the housekeeping gene were included in the same RT-PCR run to minimize variations. Each run also included standard curves for both genes using serially diluted RNA from a generic mouse tibia. LightCycler software quantitated the expression level of the gene of interest with GAPDH expression levels as a referent.

Statistical Analyses

For multiple comparisons, *t*-tests with Bonferroni corrections conservatively tested for differences in histomorphometric indices between the control and the two experimental groups. Temporal changes in body mass within a group of mice were assessed by paired *t*-tests.

The pooling of RNA from three animals within each group (run as triplicates) afforded *t*-tests (with conservative Bonferroni corrections) to compare gene expression levels between experimental (vibrated or disuse) and control mice [Kendziorski et al., 2003]. For a given gene, the true expression level in animal j of group i can be written as $y_{ij} = \mu_i + \varepsilon_j$, where μ_i is the group mean, and ε_j represents the biological variation between individual animals. The kth measurement (measurements were made in triplicates) of the jth pool (of three animals) in the ith group can be written as:

$$z_{ijk} = \mu_i + \frac{\varepsilon_{j_1} + \varepsilon_{j_2} + \varepsilon_{j_3}}{3} + \delta_k,$$

where δ_k is the measurement error. The t-test statistic to determine the difference between the control and experimental groups becomes $T = \bar{Y}_1 - \bar{Y}_2 / \left[\mathrm{SE}\left(\bar{Y}_1 - \bar{Y}_2\right)\right]$, where $\bar{Y}_i$ is the average of two pools in each group, and the denominator is the standard error of the numerator. Therefore, the variance of $\bar{Y}_1 - \bar{Y}_2$ equals $1/3*(\sigma^2_{\text{biologic}} + \sigma^2_{\text{measurement}})$, where $\sigma^2_{\text{biologic}}$ is the variance of the biological variation ε (reflected in the variation between the two pools of three animals each) and $\sigma^2_{\text{measurement}}$ is the variance of the measurement errors δ (reflected in the variation between the three measurements for each pool). Assuming $\sigma^2_{\text{biologic}}$ and $\sigma^2_{\text{measurement}}$ are similar for all six groups (3 groups*2 time points) for a given gene (i.e., the underlying distribution for the population from which the two pools were drawn is similar in shape for all groups), the standard error $1/3*(\sigma^2_{\text{biologic}} + \sigma^2_{\text{measurement}})$ is estimated by $\sum_{i=1}^{6} \left(\bar{Z}_{i1} - \bar{Z}_{i2}\right)^2 / 12$, where i is the index of the six groups from the two time points, and $\bar{Z}_{i1}$ and $\bar{Z}_{i2}$ are the average measurements of pools 1 and 2. Therefore,

TABLE I. Primer Sequences, Generated Amplicon Size, and Melting Temperature Corresponding to the 13 Candidate Genes (and GAPDH, the 'Housekeeping' Gene) Used for This Study

Target gene	Forward (5′ to 3′)	Reverse (5′ to 3′)	Size	Temperature (°C)
Genes involved in bone formation				
BMP-2	AGGATTAGCAGGTCTTTGC	GCCACGATCCAGTCATT	215	58
CBFA1	AGCAGCACTCCATATCT	CTTCCGTCAGCGTCAA	179	56
Collagen (α1)	CTGGCAAGAATGGCGA	GAAGCCACGATGACCC	161	55
Integrin-b3	AGGATTACCGACCCTCT	ATCTTGCCGAAGTCGC	121	57
IGF-1	CGCTCTGCTTGCTCACC	CCCCTCGGTCCACACA	103	58
MMP2	AGGTGTGCCAAGGTGGA	GAAGGAAACGAGCGAAGG	108	58
Osteonectin	GCATCAAGGAGCAGGACA	CGGAACAGCCAACCATC	139	58
Osterix	CCCAGAGCAGAGCAACC	CAGAGAGAGCCCCCAGA	150	57
Genes involved in bone resorption				
Cathepsin-K	GAAAATTGTGACCGTGATAATG	CGTTGTTCTTATTCCGAGC	116	56
MMP9	TCTGGCACACGCCTTTC	GGCACCATTTGAGTTTCCA	124	58
NOS2	CAGCACAAAGGGCTCAAA	CTCTCTTGCGGACCATCTC	109	57
Osteopontin	CGTCCCTACAGTCGATG	GCTGCCCCTTTCCGTTG	226	57
RANKL	CTGGTCGGGCAATTCT	CCCAAAGTACGTCGCAT	139	55
Housekeeping gene				
GAPDH	ACCAACTGCTTAGCCC	CTTCCCGTTCAGCTCT	222	55–58

They are segregated into those genes proposed to be involved in bone formation, and those important in regulating bone resorption.

the t-statistic to compare the control group to an experimental group was computed as:

$$T = \frac{\bar{Y}_{\text{control}} - \bar{Y}_{\text{exp}}}{\sqrt{\sum_{i=1}^{6} \left(\bar{Z}_{i1} - \bar{Z}_{i2}\right)^2 / 12}}.$$

The degrees of freedom for the estimate of the standard error was six, and P-values were calculated based on the underlying student-t distributions.

To contrast alterations in gene expression between the two time points, a t-statistic similar to the one above was obtained. The numerator is the difference between the experimental and control groups across the 4 and 21 days time points and the denominator is its standard error, that is, $\sqrt{\sum_{i=1}^{6} (\bar{Z}_{i1} - \bar{Z}_{i2})^2 / 6}$. Corresponding P-values were also determined with six degrees of freedom. See Kendziorski et al. [2003] for a more detailed discussion on using statistical tests for pooled samples.

Linear correlations tested the association of gene expression patterns across the three conditions and two time points. To avoid mapping of potential "noise," only genes that either (1) were differentially regulated at least at one time point (or treatment) or (2) exceeded changes in mRNA levels by 10% at least at two time points (or treatments) were included in this analysis. Expression patterns of genes which were highly correlated to each other ($R > 0.6$) were grouped into clusters. Multifactorial univariate ANOVA tested for main effects and interactions of the two fixed factors, (1) time point and (2) variations in the induced mechanical environment. Differences in the latter factor were followed-up with Tukey's post hoc tests. All tests were run separately for genes that had been categorized as bone formation or bone resorption genes. Histomorphometric data were presented as mean ± SD, and gene expression data were presented as averages of the three runs for each pool. Statistical significance was set at $P = 0.05$, and P-values between 0.05–0.07 were considered as borderline significant.

RESULTS

Body Mass and Histomorphometry

No significant differences in body mass were detected between the three experimental groups at baseline, at 4 days, or at 21 days, although disuse mice lost 4% ($P = 0.02$) of their weight over the 21 days protocol (no significant changes were observed in the other two groups).

BFR in the tibial metaphysis (trabecular bone) and the perosteal and endocortical surfaces of the diaphysis (cortical bone) were measured in the 21-day animals to characterize the tissue level response of the tibia to alterations in its normal functional environment. Averaged across trabecular and cortical bone, bone formation rates (BFR/BS) were 55% ($P = 0.04$) greater in mice exposed to the low-level mechanical signal and 60% ($P = 0.002$) lower in disuse mice when compared to control mice (Fig. 1).

Changes in BFR were relatively uniform across the trabecular and cortical surfaces. In trabecular bone, vibration stimulated a 32% ($P = 0.03$) increase in BFR, while removal of functional load bearing caused a 55% ($P = 0.001$) reduction in BFR (Table II). In diaphyseal cortical bone, BFR were 93% ($P = 0.12$) greater at the periosteal surface (62%, $P = 0.16$, at the endocortical surface) of mice subjected to 10 min/day of vibration than in control mice (Table II). In contrast, disuse caused a 60% ($P = 0.04$) decrease in BFR at the periosteal surface (69%, $P = 0.02$, at the endocortical surface). Similarly to BFR, ten minutes of daily vibrations upregulated mineralizing surface (MS/BS) in trabecular bone (18%, $P = 0.04$) and MAR at the periosteal surface (45%, $P = 0.05$). Disuse downregulated MS/BS (−20%, $P = 0.05$) and MAR (−45%, $P = 0.001$) in trabecular bone, MS/BS at the periosteal surface (−63%, $P = 0.01$), as well as MS/BS (−49%, $P = 0.03$) and MAR (−37%, $P = 0.02$) at the

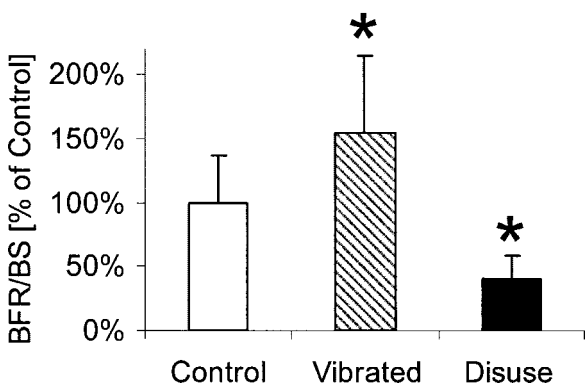

Fig. 1. Mean (±SD) tibial bone formation rates (BFR/BS) of control, vibrated, and disuse mice averaged across the trabecular proximal metaphysis and the periosteal and endocortical surfaces of the middiaphysis. Asterisks indicate significant differences between the control and an experimental group ($P < 0.05$).

TABLE II. Bone Formation Rates (BFR/BS), Mineralizing Surface (MS/BS), and Mineral Apposition Rates Measured at Trabecular (Metaphysis) and Cortical (Middiaphysis) Surfaces of Control (CTR), Vibrated (VIB), and Disuse (DIS) Mice (mean ± SD)

		BFR/BS (mm/year)	MS/BS (%)	MAR (μm/day)
Trabecular	CTR	129 ± 38	33.6 ± 5.8	1.0 ± 0.3
	VIB	170 ± 32*	39.5 ± 4.5*	1.2 ± 0.2
	DIS	58 ± 33*	27.0 ± 7.3*	0.6 ± 0.2*
Periosteal	CTR	61 ± 38	37.7 ± 17.5	0.4 ± 0.1
	VIB	118 ± 91	47.1 ± 24.2	0.6 ± 0.2*
	DIS	24 ± 24*	14.1 ± 10.2*	0.4 ± 0.2
Endocortical	CTR	72 ± 45	36.0 ± 16.4	0.5 ± 0.2
	VIB	117 ± 77	48.0 ± 18.0	0.6 ± 0.2
	DIS	22 ± 23*	18.3 ± 12.0*	0.3 ± 0.1*

Asterisks indicate significant differences with respect to the control group.

endocortical surface of the middiaphysis (Table II). Exposing the tibia to altered levels of mechanical load affected bone volume only in the trabecular metaphysis of disuse mice, which was decreased by 43% ($P = 0.003$).

Gene Expression

Four days of disuse caused the expression of several genes involved in bone formation to be downregulated, including: osterix (−36%, $P = 0.001$), BMP-2 (−26%, $P = 0.06$), collagen type I (−55%, $P = 0.003$), osteonectin (−44%, $P = 0.02$), and MMP-2 (−36%, $P = 0.05$) (Fig. 2). Of the candidate genes proposed to be critical to bone resorption, osteopontin was upregulated (+30%, $P = 0.07$) (Fig. 3). After 21 days of disuse, the expression levels of all genes, except osterix, had reestablished the levels measured in age-matched control mice. The expression of genes coding for integrin-β3, Cbfa1, RANKL, cathepsin-K, and MMP-9 were not significantly affected by disuse at either the 4 or 21 days time point (Table III).

Four days of mechanical stimulation failed to affect the mRNA levels of any of the candidate genes when compared to controls. In contrast, following 21 days of mechanical stimulation, changes in gene expression were observed. MMP-2 mRNA levels were significantly upregulated by 54% ($P = 0.03$), while osterix (+14%, $P = 0.17$), BMP-2 (+18%, $P = 0.22$), and collagen type I (+19%, $P = 0.20$) levels were modestly, but not significantly, altered. Representing genes from the "resorption" group, RANKL (+32%, $P = 0.05$), and iNos (+39%, $P = 0.07$) were significantly upregulated or were borderline significant (Fig. 3).

Using those nine genes whose expression values were either affected by vibration and/or disuse (MMP-2, osteonectin, osterix, BMP-2, collagen type I, iNOS, osteopontin, RANKL) or exceeded changes of 10% over at least two time points (Cbfa1), linear correlations (across the experimental treatments and time points) associated genes with each other such that genes with high correlation coefficients were grouped into the same cluster. Two clusters were sufficient to generate correlation coefficients above 0.6 for each gene, except RANKL. As hypothesized, genes that were anticipated to function-

TABLE III. Expression Ratios of Genes (With GAPDH Expression Levels as Referent) That Were Not Significantly Affected by Either the Anabolic Low Level Mechanical Vibration (VIB) or the Catabolic Signal, Disuse (DIS) at Either Time Point (4 and 21 days), when Compared to Values Measured in Age-Matched Control Mice (CTR)

		Cathepsin-K	Cbfa1	IGF-I	Integrin β3	MMP-9
4 days	CTR	3.43	2.18	2.37	1.52	1.58
	VIB	3.34 (−3%)	2.48 (14%)	2.34 (−1%)	1.44 (−5%)	1.80 (14%)
	DIS	3.49 (2%)	2.03 (−7%)	2.64 (12%)	1.56 (3%)	1.70 (8%)
21 days	CTR	3.35	2.31	2.40	1.67	1.91
	VIB	3.56 (6%)	2.45 (6%)	2.60 (8%)	1.79 (8%)	1.84 (−4%)
	DIS	3.53 (5%)	2.01 (−13%)	2.29 (−5%)	1.62 (−3%)	2.02 (6%)

Percentages in parentheses represent the differences in mean expression ratios between the experimental and control groups.

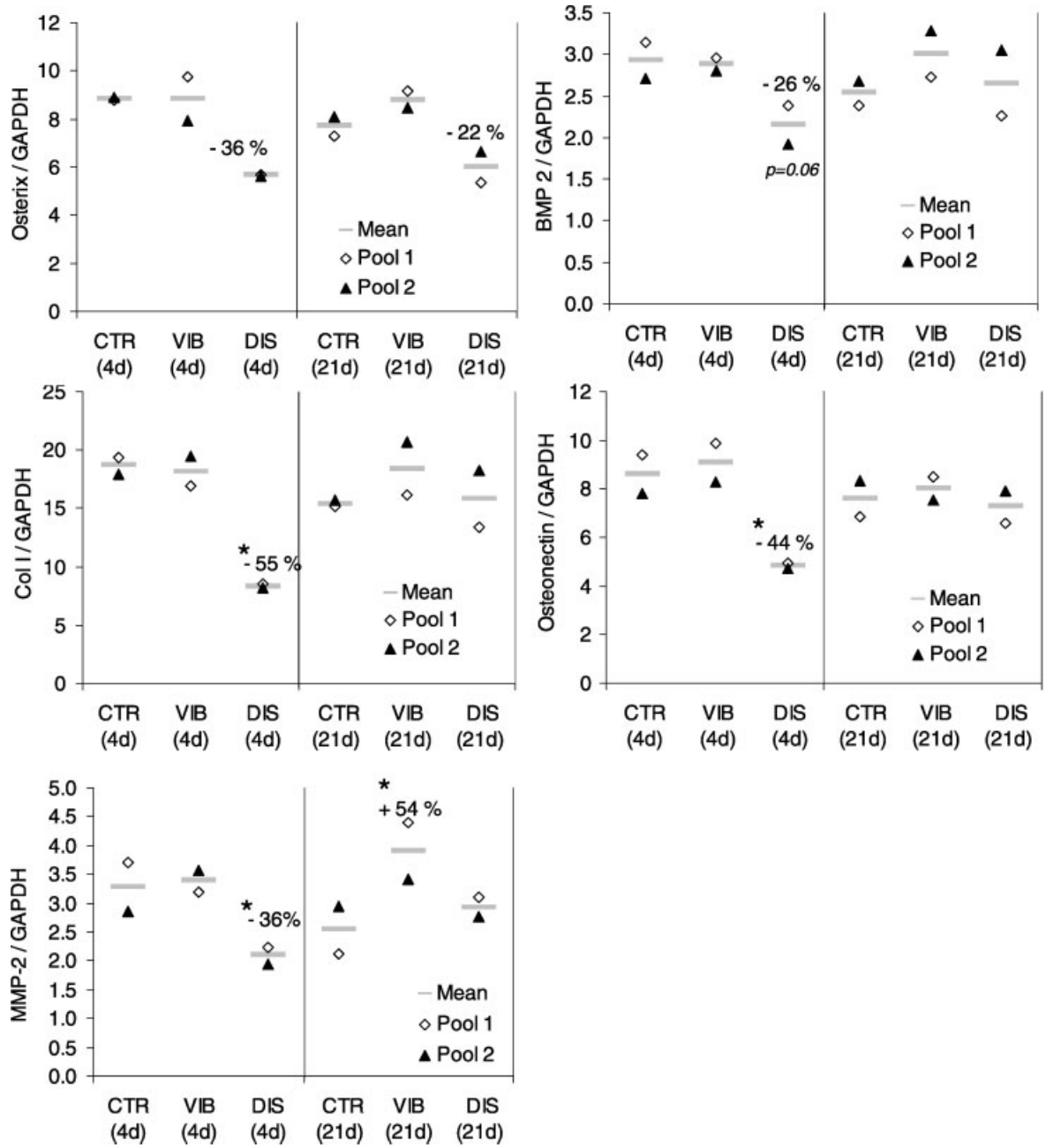

Fig. 2. mRNA levels of genes involved in *bone formation* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4 and 21 days. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($P = 0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($P < 0.05$) response between the two time points for a given gene and experimental condition.

ally relate to each other generally fell into the same cluster. Cluster 1 contained primarily bone formation genes, including Cbfa1, osterix, BMP-2, osteonectin, MMP-2, and collagen type-1 (Table IV). iNOS and osteopontin defined cluster 2 ($R = 0.68$). RANKL did not fit into any either cluster; the only gene whose expression pattern produced a correlation coefficient of above 0.35 with RANKL was iNOS ($R = 0.52$).

For further analysis, a multifactorial analysis of variance (including post hoc test) was used for the same nine genes that had been grouped

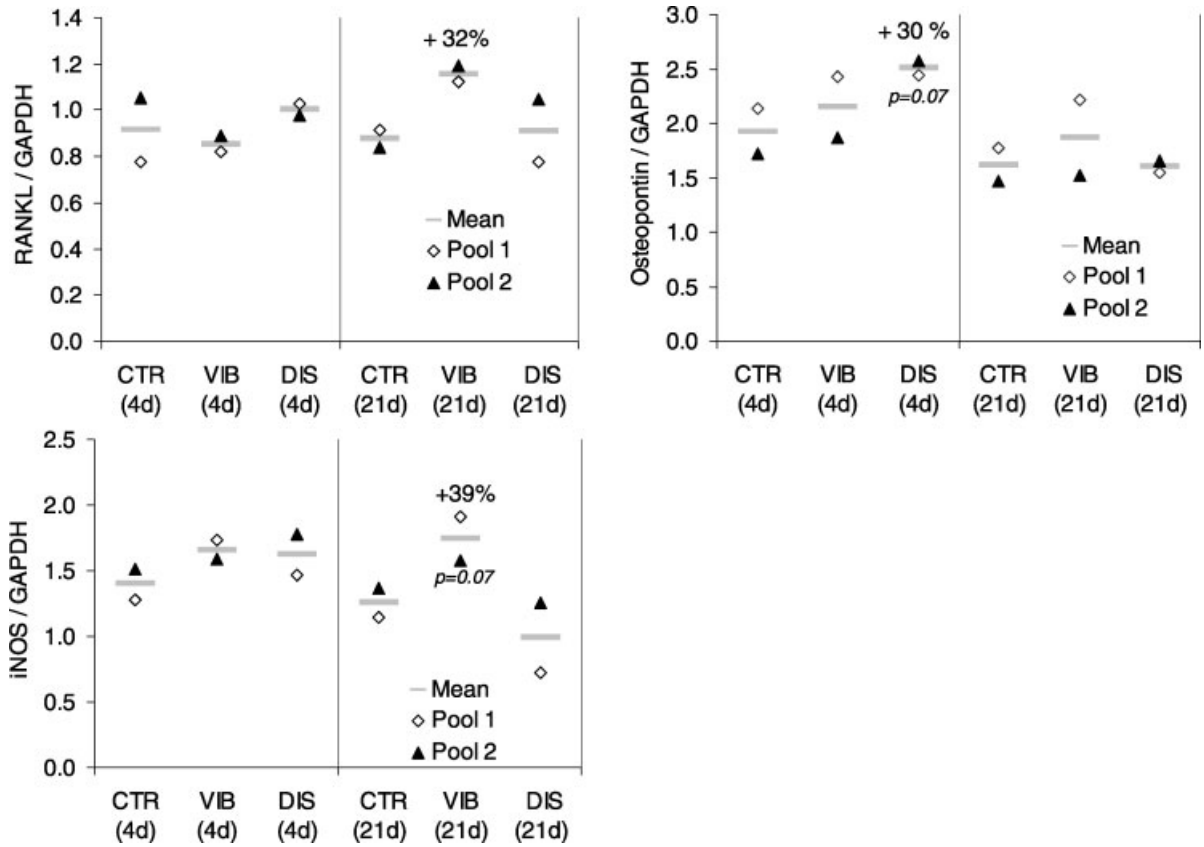

Fig. 3. mRNA levels of genes involved in *bone resorption* normalized to mRNA levels of a housekeeping gene (GAPDH) in control (CTR), vibrated (VIB), and disuse (DIS) mice after 4 and 21 days. Each graph presents the two mean values corresponding to the two pools of animals (Pool 1, Pool 2) as well as the average of these two means (Mean). Indicated percentages denote the statistically significant ($P < 0.05$, unless noted) relative differences with respect to the mean expression value of the control group. Asterisks denote a significantly different ($P = 0.05$) response between the two time points for a given gene and experimental condition.

according to their putative anabolic (Cbfa1, osterix, BMP-2, osteonectin, MMP-2, and collagen type I) or catabolic (RANKL, osteopontin, iNOS) character, testing for main effects and interactions between the two factors used in this study: (1) *time point* [4 and 21 days] and (2) *variations in the induced mechanical environment* [control, vibration, disuse]. Again, the assignment of genes to either the anabolic or catabolic category was not unique; however, several permutations in gene assignment were tested and did not substantially alter the results. The different levels of applied mechanical stimuli significantly ($P < 0.001$) affected the mRNA levels of bone forming genes, with both vibration ($P = 0.03$) and disuse ($P < 0.001$) being

TABLE IV. Correlation Coefficients Between Gene Expression Patterns Across the Experimental Groups and Time Points

Cluster 1	MMP-2	CBFA1	Osteonectin	Osterix	BMP-2	Coll-I
MMP-2	1.00					
CBFA1	0.67	1.00				
Osteonectin	0.78	0.68	1.00			
Osterix	0.78	0.83	0.86	1.00		
BMP-2	0.96	0.64	0.91	0.85	1.00	
Collagen-I	0.87	0.62	0.96	0.83	0.97	1.00

Grouping the genes based on the magnitude of their respective correlation coefficients, most genes that showed responsiveness to either vibration or disuse fell into the group above. Not surprisingly, genes within this cluster are functionally related (i.e., bone formation).

different from control. The significant ($P < 0.001$) interaction between time and the level of mechanical use (i.e., no independence between these two variables) reflected the inverse temporal expression patterns for mechanical stimulation and disuse (mRNA levels were altered by disuse primarily after 4 days and by mechanical stimulation after 21 days). Similarly, the level of mechanical load ($P = 0.04$) and the interaction between load and time ($P = 0.01$) had significant effects on the three bone resorptive genes, but significant differences in gene expression values across the two time points could only be established between control and vibrated mice.

DISCUSSION

The work presented here supports the premise that bone is sensitive to both increases and decreases in mechanical loading, and that the altered remodeling caused by such perturbations (enhanced bone formation by low-level mechanical signals, suppressed bone formation by disuse) is regulated, ultimately, by a complex, temporally dependent, interaction of a multitude of genes. Changes in gene expression stimulated by extremely low-level mechanical signals contrasted sharply with altered gene expression induced by disuse, and in both cases, the gene activity was dependent on time. The molecular events controlling the adaptive response in bone, as indicated by relative changes in expression patterns of several candidate genes coding for transcription factors, cytokines, proteins, or proteases, were distinct for increased and decreased levels of mechanical loading. While thirteen genes were examined, following these studies, it was clear that the responsive genes could be functionally grouped into two clusters; those whose primary function has been primarily linked to bone formation and those whose expression activity has been largely linked to bone resorption.

Disuse had its predominant effect on gene expression patterns early on, as reflected by the increases and decreases measured at day 4, but the anabolic signal did not influence the candidate gene pool until much later. Certainly, there is a strong likelihood that a broader pool of genes would reveal some that were perturbed by the anabolic process earlier than 3 weeks, particularly considering the morphologic changes that are observed in bone lining cells following brief bouts of loading [Chow et al., 1998]. It was surprising, however, that even with a mechanical signals that stimulated as much as a 90% increase in BFR, none of the selected genes were influenced early on and certainly implies that other genes are intricately involved in the adaptive process at these early time points. Alternatively, large expression changes in small regions within the bone may have been masked because of surrounding non-responsive regions—despite the spatial similarities in altered BFR between trabecular and cortical surfaces. Further, pooling of RNA samples reduced statistical power, and future detection of small (but significant) changes in mRNA levels will be aided by larger sample sizes.

Serum levels of molecules important for bone metabolism (e.g., calcium, PTH) were not measured in this study. While it is clear that altered circulating levels of systemic factors can affect gene expression levels, this study was performed in vivo to obtain the integrative (direct and indirect) adaptive changes in mRNA levels in a physiological system. Nevertheless, future correlations of altered gene expression levels with altered serum molecules will yield important data on the relative contributions of direct and indirect effects of anabolic and catabolic signals in bone. Further, assays to estimate changes in bone resorptive activity (e.g., TRAP staining for osteoclast products) was not addressed, and thus a direct link between the "bone resorption genes" and osteoclast activity could not be assessed. However, the large reduction in bone volume induced by disuse suggests that disuse, per se, not only suppressed bone formation activity, but also elevated resorptive activity [Judex et al., 2004]. Of course, the subset of genes, which increased their activity with disuse, might better be explained by a functional role in the recruitment and differentiation of osteoclasts rather than an active role in the suppression of formation.

None of the 13 candidate genes monitored at either time point was simultaneously affected by the catabolic and anabolic mechanical perturbations. This indicates that the functional role of the candidate genes is specific, and may be interpreted to indicate that the processes responsible for the increase in bone formation activity are independent from processes that are related to the suppression of bone formation activity. Consistent with previous investigations [Rubin et al., 2001b; Bikle et al., 2003; Oxlund et al., 2003], large increases

and decreases in mechanically mediated bone formation rates were evident. It is striking that the concomitant changes in gene expression were not particularly robust (<2-fold), indicating that while the molecular regulation of bone adaptation is exceedingly complex, it is also very subtle—particularly when compared to the much larger events such as bone repair [Hadjiargyrou et al., 2002] or the regulation of development and growth [Sodek et al., 1995; Enomoto-Iwamoto et al., 2002].

Collagen type I, the most abundant matrix protein in bone, displayed the most predictable [Ahdjoudj et al., 2002] expression pattern following the perturbation of mechanical signals. The 55% reduction in activity following just 4 days of disuse closely paralleled the mean 60% reduction in formation rates in trabecular and cortical bone. That collagen expression had normalized back to control levels following 21 days of disuse should not be completely surprising, given that the dynamics of changes in bone formation due to disuse is somewhat transient in nature [Globus et al., 1986]. In direct contrast with disuse, mechanical stimulation failed to mobilize collagen expression at 4 days, but expression patterns were moderately upregulated at 21 days. This delay, too, is not surprising considering the time it may take for the mechanical signal to recruit (and differentiate) bone cell populations such as bone lining or osteo-progenitor cells into osteoid producing osteoblasts [Chow et al., 1998].

The transcription factors Cbfa1 and osterix are essential for inducing osteoblast differentiation [Ducy et al., 1997; Otto et al., 1997; Nakashima et al., 2002]. Despite the distinct changes in BFR caused by mechanical stimulation and disuse, neither perturbation altered mRNA levels of Cbfa1. In contrast, disuse resulted in a rapid and persistent downregulation of osterix. Osterix, which acts downstream of Cbfa1, is regulated by several growth factors, including bone morphogenetic proteins (BMPs) that also play critical roles in osteoblast differentiation [Sakou, 1998]. The similar expression patterns among BMP-2, Cbfa1, osterix, and collagen type I in response to the anabolic and catabolic signals across the two time points helped to include them all in functional Cluster 1, and suggests that these genes are critical to the tight up- and downregulation of bone's anabolic activity perturbed by positive and negative mechanical events.

Demonstrating similar patterns of expression [Lockhart and Winzeler, 2000], two other genes, osteonectin and MMP-2, were found in this same functional cluster, and perhaps perform similar functions in the control of the cascade of adaptive processes. Osteonectin is a noncollagenous bone protein involved in regulatory interactions between bone cells and the extracellular matrix [Bradshaw and Sage, 2001]. Genes, such as osteonectin, are considered critical for maintaining both bone quantity and quality [Delany et al., 2003] and early control of their mRNA levels may represent a potential target for the prevention of osteopenia. Although MMP-2 has a role in the degradation of gelatin, elastin, and fibronectin within the bone matrix, it was markedly upregulated by anabolic mechanical signals and suppressed by disuse. However, it is also important to consider that MMP-2 is expressed by osteoblasts [Meikle et al., 1992; Okada et al., 1995] and important for bone mineralization [Itoh et al., 1998; Satoyoshi et al., 2001]. Thus, the upregulation of MMP-2 by mechanical signals and its downregulation by disuse may be associated primarily with anabolic events and emphasizes the importance of considering a gene's role within the integrative milieu of the in vivo environment.

In contrast to the activities of MMP-2, gene expression patterns of another member of the MMP family, MMP-9, failed to correlate with any other candidate gene and was largely unaffected by either the catabolic or anabolic stimulus. The basis for the independent relationship of these two MMP genes in mechanically mediated bone adaptation may lie with the different cell types (osteoblasts vs. osteoclasts) expressing the two molecules [Wucherpfennig et al., 1994; Okada et al., 1995], the dependency of MMP on post-translational modification, and/or the differential regulation of MMP inhibitors (TIMP).

IGF-1, a gene critical to osteoblast progenitor proliferation and regeneration [Canalis et al., 1993; Lean et al., 1996], fracture healing [Bostrom et al., 1999], and the orchestration of the anabolic response to biochemical agents [Bikle et al., 2002], did not—at least at the two time points considered—appear to play a major role in either decreases or increases in bone formation as stimulated by changes in mechanical signals. This finding is consistent with preliminary (unpublished) data from our

laboratory, which fail to show any changes in IGF-1 serum levels in the mice used for this study at either day 4 or 21. While this could easily be explained by suggesting that the two time points considered in this study simply missed the altered IGF-1 activity much earlier [Lean et al., 1995; Kawata and Mikuni-Takagaki, 1998], it is also possible that bone adaptation to the mechanical signals used in this study is such a "subtle" process that the mRNA levels of some genes typically associated with bone formation are never substantially perturbed.

Nitric oxide is a ubiquitous molecule that has been proposed as a key regulator of bone remodeling, through influencing pathways of both bone resorption and formation [Chole et al., 1998; Watanuki et al., 2002; Rubin et al., 2003]. The inducible form of nitric oxide synthase (iNOS or NOS2) is capable of producing greater quantities of nitric oxide than the constitutive form (eNOS), and its absence has been shown to prevent the catabolic events associated with menopause or mechanical disuse [van't Hof et al., 2000; Watanuki et al., 2002; Cuzzocrea et al., 2003]. In this study, the moderate correlation with RANKL expression patterns, a factor critical for osteoclast differentiation, may preferentially emphasize the resorptive role of iNOS. The upregulation of iNOS and RANKL as measured at 21 days in the mechanically stimulated animals may at first appear counterintuitive, but is consistent with the increased generation of nitric oxide in mechanical stimulation systems in vitro [Klein-Nulend et al., 1995; Pitsillides et al., 1995]. Alternatively, both nuclear factor κB and its ligand RANKL are expressed by osteoblasts, and RANKL upregulation could also reflect an increase in osteoblast number (as indicated by the increase in mineralizing surface in vibrated mice), and orchestrate not only a bone formation response, but also the coupling to a bone resorption response [Parfitt, 1994]. Perhaps, relationships such as those will help identify the cascade of genes that are critically involved in the bone remodeling process.

In summary, these data emphasize that the molecular events involved in mechanically mediated bone adaptation are both subtle and complex. Further, the similarity in expression patterns between many distinct genes responding to the catabolic and/or anabolic signals accentuates an intricate co-dependence of molecular events involved in bone's adaptation to mechanical signals. Detailed temporal assays using functional genomics and validated by histological assessment will ultimately contribute to a more complete characterization of the mechanisms, which control bone adaptation, perhaps improving our understanding of the etiology of osteoporosis, and help to identify unique molecular targets that could prevent this devastating disease.

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REFERENCES

Ahdjoudj S, Lasmoles F, Holy X, Zerath E, Marie PJ. 2002. Transforming growth factor beta2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. J Bone Miner Res 17:668–677.

Alexandre C, Vico L, Pilonchery G, Chapuy MC, Delmas PD, Chappard D. 1988. [Effects of weightlessness on phospho-calcium metabolism and its hormonal regulation in man during the 51 G Franco-American space flight]. Pathol Biol (Paris) 36:144–148.

Bikle DD, Sakata T, Leary C, Elalieh H, Ginzinger D, Rosen CJ, Beamer W, Majumdar S, Halloran BP. 2002. Insulin-like growth factor I is required for the anabolic actions of parathyroid hormone on mouse bone. J Bone Miner Res 17:1570–1578.

Bikle DD, Sakata T, Halloran BP. 2003. The impact of skeletal unloading on bone formation. Gravit Space Biol Bull 16:45–54.

Blumenfeld I, Srouji S, Peled M, Livne E. 2002. Metalloproteinases (MMPs-2, -3) are involved in TGF-beta and IGF-1-induced bone defect healing in 20-month-old female rats. Arch Gerontol Geriatr 35:59–69.

Bostrom MP, Saleh KJ, Einhorn TA. 1999. Osteoinductive growth factors in preclinical fracture and long bone defects models. Orthop Clin North Am 30:647–658.

Bradshaw AD, Sage EH. 2001. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. J Clin Invest 107:1049–1054.

Canalis E, Pash J, Gabbitas B, Rydziel S, Varghese S. 1993. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. Endocrinology 133:33–38.

Chole RA, Tinling SP, Leverentz E, McGinn MD. 1998. Inhibition of nitric oxide synthase blocks osteoclastic bone resorption in adaptive bone modeling. Acta Otolaryngol 118:705–711.

Chow JW, Wilson AJ, Chambers TJ, Fox SW. 1998. Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-week-old rats. J Bone Miner Res 13:1760–1767.

Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, Vegeto E, Caputi AP, Van De Loo FA, Puzzolo D, Maggi A. 2003. Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology 144:1098–1107.

Delany AM, Kalajzic I, Bradshaw AD, Sage EH, Canalis E. 2003. Osteonectin-null mutation compromises osteoblast formation, maturation, and survival. Endocrinology 144:2588–2596.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation [see comments]. Cell 89:747–754.

Enomoto-Iwamoto M, Kitagaki J, Koyama E, Tamamura Y, Wu C, Kanatani N, Koike T, Okada H, Komori T, Yoneda T, Church V, Francis-West PH, Kurisu K, Nohno T, Pacifici M, Iwamoto M. 2002. The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. Dev Biol 251:142–156.

Genever PG, Skerry TM. 2001. Regulation of spontaneous glutamate release activity in osteoblastic cells and its role in differentiation and survival: Evidence for intrinsic glutamatergic signaling in bone. FASEB J 15:1586–1588.

Globus RK, Bikle DD, Morey-Holton E. 1986. The temporal response of bone to unloading. Endocrinology 118:733–742.

Hadjiargyrou M, Lombardo F, Zhao S, Ahrens W, Joo J, Ahn H, Jurman M, White DW, Rubin CT. 2002. Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair. J Biol Chem 277:30177–30182.

Hatton JP, Pooran M, Li CF, Luzzio C, Hughes-Fulford M. 2003. A short pulse of mechanical force induces gene expression and growth in MC3T3-E1 osteoblasts via an ERK 1/2 pathway. J Bone Miner Res 18:58–66.

Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. 1998. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res 58:1048–1051.

Jiang GL, White CR, Stevens HY, Frangos JA. 2002. Temporal gradients in shear stimulate osteoblastic proliferation via ERK1/2 and retinoblastoma protein. Am J Physiol Endocrinol Metab 283:E383–E389.

Judex S, Zernicke RF. 2000. High-impact exercise and growing bone: Relation between high strain rates and enhanced bone formation. J Appl Physiol 88:2183–2191.

Judex S, Donahue LR, Rubin C. 2002. Genetic predisposition to low bone mass is paralleled by an enhanced sensitivity to signals anabolic to the skeleton. FASEB J 16:1280–1282.

Judex S, Boyd S, Qin YX, Turner S, Ye K, Muller R, Rubin C. 2003. Adaptations of trabecular bone to low magnitude vibrations result in more uniform stress and strain under load. Ann Biomed Eng 31:12–20.

Judex S, Garman R, Squire M, Busa B, Donahue LR, Rubin C. 2004. Genetically linked site-specificity of disuse osteoporosis. J Bone Miner Res 19:607–613.

Karsenty G. 2003. The complexities of skeletal biology. Nature 423:316–318.

Kawata A, Mikuni-Takagaki Y. 1998. Mechanotransduction in stretched osteocytes–temporal expression of immediate early and other genes. Biochem Biophys Res Commun 246:404–408.

Kendziorski CM, Zhang Y, Lan H, Attie AD. 2003. The efficiency of pooling mRNA in microarray experiments. Biostatistics 4:465–477.

Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH. 1995. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts–correlation with prostaglandin upregulation. Biochem Biophys Res Commun 217:640–648.

Lean JM, Jagger CJ, Chambers TJ, Chow JW. 1995. Increased insulin-like growth factor I mRNA expression in rat osteocytes in response to mechanical stimulation. Am J Physiol 268:E318–E327.

Lean JM, Mackay AG, Chow JW, Chambers TJ. 1996. Osteocytic expression of mRNA for c-fos and IGF-I: An immediate early gene response to an osteogenic stimulus. Am J Physiol 270:E937–E945.

Lockhart DJ, Winzeler EA. 2000. Genomics, gene expression and DNA arrays. Nature 405:827–836.

Majima T, Marchuk LL, Sciore P, Shrive NG, Frank CB, Hart DA. 2000. Compressive compared with tensile loading of medial collateral ligament scar in vitro uniquely influences mRNA levels for aggrecan, collagen type II, and collagenase. J Orthop Res 18:524–531.

Meikle MC, Bord S, Hembry RM, Compston J, Croucher PI, Reynolds JJ. 1992. Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. J Cell Sci 103(Pt 4):1093–1099.

Moalli MR, Caldwell NJ, Patil PV, Goldstein SA. 2000. An in vivo model for investigations of mechanical signal transduction in trabecular bone. J Bone Miner Res 15:1346–1353.

Morey-Holton ER, Globus RK. 2002. Hindlimb unloading rodent model: Technical aspects. J Appl Physiol 92:1367–1377.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Nomura S, Takano-Yamamoto T. 2000. Molecular events caused by mechanical stress in bone. Matrix Biol 19: 91–96.

Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: Implications for bone resorption. Lab Invest 72:311–322.

Ontiveros C, McCabe LR. 2003. Simulated microgravity suppresses osteoblast phenotype, Runx2 levels and AP-1 transactivation. J Cell Biochem 88:427–437.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Oxlund BS, Ortoft G, Andreassen TT, Oxlund H. 2003. Low-intensity, high-frequency vibration appears to prevent the decrease in strength of the femur and tibia

associated with ovariectomy of adult rats. Bone 32: 69–77.
Parfitt AM. 1994. Osteonal and hemi-osteonal remodeling: The spatial and temporal framework for signal traffic in adult human bone. J Cell Biochem 55:273–286.
Parfitt AM. 1995. Problems in the application of in vitro systems to the study of human bone remodeling. Calcif Tissue Int 56(Suppl 1):S5–S7.
Pavlin D, Gluhak-Heinrich J. 2001. Effect of mechanical loading on periodontal cells. Crit Rev Oral Biol Med 12:414–424.
Pead MJ, Skerry TM, Lanyon LE. 1988. Direct transformation from quiescence to bone formation in the adult periosteum following a single brief period of bone loading. J Bone Miner Res 3:647–656.
Peake MA, el Haj AJ. 2003. Preliminary characterisation of mechanoresponsive regions of the c-fos promoter in bone cells. FEBS Lett 537:117–120.
Pioletti DP, Muller J, Rakotomanana LR, Corbeil J, Wild E. 2003. Effect of micromechanical stimulations on osteoblasts: Development of a device simulating the mechanical situation at the bone-implant interface. J Biomech 36:131–135.
Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G, Lanyon LE. 1995. Mechanical strain-induced NO production by bone cells: A possible role in adaptive bone (re)modeling? FASEB J 9:1614–1622.
Rantakokko J, Uusitalo H, Jamsa T, Tuukkanen J, Aro HT, Vuorio E. 1999. Expression profiles of mRNAs for osteoblast and osteoclast proteins as indicators of bone loss in mouse immobilization osteopenia model. J Bone Miner Res 14:1934–1942.
Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. 1997. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 22:1082–1086.
Rubin J, Murphy T, Nanes MS, Fan X. 2000. Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells. Am J Physiol Cell Physiol 278:C1126–C1132.
Rubin C, Turner AS, Bain S, Mallinckrodt C, McLeod K. 2001a. Anabolism: Low mechanical signals strengthen long bones. Nature 412:603–604.
Rubin C, Xu G, Judex S. 2001b. The anabolic activity of bone tissue, suppressed by disuse, is normalized by brief exposure to extremely low-magnitude mechanical stimuli. FASEB J 15:2225–2229.
Rubin C, Turner AS, Muller R, Mittra E, McLeod K, Lin W, Qin YX. 2002. Quantity and quality of trabecular bone in the femur are enhanced by a strongly anabolic, noninvasive mechanical intervention. J Bone Miner Res 17:349–357.
Rubin J, Murphy TC, Zhu L, Roy E, Nanes MS, Fan X. 2003. Mechanical strain differentially regulates eNOS and RANKL expression via ERK1/2 MAP kinase. J Biol Chem 278:34018–34025.
Sakou T. 1998. Bone morphogenetic proteins: From basic studies to clinical approaches. Bone 22:591–603.
Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, Yasui N. 1999. Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. J Bone Miner Res 14: 1084–1095.
Satoyoshi M, Kawata A, Koizumi T, Inoue K, Itohara S, Teranaka T, Mikuni-Takagaki Y. 2001. Matrix metalloproteinase-2 in dentin matrix mineralization. J Endod 27:462–466.
Saunders MM, You J, Zhou Z, Li Z, Yellowley CE, Kunze EL, Jacobs CR, Donahue HJ. 2003. Fluid flow-induced prostaglandin E(2) response of osteoblastic ROS 17/2.8 cells is gap junction-mediated and independent of cytosolic calcium. Bone 32:350–356.
Sodek J, Chen J, Nagata T, Kasugai S, Todescan R, Jr., Li IW, Kim RH. 1995. Regulation of osteopontin expression in osteoblasts. Ann NY Acad Sci 760:223–241.
Tajima O, Ashizawa N, Ishii T, Amagai H, Mashimo T, Liu LJ, Saitoh S, Tokuyama K, Suzuki M. 2000. Interaction of the effects between vitamin D receptor polymorphism and exercise training on bone metabolism. J Appl Physiol 88:1271–1276.
Teitelbaum SL, Ross FP. 2003. Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649.
Turner RT. 2000. Invited review: What do we know about the effects of spaceflight on bone? J Appl Physiol 89:840–847.
van't Hof RJ, Armour KJ, Smith LM, Armour KE, Wei XQ, Liew FY, Ralston SH. 2000. Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. Proc Natl Acad Sci USA 97:7993–7998.
Watanuki M, Sakai A, Sakata T, Tsurukami H, Miwa M, Uchida Y, Watanabe K, Ikeda K, Nakamura T. 2002. Role of inducible nitric oxide synthase in skeletal adaptation to acute increases in mechanical loading. J Bone Miner Res 17:1015–1025.
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.
Wucherpfennig AL, Li YP, Stetler-Stevenson WG, Rosenberg AE, Stashenko P. 1994. Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. J Bone Miner Res 9:549–556.
You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR. 2001. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem 276: 13365–13371.