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Identification of Novel Bone-Specific Molecular Targets of Binge Alcohol and Ibandronate by Transcriptome Analysis

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Abstract

Background—Our laboratory established that binge alcohol-related bone damage is prevented by aminobisphosphonates, suggesting bone resorption increases following binge exposure. We examined the effects of binge alcohol and antiresorptive therapy on the relationship between bone damage and modulation of the vertebral transcriptome, in an attempt to determine how alcohol-induced bone damage and its prevention modulate bone-related biological pathways.

Methods—Male Sprague–Dawley rats were assigned to 1 of 6 treatment groups ($n = 12/\text{group}$). (C1) saline ip 3 d/wk for 1 week, (A1) binge alcohol, 3 g/kg, ip 3 d/wk for 1 week, (C4) saline ip, 3 d/wk for 4 weeks, (A4) binge alcohol, ip, 3 g/kg 3 d/wk for 4 weeks, (I4) ibandronate, saline ip 3 d/wk for 4 weeks, plus a single ip injection of ibandronate at 120 $\mu\text{g}/\text{animal}$, and (AI4) binge alcohol plus ibandronate as above. After 1 or 4 weeks, adjacent lumbar vertebrae were assayed for bone damage or transcriptional changes.

Results—Bone loss was not observed after 1 week of binge alcohol treatment. After 4 weeks, binge alcohol decreased vertebral BMD by 23% ($p < 0.05$) and compressive strength by 18% compared to saline controls ($p < 0.05$). Concurrent ibandronate prevented bone loss, increasing these parameters by 145 and 134% respectively compared to binge alcohol. ($p < 0.05$). Analysis of the vertebral transcriptome identified gene clusters specific for acute and chronic binge alcohol-related bone damage. Acute binge alcohol modulated the expression of integrin signaling-specific genes, while chronic binge alcohol modulated canonical Wnt signaling gene expression. Ibandronate normalized the expression of approximately 20% of the genes affected by chronic binge alcohol, allowing the identification of a unique subset of alcohol-sensitive, ibandronateresponsive genes.

Conclusions—Identification of bone-specific gene expression clusters associated with acute and chronic binge alcohol treatment allowed for the identification of cellular pathways affected by binge treatment with known involvement in bone remodeling (Integrin, Canonical Wnt signaling)

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1. Global Acute Binge Alcohol Differential Expression Gene List

Table S2. Global Chronic Binge Alcohol Differential Expression Gene List

Table S3. Targeted Acute Binge Alcohol Gene Cluster

Table S4. Targeted Chronic Binge Alcohol Gene Cluster

Table S5. Binge Alcohol/Ibandronate Co-Modulation Gene Cluster

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not previously identified as alcohol-sensitive. This data provides a basis for a plausible mechanistic explanation for the known detrimental effects of alcohol on bone formation and resorption.

Keywords

Binge Alcohol; Bone Damage; Transcriptome; Wnt Signaling; Integrin Signaling

Excessive alcohol consumption has a significant effect on morbidity and mortality in the United States each year (McGinnis and Foege, 1999). Although alcohol-related diseases are generally associated with individuals whose alcohol consumption levels are consistent with current definitions for alcoholism or alcohol abuse, there are growing numbers of individuals worldwide who engage in the practice of binge drinking. Although binge drinkers can consume large quantities of alcohol during binge episodes, these individuals are not generally classified as alcoholic or chronic alcohol abusers (Woerle et al., 2007). As such, the adverse health effects associated with excessive alcohol consumption are not limited to the alcohol dependent population, but appear to extend to a broader range of problem drinking practices including binge drinkers (Woerle et al., 2007). Binge alcohol drinking is defined as a pattern of drinking alcohol that brings blood alcohol levels (BAL) to 80 mg/dl or above. For the typical adult, this pattern corresponds to consuming 5 or more drinks (male) or 4 or more drinks (female) in about 2 hours (NIAAA-National Advisory Council). Binge drinking is prevalent among alcoholics; alcoholic binge drinkers can and do achieve extremely high BALs (Penland et al., 2001). However, binge alcohol consumption is not limited to this population. Social binge drinking of high alcohol content beverages by nonalcoholics is a commonly observed phenomenon in many cultures (Kuntsche et al., 2004; Makela et al., 2001) and in college age and older adults in the USA (Dawson et al., 2004; Miller et al., 2004), with multiple binge drinking episodes common in the young adult population (Grossberg et al., 2004).

Our laboratory has recently shown that a binge alcohol exposure model achieving high BALs produces significant decreases in bone mineral density and bone strength in adult male, female and ovariectomized rats (Callaci et al., 2004, 2006). Blood alcohol levels associated with binge alcohol treatment may be an important outcome determinant for the skeletal effects of patterned alcohol administration, as demonstrated by a previous binge alcohol study examining the effect of moderate BALs (43 to 106 mg/dl) on the rodent skeleton, which found unexpected beneficial effects on skeletal growth and BMD after a 2-d/wk alcohol binge, and no detrimental bone-related effects after a 5-d/wk alcohol binge (Sampson et al., 1999). The positive effects on bone observed by Sampson and co-workers may actually reflect the beneficial effects of moderate alcohol consumption on BMD and fracture risk previously documented (Felson et al., 1995).

Although alcohol abuse is a well-known risk factor for osteopenia in humans (Bilke et al., 1985), surprisingly little is known about the mechanisms responsible for this effect. Alcohol-related bone loss is likely caused by an imbalance in the bone remodeling cycle with effects in experimental animals observed on both bone formation (Farley et al., 1985; Friday and Howard, 1991; Wezeman et al., 1999) and bone resorption (Baran et al., 1980; Callaci et al., 2004; Farley et al., 1985; Preedy et al., 1991; Zhang et al., 2002). Recent evidence suggests that chronic alcohol-associated inhibition of bone formation in mice may be mediated by an alcohol-related increase in local TNF α production and activation of downstream TNF signaling (Perrien et al., 2003; Wahl et al., 2007). Our laboratory has shown that antiresorptive treatment with aminobisphosphonates can block binge alcohol-related bone loss, suggesting that enhanced bone resorption may occur in rodents exposed to multiple binge alcohol cycles (Callaci et al., 2004).

In the current investigation, we administered alcohol to young adult male rats in a binge-like pattern, producing BALs similar to those observed in binge drinkers. Rats exposed to longer-term binge alcohol were also treated with the antiresorptive bisphosphonate, ibandronate. Ibandronate targets bone tissue through its high affinity for hydroxyapatite and displays a long half-life (approximately 500 days) once bound to hydroxyapatite (Barrett et al., 2004). Ibandronate, like other nitrogen-containing bisphosphonates has potent antiresorptive activity mediated through inhibition of osteoclast activity and promotion of osteoclast apoptosis, thus reducing bone resorption and bone turnover rates (Barrett et al., 2004). We used gene expression array analysis to assess the effects of acute and chronic binge alcohol on the lumbar vertebral transcriptome, to gain an understanding of the relationship between alcohol-induced bone damage and modulation of bone-related cellular pathways. These experiments were performed to test the following hypotheses: 1. Bone damage caused by acute binge alcohol exposure can be identified and characterized in its early stages by unique bone-specific gene expression profiles prior to conventional bone damage assessment techniques. 2. Antiresorptive treatment will prevent chronic binge alcohol-mediated bone loss and also modulate bone-specific, alcohol-perturbed gene expression patterns and pathways. The results presented here show a connection between binge alcohol exposure and cellular pathways known to affect bone remodeling not previously identified as alcohol-sensitive.

MATERIALS AND METHODS

Binge Alcohol Model

This investigation received full approval from the Loyola University Institutional Animal Care and Use Committee (IACUC). Adult male Sprague Dawley rats were obtained at 16 weeks of age (375 to 399 g range), (Harlan, Indianapolis, IN) and acclimated to the laboratory environment for 1 week. Prior to treatment, animals were randomly assigned to one of the following treatment groups with an n of 12 animals per group, chosen based on a power analysis performed using preliminary gene array data generated in our laboratory (not shown). For animals receiving 1 week of treatment the groups were: saline control (C1), and binge alcohol-treated (A1). The one-week binge alcohol group will also be referred to in the text as acute binge alcohol-treated rats. For animals receiving 4 weeks of treatment the treatment groups were: saline control (C4), ibandronate-treated (I4), binge alcohol-treated (A4), and binge alcohol and ibandronate-treated (AI4). The 4-week binge alcohol group is also referred to in the text as chronic binge alcohol-treated rats. Animals were housed in pairs, with paired animals assigned to the same treatment group. Animals were weighed prior to the initiation of treatment and weekly throughout the study period. Animals were allowed free access to both food and water throughout the study. Ibandronate was administered as a single intraperitoneal (ip) dose of 120 μ g, 1 day prior to the start of saline or binge alcohol treatments (Bauss et al., 2002, 2004). Alcohol administration was by a single daily ip injection of a 20% (vol./vol.) ethanol/saline solution at a dose of 3 g/kg. This dose was chosen to achieve peak BALs of approximately 300 mg/dl (Nation et al., 1993). Control animals were given an ip injection of an equal volume of sterile isotonic saline at the time of alcohol group injections. Alcohol or saline injections were given starting at 9:00 AM, 3 d/wk. No ip injections were given during the remaining 4 days each week. Twenty-four hours after their last saline or alcohol injection, rats were rendered unconscious by CO₂ inhalation and killed by decapitation. Bone samples (lumbar vertebrae) were removed from each animal, dissected free of all soft tissue and either snap frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis, or wrapped in saline-soaked gauze and stored at -20°C for bone mineral density and biomechanical analysis.

Blood Ethanol Determination

Blood alcohol levels were determined by NAD⁺ reduction assay (Sigma Diagnostics, St. Louis MO.). Peak BAL was measured in 4 rats that were not included in any of the experimental protocol groups. These animals were killed by decapitation 60 minutes after a single ip injection of 20% (vol./vol.) ethanol/saline solution. The timing of peak BAL after ip injection was estimated based on a previous study that evaluated the pharmacokinetic profile of a 3 g/kg i.p. alcohol injection in rats (Nation et al., 1993).

Bone Mineral Density Measurements

Cancellous and cortical bone mineral density (BMD) of the fourth and fifth lumbar vertebrae were determined using quantitative computed tomography (QCT). Each vertebral segment was placed in a Norland Stratec bone densitometer (Orthometrix, Inc., White Plains, NY) and positioned uniformly on a support so that the instrument-scanning plane was perpendicular to the longitudinal axis of the vertebral body. Scout views were obtained to determine the midpoint of the vertebral body. Three measurements for each vertebra were performed at a resolution of 70 $\mu\text{m}/\text{voxel}$ 1 mm apart from this point. The cancellous area of the bone was defined as 45% of the total bone cross-sectional area at the midpoint of the proximal vertebral body, the remaining fraction cortical. The instrument was set to use the threshold contour mode (soft-tissue threshold set at 220 mg/cm^3) and a concentric peel algorithm (55% peel-off). Scans were made at 50 kV and 0.3 mA.

Biomechanical Structural Properties of Lumbar Vertebrae

Compressive strength tests were performed on the L4 and L5 vertebral bodies from each rat using an Instron materials testing machine (Model 5544, Canton, MA). The vertebral endplates were potted in bone cement using a previously described method that resulted in 2 parallel loading surfaces necessary to perform a uniform compression test on individual rodent vertebrae (Steinke et al., 1999). The specimens were prepared so that the posterior elements of the vertebra did not contact the loading platforms. Compression testing was performed at a crosshead speed of 0.5 mm/min to eliminate any strain rate effects. A 100 kg load-cell was used to monitor the compressive load and a precision sensor was used to measure the axial deformation of the specimen. The load-deformation data was analyzed to obtain the compressive strength of the vertebrae, defined as the maximum load sustained before failure.

Statistical Analysis of Bone Damage Parameters

Statistical analysis was performed using the SPSS software package (SPSS Inc. Chicago, IL). Analysis of statistical significance for BMD and vertebral strength data was performed by One-Way ANOVA and Tukey's HSD multiple comparison procedure. Significance was noted at $p \leq 0.05$. Multiple regression analyses were used to determine the relative contributions of body weight versus treatment condition on the effects observed in the dependent variables (BMD, strength) we studied. These analyses were performed in a 2-step hierarchical fashion. Information about treatment groups was carried by 3 dummy coded dichotomies to account for the 4 treatment conditions. All regression analyses were conducted in a similar fashion. First, the dependent variable was regressed upon post-treatment weight. Next, the treatment group dichotomies were added to the equation. The adjusted R^2 was measured in each step and the change in this statistic was tested for significance. The change in adjusted R^2 indicated the percent of variance that was explained by treatment group after accounting for the variability due to weight.

RNA Isolation

Prior to RNA isolation, the L3 lumbar vertebral body of each frozen sample was excised from the intact vertebral segment using a Dremel rotary saw. Samples were prepared for RNA extraction by first pulverizing each bone sample individually using a Freezer Mill (Spex CertiPrep, NJ). RNA was extracted from the powdered bone samples using the RiboPure RNA isolation kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. RNA samples were quantified by spectroscopy (NanoDrop Technologies, Wilmington DE) and visualized for purity and integrity using the Agilent 2100 Bioanalyzer (Santa Clara, CA) prior to gene array analysis.

Gene Expression Array Analysis

Twelve biological replicate samples per treatment group were utilized for analysis. Gene expression array analysis was performed using the Applied Biosystems 1700 Gene Expression Array System (Foster City, CA). The ABI rat genome survey microarray contains probes representing a complete, annotated, and manually curated set of approximately 27,000 rat genes from both the public and Celera databases. Analysis was performed according to the manufacturer's protocol using the Functional Genomics Core Laboratory at The University of Chicago, under the direction of Dr. Xinman Li. Briefly, Digoxigenin-UTP-labeled cRNA was generated and linearly amplified from 2 μ g of total RNA using the ABI chemiluminescent RT-IVT labeling kit. Arrays were prehybridized for 1 hour at 55°C in hybridization buffer with blocking reagent. Ten μ g of labeled cRNA was incubated in fragmentation buffer for 30 minutes at 60° and subsequently hybridized to each microarray in 0.5 ml hybridization buffer for 16 hours. Chemiluminescent detection and image acquisition were performed using the ABI chemiluminescence detection kit and 1700-microarray analyzer. Primary image analysis was performed using the ABI microarray analyzer software package.

Array Data Analysis

Data analysis was performed using the GeneSpring array analysis software package (GeneSpring GX 7.31, Agilent Corporation, Carmel, IN). Before analysis, a qualified data set was obtained by performing a bi-level quality control. First, at the replicate level, principal component analysis (PCA) and hierarchical cluster analysis were performed to identify outlier samples from within each biological replicate group that fell distal to cohort replicates comprising each treatment group. Secondly, filtering at the gene level was performed, removing those genes with signal to noise ratios (S/N) below 3.0 in 6/12 arrays per group. This quality analysis resulted in the removal of 9 total outlier arrays from the experiment and produced a qualified gene list of 12,655 genes (out of 27,000 total genes) used for all subsequent analyses. Statistical comparisons were performed using Wilcoxon-Mann-Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction. Gene signature pattern recognition was performed using Quality Threshold (QT) clustering algorithm with minimum similarity set to 0.95 and minimum cluster size set to 10 genes. Genes comprising each cluster were grouped into functionally significant clusters and biological pathways using the PANTHER (Protein Analysis THrough Evolutionary Relationships) Classification System (Thomas et al., 2003). All differentially expressed gene lists referred to in this study will be available for viewing online as supplemental data at the following address.

http://www.meddean.luc.edu/lumen/deptwebs/ortho/molecular_cellular_bone_lab.htm

Validation of Gene Array Data by Real-Time RT-PCR

Quantitative Real-Time RT-PCR was performed on a select subset of relevant genes chosen from genes showing significant differential expression by gene array analysis. A preliminary experiment was performed to select an appropriate endogenous control gene for RT-PCR analysis. Thirty-two isolated RNA samples (2 μ g) from the C4, A1, A4, and AI4 treatment groups ($n = 8$ /group) were first DNase-treated (Turbo DNA-free kit Ambion Inc., Austin, TX) and reverse transcribed into cDNA (High Capacity cDNA RT kit Applied Biosystems, Foster City, CA). Residual RNA was removed by RNase H treatment and samples were cleared of residual RNA fragments and dNTPs (MEGAclean-96 kit, Ambion Inc.). UV spectroscopy was performed to quantify cDNA samples (NanoDrop Technologies, Inc., Wilmington, DE). Thirty ng of each cDNA sample was run on 4 TaqMan Low Density Array Endogenous Control Panels on the Applied Biosystems 7900HT Gene Expression Array System, used to measure relative expression of 16 common endogenous control genes. Data analysis was performed using ABI's Sequence Detection Systems (SDS) software 2.1, and the gene showing the least deviation in Ct values across all samples (β -Tubulin, B2M) was selected as the endogenous control gene for subsequent RT-PCR validation experiments. Validation was performed ($n = 8$ /group) using the 7500 Fast Real-Time RT-PCR Gene Expression System (ABI). Each cDNA (20 ng) sample processed as above was amplified in triplicate using gene specific (β 3-integrin, Lrp5, Ctnnb1, Dkk1, OPG) and control (B2M) TaqMan Gene Expression Assay primer/probes sets. Data was analyzed with SDS software 1.4 using the $2^{-\Delta\Delta CT}$ relative quantification method (Livak and Schmittgen, 2001).

RESULTS

General Observations

All animals gained weight during the treatment period. After chronic (4-week) binge alcohol-treatment, rats showed an approximate 4% increase in body weight compared to their baseline weight, versus an 11% increase in their respective control groups (A4 vs. C4; AI4 vs. I4). This difference in post-treatment weight between control and chronic binge alcohol treated animals was significant ($p < 0.05$). Although not measured in this investigation, previous studies in our laboratory have shown no significant differences in weekly food intake between control and binge alcohol-treated rats (data not shown). A once daily alcohol-treatment protocol was used to avoid alcohol withdrawal symptoms that can occur when high doses of alcohol are administered twice daily (Penland et al., 2001). All alcohol-treated animals were monitored daily throughout the study period and no symptoms of alcohol withdrawal were observed during the 4-day period each week when alcohol was not administered. Rats exhibited a short (approximately 1 to 2 hour) period of acute alcohol intoxication immediately following each ip injection. Peak blood alcohol levels, measured 1 hour after ip alcohol injection in 4 alcohol naïve animals not included in subsequent experiments averaged 308 mg/dl, consistent with published reports of peak BAL in rats after a 3 g/kg ip alcohol administration (Nation et al., 1993). Necropsy performed on each animal revealed no apparent internal injuries from ip alcohol injections. Abdominal organs of alcohol-treated animals appeared normal by gross inspection.

Bone Damage Assessment by Bone Mineral Density and Compressive Strength Analysis

Prior to gene array analysis, alcohol-related bone damage was quantified by standard assays used previously in our laboratory. Consecutive lumbar vertebrae (L4–5) from each animal were analyzed for BMD and compressive strength-to-failure. An adjacent lumbar segment (L3) was utilized for the molecular studies described in the following section. An acute (1-week) binge alcohol treatment cycle did not significantly decrease vertebral cancellous or cortical BMD, or compressive strength compared to matched saline control values (Fig. 1A–

C). In contrast, a chronic (4-week) binge alcohol treatment cycle significantly decreased vertebral cancellous BMD ($p < 0.05$) and compressive strength values ($p < 0.05$) compared to matched saline control animals (Fig. 2A–C). Co-treatment of binge alcohol-exposed animals with ibandronate prevented this alcohol-related loss of cancellous BMD in lumbar vertebrae ($p < 0.05$) maintaining values at control levels (Fig. 2A). Ibandronate also prevented the significant decrease in compressive strength observed in binge alcohol-treated animals ($p < 0.05$) (Fig. 2C). The size of vertebral bodies as determined by cross-sectional area measurement obtained during pQCT was equivalent across treatment groups (data not shown), suggesting that the decreased BMD and vertebral compressive strength values observed after binge alcohol treatment were the result of altered mineral and biomechanical properties of the vertebrae and were not due to treatment-related differences in vertebral body area.

Because a significant effect of chronic binge alcohol treatment was observed on final post-treatment weight gain compared to control animals, we performed multiple regression analysis to determine the relative contributions of body weight versus treatment condition (i.e. binge alcohol) on the significant effects observed in the dependent variables we studied (BMD, strength). This analysis revealed that post-treatment weight accounted for only an adjusted 1% of the variance in vertebral trabecular BMD and was not statistically significant, $F(1,21) = 1.22$, $p = 0.282$. Treatment group however, accounted for an additional 39% of the variance and was significant, $F(3,18) = 5.50$, $p = 0.007$. Likewise, post-treatment weight accounted for none of the variance in vertebral compression strength and treatment group accounted for 25% of the variance observed $F(3,16) = 3.10$, $p = 0.05$.

Effects of Acute and Chronic Binge Alcohol Exposure on the Vertebral Transcriptome

After establishing the pattern and level of damage occurring in vertebral bone after acute and chronic binge alcohol treatment, we identified specific gene expression profiles associated with these treatments. We first identified global gene expression changes in L3 vertebrae after acute and chronic binge alcohol exposure. High biological replicate numbers in this experiment ($n = 12$ per group) enabled us to utilize a nonparametric statistical test, which decreased the effects of outlier data during analysis. Multiple testing correction reduced the overall false positive rate to 5%. As a first step in the analysis, we identified groups of genes that were differentially expressed ($p < 0.05$) in vertebral bone after both acute and chronic binge alcohol treatment compared to respective controls (C1 vs. A1 and C4 vs. A4). These gene lists were the basis for all subsequent bio-signatures identified in this study. We identified 1300 annotated differentially expressed genes in bone after an acute binge alcohol cycle and 1523 annotated genes after a chronic binge alcohol treatment cycle. The complete acute and chronic binge alcohol differentially expressed gene lists are shown in Tables S1 and S2 (Supplemental data) respectively. Each gene list was imported into the PANTHER classification system providing an overview of the biological effects of acute and chronic binge alcohol exposure on bone. PANTHER assigns genes to 1 of 139 regulatory and metabolic pathways. Treatment effects can result in under or over representation of genes in 1 or more PANTHER pathways, which may be classified as significant as compared to a reference gene list. Acute binge alcohol treatment significantly affected genes associated with 4 pathways including integrin, chemokine/cytokine, Ras, and PDGF signaling (Fig. 3). Depicted is a bar graph of each pathway identified with the amount of overrepresentation caused by alcohol, shown as a ratio of differentially expressed genes observed which sort into the pathway over the number of genes expected based on a reference list. Shown in Table 1 are genes differentially expressed either after acute or chronic binge alcohol, identified by PANTHER analysis as affiliated with the integrin signaling pathway.

Chronic binge alcohol exposure pathway-related effects are shown in Fig. 4. Significant effects were observed on genes affiliated with 5 pathways. Three pathways (integrin, chemokine/cytokine, and PDGF signaling) overlap with effects caused by acute binge alcohol. Two unique pathways (angiogenesis, and Wnt signaling) appear only in the chronic binge list. Differential gene expression patterns following chronic binge alcohol were strongly biased toward decreased expression. Of a total of 1523 differentially expressed genes, 1326 genes exhibited decreased expression and only 197 had increased expression levels as compared to matched control levels. PANTHER biological pathway assignment reflected this alcohol-mediated bias toward decreased expression; most chronic binge alcohol-affected genes assigned to the pathways described above displayed decreased relative expression levels. Shown in Table 2 are genes differentially expressed either after acute or chronic binge alcohol, identified by PANTHER analysis as affiliated with the Wnt signaling pathway.

Gene Clusters Associated With Acute and Chronic Binge Alcohol-Related Bone Damage

Quality Threshold (QT) clustering was used to identify a gene cluster from the acute binge alcohol exposure treatment group that would describe and identify the molecular changes occurring in bone before measurable bone damage had occurred. The criteria established for an early binge alcohol-related bone damage signature were: 1. The included genes were *only* differentially expressed in bone after acute binge alcohol treatment (before bone damage was observed). 2. The included genes were also not differentially expressed after chronic binge alcohol treatment. We identified a composite QT cluster pattern consistent with these criteria, comprised of 605 annotated genes (Table S3, Supplemental data). A subset of this signature containing 74 genes exhibiting 2-fold or greater differential expression is depicted as a heat map of control versus acute binge alcohol samples in Fig. 5.

Similarly, the list of genes differentially expressed only after a chronic binge alcohol cycle was used for identification of a gene group representing the later stages of binge alcohol-related bone loss. This cluster is comprised of only genes differentially expressed in bone after chronic binge alcohol treatment (concomitant with observed bone damage) that were not also differentially expressed after acute binge exposure. This signature includes 450 annotated genes (Table S4, Supplemental data). A subset of this cluster comprised of 114 genes exhibiting 2-fold or greater differential expression is depicted as a heat map of control versus chronic binge alcohol samples in Fig. 6.

Identification of a Binge Alcohol-Ibandronate Signature Profile

We next searched our expression dataset to determine if a gene cluster could be identified that documented whether ibandronate had a modulating effect on alcohol-related gene expression changes in bone in the co-treatment (AI) group. The criteria for the identification of this signature were as follows: 1. Genes were significantly differentially expressed after chronic (4-week) binge alcohol treatment. 2. The identical genes also exhibited modulation by ibandronate, such that their expression patterns were normalized (i.e. did not show significant differential expression compared to control) in the AI treatment group. QT clustering identified an expression profile matching these criteria. This cluster, comprised of 311 annotated genes (Table S5, Supplemental data) is a subset of the 1523 genes showing significant differential expression after chronic binge alcohol treatment described previously. Shown in Fig. 7, the signature profile depicted as a line graph is superimposed with a bar graph showing the effects of chronic binge alcohol on vertebral cancellous BMD from Fig. 2A. Each treatment group shown on the bar graph is aligned with a corresponding area of relative expression seen in the gene cluster to reinforce the concept of the dual ability of ibandronate to prevent alcohol-induced bone loss and modulate alcohol-sensitive gene expression in bone. PANTHER analysis of this signature revealed that 11 genes showing

both differential alcohol-related expression *and* normalization of expression with concurrent alcohol plus ibandronate treatment associate with the integrin-signaling pathway. In Table 3, we show the expression levels and *p* values for the 11 gene hits in the integrin pathway co-modulated by alcohol and ibandronate.

Validation of Gene Array Data by Real-Time RT-PCR

Real-Time quantitative RT-PCR (qRT-PCR) analysis was used to confirm the direction and magnitude of mRNA levels derived from array analysis for selected genes. A preliminary experiment detailed in *Methods*, identified an appropriate endogenous control gene, Beta-2 microglobulin, which displayed minimal variations in message levels between treatment groups. Genes were selected from 2 relevant groups, Wnt signaling, (Lrp5, β -catenin, Dkk1) and Integrin signaling, (Itgb3). One gene (OPG) modulated by ibandronate was also selected. The selected genes all showed significant differential expression after either acute or chronic binge alcohol treatment compared to matched control values by gene array. After performing each qRT-PCR reaction, expression data for each gene was calculated for the relevant treatment groups using the $2^{-\Delta\Delta CT}$ relative method. The RNA level data for each gene produced by both gene array and PCR analysis was plotted on a shared axis as a line graph. As seen in Fig. 8, there was excellent overall correlation between gene array and qRT-PCR with respect to the direction and magnitude of gene expression across treatment groups for each selected gene.

DISCUSSION

This study utilized a young adult rat model of binge alcohol exposure, which mimics the pattern and consumption levels characteristic of human binge drinking behavior and produces reproducible patterns of bone loss in male and female rats. We chose lumbar spine as the anatomical site to perform our studies based on clinical reports of the high frequency of vertebral compression fractures in osteoporosis patients (Genant and Jergas, 2003) coupled with the relative paucity of reports focused on alcohol and the spine (Callaci et al., 2006, Nishiguchi et al., 2001) compared to other skeletal sites such as the femur and tibia (Sampson et al., 1996, 1997, Sampson et al. 1998, Hogan et al., 1997, Wezeman et al., 1999, 2000). We report novel effects of alcohol on vertebral bone identified by relating the damage caused by binge alcohol to changes occurring in the vertebral transcriptome. Effects of a single dose of ibandronate on alcohol-related bone damage and associated transcriptional modulation are also reported. Intermittent ibandronate dosing regimens in rats are effective for prevention of estrogen-dependent bone loss and in maintaining or improving bone mass, strength, and architecture (Bauss et al., 2002, 2004).

The current study shows a clear association between the duration of binge alcohol exposure and the appearance of measurable bone damage. The effects of acute and chronic binge alcohol exposure on vertebral BMD and compressive strength reported in this study agree with previously published reports from our laboratory (Callaci et al., 2004, 2006), as well as with effects of chronic continuous alcohol exposure observed in previous investigations (Nishiguchi et al., 2000). As expected based on previous data, ibandronate administration prevented significant reductions in vertebral BMD and compressive strength in animals pretreated with a single ip injection of ibandronate prior to starting the binge alcohol treatment regimen. Ibandronate also increased cancellous BMD in control animals not receiving alcohol injections as previously demonstrated (Wezeman et al., 2007) through its inhibition of normal osteoclast-mediated bone resorption (Rogers, 2003). Multiple regression analysis performed to determine relative contributions of body weight versus treatment on measured parameters found no significant contributions of the change in body weight on BMD or compressive strength, confirming that the changes we observed in these parameters following chronic binge alcohol treatment were the result of direct (or indirect)

effects of alcohol or its metabolites on bone. These analyses (BMD, compressive strength) were performed to provide a basis for the identification of biosignatures present in bone prior to measurable alcohol-induced bone damage, concurrent with alcohol damage and in bone protected from alcohol damage by bisphosphonate treatment.

Gene array analysis was a tool utilized to gain an understanding of the biology behind the effects of acute and chronic binge alcohol exposure on vertebral BMD and compressive strength. To facilitate the analysis of data sets obtained by gene array PANTHER software was employed, allowing the sorting of differentially expressed gene lists into functional groups based on associations with known biological pathways. Several cellular signaling pathways were identified by this analysis as modulated by acute and/or chronic binge alcohol treatment in bone. Because of the complexity of potential alcohol-related modulation of signaling and its effects on bone metabolism we focus here on 2 of the alcohol-modulated pathways identified, integrin and Wnt signaling. These pathways have well-established connections to bone remodeling, but have not previously been identified as modulated by alcohol. The potential regulation of these 2 pathways in bone by alcohol exposure may provide novel mechanistic explanations for the observed effects of alcohol on the bone remodeling cycle.

Although no significant effects of acute binge alcohol on vertebral BMD or strength were observed, our results suggest that changes are occurring in bone at the molecular level in response to a 3-day alcohol binge. Approximately 1300 genes (over 10% of the genes passing quality control parameters) exhibited significant differential expression in the acute binge group (C1 vs. A1). Of the pathways affected by acute binge alcohol, the effects observed on the integrin-signaling pathway are discussed here based on the connection between integrin signaling and bone resorption. Integrins are transmembrane heterodimeric proteins consisting of α and β subunits that function by attaching cells to the extracellular matrix and transmitting matrix-derived signals to the interior of the cell. The $\alpha v \beta 3$ integrin is the principal integrin expressed by osteoclasts (McHugh et al., 2000). We found that acute alcohol exposure modulated steady-state levels of a significant number of genes involved in integrin signaling, including a significant increase in mRNA levels of $\beta 3$ integrin (Itgb3). Given the promiscuous nature of integrin heterodimer formation (Teitelbaum, 2005) an alcohol-related increase in $\beta 3$ mRNA levels could increase the formation of the $\alpha v \beta 3$ heterodimer in osteoclasts, enhancing both $\alpha v \beta 3$ -mediated matrix attachment and intracellular signaling, leading to the organization of the osteoclast cytoskeleton and formation of the protected resorptive microenvironment required for bone resorption. The significant decreases in $\beta 3$ and other integrin signaling-related genes we observed after chronic binge alcohol treatment suggests that a temporal profile of alcohol-related effects on integrin gene expression may exist in bone tissue. The finding that $\beta 3$ integrin expression and other key genes in the integrin signaling pathway are potential bone-specific targets of alcohol may represent a novel mechanism underlying an alcohol-mediated modulation of bone resorption.

Chronic binge alcohol-related differential expression also had significant effects on cellular pathways (Fig. 4). Canonical Wnt signaling is notable within this group because of its involvement in bone formation, recently uncovered based on the discovery that both loss-of-function and gain-of-function mutations occurring in the low-density lipoprotein receptor (LDLR)-related protein 5 (Lrp5), a Wnt co-receptor, are responsible for 2 rare hereditary conditions in humans; Osteoporosis pseudo-glioma syndrome (OPPG) a Lrp5 loss-of-function mutation resulting in early onset osteoporosis (Gong et al., 2001) and the high bone mass (HBM) phenotype Lrp5 gain-of function mutation resulting in hereditary increased bone mass (Boyden et al., 2002). We found that Lrp5 expression was significantly decreased in bone after binge alcohol exposure, as were the expression of several other Wnt-related

genes. Other canonical Wnt pathway-related genes of note exhibiting decreased expression following chronic binge alcohol treatment include β -catenin (ctnnb1), and Dickkopf-1 (Dkk1). β -catenin, referred to as the molecular node of the canonical Wnt pathway (Glass and Karsenty, 2006) plays a pivotal role in Wnt signal transduction to the nucleus and transcription of canonical Wnt-regulated genes. The significant decrease observed in the expression of Dkk1, a Wnt inhibitor (Glass and Karsenty, 2006) does not fit the overall pattern observed of depression of canonical Wnt signaling by alcohol, but could possibly represent a feedback mechanism activated in bone to increase canonical Wnt signaling activity in response to the repressive effects of alcohol on Lrp5 and β -catenin, similar to the feedback loop observed recently in Lrp6/ β -catenin signaling (Khan et al., 2007). Disruption of bone-related Wnt signaling could represent a novel mechanism of action responsible for alcohol-related depression of bone formation and resulting osteopenia observed in rodent chronic alcohol exposure models (Wezeman et al., 1999). The connection between alcohol-related bone damage and modulation of the canonical Wnt signaling pathway identified in this study is important because it provides a candidate molecular mechanism responsible for alcohol's suppressive effect on bone formation, long believed to be an underlying cause of alcohol-related osteopenia (Farley et al., 1985).

Another alcohol-related effect on bone formation potentially explained by a deactivation of canonical Wnt signaling includes effects on differentiation of mesenchymal progenitor cells. Signaling through Wnt/ β -catenin appears to be required for the determination of mesenchymal progenitor cells toward an osteoblast or chondrocyte lineage (Hill et al., 2005). Canonical Wnt pathway activation also inhibits adipocyte differentiation from mesenchymal progenitors, further promoting the osteoblast lineage (Bennett et al., 2005). Thus, an alcohol-related inhibition of Wnt/ β -catenin signaling could also provided a mechanistic explanation for the shift of bone marrow-derived mesenchymal stem cells (MSC) from an osteoblastic to an adipogenic lineage, recently observed in alcohol-exposed primary human and mouse MSC cultures (Cui et al., 2006, Wezeman and Gong, 2004) and for the in vivo development of alcohol-induced fatty bone marrow (Wang et al., 2003).

Quality Threshold (QT) clustering, which allowed the visualization of all existing patterns of gene expression occurring in the acute binge alcohol differentially expressed gene list was used for signature identification. A gene cluster which provided insight into the protective effects of antiresorptive treatment on alcohol-related bone loss was identified from the alcohol plus ibandronate (AI) treatment group. This cluster is comprised of genes showing both significant differential expression in the chronic binge alcohol group *and* a significant normalization of expression levels (to control levels) in the AI group. This gene expression profile parallels the pattern of bone loss and prevention of loss in the AI group shown by BMD and compressive strength analysis. The observation that ibandronate mediates both the prevention of alcohol-related bone loss and the normalization of a subset of alcohol-perturbed gene expression profiles in bone is unique and has not been previously reported. This observation is supported by a previous unrelated study which showed normalization of disease-related changes in gene expression related to therapeutic intervention in an examination of cytokine transcript levels associated with mucosal inflammatory tissue from untreated versus treated Crohn's disease patients (Stallmach et al., 2004). Taken together these observations suggest that modulation of aberrant gene expression levels associated with disease processes may be an overlooked mechanism of action for some therapeutic agents. The normalization of some integrin signaling genes by ibandronate shown in Table 3 suggests that correction of alcohol-perturbed integrin signaling gene expression by ibandronate may be a key factor in its ability to prevent alcohol-related bone loss. The presence of an alcohol-sensitive, ibandronate-responsive gene cluster comprised of many genes without apparent connections to bone remodeling suggests that ibandronate may

prevent bone loss by novel mechanisms of action in addition to the inhibition of osteoclast-mediated resorption.

In summary, the data presented in this investigation suggests that alcohol-related bone damage may be regulated, at least in part, by targeted effects on both integrin and canonical Wnt signaling pathways. These observations may provide both the basis for mechanistic explanations of the effects of alcohol on the bone remodeling cycle, and novel molecular targets for therapeutic intervention of alcohol-related osteopenia.

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Fig. 1.

Acute binge alcohol effects on vertebral BMD and compressive strength. (A) Cancellous BMD. (B) Cortical BMD. (C) Compressive strength. Treatment groups: C1, control; A1, binge alcohol. Significance noted at $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure.

Fig. 2.

Chronic binge alcohol effects on vertebral BMD and compressive strength. (A) Cancellous BMD. (B) Cortical BMD. (C) Compressive strength. Treatment groups: C4, control; A4, binge alcohol; I4, ibandronate; AI4, binge alcohol plus ibandronate. Significance noted at $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. * $p \leq 0.05$ C4 vs. A4, ** $p \leq 0.05$ A4 vs. AI4.

Fig. 3.

Classification of acute binge alcohol effects on vertebral bone results of PANTHER analysis performed as described previously. Depicted is a bar graph of each pathway identified. The amount of overrepresentation in each pathway related to acute binge alcohol treatment is depicted as a ratio of the differentially expressed genes observed in the pathway over the number of genes expected based on a reference list. Acute binge alcohol treatment significantly affected 4 pathways including integrin, chemokine/cytokine, Ras, and PDGF signaling.

Chronic Binge Alcohol Pathway Effects

Fig. 4.

Classification of chronic binge alcohol effects on vertebral bone results of PANTHER analysis performed as described previously. Depicted is a bar graph of each pathway identified. The amount of overrepresentation in each pathway related to chronic binge alcohol treatment is depicted as a ratio of the differentially expressed genes observed in the pathway over the number of genes expected based on a reference list. Significant effects were observed on 5 pathways, integrin, chemokine/cytokine, PDGF, angiogenesis, and Wnt signaling.

Fig. 5.

Heat map for acute binge alcohol differentially expressed genes expression profiles for genes changed in vertebral bone *only* between control and acute binge alcohol-treated rats were identified by QT clustering as described in *Methods*. Genes displayed in the heat map above were also identified as significantly differentially expressed ($p < 0.05$ and 2-fold changed). Shown are the expression patterns of the 74 genes matching these criteria in 12 control and 10 acute binge alcohol-treated animals. Statistical comparisons were performed using Wilcoxon–Mann–Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction.

Fig. 6.

Heat map for chronic binge alcohol differentially expressed genes expression profiles for genes changed in vertebral bone *only* between control and chronic binge alcohol-treated rats were identified by QT clustering as described in *Methods*. Genes displayed in the heat map above were also identified as significantly differentially expressed ($p < 0.05$ and 2-fold changed). Shown are the expression patterns of the 114 genes matching these criteria in 12 control and 8 chronic binge alcohol-treated animals. Statistical comparisons were performed using Wilcoxon–Mann–Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction.

Fig. 7.

Alcohol-ibandronate gene cluster expression profiles for genes differentially expressed ($p < 0.05$) in vertebral bone between control and chronic binge alcohol treatment that were also not differentially expressed between control and combined binge alcohol-ibandronate treatment were identified by QT clustering as described in *Methods*. These genes represent an ibandronate-responsive subset of chronic binge alcohol genes shown in Table 2. This cluster is depicted as a line graph of the expression profiles of 311 genes identified aligned with a bar graph of the effects of binge alcohol and ibandronate on vertebral BMD to reinforce the concept of the dual ability of ibandronate to prevent alcohol-induced bone loss and modulate alcohol-sensitive gene expression. * $p < 0.05$, Saline vs. Alcohol, ** $p < 0.001$ Alcohol vs. Combined treatment.

Fig. 8. Quantitative RT-PCR validation of micro array gene expression data. Gene expression data obtained by micro array analysis was validated by qRT-PCR for a select subset of relevant genes. Genes validated were: (A) β -catenin (Ctnnb1), (B) Lrp5, (C) Dkk1, (D) β 3 integrin (Itgb3), (E) OPG. Expression values are shown as relative expression for relevant treatment relative to control values.

Table 1

Integrin Signaling Gene List

Gene symbol	Gene name	Acute alcohol		Chronic alcohol	
		% Control	p-value	% Control	p-value
Integrin signaling genes					
Actr1a	ARPI actin-related protein 1 A	296.2	0.043	105.2	0.919
Arf3	ADP-ribosylation factor 3	306.0	0.014	116.3	0.682
Ar15	ADP-ribosylation factor 5	79.1	0.020	97.8	0.951
Col11a2	Procollagen, type XI, alpha 2	38.8	0.010	39.4	0.008
Col16a1	Procollagen, type XVI, alpha 1	73.7	0.022	56.9	0.008
Col18a1	Collagen, type XVIII, alpha 1	68.2	0.027	65.5	0.029
Col1a2	Pro-alpha-2(I) collagen	64.1	0.022	66.4	0.015
Col2a1	Procollagen, type II, alpha 1	39.2	0.017	46.3	0.029
Col4a1	Procollagen, type IV, alpha 1	47.4	0.010	53.7	0.013
Col14a2	Procollagen, type IV, alpha 2	48.7	0.010	53.6	0.008
Col14a4	Procollagen, type IV, alpha 4	52.9	0.010	87.7	0.691
Col5a1	Collagen, type V, alpha 1	41.9	0.010	36.9	0.008
Col15a2	Collagen, type V, alpha 2	65.8	0.027	48.3	0.008
Col16a2	Procollagen, type VI, alpha 2	65.9	0.027	61.9	0.010
Col9a1	Procollagen, type IX, alpha 1	40.9	0.034	56.3	0.136
Col19a3	Procollagen, type IX, alpha 3	53.3	0.043	57.0	0.217
Crkas	V-crk-associated tyrosine kinase sub.	82.8	0.043	65.9	0.029
Egfl5	EFF-like-domain, multiple 5	136.9	0.030	86.9	0.845
Elmo1	Engulfment and cell motility 1	81.0	0.049	90.1	0.533
Elmo2	Engulfment and cell motility 2	555.0	0.015	132.1	0.323
Flnb	Filamin, beta	66.2	0.011	65.7	0.040
Fn1	Fibronectin	67.7	0.038	76.0	0.153
Hras	Harvey rat sarcoma viral (v-Ha-ras)	79.9	0.012	89.7	0.241
Itga6	Integrin, alpha 6	75.1	0.024	96.8	0.845
Igfb3	Integrin beta3 subunit	369.1	0.016	36.4	0.012
Igfb7	Integrin, beta 7	71.3	0.014	104.9	0.613
Lama5	Laminin, alpha 5	74.5	0.038	37.3	0.008

Gene symbol	Gene name	Acute alcohol		Chronic alcohol	
		% Control	p-value	% Control	p-value
Lamb2	Laminin, beta 2	65.7	0.010	46.6	0.008
Lck	Lymphocyte protein tyrosine kinase	136.7	0.024	96.3	0.729
Map2k1	Mitogen-activated protein kinase 1	117.6	0.049	93.9	0.267
Map3k1	MAP kinase kinase 1	76.4	0.049	62.0	0.011
Mapk1	Mitogen-activated protein kinase 1	191.5	0.012	63.9	0.093
Mapk9	Mitogen-activated protein kinase 9	177.2	0.038	53.9	0.076
Plk3cd	Phosphatidylinositol 3-kinase CDP	72.8	0.022	80.9	0.353
Pxn	Paxilin	99.5	0.034	103.2	0.533
Raf1	Murine leukemia viral oncogene	114.3	0.038	64.8	0.046
Rap2b	Ras oncogene family member	149.1	0.030	60.3	0.013
Rhoc	Ras homolog gene family, member C	77.2	0.043	70.8	0.008
Rhod	Ras homolog gene family, member D	75.4	0.038	100.2	0.951
Sos1	Son of sevenless homolog 1	126.2	0.034	87.4	0.172
Tgfb1i1	Transforming growth factor beta 1	124.5	0.038	66.5	0.018

The table represents a list of 41 genes identified as affiliated with the integrin signaling pathway by the PANTHER classification system that were also identified as differentially expressed ($p < 0.05$) in vertebral bone between control and either acute or chronic binge alcohol-treated rats. Gene symbols discussed in text are shaded. Significant p values are shaded. Statistical comparisons were performed using Wilcoxon–Mann–Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction. Significant differential expression is noted in shaded boxes.

Table 2

Wnt Signaling Gene List

Gene symbol	Gene name	Acute alcohol		Chronic alcohol	
		% Control	p-value	% Control	p-value
Wnt signaling genes					
Acrvl	Activin type I receptor	79.0	0.510	45.8	0.025
Arid 1 a	AT rich interactive domain 1A	100.3	0.659	74.2	0.011
Catn1	Catenin alpha-like 1	95.2	0.921	52.9	0.029
Ccnd1	Cyclin D1	95.2	0.548	42.3	0.008
Ccnd2	Cyclin D2	52.9	0.010	68.2	0.404
Ccnd3	Cyclin D3	81.4	0.010	84.7	0.113
Cdh13	Cadherin 13	58.4	0.020	36.2	0.008
Cdh15	Cadherin 15	51.6	0.010	40.9	0.009
Celsr3	Cadherin EGF LAG seven-pass G-type receptor 3	82.7	0.341	64.3	0.046
Chp	Calcium binding protein p22	104.8	0.548	131.8	0.046
Chp2	Calcineurin homologous protein isoform 2	102.0	0.811	32.4	0.008
Crebbp	CREB-binding protein	111.7	0.348	63.8	0.040
Csnk2a1	Casein kinase II, alpha 1 polypeptide	151.4	0.105	64.3	0.029
Ctnnb1	Beta-catenin	92.1	0.267	33.5	0.008
Daam1	Dishevelled associated activator of morphogenesis 1	94.6	0.527	74.0	0.007
Dkk1	Dickkopf homolog 1	80.2	0.112	54.5	0.007
Dkk3	Dickkopf homolog 3	105.4	0.451	74.6	0.020
Fat	Protocadherin	70.6	0.017	51.9	0.008
Fbxw1 1	F-box and WD-40 domain protein 1 1	88.5	0.622	73.8	0.046
Fzd1	Polarity gene (frizzled)	76.9	0.055	69.6	0.021
Fzd7	Frizzled homolog 7	57.2	0.110	50.0	0.029
Gna11	Guanine nucleotide binding protein, alpha 11	80.7	0.014	76.1	0.009
Gng10	Guanine nucleotide binding protein, gamma 10	96.7	0.585	126.8	0.034
Hoxc6	Homeo box C6	100.3	1.000	63.1	0.025
Itp1	Inositol 1,4,5-triphosphate receptor 1	90.4	0.348	67.1	0.009
Itp2	Inositol 1,4,5-triphosphate receptor 2	101.0	0.921	61.7	0.029
Itp3	Inositol trisphosphate receptor subtype 3	79.8	0.105	60.9	0.018

Gene symbol	Gene name	Acute alcohol		Chronic alcohol	
		% Control	p-value	% Control	p-value
Jun	V-jun sarcoma virus 17 oncogene	95.7	0.527	53.0	0.007
Lrp5	Low density lipoprotein receptor-related protein 5	54.2	0.010	60.5	0.098
Madh1	MAD homolog 1	77.8	0.030	76.4	0.008
Myc	Myelocytomatosis viral oncogene homolog	78.9	0.039	97.5	0.909
Myh1	Type 2X myosin heavy chain	67.1	0.242	21.6	0.008
Myh1	Type 2X myosin heavy chain	68.8	0.162	24.3	0.008
Myh4	Myosin, heavy polypeptide 4, skeletal muscle	130.5	0.409	31.2	0.008
Myh7	Beta cardiac myosin heavy chain	50.3	0.179	11.3	0.008
Nfatc3	Nuclear factor of activated T-cells, cytoplasmic, cCD3	77.0	0.095	72.3	0.015
Nkd2	Naked cuticle 2	68.1	0.023	89.6	0.433
Pcdh16	Protocadherin 16 dachshous-like	80.0	0.043	64.2	0.008
Pcdh18	Protocadherin 18	88.4	0.510	71.2	0.029
Plcb3	Phospholipase C, beta 3	62.2	0.162	46.2	0.009
Ppp2r5b	Protein phosphatase 2, regulatory subunit B (B56), β	58.9	0.095	57.5	0.029
Ppp3cb	Protein phosphatase 3, catalytic subunit, beta isoform	98.4	0.698	54.5	0.034
Ppp3r1	Protein phosphatase 3, regulatory subunit B, alpha 1	176.3	0.038	42.6	0.008
Prkca	Protein kinase C, alpha	95.6	0.849	52.0	0.008
PRKCQ	Protein kinase C, theta	87.0	0.242	63.2	0.010
Sfrp2	Secreted frizzled-related protein 2	86.4	0.162	68.5	0.009
Smarca2	Smarc, subfamily a2	94.1	0.736	45.1	0.011
Smarca1	Smarc, subfamily ad1	120.5	0.955	113.4	0.046
Smarcb1	Smarc, subfamily b, member 1	102.7	0.811	50.4	0.008
Smarcc1	Smarc, subfamily c, member 1	80.2	0.022	78.8	0.040
Smarcd1	Smarc, subfamily d, member 1	52.2	0.038	59.1	0.021
Smarcd3	Smarc, subfamily d, member 3	80.9	0.319	29.8	0.010
Smefl	Transcription factor 1	80.3	0.105	48.3	0.009
Tef4	Transcription factor 4	103.7	0.591	70.2	0.007
Tefe2a	Transcription factor E2a	300.0	0.010	95.2	0.826
Tle2	Transducin-like enhancer of split 2	87.4	0.232	54.5	0.020
Tle2	Transducin-like enhancer of split 2	87.4	0.199	54.5	0.021

Gene symbol	Gene name	Acute alcohol		Chronic alcohol	
		% Control	p-value	% Control	p-value
Wisp1	WNT1 inducible signaling pathway protein 1	80.1	0.314	49.8	0.007
Wnt4	Wingless-type MMTV integration site family, member 4	85.9	0.442	70.1	0.025
Wnt8a	Wingless-related MMTV integration site 8A	155.4	0.170	218.7	0.048

The table represents a list of 60 genes identified as affiliated with the Wnt signaling pathway by the PANTHER classification system that were also identified as differentially expressed ($p < 0.05$) in vertebral bone between control and either acute or chronic binge alcohol-treated rats. Gene symbols discussed in text are shaded. Significant p values are shaded. Statistical comparisons were performed using Wilcoxon–Mann–Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction.

Table 3

Integrin Signaling Genes Co-Modulated by Binge Alcohol and Ibandronate

Gene symbol	Common name	Chronic alcohol		Alcohol + iban.	
		% Control	p-value	% Control	p-value
Alcohol-ibandronate cluster: integrin signaling genes					
Abl1	Abelson murine leukemia viral oncogene 1	82.0	0.007	94.4	0.787
Actn2	Actinin alpha2	24.4	0.002	47.2	0.116
Arpc2	Actin related protein 2/3 complex, subunit 2	126.9	0.008	105.4	0.899
Cav	Caveolin	13.2	0.002	48.7	0.062
Coll5a1	Procollagen, type XV	55.6	0.002	74.9	0.070
Itgb6	Integrin, beta 6	46.7	0.018	73.3	0.396
Lims2	LIM and senescent cell antigen domain 2	44.1	0.008	88.2	0.612
Mapk1	Mitogen-activating protein kinase 1	63.9	0.025	104.9	0.812
Mapk9	Mitogen-activating protein kinase 9	53.9	0.020	86.8	0.455
Ptpn12	Protein tyrosine phosphatase	48.9	0.016	118.0	0.710
Rho	Rho family GTPase2	60.9	0.014	81.5	0.863

The table represents a list of 11 genes associated with the integrin signaling pathway differentially expressed ($p < 0.05$) in bone after chronic binge alcohol treatment, while remaining at control levels of expression in the presence of alcohol and ibandronate. Statistical comparisons were performed using Wilcoxon-Mann-Whitney nonparametric analysis with Benjamini and Hochberg False Discovery Rate multiple testing correction. Significant p -values are shaded.