

Alcohol induced epigenetic perturbations during the inflammatory stage of fracture healing

H Wayne Sampson^{1,2,3}, Christopher D Chaput^{2,3}, Jason Brannen², Robert A Probe^{2,3}, Rakeshwar S Guleria⁴, Jing Pan⁴, Kenneth M Baker⁴ and Vincent VanBuren¹

¹Department of Systems Biology and Translational Medicine, Texas A&M Health Science Center, College of Medicine, 702 SW HK Dodgen Loop; ²Department of Orthopedic Surgery, Scott & White Clinic, Temple, TX 76504; ³Center for Bone, Joint and Spine Research, Scott & White Hospital, Temple, TX 76508; ⁴Division of Molecular Cardiology, Texas A&M Health Science Center, College of Medicine, Temple, TX 76504, USA

Corresponding author: H Wayne Sampson. Email: sampson@medicine.tamhsc.edu

Abstract

It is well recognized by orthopedic surgeons that fractures of alcoholics are more difficult to heal successfully and have a higher incidence of non-union, but the mechanism of alcohol's effect on fracture healing is unknown. In order to give direction for the study of the effects of alcohol on fracture healing, we propose to identify gene expression and microRNA changes during the early stages of fracture healing that might be attributable to alcohol consumption. As the inflammatory stage appears to be the most critical for successful fracture healing, this paper focuses on the events at day three following fracture or the stage of inflammation. Sprague–Dawley rats were placed on an ethanol-containing or pair-fed Lieber and DeCarli diet for four weeks prior to surgical fracture. Following insertion of a medullary pin, a closed mid-diaphyseal fracture was induced using a Bonnarens and Einhorn fracture device. At three days' post-fracture, the region of the fracture calluses was harvested from the right hind-limb. RNA was extracted and microarray analysis was conducted against the entire rat genome. There were 35 genes that demonstrated significant increased expression due to alcohol consumption and 20 that decreased due to alcohol. In addition, the expression of 20 microRNAs was increased and six decreased. In summary, while it is recognized that mRNA levels may or may not represent protein levels successfully produced by the cell, these studies reveal changes in gene expression that support the hypothesis that alcohol consumption affects events involved with inflammation. MicroRNAs are known to modulate mRNA and these findings were consistent with much of what was seen with mRNA microarray analysis, especially the involvement of *smad4* which was demonstrated by mRNA microarray, microRNA and polymerase chain reaction.

Keywords: alcohol, microRNA, microarray, fracture healing, inflammation

Experimental Biology and Medicine 2011; **236**: 1389–1401. DOI: 10.1258/ebm.2011.011207

Introduction

Alcoholics have increased numbers of orthopedic trauma resulting in fractures that frequently lead to retarded or non-union. Alcohol consumption has long been known to be associated with increased traumatic and non-traumatic bone fracture, to the extent that multiple rib fractures were once diagnostic of alcoholism.^{1–4} It has been shown that the relative risk of some fracture types is increased in alcoholism and this association is so strong that the fracture itself can become a marker for alcoholism.^{5,6} The high frequency of fractures is also in part due to the fact that alcohol abusers have a lower bone mass.^{7,8} Bone samples from alcoholic patients have a low trabecular bone

volume, thinner trabeculae and increased trabecular number due to trabecular perforation.^{9,10} It also reduces the density and strength of cortical bone, which it attempts to compensate for by increasing the cross-sectional area.¹¹ It has also been recognized by orthopedic surgeons^{12,13} that fractures of alcoholics are more difficult to heal successfully and have a higher incidence of non-union.^{14,15} Early suggestions have attributed this increased morbidity to a suppressed immune defense, an inhibited wound healing, general muscle atrophy or a defective osteoblastic function induced by ethanol as reviewed by Tonnesen *et al.*¹² Sears *et al.*¹⁶ have recently demonstrated suppressed serum markers of inflammation in alcohol-fed animals in response to injury.

In a prior study of the effects of alcohol on actively growing rat bone, the amount and rate of growth of the epiphyseal growth plate was dramatically reduced in alcohol-fed animals.¹⁷ The height of the last hypertrophic lacuna decreased with age from 32 μm at week 4 of the project to 26 μm at week 8, and the rate of cell proliferation per column per day was also dramatically decreased in alcohol-fed animals. In light of the fact that callus formation during fracture healing undergoes a process similar to endochondral bone formation,¹⁸ our central hypothesis is that alcohol consumption will adversely affect fracture healing through a perturbation of the normal cytokines and growth factors needed for normal fracture healing.

Normal bone healing requires coordination among many different cell types and regulatory proteins. Bone morphogenetic proteins (BMPs) are the best understood, but other factors such as PDGF (platelet-derived growth factor), IGF-1 (insulin-like growth factor 1), TGF- β (transforming growth factor β), LMP-1 (latent membrane protein 1) and bFGF (basic fibroblast growth factor) have also been documented in bone healing.^{19–21} The first event after fracture is bleeding from the damaged bone ends.²² The accumulated blood forms a clot that fills the space between the fracture surfaces. Then an acute inflammatory response begins, and inflammatory cells invade the soft tissues surrounding the fracture site, which initiate a full scale preparation for bone repair.^{19,22} TGF- β , PDGF and BMP-2/4 have been identified in the first stage of bone healing, most likely released from platelets, local osteocytes and matrix. This early stage of bone healing involves complex cellular processes including cell migration, invasion, adhesion and proliferation,²² which requires methods of study that allow for the simultaneous study of many events. TGF- α 1 expression is high during chondrogenesis and endochondral ossification, but low during intramembranous ossification.²¹ BMPs induce differentiation of mesenchymal osteoprogenitor cells, the most effective being BMP-2, -6 and -9,²³ PDGF acts early in the process by stimulating migration and proliferation of fibroblasts, and TGF- β is a stimulator of collagen production, angiogenesis²⁴ and osteoblast recruitment. Rundle *et al.*²⁵ demonstrated 6555 genes with significant changes during days 3 and 11 of normal fracture healing, with specific and unique patterns of expression during each of the first two phases covered by their experiment. However, their model did not address impaired healing, such as alcohol treatment. Callaci *et al.*²⁶ and Himes *et al.*²⁷ have associated alcohol-induced bone changes with transcriptome analysis but did not study fracture healing. At present, little is known about the altered microenvironment of fractures due to alcohol consumption. The present study is concerned with transcriptome changes during the inflammatory stages of fracture healing that can be attributed only to alcohol consumption.

It has been suggested that the required healing sequence is regulated by these signaling proteins and that aberrations lead to delayed or non-union. The therapeutic application of growth factors to stimulate fracture healing has had mixed success.²⁸ Microarray analysis is one of the most promising means of determining differences in the biological milieu. Comparing the gene expression profiles in normal and

alcohol-treated tissue may reveal valuable information concerning the pathophysiology of the healing process in the alcoholic patient. We propose to identify gene changes during the inflammatory stage of fracture healing that might be attributable to alcohol consumption in order to give focus to future alcohol research. This paper focuses on the events at day three following fracture or the stage of inflammation.

Materials and methods

Animals

Weanling, four-week-old male Sprague-Dawley rats were placed on ethanol-containing (52.8 ± 0.7 g initial wt) or pair-fed Lieber and DeCarli diets (53 ± 1.5 g initial wt) following one week of environment equilibration. They received the special diets for four weeks. The rats were thus nine weeks when fractured. This age is comparable to the adolescent growth spurt, which is a time of peak injury in humans and is also an age for which much previous work with alcohol and bone has been conducted. This research was conducted in conformity with the American Physiological Society's *Guiding Principles in the Care and Use of Animals* and all procedures were approved by Scott and White's Institutional Animal Care and Use Committee.

Diet

Ethanol-fed animals were placed on a modified Lieber and DeCarli liquid diet (Bio-Serve Inc, Frenchtown, NJ, USA; Ca 1.3 g/L; P 1.75 g/L) with ethanol (8.1–9.4% v/v) added so that the diet contained 36% ethanol-derived calories. Liquid diet (pair-fed) control animals were treated the same as the ethanol-fed rats except they were pair-fed to rats in the ethanol group and fed the liquid diet with the ethanol replaced by isocaloric and isovolumetric amounts of maltose dextrins. There were originally four animals per group. Samples that did not pass our quality control check using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) were not included in the array studies, leaving three animals in each group that were analyzed.

Surgical procedure

Preoperative and intraoperative anesthesia of the rats was accomplished by intramuscular injections of ketamine (87 mg/kg body weight) and xylazine (13 mg/kg body weight). A small skin incision was made in the right knee, and the patella deflected. A 1.0-mm diameter hole was drilled into the intercondylar notch of the femur, and a stainless steel pin was inserted into the medullary canal and recessed about 1 mm. The skin was closed with wound clips.²⁹ A closed transverse mid-diaphyseal fracture was induced using a Bonnarens and Einhorn³⁰ fracture device. Acetaminophen (110–300 mg/kg orally, every 4 h as needed) was available for administration postoperatively as an analgesic for 48 h or until no sign of pain.³¹

Postoperatively, the rats were allowed to bear weight as tolerated. The animals appeared to have little or no postoperative pain and were walking and raising up on their hind limbs as soon as they were awake.

The rats were euthanized at three days following fracture in order to study the first or inflammatory stage of healing. Euthanasia was performed using Euthanasia solution (w/ 324 mg/mL pentobarbital) at 100 mg/kg body weight and while under a plane of anesthesia. Twenty-microliter samples of blood were collected into heparinized microcapillary pipettes and placed into vials with a mixture of 0.6 N perchloric acid and 4 mmol/L *n*-propanol in double distilled water, which served as an internal control. The vials were capped tightly with a septum-sealed lid and stored at room temperature until analysis by headspace gas chromatography (Varian Associates, Palo Alto, CA, USA; model 3400) at least 24 h after collection.

The entire region of the bony fracture was harvested from the right hind-limb, frozen in liquid nitrogen and stored below -80°C for the microarray analysis.

The frozen samples were homogenized in 2 mL Trizol reagent using a Spex Freezer Mill (SPEX SamplePrep, St Metuchen, NJ, USA). The powder was quantitatively transferred to culture centrifuge tubes containing Trizol and RNA was isolated according to the manufacturer's directions for the Trizol kit (Invitrogen, Carlsbad, CA, USA). The RNA was then transferred to the Systems Biology Genomics facility (Temple, TX, USA) where they performed microarray analysis against the entire rat genome.

Sample designation

There were three replicates for each sample source, for example, A1, A2 and A3. There were six sample pairs with dye-swap replicates for each pair to make up the total of 12 two-color arrays for the experiment.

Microarray

After checking the quality and quantity of isolated total RNA from the day 3 rats using an Agilent 2100 Bioanalyzer, unclear purified total RNA samples were purified again using a MEGAcleanTM purification kit (Cat #1908; Ambion, Austin, TX, USA). Total RNA samples (1–2 μg) were then used to generate double-strand DNA (dsDNA) by two rounds of reverse transcription. Synthesized dsDNA underwent *in vitro* transcription to produce amplified antisense RNA (aRNA) comprising amino allyl-modified UTP from the dsDNA template; we used aRNA amplification kits for these amplification steps (Cat #1753; Ambion).

After purifying the aRNA, the quality of the aRNA was analyzed again using the Agilent 2100 Bioanalyzer. Subsequently, coupling reactions of qualified aRNA (8–10 μg) and Cy3 or Cy5 mono-reactive dyes (Amersham Bioscience, Piscataway, NJ, USA) were performed with the procedures of Ambion kits (Cat #1795-7).

A Rat Array Ready Oligo Set (AROS) Version 3.0 containing 26,962 longer probes representing 22,012 genes and 27,044 gene transcripts (Operon, Huntsville, AL, USA)

was spotted to UltraGAPSTM coated glass slides (Corning, Fisher Scientific, Pittsburgh, PA, USA), and went through a prehybridization process to remove the excess DNA and reduce background noises. The dye-coupled aRNA was fragmented to improve the hybridization kinetics and signals produced on microarrays through using RNA Fragmentation Reagents (Cat #8740; Ambion). The fragmented aRNA was applied to the glass arrays and hybridized overnight for 17–19 h in an environmentally controlled oven at 42°C . After hybridization, the slides were washed by using Hyb Buffer Kits (Operon), and then read using the Axon 4000A scanner (Molecular Devices, Sunnyvale, CA, USA), which is a dual scanner that scans the 635 nm (red) and 532 nm (green) wavelengths simultaneously.

Data analysis

For each analysis, the dye-swap replicates were normalized and combined in GeneSpring GX (Version 7.3; Agilent Technologies) to get six pairs of sample data from original 12 pairs of sample data. The detailed normalization order in GeneSpring GX is listed below:

- Data transformation: set measurements ≤ 1.0 ;
- Data transformation: dye swap (applied to half of the experiments);
- Per spot per chip: intensity dependent (Lowess) normalization;
- Per chip: normalize to a median or percentile;
- Per gene: normalize to median.

These six pairs of sample data were then quantile normalized in the *R* statistics environment and intensities were then $\log_2$ -transformed.

Next, the TMeV program from TIGR (The Institute for Genomic Research, Rockville, MD, USA) was used for statistics tests on the array data. *P* values were calculated by 'between subjects' *t*-test.

Differentially expressed genes (DEGs) were with the criteria that $-\log_{10}(P \text{ value})$ is more than 1.5 and the fold change is more than 2 (up-regulated) or less than 1/2 (down-regulated). *P* values for significant DEGs are thus less than $10^{-1.5}$, i.e. ~ 0.0316 , and all the effect values (absolute *t*-values) are more than 3.8, so we used effect size 3.8 and *P* value 0.0316 to do statistical power analysis with $n = 3$ to get a power value of 0.858. This indicates that we have adequate statistical power for our experimental data with sample size 3 by selecting genes based on the utilized *t*-test with a significance cut-off level of ~ 0.0316 on the *P* value and also a fold-change cut-off as described. The combined criteria gave the consequent strong statistical power. We also considered genes differentially expressed if they displayed less than a two-fold change, but the *P* value of the change was small ($P \leq 0.01$). Using a fold change of two or greater for average expression or a small *P* value (here $P \leq 0.01$) is a common practice for identifying differentially expressed genes from microarray data. Because of the relatively small sample sizes used here, more stringent approaches would resist efforts for further analysis by

producing smaller lists of differentially expressed genes. Although our approach thus has a heightened risk of producing false-positives, this risk is minimized by focusing attention on group-wise analysis in the form of Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and manual analysis of gene functional categories. These group-wise analyses are robust to individual false-positives, and we have applied stringent statistical criteria for the enrichment analyses (i.e. Benjamini–Hochberg-adjusted P values ≤ 0.05).

Enrichment analyses

Enrichment of GO terms, KEGG pathway membership and miRNA target enrichment were performed with WebGestalt (Vanderbilt University, Nashville, TN, USA). Briefly, a non-redundant list of 45 differentially expressed Unigene IDs was compiled from Tables 1 and 2 and was used as the foreground list, and the rat genome was used as the background list. Benjamini–Hochberg multiple test correction was applied to compute an adjusted P value for each enrichment test.

Polymerase chain reaction

In order to verify the microarray results, polymerase chain reaction (PCR) was conducted on several of the up- and down-regulated genes taken from the cell migration and development categories identified in the discussion section. The primer sets used for PCR were as follows: Maa1, sense 5' TGCATGCATCGGTGGAGTCGGCGG CTCAGT 3', antisense 5' GGCATGCATCAGAGTATTTAT CGTTGCTCA 3'; Mtap2, sense 5' CCTCTTCTGGAAGCAT CAAC 3', antisense 5' GTAACAATTACTACAGTTGG 3'; Actn4, sense 5' TATCACGCGGCGAACCA 3', antisense 5' TCATCCTCCTGGGCCATGT 3'; Mustn-1, sense 5' ATGC GAGAGTGTGAGCAAGCT 3', antisense 5' TTCTCAGCC GAAGACACTCTTGT 3'; Testin, sense 5' TCTAGAGAYGT NGARTGGAAYGARTGG 3', antisense 5' GGTRAGYAA GGTRAGNTGYAGAGATCT 3'; Smad4, sense 5' GTTGCA

GATAGCTTCAGGGC 3', antisense 5' GGATCCACGTA TCCATCCAC 3'.

Reverse transcription was performed in a total volume of 20 μ L with the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) using 2 μ g of RNA (final volume 10 μ L), 2 μ L of RT buffer (10 $\times$), 0.8 μ L of 100 mmol/L dNTP mix (25 $\times$), 2 μ L RT Random primers (10 $\times$), 1 μ L of 50 U MultiScribe reverse transcriptase, 1 μ L of 20 U RNase inhibitor and 3.2 μ L of nuclease-free water. The reverse transcription reactions were incubated at 25°C for 10 min and then at 37°C for 120 min followed by 85°C for five minutes in order to inactivate the reverse transcription, using an Eppendorf Mastercycler PCR system (Eppendorf, Hamburg, Germany).

Conventional PCR was carried out in a total volume of 20 μ L containing 4 μ L reverse-transcribed cDNA, 0.5 μ L of 50 pmol/L each primer, 5 μ L of nuclease-free water and 10 μ L of Universal PCR master mix (Applied Biosystems). PCR amplification was performed in the Eppendorf Mastercycler PCR system (Eppendorf). The amplification conditions used were: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, annealing at 60°C for one minute. PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide for visualization under ultraviolet light.

MicroRNA array profiling

To identify regulating miRNAs of the identified processes, miRNA array was performed on four samples from each group. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. One microgram total RNA from the sample and reference were labeled with Hy3TM and Hy5TM fluorescent labels, respectively, using the miRCURYTM LNA Array power labeling kit (Exiqon, Vedbaek, Denmark) following the procedure described by the manufacturer. The Hy3TM-labeled samples and Hy5TM-labeled samples were mixed pair-wise and

Table 1 Genes with greater than two-fold change

3 day pair-fed fracture versus alcohol fracture. 2-fold or greater		Unigene	Fold	P value
Mustn1	Musculoskeletal, embryonic nuclear protein 1	–	+5.6	0.019
H2a/x	Similar to histone H2A.x	Rn.2850	+4.7	0.016
Ccl7	Chemokine (C-C motif) ligand 7	Rn.26815	+4.2	0.019
T-box 22	Similar to T-box transcription factor protein 22	Rn.109981	+3.3	0.011
Maea	Macrophage erythroblast attacher	Rn.101181	+3.0	0.016
Olr806	Olfactory receptor 806	Rn.143116	+2.5	0.016
Sfrp1	Secreted frizzled-related protein 1 (predicted)	Rn.34957	+2.4	0.027
Chic2	Cysteine-rich hydrophobic domain 2 (predicted)	Rn.106037	+2.4	0.015
	Similar to X83328 protein (RGD1304846)	Rn.15408	+2.2	0.026
Testin	Testin gene	–	+2.1	0.011
GPCR22	G protein-coupled receptor 22 (predicted)	Rn.59212	+2.1	0.005
CENPC1	Centromere protein C 1	Rn.85211	–2.1	0.029
Smad4	MAD homolog 4 (Drosophila)	Rn.9774	–2.2	0.012
Braf	v-raf murine sarcoma viral oncogene homolog B1 (predicted)	Rn.99177	–2.2	0.031
Mtrr	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	Rn.59092	–2.4	0.013
Fes/Fps	Similar to tyrosine kinase Fps/Fes	Rn.27239	–2.6	0.023
Hist2h4a	Similar to germinal histone H4 gene	Rn.10465	–2.7	0.011
Mki67	Antigen identified by monoclonal antibody Ki-67 (predicted)	Rn.73551	–3.1	0.018
Hist1h2bn	Histone 1, H2bn (predicted)	Rn.144978	–3.1	0.023
RGD1559	Similar to FLJ20859 protein	Rn.8815	–3.6	0.005

Table 2 Genes with a *P* value of ≤ 0.01

3 day pair-fed fracture versus alcohol fracture <i>P</i> < 0.01		Unigene	Fold	<i>P</i> value
Gpr22	G protein-coupled receptor 22	Rn.59212	+2.0	0.005
	Mannosidase 1, beta	Rn.17429	+1.7	0.010
	RIKEN cDNA 9330177P20	Rn.17844	+1.7	0.004
Mtap2	Microtubule-associated protein 2	Rn.10484	+1.7	0.001
Mrpl20	Mrpl20 protein	–	+1.6	0.005
Rohn	Ras homolog gene family, member N	Rn.22162	+1.6	0.007
	Similar to T-cell receptor alpha-chain variable region (LOC501987)	–	+1.6	0.008
Syt15	Synaptotagmin-like 5	Rn.92480	+1.5	0.003
	Olfactory receptor 204	Rn.141805	+1.4	0.004
	Hypothetical protein FLJ20718	Rn.106896	+1.4	0.008
LIMB1	Limatin, actin-binding LIM protein 1 long isoform	Rn.40404	+1.4	0.008
Rdh7	Retinol dehydrogenase 7	Rn.31786	+1.4	0.008
Nqo2	NAD(P)H dehydrogenase, quinone 2	Rn.7882	+1.3	0.005
Tieg3	TGFB inducible early growth response 3	Rn.35127	+1.3	0.009
ODC	Ornithine decarboxylase	–	+1.3	0.006
Mpv17	Mpv17 transgene, kidney disease mutant-like	Rn.22842	+1.3	0.009
Ifi204	Ifi204 protein (LOC498288)	–	+1.3	0.008
Nedd1	Neural precursor cell expressed, developmentally down-regulated gene 1	Rn.23035	+1.3	0.007
Cebpe	CCAAT/enhancer binding protein, epsilon	Rn.44462	+1.3	0.008
Cacng7	Calcium channel, voltage-dependent, gamma subunit	–	+1.3	0.008
Thbs2	Thrombospondin 2	–	+1.3	0.009
Cables1	Cdk5 and Abl enzyme substrate 1	–	+1.3	0.003
	Cyclin-dependent kinase 2-interacting protein	Rn.1799	+1.3	0.003
Actn4	Actinin alpha 4	Rn.15777	+1.2	0.006
	Hypothetical protein (LOC500390)	–	+1.2	0.010
	Hypothetical protein (LOC293745)(LOC360865	Rn.129939	–1.2	0.010
	RIKEN cDNA A230078I05	Rn.145720	–1.3	0.004
	RIKEN cDNA 4933409D10	Rn.144981	–1.4	0.006
	Expressed sequence AW822216	Rn.86123	–1.4	0.004
Fut7	Fucosyltransferase 7	Rn.139741	–1.4	0.009
ILF2	Interleukin enhancer binding factor 2 (LOC310612)	Rn.137428	–1.4	0.005
Coro1c	Coronin, actin binding protein 1C	Rn.131309	–1.4	0.002
myosin-VIIb	Myosin-VIIb	Rn.154874	–1.5	0.008
	Chromosome 20 open reading frame 175	Rn.104575	–1.5	0.008
Olr255	Olfactory receptor 255	Rn.141774	–1.6	0.007
Irf3	Interferon regulatory factor 3	Rn.1499	–1.8	0.009
	FLJ20859 protein	Rn.8815	–3.6	0.005

hybridized to the miRCURY™ LNA array Version 10.0 (Exiqon), which contains capture probes targeting all miRNAs for all species registered in the miRBASE Version 10.0 at the Sanger Institute. The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station (Tecan, Grödig, Austria). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY™ LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery Inc, Segundo, CA, USA). The quantified signals were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

MicroRNA realtime PCR

Selected microRNAs (miRNAs) were converted into cDNA by reverse transcription. Then, the cDNA was amplified by realtime PCR using the miRCURY™ LNA miRNA PCR system. The experiment was performed with eight miRNAs

and three endogenous controls. The stability of the endogenous controls was evaluated using the SLqPCR R-package, which is similar to the geNorm application. The three endogenous controls were used for normalizing the quantified signal (Cp) of the miRNAs. The relative expressions were calculated based on the efficiency corrected $\Delta\Delta C_t$ method. The PCR efficiency was estimated from a serial dilution of cDNA generated from pooled RNA of the samples.

Results

Blood alcohol concentrations of the animals receiving diets containing alcohol averaged 333 ± 75 mg/dL for day 3 animals. There were 35 genes that demonstrated significant increased expression due to alcohol consumption and fracture and 20 that decreased. There were 20 genes with a greater than two-fold change: 11 with increased expression and nine with decreased expression (Table 1). Decreased expression is indicated hereafter with a negative fold change, such that a fold change of $-n$ should be interpreted as a $1/n$ change relative to control. Each of these genes had a *P* value of ≤ 0.03 as indicated in the table. Thirty-seven genes changed more than 1.3-fold, but with a *P* value of

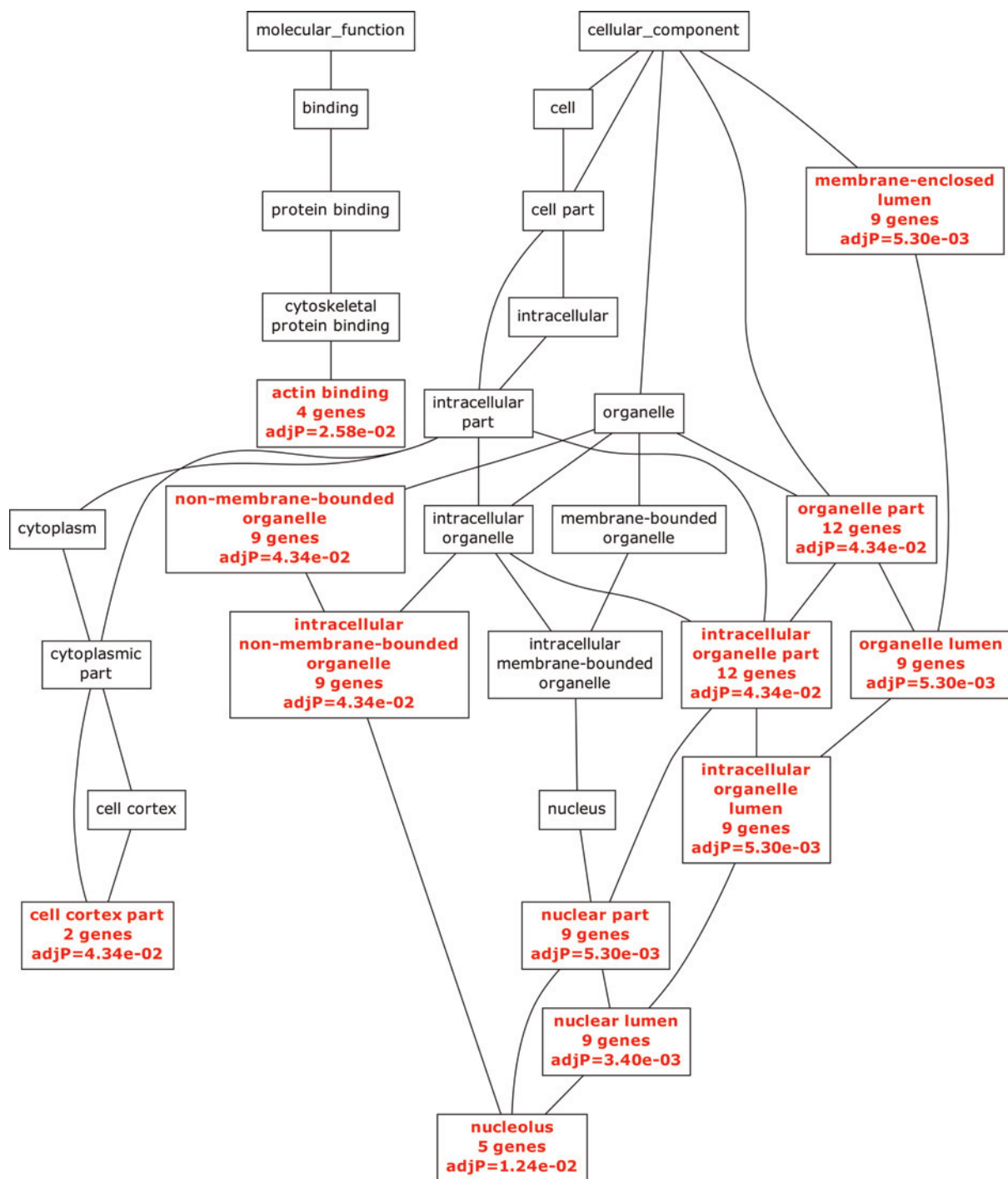


Figure 1 A non-redundant list of 45 Unigene IDs was compiled from Tables 1 and 2, and was used to test for enrichment of Gene Ontology terms. Red text indicates enriched terms with a Benjamini-Hochberg-adjusted *P* value <0.05

Table 3 Result of testing differentially expressed genes for KEGG pathway enrichment

KEGG pathway	Number of differentially expressed genes in pathway	Genes	Benjamini-Hochberg adjusted <i>P</i> value
Systemic lupus erythematosus	3	Actn4, H2afx, RGD1562378	0.0015
Adherens junction	2	Smad4, Actn4	0.0050
Axon guidance	2	Ablim1, Fes	0.0105
Regulation of actin cytoskeleton	2	Actn4, Ssh1	0.0201
Olfactory transduction	3	Olr206, Olr806, Olr255	0.0622

For this analysis, we used the same compiled list of 45 non-redundant genes as in Figure 1

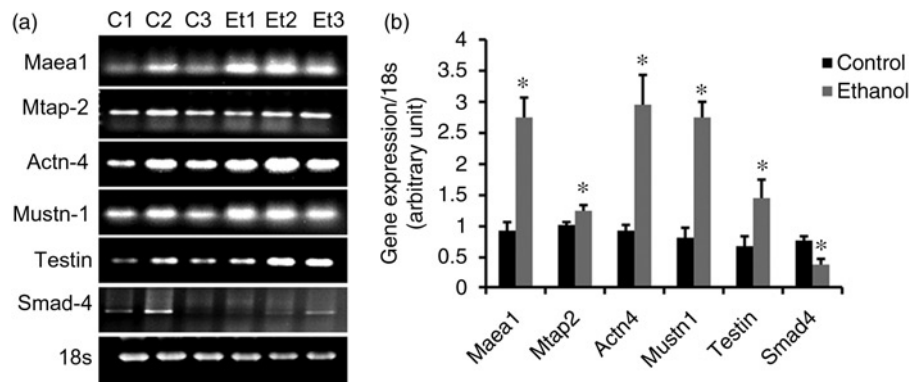


Figure 2 Polymerase chain reaction of genes selected from the cell migration and development category to verify microarray results. (a) Columns c1–c3 are from pair-fed animals and Et1–Et3 are from alcohol-fed animals. 18s is the internal control. (b) A graph of the gene expression versus 18s

≤ 0.01 (Table 2). Twenty-five had increased expression and 12 had decreased expression. We performed GO enrichment tests (Figure 1) and KEGG pathway enrichment tests (Table 3) to provide provisional summary annotations for the differentially expression gene set. PCR of the selected genes agreed with the microarray results in the direction of change (Figure 2). According to the microarray data, six vital genes (Maea, Mtap-2, Actn-4, Mustn-1, Testin and Smad-4) that regulate osteoinduction factors related to the development of the bone were selected. To validate the microarray data, the change in gene expression was evaluated by reverse transcriptase-PCR (RT-PCR). The fold change observed in the gene expression by RT-PCR versus microarray data was quite similar (Maea-1 - +2.98/+3.0; Mtap-2 -

+1.23/+1.7; Actn-4 - +3.22/+1.2; Mustn-1 - +3.44/+5.6; Testin - +2.14/+2.1 and Smad-4 - -0.49/-2.2). There were 20 miRNAs that demonstrated significant increased expression due to alcohol consumption and fracture and six that decreased (Figure 3). There was high correlation between the miRNA microarray data and quantitative PCR (Figure 4) with a Pearson correlation coefficient of 0.939. To provide an initial basis for integrating the mRNA expression data with the miRNA expression data, we performed miRNA target enrichment tests on the mRNA expression data. This analysis showed that targets of miR-26A (targets: Smad4, Map2) and miR-374 (targets: Map2, Actn4) were enriched at the 0.05 level with a Benjamini-Hochberg adjusted P value (Table 4).

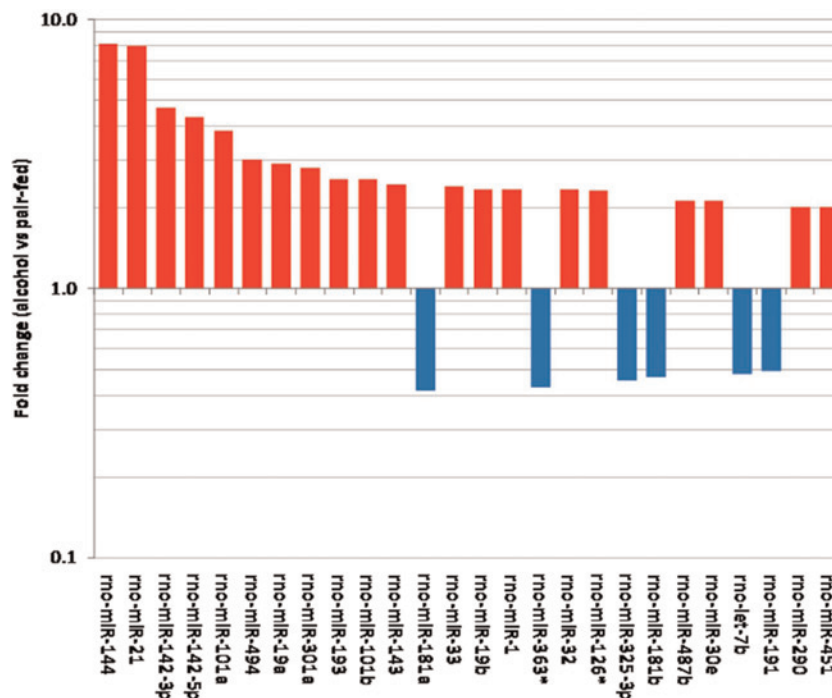


Figure 3 Differentially expressed miRNAs between alcohol and pair-fed rats. Each bar represents the fold change between the alcohol- and pair-fed animals. There are 20 miRNAs that are up-regulated in the alcohol-fed rats as indicated in red and six that were down-regulated as indicated in blue. *Statistical significance at $P \leq 0.05$ using the paired t -test. Error bars = SEM

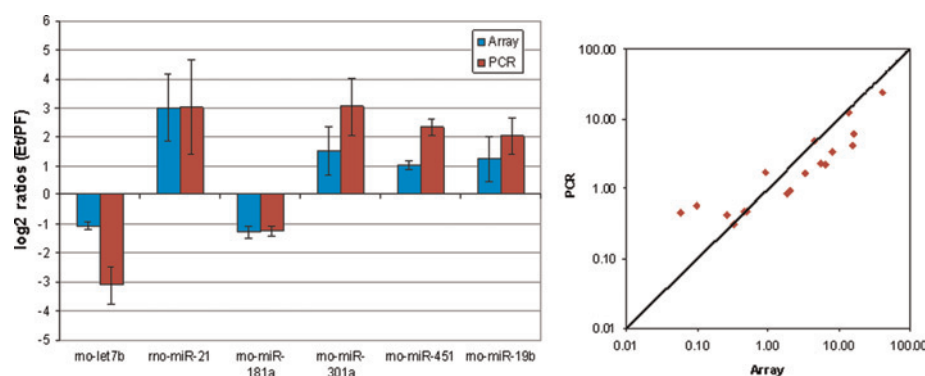


Figure 4 The diagram on the left gives the average relative expression between the two groups 'Ethanol' and 'Pair-Fed'. Positive values indicate an up-regulation in 'Ethanol' and negative values indicate a down-regulation in 'Ethanol' compared with 'Pair-Fed'. The relative expression is shown in log2 ratios and the error bars are SEM. The scatter plot on the right shows the agreement between array and quantitative polymerase chain reaction (qPCR) comparing the relative expression of array data with the relative expression of the qPCR data. The relative expressions are based on four technical replicates. The Pearson correlation of all samples and all miRNAs between array and qPCR is 0.939

Using a fold change of 2 or greater for average expression or a small P value (here $P \leq 0.01$) is a common practice for identifying differentially expressed genes from microarray data. Because of the relatively small samples sizes used here, more stringent approaches would resist efforts for further analysis by producing smaller lists of differentially expressed genes. Although our approach thus has a heightened risk of producing false-positives, this risk is minimized by focusing attention on group-wise analysis in the form of GO enrichment, KEGG pathway enrichment and manual analysis of gene functional categories. These group-wise analyses are robust to individual false-positives, and we have applied stringent statistical criteria for the enrichment analyses (i.e. Benjamini-Hochberg-adjusted P values ≤ 0.05).

The major groupings identified with the mRNA expression microarray experiment were an increased expression of genes involved in the broad category of cell migration, a decreased expression of genes that regulate proliferation and an increase in genes related to development. Each of these groupings will be considered in the Discussion section below.

Table 4 Result of testing differentially expressed genes for miRNA target enrichment

MicroRNA	Number of differentially expressed genes targeted by this miRNA	Genes	Benjamini-Hochberg adjusted P value
Rno_TACTTGA, MIR-26A, MIR-26B	2	Smad4, Map2	0.0390
Rno_TATTATA, MIR-374	2	Map2, Actn4	0.0390
Rno_CAGTATT, MIR-200B, MIR-200C, MIR-429	2	Map2, Nedd1	0.0565
Rno_GTGCCCTT, MIR-506	2	Chic2, Actn4	0.0917

For this analysis, we used the same compiled list of 45 non-redundant genes as in Figure 1

Discussion

Animal model studies of fracture healing of alcohol-consuming animals have been controversial³²⁻³⁵ and have recently been extensively reviewed by Chakkalakal.³⁶ In an attempt to unravel the mechanisms involved with alcohol-induced inhibition of bone repair, Perrien *et al.*^{37,38} demonstrated an elevation of tumor necrosis factor (TNF)- α and interleukin (IL)-1 in distraction osteogenesis and that an over-expression of IL-1 and TNF- α in alcohol-treated distraction osteogenesis fracture studies attenuated ethanol-induced inhibition of bone formation during distraction osteogenesis. These results were later duplicated in conventional transverse tibial fractures.³⁹ TNF- α was demonstrated to be expressed locally during fracture healing of chronic alcohol-consuming animals and to inhibit osteoblast proliferation.^{38,40} These effects of ethanol were later duplicated for TNF, but not for IL-1.⁴¹ A recent study⁴² using demineralized allogeneic bone matrix cylinders showed that alcohol abuse resulted in decreased bone formation within the implant, suggesting a reduced osteoinduction. It is interesting that our study did not highlight the usual cytokines and growth factors such as those seen by Rundle *et al.*²⁵ to be needed for fracture repair as reviewed in the introduction, but it must be kept in mind that this study is focused solely on the effects of alcohol on the healing process and, moreover, focused on only the early stage of inflammation. Lauing *et al.*⁴³ recently demonstrated changes in the canonical Wnt signaling pathway, which increased during callus formation in the later stages of fracture healing and then subsided, but consistent with the present study, it did not appear to be involved in the early stage of inflammation. Our studies shed additional light on the identity of osteoinduction factors that are affected by alcohol, and divide these candidates into three broad categories: cell migration, proliferation and development.

Chemotaxis and cell migration

Fracture healing is marked by key cellular events such as migration, adhesion, proliferation and differentiation, which require the tightly orchestrated activity of thousands of proteins.⁴⁴ The first stages of fracture healing involve

hematoma formation and degranulation of platelets²³ and other inflammatory cells.⁴⁵ It has been suggested that a fracture might be a triggering event for cytokine expression in skeletal tissue⁴⁵ and without this inflammatory process, bone will not heal.⁴⁶ Platelet activation leads to the release of α -granule chemokines, which attract leukocytes and further activate other platelets.⁴⁷ In our study, one of the highest fold changes seen with increased expression (+4.2) during fracture healing of alcohol-consuming animals was CCL7, a chemokine of the C-C family also known as MCP-3 (Table 5). This is a chemokine released from platelet α -granules,⁴⁸ which has been identified in other wound healing studies. It directs the migration and homing of multiple leukocyte subsets (monocytes, basophils, eosinophils, dendritic cells and natural killer cells) and maintains the inflammatory cells at the site of inflammation.^{49–52} Our studies demonstrate that this increase is accompanied by a small increase in Nqo2 (+1.3), which functions in the regulation of immune response and autoimmunity and plays a role in chemokine expression.⁵³ Also increased at this time period during fracture healing of alcohol-consuming animals, in our study, is macrophage erythroblast attracter (Maea; +3) which attaches erythroblasts with macrophages and promotes erythroid proliferation and maturation⁵⁴; and Cebpe (+1.3) which is involved in the regulation of cellular differentiation of granulocytes, macrophages and lymphocytes.^{55,56} Fut7 (–1.4) is involved in leukocyte adhesions and their recruitment into sites of inflammation.

In addition to the altered transcription of protein-coding genes caused by alcohol during the inflammatory stage of fracture healing, more importantly, miRNAs reported to regulate inflammation and hematopoietic differentiation are also changed in the present study. MicroRNA-126 and miR-21 have been identified to have an association with vascular inflammation and leukocyte adherence to endothelial cells.⁵⁷ Others, such as miR-142-3p and 5p, miR-181a and b and miR-451 have been associated with immune function

and hematopoietic differentiation.^{58–61} Additional studies will be needed in order to unravel the relationship between the specific gene and miRNAs.

A matricellular protein, thought to be secreted by platelets and fibroblasts,⁶² that was increased +1.3-fold during fracture healing of alcohol-consuming animals was thrombospondin-2 (Thbs2). Thbs2 is usually seen later in the wound healing process and has also been demonstrated to stimulate chemotaxis and diapedesis of T-cells and other leukocytes.⁶³ Interestingly, Thbs2 null mice appear to have accelerated wound healing,⁶⁴ and during glomerulonephritis, they have increased inflammation in the early stages of the disease.⁶⁵ Complementing this increase in inflammatory cell recruitment was our demonstration of the suppression (–2.6) of an anti-inflammatory gene^{66,67} tyrosine kinase Fps/Fes. Many miRNAs associated with megakaryocyte differentiation,⁶⁸ such as Let-7b, miR-30e, miR-32, miR-101a, miR-126 and miR-181b, are altered during fracture healing of alcohol-consuming animals.

In addition to increased expression of chemotactic factors in three-day alcohol-influenced fracture healing, factors required for cytokinesis and motility were also observed. These include microtubule-associated protein 2 (Mtap2) (+1.7) which is involved in the stabilization and rearrangement of microtubules and actin filaments;⁶⁹ Actn4 (+1.2), a cytoskeletal associated protein implicated in the regulation of cell motility;⁷⁰ LIMB1 (+1.4), an actin-binding protein;⁷¹ and Nedd1 (+1.3) which functions in microtubule organization;^{72,73} all of which might participate in the movement of leukocytes to the inflammatory site.

Other up-regulated genes related to microtubule polymerization, cell shape changes or migration that were seen in this study were ODC (+1.3), the first rate-limiting step in polyamine synthesis, which is required for microtubule formation during gastric mucosal healing⁷⁴ and Rohn (+1.6), which is involved in the organization of cytoskeletal components. Sytl5 (+1.5) is involved in exocytosis and

Table 5 Genes associated with chemotaxis and migration

3 day pair-fed fracture versus alcohol fracture (A versus C)		Unigene	Fold	P value
Genes affecting chemotaxis and inflammation				
CCL7	Chemokine (C-C motif) ligand 7	Rn.26815	+4.2	0.019
Maea	Macrophage erythroblast attracter	Rn.101181	+3.0	0.016
Cebpe	CCAAT/enhancer binding protein, epsilon	Rn.44462	+1.3	0.008
Fut7	Fucosyltransferase 7	Rn.139741	–1.4	0.009
ILF2	Interleukin enhancer binding factor 2 (LOC310612)	Rn.137428	–1.4	0.005
Nqo2	NAD(P)H dehydrogenase, quinone 2	Rn.7882	+1.3	0.005
GPCR22	G protein-coupled receptor 22	Rn.59212	+2.1	0.005
Thbs2	Thrombospondin 2	–	+1.3	0.009
Fes/Fps	Tyrosine kinase Fps/Fes	Rn.27239	–2.6	0.023
Genes affecting microtubule/migration				
Mtap2	Microtubule-associated protein 2	Rn.10484	+1.7	0.001
Actn4	Actinin alpha 4	Rn.15777	+1.2	0.006
LIMB1	Limatin, actin-binding LIM protein 1 long isoform	Rn.40404	+1.4	0.008
Nedd1	Neural precursor cell expressed, developmentally down-regulated gene 1	Rn.23035	+1.3	0.007
ODC	Ornithine decarboxylase	–	+1.3	0.006
Rohn	Ras homolog gene family, member N	Rn.22162	+1.6	0.007
Chic2	cysteine-rich hydrophobic domain 2	Rn.106037	+2.4	0.015
Sytl5	Synaptotagmin-like 5	Rn.92480	+1.5	0.003
Coro1c	Coronin, actin binding protein 1C	Rn.131309	–1.4	0.002
myo-VIib	Myosin-VIib	Rn.154874	–1.5	0.008

Table 6 Genes associated with proliferation

3-day pair-fed fracture versus alcohol fracture (A versus C)		Unigene	Fold	P value
Mki-67	Antigen identified by monoclonal antibody Ki-67	Rn.73551	−3.1	0.018
CENPC1	Centromere protein C 1	Rn.85211	−2.1	0.029
Braf	v-raf murine sarcoma viral oncogene homolog B1	Rn.99177	−2.2	0.031
Cables1	Cdk5 and Abl enzyme substrate 1	–	+1.3	0.003
Ifi204	Ifi204 protein (LOC498288)	–	+1.3	0.008
Hist1h2bn	Histone 1, H2bn	Rn.144978	−3.1	0.023
Hist2h4a	Similar to germinal histone H4 gene	Rn.10465	−2.7	0.011
H2a/x	Histone H2A.x	Rn.2850	+4.7	0.016

Rab27-dependent membrane trafficking⁷⁵ as is Chic2 (+2.4).⁷⁶

This study demonstrated also proteins involved in cytokinesis and motility whose gene expression was down-regulated, such as Corolc (−1.4) which is an actin-binding protein involved in cell locomotion and whose absence strongly inhibited wound closure in wound healing assays,^{77,78} and myosin-VIIb that is thought to play a role in linking the actin cytoskeleton to adhesion receptors on the cell surface^{79,80} (−1.5). These data are consistent with decreased fracture healing as a consequence of alcohol consumption.

Proliferation

A second major area affected by alcohol consumption during fracture healing, as demonstrated in this study, was a decreased expression of genes involved in proliferation (Table 6). Mki-67 (−3.1), recently reviewed by Scholzen and Gerdes,⁸¹ is a non-histone protein that is required in the active phases of the cell cycle, but is absent in quiescent or resting cells.^{82,83} It is considered to be a marker of proliferative cells^{82,84} and is believed to play a role in the regulation of higher-order chromatin structure.⁸⁵ Its marked decrease in this study may signal cell-cycle arrest as a result of alcohol consumption. This hypothesis is supported by the decreased expression of CENPC1 (−2.1), an RNA-associating protein that binds α -satellite RNA, which is responsible for the regulation of the metaphase centromere,⁸⁶ and B-raf (−2.2), which is an oncogene involved in the transduction of mitogenic signals from the cell membrane to the nucleus.⁸⁷ B-raf is also of significance because its absence impairs megakaryocytopoiesis which is important for platelet formation.⁸⁸ Cables1 (+1.3) and Ifi204 (+1.3), genes that inhibit cell cycle progression,^{89,90} were found to be increased in expression. In addition to a decrease in these cell-cycle genes and their role in the

regulation of chromatin structure is the decrease in Hist1, h2bn (−3.1) and H4 (−2.7), which are key components of nucleosomes. These taken together might indicate that chromatin remodeling is decreased in the region of fracture healing in alcohol-consuming animals. Interestingly, H2a/x (+4.7), which plays a critical role in recruitment of repair factors during DNA repair,⁹¹ is up-regulated almost five-fold, possibly indicating an attempt to repair the chromosome damage in the alcohol-treated animals.

Similarly, Let-7b, miR-21, miR-19a,b, miR-32 and miR-181a have been reported to be associated with cell cycle and multiple myeloma,^{92–94} and miR-144, Let-7b and miR-19s have been reported to be involved in activation of caspase cascade and apoptosis, indicating that alcohol could possibly interfere with the regulation of cell cycle gene expression.

Development

The gene identified in the present study with the greatest fold increase due to alcohol consumption and fracture healing was Mustang 1 (Mustn1) (+5.6), a gene with a nuclear localization that is highly expressed in embryogenesis and bone regeneration, but not in adult tissue⁹⁵ (Table 7). It has been reported to increase in fold change through day 5 postfracture and then decrease thereafter and is believed to be a regulatory protein.⁹⁵ Mustang has been reported to be activated by the binding of c-fos, JunD and Fra-2 to its promoter.⁹⁶ Testin (+2.1), a gene identified in the embryonic apical ectodermal ridge and other early embryonic bone regions,⁹⁷ is up-regulated, as is T-box 22 (+3.3), a gene involved in the regulation of developmental processes, which might function in directed healing. Wnt-5A has been reported in normal fracture healing to increase sharply at day 3 decline at days 5–7, rise again at days 10 and 14 and decline back down to intact bone levels at day 21, suggesting that it may be

Table 7 Genes associated with development

3-day pair-fed fracture versus alcohol fracture (A versus C)		Unigene	Fold	P value
Mustn1	Musculoskeletal, embryonic nuclear protein 1	–	+5.6	0.019
Testin	Testin gene	–	+2.1	0.011
T-box 22	Similar to T-box transcription factor protein 22	Rn.109981	+3.3	0.011
Smad4	MAD homolog 4 (Drosophila)	Rn.9774	−2.2	0.012
Rdh7	Retinol dehydrogenase 7	Rn.31786	+1.4	0.008
Mtrr	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	Rn.59092	−2.4	0.013

3' cgAAACUGUUAUGAUACGUGAc 5' rno-miR-301a
 | | | | : | | : | | | | | : |
 67:5' aaUCUGAAGAU- UUAUUGCAUu 3' Smad4

Figure 5 A sequence alignment between miR-301a and smad4 which has an alignment score of 151

activated during inflammation and chondrogenesis.^{44,98} This increase is not seen in alcohol-consuming animals in the present study, but there is a 2.4-fold increase in Sfrp1 which is a Wnt antagonist that directly interacts with the Wnt ligand,⁹⁹ indicating alcohol may antagonize the Wnt pathway, which has previously been implicated in fracture healing. miR-290 has been reported to be dominant in embryonic stem cells¹⁰⁰ and miR-126 has been reported to regulate HOXA9, a developmental gene.¹⁰¹

Smad4 (−2.2) is a developmental gene whose expression is decreased during fracture healing in the present study with alcohol-treated animals, but is greatly increased during fracture healing of normal animals.¹⁰² Smad4 (−2.2) interacts with phosphorylated Smad 1 or 5 and enters the nucleus to activate transcriptional machinery for early BMP response genes involved in cartilage and bone formation^{102–104} or with Smad 2 or 3 to induce TGF-β for later stages of fracture healing.¹⁰² Interestingly, Smad4 has been reported to be a target for miR-301a, which this study identifies as up-regulated. As a result, we observed a down-regulation of the Smad4 gene in the alcohol-treated rats. Interestingly, Mustn1 is also a target for miR-301a and we observed an up-regulation of the gene expression in the alcohol-treated rats. It will be a point of interest that under similar pathology, miR-301a is a negative regulator for Smad4 and a positive regulator for Mustn1 gene expression. The current finding becomes more intriguing since both these genes are regulating osteoinduction factors related to the development of the bone.

Figure 5 demonstrates alignment between Smad4 and miR-301a. Since miRNAs usually inhibit their target gene, this up-regulation of miR-143 is consistent with the down-regulation of the Smad4 gene. Since, Mtrr has been found to have a significant correlation with osteocalcin levels and bone turnover,¹⁰⁵ it is interesting that we found a 2.4-fold decrease in fracture healing of alcohol-consuming animals.

In summary, while it is recognized that mRNA levels may or may not represent protein levels successfully produced by the cell, these studies reveal changes in gene expression that support the hypothesis that alcohol consumption affects events involved with inflammation. MicroRNAs are known to modulate mRNA and confirmed much of what was seen with mRNA microarray analysis, especially the involvement of smad4 which was demonstrated by mRNA microarray, miRNA and PCR.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. HWS designed the experiments, collected samples and wrote the manuscript; CDC and JB performed the surgical procedures; RAP and KMB contributed to the design of the studies and reviewed

the manuscript; RSG and JP performed the PCR; and VV performed the microarrays.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Association Internationale pour Osteosynthese Dynamique (AIOD) and the Center for Bone, Joint and Spine Research, Department of Orthopedics, Scott and White Hospital, Temple, TX, USA. The authors express appreciation to Alaina C Dearman MS who isolated the RNA, and Usha Chowdhury MS and Hung-Chung Huang who ran the hybridizations.

REFERENCES

- Israel Y, Orrego H, Holt S, Macdonald DW, Meema HE. Identification of alcohol abuse: thoracic fractures on routine chest X-rays as indicators of alcoholism. *Alcoholism Clin Exp Res* 1980;4:420–2
- Kristensson H, Lunden A, Nilsson BE. Fracture incidence and diagnostic roentgen in alcoholics. *Acta Orthop Scand* 1980;51:205–7
- Johnell O, Kristensson H, Johnell I. Lower limb fractures and registration for alcoholism. *Scand J Soc Med* 1985;13:95–7
- Laitinen K, Valimäki M. Alcohol and bone. *Calcif Tissue Int* 1991;49:S70–73
- Nordqvist A, Petersson CJ. An analysis of 413 injuries. *Acta Orthop Scand* 1996;67:364–6
- Cawthon PM, Harrison SL, Barrett-Connor E, Fink HA, Cauley JA, Lewis CE, Orwoll ES, Cummings SR. Alcohol intake and its relationship with bone mineral density, falls, and fracture risk in older men. *J Am Geriatr Soc* 2006;54:1649–57
- Saville PD. Changes in bone mass with age and alcoholism. *J Bone Joint Surg* 1965;47-A:492–9
- Saville PD. Alcohol-related skeletal disorders. *Ann N Y Acad Sci* 1975;252:287–91
- Hogan HA, Sampson HW, Cashier E, Ledoux N. Alcohol consumption by young actively growing rats. A study of cortical bone histomorphometry and mechanical properties. *Alcoholism Clin Exp Res* 1997;21:809–16
- Sampson HW. Effect of alcohol consumption on adult and aged bone: a histomorphometric study of the rat animal model. *Alcoholism Clin Exp Res* 1998;22:2029–34
- Hogan HA, Groves JA, Sampson HW. Long-term alcohol consumption in the rat affects femur cross-sectional geometry and bone tissue material properties. *Alcoholism Clin Exp Res* 1999;23:1825–33
- Tonnesen H, Pedersen A, Jensen MR, Møller A, Madsen JC. Ankle fractures and alcoholism. The influence of alcoholism on morbidity after malleolar fractures. *J Bone Joint Surg* 1991;73-B:511–3
- Nyquist F, Berglund M, Nilsson BE, Obrant KJ. Nature and healing of tibial shaft fractures in alcohol abusers. *Alcohol Alcoholism* 1997;32:91–5
- Foulk DA, Szabo RM. Diaphyseal humerus fractures: natural history and occurrence of nonunion. *Orthopedics* 1995;18:333–5
- Nyquist F, Overgaard A, Dürpe H, Obrant KJ. Alcohol abuse and healing complications after cervical hip fractures. *Alcohol Alcohol* 1998;33:373–80
- Sears BW, Volkmer D, Yong S, Himes RD, Lauing K, Morgan M, Stover MD, Callaci JJ. Binge alcohol exposure modulates rodent expression of biomarkers of the immunoinflammatory response to orthopaedic trauma. *J Bone Joint Surg [Am]* 2011;93:739–49
- Sampson HW, Chaffin C, Lange J, Defee B II. Alcohol consumption by young actively growing rats. A histomorphometric study of cancellous bone. *Alcoholism Clin Exp Res* 1997;21:352–9
- Ham AW. Bone and bones. In: Ham AW, Cormack DH, eds. *Histology*. 8 edn. Philadelphia: JB Lippincott Co, 1979:377–462
- Bolander ME. Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* 1992;200:165–70

- 20 Jingushi S, Joyce ME, Bolander ME. Genetic expression of extracellular matrix proteins correlates with histologic changes during fracture repair. *J Bone Miner Res* 1992;7:1045–55
- 21 Themistocleous GS, Kontou SE, Lembessis P, Katopodis HA, Kaseta MA, Themistocleous MS, Koutsilieris M. Skeletal growth factor involvement in the regulation of fracture healing process. *In Vivo* 2003;17:489–504
- 22 Li X, Quigg RJ, Zhou J, Ryaby JT, Wang H. Early signals for fracture healing. *J Cell Biochem* 2005;95:189–205
- 23 Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC. Osteogenic activities of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg [Am]* 2003;85-A:1544–52
- 24 Goumans MJ, Lebrin F, Valdimarsdottir G. Controlling the angiogenic switch. A balance between two distinct TGF- β receptor signaling pathways. *Trends Cardiovasc Med* 2003;13:301–7
- 25 Rundle CH, Wang H, Yu H, Chadwick RB, Davis EI, Wergedal JE, Lauk HW, Mohan S, Ryaby J, Baylink DL. Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. *Bone* 2006;38:521–9
- 26 Callaci JJ, Himes R, Lauing K, Wezeman FH, Brownson K. Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. *Calcif Tissue Int* 2009;84:474–84
- 27 Himes R, Wezeman FH, Callaci JJ. Identification of novel bone-specific molecular targets of binge alcohol and ibandronate by transcriptome analysis. *Alcoholism Clin Exp Res* 2008;32:1167–80
- 28 Heckman JD, Ehler W, Brooks BP, Aufdemorte TB, Lohmann CH, Morgan T, Boyan-Salyers BD. Bone morphogenetic protein but not transforming growth factor- β enhances bone formation in canine diaphyseal nonunions implanted with a biodegradable composite polymer. *J Bone Joint Surg [Am]* 1999;81-A:1717–29
- 29 Desai BJ, Meyer MH, Porter S, Kellam JF, Meyer RA Jr. The effect of age on gene expression in adult and juvenile rats following femoral fracture. *J Orthop Trauma* 2003;17:689–98
- 30 Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. *J Orthop Res* 1984;2:97–101
- 31 Hawk C, Leary S. *Formulary for Laboratory Animals*. 3rd edn. Malden, MA: Wiley-Blackwell, 2005
- 32 Nyquist F, Halvorsen V, Madsen JE, Nordsletten L, Obrant KJ. Ethanol and its effects on fracture healing and bone mass in male rats. *Acta Orthop Scand* 1999;70:212–6
- 33 Chakkalakal DA, Novak JR, Fritz ED, Mollner TJ, McVicker DL, Lybarger DL, McGuire MH, Donohue TM Jr. Chronic ethanol consumption results in deficient bone repair in rats. *Alcohol Alcohol* 2002;37:13–20
- 34 Elmali N, Ertem K, Ozen S, Inan M, Baysal T, Güner G, Bora A. Fracture healing and bone mass in rats fed on liquid diet containing ethanol. *Alcoholism Clin Exp Res* 2002;26:509–13
- 35 Chakkalakal DA, Novak JR, Fritz ED, Mollner TJ, McVicker DL, Garvin KL, McGuire MH, Donovan JM. Inhibition of bone repair in a rat model for chronic and excessive alcohol consumption. *Alcohol* 2005;36:201–14
- 36 Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. *Alcoholism Clin Exp Res* 2005;29:2077–90
- 37 Perrien DS, Brown EC, Fletcher TW, Irby DJ, Aronson J, Gao G, Skinner RA, Hogue WR, Feige U, Suva LJ, Ronis MJJ, Badger TM, Lumpkin CK Jr. Interleukin-1 and tumor necrosis factor antagonists attenuate ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis. *J Pharmacol Exp Ther* 2003;303:904–8
- 38 Perrien DS, Liu Z, Wahl EC, Bunn RC, Skinner RA, Aronson J, Fowlkes J, Badger TM, Lumpkin CK Jr. Chronic ethanol exposure is associated with a local increase in TNF- α and decreased proliferation in the rat distraction gap. *Cytokine* 2003;23:179–89
- 39 Perren SM, Wahl EC, Hogue WR, Feige U, Aronson J, Ronis MJJ, Badger TM, Lumpkin CK Jr. IL-1 and TNF antagonists prevent inhibition of fracture healing by ethanol in rats. *Trans Ann Orthop Res Soc* 2006;82:656–60
- 40 Wahl EC, Liu L, Perrien DS, Aronson J, Hogue WR, Skinner RA, Hildestrand M, Ronis MJJ, Badger TM, Lumpkin CK Jr. A novel mouse model for the study of the inhibitory effects of chronic ethanol exposure on direct bone formation. *Alcohol* 2006;39:159–67
- 41 Wahl EC, Perrien DS, Aronson J, Liu Z, Fletcher TW, Skinner RA, Feige U, Suva LJ, Badger TM, Lumpkin CK Jr. Ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis: a role for the tumor necrosis factor signaling axis. *Alcoholism Clin Exp Res* 2006;29:1466–72
- 42 Trevisiol CH, Turner RT, Pfaff JE, Hunter JC, Menagh PJ, Hardin K, Ho E, Iwaniec UT. Impaired osteoinduction in a rat model for chronic alcohol abuse. *Bone* 2007;41:175–80
- 43 Lauing K, Himes R, Callaci JJ. Binge alcohol exposure impairs fracture healing by disruption of the canonical wnt signaling pathway. *Alcohol Clin Exp Res* 2009;33S:33A
- 44 Hadjiargyrou M, Lombardo F, Zhao S, Ahrens W, Joo J, Ahn H, Jurman M, White DW, Rubin CT. Transcriptional profiling of bone regeneration. insight into the molecular complexity of wound healing. *J Biol Chem* 2002;277:30177–82
- 45 Einhorn TA, Majeska RJ, Rush EB, Levine PM, Horowitz MC. The expression of cytokine activity by fracture callus. *J Bone Miner Res* 1995;10:1272–81
- 46 Phillips AM. Overview of the fracture healing cascade. *Injury* 2005;36S:S5–S7
- 47 Gear ARL, Camerini D. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. *Microcirculation* 2003;10:335–50
- 48 Reed G. Platelet secretion. In: Michelson AD, ed. *Platelets*. 2nd edn. Boston: Elsevier, 2007: 309–18
- 49 McQuibban CA, Gong J-H, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties *in vivo*. *Blood* 2002;100:1160–7
- 50 Chakravarti S, Wu F, Vij N, Roberts L, Joyce S. Microarray studies reveal macrophage-like function of stromal keratocytes in the cornea. *Invest Ophthalmol Vis Sci* 2004;45:2475–3484
- 51 Proost P, Wuyts A, Van Damme J. Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. *J Leukoc Biol* 1996;59:67–74
- 52 Menten P, Wuyts A, Van Damme J. Monocyte chemotactic protein-3. *Eur Cytokine Netw* 2001;12:554–60
- 53 Iskander K, Li J, Han S, Zheng B, Jaiswal AK. NQO1 and NQO2 regulation of humoral immunity and autoimmunity. *J Biol Chem* 2006;281:30917–24
- 54 Hanspal M, Hanspal JS. The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact. *Blood* 1994;84:3494–504
- 55 Tavor S, Vuong PT, Park DJ, Gombart AF, Cohen AH, Koeffler HP. Macrophage functional maturation and cytokine production are impaired in C/EBP ϵ -deficient mice. *Blood* 2002;99:1794–801
- 56 Hirai H, Zhang P, Dayaram T, Hetherington CJ, Mizuno S, Imanishi J, Akashi K, Tenen DG. C/EBP β is required for 'emergency' granulopoiesis. *Nat Rev* 2006;7:732–9
- 57 Urbich C, Kuehnbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008;79:581–8
- 58 Sonkoly E, Stahle M, Pivarcsi A. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. *Semin Cancer Biol* 2008;18:131–40
- 59 Moschos SA, Williams AE, Perry MM, Birrell MA, Belvisi MG, Lindsay MA. Expression profiling *in vivo* demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids. *BMC Genomics* 2007;8:240–52
- 60 Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303:83–6
- 61 Foshay KM, Gallicano GI. Small RNAs, big potential: the role of microRNAs in stem cell function. *Curr Stem Cell Res Ther* 2007;2: 264–71
- 62 Kopp KG, Hooper AT, Broekman MJ, Aveilla ST, Petit I, Luo M, Milde T, Ramos CA, Zhang F, Kopp T, Borstein P, Jin DK, Marcus AJ, Rafii S. Thrombospondins deployed by thrombopoietic cells determine angiogenic switch and extent of revascularization. *J Clin Invest* 2006;116:3277–91

- 63 Li Z, Calzada MJ, Sipes JM, Cashel JA, Krutzsch HC, Annis DS, Mosher DF, Roberts DD. Interactions of thrombospondins with $\alpha 4 \beta 1$ integrin and CD47 differentially modulate T cell behavior. *J Cell Biol* 2002;157:509–19
- 64 Kyriakides TR, Bornstein P. Matricellular proteins as modulators of wound healing and the foreign body response. *Thromb Haemost* 2003;90:986–92
- 65 Daniel D, Amann K, Hohenstein B, Bornstein P, Hugo C. Thrombospondin 2 functions as an endogenous regulator of angiogenesis and inflammation in experimental glomerulonephritis in Mice. *J Am Soc Nephrol* 2007;18:788–98
- 66 Parsons SA, Greer PA. The Fps/Fes kinase regulates the inflammatory response to endotoxin through down-regulation of TLR4, NF- κ B activation, and TNF- α secretion in macrophages. *J Leukoc Biol* 2006;80:1522–8
- 67 Parsons SA, Mewburn JD, Truesdell P, Greer PA. The Fps/Fes kinase regulates leucocyte recruitment and extravasation during inflammation. *Immunology* 2007;122:542–50
- 68 Garzon R, Pichiorri F, Palumbo T, Luliano R, Cimmino A, Aqeilan R, Volinia S, Bhatt D, Alder H, Marcucci G, Calin GA, Liu CG, Bloomfield CD, Andref M, Croce CM. MicroRNA fingerprints during human megakaryocytopoiesis. *Proc Natl Acad Sci* 2006;103:5078–83
- 69 Dehmelt L, Smart FM, Ozer RS, Halpain S. The role of microtubule-associated protein 2c in the reorganization of microtubules and lamellipodia during neurite initiation. *J Neurosci* 2003;23:9479–90
- 70 Barbolina MV, Adley BP, Kelly DL, Fought AJ, Scholtens DM, Shea LD, Stack MS. Motility-related actin alpha-4 is associated with advanced and metastatic ovarian carcinoma. *Lab Invest* 2008;88:1–13
- 71 Kim AC, Peters LL, Knoll JHM, Van Huffel C, Ciciotte SL, Kleyn PW, Hishti AH. Limatin (LIMAB1), an actin-binding LIM protein, maps to mouse chromosome 19 and human chromosome 10q25, a region frequently deleted in human cancers. *Genomics* 1997;46:291–3
- 72 Manning J, Kumar S. NEDD1: function in microtubule nucleation, spindle assembly and beyond. *Int J Biochem Cell Biol* 2007;39:7–11
- 73 Liu L, Wiese C. *Xenopus* NEDD1 is required for microtubule organization in *Xenopus* egg extracts. *J Cell Sci* 2008;121:578–89
- 74 Banan A, McCormack SA, Johnson LR. Polyamines are required for microtubule formation during gastric mucosal healing. *Am J Physiol Gastrointest Liver Physiol* 1998;274:G879–G885
- 75 Tsuboi T, Fukuda M. The Slp4-a linker domain controls exocytosis through interaction with Munc18-1-Syntaxin-1a complex. *Mol Biol Cell* 2006;17:2101–12
- 76 Cools J, Mentens N, Marynen P. A new family of small, palmitoylated, membrane-associated proteins characterized by the presence of a cysteine-rich hydrophobic motif. *FEBS Lett* 2001;492:204–9
- 77 Rosentreter A, Hofmann A, Xavier CP, Stumpf M, Noegel AA, Clemen CS. Coronin 3 involvement in F-actin-dependent processes at the cell cortex. *Exp Cell Res* 2007;313:878–95
- 78 Mishima M, Nishida E. Coronin localizes to leading edges and is involved in cell spreading and lamellipodium extension in vertebrate cells. *J Cell Sci* 1999;112:2833–42
- 79 Chen Z-Y, Hasson T, Zhang D-S, Schwender BJ, Derfler BH, Mooseker MS, Corey DP. Myosin-VIIb, a novel unconventional myosin, is a constituent of microvilli in transporting epithelia. *Genomics* 2001;72:285–96
- 80 Henn A, De La Cruz EM. Vertebrate myosin VIIb is a high duty ratio motor adapted for generating and maintaining tension. *J Biol Chem* 2005;280:39665–76
- 81 Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 2000;182:311–22
- 82 Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad HD. Immunobiochemical and molecular biological characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am J Pathol* 1991;138:867–73
- 83 Endl E, Kausch I, Baack M, Knippers R, Gerdes J, Scholzen T. The expression of Ki-67, MCM3, and p27 defines distinct subsets of proliferation, resting, and differentiated cells. *J Pathol* 2001;195:457–62
- 84 Gerlach C, Golding M, Larue L, Alison MR, Gerdes J. Ki-67 immunoreactivity is a robust marker of proliferative cells in the rat. *Lab Invest* 1997;77:697–8
- 85 Scholzen T, Endl E, Wohlenberg C, van der Sar S, Cowell IG, Gerdes J, Singh PB. The Ki-67 protein interacts with members of the heterochromatin protein 1 (HP1) family: a potential role in the regulation of higher-order chromatin structure. *J Pathol* 2002;196:135–44
- 86 Wong LH, Brettingham-Moore KH, Chan L, Quach JM, Anderson MA, Northrop EL, Hannan R, Saffery R, Shaw ML, Williams E. Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere. *Genome Res* 2008;17:1146–60
- 87 Deluca AM, Srinivas A, Alani RM. BRAF kinase in melanoma development and progression. *Expert Rev Mol Med* 2008;10:1–14
- 88 Kamata T, Kang J, Lee TH, Wojnowski L, Pritchard CA, Leavitt AD. A critical function for B-Raf at multiple stages of myelopoiesis. *Blood* 2005;106:833–40
- 89 Lee H, Sakamoto H, Luo H, Skaznik-Eikel M, Friel A, Niikura T, Tilley J, Niikura Y, Klein R, Styer A, Zukerberg L, Tilley J, Rueda B. Loss of CABLES1, a cyclin-dependent kinase-interacting protein that inhibits cell cycle progression, results in germline expansion at the expense of quality in adult female mice. *Cell Cycle* 2007;6:2678–84
- 90 Luan Y, Lengyel P, Liu CJ. p204, a p200 family protein, as a multifunctional regulator of cell proliferation and differentiation. *Cytokine Growth Factor Rev* 2008;19:357–69
- 91 Paull TT, Rogakou EP, Yamazaki Y, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10:886–95
- 92 Carleton M, Cleary MA, Linsley PS. MicroRNAs and cell cycle regulation. *Cell Cycle* 2007;6:2127–32
- 93 Bueno MJ, de Castro IP, Malumbres M. Control of cell proliferation pathways by microRNAs. *Cell Cycle* 2008;7:3143–8
- 94 Chivukula RR, Mendell JT. Circular reasoning: microRNAs and cell-cycle control. *Trends Biochem Sci* 2008;33:474–81
- 95 Lombardo F, Komatsu D, Hadjiargyrou M. Molecular cloning and characterization of Mustang, a novel nuclear protein expressed during skeletal development and regeneration. *FASEB J* 2004;18:52–61
- 96 Liu C, Hadjiargyrou M. Identification and characterization of the Mustang promoter: regulation by AP-1 during myogenic differentiation. *Bone* 2008;39:815–24
- 97 Bekman E, Henrique D. Embryonic expression of three mouse genes with homology to the *Drosophila melanogaster prickle* gene. *Mech Dev* 2002;119S:S77–S81
- 98 Zhong N, Gersch RP, Hadjiargyrou M. Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. *Bone* 2006;39:5–16
- 99 Satoh W, Matsuyama M, Takemura H, Aizawa S, Shimono A. Sfrp 1, Sfrp 2, and Sfrp 5 regulate the Wnt/ β -catenin and the planar cell polarity pathways during early trunk formation in mouse. *Genesis* 2008;46:92–103
- 100 Wang Y, Baskerville S, Shenoy A, Babiarz JE, Baehner L, Belloch R. Embryonic stem cell-specific microRNAs regulate the G1–S transition and promote rapid proliferation. *Nat Genet* 2008;40:1478–83
- 101 Shen WF, Hu YL, Uttarwar L, Passegue E, Largman C. MicroRNA-126 Regulates HOXA9 by binding to the Homeobox. *Mol Cell Biol* 2008;28:4609–19
- 102 Yu Y, Yang JL, Chapman-Sheath PJ, Walsh WR. TGF- β , BMPs, and their signal transducing mediators, Smads, in rat fracture healing. *J Biomed Mater Res* 2002;60:392–7
- 103 Reddi AH. Initiation of fracture repair by bone morphogenetic proteins. *Clin Orthop* 1998;355:S66–S72
- 104 Farhadieh RD, Gianoutsos MP, Yn Y, Walsh WR. The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis on the mandible. *J Craniofacial Surg* 2004;15:714–8
- 105 Kim DJ, Park BL, Koh JMK, Kim GS, Kim LH, Cheong HS, Shin HD, Hong JM, Kim TH, Shin HI, Park EK, Kim SY. Methionine synthase reductase polymorphisms are associated with serum osteocalcin levels in postmenopausal women. *Exp Mol Med* 2006;38:519–24

(Received June 9, 2011, Accepted August 16, 2011)