Available online at www.sciencedirect.com
ScienceDirect
Toxicology and Applied Pharmacology 223 (2007) 225–233
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Differential gene expression and lipid metabolism in fatty liver induced by acute ethanol treatment in mice

Hu-Quan Yin [a], Mingoo Kim [b], Ju-Han Kim [b,c], Gu Kong [d], Kyung-Sun Kang [e], Hyung-Lae Kim [f], Byung-IL Yoon [g], Mi-Ock Lee [a], Byung-Hoon Lee [a,*]

[a] College of Pharmacy, Seoul National University, San 56-1, Sillim-dong, Gwanak-gu, Seoul 151-742, Republic of Korea
[b] Seoul National University Biomedical Informatics, Seoul National University, Seoul 110-799, Republic of Korea
[c] Human Genome Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea
[d] College of Medicine, Hanyang University, Seoul133-791, Republic of Korea
[e] College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea
[f] College of Medicine, Ewha Womans University, Seoul 158-710, Republic of Korea
[g] College of Veterinary Medicine, Kangwon National University, Chuncheon 200-791, Republic of Korea

Received 2 January 2007; revised 5 May 2007; accepted 19 June 2007
Available online 4 July 2007

Abstract

Ethanol induces cumulative liver damage including steatosis, steatohepatitis and cirrhosis. The aim of this study is to investigate the global intrahepatic gene expression profile in the mouse liver treated with ethanol. A single oral dose of 0.5 or 5 g/kg ethanol was administered to male ICR mice, and liver samples were obtained after 6, 24 and 72 h. Histopathological evaluation showed typical fatty livers in the high-dose group at 24 h. Microarray analysis identified 28 genes as being ethanol responsive (two-way ANOVA; $p<0.05$), after adjustment by the Benjamini–Hochberg multiple testing correction; these genes displayed ≥2-fold induction or repression. The expression of genes that are known to be involved in fatty acid synthesis was examined. The transcript for lipogenic transcription factor, sterol regulatory element (SRE)-binding factor 1 (*Srebf1*), was upregulated by acute ethanol exposure. Of the genes known to contain SRE or SRE-like sequences and to be regulated by SRE-binding protein 1 (SREBP1), those encoding malic enzyme (*Mod1*), ATP-citrate lyase (*Acly*), fatty acid synthase (*Fasn*) and stearyl-CoA desaturase (*Scd1*) were induced by ethanol. Quantitative real-time PCR confirmed the changes in the expression levels of the selected genes. The change in the *Srebf1* mRNA level correlates well with that of the SREBP1 protein expression as well as its binding to the promoters of the target genes. The present study identifies differentially expressed genes that can be applied to the biomarkers for alcohol-binge-induced fatty liver. These results support the hypothesis by which ethanol-induced steatosis in mice is mediated by the fatty acid synthetic pathway regulated by SREBP1.

Keywords: Ethanol; Fatty liver; Toxicogenomics; Fatty acid synthesis; Sterol regulatory element-binding protein (SREBP); Microarray

Introduction

The liver is a primary site for xenobiotic metabolism and is the most common target organ for chemically induced injuries. The type of hepatobiliary injury is mostly determined by the nature of the xenobiotic chemicals to which the organism has been exposed. Some substances produce a very specific type of damage, whereas other chemicals induce cumulative damage or a combination of damaging events (Treinen-Moslen, 2001). A representative example of the latter type of chemical is ethanol. Fatty infiltration, which is the first manifestation of ethanol-induced liver injury, is usually followed by inflammation, focal necrosis and terminal venular sclerosis, that can ultimately develop into cirrhosis (Diehl, 1997; Neuman, 2003).

The phenomenon of fatty liver, also known as steatosis, refers to the abnormal accumulation of fat in hepatocytes. Although many toxicants can cause lipid accumulation in the liver, the mechanisms may be different. Lipid accumulation

* Corresponding author. Fax: +82 2 874 7843.
E-mail address: lee@snu.ac.kr (B.-H. Lee).

0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2007.06.018

is related to one or more of the following events: an oversupply of free fatty acids to the liver, interference with the triglyceride cycle, increases in the synthesis or esterification of fatty acids, decreased fatty acid oxidation, decreased apoprotein synthesis and decreased synthesis or secretion of very low density lipoprotein (Treinen-Moslen, 2001). Although it was formerly considered a benign and fully reversible condition, hepatic fatty infiltration is an important pathogenic factor in the development of alcoholic liver disease. Enhanced hepatic fatty acid synthesis and reduced fatty acid oxidation act together in the development of an alcohol-induced fatty liver (Feinman and Lieber, 1999; You et al., 2002; You and Crabb, 2004).

Sterol regulatory element-binding proteins (SREBPs) are a family of membrane-bound transcription factors that regulate lipid homeostasis by controlling the expression of genes for cholesterol and fatty acid metabolism. Three members of the SREBP family have been described in mammalian species: SREBP-1a and 1c are encoded by a single gene (*Srebf1*) located on human chromosome 17p11.2 (Hua et al., 1995), and SREBP-2 is encoded by a separate gene, *Srebf2*, which is located on human chromosome 22q13 (Miserez et al., 1997). SREBPs are synthesized as precursors that are bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBPs are transported to the nucleus, where they bind to sterol regulatory elements in the promoters of specific genes, recruit co-activators to the promoters and stimulate gene transcription (Nagoshi et al., 1999; Bennett et al., 2004). Specific analysis of individual isoforms has suggested that SREBP-1 is selectively involved in the activation of genes associated with fatty acid metabolism and *de novo* lipogenesis, whereas SREBP-2 may be more selective for genes that are involved directly in cholesterol homeostasis (Horton et al., 1998; Pai et al., 1998). SREBP transcription factors are regulated by many experimental and physiological conditions, including insulin, activation of the liver X receptor, glucose and steroids (reviewed by Eberlé et al., 2004). Recently, You et al. (2002) have reported that chronic ethanol feeding induces the fatty acid synthesis pathway by the activation of SREBP-1, which contributes to the development of an alcoholic fatty liver.

DNA microarray technology has been used in a number of studies to identify the global gene expression pattern and to elucidate the mechanisms of liver disease associated with chronic ethanol administration (Tadic et al., 2002; Deaciuc et al., 2004a,b; French et al., 2005). However, the literature does not contain any investigation of changes in hepatic gene expression following binge alcohol exposure. To identify the gene expression changes that cause hepatotoxicity and to characterize further the spectrum of fatty liver-related transcripts, the temporal and dose-dependent effects of acute ethanol intoxication on hepatic gene expression were examined in the context of complementary histological and clinical chemistry end-points. The present study identifies differentially expressed genes that can be applied to the biomarkers for alcohol-binge-induced fatty liver.

Materials and methods

Animal treatments. Specific pathogen-free male ICR mice (20–25 g) were obtained from Jung-Ang Laboratory Animal Co. (Seoul, Korea) and allowed free access to standard chow and tap water. They were kept in temperature-controlled and filter-sterilized animal quarters under a 12-h light:12-h dark cycle. The use of animals was in compliance with the guidelines established by the Animal Care Committee of this institute. A total of 27 mice (9 per group) was given a single oral dose of either 0.5 g/kg (low-dose group, L), 5 g/kg (high-dose group, H) or distilled water (control group, C) and three from each group were subsequently killed at 6 (C6, L6 and H6), 24 (C24, L24 and H24) or 72 h (C72, L72 and H72). A cross section of the left lateral lobe of the liver was collected in 10% neutral buffered formalin for histopathology. The remaining liver tissue was collected in RNase-free tubes and snap-frozen in liquid nitrogen. Frozen tissues were stored at −70 °C until processed for RNA extraction.

Clinical chemistry and histological analysis. Serum levels of alanine aminotransferase (ALT), alkaline phosphatase, blood urea nitrogen, creatinine, total bilirubin, triglyceride and albumin were monitored by standard clinical chemistry assays on an Automated Chemistry Analyzer (Prestige 24I; Tokyo Boeki Medical System, Tokyo, Japan). Liver tissues collected in formalin were dehydrated, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H&E). Histopathologic examinations of the liver sections were conducted by a pathologist and were peer-reviewed.

RNA isolation. Total RNA was extracted using an Easy-Blue™ total RNA extraction kit (Intron Biotech, Sungnam, Korea), purified using Qiagen RNeasy® Mini Kits (Qiagen, Basel, Switzerland) and examined for integrity using an Agilent 2100 Bioanalyzer (Ambion, Austin, TX).

Microarray analysis of differential gene expression. Applied Biosystems (Foster City, CA) Mouse Genome Survey Microarrays were used to analyze differential gene expression profiles. Microarray analysis was performed as described in our previous report (Yin et al., 2006). Briefly, each microarray was first prehybridized at 55 °C for 1 h in hybridization buffer with blocking reagent. Ten micrograms of digoxigenin-UTP labeled cRNA targets were cut with fragmentation buffer at 60 °C for 30 min and hybridized to each microarray in a 0.5-ml volume at 55 °C for 16 h. Images were auto-gridded and spots were spatially normalized. Chemiluminescent signals were quantified, corrected for background and the final images and feature data were processed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer v1.1.

Data analysis. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. For faultless results, instead of using raw data of bad spots, we removed the bad spots and then imputed their values using KNN imputation algorithm (Troyanskaya et al., 2001). The bad spots are identified according to the manufacturer's recommendation: the value of quality flag is less than 100. And we used "assay-normalized signals" feature as a measurement for mRNA abundance according to the manufacturer's protocol. Next we performed log-scale data transformation using the variance stabilizing normalization method to attain more maneuverable value range and log-normal assumption. Then we equalized the each array's value distribution for fair across-chip comparison using quantile-normalization (Bolstad et al., 2003). The overall flow of data analysis is illustrated in Fig. 1. Two-way analysis of variance (ANOVA) was applied to determine both time and dose effects and differentially expressed sets of genes. Since the ANOVA test is repeated for each gene, we did Benjamini–Hochberg multiple-testing correction for FDR control of the result. Through two-way ANOVA test, we identified genes with plausible biological interpretation according to the significance of the terms in ANOVA models. For further analysis, these genes are divided into gene clusters according to their expression profile using Cluster/TreeView analytic package, so that genes with similar expression behavior are in same cluster (Eisen, Stanford University, CA). Finally, we tried to understand the meaning of these expression clusters using existing knowledge such as annotations in Panther database (www.pantherdb.org) and the pathway information in ArrayXPath (www.snubi.org/software/ArrayXPath/).

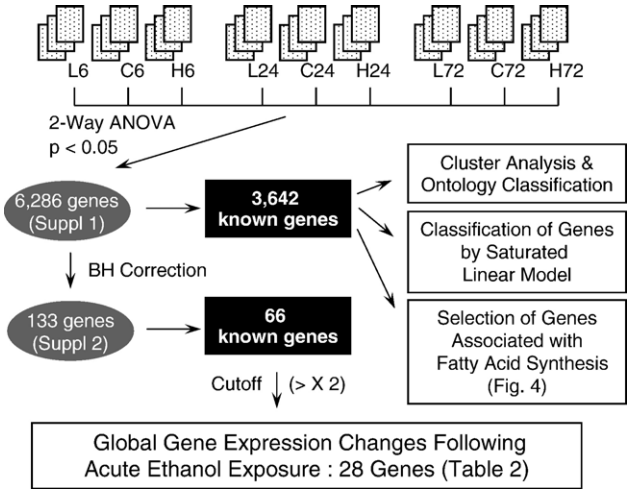

Fig. 1. Flow chart for data analysis. The microarray data were analyzed by two-way ANOVA, adjusted by the Benjamini–Hochberg multiple testing correction and subjected to a cutoff of 2-fold or greater induction or repression. C, L and H indicate the control, low-dose group (0.5 g/kg) and high-dose group (5 g/kg) and the numbers after the symbol indicate the time point when the animals were sacrificed. Suppl. 1 and 2: Supplementary data 1 and 2, respectively.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was prepared using the Easy-Blue™ Total RNA Extraction Kit (Intron Biotech, Korea) and single-strand cDNA was synthesized from the RNA in a reaction mixture containing random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Gene-specific primers designed using Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) were used (Table 1). qRT-PCR amplifications were performed using Fast Start DNA Master SYBR Green I Mixture Kit (Roche Diagnostics, USA) in a Light Cycler system (Roche Diagnostics, USA) following manufacturer's protocol. To determine the specificity of amplification, melting curve analysis was applied to all final PCR products.

Western blot analysis. Nuclear extracts from mouse livers were prepared as described previously (You et al., 2002). Eighty micrograms of the protein was separated by 10% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunoblotted with rabbit polyclonal Srebf1 antibody (Abcam, Cambridge, MA). Detection was performed by using enhanced chemiluminescence Western Blotting Detection Reagents (Amersham, Piscataway, NJ).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed as described elsewhere (Nelson et al., 2006a) with some modifications. Briefly, liver tissue homogenate was fixed with 1% formaldehyde in PBS and the cross-linking was stopped with 125 mM glycine at room temperature. Chromatin was sheared by sonication and immunoprecipitated with anti-SREBP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or non-immune IgG antibody at 4 °C. Immune complexes were collected with protein A-Sepharose beads (Amersham Biosciences). Total DNA was purified by Chelex 100-based method (Nelson et al., 2006b). Promoter-specific primer pairs used in qRT-PCR are as follows: Acly-sense, 5′-TGCGCTCACATCCATCACT, Acly-antisense, 5′-GAAGTTGCGTCAGGTCTCG; Fasn-sense, 5′-ACGTCCTTCCTCCCTGTCTT, Fasn-antisense, 5′-GCCCTCCTGTTGCTTTGTC; Mod1-sense, 5′-CTC-TGGCAAGCACTTTGTCCC, Mod-antisense, 5′-GAAATGGCATGGCGTACT-CAC; Scd1-sense, 5′-GAGTACCAGACTAGGACCAATG, Scd1-antisense, 5′-TGACCTGAAAGCCGAGAA.

Results

Clinical chemistry and histological end-points

ICR mice were treated with 0.5 or 5 g/kg ethanol, and acute hepatotoxicity was evaluated by measuring plasma ALT and histology. No significant changes in hepatic enzyme activity were observed between the control and ethanol-treated mice (Fig. 2A). In the histological analysis, no biologically significant liver damage was noted, except in the high-dose ethanol group at 24 h, which presented with a diffuse, mild fatty liver (Fig. 2B). The other serum biomarkers tested in this study were not significantly changed by the ethanol treatment (data not shown).

Global gene expression analysis following ethanol treatment

To identify global gene expression changes associated with acute ethanol-induced hepatotoxicity, two-way ANOVA was conducted on the dose- and time-response data. From a total of 32,381 probe sets, 6286 genes were identified as being ethanol-modulated in a statistically significant manner ($p<0.05$, Supplementary data 1). Functional categorization of the data was performed using the annotation information in the PANTHER database for ontology. The ethanol-induced changes in expression included genes that are involved in a variety of biological processes. The largest group of genes comprised

Table 1
Gene-specific primers used in quantitative real-time PCR

Gene	NCBI RefSeq	Forward primer (5′-3′)	Reverse primer (5′-3′)
Auh	NM_016709	GCCATGTGTTAGAACAGAACCA	TCTGAGCATAGCAGGCTTCTT
Ctgf	NM_010217	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA
Gga1	NM_145929	GGAGGCACGAATCAACAGAG	CACGTCTCCAGAACCGTCAAG
Helb	NM_080446	CTGCACCCGTACAAGAGCG	TCATCAGGGATAGACACTCGC
Rpp14	NM_025938	CCCCTCAGAGTATCACTACATGA	TCGTAGGTGAGAACATCCACAG
Rps6ka2	NM_011299	CCCTCCCAGTTCGAGCTACTA	CTCGCACTTTCAAGGTGGCT
Zfhx1b	NM_015753	AGCGACACGGCCATTATTTAC	GTTGGGCAAAAGCATCTGGAG
Acly	NM_134037	CAGCCAAGGCAATTTCAGAGC	CTCGACGTTTGATTAACTGGTCT
Fasn	NM_007988	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
Mod1	NM_008615	TGGACCTGCAAGACAGAAATG	AACTGAAGCAATGTGCCCTTT
Scd1	NM_009127	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Srebf1	NM_011480	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
6820449I-09Rik	NM_177128	AACCCGAGCAATTCCTAAGTAGA	GAGGTTTGGTTCCACCGATAAA
Gapdh	NM_001001303	CGGTGCTGAGTATGTCG	TTCTGGGTGGCAGTGAT

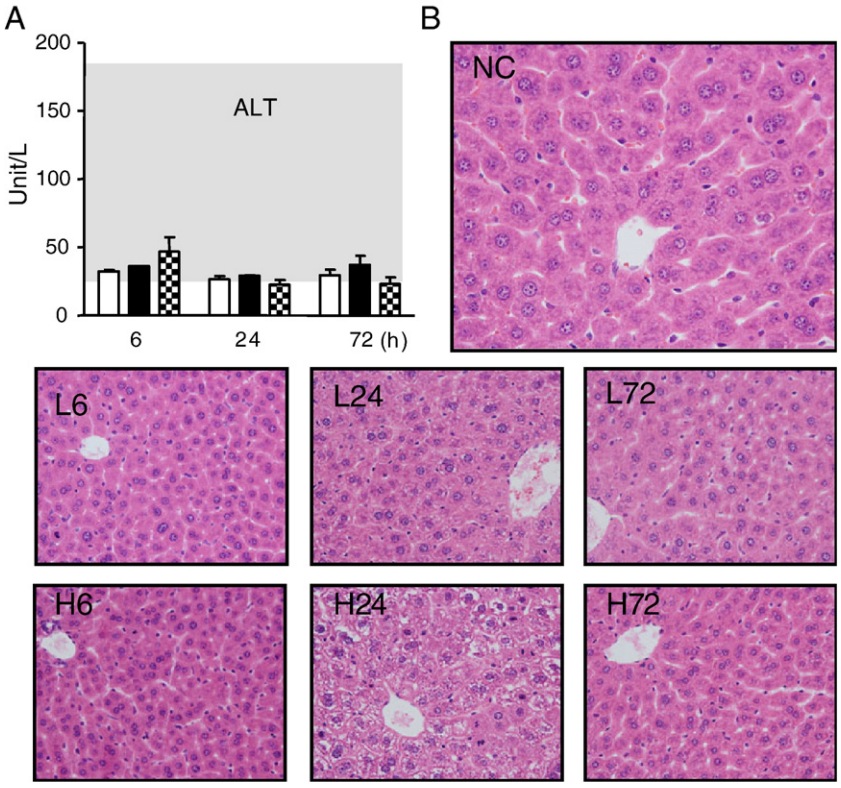

Fig. 2. Biochemical and pathological analyses of the sera and livers obtained from ICR mice after oral exposure to ethanol. (A) The levels of serum ALT were measured at each time point in the vehicle (□), low-dose ethanol (■) and high-dose ethanol (⊠) treatment groups. The normal range of ALT in ICR mice is indicated in gray. The results shown are the mean±SD of three mice. (B) Histopathological analysis of hematoxylin and eosin-stained paraffin sections (×400).

those genes associated with signal transduction, while genes involved in nucleic acid metabolism, protein metabolism and developmental processes were the next most abundant annotation groups. Tree analysis of the genes classified the patterns of responses for all the treatment conditions relative to the controls. The greatest distance between nodes was between the low-dose 6-h (L6) group and the high-dose 72-h (H72) group. The low- and high-dose groups at each time point were in the same node, and the distances between the nodes increased relative to the duration of treatment, which indicates a stronger influence of time than dose in our experimental regimen.

Adjustment of the data by the Benjamini–Hochberg multiple testing correction (Benjamini and Hochberg, 1995) reduced the number of affected genes to 133 (Supplementary data 2). After removal of the unidentified probe sets, 66 known genes were selected. When the data were filtered on the basis of 2-fold up- or downward changes in expression level, 28 genes were extracted, which were considered to be ethanol-responsive genes in the liver. Functional categorization of the genes using the PANTHER database for ontology is presented in Table 2. In a broader sense, ethanol-responsive genes are not limited to these 28 genes, but include all of the 6286 genes selected by the two-way ANOVA.

Validation of the microarray data

The microarray results were confirmed for a subset of genes using qRT-PCR. In the one-point validation, the expression levels of eight genes with various degrees of up- or down-regulation were verified in the H6 group (Table 3). In the kinetic validation, the expression levels of *Srebf1* and four target genes were evaluated at each time point (Fig. 3). In general, the results obtained from the microarray experiments correlated well with the qRT-PCR data. Of all the genes quantified, only the results for qRT-PCR of *Ctgf* differed from the microarray results. Although the *Ctgf* transcript showed slight upregulation in the qRT-PCR analysis, it showed significant downregulation in the microarray analysis.

Expression of genes associated with fatty acid synthesis

The effects of ethanol on the expression of genes associated with fatty acid synthesis were investigated. In general, the expression of several genes involved in fatty acid synthesis was upregulated in the H6 group. The expression of *Srebf1* was increased 2.7-fold in the H6 group and was decreased to the level of the control in the H24 and H72 groups (Figs. 3 and 4). Of the genes known to contain a sterol regulatory element in their promoters and to be regulated by SREBP1, the genes for ATP-citrate lyase (*Acly*), fatty acid synthase (*Fasn*), malic enzyme (*Mod1*) and for stearyl-CoA desaturase (*Scd1*) were all upregulated. In the elongation process, the expression levels of *Hadhsc* and *Ehhadh*, which have 3-hydroxyacyl-CoA dehydrogenase activities, were increased by ethanol treatment. As shown in Fig. 4, the overall net effect of altered gene expression associated with fatty acid synthesis was to increase fatty acid

Table 2
Genes altered more than 2-fold ($p<0.05$) in the liver of mice treated with ethanol

Gene symbol	Grip query	L6	L24	L72	H6	H24	H72	Biological process
Trpc1	NM_011643	−0.72	0.45	0.48	0.37	1.02	0.69	*SP, ST*
Auh	NM_016709	−0.92	0.96	−0.46	−0.10	0.89	−1.19	*CM, LM*
Whsc1l1	NM_001001735	−1.64	−0.40	0.08	−0.75	0.26	−1.03	*NM*
Csf1	M21149	−1.26	−0.45	0.29	−0.55	−0.40	0.51	*DP, ID, ST*
Hpse	NM_152803	−0.05	−0.18	−0.78	−0.45	0.13	−1.54	*DP*
Srd5a2l	NM_020611	0.04	−0.10	0.85	0.03	1.04	1.16	*DP*
Olfr976	NM_146367	0.14	−0.60	0.96	0.04	−0.15	1.11	*SP, ST*
Kif2c	NM_134471	1.12	0.56	1.02	−0.23	0.48	1.12	*DP*
Hlf	NA	0.57	−0.47	−1.07	0.25	−0.70	−1.04	*DP, NM*
Fbxo16	NM_015795	1.49	0.45	−0.72	−0.19	0.72	−0.60	*OM*
Man1b	NM_010763	−0.69	1.25	1.83	−0.29	0.88	0.25	*CM, PM*
9430095K15Rik	AK035166	1.00	1.35	−0.43	0.45	0.72	−0.54	*DP, ST*
Pla2g7	NM_013737	−1.32	−0.02	−0.40	0.07	1.11	0.54	*ID, LM*
Rpl23	AK002579	0.07	0.15	−1.34	−0.25	−0.72	−1.17	*PM*
Gga1	NM_145929	−0.97	1.36	0.09	−0.47	1.03	1.20	*PM, PT*
Rps6ka2	NM_011299	0.67	0.74	0.02	0.44	0.88	−1.26	*CC, DP, PM, ST*
Ctgf	NM_010217	−0.73	−0.69	0.35	−0.64	−1.29	−0.13	*ST*
2810037C14Rik	AK015272	0.34	−0.14	−0.51	−0.78	−0.08	−1.09	*ON*
2310007F12Rik	NM_175146	−0.64	0.35	−0.96	−0.06	−0.10	−1.05	Unclassified
2810032G03Rik	NM_028318	0.29	−1.55	0.63	0.13	−1.57	0.21	Unclassified
6820449I09Rik	NM_177128	0.90	−1.10	0.19	0.82	0.59	−0.36	Unclassified
A930016D02Rik	NM_176920	0.53	−0.44	1.66	0.26	−0.17	0.70	Unclassified
Helb	NM_080446	−1.10	−0.68	−0.18	0.25	−1.20	−0.30	Unclassified
MGC86136	NM_207245	0.15	−0.39	0.99	−0.14	−0.66	1.13	Unclassified
Olfr1038	NM_147013	−0.73	0.10	0.60	−0.07	0.11	1.13	Unclassified
Rpp14	NM_025938	−0.70	0.31	−1.05	−0.89	−0.39	−0.73	Unclassified
Taf1a	NM_021466	−0.73	−0.30	−1.02	−0.81	0.08	−0.37	Unclassified
Zfhx1b	NM_015753	−1.02	0.88	0.07	0.01	1.04	0.50	Unclassified

Values represent fold-changes in $\log_2$ scale compared to corresponding vehicle control.
CC, cell cycle; CM, carbohydrate metabolism; DP, developmental process; ID, immunity and defense; LM, lipid metabolism; NM, nucleic acid metabolism; OM, other metabolism; ON, oncogenes; PM, protein metabolism and modification; PT, protein traffic; SP, sensory perception; ST, signal transduction.

biosynthesis, which may contribute to the induction of acute alcoholic fatty liver. Interestingly, the kinetic changes in the expression of Srebf1 and the SREBP target genes correlated well in the microarray and qRT-PCR analyses (Fig. 3). The change in the *Srebf1* mRNA level correlates well with that of the SREBP1 protein expression as well as its binding to the promoters of the target genes (Fig. 5). The increases in expression of these genes (H6) preceded ethanol-induced development of a fatty liver (H24). These results suggest the potential use of these genes as predictive biomarkers of alcohol binge-induced steatogenic hepatotoxicity.

Table 3
Quantitative real-time PCR validation of selected genes from microarray data in H6 group

Gene	Microarray	qRT-PCR
Auh	−0.10±0.12	0.16±0.07
Ctgf	−0.64±0.20	0.30±0.07
Gga1	−0.47±0.08	−0.69±0.14
Helb	0.25±0.08	0.41±0.04
Rpp14	−0.89±0.09	−1.18±0.09
Rps6ka2	0.44±0.14	0.30±0.05
Zfhx1b	0.01±0.10	−0.17±0.08
6820449I09Rik	0.82±0.62	0.94±0.05

Data are mean±SEM of three independent measurements, which represent fold change on log2 scale compared to data obtained from C6 group.

Discussion

Chemical-induced hepatotoxicity is manifested by various types of injury, depending on the nature and dose of the chemical. Ethanol is categorized as a mixed-type hepatotoxicant that induces cumulative damage, starting from mild steatosis and ending in irreversible end-stage liver lesions. Despite the enormous amount of information in the literature on the properties and mechanism of ethanol-induced hepatic steatosis, it is still difficult to discern what exactly happens in binge drinkers and how the steatotic hepatotoxicity associated with this type of alcohol consumption can be predicted. The analysis of short-term toxicogenomic data to predict the results of long-duration studies requires the development of a database of compounds with known chronic toxicities. Although many technical problems currently limit the usefulness of this concept, pattern recognition experiments appear to hold considerable promise (Hamadeh et al., 2002). For this purpose, the Korean Toxicogenomic Research Consortium has embarked on a project to construct a toxicogenomic database of known hepatotoxicants, including tetracycline (Yin et al., 2006), diclofenac (Chung et al., 2006a), D-galactosamine (Chung et al., 2006b), valproic acid, methotrexate, α-naphthylisothiocyanate, 4,4′-methylene dianiline, 6-mercaptopurine, phenylbutazone and ethanol.

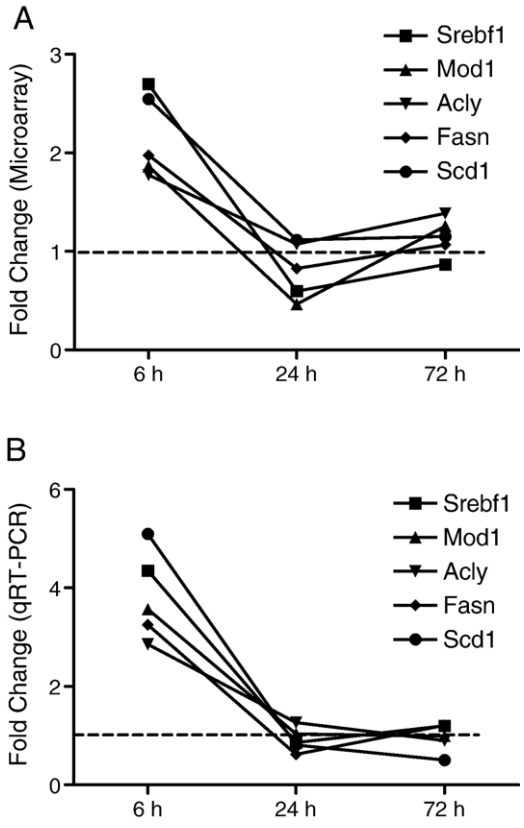

Fig. 3. Temporal changes in the expression levels (A, microarray; B, qRT-PCR) of *Srebf1* and four target genes in the high-dose group, expressed as fold-changes compared to the corresponding control groups.

Ethanol-induced changes in global gene expression

When the microarray data from the nine experimental groups were analyzed by two-way ANOVA, the expression levels of about 19% (6286 of 32,381) of the whole genes showed statistically significant changes. To control the increased chance of discovering false-positives by applying false discovery rate (FDR), we applied the Benjamini–Hochberg multiple testing correction method (FDR <0.05). Among the 133 genes called significant, about less than seven were expected to be false-positive. The 26 genes having ethanol-specific gene expression pattern can be functionally categorized into critical cellular processes, like the metabolic processes of lipids, carbohydrates, nucleic acids and proteins, as well as developmental processes.

Transient receptor potential canonical 1 (Trpc1), an extensive family of mammalian transient receptor potential channels, has been found to possess sensing capabilities for a range of factors including redox status, arachidonic acid metabolites and growth factors (Clapham, 2003). Upregulated TRPC1 is a general feature of smooth muscle cells in occlusive vascular disease and that TRPC1 inhibitors have potential as protective agents against human vascular failure (Kumar et al., 2006). In the liver cells, Trpc1 mediates the inflow of Na^+ and Ca^{2+} to the cytoplasmic space and hence modulates the intracellular concentrations of Ca^{2+} and Na^+, which may regulate cell volume directly or indirectly (Chen and Barritt, 2003). AU RNA-binding protein/enoyl-coenzyme A hydratase (Auh) protein binds to the AU-rich element of RNA, which has a role in directing RNA to rapid degradation and deadenylation. Auh is also homologous to enoyl-CoA hydratase, an enzyme

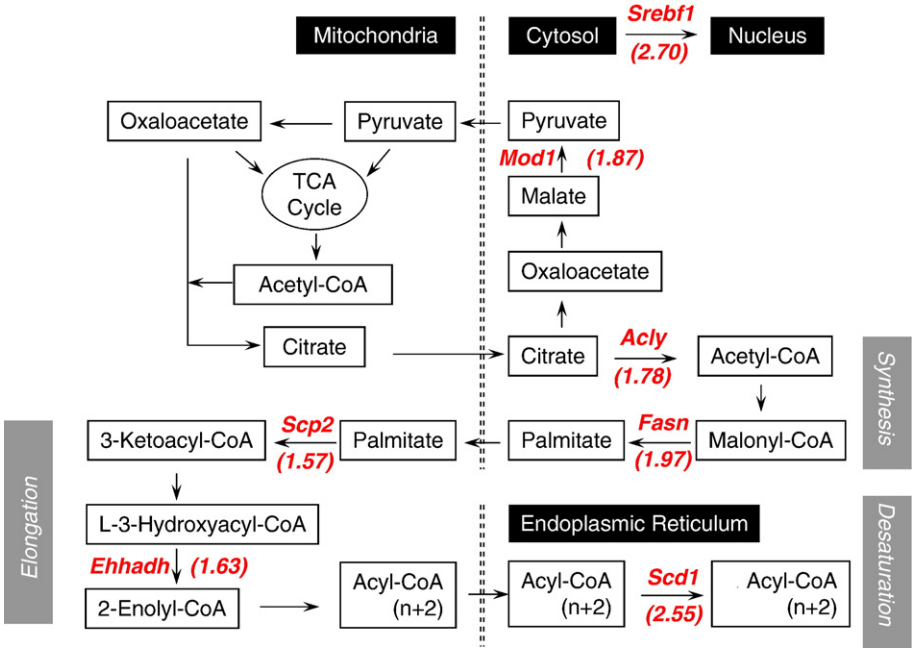

Fig. 4. The pathway for fatty acid synthesis and the relative induction or repression of genes with statistically significant differences ($p<0.05$) from the vehicle control (two-way ANOVA). The numbers in parenthesis represent the fold-changes in H6 compared to the C6 group. To make the data more intelligible, they are presented in natural numbers (not in a log2 scale). *Scp2*, sterol carrier protein 2; *Ehhadh*, enoyl-coenzyme A hydratase/3-hydroxyacyl coenzyme A dehydrogenase; *Srebf1*, sterol regulatory element-binding factor 1; *Mod1*, malic enzyme; *Acly*, ATP citrate lyase; *Fasn*, fatty acid synthase; *Scd1*, stearyl-coenzyme A desaturase 1.

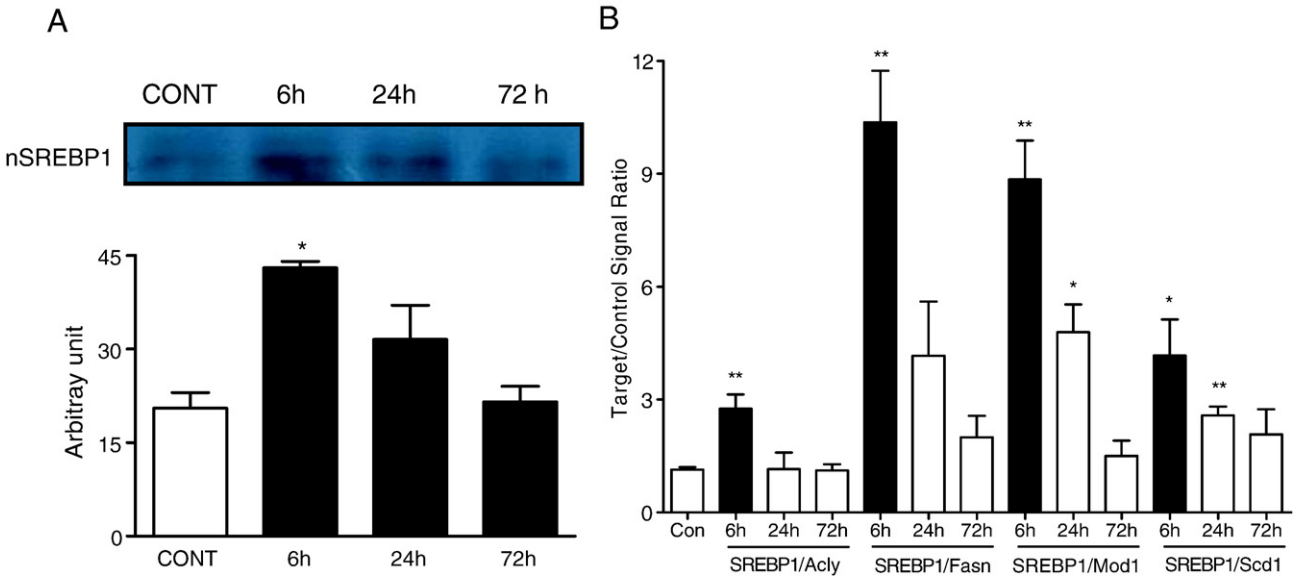

Fig. 5. Kinetic changes of the expression of SREBP1 protein and the binding to the promoters of the target genes. (A) Representative Western blots show the kinetic changes of SREBP1 expression following ethanol treatment. Each bar represents the mean intensity with standard deviation obtained from 3 mice. A single dose of ethanol (5 g/kg) was administrated to the ICR mice and nuclear fraction of liver sample was separated on a 10% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with the rabbit polyclonal SREBP1 antibody. (B) ChIP assays were performed using liver samples from ICR mice after oral exposure to ethanol. Following immunoprecipitation, DNA was extracted and analyzed for the presence of the SREBP1-binding region of each target gene by real-time PCR. Each bar represents the mean intensity with standard deviation obtained from 3 mice (*$p<0.05$;**$p<0.01$).

involved in fatty acid degradation, and has been shown to have intrinsic hydratase enzyme activity (Nakagawa et al., 1995). Ribosomal protein S6 kinase, polypeptide 2 (Rps6ka2), also known as MAPKAPK1C, p90rsk, pp90rsk, Rps6ka-rs1, Rsk3, RSK-3 or S6K-alpha 2, encodes a member of the ribosomal S6 kinase family of serine/threonine kinases. This kinase contains 2 nonidentical kinase catalytic domains and phosphorylates various substrates, including members of the mitogen-activated kinase (MAPK) signalling pathway. The activity of this protein has been implicated in controlling cell growth and differentiation (Blenis, 1993; Bonni et al., 1999). Considering these results, the global gene expression profile of mouse liver after acute ethanol exposure may serve as early biomarker for ethanol-induced liver injury.

Ethanol-induced changes in gene expression associated with fatty acid synthesis

The animal model for acute ethanol-induced hepatotoxicity in human binge drinkers was developed by Carson and Pruett (1996) and modified by Song et al. (2003). In the present study, we administered ICR mice with a single oral dose of 0.5 or 5 g/kg ethanol. A typical fatty liver change was noted in the H24 group, which recovered by 72 h. Therefore, our model is appropriate for studies of the global and temporal changes in gene expression associated with acute ethanol-induced hepatotoxicity. Many studies of hepatic gene expression profiling have been performed with chronic ethanol treatment models that use intragastric alcohol infusion or the administration of alcohol-containing liquid diets (Tadic et al., 2002; Deaciuc et al., 2004a,b; French et al., 2005). However, a hepatic genome-wide analysis following the acute administration of ethanol has not been performed previously.

Although the mechanisms by which ethanol induces hepatic steatosis are complex and not yet fully understood, the following factors have been proposed as potential mechanisms: an accelerated supply of free fatty acids (Lieber et al., 1966); impaired β-oxidation of fatty acids (Rabinowitz et al., 1991); and inhibition of lipoprotein secretion from the liver (Venkatesan et al., 1988). Recently, You et al. (2002) have reported that the metabolism of ethanol increased hepatic lipogenesis by activating SREBP-1, and that this effect of ethanol may contribute to the development of an alcoholic fatty liver. These authors also identified the AMP-activated protein kinase (AMPK) as a key regulator of SREBP-1 activation (You et al., 2004). The role of AMPK in the expression of hepatic SREBP-1, fatty acid synthesis and steatogenesis was further verified using the AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (Tomita et al., 2005).

Several studies of animals treated chronically with ethanol have revealed a significant increase in hepatic lipogenesis (Lieber et al., 1966; Carrasco et al., 2001), which is closely associated with an increase in many lipogenic enzymes, such as fatty acid synthase (*Fasn*), malic enzyme (*Mod1*), acetyl-CoA carboxylase (*Acc*), ATP citrate lyase (*Acly*) and 6-phosphogluconate dehydrogenase (Joly et al., 1973; Arakawa et al., 1975; Muramatsu et al., 1981). Although there exist some controversies over the changes in lipogenic enzyme activities by ethanol, such as ATP citrate lyase and malic enzyme, most of the discrepancies are due to the different treatment conditions and animal models that have been used (Simpson et al., 1994; Oyama et al., 2000). The most notable change in gene

expression associated with an ethanol-induced fatty liver in the present study was the upregulation of the *Srebf1* gene in the L6 and H6 groups. This is the first report that the expression of *Srebf1* is increased in an acute ethanol-induced hepatic steatosis animal model. Consequently, the known target genes of SREBP1, which include *Mod1, Acly, Fasn* and *Scd1*, were also upregulated. Dynamic changes in the expression of these genes preceded the pathologic changes manifested by ethanol (Fig. 3), which suggests the potential use of these genes as biomarkers for acute ethanol-induced steatosis.

Evidence exists that other steatogenic chemicals, such as orotic acid and conjugated linoleic acid, exert their actions by inducing lipogenesis through the transactivation of SREBP-1 and the subsequent expression of genes associated with fatty acid synthesis (Clement et al., 2002; Buang et al., 2005). Transgenic mice that overexpress the NH_2-terminal domain of SREBP-1a, which does not need to be cleaved proteolytically to activate transcription, develop massive enlargement of the liver owing to the increased expression of the gene for lipogenesis (Shimano et al., 1996a,b). In contrast, mice that are deficient for leptin and SREBP-1 [ob/ob × Srebp-$1^{(-/-)}$] have been found to have a markedly attenuated fatty liver, as found for ob/ob mice, with a concurrent decrease in the level of mRNA for fatty liver synthase (Yahagi et al., 2002). Similar results have been found in many experiments in which dietary components or the fat-derived hormone adiponectin reduce the fatty liver via the downregulation of SREBP-1 in normal, ob/ob or chronic alcohol-fed mice (Sekiya et al., 2003; Xu et al., 2003; Ascencio et al., 2004).

In summary, we document a global gene expression profile for the ICR mouse liver following acute ethanol treatment, as determined using oligonucleotide microarrays. We identify 26 ethanol-responsive genes that are significantly up- or down-regulated more than 2-fold. We also show that acute ethanol affects the expression levels of *Srebf1* and many other SREBP target genes, thereby increasing fatty acid synthesis.

These results support the hypothesis by which ethanol-induced steatosis in mice is mediated by the fatty acid synthetic pathway regulated by SREBP1. These results contribute to the production of a database that can be used for the prediction of toxicity through pattern recognition.

Acknowledgment

This work was supported by a Korea Food and Drug Administration grant (KFDA-05122-TGP-584).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2007.06.018.

References

Arakawa, M., Taketomi, S., Furuno, K., Matsuno, T., Iwatsuka, H., 1975. Metabolic studies on the development of ethanol-induced fatty liver in KK-Ay mice. J. Nutr. 105, 1500–1508.

Ascencio, C., Torres, N., Isoard-Acosta, F., Gomez-Perez, F.J., Hernandez-Pando, R., Tovar, A.R., 2004. Soy protein affects serum insulin and hepatic SREBP-1 mRNA and reduces fatty liver in rats. J. Nutr. 134, 522–529.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc., Ser. A Stat. Soc. 57, 289–300.

Bennett, M.K., Toth, J.I., Osborne, T.F., 2004. Selective association of SREBP isoforms with target promoters in vivo. J. Biol. Chem. 279, 37360–37367.

Blenis, J., 1993. Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. U.S.A. 90, 5889–5892.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19, 185–193.

Bonni, A., Brunet, A., West, AE., Datta, SR., Takasu, MA., Greenberg, ME., 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286, 1358–1362.

Buang, Y., Wang, Y.M., Cha, J.Y., Nagao, K., Yanagita, T., 2005. Dietary phosphatidylcholine alleviates fatty liver induced by orotic acid. Nutrition 21, 867–873.

Carrasco, M.P., Marco, C., Segovia, J.L., 2001. Chronic ingestion of ethanol stimulates lipogenic response in rat hepatocytes. Life Sci. 68, 1295–1304.

Carson, E., Pruett, S., 1996. Development and characterization of a binge drinking model in mice for evaluation of the immunological effects of ethanol. Alcohol Clin. Exp. Res. 20, 132–138.

Chen, J., Barritt, G.J., 2003. Evidence that TRPC1 (transient receptor potential canonical 1) forms a Ca(2+)-permeable channel linked to the regulation of cell volume in liver cells obtained using small interfering RNA targeted against TRPC1. Biochem. J. 373 (Pt2), 327–336.

Chung, H., Kim, H.J., Jang, K.S., Kim, M., Yang, J., Kim, J.H., Lee, Y.S., Kong, G., 2006a. Comprehensive analysis of differential gene expression profiles on diclofenac-induced acute mouse liver injury and recovery. Toxicol. Lett. 166, 77–87.

Chung, H., Kim, H.J., Jang, K.S., Kim, M., Yang, J., Kang, K.S., Kim, H.L., Yoon, B.I., Lee, M.O., Lee, B.H., Kim, J.H., Lee, Y.S., Kong, G., 2006b. Comprehensive analysis of differential gene expression profiles on D-galactosamine-induced acute mouse liver injury and regeneration. Toxicology 227, 136–144.

Clapham, D.E., 2003. TRP channels as cellular sensors. Nature. 426, 517–524.

Clement, L., Poirier, H., Niot, I., Bocher, V., Guerre-Millo, M., Krief, S., Staels, B., Besnard, P., 2002. Dietary trans-10, cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. J. Lipid Res. 43, 1400–1409.

Deaciuc, I.V., Arteel, G.E., Peng, X., Hill, D.B., McClain, C.J., 2004a. Gene expression in the liver of rats fed alcohol by means of intragastric infusion. Alcohol 33, 17–30.

Deaciuc, I.V., Doherty, D.E., Burikhanov, R., Lee, E.Y., Stromberg, A.J., Peng, X., de Villiers, W.J.S., 2004b. Large-scale gene profiling of the liver in a mouse model of chronic, intragastric ethanol infusion. J. Hepatol. 40, 219–227.

Diehl, A.M., 1997. Alcoholic liver disease: natural history. Liver Transpl. Surg. 3, 206–211.

Eberlé, D., Hegarty, B., Bossard, P., Ferré, P., Foufelle, F., 2004. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 86, 839–848.

Feinman, L., Lieber, C.S., 1999. Ethanol and lipid metabolism. Am. J. Clin. Nutr. 70, 791–792.

French, B.A., Dedes, J., Bardag-Gorce, F., Li, J., Wilson, L., Fu, P., Nan, L., French, S.W., 2005. Microarray analysis of gene expression in the liver during the urinary ethanol cycle in rats fed ethanol intragastrically at a constant rate. Exp. Mol. Pathol. 79, 87–94.

Hamadeh, H.K., Bushel, P.R., Jayadev, S., Martin, K., DiSorbo, O., Sieber, S., Bennett, L., Tennant, R., Stoll, R., Barrett, J.C., Blanchard, K., Paules, R.S., Afshari, C.A., 2002. Gene expression analysis reveals chemical-specific profiles. Toxicol. Sci. 67, 219–231.

Horton, J.D., Shimomura, I., Brown, M.S., Hammer, R.E., Goldstein, J.L., Shimano, H., 1998. Activation of cholesterol synthesis in preference to fatty

acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J. Clin. Invest. 101, 2331–2339.

Hua, X., Wu, J., Goldstein, J.L., Brown, M.S., Hobbs, H.H., 1995. Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13. Genomics 25, 667–673.

Joly, J.G., Feinman, L., Ishii, H., Lieber, C.S., 1973. Effect of chronic ethanol feeding on hepatic microsomal glycerophosphate acyltransferase activity. J. Lipid Res. 14, 337–343.

Kumar, B., Dreja, K., Shah, S.S., Cheong, A., Xu, S.Z., Sukumar, P., Naylor, J., Forte, A., Cipollaro, M., McHugh, D., Kingston, P.A., Heagerty, A.M., Munsch, C.M., Bergdahl, A., Hultgardh-Nilsson, A., Gomez, M.F., Porter, K.E., Hellstrand, P., Beech, D.J., 2006. Upregulated TRPC1 channel in vascular injury in vivo and its role in human neointimal hyperplasia. Circ. Res. 98, 557–563.

Lieber, C.S., Spritz, N., DeCarli, L.M., 1966. Role of dietary, adipose, and endogenously synthesized fatty acids in the pathogenesis of the alcoholic fatty liver. J. Clin. Invest. 45, 51–62.

Miserez, A.R., Cao, G., Probst, L.C., Hobbs, H.H., 1997. Structure of the human gene encoding sterol regulatory element binding protein-2 (SREBF2). Genomics 40, 31–40.

Muramatsu, M., Kuriyama, K., Yuki, T., Ohkuma, S., 1981. Hepatic lipogenesis and mobilization of peripheral fats in the formation of alcoholic fatty liver. Jpn. J. Pharmacol. 31, 931–940.

Nagoshi, E., Imamoto, N., Sato, R., Yoneda, Y., 1999. Nuclear import of SREBP-2, a basic helix-loop-helix-leucine zipper (bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin-β with HLH-Zip. Mol. Biol. Cell. 10, 2221–2223.

Nakagawa, J., Waldner, H., Meyer-Monard, S., Hofsteenge, J., Jeno, P., Moroni, C., 1995. AUH, a gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydratase activity. Proc. Natl. Acad. Sci. U.S.A. 92, 2051–2055.

Nelson, J.D., Denisenkoko, O., Bomsztyk, K., 2006a. Protocol for the fast chromatin Immunoprecipitation (ChIP) method. Nat. Protoc. 1, 179–185.

Nelson, J.D., Denisenko, O., Sova, P., Bomsztyk, K., 2006b. Fast chromatin immunoprecipitation assay. Nucleic Acids Res. 34, e2.

Neuman, M.G., 2003. Cytokine-central factors in alcoholic liver disease. Alcohol Res. Health 27, 307–316.

Oyama, L.M., Couto, R.C., Couto, G.E., Damaso, A.R., Oller do Nascimento, C.M., 2000. Ethanol intake during lactation. II. Effects on pups' liver and brain metabolism. Alcohol 21, 201–206.

Pai, J., Guryev, O., Brown, M.S., Goldstein, J.L., 1998. Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. J. Biol. Chem. 273, 26138–26148.

Rabinowitz, J.L., Staeffen, J., Hall, C.L., Brand, J.G., 1991. A probable defect in the β-oxidation of lipids in rats fed alcohol for 6 months. Alcohol 8, 241–246.

Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., Nakakuki, M., Nagai, R., Ishibashi, S., Osuga, J., Yamada, N., Shimano, H., 2003. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. Hepatology 38, 1529–1539.

Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, I., Brown, M.S., Goldstein, J.L., 1996a. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J. Clin. Invest. 98, 1575–1584.

Shimano, H., Horton, J.D., Hammer, R.E., Shimomura, I., Brown, M.S., Goldstein, J.L., 1996b. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J. Clin. Invest. 98, 1575–1584.

Simpson, K.J., Venkatesan, S., Peters, T.J., 1994. Fatty acid synthesis by rat liver after chronic ethanol feeding with a low-fat diet. Clin. Sci. 87, 441–446.

Song, Z., Zhou, Z., Chen, T., Hill, D., Kang, J., Barve, S., McClain, C., 2003. *S*-Adenosylmethionine (SAMe) protects against acute alcohol induced hepatotoxicity in mice. J. Nutr. Biochem. 14, 591–597.

Tadic, S.D., Elm, M.S., Li, H.S., Van Londen, G.J., Subbotin, V.M., Whitcomb, D.C., Eagon, P.K., 2002. Sex differences in hepatic gene expression in a rat model of ethanol-induced liver injury. J. Appl. Physiol. 93, 1057–1068.

Tomita, K., Tamiya, G., Ando, S., Kitamura, N., Koizumi, H., Kato, S., Horie, Y., Kaneko, T., Azuma, T., Nagata, H., Ishii, H., Hibi, T., 2005. AICAR, an AMPK activator, has protective effects on alcohol-induced fatty liver in rats. Alcohol. Clin. Exp. Res. 29, 240S–245S.

Treinen-Moslen, M., 2001. Toxic responses of the liver, In: Klassen, C.D. (Ed.), Casarett & Doull's Toxicology: The Basic Science of Poisons, 6th ed. McGraw-Hill, International Edition, pp. 471–489.

Troyanskaya, O., Cantor, M., Sherlock, G., Brown, P., Hastie, T., Tibshirani, R., Botstein, D., Altman, R.B., 2001. Missing value estimation methods for DNA microarrays. Bioinformatics 17, 520–525.

Venkatesan, S., Ward, R.J., Peters, T.J., 1988. Effect of chronic ethanol feeding on the hepatic secretion of very-low-density lipoproteins. Biochim. Biophys. Acta 960, 61–66.

Xu, A., Wang, Y., Keshaw, H., Xu, L.Y., Lam, K.S., Cooper, G.J., 2003. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver disease in mice. J. Clin. Invest. 112, 91–100.

Yahagi, N., Shimano, H., Hasty, A.H., Matsuzaka, T., Ide, T., Yoshikawa, T., Amemiya-Kudo, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., Yamada, N., 2002. Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. J. Biol. Chem. 277, 19353–19357.

Yin, H.Q., Kim, M., Kim, J.H, Kong, G., Lee, M.O., Kang, K.S., Yoon, B.I., Kim, H.L., Lee, B.H., 2006. Hepatic gene expression profiling and lipid homeostasis in mice exposed to steatogenic drug, tetracycline. Toxicol. Sci. 94, 206–216.

You, M., Crabb, D.W., 2004. Recent advances in alcoholic liver disease. II. Minireview: molecular mechanisms of alcoholic fatty liver. Am. J. Physiol.: Gastrointest. Liver Physiol. 287, G1–G6.

You, M., Fischer, M., Deeg, M.A., Crabb, D.W., 2002. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). J. Biol. Chem. 277, 29342–29347.

You, M., Matsumoto, M., Pacold, C.M., Cho, W.K., Crabb, D.W., 2004. The role of AMP-activated protein kinase in the action of ethanol in the liver. Gastroenterology 127, 1798–1808.