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Distinct methylation patterns in histone H3 at Lys-4 and Lys-9 correlate with up- & down-regulation of genes by ethanol in hepatocytes

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Abstract

Ethanol induced liver injury is associated with a global change in gene expression but its mechanisms are not known. We studied whether alcohol induced gene expression are associated with post-translational methylations of histone H3. Primary culture of rat hepatocytes were treated with ethanol (50 or 100 mM) for 24 hr and the status of methylation of H3 at lys 4 (H3dimeK4) or lys 9 (H3dimeK9) were monitored by western blotting using antibodies to dimethylated histone H3 at lys 4 or lys 9. The cells exposed to ethanol showed strikingly opposing behaviors in methylation patterns; H3dimeK9 methylation was decreased whereas H3dimeK4 increased. Similar results were obtained in the interphase nuclei. Their binding on the metaphase chromosomes exhibit distinct site specific pattern of accumulation. Next, chromatin immuno-precipitation of the ethanol treated samples with antibodies for methylated lys 4 or lys 9 histone H3 followed by amplification of the immuno-precipitated DNA, was used to determine their association with the promoters of genes up-or down regulated by ethanol. Lys4 methylation was associated with ethanol up-regulated genes (Adh, GST-yc2) whereas lys 9 methylation with down regulated genes (Lsdh, cytP4502c11) demonstrating a difference between these two methylations. These results suggest that exposure of hepatocytes to ethanol changes the expression of several susceptible genes which are associated with site specific modification of dimethylated forms of histone H3 amino termini at their regulatory regions.

Keywords

Ethanol; Histone; Methylation; Epigenetics; Liver; Hepatocytes

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Introduction

Alcohol affects virtually all the major organs in the human body but liver is the most affected. Ethanol causes oxidative and metabolic stress (Thurman et al., 1998; Lieber, 2000). Liver injury is also a complex multi-factorial process involving both parenchymal and non-parenchymal cells, hepatocytes, hepatic stellate/Ito cells, mononuclear and Kupffer cells (Nagy, 2004). Continued chronic exposure to ethanol may impair liver function via pathways that are dependent or independent on ethanol metabolism. Ethanol also alters signal transduction pathways that include G protein coupled receptors and protein kinases (e. g. PKC, Src, MAPKs) as well as transcription factors (Peoples et al., 1996; Nagy, 2004; Aroor & Shukla, 2004).

Prolonged exposure to ethanol impairs liver function via different pathways and exhibits global change in hepatic gene expression covering a wide spectrum of cellular function (Tadic et al., 2002; Deaciuc et al., 2004a,b; French et al., 2005). Thus changes in the expression of genes have been implicated in the development of alcoholic liver diseases (ALD). Genes that are involved in ethanol metabolism (Morimoto et al., 1993; Ronis et al., 1993; Deaciuc et al., 2004a,b) cell signaling (Mochly-Rosen et al., 1988; Gordon et al., 1986; Hong et al., 2002a,b) and apoptosis (Yacoub et al., 1995; Deaciuc et al., 1999; Deaciuc et al., 2002a,b; Koteish et al., 2002) are highly sensitive to alcohol. Micro-array studies have also shown profile of hepatic gene expressions induced by alcohol consumption (Tadic et al., 2002; Deaciuc et al., 2004a,b; French et al., 2005). These changes in liver transcriptomes affect different transcriptional factors, enzymes of several metabolic pathways, receptors, cytokines, signaling molecules, immuno-modulators, antioxidant enzymes and modifiers of proteins secretors etc. (Mochly-Rosen, et al., 1988; Hong et al., 2002; Deaciuc et al., 2004a,b). The changes of several unrelated gene families imply a major transition in chromatin structure or organization. Post-translational modifications of histone amino-termini, such as acetylation, phosphorylation and methylation are in most cases correlated with functional organization of chromosomes and chromatin dynamics (Kohlmaier et al., 2004; Jenuwein and Allis, 2001; Wilkins, 2005). An in vivo study on cell morphology reported that the size and shape of alcohol fed hepatocytes were reduced substantially relative to untreated cells (Noble and Tewari, 1973). Initially, it was reported that chromosomes of chronic alcohol treated hepatocytes have highly condensed and altered nonhistone nuclear proteins that may contribute as epigenetic signals (Mahadev and Vemuri, 1998; Park et al., 2003). Later, in vitro treatment of hepatocytes with alcohol showed an increase in histone H3 acetylation at lysine 9 residue that was transient but positive for transcriptional activation (Park et al., 2003; Park et al., 2005). In contrast to transient nature of acetylation and phosphorylation, the methylation of histone appears to be a relatively stable signature for long-term maintenance of epigenetic chromatin state (Boggs, et al., 2002; Peters et al., 2002). The interrelationship between changes in histone H3 lysine methylation and alteration of gene expression is increasingly being appreciated (Jenuwein and Allis 2001; Boggs et al, 2002). Epigenetics is proposed to play role in the cellular actions of ethanol (Shukla and Aroor, 2006). We have therefore analyzed the role of histone H3 methylation of Lysine 9 and Lysine 4 in relation to alcohol related gene expression to determine the potential link between transcriptional changes and covalent modifications of histone tails.

MATERIALS AND METHODS

Isolation of hepatocytes and alcohol treatment

Hepatocytes were isolated from male Sprague–Dawley rats (200–250 gms) using collagenase-perfusion protocol as described previously (Weng and Shukla, 2000). Viability of isolated hepatocytes was routinely about 95 %. Isolated hepatocytes were plated on collagen-coated dishes (7.5×10^6 cells/100 mm dish) in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Hepatocytes were allowed to attach to culture dishes

for 2 h and then treated with different concentrations of ethanol in DMEM containing 0.1% FBS for indicated time periods. We have shown earlier that at 24 hrs of ethanol treatment under these conditions about 15–20 % cells show apoptosis (Lee & Shukla, 2005).

Interphase cell indirect immunofluorescence and FISH analyses

Primary culture of hepatocytes were treated with ethanol or with culture media and fixed in 4% formaldehyde for 15 min at room temperature (RT). Cells were then permeabilized by a thorough wash in PBS. We washed cells three times in 50% formamide and 2X SSC, three times in 2X SSC and then once with PBS and 0.5% BSA before carrying out immunofluorescence staining. Cells were incubated with anti-rabbit histone H3 dimethylated Lysine-4 or Lysine-9 antibodies at 4°C overnight. After hybridization, cells were incubated in PBT solution for 30 min. Cells were further treated with Cy3-conjugated goat anti-rabbit secondary antibodies for 3 hr at room temperature. Cells were further counterstained with DAPI. Images were acquired using a Zeiss Axioplan 2 fluorescence microscope with an Orca 2 CCD camera (Hamamatsu) and Improvision software (IPLab). The amount of antibodies in the interphase nuclei was estimated by determining gray scale level using Metamorph version 4.6 (Universal Imaging, Westchester, PA).

Metaphase chromosome immunofluorescence

Primary hepatocytes were grown, harvested and collected on the microscope slides using Cytospin 3 centrifuge. Cells on the slides were fixed with 4% paraformaldehyde and washed twice with PBS. Modified histones were detected by indirect immunofluorescence as described (Kohlmaier et al., 2004). Briefly, cells were incubated for 1 hr at 37°C in a humid chamber with serial dilutions of either primary Lys-9- or Lys-4-dimethyl H3 or β -actin antisera, and washed in KCM (120 mM KCl, 20mM NaCl, 10 mM Tris-Cl, pH 8.0, 0.5 M EDTA, 0.1% Triton). We then added Cy3-conjugated, affinity-purified, donkey anti-rabbit IgG antibody (Jackson Immuno-Research) diluted 1:40 in KCM, and incubated the mixture for 30 min at room temperature. Cells were again washed with KCM and fixed in 4% formaldehyde for 10 min at room temperature. After washing with sterile water, chromosomes were counterstained with DAPI, mounted on the coverslips with anti-fade media (Vectashield) and viewed in a Zeiss Axiophot fluorescence microscope.

Immunoprecipitation of Chromatin

Chromatin immunoprecipitation (CHIP) assays were conducted as described previously (Park et al., 2005). In this case, formaldehyde-fixed chromatin was isolated from hepatocytes (Peters et al., 2002). In each immunoprecipitation reaction, we used approximately 3×10^6 cells-worth of sonicated chromatin and the antibodies as indicated in the text. After extensive washing, reverse cross-linking, RNase A and proteinase K digestions, we analyzed the immunoprecipitated DNA by semi quantitative PCR using primers specific for the promoter regions of rat Adh-1, GST-Yc2 (gene code S72506) CYP2C11 (X79081), Lsdh (J03863) and GAPDH genes. Primers sequences for the first four genes were derived from series of PCR amplification or previously described sequences (Park et al., 2005). For PCR, immuno-precipitated and input DNA were amplified in a same reaction for 35 cycles at 94°C for 45 sec, 58°C for 45 sec, 72°C for 1 min using two primer pairs spanning different promoter regions. The PCR products were electrophoresed in 2% agarose gel. We used following oligonucleotides for amplification of DNA from chromatin immuno-precipitated by the histone H3 methylated Lysine-9 and Lysine-4 antibodies (For additional primers see supplementary Table-1). Adh-1 gene forward primer 5' tgccacctgtctctctct 3' and reverse primer 5' tcccccttctcaactgtc 3' Glutathione-S transferase gene forward primer 5' ggggcagtgtcttgaag 3' and reverse primer 5' gcacaggcaaaacaagatg 3' Cytochrome P450 2C11 gene, forward primer 5' ctcgaagtagcattatgcattta 3' and reverse primer 5' gggtgtaaaagagaagatcaaaga 3' L-serine

dehydrogenase gene forward primer 5' acgagctgtacgcgttttaagt 3' and reverse primer 5' tcccaagactaactgtggacaa 3' GAPDH gene forward primer 5' tatgatgacatcaagaaggtgg 3' and reverse primer 5' caccaccctgttgctga 3'

Real time PCR analysis

For better quantification, same DNA from each sample was also amplified by real-time PCR. The amplification was performed using QPCR SYBR green master mix at the following conditions: 50°C for 2 min, 95°C for 15 min, followed by 35 cycles at 95°C for 25 sec and 60°C for 1 min. The estimated products amplified from the CHIP assay were normalized against that derived from the input DNA. Same set of primers was used for PCR and real time PCR amplification.

Northern blot hybridization

Total cellular RNA from primary rat hepatocyte was used for northern analysis based on methods described earlier (Bhadra et al. 1997 and following Trizol method). Hybridization with different [³²P] labeled RNA probes (Adh, GSTYc2, Lsdh, CYP2C11 and GAPDH as a loading control) was carried out as previously described (Bhadra et al. 1997).

Western blot Hybridization

Primary culture of hepatocytes were incubated with 50mM or 100mM ethanol, washed with ice cold PBS and lysed with 1X Lamelli buffer. The protein concentration was estimated by Lowry's method. The 50 µg protein/per lane was fractionated in 12% SDS-PAGE gel. Separated proteins were transferred to a PVDF membrane. The membranes were probed with anti-rabbit histone H3 dimethyl Lysine-4, anti-rabbit histone Lysine-9 (Upstates, Virginia, USA), and with anti-rat β-Actin antibody (1:500 dilution) (Progen, Queensland, Australia). The blots was treated with goat anti rabbit or goat anti rat alkaline phosphatase conjugated secondary antibody (Roche, Germany) in 1:2000 dilution and developed for color detection using NBT and BCIP.

RESULTS

Changes in histone H3 Lysine 4 and 9 methylation levels in alcohol treated rat hepatocytes

We first examined histone methylation levels in the interphase nuclei of rat hepatocytes exposed to alcohol. Primary hepatocytes from rat liver were allowed to settle at the surface of culture dishes for 2 hrs and subsequently treated with ethanol (50mM, 100mM) and control media. Histone H3 acetylation by ethanol occurs maximally at 24 hours treatment (Park et al., 2003; Park et al., 2005). The histone proteins were isolated from nuclei of hepatocytes treated for 24 hrs either with control medium or ethanol. To determine the changes in histone methylation level in histone H3 Lysine 4 (K4) and Lysine 9 (K9), western blot hybridization was performed by probing with dimethylated forms of Lysine-4 or Lysine-9 histone H3 antibodies. Same blots were reprobed with β-actin protein that also served as a gel loading control. The histone H3 Lysine 4 or Lysine 9 methylation signals relative to β-actin protein in each lane showed opposite effects in the alcohol fed cells. The Lysine-9 methylation was reduced at both concentrations of alcohol together with an elevation of histone Lysine-4 methylation in the hepatocytes (Figure 1a). These results indicated that histone dimethyl Lysine 4 and Lysine 9 (H3me2K4 and H3me2K9) are sensitive to alcohol in hepatocytes. The ratios of three independent blots showed that enhancement of Lysine 4 methylation is greater than the degree of reduction of H3 Lysine 4 methylation in the same batch of alcohol treated cells.

The opposite patterns in Lysine 4 and Lysine 9 methylation by ethanol could reflect complex epigenetic marks of the chromatin organization. To test whether the effect of alcohol is

permanent or transient, the opposing pattern of histone H3 methylation in two different lysine residues were tested by extending the ethanol exposure time. Ethanol induces contrasting changes at Lysine-9 and Lysine-4 methylation of histone H3 (Figure 1a). A prolonged alcohol treatment for 72 hours, or hepatocytes treated with ethanol for 24 hours followed by additional incubation for 24 hours in control culture media, did not restore normal (basal) methylation pattern (data not shown). It is therefore possible that distinct changes in the methylation of histone H3 in two lysine residues form a complex 'histone code' that may provide a long-term signal for epigenetic marks in chromatin structure and state of the upregulatory genes in alcohol exposed hepatocytes.

Double immuno-staining of nuclei using Histone H3 Lysine 4 and Lysine 9 antibodies

To test whether changes in histone H3 methylation in ethanol exposed hepatocytes correlated with their accumulation in interphase nuclei, we examined a collection of nuclei from primary cultures of rat hepatocytes treated either with culture media or ethanol by an indirect immunofluorescence using histone H3 dimethyl Lys antibodies. The immuno-staining of different sets of nuclei showed that accumulation of Lysine-9 methylation was considerably reduced in alcohol treated cells (Figure 1b). In contrast, staining with an antibody specific for histone H3 methylated at Lys-4 showed that dimethylated form of H3K4 was increased conspicuously in most of the nuclei (Figure 1b). A statistical evaluation of antibody accumulation between nuclei of ethanol exposed and unexposed cells was performed based on the intensity of fluorescent dyes using the same parameters including exposure time. The data revealed that at both concentrations, the accumulation of methylation of two separate lysine residues of histone H3 tails in the interphase nuclei correlated with changes seen visually (Figure 1c). As expected, it also correlates with the total estimation of histone lysine methylation detected by Western hybridization. It was also noted that the accumulation of Lysine 4 methylation was uniform throughout the nuclei and did not exhibit sub-nuclear compartmentalization. It therefore indicates that ethanol causes uniform enrichment of Lysine-4 methylated histone H3 in liver interphase nuclei but it decreases Lysine-9 dimethylated H3 proteins in the same nuclei (see, Figure 1b). Histone H3 dimethyl lysine in control nuclei is not evenly distributed. It forms a variegated pattern marking an area of the subnuclear compartment where accumulation is very weak. The hepatocyte nuclei with reduced histone H3 lysine 9 methylation, due to ethanol exposure, also exhibits similar distribution indicating an uniform reduction.

For further understanding of their chromosomal association, we also immuno-stained the condensed chromosomes of metaphase spreads from ethanol treated cells. Staining of histone H3 Lysine 9 antibodies in control and ethanol exposed cells revealed that overall distribution of H3me2K9 antibodies on all chromosomes in control cells are more intense than that of alcohol exposed cells (Figure 2). However, there are no differences in antibody binding intensity at the centromeric regions of the chromosome at both cell types. It suggests that a loss of Histone H3K9 might be limited to euchromatic regions. In addition, marginal loss of H3me2K9 at the chromocenter does not reflect any changes in visible intensity. In addition, lack of significant differences in DNA staining of metaphase chromosomes between alcohol treated and untreated cells reflects that the change in H3K9 methylation does not contribute in de-repression of chromosomal condensation (Figure 2).

On the contrary, analyses of metaphase chromosomes using antibody specific for Lys-4 methylated H3 showed that antibody was intensely associated with all chromosomes in alcohol treated hepatocytes (Figure 3). In control cells staining is almost absent in the chromosomes except for few hot spots (present study; Boggs et al., 2002; Peters et al., 2003). But, apart from overall increments of histone H3 Lys-4 methylation on the chromosome, alcohol also intensifies its binding at the hot spots. It indicates that the pattern of distribution of H3K4

antibodies is different from uniform distribution pattern of H3K9 antibodies (Figure 3 enlarged view). These findings suggest that differential distribution of dimethylated form of histone H3 on different chromosomal locations of alcohol treated nuclei may demark their functional distinction of chromatin organization required for modulation of gene expression. It also revealed that the effect of ethanol on H3K9 methylation is much broader than their impact on H3 K4 methylation on the chromosomal sites (Figures 2 & 3 enlarged view).

Ethanol induced changes in genes expressed in liver

Global gene expression profile of ethanol fed rat revealed that it causes alterations in several genes expressed in liver. Nearly 35% of genes expressed in liver exhibit more than two fold changes in the presence of ethanol (Deaciuc et al., 2004a). Using same gene chip approach, other studies have confirmed a similar trend of alcohol induced gene expression (Tadic et al., 2002; Deaciuc et al., 2004b; French et al., 2005). To validate this ethanol sensitivity we selected four genes; two genes that are upregulated, [Alcohol dehydrogenase-1 (Adh-1) and GlutathionS-transferase Yc2 (GST-Yc2)], and two genes that are downregulated, [L-serine dehydratase (Lsdh) and Cytochrome P450 2C11 (CYP2C11)], by ethanol (Tadic et al., 2002; Park et al., 2005). We performed quantitative Northern blot analysis using primary hepatocytes exposed to alcohol and normal media. Each blot was rehybridized with GAPDH probe. Expression of GAPDH provides a valid measure of the loading difference in each lane. The ratios between each transcript and GAPDH were estimated from three independent experiments. The transcripts of upregulatory Adh and GST-Yc2 genes were considerably increased in rat hepatocytes exposed to ethanol for at least 24 hrs (Figure 4).

These results are distinct from gene chip assay but follows same upregulatory trend in both cases. In downregulatory genes Lsdh and CYP2C11 expression were reduced as expected after ethanol consumption (Figure 4; Tadic et al., 2002; Deaciuc et al., 2004a). Thus, similar results of alcohol related gene expression were obtained employing two independent detection techniques and established that ethanol exposure elicits stable changes in gene expression both in primary hepatocytes culture as well as liver invivo (present study and Deaciuc et al., 2004a,b).

Delocalization and accumulation of histone methylation in selective gene promoters

To determine whether methyl marks at the regulatory regions of those genes, up- or down-regulated by the ethanol, correlated with their chromosomal accumulation on the metaphase spreads, the alcohol treated and untreated cells were used for CHIP assay. Chromatin was immunoprecipitated from hepatocytes using antibodies against Lysine-4, or Lysine-9 methylated histone H3. The immuno-precipitated DNA was analysed by real time and semi quantitative PCR amplification using primer sets of each gene. Initially, the sequence required for binding to antibodies was determined by amplifying DNA from immuno-precipitated chromatin from control cell extract. We monitored the amplified fragment of the upregulatory Adh-1 and GSTYc2 and downregulatory Lsdh, CYP2C11 genes (Figure 5; Tadic et al., 2002; Park et al., 2005) and GAPDH, a house-keeping gene that is not altered after ethanol treatment (Kohlmaier et al., 2004; Park et al., 2005; see Figure 5). Treatment of hepatocytes with ethanol reduces H3 Lysine-9 methylation with subsequent increase of H3 Lysine-4 methylation in the regulatory region of the upregulatory genes. Whereas, in down regulatory genes, the dimethyl Lysine-9 accumulated at the promoter with negligible trace of Lysine-4 methylated histone H3 (Figure 5). Increased association of dimethyl H3 Lysine-4 and decreased association of dimethyl H3 Lysine-9 antibodies at the promoter of the upregulatory genes strongly correlated to the changes in histone H3 methylation pattern elicited by alcohol treatment. However, this interrelationship was rather complex with genes repressed by ethanol. In this case, a greater accumulation of Lysine 9 methylation and subsequent reduction of Lysine 4 accumulation at distinct promoter sites was noticed in down regulated genes (i.e. Lsdh &

Cyp2C11; Figure 5). However, the accumulation on the first exon of each transcript is less. Thus accumulation of methylated histone on the regulatory regions of each susceptible transcript might be responsible for its activity. Ethanol therefore, promotes locus specific chromatin modification that is not clearly reflected by the immunostaining of metaphase chromosomes and interphase nuclei. These results suggest that apart from overall modulation, as exemplified by immunostaining, ethanol facilitates localized changes of histone codes both in downregulatory and upregulatory genes. Such regulatory effect of alcohol on repressed genes would be useful for better understanding of the complete spectrum of alcohol effect on gene regulation related to liver injury.

DISCUSSION

This work established that methylated forms of histone H3 are epigenetic imprints of ethanol action in liver. These changes can be interdependent on other transcriptional factors and epigenetic signals (Jenuwein and Allis, 2001; Wilkins, 2005; Peters et al., 2002) for modulation of gene expression. Altered gene expressions may culminate into the progression of fatty liver, inflammation and liver cirrhosis.

In addition to other changes, reduction of H3meK9 may contribute to transcriptional upregulation of a range of gene population in hepatocytes that correspond likely to a major fraction of 798 genes with altered expression due to ethanol exposure (Deaciuc et al., 2004a,b; French et al., 2005). It was reported that expression of nearly 35% of 2259 genes expressed in liver were altered by the exposure to ethanol. These epigenetic changes mostly occur through a positive interaction between chromatin state and covalent modifications of the H3 amino-terminal tail. Earlier it was reported that alcohol causes hyperacetylation of H3K9 (Park et al., 2003; Park et al., 2005). Now we are demonstrating major changes of histone H3 methylation forms in two distinct sites of lysine residues (Lysine-4 and Lysine-9). It is reasonable to assume that acetylation and methylation processes at the regulatory regions occur at the same nucleosome. It is proposed that modifications in methylation are either coupled or orchestrated with hyperacetylation for the fine-tuning of the chromatin states of hepatocyte exposed to alcohol that is required for modulation of complex 'histone code' related to transcriptomes. Alternatively, acetylation and methylation at the same lysine residue may occur sequentially. For upregulatory genes acetylation may be predominant over demethylation of H3 lysine 9 in the same nucleosome, whereas, in down regulatory genes the situation may be opposite. However, it is unlikely that the epigenetic signals prolong their effect for promoting cellular memory of chromatin architecture in the liver. Lack of any recovery in histone methylation and chromatin architecture in cells after 24 hrs of ethanol withdrawal, raise the possibility that modifications in both Lysine-4 and Lysine-9 are retained firmly during chromosome condensation in metaphase spreads. Such specific modifications may serve as stable epigenetic signature and retained in memory of cells for at least 24 hrs after withdrawal of alcohol (Martens et al., 2003; Peters et al., 2003). Finally, the data from chromatin immunoprecipitation demonstrate, at the molecular level, a reciprocal association between Lys-9 and Lys-4 methylated histone H3 in different regulatory sites of effected genes related to the transcriptional status of the genes (Boggs et al., 2003; Peters et al., 2002; Martens et al., 2003). Collectively, our data show that differential methylation of two different Lysine residues of histone H3 at two distinct sites may set up domains of transcriptionally competent chromatin, which may carry out different cellular functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Histone H3 dimethyl Lysine-4 and Lysine-9 levels were determined by western blotting in ethanol treated rat hepatocytes. The nuclei of male rat hepatocyte treated 24 hrs with 50mM, 100mM ethanol were used. The blots were probed with dimethylated Lysine-4 and Lysine-9 histone H3 antibodies. Same blots were reprobed with β-Actin antibody for estimating the loading difference in each lane. The ratios (H3me2K9/β-Actin and H3me2K4/β-Actin) from three independent experiments were plotted in a graph above the gel panel. The ratios with asterisk were different from ratio derived from control hepatocytes at the 95% level of confidence. (b) Alteration of histone H3 Lysine-4 and Lysine-9 methylation in alcohol treated rat hepatocytes. Hepatocytes were treated with PBS, 50mM or 100mM ethanol for 24 hrs. The

treated cells were immunostained with histone H3 Lysine- 4 and Lysine-9 antibodies. Same set of cells was counterstained with DAPI. Merge figures of the nuclei are presented at the right side of the panel.

(c) Quantitative estimation of histone H3 dimethyl Lysine-4 and Lysine-9 antibodies hybridized with interphase nuclei. Histogram represents intensity of antibodies calculated by the gray scale values accumulated in each pixel of the nuclei. Number of nuclei (n) that are analyzed in each cell type and concentration of alcohol are shown.

Figure 2. Ethanol reduces chromosomal association of Lysine-9 methylated form of histone H3 in the hepatocyte metaphase spreads

Metaphase chromosomes from rat hepatocytes were immuno-stained with H3me2K9 antibodies after 24 hrs post incubation with PBS, 100mM alcohol. Below is the enlarged view of the single chromosome hybridized with Lys-9 dimethyl H3 antibody. Arrow indicates the accumulation of H3me2K9 antibodies on different chromosomal locations. Corresponding DAPI stained images of the same metaphase spreads are shown. Magnification is 600X. Note that the frequency of metaphase spreads was considerably lower in undivided primary hepatocytes.

Figure 3.

Enrichment of H3me2K4 antibody labeling on the chromosomes of metaphase spreads derived from rat hepatocytes treated with culture media and 100mM ethanol alone for 24 hrs. Same chromosomes were counterstained with DAPI as shown. Enlarged view of the single chromosome immunostained with Lysine-4 dimethyl histone H3 antibody was shown below. Arrows indicate intense labeling of the few hot spots on the single chromosome. Magnification is 600 X.

Figure 4. Northern transfers of primary rat hepatocyte cells treated with alcohol

The cells were incubated with 50mM or 100mM of ethanol for 24 hrs. Total cellular RNA isolated from control and alcohol treated cells were blotted and probed in triplicate for four independent probes as noted in the figure. Each blot was reprobed with GAPDH as a loading control. The bar diagram above the panel was drawn from three relative ratios between each transcripts and GAPDH calculated from triplicate gels. The values with asterisks are significantly different from control lanes at the 95% confidence level.

Figure 5. CHIP assay demonstrating association of histone H3me2K9 and H3me2K4 methylation at the promoter of the genes up and down regulated by ethanol in hepatocytes

Chromatin immunoprecipitation (see Methods) was performed using hepatocytes treated with media, 50mM or 100 mM EtOH for 24 hrs employing antibodies noted in the panel.

Immunoprecipitated DNA contained promoter DNA sequence of two up-regulated (*Adh-1* and *GST-Yc2*) and two down-regulated (*CYP2C11*, *Lsdh*) genes and a house keeping gene *GAPDH*. The DNA was amplified by semi quantitative PCR and further validated by real-time PCR analyses. The PCR products were separated by 2% agarose gel in which *GAPDH* DNA served as an internal control.

A graph was plotted using the quantitative measurements of each amplified product using real-time PCR from two independent experiments. The mean ratios marked with asterisks were statistically different from the ratios derived from hepatocytes exposed to culture media at the 95% confidence level.