Gene expression and pathway analysis of human hepatocellular carcinoma cells treated with cadmium

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Abstract

Cadmium (Cd) is a toxic and carcinogenic metal naturally occurring in the earth's crust. A common route of human exposure is via diet and cadmium accumulates in the liver. The effects of Cd exposure on gene expression in human hepatocellular carcinoma (HepG2) cells were examined in this study. HepG2 cells were acutely-treated with 0.1, 0.5, or 1.0 μM Cd for 24 hours; or chronically-treated with 0.01, 0.05, or 0.1 μM Cd for three weeks and gene expression analysis was performed using Affymetrix GeneChip® Human Gene 1.0 ST Arrays. Acute and chronic exposures significantly altered the expression of 333 and 181 genes, respectively. The genes most upregulated by acute exposure included several metallothioneins. Downregulated genes included the monooxygenase CYP3A7, involved in drug and lipid metabolism. In contrast, CYP3A7 was upregulated by chronic Cd exposure, as was DNAJB9, an anti-apoptotic J protein. Genes downregulated following chronic exposure included the transcriptional regulator early growth response protein 1. Ingenuity Pathway Analysis revealed that the top networks altered by acute exposure were lipid metabolism, small molecule biosynthesis, and cell morphology, organization, and development; while top networks altered by chronic exposure were organ morphology, cell cycle, cell signaling, and renal and urological diseases/cancer. Many of the dysregulated genes play important roles in cellular growth, proliferation, and apoptosis, and may be involved in carcinogenesis. In addition to gene expression changes, HepG2 cells treated with cadmium for 24 hours indicated a reduction in global levels of histone methylation and acetylation that persisted 72 hours post-treatment.

Introduction

Cadmium is a toxic and carcinogenic transition metal naturally occurring in the earth's crust. It can also be introduced into the environment via anthropogenic sources. Cadmium is easily

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

absorbed by plants from the soil, thus a common non-occupational route of human exposure is through diet [1]. Levels in the US food supply can range from 2-40 ppb [2]. Additionally, smokers are exposed to high levels of cadmium from tobacco, about 1.7 μg per cigarette [1]. Most cadmium accumulates in the liver and kidneys of individuals with low environmental exposures [3]. Occupational exposure to cadmium has been linked to cancers of the lung, prostate, kidney, liver, hematopoietic system, bladder, pancreas, testis, and stomach [4, 5], hence cadmium is classified as a class I human carcinogen by IARC [1]. The mechanisms of cadmium-induced carcinogenesis however, are not fully understood. While cadmium can be genotoxic at high doses, it is not highly mutagenic and does not form DNA adducts [6]. Cadmium is thought to induce oxidative stress by depleting glutathione and protein-bound sulfhydryl groups, leading to increased production of reactive oxygen species (ROS) and apoptosis [7, 8]. Cadmium is predominately non-genotoxic and therefore may induce carcinogenesis via epigenetic mechanisms by altering the expression levels of various critical genes [4] .

Carcinogenesis is a complex, multi-step process that involves many alterations to the cell. DNA damage, if left unrepaired, can lead to gene mutations. These mutations may be deleterious if they occur in genes controlling cell cycle, cell growth, or other critical processes. In addition, expression of various genes can be altered via epigenetic mechanisms. Epigenetic modifications alter chromatin structure, leading to changes in gene expression without affecting DNA structure or sequence. If these changes are inherited, then this process is considered an epigenetic effect. These epigenetic mechanisms include DNA methylation, micro RNAs, noncoding RNAs and post-translational modifications to histones [9, 10]. The N-terminal tails of histones can be altered post-translationally via methylation, acetylation, phosphorylation, SUMOylation, or other modifications [11]. These modifications can lead to relaxation or condensation of chromatin. Modifications can also recruit other histone modifying enzymes to initiate further alterations to the chromatin. These changes can dramatically alter gene expression, which can dysregulate normal cellular functions and lead to disease, transformation, and possibly carcinogenesis.

A common route of exposure to cadmium is via diet, and cadmium accumulates in the liver. Exposure has also been linked to liver disease and cancers of the liver [12, 13]. It is hypothesized that both acute and chronic exposures to cadmium induce gene expression changes in human hepatocarcinoma HepG2 cells. These differentially expressed genes will likely be involved in various carcinogenesis pathways. Previous gene expression studies on cadmium exposure in HepG2 studies have focused on higher, non-biologically relevant concentrations or very short exposure times (6 hours or less) [14, 15], and indicate an enrichment in carcinogenesis related genes and pathways. This study explored the changes in gene expression caused by acute and chronic treatment with low doses of $CdCl_2$ in HepG2 cells and investigated the biological functions of differentially modified genes. HepG2 cells were treated with 0.1, 0.5, or 1.0 μM cadmium chloride for 24 hours, or 0.01, 0.05, or 0.1 μM cadmium chloride for three weeks and gene expression analysis was performed using Affymetrix GeneChip Human Gene 1.0 ST Arrays.

Gene expression changes are often accompanied by both gene-specific and global changes to histone modifications. Western blot analysis was used to investigate global changes to

histone methylation and acetylation levels caused by acute treatment with 0, 0.5, 1.0, 2.5, and 5 μM cadmium chloride, as well as after a 72-hour recovery period to look for persistence of the changes. Cadmium has a half-life in the liver of up to 20 years, and levels can easily reach micromolar concentrations [16]. Additionally, uptake of insoluble cadmium particles can lead to high concentrations in the liver, as these particles are accumulated over time and not readily eliminated.

Materials and methods

Cell culture

Human hepatocarcinoma (HepG2) (ATCC) were cultured in Dulbecco's modified minimum essential media (DMEM), low glucose (1 g/L) (Invitrogen), supplemented with 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). For cadmium exposures, cells were seeded one day prior to treatment. Cadmium chloride hemipentahydrate (Acros Organics) was added to the media and evenly applied to the cultured cells. Cells were treated with 0.1, 0.5, or 1.0 μM $CdCl_2$ for 24 hours (acute treatment), or 0.01, 0.05, or 0.1 μM $CdCl_2$ for three weeks (chronic treatment). For chronic treatment, $CdCl_2$ concentrations were maintained in the cell culture media. Both untreated control cells and treated cells were cultured in a 37 °C incubator with 5% CO_2 until harvesting. For Western blots, cells were treated with 0.5, 1.0, 2.5, or 5 μM $CdCl_2$ for 24 hours before cells were lysed for protein extraction. After 24 hour treatment with 5 μM $CdCl_2$, cell culture media was replaced with fresh media containing no cadmium for washout samples. Cells were lysed for protein extraction after a 72 hour washout period.

RNA extraction and microarray hybridization

At the end of the treatment, cells were collected in Trizol (Invitrogen) and RNA was isolated and purified using RNeasy PlusMicro Kit (Qiagen, Valencia, CA). 100 ng of total RNA was used to synthesize double-stranded cDNA (dsDNA). cRNA was synthesized from dsDNA template, and subsequently used to produce sense single-stranded cDNA (ssDNA) with incorporated deoxyuridine triphosphate. The ssDNAs were fragmented, end-labeled, and hybridized to Affymetrix Human Gene 1.0 ST Array (Affymetrix). Arrays were hybridized and scanned using a standard protocol.

Microarray data analysis

Microarray data analysis was performed using GeneSpring v12.0 (Agilent Technologies). Differentially expressed genes were identified using one-way ANOVA (p<0.05). Hierarchical cluster analysis using Euclidean distance was performed to cluster genes and samples to generate a heat map. Gene network and pathway analysis was performed using Ingenuity Pathway Analysis (http://www.ingenuity.com).

Real time PCR

250 ng of total RNA extracted from control and cadmium-treated cells was converted to single stranded cDNA using Superscript® III (Invitrogen). Quantitative real-time PCR analysis was performed on 20 ng of each cDNA using SYBR green (Applied Biosystems) on ABI prism 7900HT (Applied Biosystems). Relative gene expression levels were

normalized to β-actin expression. All PCR reactions were performed in triplicate. Primers were obtained from Invitrogen. Results were presented as fold change relative to the level expressed in untreated control cells using the CT method. The primer sequences are listed in table 1.

Whole cell lysis and protein isolation

HepG2 cells were lysed using boiling buffer (1% SDS, 1 mM Na_3VO_4, 10 mM Tris-Cl, pH 7.4). 0.5 mL of 100 °C preheated boiling buffer was added to 10 cm cell culture dishes at a sub-confluent density after two washes with 1X PBS. The lysate was denatured at 100 °C for 5 minutes then sonicated using a Diagenode Bioruptor at a maximum setting for 10 minutes. Samples were then centrifuged at 4 °C for 15 minutes at 21,000 rpm. Protein was quantified using a Nanodrop 2000 Spectrophotometer.

Western Blot

Mini-PROTEAN TGX precast gels (Bio-Rad) were loaded with protein in Laemmli sample buffer (Bio-Rad) containing 5% (v/v) 2-mercaptoethanol (Sigma). The Precision Plus Protein Kaleidoscope standard (Bio-Rad) was used to determine protein size. Electrophoresis took place in 1X Tris/glycine/SDS buffer (Bio-Rad) at 100 volts at room temperature. The protein was transferred onto Immuno-Blot PVDF Membrane (Bio-Rad) in 1X Tris/glycine buffer (Bio-Rad) at 22 volts overnight at 4 °C. The membranes were blocked for 30 minutes with 5% (w/v) Blotting-Grade Blocker (Bio-Rad) in TBST at room temperature. Membranes were incubated with primary antibodies for one hour at room temperature or overnight at 4 °C. Membranes were incubated with secondary antibodies (goat anti-mouse IgG-HRP [sc-2005, Santa Cruz Biotechnology, Dallas, TX] or goat anti-rabbit IgG-HRP [sc-2004, Santa Cruz Biotechnology]) for one hour at room temperature. Protein bands were detected using the Pierce ECL Western Blotting Substrate (Thermo). Relative band intensities were determined using ImageJ software (NIH).

Results

A distinct set of genes were up- and downregulated due to acute and chronic treatment with cadmium

Heatmaps of differentially expressed genes are shown in figure 1 for all treatment groups (left, acute treatment; right, chronic treatment). Differential genes are shown either in red (upregulation) or blue (downregulation) ($p > 0.05$). The intensity of the color indicates the level up/down regulation. The samples were grouped by treatment dose and showed an overall global change in expression for all genes. Hierarchical clustering analysis indicated a detectable difference in gene expression patterns between sample groups. The fifteen top up- and down-regulated genes are listed for the 0.5 μM acute treatment (table 2) and 0.1 μM chronic treatment (table 3), as these were the treatment groups with the highest number of genes with altered expression for each exposure duration. Expression levels for those top genes are also given for the other treatment doses. Expression of 333 genes was significantly changed by acute exposure in HepG2 cells, while 181 genes were altered by chronic exposure. Of the 333 genes significantly altered by acute cadmium exposure, 171 genes were upregulated and 162 were downregulated. Of the 181 genes significantly altered by

chronic exposure, 88 genes were upregulated while 93 genes were downregulated. There were four genes significantly upregulated by both acute and chronic exposures and no genes were commonly downregulated by the treatments.

Gene Expression Validation

Microarray results for both acute and chronic exposures were validated for three top up- and down-regulated genes by quantitative real-time PCR (qRT-PCR) of RNA from treated and untreated HepG2 cells. Results are shown in table 4. qRT-PCR results shown represent means of triplicates from two independent experiments, represented as fold change to level of expression in control HepG2 cells. qPCR is a more robust and quantitative measure of mRNA levels, thus fold changes are not identical to those measured in the microarrays. The direction of the change, however, was consistent between microarray and qPCR for all of the genes studied.

Genes altered by 24 hour treatment with cadmium

Several metallothionein (MT) genes were among the genes most upregulated with acute treatment with cadmium, including MT1G, MT1F, MT1M, MT1B, MT1X and MT1H (table 2). Not surprisingly, ten of the top 15 upregulated genes due to acute treatment were MT genes or pseudogenes. Downregulated genes included the cytochrome p450 monooxygenase CYP3A7, 5'-nucleotidase ecto (NT5E/CD73), serine peptidase inhibitor, Kazal type 1 (SPINK1), and the transcription factor zinc finger protein 224 (ZZF224).

Genes altered by chronic treatment with cadmium

Genes most upregulated by three week chronic treatment with cadmium included CYP3A7, the extracellular matrix protein nephronectin (NPNT), the J family member of 70 kD heat shock protein (Hsp70) chaperones DNAJB9, and transcriptional activator zinc finger and BTB domain containing 38 ZBTB38 (table 3). Downregulated genes included transcriptional regulator early growth response regulator EGR1, alcohol dehydrogenase ADH4, helix-loop-helix protein inhibitor of DNA binding 1 (ID1), transporters SLC16A6 and SLC2A3, and connective tissue growth factor CTGF.

Gene expression and pathway analysis

Heatmaps of differentially expressed genes are shown in figure 1. Genes highlighted in red indicate upregulation versus untreated control cells, while blue indicates downregulation in the treated cells. Hierarchical clustering analysis shows a detectable difference in gene expression patterns between sample groups. Genes whose expression was altered at least 1.25-fold were uploaded into Ingenuity Pathway Analysis for further investigation. Top networks altered by acute exposure to 0.5 μM cadmium, as determined by IPA, were lipid metabolism, small molecule biochemistry; vitamin and mineral metabolism; and cell morphology, assembly, organization, and development (figure 2). Top molecular and cellular functions altered by acute exposure included cell death and survival, and cellular assembly and organization. Top diseases and disorders that were enriched were cancer, gastrointestinal diseases, and renal and urological disease. Top canonical pathways were retinol biosynthesis, nicotine degradation, and lipopolysaccharide (LPS)/interleukin-1 (IL-1)

mediated inhibition of retinoid X receptor (RXR) function. Top toxicological functions were liver hyperplasia/hyperproliferation, hepatocellular carcinoma, liver proliferation, degeneration, and cirrhosis.

Top networks altered by chronic exposure were organ morphology, cell cycle, and cancer; developmental disorder, renal and urological diseases/cancer; and cellular growth and proliferation, and cancer (figure 3). Top molecular and cellular functions altered by chronic exposure included cell-to-cell signaling and interaction, cell death and survival, and cellular growth and proliferation. Top diseases and disorders were cancer, reproductive system disease, and endocrine system disorders. Top canonical pathways were tyrosine degradation I, pregnane X receptor/retinoid X receptor (PXR/RXR) activation, and pyrimidine deoxyribonucleotides de novo biosynthesis I. Top upstream regulators were IL-6, hypoxia inducible factor 1α (HIF1α), platelet-derived growth factor (PDGF) BB, tumor necrosis factor (TNF), and transcription factor E2F4. Top toxicological functions were liver hyperplasia/hyperproliferation, hepatocellular carcinoma, liver regeneration, liver necrosis, and liver proliferation.

Global changes in histone methylation and acetylation marks

The concentrations used for Western blot analysis were determined based on preliminary experiments in which doses below 0.5 μM cadmium chloride did not yield detectable changes to histone modifications (data not shown). Due to the slow growth rate of HepG2 cells, a washout (WO) period of 72 hours, in which the cadmium chloride was completely removed from the cell culture media, was used to ascertain the persistence of the epigenetic changes for at least three generations post-treatment.

Both histone H3 and H4 pan acetylation levels decreased with cadmium levels in HepG2 cells, however neither decrease was observed at the 0.5 μM dose. After a 72 hour washout period, the acetylation levels remained diminished, but not to the levels seen directly following treatment. H4K16ac levels decreased with increasing cadmium dose and were nearly depleted with both 2.5 and 5 μM cadmium. A reduction in protein expression of nearly 20% was observed at 0.5 μM. Diminished acetylation levels did not recover 72 hours post-treatment. Global H3K4me3 levels decreased with cadmium dose in HepG2 cells, however this effect was not entirely dose dependent, as levels were higher after treatment with 1.0 versus 0.5 μM cadmium. Levels remained slightly reduced 72 hours post-treatment. Reduced global H3K9me2 levels were observed after treatment with 2.5 and 5 μM cadmium. This decrease was sustained even 72 hours after the metal was removed from the media (figure 4).

Discussion

The genes most upregulated by acute exposure to cadmium chloride included several metallothioneins (MTs), including MT1G, MT1F, MT1M, MT1B, MT1X and MT1H. MT genes are usually expressed at low levels, but their expression is often induced in the presence of metal ions, as their encoded cysteine-rich proteins are involved in metal storage, transport, and detoxification. MTs are also involved in regulation of homeostasis of essential trace metals [17]. Dysregulated MT expression has been observed in many cancers [10, 17],

including hepatocellular carcinoma [18] as well as liver disease [19]. Upregulated MT expression indicates a cellular response to the cadmium treatment and mechanism to ameliorate the toxic effects of the metal.

Downregulated genes included the cytochrome p450 monooxygenase CYP3A7, which is involved in drug metabolism and lipid synthesis [20]. NT5E [5'-nucleotidase, ecto (CD73)] was also downregulated following to acute exposure. NT5E encodes a plasma membrane protein that catalyzes the conversion of extracellular nucleosides to membrane permeable nucleosides. NT5E functions as the rate-limiting enzyme in the conversion of pro-inflammatory ATP to immunosuppressive adenosine [21]. Its downregulation may contribute to a pro-inflammatory state. NT5E appears to play key regulatory roles in cancer cell proliferation, migration, and invasion and in tumor angiogenesis. Its upregulation has been reported in some carcinomas including gallbladder [22], prostate [23], and those of the gastrointestinal tract [24]. It has recently been identified as a novel prognostic indicator of colorectal cancer [21]. Its expression can be regulated by cytokines or by hypoxia [25], perhaps via a potential HIF-1 binding site [26]. The role of NT5E in human liver cancers outside of metastases has not been widely reported.

Serine peptidase inhibitor, Kazal type 1 (SPINK1), also downregulated following acute exposure, is secreted by pancreas cells to inhibit activation of zymogens. SPINK1 is aberrantly expressed in a number of human cancers and was found to promote epithelial to mesenchymal transition in prostate cancer [27]. SPINK1 was also found to increase breast cancer invasiveness and chemoresistance [28]. It has recently been identified as a possible marker for hepatocellular carcinoma (HCC) [29]. While downregulation of SPINK1 as it relates to HCC has not been reported, loss of SPINK1 was found to correlate with aggressive urothelial carcinomas of the bladder [30].

Transformer 2 alpha homolog (TRA2A) was also downregulated with acute cadmium treatment. This nuclear protein with several RNA recognition motif domains plays a role in the regulation of pre-mRNA splicing. TRA2A, as well as other proteins in the spliceosome pathway, was found to be dysregulated in a meta-analysis of human HCC [31]. Thus, cadmium may affect proper mRNA splicing, which could lead to aberrant gene expression.

While CYP3A7 expression was significantly downregulated following acute exposure, this monooxygenase gene was found to be one of the most upregulated due to chronic exposure. CYP3A7 has been observed to be upregulated in non-alcoholic steatohepatitis (fatty liver disease) and cirrhosis, as well after exposure to hepatotoxic compounds. The upregulation of CYP3A7 appears to be a response of stressed hepatocytes both *in vitro* and *in vivo* [20].

Nephronectin (NPNT), also upregulated due to chronic exposure, is an extracellular matrix protein that regulates cell adhesion, spreading, and survival in the kidney. While the role of NPNT in the liver is not known, its expression is upregulated in hepatitis mouse models and in liver samples from patients exhibiting hepatitis, fatty liver, and cirrhosis [20]. NPNT may play a role in inflammation, as it localizes to inflammatory foci [32]. Inflammation, especially as induced by hepatitis B infection, is a known precursor to HCC, thus the

upregulation of NPNT during inflammatory injury to the liver as well as HCC warrants further study.

DNAJB9, a J protein family member, was also upregulated due to chronic treatment. J proteins function as part of the heat shock 70 kD protein (Hsp70) machinery that guides proper protein folding, drives protein transport, and mediates protein-protein interactions [33]. DNAJB9 is induced by endoplasmic reticulum (ER) stress and helps protect stressed cells from apoptosis. DNAJB9 was found to be induced by p53 under genotoxic conditions and to inhibit the pro-apoptotic function of p53 [34]. The upregulation of DNAJB9 may inhibit p53-induced apoptosis of cells under stress and allow for transformation to occur after exposure to cadmium.

CREB3 Regulatory Factor (CREBRF/C5orf41) was also upregulated by chronic exposure to cadmium. This protein, induced by ER stress, acts as a negative regulator of the ER stress response and unfolded protein response by repressing the transcriptional activity of CREB3 transcription factor [35]. CREB3 is involved in cell proliferation and migration, tumor suppression, and the inflammatory response. It promotes cell survival against ER stress-induced apoptotic cell death during the unfolded protein response. Upregulation of CREB3RF can lead to repression of CREB3, which could cause aberrant cell proliferation or dysregulate the unfolded protein response, leading to apoptosis.

Genes downregulated due to chronic exposure included the transcriptional regulator Early Growth Response 1 (EGR1). EGR1 encodes an ERK-induced transcriptional regulator whose target genes have various roles in differentiation and mitogenesis. EGR1 was found to arrest cell mobility, inhibit migration and induce apoptosis in human non-small-cell lung carcinoma cells [36]. It has previously been observed to be downregulated in HCC [20]. It has been suggested that EGR1 is a tumor suppressor gene, thus its downregulation due to cadmium exposure requires further investigation.

ADH4 was also downregulated by chronic exposure. ADH4, an alcohol dehydrogenase, metabolizes a wide array of substrates including ethanol, retinol, hydroxysteroids, and lipid peroxidation products. Diseases associated with ADH4 include alcoholic liver cirrhosis and alcohol dependence. Reduced expression of ADH4 has been suggested as a potential prognostic indicator of HCC [37].

Inhibitor of DNA binding 1 (ID1), also downregulated due to chronic cadmium treatment, is a helix-loop-helix protein that interacts with other HLH transcription factors. ID1 inhibits DNA binding and transcriptional activation activity of its interacting proteins. ID1 plays a role in cell growth, differentiation, and senescence. It has been observed to be downregulated in cirrhosis, HCC, and other carcinomas [20]. Reduced expression of ID1 can inhibit transcriptional activation of downstream genes, leading to irregular cell growth or senescence.

C8orf4, also known as human thyroid cancer protein 1 (TC-1), was also downregulated with chronic treatment. TC-1 protein functions as a positive regulator of the Wnt/β-catenin signaling pathway that regulates cell migration, polarity, and fate determination during embryogenesis. Mutations or epigenetic inactivation of elements of this pathway can lead to

birth defects or cancer [38]. TC-1 is thought to competitively block interactions between β-catenin and a repressor protein, leading to upregulation of β-catenin target genes [39]. TC-1 may also play a role in NF-κB and ERK signaling pathways [40]. Downregulation TC-1 can thus lead to aberrant signaling, causing disrupted embryonic development or carcinogenesis.

A distinct set of genes was altered due to acute and chronic treatment in HepG2 cells. Acute exposure to cadmium altered the expression of genes encoding proteins involved in detoxification of metals and xenobiotics and metabolism of drugs, lipids, nucleosides, and other small molecules. Thus, acute exposure may disrupt metabolism and upregulate genes involved in detoxification and sequestration of the cadmium. Alternatively, chronic treatment caused dysregulation of genes involved in various cell signaling pathways, including immune, NFAT, and insulin signaling. Genes involved in the inflammatory response, unfolded protein response, and stress response were also dysregulated, indicating increased cellular stress and the initiation of an inflammatory state. Disruption of signaling pathways, stress response, proliferation, and cell cycle, as well as increased cellular stress and inflammation are all potential mechanisms to induce carcinogenesis in HepG2 cells chronically exposed to cadmium.

IPA revealed that top networks altered by acute exposure were lipid metabolism, small molecule biochemistry; vitamin and mineral metabolism; and cell morphology, assembly, organization, and development. Top molecular and cellular functions altered by acute exposure included cell death and survival, and cellular assembly and organization. Top networks altered by chronic exposure were organ morphology, cell cycle, and cancer; developmental disorder, renal and urological diseases/cancer; and cellular growth and proliferation, and cancer. Top molecular and cellular functions altered by chronic exposure included cell-to-cell signaling and interaction, cell death and survival, and cellular growth and proliferation.

Gene expression can be modulated epigenetically via DNA methylation, microRNAs or posttranslational modifications to histones. While cadmium has been found to alter global methylation levels in various human cell lines [6, 41, 42], changes to histone modifications have not been reported in HepG2 cells. Both methylation and acetylation are important histone posttranslational modifications that can have dramatic effects on gene expression. Methylation of histones may activate or suppress gene expression, depending on the particular residue modified and the level of methylation. Trimethylated H3 lysine 4 (H3K4me3) is found in promoter regions of transcriptionally active genes, [43] while dimethylated H3K9 (H3K9me2) is localized to regulatory regions of transcriptionally silent genes [44]. Altered global levels of H3K4me3 and H3K9me2 are associated with aberrant gene expression and have been linked with several human cancers [45, 46]. Cadmium treatment increased global levels of both histone marks in human bronchial epithelial cells [47]. Effects of cadmium on global H3K4me3 and H3K9me2 levels have not been previously reported in HepG2 cells.

Histone acetylation is typically associated with transcriptional activation. Addition of an acetyl group to a lysine reside on a histone N-terminal tail neutralizes its positive charge. This weakens contact between the lysine and DNA backbone, opening up the chromatin and

facilitating loading of the transcription machinery onto DNA [48]. Both hyperacetylation and deacetylation of histones have been observed in human cancers and depend on the target gene or genes affected [49]. Acetylated histone H4K16 (H4K16ac) regulates chromatin remodeling by inhibiting the formation of condensed 30 nm fiber structures [50]. Loss of H4K16 is commonly seen in human cancers [51]. Effects of cadmium on H4K16ac levels have not been reported.

In the current study, cadmium treatment caused a decrease in histone H3 and H4 acetylation levels. After a 72 hour recovery period, the acetylation levels remained slightly diminished. H4K16ac levels decreased with increasing cadmium dose and were nearly depleted at the higher concentrations. Acetylation levels did not recover 72 hours post-treatment. Global H3K4me3 and H3K9me2 levels decreased with increased cadmium concentrations. H3K4me3 levels remained slightly reduced 72 hours post-treatment. Reduced H3K9me2 persisted 72 hours post treatment. These results indicate that while acute cadmium treatment can cause significant changes to the global levels of methylation and acetylation of histones, these changes do not always persist after the cadmium is removed. Changes to H4K16ac and H3K9me2 did appear persistent; at least 72 hours post treatment. These results suggest a change to the epigenetic landscape induced by acute exposure to cadmium.

Conclusion

Treatment with low concentrations of cadmium, both acute and chronic, caused significant changes to expression levels of hundreds of different genes. A different set of genes was altered depending on the length of the treatment. Dysregulated genes were involved in many important pathways and processes including metabolism, cell death and survival, and cell-to-cell signaling. Changes to global histone modifications were also observed with acute treatment with various doses of cadmium. Global acetylation and methylation levels both decreased after exposure. Some of these changes persisted 72 hours after treatment. Histone methylation and acetylation can dramatically alter gene expression via the unwinding or condensing of chromatin. It is clear that cadmium can induce significant changes to both gene expression and epigenetic marks. Further studies into whether these changes occur in the promoter or regulatory regions of dysregulated genes will provide further insight into the epigenetic mechanisms by which cadmium can affect gene expression.

Acknowledgments

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Highlights

- A common route of human exposure to the carcinogenic metal cadmium is via diet

- HepG2 cells were treated acutely or chronically with varying doses of cadmium

- Gene expression analysis was performed using Affymetrix Human Gene 1.0 Arrays

- Acute and chronic exposures altered the expression of 333 and 181 genes, respectively

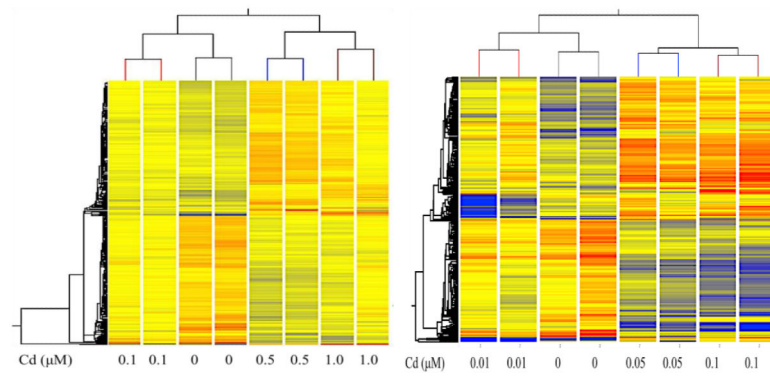- Acute cadmium exposure altered global levels of histone methylation and acetylation

Figure 1.
Heatmaps of differentially expressed genes due to acute (left) and chronic (right) cadmium treatment in HepG2 cells (over 1.25-fold change, p>0.05). Red indicates upregulation while blue indicates downregulation of gene expression relative to control, untreated cells.
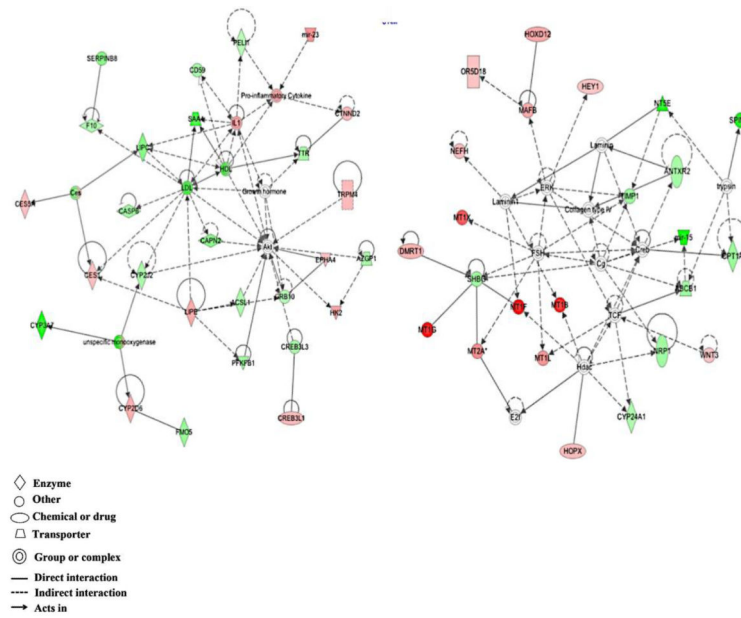
Figure 2.
Top networks altered by acute exposure to 0.5μM cadmium in HepG2 cells were lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism (left), and cellular morphology, assembly, organization, and development (right). Red – upregulation; green - downregulation.
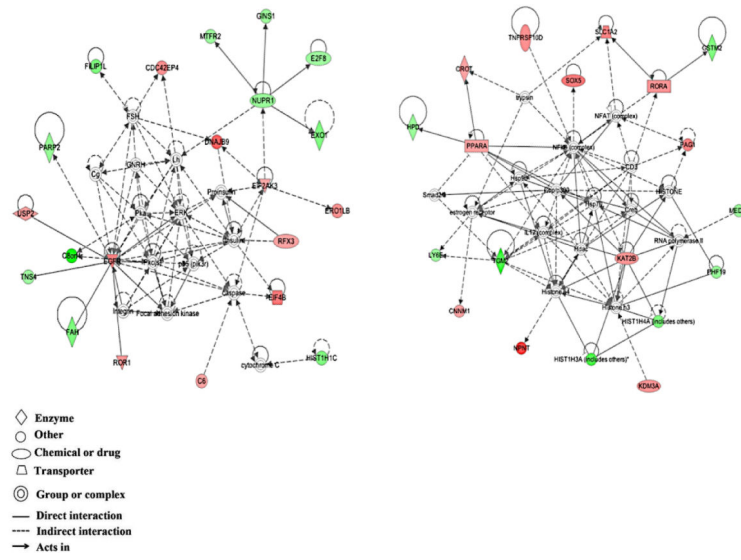
Figure 3.
Top networks altered by chronic exposure to 0.1μM cadmium in HepG2 cells were organ morphology, cell cycle, and cancer (left), and developmental disorder, renal and urological diseases and cancer (right). Red – upregulation; green – downregulation
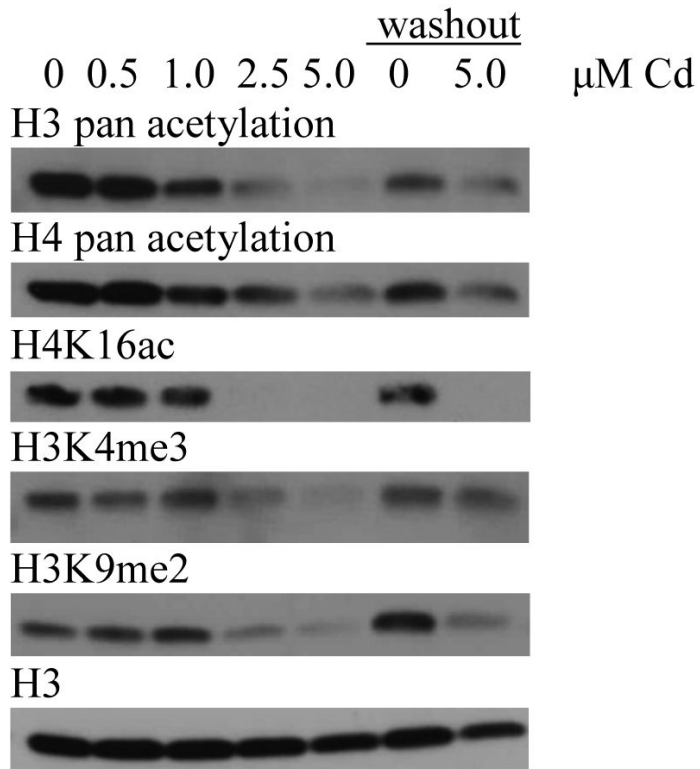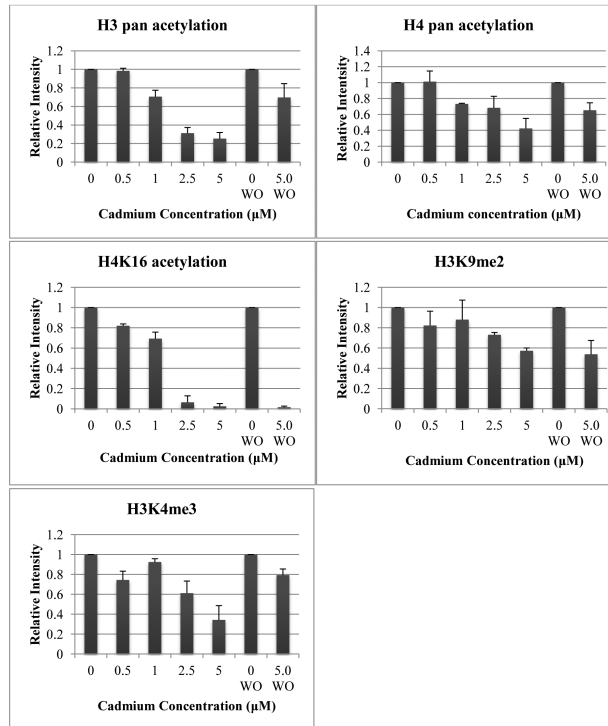
Figure 4.

Western blot images (A) and graphical representations (B) of relative intensities calculated by ImageJ for cadmium treatment in HepG2 cells. Cells were treated for 24 hours with varying doses of cadmium and cells were lysed post-treatment. Cells treated with 5 μM cadmium were allowed to recover for 72 hours after treatment before lysis (washout). Blots and graphs are representative of two independent experiments. WO; washout

Table 1

Primer sequences used for qPCR validation of microarray results

MT1G-F:	GCCAGCTCCTGCAAGTGCAA	**MT1G-R:**	TCTCCGATGCCCTTTGCAG
MT1F-F:	TCCTGCAAGTGCAAAGAGTG	**MT1F-R:**	TCCTGCAAGTGCAAAGAGTG
MT1M-F:	TCCGGGTGGGCCTAGCCAGTCG	**MT1M-R:**	AATGCAGCAAATGGCTCAGTATCGTATTG
CYP3A7-F:	GATCTCATCCCAAACTTGGCCG	**CYP3A7-R:**	CATAGGCTGTTGACAGTCATAAATA
NT5E-F:	GGGCGGAAGGTTCCTGTAG	**NT5E-R:**	GAGGAGCCATCCAGATAGACA
SPINK1-F:	TGTCTGTGGGACTGATGGAA	**SPINK1-R:**	TCAACAATAAGGCCAGTCAGG
NPNT-F:	GCTGGTATCCTCGCTCTACC	**NPNT-R:**	TCCTCCCACCATAACGACAT
DNAJB9-F:	GCCATGAAGTACCACCCTGACA	**DNAJB9-R:**	TCGTCTATTAGCATCTGAGAGTGT
EGR1-F:	CTTCAACCCTCAGGC GGACA	**EGR1-R:**	GGAAAGCGGCCAGTATAGGT
ADH4-F:	GAAACCATGAAAGCAGCCCT	**ADH4-R:**	CCAACCACCAAAGAATGTTCC
ID1-F:	CCAACGCGCCTCGCCGGATC	**ID1-R:**	CTCCTCGCCAGTGCCTCAG
β actin-F:	CACCATTGGCAATGAGCGGTTC	**β actin-R:**	AGGTCTTTGCGGATGTCCACGT

Table 2

Top fifteen up- and downregulated genes ($p<0.05$) by 24 hour treatment with 0.5 μM CdCl$_2$ in HepG2 cells. Fold changes for the other treatments also listed. # fold change >1.25

Gene Symbol	Gene Description	Fold Change (0.5 μM)	Fold Change (0.1 μM)	Fold Change (1.0 μM)	Regulation
MT1G	metallothionein 1G	**2.215775**	1.5027326	2.6238236	up
MT1F	metallothionein 1F	**1.9121428**	1.405878	2.2424114	up
MT1M	metallothionein 1M	**1.8444134**	#	6.988504	up
MT1B	metallothionein 1B	**1.7858652**	#	5.00817	up
MT1X	metallothionein 1X	**1.6122478**	1.4785146	1.8759271	up
MT1P3	metallothionein 1 pseudogene 3	**1.5198572**	1.307409	1.7234105	up
CSH2	chorionic somatomammotropin hormone 2	**1.5099572**	#	1.3089397	up
KRTAP10-2	keratin associated protein 10-2	**1.5006515**	#	1.2620568	up
MT1JP|MT1M	metallothionein 1J (pseudogene) | metallothionein 1M	**1.4997663**	1.2292569	1.8385758	up
MT1P2	metallothionein 1 pseudogene 2	**1.4846494**	1.2776792	#	up
MT1H	metallothionein 1H	**1.4614742**	#	2.0551221	up
SCXA|SCXB	scleraxis homolog A | scleraxis homolog B	**1.4582701**	#	1.2557648	up
SCXA|SCXB	scleraxis homolog A | scleraxis homolog B	**1.4572202**	#	1.25512	up
TMEM61	transmembrane protein 61	**1.4298121**	#	#	up
MT1DP	metallothionein 1D (pseudogene)	**1.4165779**	1.2316265	#	up
GBP2	guanylate binding protein 2, interferon inducible	**-1.5112702**	-1.4250351	-1.4131097	down
MIR21	microRNA 21	**-1.5275458**	-1.2404983	-1.6964701	down
ZNF224	zinc finger protein 224	**-1.572604**	-1.2397038	-1.3674877	down
SAA4	serum amyloid A4, constitutive	**-1.573077**	-1.3437153	-1.5190501	down
ACTR3C	ARP3 actin-related protein 3 homolog C (yeast)	**-1.5773983**	-1.2445956	#	down
SNORD50B	small nucleolar RNA, C/D box 50B	**-1.5776544**	#	-1.527359	down
TRA2A	transformer 2 alpha homolog (Drosophila)	**-1.6224118**	-1.2303989	-1.5386964	down
CD109		**-1.659105**	#	-1.5179392	down
CYP3A7	cytochrome P450, family 3 subfamily A, polypeptide 7	**-1.664616**	-1.3983974	-1.483224	down
NT5E	5′-nucleotidase, ecto (CD73)	**-1.691666**	-1.5449091	-1.5966983	down
SPINK1	serine peptidase inhibitor, Kazal type 1	**-1.700486**	-1.39644	-1.4552168	down
SNORD14E	small nucleolar RNA, C/D box 14E	**-1.7864679**	#	-1.8290889	down
SNORD75	small nucleolar RNA, C/D box 75	**-1.8150924**	#	-1.5868542	down
UCA1	urothelial cancer associated 1 (non-protein coding)	**-1.8260639**	-1.5522093	-1.6103948	down
MIR15A	microRNA 15a	**-1.9946613**	-1.3650888	-1.779063	down

Table 3

Top fifteen up- and downregulated genes (p<0.05) by three week treatment with 0.1 μM CdCl$_2$ in HepG2 cells. Fold changes for other treatments are also listed. ## fold change <1.25

Gene Symbol	Gene Description	Fold Change (0.1 μM)	Fold Change (0.05 μM)	Fold Change (0.01 μM)	Regulation
GSTTP1|GSTTP2|	glutathione S-transferase theta pseudogene 1	**1.72073**	1.4901183	1.379154	up
SNRPN	small nuclear ribonucleoprotein polypeptide N	**1.6563919**	1.6350169	##	up
CYP3A7	cytochrome P450, family 3, subfamily A, polypeptide 7	**1.5384295**	1.3135703	##	up
NPNT	NPNT nephronectin	**1.5199842**	1.4056195	##	up
CAB39L	calcium binding protein 39-like	**1.5002468**	1.240774	##	up
ZBTB38	zinc finger and BTB domain containing 38	**1.4971563**	1.3198949	##	up
SNORD116-13	Small Nucleolar RNA, C/D Box 116-13	**1.4758297**	1.2465166	1.2062092	up
FBLN5	fibulin 5	**1.4431939**	1.4760754	1.3243747	up
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	**1.4407821**	1.4589071	1.2065107	up
C6orf138	chromosome 6 open reading frame 138	**1.4336088**	1.3983722	##	up
C5orf41	CREB3 regulatory factor	**1.422349**	1.3452742	##	up
MGAM	maltase-glucoamylase (alpha-glucosidase)	**1.4202621**	1.3677275	##	up
SNORD109A|SNORD109B	small nucleolar RNA, C/D box 109A/109B	**1.4162974**	1.3215218	1.3739192	up
SNORD109A|SNORD109B	small nucleolar RNA, C/D box 109A/109B	**1.4149537**	1.3213891	1.3719118	up
SCARNA9L	small Cajal body-specific RNA 9-like	**1.4086239**	1.220644	1.3410316	up
RRM2	ribonucleotide reductase M2	**−1.4714146**	−1.3555955	##	down
BTBD11	BTB (POZ) domain containing 11	**−1.4720253**	−1.4257193	##	down
CTGF	connective tissue growth factor	**−1.4752284**	−1.3320917	##	down
HIST1H3B	histone cluster 1, H3b	**−1.485579**	−1.3974302	##	down
EGR1	early growth response 1	**−1.517825**	−1.4408088	##	down
TGM2	transglutaminase 2	**−1.5214871**	−1.4627025	##	down
ANKRD1	ankyrin repeat domain 1	**−1.5234282**	−1.4508984	##	down
HIST1H2AK|HIST1H2BN	histone cluster 1, H2ak/H2bn	**−1.537931**	##	##	down
MIXL1	Mix paired-like homeobox	**−1.5498296**	−1.3671521	##	down
SLC16A6	solute carrier family 16, member 6	**−1.5749238**	−1.6012409	−1.322911	down
SLC2A3	solute carrier family 2 member 3	**−1.5907145**	−1.4265127	##	down
ADH4	alcohol dehydrogenase 4 (class II)	**−1.6070219**	−1.3255012	−1.2636199	down
LOC1720	dihydrofolate reductase pseudogene	**−1.632394**	−1.3695079	−1.207613	down
ID1	inhibitor of DNA binding 1	**−1.6802144**	−1.5311089	##	down
C8orf4	chromosome 8 open reading frame 4	**−1.7252662**	−1.5806782	##	down

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Table 4

qRT-PCR validation of microarray results. qRT-PCR data represents means of triplicates from two independent experiments, represented as fold change to level of expression in control HepG2 cells.

Gene	0.5 μM Acute Treatment	
	Microarray Fold Change	qRT-PCR Fold Change
MT1G	2.216	2.074
MT1F	1.912	3.510
MT1M	1.844	7.162
CYP3A7	−1.665	−2.098
NT5E	−1.692	−2.306
SPINK1	−1.701	−1.523
Gene	**0.1 μM Chronic Treatment**	
	Microarray Fold Change	qRT-PCR Fold Change
CYP3A7	1.538	1.871
NPNT	1.520	3.680
DNAJB9	1.441	1.776
EGR1	−1.518	−2.413
ADH4	−1.607	−1.269
ID1	−1.680	−1.258