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Dose- and Time-Dependent Epigenetic Changes in the Livers of Fisher 344 Rats Exposed to Furan

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The presence of furan in common cooked foods along with evidence from experimental studies that lifetime exposure to furan causes liver tumors in rats and mice has caused concern to regulatory public health agencies worldwide; however, the mechanisms of the furan-induced hepatocarcinogenicity remain unclear. The goal of the present study was to investigate whether or not long-term exposure to furan causes epigenetic alterations in rat liver. Treating of male Fisher 344 rats by gavage 5 days per week with 0, 0.92, 2.0, or 4.4 mg furan/kg body weight (bw)/day resulted in dose- and time-dependent epigenetic changes consisting of alterations in DNA methylation and histone lysine methylation and acetylation, altered expression of chromatin modifying genes, and gene-specific methylation. Specifically, exposure to furan at doses 0.92, 2.0, or 4.4 mg furan/kg bw/day caused global DNA demethylation after 360 days of treatment. There was also a sustained decrease in the levels of histone H3 lysine 9 and H4 lysine 20 trimethylation after 180 and 360 days of furan exposure, and a marked reduction of histone H3 lysine 9 and H3 lysine 56 acetylation after 360 days at 4.4 mg/kg bw/day. These histone modification changes were accompanied by a reduced expression of *Suv39h1*, *Prdm2*, and *Suv4-20h2* histone methyltransferases and *Ep300* and *Kat2a* histone acetyltransferases. Additionally, furan at 2.0 and 4.4 mg/kg bw/day induced hypermethylation-dependent down-regulation of the *Rassf1a* gene in the livers after 180 and 360 days. These findings indicate possible involvement of dose- and time-dependent epigenetic modifications in the furan hepatotoxicity and carcinogenicity. *Key words*: Furan; liver; rat; epigenetic changes.

Furan is a volatile heterocyclic compound widely used in the chemical manufacturing industry and is also found in a variety of common heat-processed foods (Bakhiya and Apple, 2010; Morehouse *et al.*, 2008; Moro *et al.*, 2012). The average exposure of the U.S. consumer to furan is ~0.25 μg/kg body weight (bw)/day (Morehouse *et al.*, 2008). Importantly, relatively high furan levels have been detected in infant formulas and baby foods, and it has been estimated that the average consumption by children during their first year of life and for infants consuming only formula is 0.41 and 0.9 μg/kg bw/day, respectively (Morehouse *et al.*, 2008). Additionally, it has been reported that furan is a constituent in tobacco smoke (Charles *et al.*, 2008). The presence of furan in food has caused concern to regulatory public health agencies such as the U.S. Food and Drug Administration and European Food Safety Authority and led to the research and evaluation of furan by organizations, including the National Toxicology Program (NTP, 1990) and the International Agency for Research on Cancer (IARC, 1995). Based on findings from high-dose animal studies, furan is considered to be a "reasonably anticipated to be a human carcinogen" by the NTP and a "possible human carcinogen (Group 2B)" by the IARC.

Furan is metabolized in the liver by CYP2E1 to a highly reactive *cis*-2-butene-1,4-dial that may react with various biological macromolecules, including proteins and DNA (Peterson, 2006). A lifetime exposure of rats and mice to furan produced substantial evidence of carcinogenic activity of furan (Moser *et al.*, 2009; NTP, 1990). Specifically, exposure to a low dose of furan (2 mg/kg bw/day) resulted mainly in the development of liver cholangiocarcinomas in male and female Fisher 344 (F344) rats, whereas higher doses of furan (4 mg/kg bw/day and greater) induced hepatocellular adenomas, hepatocellular carcinomas, and mononuclear cell leukemia in both sexes of F344 rats, and hepatocellular adenoma and carcinoma in male and female B6C3F1 mice (Cordelli *et al.*, 2010; Moser *et al.*, 2009; NTP, 1990).

Despite a large body of evidence for furan-induced hepatocarcinogenicity, the mechanisms leading to the liver tumor development are still unclear (Bakhiya and Apple, 2010). Although some reports have demonstrated furan genotoxicity in both *in vitro* and *in vivo* model systems (Banda *et al.*, 2013; Cordelli *et al.*, 2010; Johansson *et al.*, 1997; Kellert *et al.*, 2008; Neuwirth *et al.*, 2012), other genotoxicity studies have been

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negative or produced equivocal results (Durling *et al.*, 2007; McDaniel *et al.*, 2012) suggesting a predominantly nongenotoxic mode of furan carcinogenicity. This is further evidenced by results of several studies indicating that furan may cause liver carcinogenesis via the induction of cytotoxicity, inflammation, oxidative stress, inactivation of protein functions through covalent binding of the furan reactive metabolites, increased cell proliferation, and liver injury (Hickling *et al.*, 2010; Jackson *et al.*, 2014; Moro *et al.*, 2012; Moser *et al.*, 2009).

Accumulating evidence indicates that some adverse biological effects caused by exposure to toxicants, regardless of their DNA reactivity, may be attributed to the ability of chemical compounds to provoke epigenetic alterations, including DNA hypomethylation, promoter region DNA hypermethylation of tumor suppressor genes, histone modifications, and changes in the function of chromatin modifying enzymes (Baccarelli and Bollati, 2009). Consequently, it has been hypothesized that chemically induced epigenetic alterations may play an important role in the carcinogenic process in some instances (Goodman and Watson, 2002; Pogribny and Beland, 2013). The results of several studies support the link between the adverse biological effects of furan and nongenotoxic epigenetic abnormalities (Chen *et al.*, 2010, 2012; Jackson *et al.*, 2014; Recio *et al.*, 2013). For example, Chen *et al.* (2010) demonstrated that exposure of male F344 rats to low doses of furan (0.1–2 mg/kg bw/day) for 28 days altered the expression profiles of critical cancer-related genes, including those controlling cell cycle and cell death, which were independent of the DNA methylation status. More recently, Chen *et al.* (2012) reported that the exposure of male Sprague Dawley rats to a high concentration of furan (30 mg/kg bw/day) for 3 months resulted in noticeable changes in gene expression accompanied by hypermethylation and hypomethylation of specific gene promoter regions. Additionally, Recio *et al.* (2013) reported an altered expression of long noncoding RNAs in the livers of female B6C3F1 mice exposed to furan at 4 and 8 mg/kg bw/day for 3 weeks. Although these data suggest the possible involvement of epigenetic mechanisms in furan hepatotoxicity and carcinogenicity, there is a lack of conclusive information to clarify the role of epigenetic aberrations in their pathogenesis. In light of this, we investigated changes in the status of global DNA methylation and histone modifications, promoter methylation, and the expression of tumor suppressor and chromatin modifying genes in the livers of male F344 rats exposed to furan for 90, 180, and 360 days at doses 0, 0.92, 2.0, and 4.4 mg/kg bw/day.

MATERIALS AND METHODS

Animals, experimental design, and treatments. Male F344 rats (3 weeks of age) were obtained from the breeding colony at the National Center for Toxicological Research (NCTR). This strain of rats was used in a furan carcinogenicity bioassay conducted by the National Toxicology Program (NTP, 1990). Rats were housed in sterilized cages in a temperature controlled room (24°C) with a 12 h light/dark cycle, and given *ad libitum* access to water and a NIH-41 irradiated pelleted diet. At 7 weeks of age, rats were treated by gavage 5 days per week with 0, 0.92, 2.0, or 4.4 mg furan/kg bw/day dissolved in corn oil, a protocol identical that used in the previous furan carcinogenicity bioassay (NTP, 1990). Five experimental rats and five control rats from each treatment group were euthanized by exsanguination following deep isoflurane anesthesia after a period of 90, 180, or 360 days of treatments. The livers were excised, and a slice of the median lobe was fixed in neutral buffered formalin for 48 h for histopathological examination. The remaining median lobe was snap-frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses. All experimental procedures were reviewed and approved by the NCTR Animal Care and Use Committee.

Global DNA methylation. The methylation status of genomic DNA was evaluated by fluorometric MethylFlash Methylated DNA Quantification kits (Epigentek, Farmingdale, NY) according to the manufacturer's protocol.

Western blotting. The level of trimethylation of histone 3 lysine 4 (H3K4me3), histone 3 lysine 9 (H3K9me3), histone 3 lysine 27 (H3K27me3), and histone 4 lysine 20 (H4K20me3), and of acetylation of histone 3 lysine 9 (H3K9ac) and histone H3 lysine 56 (H3K56ac) were determined by Western blot analysis. Primary antibodies against histone H3K4me3, H3K9me3, H3K27me3, H4K20me3, and H3K56ac were purchased from Millipore Corporation (Billerica, MA); primary antibodies against H3K9ac were obtained from Abcam (Cambridge, MA). All primary antibodies were diluted to 1:1000. Horseradish peroxidase (HRP)-coupled secondary antirabbit antibodies (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA) were used for visualization. Chemiluminescence was detected with the HRP Substrate (Millipore Corporation) and measured directly by a BioSpectrum AC Imaging System (UVP, Upland, CA). Signal intensities were analyzed using ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA). Equal protein loading was confirmed by immunostaining with antihistone H3 and antihistone H4 antibodies (Millipore Corporation).

RNA extraction and quantitative reverse transcription PCR. Total RNA was extracted from liver tissue using RNeasy Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using random primers and High Capacity cDNA Reverse Transcription kits (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. cDNA were analyzed in a 96-well plate assay format using a 7900HT Fast Real-Time PCR System (Life Technologies). Each plate contained one experimental gene and a housekeeping gene. All primers for the gene expression analysis were obtained from Applied Biosystems and are listed in Supplementary table 1. The cycle threshold (C_t) for

each sample was determined from the linear region of the amplification plot. The ΔC_t values for all genes were determined relative to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) or β-actin (*Actb*). The $\Delta\Delta C_t$ values were calculated using treated group means relative to the control group means. The fold change data were calculated from the $\Delta\Delta C_t$ values. A gene was considered to be differentially expressed if the change in expression corresponded to the following criteria: a p-value <0.05 and a fold change >1.5. All quantitative reverse transcription-PCR (qRT-PCR) reactions were conducted in triplicate and the experiments were repeated twice.

Methylation-specific PCR. The methylation status of CpG sites located in the promoter regions of Ras association (RalGDS/AF-6) domain family member 1 (*Rassf1a*), cyclin-dependent kinase inhibitor 2A (*Cdkn2a*; $p16^{INK4A}$), and E-cadherin (*Cdh1*) was determined by methylation-specific PCR (MSP). Briefly, genomic DNA (1 μg) was modified by a standard bisulfite treatment, followed by PCR amplification using two sets of primers specific to unmethylated and methylated cytosine residues in the promoter region of the gene of interest. The sequence of gene-specific unmethylated and methylated primers used in this study are listed in Supplementary table 2. The MSP products were then visualized on a 3% high-resolution agarose gel. The presence or absence of a band after PCR amplification with the methylated primer set was indicative of methylation status. Negative control PCR amplifications were performed by using both sets of primers with unmodified DNA.

Statistical analyses. Results are presented as mean ± SD ($n = 5$ from each treatment group at each time interval). Data were analyzed by one-way analysis of variance (ANOVA), with pairwise comparisons being made by the Student–Newman-Keuls method. Linear regression analysis was used to determine dose-related trends. When necessary, the data were natural log transformed before conducting the analyses to maintain a more equal variance or normal data distribution. p-values <0.05 were considered significant.

RESULTS

Liver Global DNA Methylation and Expression of DNA Methyltransferases

Accumulating evidence indicates that exposure to well-known chemical carcinogens may perturb the status of DNA methylation in target organs (Baccarelli and Bollati, 2009; Goodman and Watson, 2002; Herceg *et al.*, 2013; Pogribny and Beland, 2013). Furthermore, it has been suggested that the disruption of normal DNA methylation patterns may be responsible for the induction and promotion of tumor development (Feinberg, 2004; Jaffe, 2003). In light of this, the status of global DNA methylation in the livers of the F344 rats exposed to furan was determined. Figure 1A shows that genomic DNA methylation in the livers of furan-treated rats at 0.92, 2.0, and 4.4 mg/kg bw/day decreased in a dose-related fashion after a 360-day treatment period. At that time, the level of genomic DNA methylation in the livers of rats treated with furan at the doses 0.92, 2.0, and 4.4 mg/kg bw/day was lower by 30, 36, and 49%, respectively, as compared to the control rats (Fig. 1A).

The expression of *Dnmt1* did not change at any time interval or dose in the livers of furan-treated rats (Fig. 1B). In contrast, the expression of *Dnmt3a* in the livers of rats treated with furan demonstrated a dose-related decreasing trend at 360 days, with the 4.4 mg/kg bw/day dose causing a significant reduction (Fig. 1C). Likewise, *Dnmt3b* was significantly down-regulated at 90, 180, and 360 days in rats treated with 4.4 mg/kg bw/day (Fig. 1D).

Status of Liver Histone Lysine Modifications

In addition to chemically induced alterations in DNA methylation, the disruption of a normal pattern of covalent histone modifications is another epigenetic change frequently observed upon chemical exposure. In order to determine whether or not furan treatment causes any alterations in histone lysine modifications, the levels of H3K4me3, H3K9me3, H3K27me3, H4K20me3, H3K9ac, and H3K56ac in the livers of control rats and rats exposed to furan were examined using Western immunoblotting (Fig. 2A). It is well established that these histone lysine modifications play a crucial role in the maintenance of genomic stability, chromatin assembly and organization, DNA damage and repair, and regulation of gene transcription (Jenuwein, 2006; Tanaka *et al.,* 2012).

Figure 2B shows that the levels of histone H3K9 and H4K20 trimethylation decreased in a dose-dependent manner after 180 and, especially, 360 days of exposure to furan. After 360 days, the levels of histone H3K9me3 and H4K20me3 in the livers of rats treated with furan decreased by 35 and 36%, respectively, as compared to the control rats. Treatment with furan had little to no effect on histone H3K4 or H3K27 trimethylation in the rat livers (Supplementary fig. 1).

The levels of histone H3K9ac and H3K56ac in livers of F344 rats treated with furan at 4.4 mg/kg remained unchanged after 90 and 180 days, but decreased significantly after 360 days of exposure (Fig. 2). At that time, the levels of histone H3K9ac and H3K56ac decreased by 45 and 32%, respectively.

Expression of Chromatin Modifying Genes in Liver

To investigate further the mechanisms of epigenetic alterations, the expression of histone modifying genes was determined by qRT-PCR. Exposure to furan at 4.4 mg/kg bw/day causes a significant down-regulation of *Prdm2*, *Suv39h1*, *Suv4-20h2*, and *Ehmt2* histone methyltransferases and dose-dependent down-regulation of *Ep300* and *Kat2a* histone acetyltransferases after a 360-day treatment period (Fig. 3).

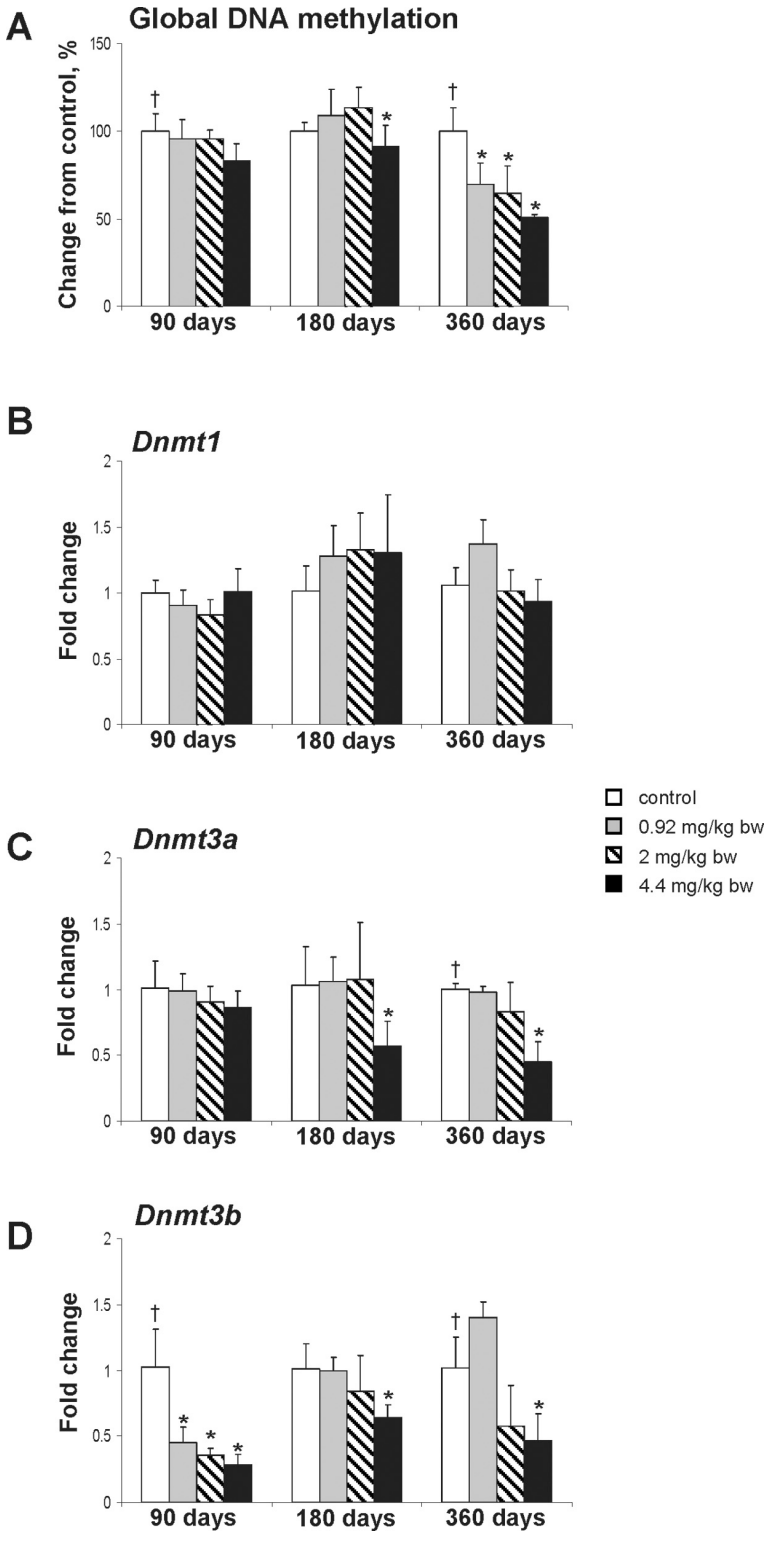

FIG. 1. DNA methylation and the expression of DNA methyltransferases in the livers of control rats and rats treated with furan. (A) The extent of DNA methylation. The results are presented as an average percentage change in the degree of DNA hypomethylation in the livers of rats from the furan-treated group relative to that in the control group, which was assigned a value of 100%. The expression of *Dnmt1* (B), *Dnmt3a* (C), and *Dnmt3b* (D) genes was determined by qRT-PCR as detailed in the Materials and Methods section. The results are presented as an average fold change in the expression of each gene in the livers of rats from each experimental group relative to that in the control group, which were assigned a value 1. Values are mean ± SD ($n = 5$). * denotes a significant ($p < 0.05$) difference from the control rats; † denotes significant ($p < 0.05$) trend.

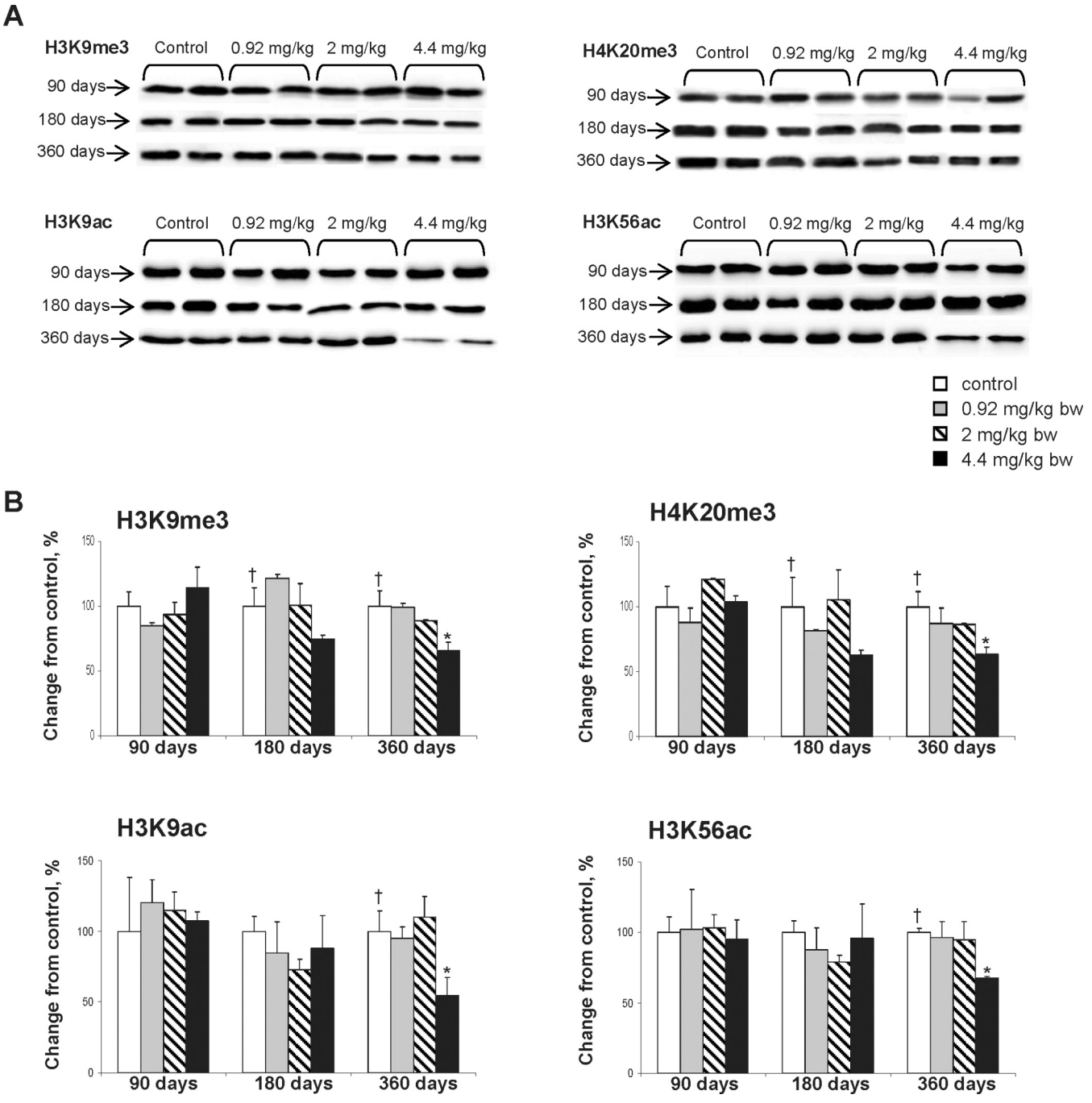

FIG. 2. Westernblot analysis of histone H3K9, and H4K20 trimethylation and H3K9 and H3K56 acetylation in the livers of control rats and rats treated with furan. (A) Representative Western blot images. (B) Densitometric analysis of the immunostaining results, shown as percent change in histone modification level in the each experimental group relative to the corresponding values in the control group. Values are mean ± SD ($n = 5$). * denotes a significant ($p < 0.05$) difference from the control rats; † denotes significant ($p < 0.05$) trend.

Gene-Specific DNA Methylation Changes in the Liver of Fisher 344 Rats Exposed to Furan

One of the most compelling examples of the mechanistic link between epigenetic alterations and carcinogenesis is silencing of critical tumor-suppressor genes by hypermethylation of promoter CpG islands (Jones and Baylin, 2007). Hence, the promoter methylation and expression status of $p16^{INK4a}$, *Rassf1a*, and *Cdh1*, tumor suppressor genes that are frequently silenced epigenetically in human and rodent liver carcinogenesis (Pogribny and Rusyn, 2014), was investigated.

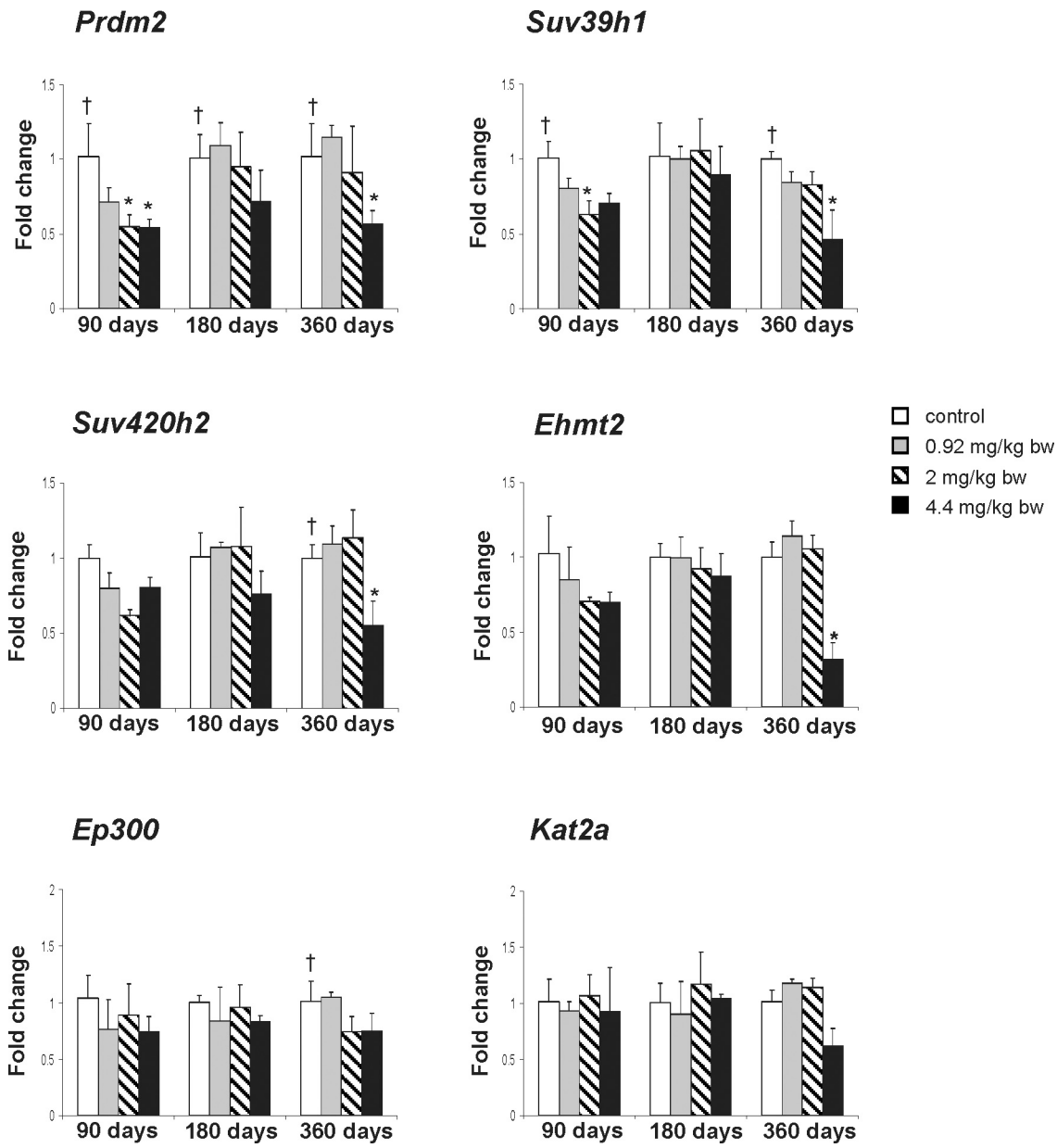

FIG. 3. The expression of chromatin-modifying genes in the livers of control rats and rats treated with furan. The expression of *Prdm2*, *Suv39h1*, *Suv420h2*, *Ehmt2*, *Ep300*, and *Kat2a* genes was determined by qRT-PCR as detailed in the Materials and Methods section. The results are presented as an average fold change in the expression of each gene in the livers of rats from each experimental group relative to that in the control group, which were assigned a value 1. Values shown are mean ± SD ($n = 5$). * denotes a significant ($p < 0.05$) difference from the control rats; † denotes significant ($p < 0.05$) trend.

Exposure to furan at a dose 4.4 mg/kg bw/day caused hypermethylation of $p16^{INK4a}$ and *Rassf1a* after 180 days, and dose-dependent hypermethylation of these genes after 360 days in the livers rats treated with furan at 2.0 and 4.4 mg/kg (Table 1, Figs. 4A and 4C), whereas no methylation changes were found at the earlier, 90-day time interval. In contrast, furan treatment did not change the methylation status of the *Cdh1* gene at any time interval (Table 1, Fig. 4C). qRT-PCR analysis demonstrated that only the expression of *Rassf1a* (Fig. 4B) significantly decreased in association with its promoter hypermethylation, whereas the expression of hypermethylated $p16^{INK4a}$ (Fig. 4D) or unmethylated *Cdh1* (Fig. 4E) did not change.

DISCUSSION

It is widely believed that continuous exposure to certain natural and man-made chemical and physical agents plays a causative role in the development of cancer. This suggestion

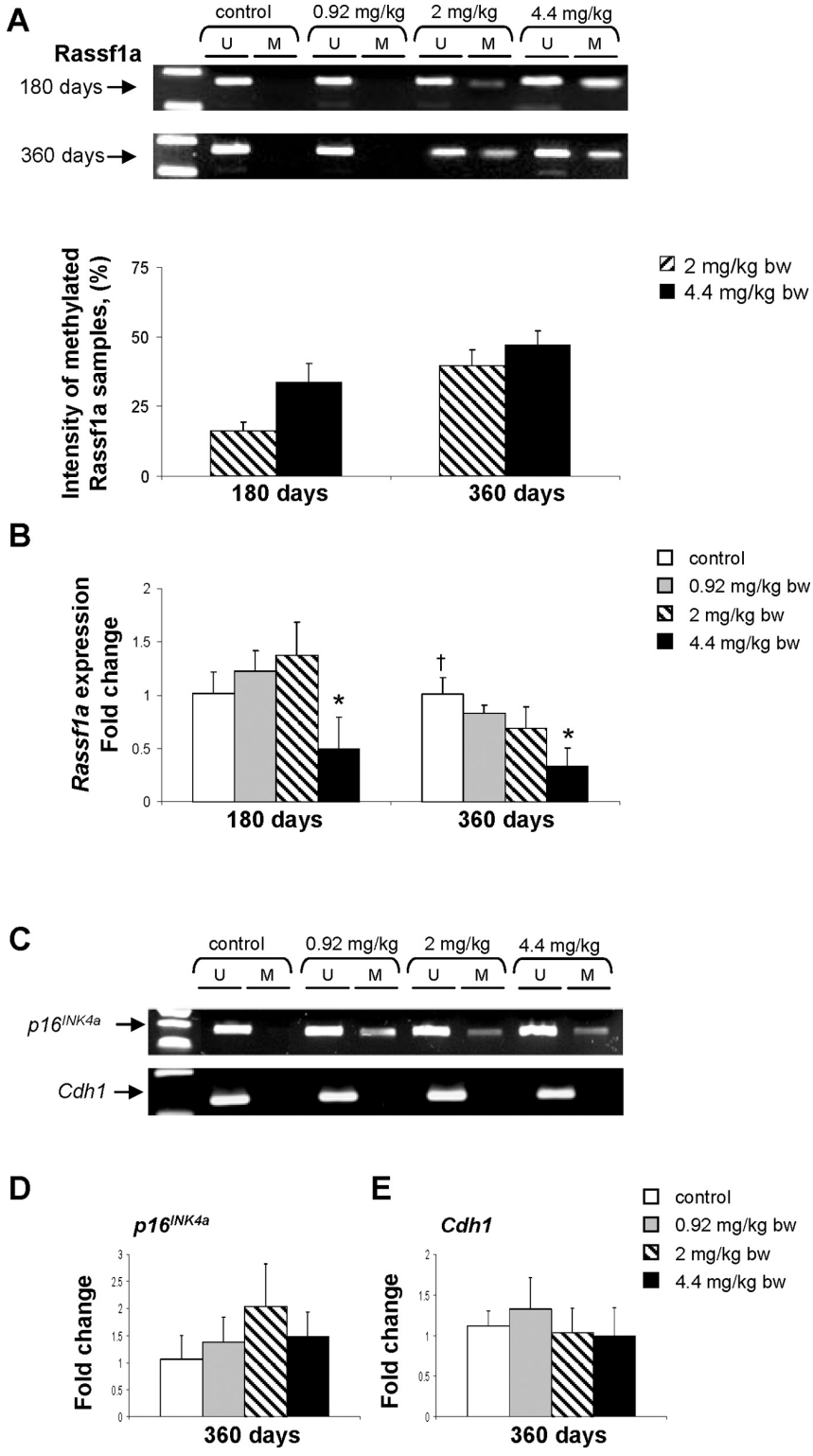

FIG. 4. Promoter methylation and expression of *Rassf1a*, and *Cdh1* tumor suppressor genes in the livers of control rats and rats treated with furan. (A) Methylation-specific PCR analysis of *Rassf1a* promoter methylation. Bisulfite-modified DNA was amplified with two sets of primers specific to unmethylated (U) and methylated (M) CpG sites in the promoter region. Presence of methylated PCR product band after amplification with methylation-specific primers indicates appearance of *de novo* methylation. (B) The expression of *Rassf1a* as determined by qRT-PCR. (C) Methylation-specific PCR analysis of $p16^{INK4a}$ and *Cdh1* promoter methylation. (D) The expression of $p16^{INK4a}$ as determined by qRT-PCR. (D) The expression of *Cdh1* as determined by qRT-PCR. Values are mean ± SD ($n = 5$). * denotes a significant ($p < 0.05$) difference from the control rats; † denotes significant ($p < 0.05$) trend.

TABLE 1
Percentage of Methylated Samples in Liver Rats Treated with Furan

Gene name	180 days			360 days		
	0.92 mg/kg	2 mg/kg	4.4 mg/kg	0.92 mg/kg	2 mg/kg	4.4 mg/kg
Cdh1	0% (0/5)[a]	0% (0/5)	0% (0/6)	0% (0/4)	0% (0/4)	0% (0/5)
p16^{INK4a}	0% (0/5)	0% (0/5)	80% (4/5)	50% (2/4)	75% (3/4)	100% (4/4)
Rassf1a	0% (0/5)	0% (0/5)	83% (5/6)	0% (0/4)	75% (3/4)	100% (5/5)

Note. Samples in control groups were unmethylated.
[a]Methylated/total samples ratio.

is based on a wealth of data showing the ability of some these agents to trigger the mutational and/or nonmutational (i.e., epigenetic) events that may affect the proper expression of genetic information leading to the accumulation of multiple cancer-specific phenotypes in target organ. Recent work on mechanisms of chemical carcinogenesis indicates that both genotoxic and nongenotoxic carcinogens affect the cellular epigenetic state via induction of various types epigenotoxic abnormalities (Baccarelli and Bollati, 2009; Herceg *et al.*, 2013; Pogribny and Beland, 2013).

The present study demonstrated that long-term exposure of F344 rats to furan produced prominent dose- and time-dependent epigenetic changes in the liver consisting of alterations in the global pattern of DNA methylation and histone lysine methylation and acetylation, gene-specific methylation, and altered expression of chromatin modifying genes. Specifically, exposure to furan caused global demethylation of hepatic DNA after 360 days of treatment. We also observed a sustained decrease in the levels of histone H3K9 and H4K20 trimethylation in the livers after 180 and 360 days of furan exposure, and a marked reduction of histone H3K9 and H3K56 acetylation after 360 days at the highest dose of 4.4.mg/kg bw/day. These histone modification changes were accompanied by a reduced expression of *Suv39h1*, *Prdm2*, and *Suv4-20h2* histone methyltransferases and *Ep300* and *Kat2a* histone acetyltransferases. Additionally, treatment with furan induced hypermethylation-dependent down-regulation of the *Rassf1a* tumor suppressor gene in the livers after 180 and 360 days of treatment at the highest furan dose.

Previous reports have demonstrated that carcinogen-induced demethylation of the genome in target organs is one of the main epigenetic responses to a range of well-known chemical liver carcinogens, including arsenic, 2-acetylaminofuorene, and aflatoxin B_1 (Chen *et al.*, 2004; Wu *et al.*, 2013). In contrast, Chen *et al.* (2010, 2012) previously reported that exposure of male F344 rats to furan at 0.1 and 2.0 mg/kg bw/day for 28 days, or male Sprague Dawley rats to 30 mg/kg day/bw for 3 months did not alter the status of global DNA methylation in the livers. Similarly, in the present study, the status of genomic DNA methylation remained unaffected in the livers of furan-treated rats at any dose at 90 days; however, after 360 days we found substantial decrease in genomic DNA methylation in the livers of F344 rats exposed to all three dose levels (0.92, 2.0, and 4.4 mg/kg bw/day).

It is well-established that the normal pattern of DNA methylation is closely related to the functioning of the DNA methylation machinery, including DNA methyltransferase DNMT1, DNMT3a and DNMT3b, DNA demethylases, DNA integrity, and the status of one carbon metabolism. The results of the present study indicate that furan-induced loss of genomic DNA methylation may be explained by a reduced expression of both *de novo Dnmt3a* and *Dnmt3b* after 180 and 360 days of treatment. This suggestion corresponds to previous findings demonstrating that inactivation of either *Dnmt3a* or *Dnmt3b* resulted in global DNA hypomethylation (Dodge *et al.,* 2005; Raddatz *et al.,* 2012).

The decrease in global DNA methylation observed upon furan treatment was accompanied by a decrease in histone H3K9me3, H4K20me3, H3K9ac, and H3K56ac. Trimethylated states of histone H3K9 and H4K20 have been shown to play a central role in the formation of heterochromatin and the maintenance of genomic stability and chromatin integrity (Jenuwein, 2006). Additionally, H3K56 acetylation is a key player in DNA replication and repair (Tanaka *et al.,* 2012). Therefore, it is possible that a loss of any of these histone marks may compromise genomic stability and integrity and impair the viability of cells.

The mechanisms responsible for the furan exposure-related histone alterations may be attributed, in part, to an altered expression of histone-modifying genes. Specifically, decreased time- and dose-dependent levels of histone H3K9 and H4K20 trimethylation in the livers of rats exposed to furan were accompanied by a reduced expression of *Suv39h1*, *Prdm2*, and *Suv4-20h2* histone methyltransferases. Likewise, the decrease in acetylation of histone H3K9 and H3K56 can be explained by down-regulation of *Ep300* and *Kat2a* histone acetyltransferases.

An additional important finding in this study is promoter hypermethylation of *p16^{INK4a}* and *Rassf1a* tumor suppressor genes in the livers of furan-treated rats; however, of these hypermethylated genes, only the expression of *Rassf1a* changed. This is unexpected but not unusual observation since any change in gene expression or even a greater expression of hypermethylated genes has been reported during hepatocarcinogenesis and in human HCC (Revil *et al.*, 2013). These findings suggest that not all DNA methylation changes are of equal importance dur-

ing carcinogenesis and that some DNA methylation changes may be purely "passenger events" as is the case with genetic mutations (Kalari and Pfeifer, 2010). The results of this study demonstrate that the expression of *Rassf1a* was significantly decreased in association with its promoter methylation. This indicates that hypermethylation of *Rassf1a* may be a significant exposure-related epigenetic event in the pathogenesis of furan-induced liver carcinogenesis. This suggestion is supported by the wealth of data that has established a role of *Rassf1a* down-regulation in the loss of cell cycle control, resistance to apoptosis, and enhanced genomic instability (Richter *et al.*, 2009). Additionally, several reports have documented that hypermethylation of *Rassf1a* is not only one of the most common epigenetic alterations in full-fledged liver tumors, but also a frequent epigenetic abnormality found in the early stages of aflatoxin B_1- and hepatitis B virus-related human hepatocarcinogenesis (Um *et al.*, 2011; Zhang *et al.*, 2002).

In the present study, epigenetic changes in the livers of furan-exposed rats occurred in the absence of the evident morphological neoplastic abnormalities. This suggests that these epigenetic alterations are directly related to the effect of furan exposure and are not a consequence of neoplastic morphological changes; however, we cannot exclude the possibility that nonneoplastic morphological lesions, e.g., cellular proliferation, cell death, inflammation, could have contributed, in part, to the observed epigenetic alterations. Further research is needed to determine the mechanistic role of epigenetic alterations in furan-induced liver carcinogenesis.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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