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Epigenetic Events Determine Tissue-Specific Toxicity of Inhalational Exposure to the Genotoxic Chemical 1,3-Butadiene in Male C57BL/6J Mice

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ABSTRACT

1,3-Butadiene (BD), a widely used industrial chemical and a ubiquitous environmental pollutant, is a known human carcinogen. Although genotoxicity is an established mechanism of the tumorigenicity of BD, epigenetic effects have also been observed in livers of mice exposed to the chemical. To better characterize the diverse molecular mechanisms of BD tumorigenicity, we evaluated genotoxic and epigenotoxic effects of BD exposure in mouse tissues that are target (lung and liver) and non-target (kidney) for BD-induced tumors. We hypothesized that epigenetic alterations may explain, at least in part, the tissue-specific differences in BD tumorigenicity in mice. We evaluated the level of *N*-7-(2,3,4-trihydroxybut-1-yl)guanine adducts and 1,4-bis-(guan-7-yl)-2,3-butanediol crosslinks, DNA methylation, and histone modifications in male C57BL/6 mice exposed to filtered air or 425 ppm of BD by inhalation (6 h/day, 5 days/week) for 2 weeks. Although DNA damage was observed in all three tissues of BD-exposed mice, variation in epigenetic effects clearly existed between the kidneys, liver, and lungs. Epigenetic alterations indicative of genomic instability, including demethylation of repetitive DNA sequences and alterations in histone-lysine acetylation, were evident in the liver and lung tissues of BD-exposed mice. Changes in DNA methylation were insignificant in the kidneys of treated mice, whereas marks of condensed heterochromatin and transcriptional silencing (histone-lysine trimethylation) were increased. These modifications may represent a potential mechanistic explanation for the lack of tumorigenesis in the kidney. Our results indicate that differential tissue susceptibility to chemical-induced tumorigenesis may be attributed to tissue-specific epigenetic alterations.

Key words: 1,3-butadiene, epigenetics, genotoxicity

1,3-Butadiene (BD), an industrial chemical used in the synthesis of plastics and rubber (White, 2007), has been classified as a "Group 1 carcinogen" (carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2012). BD poses an occupational health hazard to humans exposed in industrial settings, with BD exposure most closely associated with an increased cancer risk in hematolymphatic organs, including lymphosarcoma, reticulosarcoma, and leukemia (Delzell *et al.*, 1996). Additionally, BD is present in automobile exhaust and cigarette smoke, further contributing to the

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ubiquity of this chemical in the environment, and further raising concern for human health (Hecht, 1999; Pelz *et al.*, 1990). In rodents, in addition to the tumors in the hematopoietic system, BD exposure causes tumors in liver and lungs (Melnick and Sills, 2001). Although many comprehensive reports of the carcinogenicity of BD have been presented (Kirman *et al.*, 2010; Swenberg *et al.*, 2011), the understanding of the underlying mechanisms associated with the variation in effects of exposure across tissues is lacking.

The carcinogenicity of BD is mediated by DNA-reactive epoxides formed during butadiene metabolism (Goggin *et al.*, 2009; Swenberg *et al.*, 2011). BD is metabolized by cytochrome P450 monooxygenases to form 1,2-epoxy-3-butene, 1,2,3,4-diepoxybutane, and 1,2-epoxy-3,4-butanediol (Swenberg *et al.*, 2011). These reactive metabolites are directly mutagenic, and the interaction of these intermediates with DNA is considered a key step in the mechanism of BD carcinogenicity (Bond and Medinsky, 2001; Kirman *et al.*, 2010). Point mutations and sister chromatid exchanges, common consequences of bulky DNA adducts, have been observed as a consequence of exposure to butadiene (IARC, 2008).

In addition to the well-established and widely recognized genotoxicity of BD, other mechanisms have been postulated (Kirman *et al.*, 2010). A previous report by Koturbash *et al.* (2011b) demonstrated that BD also causes epigenetic effects in the mouse liver. It has been shown that exposure to a number of chemical carcinogens may impair the cellular epigenome in tissues that are targets for chemical-induced tumorigenesis by altering the DNA methylation and histone modification patterns (Baccarelli and Bollati, 2009; Pogribny and Beland, 2014; Pogribny and Rusyn, 2013). Such epigenetic alterations may ultimately compromise the proper expression of genetic information, a feature that is considered one of the hallmarks of cancer (Hanahan and Weinberg, 2011).

The tissue-specific variations in the response to DNA-damaging agents, including BD, are well known (Melnick and Sills, 2001); however, there is a lack of knowledge on the tissue-specific effects of BD exposure beyond the formation of DNA adducts and crosslinks (Goggin *et al.*, 2009; Swenberg *et al.*, 2011). Delineation of additional molecular events associated with tissue-specific BD damage may better characterize the underlying mechanisms of tissue-specific tumorigenicity and identify molecular drivers of the tumorigenic process.

The goal of the present study was to use a male mouse subacute inhalational study design to investigate whether or not exposure to BD results in distinct genotoxic effects and epigenetic alterations in various solid tissues. Such tissue-specific alterations may provide insight into the molecular characteristics of BD-induced tumorigenesis.

MATERIALS AND METHODS

Animals and experimental design. Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were housed in sterilized cages in a temperature-controlled (24°C) room with a 12/12-h light/dark cycle, and given *ad libitum* access to purified water and NIH-31 pelleted diet (Purina Mills, Richmond, Indiana). This strain was selected for the present study based on previously reported findings that demonstrated that C57BL/6J mice are highly susceptible to BD exposure, as evidenced by the greatest extent of both DNA damage and epigenetic alterations, relative to six other inbred mouse strains (Koturbash *et al.*, 2011a). After 2 weeks of acclimation, the mice (9–13 weeks of age) were allocated randomly into a control group exposed to filtered air, or an experimental group exposed to 425 ppm BD. Exposures were conducted 6 h/day, 5 days/week (Monday-Friday) for 2 consecutive weeks. Each experimental day, mice were placed in a cylindrical metal mesh holder for the duration of exposure and then returned to their cages. The mice were weighed daily. The concentration of BD in the exposure chambers was monitored at the beginning and at the end of each exposure period using gas chromatography as detailed below. Following the final exposure, mice were euthanized by exsanguination following deep nembutal (100 mg/kg intraperitoneal injection) anesthesia. Livers, lungs, and kidneys were excised and snap-frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses. The animals were treated humanely and with regard for alleviation of suffering. All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Measurement of BD chamber concentrations by gas chromatography. The concentration of BD in the exposure chambers was monitored twice a day: an hour after the beginning and an hour before the end of each exposure. An air sample was taken from each of the chambers and analyzed with a CP-3800 (Varian, Inc, Walnut Creek, California) gas chromatograph. The gas chromatograph utilized a 10 ml gas sample loop injector flushed from a sample Teflon bag, purged with a sample of the exposure gas injected into a separation column to isolate the BD from the air for integration of the response from a flame ionization detector. The 30 foot long, 1/8 in. in diameter stainless steel packed column was a Supelco (Bellefonte, Pennsylvania) part number 12809-U using 23% SP-1700 active phase on 80/100 Chromosorb PAW support. Calibration of the instrument was accomplished with analysis of a commercial gas standard of an eight-component hydrocarbon mixture (Ref#88-104317) prepared by National Specialty Gases (Morrisville. North Carolina) and certified using National Institute of Standards and Technology traceable standards with 5% uncertainty.

Determination of N7-guanine adduct formation. Genomic DNA was isolated from mouse liver, lung, and kidney tissues using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, California). The analysis of N-7-(2,3,4-trihydroxybut-1-yl)-guanine (THB-Gua) was performed following neutral thermal hydrolysis by liquid chromatography/positive ion electrospray ionization/tandem mass spectrometry (LC/ESI+MS/MS) as described in Goggin *et al.* (2009) with minor modifications. Briefly, DNA (100 μg) from each sample was spiked with 500 fmol stable isotope labeled THB-Gua internal standard (racemic [15_{N5}]-THB-Gua), and adjusted with deionized water to a volume of 400 μl. Neutral thermal hydrolysis was performed at 95°C for 30 min, and the samples were centrifuged through an Amicon 10K filter (Millipore Corporation, Billerica, Massachusetts). The samples were then purified by off-line HPLC using a Sunfire 4.6 × 250 mm column (Waters, Milford, Massachusetts) at a flow rate of 1 ml/min with 10 mM ammonium formate at a pH of 4.3 serving as mobile phase A, and methanol as mobile phase B. Collected fractions were dried down completely by vacuum centrifugation and then reconstituted in 20 μl of water before injection into the ACQUITY UPLC HSS T3 C18 column (2.1 × 100 mm, 1.8 μm at 200 μl/min) (Waters) coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts). Mobile Phase A was 0.1% acetic acid in water while methanol was used as mobile phase B. A gradient elution method from 1% B to 80% B over 5 min was used to achieve separation with a total cycle time of 15 min per injection. Quantitation of the THB-Gua analyte and

internal standard was performed by selected reaction monitoring using precursor to fragment ion transitions of *m/z* 256.1→152.1 (THB-Gua) and *m/z* 261.1→157.1 ([15_{N5}]-THB-Gua) with collision energy of 20 V for both.

Determination of bis-N7-guanine crosslinks formation. The levels of 1,4-bis-(guan-7-yl)-2,3-butanediol crosslinks (bis-N7G-BD) in DNA were evaluated using the method outlined in Sangaraju *et al.* (2012). Briefly, the aliquots were spiked with racemic 15_{N10}-*bis*-N7G-BD internal standard, subjected to neutral thermal hydrolysis (70°C for 1 h) followed by ultrafiltration through a Nanosep 10K filter (Pall Life Sciences, Ann Arbor, Michigan) and off-line HPLC purification. Off-line HPLC purification used a Zorbax Eclipse XDBC18 column (4.6 × 150 mm, 5 μm, from Agilent Technologies Palo Alto, California) eluted at 1 ml/min with a gradient of 0.4% formic acid in Milli-Q water (mobile phase A) and HPLC grade acetonitrile (mobile phase B). *Bis*-N7G-BD containing fractions, collected between 14 and 18 min, were dried completely and dissolved in 25 μl water. For nano-HPLC-nano ESI + MS/MS analysis, a nanoAcquity UPLC system (Waters) was interfaced to a TSQ Quantum UltraAM mass spectrometer (Thermo Fisher Scientific Corp, Waltham, Massachusetts) and used for all analyses with 0.01% acetic acid in LC-MS grade water used as mobile phase A and methanol:acetonitrile (1:1) as mobile phase B. Samples were first loaded on a trapping column (Symmetry C18 nanoAcquity, 0.18 × 20 mm, Waters) at 10 μl/min, which was followed by chromatographic separation using nano-HPLC column (75 μm × 200 mm) manually packed with Zorbax SB-C18, 5 μm chromatographic packing (Agilent Tech, Santa Clara, California). The column was eluted at 0.4 μl/min, and *bis*-N7G-BD eluted at approximately 15 min. Quantitation was based on the area ratios of the analyte peaks and the internal standard peaks on the extracted ion chromatograms corresponding to *bis*-N7G-BD and $^{15}N_{10}$-*bis*-N7G-BD, respectively. Nano-HPLC-nano ESI^{+}-MS/MS method standard curves were constructed by analyzing solutions with known amounts of internal and analyte standards followed by regression analysis of the actual and observed amounts of *bis*-N7G-BD.

Quantitative reverse-transcription polymerase chain reaction. Total RNA was extracted from frozen liver, lung, and kidney tissues using a Qiagen RNeasy kit. Complementary DNA (cDNA) was synthesized from 10 μg total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California). Gene expression was then determined by quantitative reverse-transcription PCR (qRT-PCR) using gene expression assays (Applied Biosystems). All genes and primers are listed in Supplementary Table 1. Two to four replicates were run for each sample. Reactions were performed in a 96-well assay format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA level of the housekeeping gene *Gusb1* was evaluated in tandem with each experimental run. The relative amount of each mRNA transcript was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

DNA Methylation of Repetitive Sequences. The methylation status of short interspersed nucleotide elements (SINEs) B1 and B2, and minor and major satellite repetitive sequences was determined by a McrBC-methylation sensitive quantitative PCR assay as described in Martens *et al.* (2005). Additionally, because that assay does not distinguish between 5-methylcytosine (5-mC) and 5-hydroxymethycytocise (5-hmC), we also assessed the level of 5-hmC in repetitive sequence by hydroxymethylated DNA immunoprecipitation (hMeDIP) using the hMeDIP kit (Diagenode, Denville, New Jersey).

Western blot analysis of histone modifications. The level of trimethylation of histones H3 lysine 9 (H3K9me3), H3 lysine 27 (H3K27me3), and H4 lysine 20 (H4K20me3), as well as acetylation of histones H3 lysine 27 (H3K27ac), H3 lysine 56 (H3K56ac), and H4 lysine 16 (H4K16ac) in the livers, lungs, and kidneys of both control and BD-exposed mice was analyzed by western blot analysis as described (Tryndyak *et al.*, 2006).

Statistical analyses. Results are presented as mean ± SD. Differences between control and BD-exposed mice for each tissue and each endpoint were evaluated by Student's *t* test. Pearson product-moment correlation coefficients were used to determine the relationship between levels of BD-induced DNA adducts and histone modifications. Data were natural log-transformed before conducting analysis to ensure equal variance or normal data distribution, when necessary. $P < 0.05$ was considered significant.

RESULTS

Air Concentrations of BD in the Exposure Chambers

The concentration of BD was monitored by gas chromatography of an air sample taken from the exposure chamber an hour into and an hour before the conclusion of the 6 h exposure each day. Over the 10 day duration of the experiment, the average concentration of BD in the exposure chamber was 425.8 ± 162.0 ppm. This dose is within the range of concentrations that have been reported to cause tumors in the liver and the lung of BD-exposed B6C3F1 mice in chronic inhalation studies (6.25–1250 ppm) (IARC, 2008; Melnick and Sills, 2001). Further, BD has been shown to have a supralinear exposure-response curve in mice exposed to BD at levels between 0 and 625 ppm (Melnick and Sills, 2001), indicating that 425 ppm is an applicable exposure concentration for the study of potential mechanisms of BD-induced tumorigenicity.

Levels of BD-DNA Adducts in Lung, Liver, and Kidney

Several comprehensive studies of adverse health effects of BD (Goggin *et al.*, 2009; Swenberg *et al.*, 2011) demonstrated that exposure to BD results in the formation of BD-specific DNA adducts. This was evidenced by the presence of several BD-induced DNA adducts, including THB-Gua adducts and *bis*-N7G-BD crosslinks, found in both rats and mice exposed to BD by inhalation (Goggin *et al.*, 2009; Swenberg *et al.*, 2011). Therefore, the presence of these DNA adducts in the lung, liver, and kidney tissues of mice exposed to BD inhalation and control animals was investigated.

Figure 1 shows that in mice exposed to BD THB-Gua adducts were present at the highest level in the lung tissue (8.48 adducts/10^6 nucleotides), followed by kidney (6.15 adducts/10^6 nucleotides), and then liver (3.28 adducts/10^6 nucleotides). THB-Gua adducts were undetectable in tissues from control (exposed to clean air) animals. The difference in the formation of THB-Gua adducts was statistically significant between the lung and liver ($P < 0.0001$), but did not differ between kidney and either lung or liver.

Similar to the results seen in the THB-Gua adduct analysis, *bis*-N7G-BD crosslinks were undetectable in tissues from control animals, but were observed in all three of the tissues in mice exposed to BD, with the highest level of *bis*-N7G-BD crosslinks, 3.07/10^7 nucleotides, found in lung tissue (Fig. 1). The number of *bis*-N7G-BD crosslinks was significantly greater in the lung

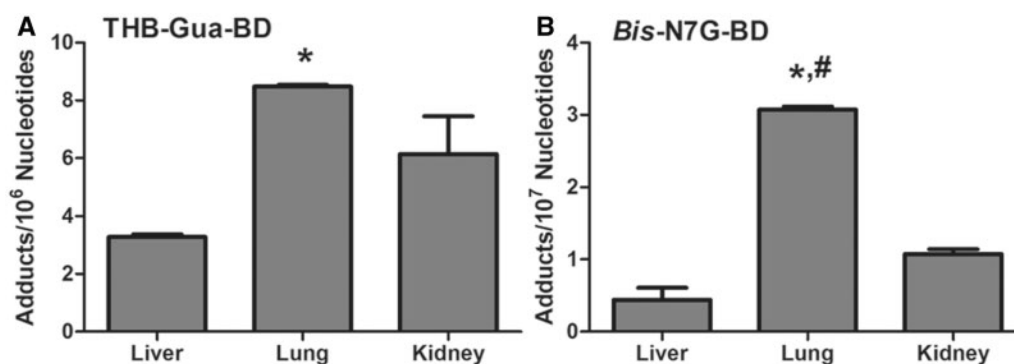

FIG. 1. Amounts of THB-Gua-BD adducts **(A)** and Bis-N7G-BD crosslinks **(B)** in tissues from mice exposed to 425 ppm of BD. Data are presented as mean ± SD ($n = 3$). Asterisk and pound (* and #) denote significant ($P < 0.05$) differences in the amount of the same adduct between the lung and liver, and lung and kidney, respectively.

when compared with the liver ($P < 0.05$), as well as when compared with that of the kidney ($P < 0.01$).

DNA Methylation at Repetitive Sequences

Substantial evidence exists indicating that exposure to certain chemical carcinogenic agents, including BD, may perturb the status of DNA methylation (Koturbash *et al.*, 2011b). Furthermore, it has been suggested that the disruption of normal DNA methylation patterns may be responsible for tumor induction and development (Feinberg and Tycko, 2004). In light of this, the methylation status of SINE B1 and B2 repetitive elements, as well as minor and major satellites, a sensitive indicator of global DNA methylation (Yang *et al.*, 2004), was determined in BD-associated cancer target (livers and lungs) and non-target (kidneys) tissues, as have been established in a 2-year BD mouse carcinogenicity study (IARC, 2012). Figure 2 shows that exposure to BD caused a significant decrease in the methylation of SINE B1 and B2 repetitive elements, and minor and major satellites in the livers, and SINE B2 and major satellites in the lungs. In contrast, the methylation status did not change in the kidneys of BD-exposed mice of any of the evaluated repetitive sequences. Furthermore, one of the oxidized derivatives of 5-mC, 5-hmC, displayed a similar pattern, as shown in Fig. 3. The level of 5-hmC was decreased more noticeably in the liver and lung than in the kidney, with the exception of major satellites, in which 5-hmC was similar across all tissues. However, the decrease in 5-hmC was only significant in SINE B2 in the lung.

Tissue-Specific Effects of BD Exposure on Histone Modifications

In addition to affecting the DNA methylome, compelling data demonstrate that chemical carcinogens may also disturb the normal pattern of covalent histone modifications (Baccarelli and Bollati, 2009; Thomson *et al.*, 2014). Based on this evidence, several types of histone modifications, particularly those associated with chromatin structure, DNA damage response and repair, genomic stability, and gene transcription, were investigated. Figure 4 shows that the levels of H3K9me3, H3K27me3, and H4K20me3, predominant histone lysine methylation marks of heterochromatin (Dillon, 2004; Martin and Zhang, 2005), in the livers and lungs of mice exposed to BD did not differ from the levels observed in control mice. In contrast, the level of H3K9me3, H3K27me3, and H4K20me3 in the kidneys of BD-exposed mice was substantially greater, 2.0, 2.6, and 1.4 times, respectively, than that observed in control mice. The level of H3K27ac was greatly increased, 2.2 times, in the livers of mice exposed to BD, whereas the level of this histone modification was unaffected in the lungs and kidneys.

Exposure to BD also led to a marked decrease in acetylation of histones H3K56 and H4K16 in the lungs (Fig. 4). In contrast, no differences were found in the livers and kidneys between BD-exposed and control mice. Interestingly, the level of histone H3K56ac was strongly negatively correlated with the extent of THB-Gua adducts and *bis*-N7G-BD crosslinks (Fig. 1), in the lungs ($r = -0.89$, $P < 0.05$).

Expression of Chromatin-Modifying Genes

To investigate a potential mechanism of the variation in the extent of DNA methylation and histone modification among tissues in BD-exposed mice, the expression of several genes that encode enzymes responsible for DNA methylation and relevant histone modifications was evaluated. Figure 5 shows a significant decrease in the expression of *Dnmt1* and *Dnmt3a* genes in the livers that correlated with hypomethylation of DNA observed in repetitive sequences in the liver. *Tet1* and *Tet2* genes encode enzymes that catalyze the conversion of 5-mC to 5-hmC (Tahiliani *et al.*, 2009). The expression of these two genes was not significantly different between the treatment and control groups, with the exception of a decrease in *Tet1* in the lungs of BD-exposed mice. This may explain the significant decrease in 5-hmC in SINEB2 sequences in the lungs. Figure 6 shows an increase in the expression of *Ezh2* gene in the lungs of mice exposed to BD, as well as a substantial increase, more than 2-fold, in the expression of the histone deacetylase gene *Sirt1* in the liver, lungs, and kidneys of BD-exposed mice. Interestingly, the expression of the histone acetyltransferase gene *Kat8* was also increased in the livers, lungs, and kidneys of BD-exposed mice; however, the magnitude of this increase in the lungs was substantially lower (Fig. 6). A similar pattern was also observed in the expression of the histone acetyltranferase gene *Kat2b* in the livers and lungs (Fig. 6). The expression of other chromatin modifying genes in the livers, lungs, and kidneys was not significantly affected by BD exposure.

DISCUSSION

Convincing evidence exists showing that chemical carcinogens affect the epigenetic state of cells (Baccarelli and Bollati, 2009; Pogribny and Beland, 2014; Pogribny and Rusyn, 2013). Such epigenetic reprogramming has been proposed to be an essential causal component of "genomic instability," an enabling characteristic of cancer cells (Hanahan and Weinberg, 2011). Epigenetic changes have been proposed as biomarkers of

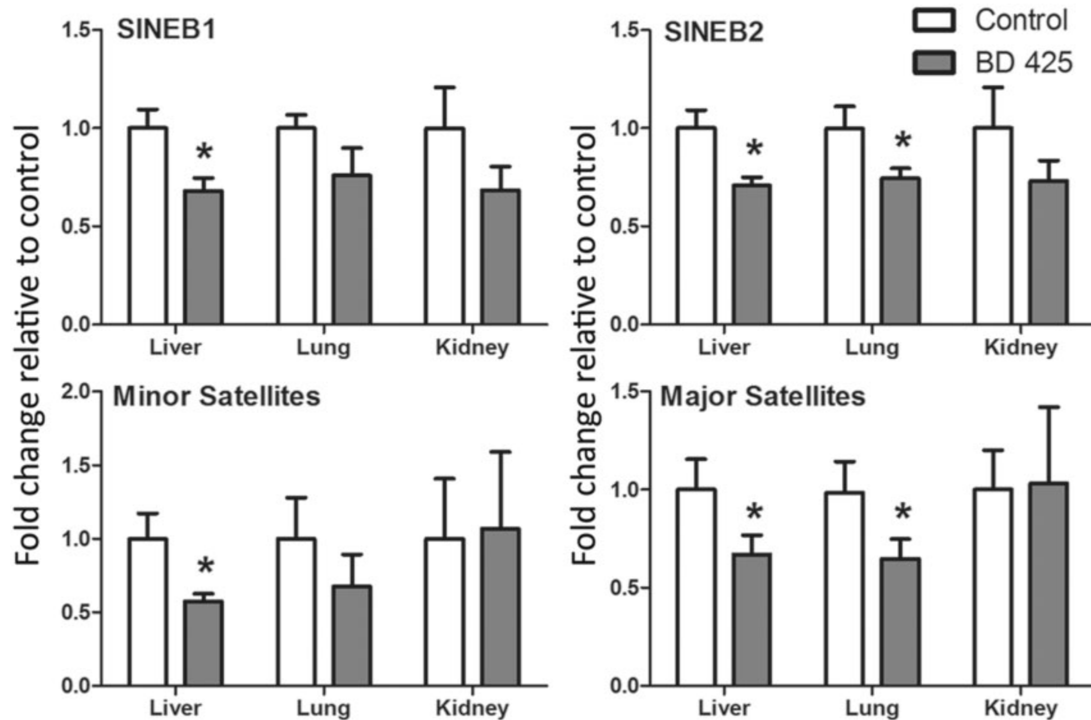

FIG. 2. Effects of BD exposure on the extent of DNA methylation in mouse tissues. Loss of methylation at SINE B1, SINE B2, major and minor satellite repetitive elements in the tissues of BD-exposed mice as measured by McrBC-methylation sensitive quantitative PCR (qPCR). The results are presented as the average fold change in the degree of DNA hypomethylation relative to the control values of the corresponding tissues. Data are presented as mean ± SD ($n = 3$). Asterisks (*) denote significant ($P < 0.05$) differences from the controls.

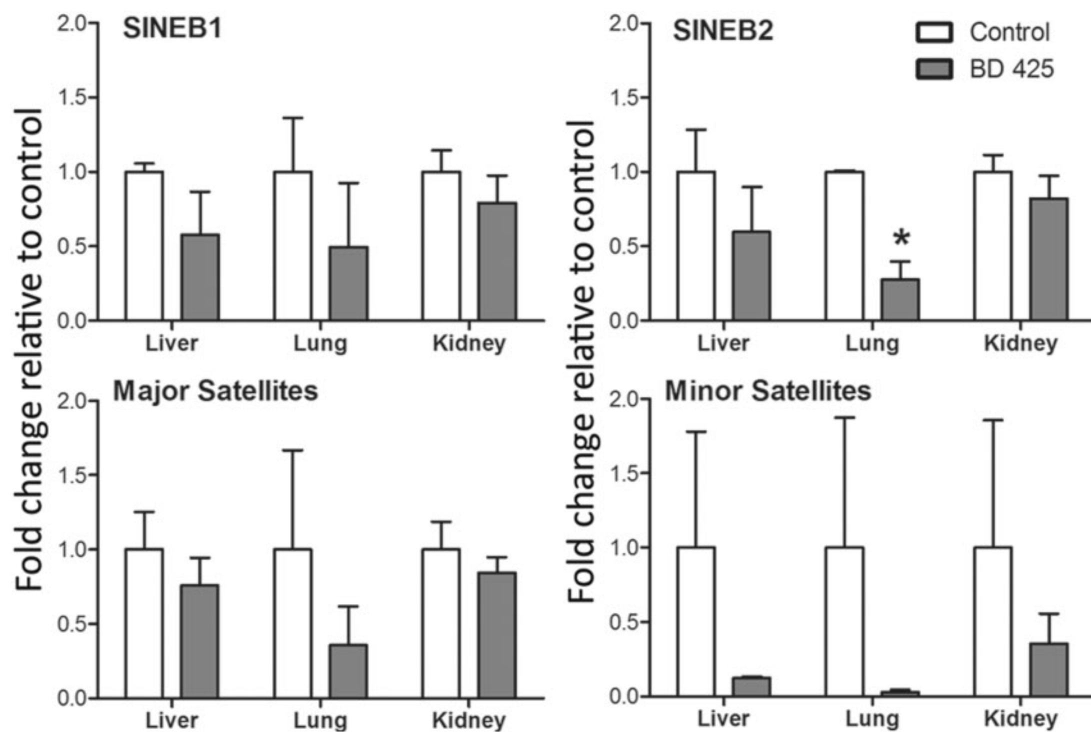

FIG. 3. Effects of BD exposure on the extent of 5-hydroxymethylcytosine in mouse tissues. Loss of methylation at SINE B1, SINE B2, major and minor satellite repetitive elements in the tissues of BD-exposed mice as determined by hydroxymethylated DNA immunoprecipitation (hMeDIP). The results are presented as the average fold change in the degree of DNA hypomethylation relative to the control values of the corresponding tissues. Data are presented as mean ± SD ($n = 3$). Asterisks (*) denote significant ($P < 0.05$) differences from the controls.

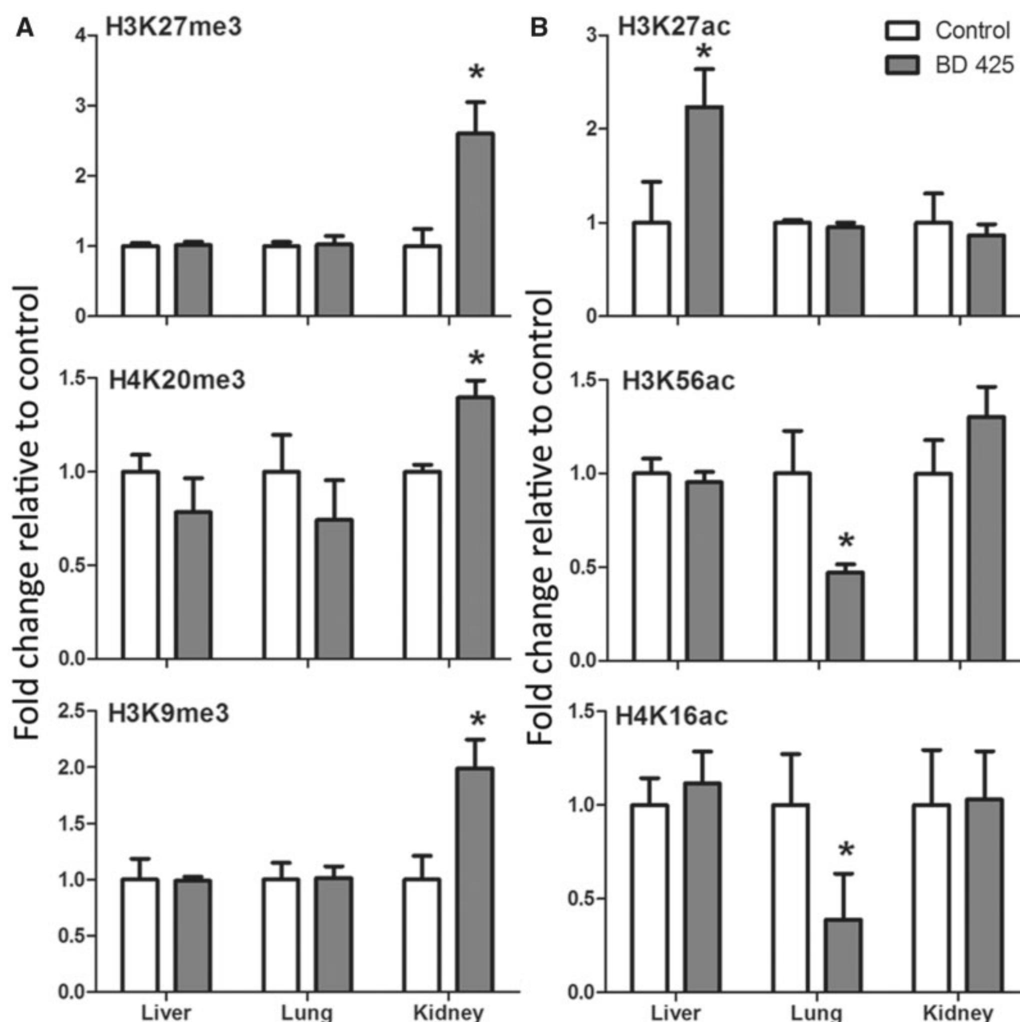

FIG. 4. Effects of BD exposure on histone modifications. H3K27me3, H4K20me3, H3K9me3, H3K27ac, H3K56ac, and H4K16ac levels were assessed by immunostaining using specific antibodies against trimethylated or acetylated histones. Equal sample loading was confirmed by immunostaining against total histone H3 or H4. Densitometry analysis of the immunostaining results is shown as change in methylation or acetylation level relative to the tissue-matched controls after correction for the total amount of each histone in the individual samples. Results are presented as the average fold change relative to the control values of the corresponding tissues. Data are presented as mean ± SD ($n = 3$). Asterisks (*) denote significant ($P < 0.05$) differences from the controls.

exposure to carcinogens, as well as markers of effect, because of the impact that epigenetic effects of chemicals have on toxicity phenotypes (Herceg *et al.*, 2013; Marlowe *et al.*, 2009). Alterations in DNA methylation, histone/chromatin remodeling, and expression of non-coding RNAs represent the most frequently reported changes to the epigenome induced by exposure to toxic chemicals (Herceg *et al.*, 2013; Marlowe *et al.*, 2009; Pogribny and Beland, 2014; Pogribny and Rusyn, 2013). Furthermore, the role of epigenetic alterations in chemical toxicity may or may not be associated with DNA damage; such changes to the epigenome potentially occur as a consequence of DNA damage (Khobta and Epe, 2012), or may represent a non-genotoxic mechanism of carcinogenesis (Herceg *et al.*, 2013; Pogribny and Rusyn, 2013). Recent studies have indicated an association between DNA damage responses and changes to chromatin structure as a result of chemically induced DNA damage, particularly histone modifications and post-repair chromatin restoration at sites of DNA damage (Vempati and Haldar, 2012; Zhu and Wani, 2010).

Transcription is also affected by damage-induced chromatin alterations; localized chromatin condensation in response to DNA double-strand breaks can occur within regions of several kilobases from sites of damage, and can induce inactivation of transcription and epigenetic silencing of the nearby genes (Shanbhag *et al.*, 2010). Additionally, it has been shown that the methylation status of H3K9, especially of H3K9me3, is associated with mutation rate in cancer cells, which suggests that mutation rates in cancer genomes are closely related to chromatin organization state (Schuster-Bockler and Lehner, 2012). Such modulation of the epigenetic status of loci with DNA damage indicates the involvement of DNA damage in the regulation of gene expression (Khobta and Epe, 2012).

Recent work by our group addressed the relationship between genotoxic (eg, formation of DNA adducts) and epigenetic (eg, alterations in marks of chromatin regulation) mechanisms of carcinogenesis. First, using a multistrain mouse model to emulate the genetically diverse human population, we observed variation in the response to the classic genotoxic carcinogen BD, as well as to other chemicals (Koturbash *et al.*, 2011a; Rusyn *et al.*, 2010), confirming that important interstrain differences exist in both genotoxic and epigenotoxic effects of chemical carcinogens. Next, we confirmed that chromatin

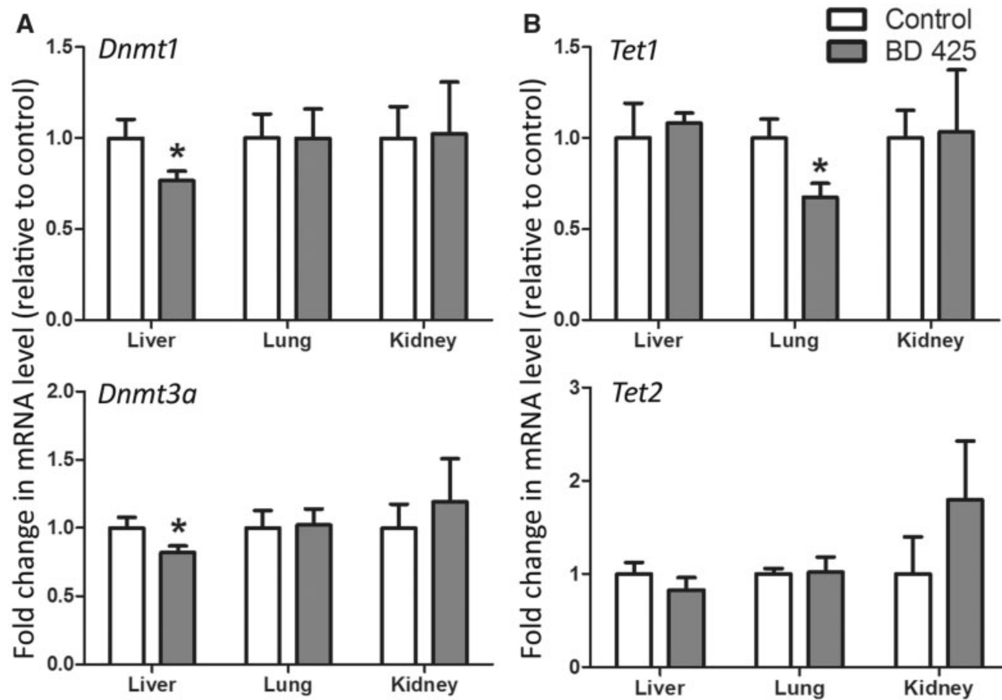

FIG. 5. Effects of BD exposure on the expression of DNA methylation and DNA demethylation genes. mRNA levels of DNA methyltransferase genes **(A)** and methylcytosine dioxygenase genes **(B)** were evaluated by qPCR. Results are presented as the average fold change relative to the control values of the corresponding tissue. All experimental genes were run at least in triplicate. Data are presented as mean ± SD ($n = 3$). Asterisks (*) denote significant ($P < 0.05$) differences from the controls.

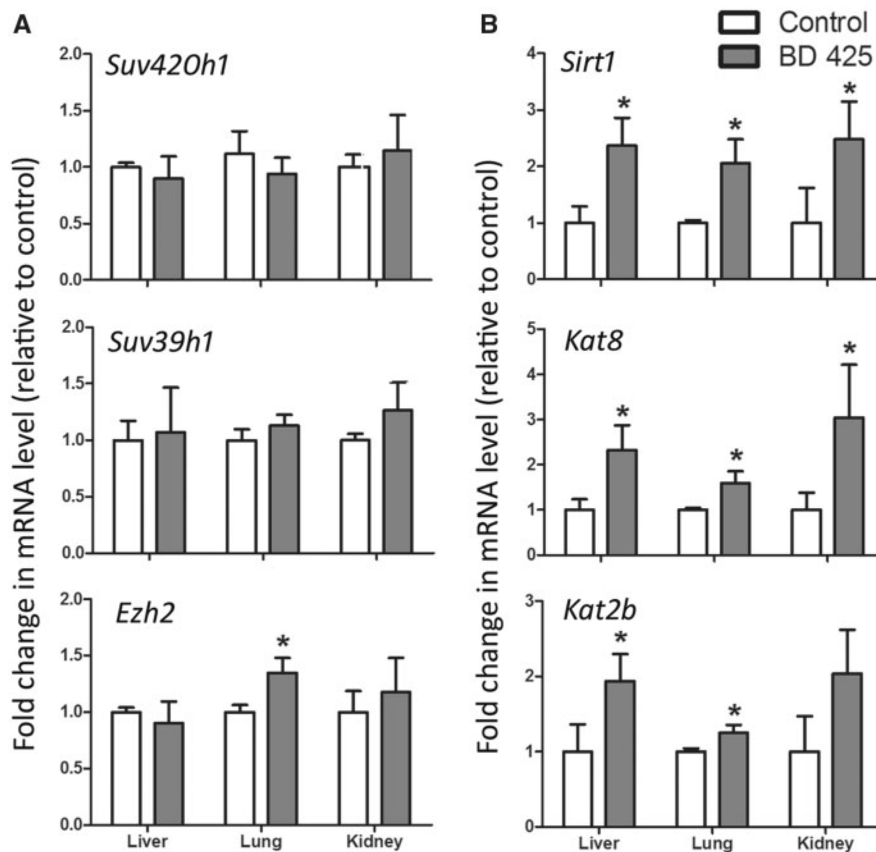

FIG. 6. Effects of BD exposure on the expression of histone modifying genes. mRNA levels of histone methyltransferase genes **(A)** and genes related to histone acetylation **(B)** were evaluated by qPCR. Results are presented as the average fold change relative to the control values of the corresponding tissue. All experimental genes were run at least in triplicate. Data are presented as mean ± SD ($n = 3$). Asterisks (*) denote significant ($P < 0.05$) differences from the controls.

remodeling response is a mechanism that largely, if not completely, explains the interstrain differences in BD-induced DNA damage (Koturbash *et al.*, 2011a).

Although profound genotoxic and non-genotoxic changes in target organs of tumorigenesis have been demonstrated as a consequence of exposure of experimental animals to certain carcinogens, including BD (Koturbash *et al.*, 2011a,b), information on the extent of these alterations in non-target organs of carcinogenesis is lacking. The results of the present study demonstrate that inhalational exposure of male C57BL/6J mice, a strain previously reported to have high levels of BD-induced DNA damage and epigenetic alterations in the liver, to BD induced a substantial accumulation of genotoxic lesions in DNA in both target (liver and lungs) and non-target (kidneys) tissues. This was evidenced by the extensive formation of THB-Gua adducts and *bis*-N7G-BD crosslinks in all three tissues, with the greatest prevalence in the lungs (Fig. 1). Interestingly, the number of BD-induced DNA adducts in the non-target organ (kidneys) of BD-exposed mice was comparable (*bis*-N7G-BD crosslinks) or even significantly greater (THB-Gua adducts) than in the livers. The pattern of *bis*-N7G-BD crosslinks formation observed in the present study differs from previous studies. Specifically, Goggin *et al.* (2009) demonstrated that the liver had a higher prevalence of these adducts than the kidneys and lungs. This discrepancy may be attributable to the use of a different mouse strain (B6C3F1), or the use of female mice instead of males, as previous work has shown that the prevalence of DNA adducts varies significantly across inbred mouse strains (Koturbash *et al.*, 2011a) and between sexes (Meng *et al.*, 2007). Our observation of similar levels of BD adducts in a target (liver) and non-target (kidney) organs indicate that mechanisms other than, or in addition to, genotoxic lesions may play a critical role in the adverse effects of BD exposure.

It has been previously reported that inhalational exposure to BD results in a loss of DNA methylation in the liver (Koturbash *et al.*, 2011b). In concordance with that report, in the present study, we observed an increase in the level of DNA repetitive sequences hypomethylation in the livers and lungs, two target organs for BD-induced carcinogenesis. In contrast, the extent of DNA methylation in a non-target organ (kidneys) was not affected by BD exposure. In addition to carcinogen-induced changes in DNA methylation, alterations in chromatin organization, as a consequence of a chemical challenge, are a key component of the tumorigenic process (Baccarelli and Bollati, 2009; Thomson *et al.*, 2014). The present study also demonstrated that exposure to BD caused tissue-specific alterations in histone modifications, including increased histone H3K27 acetylation in the livers, deacetylation of histones H3K56 and H4K16 in the lungs, and increased trimethylation of histones H3K9, H3K27, and H4K20 in the kidneys. In our previous study on the genotoxic effects and epigenetic alterations of BD exposure in mice (Koturbash *et al.*, 2011b), we demonstrated that BD-induced DNA hypomethylation was accompanied by a decrease in trimethylation of histones H3K9, H3K27, and H4K20 in the livers. It has been established that decreases in DNA methylation and in histone methylation at H3K9, H3K27, and H4K20 are associated with the formation of relaxed chromatin. Surprisingly, we did not detect a reduction of these histone lysine methylation marks in response to BD exposure in the present study. This may be explained, in part, by relatively lower air concentrations of BD in the exposure chambers when compared with those reported previously (Koturbash *et al.*, 2011a,b), resulting in a lower cumulative dose of BD. However, we detected a substantial increase in the level of hepatic histone H3K27 acetylation, which, in addition to H3K9, H3K27, and H4K20 demethylation, is an indicator of transcriptionally active (relaxed) chromatin (Szulwach *et al.*, 2011).

Another novel observation of the present study was the extensive deacetylation of H3K56 and H4K16 in the lungs of BD-exposed mice. It is well accepted that H3K56ac and H4K16ac are critical for the organization of chromatin, genomic stability, and DNA damage response (Masumoto *et al.*, 2005; Taipale *et al.*, 2005). Specifically, Li *et al.* (2008) have demonstrated that H3K56ac regulates replication-coupled nucleosome assembly, and Vempati and Haldar (2012) reported that increased acetylation of both histones H3K56 and H4K16 is associated with DNA damage induced by genotoxic chemicals, indicating an important role for both of these modifications in DNA damage repair. Furthermore, it is well documented that loss of H3K56ac and/or H4K16ac is associated with impaired DNA repair, major chromosomal rearrangements, and genomic instability (Prado and Clemente-Ruiz, 2012; Taipale *et al.*, 2005; Yang and Freudenreich, 2010). These findings, in addition to our observation of a strong negative correlation between the formation of DNA adducts and a loss of acetylation of histones H3K56 and H4K16 in the lungs of BD-exposed mice, suggest a critical role of H3K56ac and H4K16ac in BD-induced lung tumorigenesis. Specifically, reduction of histone H3K56ac and H4K16ac may compromise the proper repair of BD-induced DNA lesions, leading to their extensive accumulation and, ultimately, genomic instability. The loss of H3K56ac and H4K16ac in the lungs of BD-exposed mice may be mechanistically explained by the substantial up-regulation of the histone deacetylase gene *Sirt1* relative to the up-regulation of the histone acetyltransferases genes *Kat2b*, the mouse homolog of yeast human genes known to acetylate H3K56 (Das *et al.*, 2009), and *Kat8*, which primarily acetylates H4K16 (Taylor *et al.*, 2013), favoring the histone deacetylation process.

In addition, we observed a striking increase in trimethylation of histones H3K9, H3K27, and H4K20 in the kidneys of BD-exposed mice. It is well accepted that these epigenetic marks are integral in the maintenance of genomic stability and chromatin structure (Dillon, 2004; Martin and Zhang, 2005). The results of our previous study on molecular determinants of mouse interstrain variability to BD exposure indicated a key role of these histone modifications in BD genotoxicity (Koturbash *et al.*, 2011a). Specifically, CAST/EiJ mice, the strain that exhibited the lowest formation of hepatic BD-induced DNA adducts and no evidence for hepatotoxicity, was characterized by a substantial increase in the levels of H3K9me3, H3K27me3, and H4K20me3 upon BD exposure. Similarity between the changes in the extent of H3K9, H3K27, and H4K20 trimethylation and DNA methylation in the kidneys of C57BL/6J mice in the present study, and that observed in the livers of the most resistant strain to BD exposure found in the previous study suggest that this epigenetic response may exert a protective effect on tissue, minimizing the damage caused by BD.

"In conclusion," our results indicate that BD elicits both genotoxicity and epigenetic effects, but that the tissue-specific toxicity of BD may be largely due to epigenetic alterations. Figure 7 shows a summary of the most notable alterations induced by exposure to BD. It can be clearly visualized that although all tissues sustained DNA damage, differing responses in DNA methylation and histone modifications existed across tissues, as depicted by the arrows in the chart. Such interplay between genotoxic and epigenetic effects may be generalizable to other DNA-damaging chemicals. For example, it has been previously demonstrated that epigenetic alterations play a role in

FIG. 7. Summary of genotoxic and epigenetic changes in the tissues of BD-exposed mice. This chart highlights the significant alterations observed in each of the tissues as presented in Figs. 1–6. Arrows indicate a direction of the effect (increase or decrease) observed for each of the marks listed in the same box, when compared with the same tissues in control mice.

hepatocarcinogenesis after exposure to 2-acetylaminofluorene, another genotoxic chemical carcinogen (Pogribny *et al.*, 2011). Although inter-species differences, as well as within-species tissue differences, in the carcinogenicity of BD have been generally attributed to differences in metabolism and DNA damage repair (Kirman *et al.*, 2010), we argue that epigenetic mechanisms are equally, if not more, accountable for the tissue-specific variation in the response to chemical exposure. We observed prominent differences in both DNA methylation and histone modification response between target and non-target tissues of BD in the mouse: alterations in histone modifications associated with genomic instability and loss of DNA methylation at repetitive sequences in target organs (liver and lungs), and the absence of DNA methylation changes along with the formation of genome protecting chromatin condensation in a non-target tissue (kidneys). It should be noted that assumptions regarding tissue-specific tumorigenesis of BD were drawn based on previous studies in B6C3F1 mice (IARC, 2008) and to confirm the relationship between tissue-specific BD-induced epigenetic alterations and carcinogenesis, longer-term exposures are required in C57BL/6J mice. Additionally, although the present study focused on global changes in the epigenome as a result of BD exposure, evaluating changes in the local chromatin landscape at specific genes using techniques such as ChIP may enable a better understanding of the mechanisms of BD-induced genomic alterations.

SUPPLEMENTARY DATA

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

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