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Effects on specific promoter DNA methylation in zebrafish embryos and larvae following benzo[a]pyrene exposure

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Abstract

Benzo[a]pyrene (BaP) is an established carcinogen and reproductive and developmental toxicant. BaP exposure in humans and animals has been linked to infertility and multigenerational health consequences. DNA methylation is the most studied epigenetic mechanism that regulates gene expression, and mapping of methylation patterns has become an important tool for understanding pathologic gene expression events. The goal of this study was to investigate aberrant changes in promoter DNA methylation in zebrafish embryos and larvae following a parental and continued embryonic waterborne BaP exposure. A total of 21 genes known for their role in human diseases were selected to measure percent methylation by multiplex deep sequencing. At 96 hours post fertilization (hpf) compared to 3.3 hpf, *dazl*, *nqo1*, *sox3*, *cyp1b1*, and *gstp1* had higher methylation percentages while *c-fos* and *cdkn1a* had decreased CG methylation. BaP exposure significantly reduced egg production and offspring survival. Moreover, BaP decreased global methylation and altered CG, CHH, and CHG methylation both at 3.3 and 96 hpf. CG methylation changed by 10% or more due to BaP in six genes (*c-fos*, *cdkn1a*, *dazl*, *nqo1*, *nrf2*, and *sox3*) at 3.3 hpf and in ten genes (*c-fos*, *cyp1b1*, *dazl*, *gstp1*, *mlh1*, *nqo1*, *pten*, *p53*, *sox2*, and *sox3*) at 96 hpf. BaP also induced gene expression of *cyp1b1* and *gstp1* at 96 hpf which were found to be hypermethylated. Further studies are needed to link aberrant CG, CHH, and CHG methylation to heritable epigenetic consequences associated with disease in later life.

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Keywords

benzo[a]pyrene; DNA methylation; embryo; larvae; zebrafish

1. Introduction

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon ubiquitous in the environment and derived from the incomplete combustion of organic compounds (Latimer and Zheng, 2003). The 2011 CERCLA's Priority List of Hazardous Substances ranks BaP # 8, and in the 2012 IARC Monographs, BaP is a Group 1 animal and human carcinogen (<http://monographs.iarc.fr/ENG/Classification/>). BaP also is an established reproductive and developmental toxicant. BaP exposure in humans is linked to altered sperm morphology, and decreased sperm and egg numbers (Zenzes et al., 1998; Zenzes et al., 1999; Gaspari et al., 2003). In animal models, BaP reduced gonad weights, damaged ovarian follicles, and led to infertility (Mohamed et al., 2010). BaP exposure during pregnancy resulted in increased fetal death, low birth weights, and birth defects (Legraverend et al., 1984; Barbieri et al., 1986; Archibong et al., 2002).

Furthermore, BaP exposure resulted in long-lasting health consequences on the offspring of BaP exposed parents. For example, *in utero* BaP exposure increased incidence of lung adenomas in five subsequent generations of mice (Turusov et al., 1990). Male offspring were more prone to develop liver tumors compared to female offspring in later life (Wislocki et al., 1986). In mice, reduced numbers of sperm and egg follicles were found in the offspring of exposed adults (MacKenzie et al., 1981; Kristensen et al., 1995). Paternal BaP exposure also adversely impacted sperm function and fertility in at least three generations of mice (Mohamed et al., 2010). Evidence of BaP's persistent neurotoxicity includes impaired spatial learning in adult animals after early postnatal exposure (Chen et al., 2012), decreased reflexes in lactationally exposed animals (Bouayed et al., 2009), and deficits in a novelty discrimination behavior in offspring following *in utero* exposure (Li et al., 2012). The molecular mechanism(s) that mediate the long-term effects of BaP remain unknown.

Increasing evidence shows that disruption of the intrauterine environment by nutritional or chemical factors may alter epigenetic mechanisms that play a key role in the fetal programming of adult diseases (Heindel, 2008; Szyf, 2009; Choudhuri et al., 2010). Notable examples include bisphenol A, phthalates, diethylstilbestrol, vinclozolin, and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Heindel 2006; LeBaron et al., 2010; Singh et al., 2012; Manikkam et al., 2012). Our previous study found that global methylation was decreased after BaP exposure in zebrafish embryos (no parental exposure), suggesting that epigenetic mechanisms especially aberrant DNA methylation could be involved in the BaP-induced toxicity (Fang et al., 2013a). Therefore, we hypothesized that BaP alters DNA methylation in critical cancer and developmental genes, which could lead to abnormal gene regulation and disease development in later life.

Gene expression is regulated by the access of transcription factors and enhancers to gene promoters which is, in turn, controlled by epigenetic mechanisms, such as molecular

modifications of DNA (DNA methylation) or histones (acetylation, deacetylation, methylation, and phosphorylation) (Bollati and Baccarelli, 2010). DNA methylation, associated with cytosine methylation in CpG dinucleotides, is the most studied of epigenetic mechanisms. However, methylation of cytosine can also occur in non-CpG sites including CHG and CHH sequence contexts, where H is an A, C, or T (Feng et al., 2010). Mapping of methylation patterns in CpG islands has become an important tool for understanding both normal and pathologic gene expression events (Campion et al., 2009). For example, global hypomethylation and specific promoter hypermethylation have been linked with genomic instability and inactivation of tumor suppressor genes (Kisseljova and Kiseeljev, 2005; Wadjet et al., 2001).

Recently, zebrafish (*Danio rerio*) has become a preferred animal model for human disease due to its rapid life cycle, high fecundity, transparent development, and because the embryos are amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Santoriello and Zon, 2012). In support of the validity of the zebrafish model, genomic comparison analyses between zebrafish and human genes showed that 76% of human genes have at least one zebrafish ortholog (Howe et al., 2013). In addition, 58% of genes currently associated with human disease in genome-wide association studies have zebrafish orthologs (Kettleborough et al., 2013).

The goal of this study was to investigate aberrant changes in promoter DNA methylation in zebrafish whole embryos and larvae following a parental and continued embryonic waterborne BaP exposure. A total of 21 genes were selected for this study based on their known role in human diseases such as cancer, neurodegenerative disorders, and infertility (Table 1). BaP exposure caused >10% hypo- or hypermethylation in 10 genes at 96 hpf (hours post fertilization) and at both 3.3 and 96 hpf caused hypermethylation and hypomethylation of two and three genes, respectively. In addition to the BaP effects, this study provides important information related to constitutive gene-specific complexity in promoter methylation during zebrafish development.

2. Materials and methods

2.1. Zebrafish care

AB line wild-type zebrafish (*Danio rerio*) were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR, USA) and raised under the approved IACUC (Institutional Animal Care and Use Committee) protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL, USA) with zebrafish water (pH 7.0-7.5, 60 parts per million (ppm), Instant Ocean, Cincinnati, OH, USA) at 25–28 °C, 14:10 light-dark cycle. Fish were fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. Sexually mature fish without any deformities or signs of disease were selected as breeders for the parental exposure.

2.2 Parental and embryonic waterborne exposure

Adult zebrafish (2 males × 4 females, N=6 replicate tanks for each treatment, see Supplemental Figure 1 for a diagram of the experimental design) were acclimated for 7 days

in an 818 Low Temp Illuminated Incubator (Precision Scientific, Chennai, India) at 28.5°C. During the acclimation, fish tanks were covered to obtain darkness all day except from 8:00–9:00 a.m. to trigger spawning, and eggs were collected daily from each tank during this pre-exposure period to ensure successful reproduction. Thereafter, parental fish were waterborne exposed to control or 50 µg/L (ppb) BaP for 7 days before collecting eggs; ethanol was used as vehicle solvent, and final ethanol concentration was 0.1 µl/mL (100 parts per million, ppm) in all treatment groups. This dose of ethanol is not teratogenic to zebrafish (Ali et al., 2011). BaP is teratogenic to zebrafish embryos with EC₅₀ of 0.52 µM (131 µg/L) (Weigt et al., 2011), and 24 µg/L BaP reduced survival, hatching success, and global DNA methylation in an embryo waterborne exposure in zebrafish (Fang et al. 2013a). Therefore, 50 µg/L BaP was within this range. From day 7 to day 11 of the parental exposure, eggs were collected, counted, and raised in normal conditions (control) or continuously exposed to 50 µg/L BaP with 30 embryos in 10 mL zebrafish water until 3.3 and 96 hpf. At 3.3 or 96 hpf, embryos or larvae were collected for molecular analysis as described in sections 2.5 and 2.10, respectively.

2.3. Extractions and chemical analysis

Water was changed and/or re-dosed daily during the adult zebrafish acclimation, adult exposure and embryo exposure. Both control and BaP-treated water were sampled three times during the first 7 days of the exposure for actual BaP quantitation. Water samples (20–200 mL) were collected 20 minutes after dosing and analyzed to confirm control and BaP concentrations from each adult zebrafish exposure tank. Sep-Pak® Vac RC C18 cartridges (500 mg) (Waters Corp., Milford, MA, USA) were pre-washed with 50 mL of 75% methanol and water samples added. Methylene chloride (7.5 mL, 2X) was added to the columns to elute BaP. Then, solvents were evaporated under a gentle flow of nitrogen gas, and samples were re-constituted in iso-octane. BaP concentrations in the water extracts were measured by gas chromatography (Agilent 6890, Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometry (Agilent 5973N) in selected ion monitoring mode for ions 252 and 253. BaP standards (0.1, 0.2, 0.5, 1, and 2 ppm in iso-octane) made up the standard curve. BaP was not identified in the ethanol-treated samples. The actual BaP concentration of the water was: 42.0 ± 1.9 µg/L.

2.4. Embryo survival

At the time of egg collection, 30 fertilized eggs per treatment group were pooled and placed in a 10-mL vial containing control or BaP-treated zebrafish water. Each pool had three to four replicate vials giving a total of 90 to 120 fertilized eggs per treatment group. To determine effects of BaP on survival, the number of dead embryos and larvae were recorded at 24, 48, 72, and 96 hpf.

2.5. Genomic DNA extraction

To determine global DNA methylation status, genomic DNA from zebrafish embryos (3.3 hpf; n=3 pools, 200 embryos/pool) and larvae (96 hpf; n=3 pools, 30 larvae/pool), was extracted as previously described (Fang et al., 2013b). Briefly, for 3.3 hpf embryos, DNA was extracted with DNAzol (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol except the following modifications: homogenized embryos were

digested overnight with proteinase K (120 mg/mL) to eliminate the high content of yolk protein; PolyAcryl carrier (Molecular Research Center) was used to isolate small quantities of DNA, such as in embryos; and DNA was further purified with the DNA Clean & Concentrator kit (ZYMO Research, Irvine, CA, USA). For 96 hpf larvae, DNA was extracted with the DNeasy Blood & Tissue Easy Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. For both methods, DNA was treated with RNAase to remove RNA contaminants. DNA concentrations were quantitated with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.6. Global DNA methylation

The Methylamp™ Global DNA Methylation Quantification Kit (Epigentek Group) was used to quantitate the methylated cytosine percentage in genomic DNA samples. In an ELISA-like reaction, a 5-methylcytosine monoclonal antibody recognized methylated DNA and then bound to a secondary antibody linked with a substrate enzyme. After the colorimetric reaction, the methylated cytosine amount was measured from the OD intensity at 450 nm with a Biotek ELx800 plate reader (Winooski, VT, USA). The cytosine methylation percentage was calculated by using the formula: cytosine methylation % = (OD (sample-negative control)/36.5%)/(OD (positive control-negative control) X 10); 36.5% is the GC content in zebrafish genome (Han et al., 2008). Ten was the dilution factor of positive control. Each biological triplicate sample was measured in duplicate. Statistical differences in 5-methylcytosine percentage between control and 3.3 or 96 hpf groups was determined by the Student *t*-test (* $p < 0.05$, $n = 3$ pools and each pool had 200 embryos at 3.3 hpf or 30 larvae at 96 hpf).

2.7. PCR of sodium bisulfite-converted DNA for downstream sequencing

Genomic DNA from the same treatment groups ($n = 3$ per group) were pooled at equal molar ratio. This resulted in four DNA pools: 3.3 hpf control, 3.3 hpf BaP, 96 hpf control, and 96 hpf BaP. Genomic DNA was treated with sodium metabisulfite as previously described (Fang et al., 2013a). Bisulfite specific PCR primers for the target genes were designed with Methyl Primer Express V1.0 (Applied Biosystems, Supplemental Table 1). Target gene regions were amplified from bisulfite-converted DNA with ZymoTaq™ PreMix (Hot start DNA taq polymerase, ZYMO Research). PCR products were cleaned with the ZR-96 DNA Clean & Concentrator™-5 kit (ZYMO Research).

2.8. Illumina TruSeq library preparation and next generation sequencing

PCR products from the same DNA pool were made into an indexed sequencing library with the TruSeq DNA Sample Prep kit (Product number FC-121-2001, Illumina, San Diego, CA, USA) by Global Biologics (Columbia, MO, USA). Briefly, amplicons were quantitated using Qubit dsDNA BR Assay and Qubit 2.0 fluorometer (Life Technologies Inc., Carlsbad, CA, USA). Amplicons were pooled at equal molar ratio and fragmented to ~100–200 bp with a single shearing by sonication. The resulting DNA was prepared for sequencing by blunt end repair, 3' adenylation, multiplex compatible adapter ligation (containing TruSeq indexes), and PCR amplification. Library validation was performed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Illumina-adapted library

pools prepared were further size-fractionated on a Caliper LabChip XT with a DNA 750 Assay Kit (Product number 760541, PerkinElmer, Waltham, MA, USA) to remove residual adapter dimers. For each pool, the DNA was collected and then assayed by an Illumina library quantification kit (Product number KK4854, Kapa Biosystems, Inc, Woburn, MA, USA) on a qPCR instrument (LightCycler 480, Roche Applied Science, Indianapolis, IN, USA). Each pool was clustered via cBot (Illumina) on a single lane of a TruSeq PE Cluster Kit v3 paired-end flowcell (Product number PE-401-3001, Illumina). Paired-end 2 × 100 bp sequencing was carried out on an Illumina HiSeq 2000 with TruSeq SBS Kit v3 (Product number FC-401-3001, Illumina) chemistry.

2.9. Sequencing data analysis

Sequence adaptors and PCR primers were removed with the program cutadapt (Martin, 2011). Low quality trimming and filtering were performed using fastq_quality_trimmer and fastq_quality_filter from FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). After quality control, sequence reads were synchronized with in house Perl scripts. Only paired-end reads were used for downstream analysis. Cytosine methylation was determined and counted using Bismark Bisulfite Mapper (version 0.7.4,0.7.7) (Krueger et al., 2011). Briefly, a reference genome with the amplicon sequences was built and bisulfite converted *in silico*, followed by the alignment using Bowtie 1. Cytosine methylation counting for each methylation type, i.e. CG, CHH, CHG, was performed with the Bismark methylation extractor. Data mining was completed by excluding CpGs that had less than 100 sequence reads per site. Percent methylation was calculated by the number of methylated cytosines with in CG, CHH, CHG divided by the total number of the corresponding type of cytosines within the same gene region.

2.10. Quantitative reverse transcription real time PCR (qPCR)

To evaluate if changes in DNA methylation were associated with changes in gene expression due to BaP, nine genes were selected to measure mRNA expression using qPCR. These genes (*c-fos*, *mlh1*, *p53*, *nqo1*, *gstp1*, *cyp1b1*, *sox2*, *sox3*, and *dazl*) exhibited the most change in CG methylation due to BaP. RNA was isolated with RNeasy (Molecular Research Center, Cincinnati, OH, USA) and purified with RNeasy Mini Kit (Qiagen) by following the manufacturer's protocols. Total RNA (250 ng) was reverse transcribed to double stranded cDNA libraries by using TaqMan® Reverse Transcription Reagents (Applied Biosystems). qPCR primers were designed with Primer Express® Software v2.0 (Applied Biosystems) (Supplemental Table 1). Relative abundance of target genes to 18S rRNA transcripts was determined by qPCR with SYBR®Green in a GeneAmp 7500 Sequence Detection System (Applied Biosystems). Statistical differences between treatments were determined on the linearized 2^{-Ct} values by Student t-test or one-way ANOVA. Amplification efficiencies of the target genes and 18S rRNA primer pairs were determined using the formula $E = 10^{(-1/\text{slope})-1}$ (Higuchi et al., 1993) and were between the accepted range of 80 to 117.

3. Results

3.1. BaP exposure decreased egg production

Before exposure began, the average egg production for zebrafish (2 males and 4 females per tank, $n=12$) was 95.6 ± 12.8 eggs/tank/day (Fig. 1A). During the exposure, the average number of eggs collected was 152.6 ± 3.6 eggs/tank/day in the control group and 68.2 ± 24.1 eggs/tank/day in the BaP (42.0 ± 1.9 $\mu\text{g/L}$) group ($n=6$). Therefore, after acclimation (i.e., 7-day period before the exposure) the number of eggs significantly increased to 160% of the preexposure period. However, BaP exposure significantly decreased the number of eggs produced by 55% compared to controls.

3.2. BaP adversely affected embryo survival

Before exposure, percent survival of zebrafish embryos ranged from $98.8 \pm 0.8\%$ at 24 hpf to $97.2 \pm 1.4\%$ at 96 hpf (Fig. 1B). When parents and their embryos were continually exposed to 42.0 ± 1.9 $\mu\text{g/L}$ BaP, embryo survival was significantly lowered at all times points in comparison to controls and pre-exposure. The majority of deaths occurred within the first 24 hpf when embryo survival significantly decreased from $89.0 \pm 3.0\%$ in controls to $33.8 \pm 3.3\%$ in the BaP-treated group. In addition, at 96 hpf (point at which organogenesis is complete) the percent survival significantly decreased from $86.4 \pm 3.8\%$ in controls to $27.2 \pm 6.2\%$ in the BaP-treated group, which is a 68.5% decrease. At 24 and 48 hpf, the percent survival in the control group was significantly lower than before exposure by $\sim 10.1\%$; however, at 72 and 96 hpf the difference in percent survival between controls and before exposure was not significant.

3.3. Global DNA methylation

Because of the small size of the zebrafish embryos and larvae at 3.3 and 96 hpf, throughout this study pools of whole embryos were used, and thus, it was not possible to study tissue specific changes in DNA methylation and gene expression. At 3.3 hpf, BaP non-significantly reduced total DNA methylation in zebrafish embryos, but at 96 hpf, BaP significantly reduced DNA methylation by 49.0% in larvae (Fig. 2).

3.4. Constitutive gene specific DNA methylation during development

Overall as expected, percent cytosine methylation, was highest at CG compared to the CHG or CHH contexts for all genes (Fig. 3). At 3.3 hpf, constitutive percent CG methylation was the highest in *apc*, *h-ras*, *nos2b*, *drd2l*, *msh3*, *bdnf*, and *brca1* ranging from approximately 87% to 96% (Fig. 3). For *p53* and *dazl*, the percent CG methylation was 18% and 10%, respectively. The rest of the genes (*cyp1b1*, *nrf2*, *cdkn1a*, *sox2*, *sox3*, *gtsp1*, *c-fos*, *nqo1*, and *cdh2*) had less than 10% constitutive CG methylation. Both percent CHG and CHH methylation ranged approximately between 0.1% and 7%, and genes *msh3* and *cyp1b1* showed the highest percent CHG and CHH methylation (data not shown).

At 96 hpf, constitutive percent CG methylation was also highest in *apc*, *h-ras*, *nos2b*, *msh3*, *drd2l*, *brca1*, *bdnf*, in addition to *esr1* ranging from approximately 82% to 95% (Fig. 3). Percent CG methylation in *dazl*, *p53*, and *cyp1b1* was 39%, 16%, and 12%, respectively. As observed at 3.3 hpf, less than 10% CG methylation was observed in *cdkn1a*, *sox3*, *sox2*,

gstp1, *nqo1*, *cdh2*, and *c-fos*, in addition to *mlh1* and *pten*. Also, similar to 3.3 hpf, percent CHG and CHH methylation ranged between 0.1% and 10%, and *cyp1b1* showed the highest percent CHG and CHH methylation.

Constitutive changes in percent CG methylation during development between 3.3 and 96 hpf are shown in Table 2. The biggest developmental change from 3.3 hpf to 96 hpf was an increase of ~310% in *dazl* CG methylation. In addition, CG methylation in *nqo1* increased by 122% at 96 hpf. Also, an increase in CG methylation of ~52%, 48%, and 31% in *sox3*, *cyp1b1*, and *gstp1*, respectively, was detected while there was a decrease of ~30% and 28% in *c-fos* and *cdkn1a*, respectively. Constitutive changes in CG methylation in the rest of the genes during development were less than 10%.

Constitutive changes in percent CHG and CHH methylation did not necessarily correspond with changes in percent CG methylation (Table 2). For example, the biggest changes in CHG methylation were increases of ~190% in *nqo1* and ~116% in *sox3* CHG methylation. All the genes except for *drd2l* and *gstp1* showed changes greater than 10% in CHG methylation between 3.3 and 96 hpf. Similar to the CHG context, the biggest increase in percent CHH methylation was seen in *sox3* (~109%) and *nqo1* (~93%). Also similar to the CHG context, only *drd2l*, *gstp1*, and *cdh2* showed less than 10% change in CHH methylation between 3.3 and 96 hpf.

3.5. BaP effects on hyper- and hypomethylation at 3.3 hpf

At 3.3 hpf, BaP exposure caused a 60.8% CG hypermethylation in *nqo1* and a 39.2% CG hypermethylation in *dazl*. BaP caused CG hypomethylation of 32.6% in *cdkn1a*, 26.2% in *sox3*, 14.9% in *c-fos*, and 11.8% in *nrf2* (Table 3 and Supplemental Table 2). In the rest of the genes at 3.3 hpf, BaP caused less than 10% change in CG methylation. The least affected gene by BaP in the CG context was *apc* (0.02% CG hypomethylation), but it was the most affected gene in both the CHG context (67.4% CHG hypomethylation) and the CHH context (65.9% CHH hypomethylation) (Supplemental Table 2).

The particular CG site primarily causing the overall percent change in methylation for *nqo1* (60.8%) was at position 418 of the amplicon; the last CG in the amplified PCR product (Fig. 4). However, the mean BaP-induced CG hypermethylation of 39.2% observed in *dazl* was not due an increase in methylation at any single CG site, but instead, the increase was across many different CG sites (Fig. 4). This same BaP-mediated change in methylation across various CG sites was observed in *cdkn1a*, *sox3*, *c-fos*, and *nrf2*.

3.6. BaP effect on hyper- and hypomethylation at 96 hpf

At 96 hpf, BaP caused 10% CG methylation change in ten genes, six of them were CG hypermethylated and four were CG hypomethylated (Table 3). In comparison to 3.3 hpf, CG methylation was altered in more genes at 96 hpf. The biggest CG methylation change was 77.8% CG hypermethylation in *c-fos* followed by 73.0% CG hypermethylation in *mlh1*. BaP-mediated hypermethylation was also found for *nqo1* (28.1%), *gstp1* (25.1%), *cyp1b1* (24.7%), and *dazl* (18.4%). CG hypomethylation was detected in *p53* (35.5%), *sox2* (21.1%), *sox3* (19.9%) and *pten* (10.2%) (Table 3 and Supplemental Table 3). The biggest

percent methylation change due to BaP at 96 hpf in the CG context (77.8% CG hypermethylation in *c-fos*) did not correspond to the most affected gene in the CHG context (92.0% CG hypermethylation in *mlh1*) or the CHH context (89.7% CG hypermethylation in *gstp1*) (Supplemental Table 3); however, all three genes (*c-fos*, *mlh1* and *gstp1*) exhibited a more than 19% increase in all methylation contexts (CG, CHG, and CHH). In general at 96 hpf, changes in CG methylation due to BaP were distributed across various CG sites in each gene.

3.7. Constitutive and BaP effects on mRNA expression at 3.3 hpf and 96 hpf

To investigate if DNA methylation changes were associated with an alteration in whole embryo/larvae gene expression, mRNA expression of *c-fos*, *mlh1*, *p53*, *nqo1*, *gstp1*, *cyp1b1*, *sox2*, *sox3*, and *dazl* was measured. When relative constitutive mRNA expression was compared at 3.3 hpf, *sox2* and *gstp1* had the lowest and highest constitutive expression, respectively (Fig. 6). *dazl* and *gstp1* had the lowest and highest constitutive expression at 96 hpf, respectively. *cyp1b1*, *nqo1*, *sox2*, and *mlh1* constitutive mRNA expression was significantly increased at 96 hpf compared to their expression at 3.3 hpf. In contrast, *dazl* constitutive mRNA expression was significantly decreased at 96 hpf compared to 3.3 hpf. BaP relative to controls did not significantly change gene expression in zebrafish embryos at 3.3 hpf (Fig. 7A). However, at 96 hpf, expression of *cyp1b1* and *gstp1* was induced by BaP exposure in zebrafish larvae (Fig. 7B).

4. Discussion

BaP is teratogenic to zebrafish development by causing growth retardation and abnormalities in the head and tail. After waterborne BaP exposure to zebrafish embryos, the LC₅₀ is 5.1 μ M (1285 μ g/L) and the EC₅₀ for teratogenesis is 0.52 μ M (131 μ g/L) (Weigt et al., 2011). In our study, after an adult waterborne BaP (42.0 ± 1.9 μ g/L) exposure, reproductive success as reflected by egg production was significantly reduced (Fig. 1A). When the offspring were continuously exposed to BaP, their survival rate dropped significantly (Fig. 1B) and some deformities were observed (data not shown). Our dose of BaP caused higher incidence of mortality than previously reported in a similar zebrafish study but without the parental exposure (Weigt et al., 2011). This suggests that parental BaP exposure in addition to embryonic exposure enhances the toxic effects of BaP. The deformities observed included delayed hatching, curved tail, abnormal head and pericardial edema growth. This study further supports the classification of BaP as a reproductive and developmental toxicant.

Consistent with our earlier findings (Fang et al., 2013a), global DNA methylation percentage was decreased by BaP in the 96 hpf larval offspring (Fig. 2). DNA hypomethylation may increase the vulnerability to many diseases by altering gene expression, elevating mutation rates, increasing genome instability or triggering apoptosis (Lichtenstein et al., 2001; Kisseljova et al., 2005). In order to evaluate DNA methylation changes in specific genes, multiplex bisulfite deep sequencing was employed to analyze DNA methylation patterns in 21 gene promoters in four treatment groups, namely 3.3 hpf control, 3.3 hpf BaP, 96 hpf control, and 96 hpf BaP, using Illumina next generation

sequencing technologies. Advantages of multiplex sequencing include that it was subcloning-free and provided thousands of reads per CG in each sample versus 12–20 bacteria colonies that may be measured by traditional Sanger sequencing. These 21 candidate genes were carefully selected based on literature reports that their expression has been affected by BaP exposure and/or they are important cancer or developmental related genes (Table 1). Furthermore, this study provides an indication of the methylation status of these genes at two important developmental stages. The first time point (3.3 hpf) is consistent with the mid-blastula transition in zebrafish (Kimmel et al., 1995; Andersen et al., 2012) and reflects consequences of BaP exposure during gametogenesis and early embryogenesis, while 96 hpf is consistent with the completion of organogenesis, and thus, reflects the effects of BaP during the whole course of embryogenesis and organogenesis.

At 3.3 hpf, BaP markedly increased CG methylation in *nqo1* and *dazl*, and reduced CG methylation in *sox3* and *cdkn1a* by more than 20% when compared to control. Expression of *cdkn1a* and *nqo1* was altered by BaP in MCF-7 breast cancer cells and the methylation of these genes was altered in different cancers (Hockley et al., 2006; Habano et al., 2009). Thus, abnormal methylation in *nqo1* and *cdkn1a* may contribute to increased risks of carcinogenesis. On the other hand, our previous work found that BaP exposure to zebrafish embryos decreased promoter methylation in the germ cell marker gene *vasa* (Fang et al., 2013a). Like *vasa*, *dazl* is maternally deposited and its constitutive methylation status matches gene expression changes over development. However, BaP's increase in methylation at 3.3 hpf was not reflected in a decrease in mRNA expression. *dazl* is an important gene for spermatogenesis (Rengaraj et al., 2010). In mammals, *dazl* deficiency leads to spermatogenic arrest and alterations in promoter DNA methylation of *dazl* have been found in poor quality sperm (Li et al., 2013; Krausz et al., 2012; Navarro-Costa et al., 2010). Therefore, aberrant methylation of *dazl* may contribute to impaired fertility in adulthood.

BaP induced more changes at 96 hpf than at 3.3 hpf in that both global DNA methylation was significantly decreased and ten genes (6 hyper- and 4 hypomethylated) showed percent CG methylation changes of more than 10%. This suggests that during completion of embryogenesis and organogenesis, changes in DNA methylation are more susceptible to BaP exposure. These genes include cancer related genes *c-fos*, *mlh1*, *pten*, and *p53*, metabolic genes *nqo1*, *gstp1*, and *cyp1b1*, and developmental and reproductive genes *sox2*, *sox3* and *dazl*. CG hypermethylation was observed in *c-fos*, *mlh1*, *nqo1*, *gstp1*, and *cyp1b1*, which agrees with previous studies that found hypermethylation in these genes in various cancer tissues (Table 1). The abnormal modification of methylation patterns in these genes may contribute to carcinogenesis in later life. BaP decreased CG methylation in *sox2* and *sox3*. These two genes are essential for maintaining undifferentiated embryonic stem cells, and they have increased promoter methylation in differentiated cells (Lindeman et al., 2010). The reduction of CG methylation in *sox2* and *sox3* may be due to delayed development caused by BaP. Similar to the changes at 3.3 hpf, BaP increased CG methylation in *dazl* at 96 hpf, which may affect male fertility in the long-term.

In addition to CG methylation, BaP affected non-CG methylation at CHH and CHG sites. The biological function of non-CG methylation is currently unclear. It is known that non-CG

methylation is abundant in stem cells, and it has a potential role in regulating gene transcription (Pulverer et al., 2012; Ichiyanagi et al., 2013; Shirane et al., 2013). Therefore, the physiological significance of BaP effects on non-CG methylation is unknown and requires further investigation.

In 96 hpf larvae, BaP treatment significantly increased *cyp1b1* and *gstp1* mRNA expression, but not transcription of the other seven genes even though their promoter CpG methylation percentage was affected. *cyp1b1* and *gstp1* are well established biomarker genes induced by BaP exposure (Bowes et al., 1996; de Waard et al., 2008). Other studies have reported that high concentrations of BaP exposure significantly increased expression of *nqo1*, *p53*, and *c-fos* (Hockley et al., 2006; Qin et al., 2010). However, induction in these three genes was not seen in our study possibly due to differences in the BaP doses tested or developmental stage or tissue specificity. Generally, promoter DNA methylation and gene expression are inversely correlated (Kass et al., 1997) which means DNA hypermethylation will effectively repress gene expression and *vice versa*. In our study, however, *cyp1b1* and *gstp1* were hypermethylated and gene expression was induced. In fact, many promoter methylation patterns do not correlate well with gene expression. For example, hypomethylated promoters can sometimes be associated with gene silencing (Weber et al., 2007). It is possible that other mechanisms, such as transcription factors and hormones, may be mediating the BaP induction of *cyp1b1* and *gstp1* even though hypermethylation was observed in these genes.

DNA methylation patterns are established in embryogenesis and stably inherited during DNA replication. After fertilization, genomic DNA in the inner cell mass undergoes rapid demethylation and remethylation (Weaver et al., 2009; Sanz et al., 2010). In zebrafish, DNA methylation patterns are re-established from 4.3 to 6 hpf (Fang et al., 2013b). During cellular differentiation, lineage-specific DNA methylation patterns undergo further remodeling (Mohn et al., 2008). In primordial germ cells, genome-wide demethylation occurs after sex determination (Popp et al., 2010). The methylation level remains low in the immature germ cells until *de novo* methylation occurs during germ cell maturation and gametogenesis (Rousseaux et al., 2005; Lees-Murdock et al., 2008). Globally, zebrafish genomic DNA has low methylation at 3.3 hpf and normal methylation levels at 96 hpf (Fang et al., 2013b). In this study, we found that five of our target genes, i.e. *dazl*, *nqo1*, *sox3*, *cyp1b1*, and *gstp1*, followed the wave of DNA remethylation and had increased methylation percentage at 96 hpf. Notably, the dramatically increased DNA methylation in *dazl* correlates with significant reduction in gene expression, indicating that DNA hypermethylation may be the mechanism repressing transcription of *dazl*. In contrast, *c-fos* and *cdkn1a* had decreased CG methylation at 96 hpf compared to that at 3.3 hpf, and for *c-fos* this corresponded to a constitutively higher gene expression at 96 hpf. However, the increased CG methylation in *cyp1b1*, *nqo1*, *sox3* was coupled with increased gene expression, indicating that the transcriptional regulation of these genes may not be affected by DNA methylation at this developmental stage or that a more severe change in CG methylation is needed to suppress gene expression as observed in *dazl*. Additionally, these studies represent effects at the whole embryo/larvae, and changes in methylation or gene transcription at the individual tissue level may be being masked by dilution. One limitation of this study is that the biological replicates were pooled for multiplex sequencing, and thus

statistical analyses were not possible. However, 600 embryos were pooled per treatment group for the ultradeep sequencing, increasing the biological significance to a degree not possible for *in vivo* mammalian studies. The other limitation is that the physiological significance of CG, CHH, CHG methylation changes during development or after BaP exposure is not understood. In fact, to our knowledge our results are the first report of BaP exposure-induced changes in CHH and CHG status. Clearly, BaP is a DNA methylation modifier and further studies should be done to carefully map the genome-wide changes of each type of cytosine methylation after BaP exposure and then determine the biological impact on development.

In a previous study by our laboratory, parental dietary BaP exposure alone (no embryonic exposure) resulted in multigenerational developmental deformities in body shape, tail and pectoral fin shape, and brain size at 96 hpf (Corrales et al., 2014). Although these two studies represent two different exposure routes, BaP exposure consistently had both genetic and phenotypic adverse effects on zebrafish development. Furthermore, in an effort to link adverse effects due to environmental exposure during development to hereditary epigenetics and DNA methylation patterns in later life, we will investigate multigenerational DNA methylation changes in adults after parental BaP exposure.

Conclusion

In this study, we found that parental followed by embryonic BaP exposure significantly reduced egg production and offspring survival in zebrafish. BaP caused global DNA hypomethylation in 96 hpf larvae. Constitutively, an increase in CG methylation and decrease in gene expression were observed in *dazl* at 96 hpf compared to 3.3 hpf. In contrast, decreased CG methylation and increased gene expression were found in *c-fos*. BaP altered CG, CHH, and CHG methylation in many of the target cancer and developmental genes both at 3.3 and 96 hpf (Tables 1 and 3). The changes were greater at 96 hpf than that at 3.3 hpf, suggesting that DNA methylation is more susceptible to BaP modification during late embryogenesis or organogenesis than that during gametogenesis and early embryogenesis. In sum, BaP exposure in early life alters promoter CG methylation and gene expression in cancer and development related genes, possibly leading to higher risk of adult diseases in later life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

apc	adenomatous polyposis coli
bdnf	brain-derived neurotrophic factor
BaP	benzo[a]pyrene
brca1	breast cancer 1
cdh2	cadherin-2
cyp1b1	cytochrome P450, family 1, subfamily B, polypeptide 1
dazl	deleted in azoospermia-like
drd1	dopamine receptor D1
er1	estrogen receptor 1
gstp1	glutathione <i>S</i> -transferase pi 1
hpf	hours post fertilization
h-ras	Harvey rat sarcoma virus oncogene1
mlh1	human mutL homolog 1
msh3	mutS homolog 3
nos2b	nitric oxide synthase b
nqo1	NAD(P)H dehydrogenase quinone 1
nrf2	nuclear factor erythroid 2-related factor 2
cdkn1a	cyclin-dependent kinase inhibitor 1A or p21
p53 or tp53	tumor protein
pten	phosphatase and tensin homolog
sox2	sex determining region Y-box 2
sox3	sex determining region Y-box 3

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Figure 1.

BaP effects on parental zebrafish egg production and cumulative survival in offspring. (A) Eggs were collected before and after $42.0 \pm 1.9 \mu\text{g/L}$ (ppb) BaP waterborne exposure to adult zebrafish. The average number of eggs collected per day per tank was calculated after 7 to 11 days of exposure ($n = 12$ tanks pre-exposure, $n = 6$ tanks/treatment group during exposure, $p < 0.05$ one-way ANOVA and Neumann Keuls multiple comparison tests). (B) Percent offspring survival was determined at 24, 48, 72, 96 hp. Sample sizes ($n=3$ or 4)

were 30 embryos/pool with a total of 90–120 fertilized eggs per treatment group; $p < 0.05$, one-way ANOVA followed by Tukey's post hoc test.

Figure 2.

Global DNA methylation effects in the offspring after parental and continued embryonic BaP ($42.0 \pm 1.9 \mu\text{g/L}$) exposure. Three pools of embryos (each pool 200 embryos) or larvae (each pool 30 larvae) were used to extract DNA and determine 5-methylcytosine percentage ($n=3$, * $p<0.05$, Student t -test).

Figure 3.

Constitutive percent promoter methylation in CG, CHG, and CHH sites of 21 genes at 3.3 and 96 hpf in zebrafish. Data is presented from highest to lowest % CG DNA methylation. Percent CHG and CHH methylation at 3.3 hpf (not shown) was similar to that at 96 hpf.

Figure 4.
Site-specific % CG methylation profile at 3.3 hpf in zebrafish embryos. Blue = methylated cytosine, yellow = unmethylated cytosine, and in parenthesis is the mean % CG DNA methylation change for each gene due to BaP exposure.

Figure 5.

Site-specific % CG methylation profile at 96 hpf in zebrafish larvae. Blue = methylated cytosine, yellow = unmethylated cytosine, and in parenthesis is the mean % CG DNA methylation change for each gene due to BaP exposure.

Figure 6.

Constitutive mRNA expression in 3.3 and 96 hpf zebrafish as measured by qRT/RT-PCR.

Fold change of expression was normalized to 18S rRNA expression and relative to *cyp1b1* expression at 3.3 hpf (n = 3 pools, 200 or 30 embryos or larvae, respectively/pool; *p<0.05; Student T-test between 3.3 and 96 hpf within each gene).

Figure 7.

BaP induced effects on mRNA expression in embryos at 3.3 hpf (A) and in larvae at 96 hpf (B) as measured by qRT/RT-PCR. Fold change was normalized to 18S rRNA expression and relative to controls (n = 2–3 pools, 200 or 30 embryos or larvae, respectively/pool; * $p < 0.05$).

Table 1

List of genes and diseases associated with DNA methylation changes

Gene ¹	Name	Biological function	DNA methylation & disease	BaP effect at 96 hpf in zebrafish ²
<i>apc</i>	adenomatous polyposis coli	inhibitor of β -catenin, tumor suppressor	Hypermethylation in cancer	-
<i>bdnf</i>	brain-derived neurotrophic factor	neuron development	Hypermethylation in neurodegenerative and neuropsychiatric disorders: depression, bipolar disorder, schizophrenia, suicidal behavior	-
<i>brca1</i>	breast cancer 1	DNA repair, cell-cycle control, chromatin remodeling	Hypermethylation in breast and ovarian cancer	-
<i>cdh2</i>	cadherin-2	cell adhesion	Hypermethylation in cancer	-
<i>cdkn1a</i>	cyclin-dependent kinase inhibitor 1A or p21	cell-cycle arrest	Hypermethylation in cancer	-
<i>c-fos</i>	FBJ murine osteosarcoma viral oncogene homolog	proto-oncogene involved in signal transduction, cell proliferation and differentiation	Hypermethylation in cancer	↑
<i>cyp1b1</i>	cytochrome P450, family 1, subfamily B, polypeptide 1	xenobiotic and steroid metabolism	Hypo- or hypermethylation in cancer	↑
<i>dazl</i>	deleted in azoospermia-like	gametogenesis	Hypermethylation in poor quality sperm	↑
<i>drd2l</i>	dopamine receptor D2 like	inhibitor of adenylyl cyclase activity in neuronal signaling	Hypermethylation in neuropsychiatric disorders: eating disorders, bipolar disorder, schizophrenia	-
<i>esr1</i>	estrogen receptor 1 (estrogen receptor α)	estrogen hormone response	Hypermethylation in hormone-mediated cancers and in cardiovascular disease	-
<i>gstp1</i>	glutathione S-transferase pi 1	xenobiotic metabolism	Hypermethylation in cancer	↑
<i>h-ras</i>	Harvey rat sarcoma virus oncogene1	proto-oncogene	Hypermethylation in poor quality sperm	-
<i>mlh1</i>	human mutL homolog 1	mismatch-repair gene	Hypermethylation in cancer	↑
<i>msh3</i>	mutS homolog 3	mismatch-repair gene	Hypermethylation in cancer	-
<i>nos2b</i>	nitric oxide synthase b	synthesis of nitric oxide	Hypo- or hypermethylation in respiratory diseases	-
<i>nqo1</i>	NAD(P)H dehydrogenase quinone 1	xenobiotic metabolism	Hypermethylation in cancer	↑
<i>nrf2</i>	nuclear factor (erythroid-derived 2)-like 2	oxidative stress response	Hypermethylation in cancer	-
<i>p53</i>	tumor protein 53	DNA repair, cell-cycle arrest, apoptosis	Hypermethylation in cancer	↓
<i>pten</i>	phosphatase and tensin homolog	apoptosis and cell movement and adhesion	Hypermethylation in cancer	↓
<i>sox2</i>	sex determining region Y-box 2	embryogenesis, neuronal development	Hypermethylation in cancer	↓
<i>sox3</i>	sex determining region Y-box 3	neuronal development	Hypermethylation in cancer	↓

¹References: *apc* (Richiardi et al., 2013; Heller et al., 2010; Hernandez-Vargas et al., 2010); *bdnf* (D'Addario et al., 2012; Fuchikami et al., 2011; Keller et al., 2010; Autry and Monteggia, 2009); *brca1* (Esteller et al., 2000; Esteller et al., 2001; Xu et al., 2009); *cdh2* (Berx and van Roy 2009; Sui et al., 2012; Loo et al., 2010); *cdkn1a* (Campion et al., 2009); *c-fos* (Liu et al., 2003; Wainfan and Poirier, 1992); *cyp1b1* (DiNardo et al., 2013; Habano et al., 2009; Sissung et al., 2006; Tokizane et al., 2005); *dazl* (Li et al., 2013; Krausz et al., 2012; Navarro-Costa et al., 2010); *drd2l*

(Abdolmaleky et al., 2005); *esr1* (Majumdar et al., 2011; Campion et al., 2009); *gstp1* (Richiardi et al., 2013; Muggerud et al., 2010); *h-ras* (Hass et al., 1993; Campion et al., 2009); *mlh1* (Muggerud et al., 2010; Heller et al., 2010; Esteller et al., 2001); *msh3* (Lahtz and Pfeifer, 2011); *nos2b* (Breton et al., 2012); *nqo1* (Tada et al., 2005); *nrf2* (Khor et al., 2011; Yu et al., 2010); *p53* (Intarasunanont et al., 2012; Zeng et al., 2011; Hernandez-Vargas et al., 2010; Campion et al., 2009); *pten* (Campion et al., 2009; Muggerud et al., 2010); *sox2* (Farthing et al., 2008; Wong et al., 2010; Hirabayashi and Gotoh, 2010); *sox3* (Hirabayashi Gotoh, 2010).

² BaP-induced 10% DNA methylation change following a parental and continued embryonic waterborne exposure in zebrafish (this study).

Table 2

Constitutive changes in DNA methylation during development. The numbers represent percent methylation change in the CG, CHG, and CHH contexts at 96 hpf relative to 3.3 hpf in zebrafish.

Gene	% methylation change		
	CG*	CHG	CHH
dazl	309.9	61.4	69.4
nqo1	122.0	189.9	93.2
sox3	51.8	116.2	108.9
cyp1b1	48.4	42.6	43.8
gstp1	30.8	-8.5	-8.4
c-fos	-30.4	-51.2	-14.0
cdkn1a	-28.1	-53.5	-51.5
p53	-7.9	-10.5	-16.7
drd2l	-6.3	-9.2	-0.4
bdnf	-5.3	-59.2	-57.3
cdh2	-5.0	14.0	0.8
nos2b	-3.3	-55.8	-55.4
msh3	-1.9	-32.8	-26.3
h-ras	-1.6	-34.7	-34.1
brca1	-1.4	-21.4	-34.0
sox2	-0.8	10.1	13.9
apc	-0.3	-51.2	-51.9

* Genes sorted from highest to lowest absolute CG methylation change.

Table 3

BaP-induced mean percent DNA CG methylation change following a parental and continued embryonic waterborne exposure at 3.3 and 96 hpf in zebrafish.

Gene	BaP-induced % CG methylation at 3.3 hpf	BaP-induced % CG methylation at 96 hpf*
<i>c-fos</i>	-14.9	77.8
<i>mlh1</i>	-	73.0
<i>p53</i>	-7.8	-35.5
<i>nqo1</i>	60.8	28.1
<i>gstp1</i>	-4.0	25.7
<i>cyp1b1</i>	-6.3	24.7
<i>sox2</i>	-8.8	-21.1
<i>sox3</i>	-26.2	-19.9
<i>dazl</i>	39.2	18.4
<i>nrf2</i>	-11.8	-
<i>pten</i>	-	-10.2
<i>esr1</i>	-	2.7
<i>cdh2</i>	-6.3	-2.5
<i>bdnf</i>	-0.7	2.5
<i>brca1</i>	2.8	2.4
<i>cdkn1a</i>	-32.6	2.0
<i>drd2l</i>	0.2	1.8
<i>nos2b</i>	0.5	0.4
<i>h-ras</i>	0.1	0.4
<i>msh3</i>	0.04	0.3
<i>apc</i>	-0.02	0.1

* Genes sorted from highest to lowest absolute change in CG methylation at 96 hpf.