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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 hpf (hours post fertilization) 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.

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, 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 and Angevine, 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

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; *esr1*, estrogen receptor 1; *gstp1*, glutathione *S*-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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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; Manikkam et al., 2012; Singh and Li, 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 (Wadjed et al., 2001; Kisseljova and Kisseljov, 2005).

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 71% of human genes have at least one zebrafish ortholog (Howe et al., 2013). In addition, 76% of genes currently associated with human disease in genome-wide association studies have zebrafish orthologs.

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.

Table 1
List of genes and diseases associated with DNA methylation changes.

Gene[1]	Name	Biological function	DNA methylation and disease	BaP effect at 96 hpf in zebrafish[2]
apc	Adenomatous polyposis coli	Inhibitor of β-catenin, tumor suppressor	Hypermethylation in cancer	–
bdnf	Brain-derived neurotrophic factor	Neuron development	Hypermethylation in neurodegenerative and neuropsychiatric disorders: depression, bipolar disorder, schizophrenia, suicidal behavior	–
brca1	Breast cancer 1	DNA repair, cell-cycle control, chromatin remodeling	Hypermethylation in breast and ovarian cancer	–
cdh2	Cadherin-2	Cell adhesion	Hypermethylation in cancer	–
cdkn1a	Cyclin-dependent kinase inhibitor 1A or p21	Cell-cycle arrest	Hypermethylation in cancer	–
c-fos	FBJ murine osteosarcoma viral oncogene homolog	Proto-oncogene involved in signal transduction, cell proliferation and differentiation	Hypermethylation in cancer	↑
cyp1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Xenobiotic and steroid metabolism	Hypo- or hypermethylation in cancer	↑
dazl	Deleted in azoospermia-like	Gametogenesis	Hypermethylation in poor quality sperm	↑
drd2l	Dopamine receptor D2 like	Inhibitor of adenylyl cyclase activity in neuronal signaling	Hypermethylation in neuropsychiatric disorders: eating disorders, bipolar disorder, schizophrenia	–
esr1	Estrogen receptor 1 (estrogen receptor α)	Estrogen hormone response	Hypermethylation in hormone-mediated cancers and in cardiovascular disease	–
gstp1	Glutathione *S*-transferase pi 1	Xenobiotic metabolism	Hypermethylation in cancer	↑
h-ras	Harvey rat sarcoma virus oncogene1	Proto-oncogene	Hypermethylation in poor quality sperm	–
mlh1	Human mutL homolog 1	Mismatch-repair gene	Hypermethylation in cancer	↑
msh3	mutS homolog 3	Mismatch-repair gene	Hypermethylation in cancer	–
nos2b	Nitric oxide synthase b	Synthesis of nitric oxide	Hypo- or hypermethylation in respiratory diseases	–
nqo1	NAD(P)H dehydrogenase quinone 1	xenobiotic metabolism	Hypermethylation in cancer	↑
nrf2	Nuclear factor (erythroid-derived 2)-like 2	Oxidative stress response	Hypermethylation in cancer	–
p53	Tumor protein 53	DNA repair, cell-cycle arrest, apoptosis	Hypermethylation in cancer	↓
pten	Phosphatase and tensin homolog	Apoptosis and cell movement and adhesion	Hypermethylation in cancer	↓
sox2	Sex determining region Y-box 2	Embryogenesis, neuronal development	Hypermethylation in cancer	↓
sox3	Sex determining region Y-box 3	Neuronal development	Hypermethylation in cancer	↓

[1] References: *apc* (Heller et al., 2010; Hernandez-Vargas et al., 2010; Richiardi et al., 2013); *bdnf* (Autry and Monteggia, 2009; Keller et al., 2010; Fuchikami et al., 2011; D'Addario et al., 2012); *brca1* (Esteller et al., 2000, 2001; Xu et al., 2009); *cdh2* (Berx and van Roy, 2009; Loo et al., 2010; Sui et al., 2012); *cdkn1a* (Campion et al., 2009); *c-fos* (Wainfan and Poirier, 1992; Liu et al., 2003); *cyp1b1* (Tokizane et al., 2005; Sissung et al., 2006; Habano et al., 2009; DiNardo et al., 2013); *dazl* (Navarro-Costa et al., 2010; Krausz et al., 2012; Li et al., 2013); *drd2l* (Abdolmaleky et al., 2005); *esr1* (Campion et al., 2009; Majumdar et al., 2011); *gstp1* (Muggerud et al., 2010; Richiardi et al., 2013); *h-ras* (Hass et al., 1993; Campion et al., 2009); *mlh1* (Esteller et al., 2001; Heller et al., 2010; Muggerud et al., 2010); *msh3* (Lahtz and Pfeifer, 2011); *nos2b* (Breton et al., 2012); *nqo1* (Tada et al., 2005); *nrf2* (Yu et al., 2010; Khor et al., 2011); *p53* (Campion et al., 2009; Hernandez-Vargas et al., 2010; Zeng et al., 2011; Intarasunanont et al., 2012); *pten* (Campion et al., 2009; Muggerud et al., 2010); *sox2* (Farthing et al., 2008; Hirabayashi and Gotoh, 2010; Wong et al., 2010); and *sox3* (Hirabayashi and Gotoh, 2010).

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