SIGNIFICANCE OF EPIGENETIC DATA IN ENVIRONMENTAL RISK ASSESSMENT: USING HUMAN HEALTH AND ECOLOGICAL MODELS

by

RAMYA TULASI KOLLI

(Under the Direction of Brian S. Cummings)

ABSTRACT

The role of epigenetic mechanisms in gene regulation is well studied in basic science and cancer pathology. However, our knowledge on toxicant-induced alterations of gene expression via epigenetic alterations is meager. Having this knowledge is key to estimating the risk of adverse and long-term effects of toxicants on human health and ecosystem. This series of studies tested the epigenetic effects of sub-chronic exposure to low-dose bromate ($BrO_3^-$) on human and rat renal cells. $BrO_3^-$ is a drinking water disinfection byproduct regulated by the US EPA. Based on our previous data, we tested the hypothesis that $BrO_3^-$-induced renal p21 expression is mediated by epigenetic mechanisms.

Our data demonstrated that expression of rat renal p21 was regulated by histone acetylation and not DNA methylation of the regions analyzed, after sub-chronic exposure to $BrO_3^-$. These data also demonstrated that $BrO_3^-$-induced p21 expression in human renal cells was neither regulated by DNA methylation nor histone acetylation. Finally, these data demonstrated species-specific differences in epigenetic regulation of p21 and

suggested an uncertainty in extrapolating rat epigenetic data for assessing the risk of toxicants in humans.

17α-ethynylestradiol (EE2), an orally active synthetic estradiol used in contraceptives, it is a water contaminant that presents concerns for both human and ecological health significance. Vitellogenin (Vtg) is an egg yolk precursor protein and can be a molecular marker of exposure to estrogenic endocrine disrupting chemicals (EDCs). Our data demonstrated that adult male zebrafish exposed to EE2 showed a significant increase in Vtg mRNA as early as 0.25 days and promoter hypomethylation at any CpG sites analyzed only after 4 days. These decreases brought the methylation of v*tg1* in male zebrafish to same level as that of female controls, suggesting that it may lead to feminization. We also observed that EE2-induced decrease in DNA methylation persisted after EE2 removal, unlike mRNA levels which returned to baseline by 7 days. These data suggested a role for DNA methylation in Vtg induction and identified a novel epigenetic mark of feminization that may serve as an indicator of previous exposure to EE2, which will aid in ecological risk assessment of EDCs.

INDEX WORDS: Epigenetics, DNA Methylation, Histone Acetylation, Risk Assessment, Bromate, Nephrotoxicity, Mechanism of Toxicity, Endocrine Disrupting Chemicals, Zebrafish

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

| | |
|---|---|
| 5-Aza | 5-aza-2’-deoxycytidine |
| BLAST | basic local alignment search tool |
| $BrO_3^-$ | bromate |
| CDK | cyclin-dependent kinase |
| CDKN1a | cyclin-dependent kinase inhibitor 1a |
| ChIP | chromatin immunoprecipitation |
| DBP | disinfection byproduct |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxynucleoside 5’-triphosphate |
| *E. coli* | *Escherichia coli* |
| EDC | endocrine disrupting chemical |
| EE2 | 17α-ethynylestradiol |
| ERE | estrogen response element |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GGF | Georgia genomics facility |
| H3K9/14 Ac | histone 3 lysine 9 and 14 acetylation |
| HEK293 | human embryonic kidney cells |

| | |
|---|---|
| IDT | integrated DNA technologies |
| $KBrO_3$ | potassium bromate |
| MAPK | mitogen-activated protein kinase |
| $MgCl_2$ | magnesium chloride |
| MID | molecular identifier |
| NGS | next-generation sequencing |
| NRK | normal rat kidney cells |
| SIE-1 | sis-inducible element-1 |
| TGBS | targeted gene bisulfite sequencing |
| TSA | trichostatin A |
| TSS | transcription start site |
| Vtg | vitellogenin |
| WGBS | whole genome bisulfite sequencing |

# CHAPTER 1

# INTRODUCTION

## 1. Epigenetics

Epigenetics is the study of heritable changes in gene activity without any changes in the DNA sequence (Waddington, 1942, Russo *et al.*, 1996). Major mechanisms involved in epigenetic alterations are DNA methylation, histone modifications and regulation by non-coding RNAs (ncRNAs) (Costa, 2008). Epigenetic profiles are tissue-specific. Epigenetic modifications naturally manifest for terminal differentiation of cells into skin cells, brain cells, liver cells, etc. Monozygotic twins with similar genetic make-up have very different epigenetic profiles, rendering them differentially susceptible to disease and exposures (Fraga *et al.*, 2005).

### 1.1. DNA Methylation

DNA methylation is a biochemical process catalyzed by DNA methyltransferases (DNMT) where methyl groups are added to the cytosine nucleotides forming 5-methylcytosine residues (Holliday *et al.*, 1975). This is seen predominantly with cytosines in the CpG dinucleotides in multicellular eukaryotes. DNA methylation decreases the transcription of genes by impeding the access of transcription factors to their binding sites (Choy *et al.*, 2010). The extent and pattern of methylation controls the transcriptional activity of a gene, that is, methyl groups act as switchboard operators for gene expression. The addition of methyl groups by DNMTs can either be by *De novo* methylation where they recognize signals to newly methylate cytosines, or it can be by maintenance

methylation where hemimethylated CpGs are identified during DNA replication and methyl groups are added to the unmethylated counterparts. In mammals, DNMT1 is a maintenance methyltransferase and DNMT3A and 3B are *de novo* methyltransferases (Li *et al.*, 2007, Jones *et al.*, 2009).

### 1.2. Histone Modifications

Eukaryotic DNA is structured into nucleosomes by packages around histone proteins (Bhasin *et al.*, 2006). Histones are post-translationally modified by various processes like acetylation, methylation, phosphorylation, ubiquitination, sumoylation, glycosylation, biotinylation, etc. Each regulates gene expression differently (Bannister *et al.*, 2011). Histone acetylation is one of the most extensively studied histone modifications, which leads to loosening of histone-DNA bonds thereby facilitating the accessibility of transcriptional machinery to the DNA hence regulating expression (Bannister *et al.*, 2001).

Histone modifications have a bilateral relationship, which means agents affecting DNA methylation may indirectly drive histone modifications (D'Alessio *et al.*, 2006). Certain microRNAs also target DNA methyltransferases and histone deacetylases (Tuddenham *et al.*, 2006, Zhou *et al.*, 2010). This suggests that the epigenetic machinery may function in a reciprocal network of events that need to be understood for deciphering the mechanisms of toxicant-induced cellular adverse effects.

### 1.3. Non-coding RNAs

Non-coding RNAs (ncRNA) are small RNA molecules that are not translated to proteins. The ncRNAs that are known to have a role in epigenetic gene regulation are microRNA (miRNA), short interfering RNA (siRNA), piwi interacting RNA (piRNA) and

long non-coding RNA (lncRNA) (Carthew *et al.*, 2009, Kaikkonen *et al.*, 2011). These ncRNAs play key roles in DNA methylation targeting, histone modifications and gene silencing. The most studied ncRNAs are miRNAs and siRNAs that target a specific mRNA sequence to block translation and are known to cause aberrant expression patterns in cancer (Mraz *et al.*, 2012).

Epigenetic changes can be mitotically stable through cell division, transgenerationally inherited and can persist through biological memory even after removal of the stressors (Richards, 2006). Until less than two decades ago DNA methylation was believed to be irreversible post-development, but Ramchandani *et al* demonstrated otherwise (Ramchandani *et al.*, 1999). The reversibility of methylation is an extremely important finding for reproductive toxicoepigenomics and toxicomethylomics studies as environmental exposures could impact DNA methylation not only during pregnancy but also through-out life (Szyf, 2011). Unlike DNA methylation, histone acetylation is a transient mechanism (Rogge *et al.*, 2013). Alterations in gene expression via ncRNAs are implicated to be stably inherited (Blomen *et al.*, 2011).

## 2. Epigenetics in Toxicology

The epigenome can be influenced by toxicants, nutrients, pharmaceuticals, lifestyles and demographics (Gore, 2007). Stimuli that have this effect are termed as epimutagens. Epigenetic endpoints are abundantly studied in cancer biology in terms of chemical and non-chemical carcinogenesis. Epigenetic abnormalities in human cancers are proposed to be used as potential biomarkers for diagnosis (Suijkerbuijk *et al.*, 2011). Global DNA hypomethylation and critical gene hypermethylation and hypormethylation and aberrant histone modifications have been shown to paint the landscape of cancerous

cells (Feinberg, 1983, Sharma *et al.*, 2010, Jones *et al.*, 2007). For instance, in breast cancer alone, hundreds of critical genes, including tumor suppressor genes, were observed to be transcriptionally silenced by DNA hypermethylation (Hinshelwood *et al.*, 2008). Other studies showed association of histone deacetylation and methylation with such gene silencing (Ropero *et al.*, 2007, Iizuka *et al.*, 2003).

Various chemical-induced cancers have shown aberrations in epigenetic landscapes. Phenobarbital, an epilepsy medication, is known to induce liver carcinomas in rats by hypermethylation of p53 and p16 promoters (Phillips *et al.*, 2008, Kostka *et al.*, 2007). Tobacco smoke is considered the most widespread carcinogen that contains polycyclic aromatic hydrocarbons (PAHs), benzene, N-nitrosamines, 1,3-butadiene, etc (Taioli, 2008, Hoffmann *et al.*, 2001). Smoking-induced lung cancers showed methylation-induced transcriptional silencing of p53, p16, cadherin-1, death associated protein kinase 1 (DAPK1) and Ras effector proteins (Divine *et al.*, 2005, Russo *et al.*, 2005, Belinsky, 2005). Chronic exposure to various metals has been shown to correlate to cancer induction (Salnikow *et al.*, 2008, Beyersmann, 2002). Arsenic exposures showed global DNA hypomethylation and hypermethylation of tumor suppressor genes (Zhao *et al.*, 1997, Mass *et al.*, 1997, Chanda *et al.*, 2006). Chromium, the strongest known carcinogenic metal mutagen (Beyersmann, 2002), induced lysine 9 dimethylation in histone leading to downregulation of mutL homolog 1 (MLH1), which directly correlated to the occurrence of lung cancer (Takahashi *et al.*, 2005).

Epigenetic toxicants are known to have many non-cancer endpoints. Benzo(a)pyrene (BaP), and other PAHs are byproducts of incomplete combustion of organic matter and are popularly known to be produced from smoking and grilling meat

(Phillips, 1999). BaP is known to hypermethylate CpG islands in the acyl-CoA synthetase long chain family of genes which further correlate to environmentally related childhood asthma (Perera *et al.*, 2009). Phthalates, including di(2-ethylhexyl) phthalate (DEHP) are industrial plasticizers that are classified as endocrine disruptors (Swan *et al.*, 2005). DEHP was able to induce hypermethylation in mouse testis, which correlated to developmental malformations and insulin imbalance (Wu *et al.*, 2010). Bisphenol A (BPA) is another popular endocrine disruptor known to alter DNA methylation, histone trimethylation and miRNA induction leading to hormonal imbalance and developmental toxicities (Weng *et al.*, 2010, Avissar-Whiting *et al.*, 2010, Doherty *et al.*, 2010).

**3. Toxicoepigenomics and Risk Assessment**

Toxicoepigenomics is the study of the relationship between adverse effects induced by exposure to toxic agents and epigenetic alterations. The epigenome, unlike the genome, is variable across cell types, developmental stage, age and environmental exposures. This plasticity poses various levels of challenges in the incorporation of epigenetic data in risk assessment. Risk assessment is the quantitative and/or qualitative estimation of risk related to a recognized hazard. The efforts of the International Human Epigenome Consortium and their reference epigenome focus on resolving the challenges of incorporating epigenetic data in risk assessment (IHEC, 2017). Epigenetic changes could serve as surrogate markers where environmental exposure samples have short half-lives and low biological doses (Ladd-Acosta *et al.*, 2016). They accumulate gradually and hence provide for early detection strategies for risk prediction (Pashayan *et al.*, 2016). For instance, in a cervical cancer study, normal cells showed epigenetic changes years prior to neoplastic transformation (Teschendorff *et al.*, 2012). Williams *et al* proposed that

the risk assessment of an epigenetic carcinogen should be based on the no observed effect level (NOEL) approach including the uncertainty factors (Williams, 1996). It is key to identify if the epigenetic mark is associative or causative to the disease or toxicity (Verma *et al.*, 2013). For transgenerational assessment of epigenetic effects, it had been proposed that the assessment at the NOEL and lowest observed effect level (LOEL) for the most sensitive phenotypic endpoint is very important (Shaw *et al.*, 2017). For instance, Alyea *et al.*, 2014 observed DNA methylation changes induced by 1,3-butadiene at concentrations below the NOEL upon prolonged exposure (NTP, 1993). Such studies suggest that the existing regulatory values are not protective, hence encouraging the consideration of accumulative epigenetic effects in risk assessment.

Epigenetic data alone do not suffice for risk assessment. The FORECEE (four cancers one test) program conducted by a consortium of scientists across Europe was designed to predict the risk of developing various cancers in women by incorporating epigenomics with genomics data (Pashayan *et al.*, 2016). The justification of incorporation of epigenetic data in cancer risk assessment is stronger than in noncancer risk assessment. It is established that in many cancers there is evidence of promoter hypermethylation of tumor suppressor genes and global DNA hypomethylation. This would lead to destabilization of the genome by inducing chromosome instability, oncogene activation, etc. (Sharma *et al.*, 2010, Jones *et al.*, 2007). The stability, heritability and specificity of epigenetic alterations makes them better biomarkers of exposure to carcinogens than the transcript and protein endpoints. More importantly, the reversibility of epigenetic marks renders it a tool not only for therapeutic but also preventive purposes (Koturbash *et al.*, 2011).

Shaw *et al.*, 2017 suggest a structured risk assessment experimental design to maximize the usefulness of epigenetic data generated. The authors proposed a multi-tiered assessment with tier 1 assessing acute toxicity (hours – days) and dose-response relationships, tier 2 assessing subacute toxicity (days – weeks), tier 3 assessing chronic toxicity (two generations) and reproductive effects, tier 4 assessing long-term effects (more than two generations) and epigenetic analysis of parental (P0) generation and tier 5 assessing transgenerational toxicity (more than three generations) and recovery time or epigenetic memory. As proposed, we could obtain key risk factors from tiers 4 and 5 where environmentally relevant concentrations are focused. The authors also bring up an interesting point that it might be irrelevant to assess epigenetic effects of higher, environmentally unrealistic concentrations of toxicants as they might have already been regulated. The effects of higher concentrations can usually be assessed by morphological endpoints that are cheaper to assess than epigenetic endpoints.

**4. Challenges in Incorporating Epigenetic Data in Risk Assessment**

There exist various challenges for using epigenetic marks to estimate cumulative risks. A major challenge being the influence of confounding factors like age, sex, ancestry, cell type and inter-individual variability. Using suitable model organisms with adequate amounts of replication is key to minimizing these confounding factors. Due to differences in epigenome among cell types, the heterogeneity in tissue samples makes it extremely challenging to consider tissue specificity of the epigenetic measurements. Time of exposure and design of experiment play key roles in influencing the epigenome, whether the exposure was at germline development or within or outside of the developmental

tissue-specific programming, or if it is during adulthood (Foley *et al.*, 2009, Fleisch *et al.*, 2012).

Currently, evidence for epigenetic alterations modulating adverse phenotypic outcomes to many toxicant exposures is lacking for establishing causality (Alyea *et al.*, 2014). Hence future studies need revised designs to address correlative versus causal relationships between adverse outcomes and molecular end-points. However, there exists a literature bias in outcome reporting due to the gaps in publishing negative results in which this causal relationship might often be lost (Dwan *et al.*, 2013). There also exists experimental irreproducibility of epigenetic data. This can possibly be minimized by providing detailed technical descriptions of the methods.

Anway *et al.*, 2005 studied transgenerational epigenetic alterations induced by endocrine disruptors in rats and observed that the effects were not only inherited to F1 offspring but also were identified in the F3 and F4 non-exposed generations. Such studies state that if the alterations are measured in the F1 offspring (direct gamete exposure), they might not conclusively be inherited unless observed in the F3+ generations. However, some studies suggest that the alterations are heritable only after repeated exposure of multiple generations (Schultz *et al.*, 2016). This might be an indication of existence of an epigenetic tipping point above which the toxicant-induced alterations shift from adaptive to transgenerationally stable (Shaw *et al.*, 2017).

**5. Concepts in Risk Assessment**

Based on the environmental component that is impacted, risk assessment is categorized into two areas: Human Health and Ecological. Chapters in this dissertation discuss components in the human health category, specifically studies on the ability of

the disinfection byproduct (DBP) bromate ($BrO_3^-$) to induce cell death and epigenetic changes in human and rat renal cells. Another chapter focused on ecological risk assessment by focusing on the mechanism mediating the feminizing of zebrafish by the synthetic estrogen 17α-ethynylestradiol (EE2).

### 5.1. Human Health Risk Assessment

The advance in technology and industrialization seen in the earlier 20$^{th}$ century resulted in humans being continuously exposed to a variety of chemical compounds through air, water, food and other sources. The challenge is to determine whether these chemicals cause any adverse effects and the bigger challenge is determining what dose makes them poisonous, hence defining the risk. As per the Integrated Risk Information System (IRIS) at the US-EPA, risk assessment is a four-step process and the steps include hazard identification, dose-response assessment, exposure assessment and risk characterization (US-EPA, 2012 and 2014).

For hazard identification, the toxicity data is validated and a weight-of-evidence summary is assembled for the association of substance with its toxic effects (Baynes, 2012). Dose-response assessment investigates the numerical association between exposure and its effects. Exposure assessment evaluates the mode, level, frequency and the timing of exposure. The risk characterization examines the transparency and consistency of the data and states any uncertainties to help the assessors in policy making (US-EPA, 2000).

One of the broad classifications of risk assessment is based on whether the chemical generates a cancerous or a noncancerous response. The cancer risk assessment assumes a non-threshold mechanism, meaning there is no safe level of

exposure that does not generate a carcinogenic response. This mechanism includes a weight-of-evidence evaluation and a slope factor calculation (US-EPA, 2005). The weight-of-evidence determines whether the chemical agent has the likelihood of being a human carcinogen with the characterization ranging from a sufficient to inadequate evidence. The slope factor is a probability estimate of the response and is used alongside the weight-of-evidence classification to determine the strength of evidence whether the chemical is a human carcinogen.

The noncancer risk assessment has two approaches, the no observed adverse effect (NOAEL) and the bench mark dose (BMD) approach. The NOAEL approach assumes a threshold that needs to be met before the noncancer effect is expressed. That means, an adverse effect can be manifested after overcoming a protective mechanism. BMD is a statistical lower confidence limit of a dose that produces a predetermined change in response rate of an adverse effect (benchmark response or BMR) compared to the background. BMR must be specified before calculating the BMD and is generally set near the lower limit of responses that can be measured directly. The goal of BMD is to define a starting point of departure (POD), which is an estimated or experimental point that marks the beginning of extrapolation to determine risk of exposure. The NOAEL is a better approach for the human health aspect like our work on bromate where environmentally relevant doses and timelines were investigated. NOAEL is more sensitive to small changes in the data when compared to BMD. The predictions made by statistical models proposed for the BMD approach are proposed to pose serious errors when extrapolating to low doses.

While the NOAEL is driven by the dose selected, BMD depends on the dose-response curve. BMD is not constrained by experimental design and can theoretically assume any value while the NOAEL is one of the experimental doses and is sensitive to sample size. The goal of BDM is to define a starting POD. Both the NOAEL and BMD approaches are used to determine the reference dose for characterizing noncancer effects of various exposures. However, NOAEL is traditionally used for effects that are expected to have threshold whereas BMD considers doses that are above the threshold. The reference dose is an estimate of daily oral exposure (RfD) or inhalation exposure (RfC) to the human population with consideration of the uncertainty factors (UFs). The UFs are applied in risk assessment to compensate for limited data, animal model to human data extrapolation and inter-individual variations in age, sex, susceptibilities, etc. In the following few chapters we focus on the dose-response aspect of risk assessment mainly focusing on mechanism of action and the dose-time and dose-response concurrence.

### 5.1.1. Drinking Water Disinfection and $BrO_3^-$

Drinking water is disinfected with one or more chemical agents to render it safe and free from disease-causing microorganisms. More than 200 million people consume disinfected drinking water in the US (US-EPA, 1998a, Richardson *et al.*, 2007). However, the undesirable outcomes of these treatments are the DBPs of various chemical classifications (Richardson SD *et al* 2007). Disinfection processes such as chlorination and chloramination generate a majority of the known byproducts called trihalomethanes (THMs) that have shown to be associated with the risk for bladder cancer (Cantor, 1997, Villanueva *et al.*, 2007). Ozonation has been an alternative method to chlorination for

disinfection of drinking water in the US since the 1993 outbreak of Cryptosporidium, which is resistant to chlorination, in Milwaukee (Cope *et al.*, 2015, US-EPA, 1998a). About 264 operating plants each serving more than 10,000 people in the US use ozonation (Cotruvo *et al.*, 1999). Ozonation reduced the generation of chlorinated DBPs but is not without side-effects. $BrO_3^-$ is a byproduct formed by the reaction of ozone with the naturally occurring bromide ($Br^-$) in the ground and source water. $BrO_3^-$ is designated as a probable human carcinogen by the International Agency for Research on Cancer (IARC, 1999). It is known to cause renal cancers in female and male rats, and also thyroid and testicular cancers in male rats (Kurokawa *et al.*, 1990, DeAngelo *et al.*, 1998, Wolf *et al.*, 1998). While there is no epidemiologic data on $BrO_3^-$-induced carcinogenesis in humans, there is an uncertainty with regards to species susceptibility. UV-treatment would be an alternative disinfection process to replace ozonation and avoid $BrO_3^-$ in drinking water, but since it requires direct contact with the microbial matter, a pre-filtration unit is necessary (Stewart, 1990), which is not economical in most situations.

### 5.1.2. Risk Assessment of $BrO_3^-$

The grand challenge in risk assessment of DBPs is to evaluate an acceptable level of DBPs in the source water while maintaining the required levels for protection against microbial diseases. The regulated level for $BrO_3^-$ established by the US-EPA is 0.01 ppm (US-EPA, 1998b), which is usually less than what is formed after ozonation of fresh water. EPA standards and the starting POD for the regulation of $BrO_3^-$ is based on various studies but primarily on the critical study by DeAngelo *et al.*, 1998. Here, various concentrations of potassium bromate ($KBrO_3$) was administered through drinking water to male F344 rats and male B6C3F1 mice for 100 weeks. Dose- dependent measures of

body weight, organ weight, serum chemistry, histopathology and survival were evaluated. Various adverse effects were observed in the treatment groups, the most critical one being urothelial hyperplasia in the 6.1mg/kg-day dose group. These rats showed non-neoplastic kidney lesions, renal papilla mineralization and eosinophilic droplets in proximal tubule epithelium. Based on these renal effects, a NOAEL of 1.1mg/kg-day and a LOAEL of 6.1mg/kg-day was identified in rats. The study also indicated that mice might be less sensitive than rats to $BrO_3^-$ . Mice showed no changes in body weight, organ weight and survival and no incidence of non-neoplastic lesions. Hence a NOAEL of 59.6mg/kg-day, which was the highest tested dose, was identified in mice. The adverse effect used by EPA was urothelial hyperplasia and the POD being the LOAEL value 6.1mg $BrO_3^-$/kg-day. DeAngelo and colleagues also studied the cancerous effects of $BrO_3^-$. Dose-dependent increases were observed in incidence of tumors in kidney (adenomas and carcinomas), thyroid (adenomas and carcinomas) and tunica vaginalis testis (mesotheliomas). The only tumor incidence in mice was in kidneys and were not dose dependent.

### 5.1.3. Mechanisms of Action of $BrO_3^-$

The kidney is the major target organ for $BrO_3^-$ -induced toxicity (Kurokawa *et al.*, 1982). Understanding the mechanism of $BrO_3^-$ -induced toxicity is important for extrapolating toxicological data to humans (Moore *et al.*, 2006). As such, $BrO_3^-$ is known to induce DNA damage by 8-hydroxyguanosine (8-OHdG) production, which is a measure of oxidative stress *in vitro* and *in vivo* (Kawanishi *et al.*, 2006). $BrO_3^-$ also induces G2/M cell cycle arrest prior to the occurrence of cell death, and increases the expression of

stress response kinases and DNA damage response proteins like p38, a mitogen-activated protein kinase (MAPK) (Zhang *et al.*, 2010 and 2011).

Inhibition of cell cycle progression by p21 occurs at various checkpoints, thereby inducing cell cycle arrest and restraining proliferation. Our laboratory previously showed that $BrO_3^-$ induced concentration- and time-dependent increases in renal toxicity in both *in vitro* and *in vivo* models that correlated to the induction of p21 (Zhang *et al.*, 2010, Kolisetty *et al.*, 2013a). p21 activation is protective against various nephrotoxic effects including the effects of other nephrotoxicants like cisplatin (Jiang *et al.*, 2008, Price *et al.*, 2009, di Pietro *et al.*, 2012).

Our previous *in vitro* studies showed that high doses of $BrO_3^-$ induced p21 activation by mechanisms involving DNA damage and p53 activation (Zhang *et al.*, 2010 and 2011). Our more recent *in vivo* studies showed that $BrO_3^-$ increased p21 expression in the renal proximal tubule region in rats via mechanisms independent of p53 (Kolisetty *et al.*, 2013a and 2013b). Activation of p21 correlated with other markers of renal dysfunction. Our recently published work (Scholpa *et al.*, 2014) showed that concentrations of $BrO_3^-$ exposure shown to not induce DNA damage, p53 activation, or other markers of cell death, also induced p21 expression. In fact, chronic exposure of cells to $BrO_3^-$ at concentrations as low as 0.01 ppm (the EPA established maximum contaminant level, MCL) increased p21 expression. Interestingly, higher concentrations of $BrO_3^-$ (10 ppm) did not induce p21, but did induce DNA damage. The changes in p21 expression at lower concentrations correlated to changes in methylation within the DNA coding region (Scholpa *et al.*, 2014). Further, inhibition of DNA methylation or histone

deacetylation using 5-azacytidine (5-Aza) or trichostatin A (TSA), respectively, increased p21 expression and altered cell death.

### 5.1.4. p21 Regulation by DNA Methylation

Down-regulation of p21 expression has been indicated in the initiation and progression of many cancer types. The major mechanism mediating the loss of p21 expression, especially in non-small cell lung cancer cell lines, is reported to be aberrant methylation (Teramen *et al.*, 2011). Changes in promoter region hypermethylation and inactivation for p21 was also shown in prostate cancer cell lines by Bott *et al.*, 2005, in aneuploidy cancer cells by Zheng *et al.*, 2012, in odontogenic keratocysts by Moreira *et al.*, 2009, in lymphomas and carcinomas by Ying *et al.*, 2004 and in human lung cancer cell line by Zhu *et al.*, 2003. Hypermethylation of p21 strongly correlated with the decreases in mRNA levels in tumor cells in acute lymphoblastic leukemia (Roman-Gomez *et al.*, 2002) and could be an important factor in disease prognosis. Methylation-specific PCR (MSP) data also showed hypermethylation of p21 promoter region CpG islands in glioma samples from a south Indian patient population (Palani *et al.*, 2011).

p53-dependent regulation of p21 is induced by DNA damage, such as that induced by UV or X-ray treatment, which can lead to apoptosis or cell cycle arrest (el-Deiry *et al.*, 1994). Rat-1 fibroblast cells express wild-type p53 but lack p21 expression. DNA methylation analysis of these cells showed hypermethylation in the p21 promoter region. Exposure of these cells to X-rays did not induce apoptosis or cell cycle arrest (Allan *et al.*, 2000). This study also shows that 5-Aza (a DNA methyltransferase inhibitor) increased the expression of p21, suggesting that DNA methylation is a regulator of p21 expression in these cells.

### 5.1.5. p21 Regulation by Histone Modifications

Contrary to above hypothesis regarding DNA methylation of p21, the promoter region of this gene was not methylated in gastric cancer cells (Shin *et al.*, 2000). This study suggested that another mechanism for inactivation of p21 was histone deacetylation. The deacetylation of histones would lead to the dysregulation of a G1/S checkpoint, a subsequent lack of growth arrest and to the induction of cancer. Several studies suggest that p21 is a target for HDAC1 in human cancers (Senese *et al.*, 2007), but few suggest similar mechanism in non-cancer cells and none have suggested it in kidney cells. HDAC1 shares a common binding domain with p53 on the C-terminus of Sp1 (Lagger *et al.*, 2003). Previous studies have demonstrated that p53 competes with HDAC1 to form a p53-Sp1 complex, leading to the dissociation of HDAC1, histone acetylation and hence expression of p21. Zupkovitz *et al.*, 2010 suggested this to be the validation behind HDAC1 being the target for HDAC inhibitors' role as anti-cancer agents. This was proven using HDAC1$^{-/-}$ mouse embryonic fibroblasts.

Exposure of cells to the HDAC inhibitor TSA or sodium butyrate increased the transcription of p21 by inducing the acetylation of histones H4 and H3 (Fang *et al.*, 2004). Inhibition of HDAC by TSA in human keratinocytes also resulted in statistically significant decreases in proliferation and increases in acetylated p53 and p21 (LeBoeuf *et al.*, 2010). It was also observed that molecular inhibition of p21 in TSA treated cells did not affect total p53 expression, but the knockdown of p53 prevented p21 accumulation. In non-small cell lung cancer lines, TSA treatment in combination with silibinin (a flavonolignan with anti-lung cancer efficacy) dramatically increased p21 expression with increases in acetyl-H3 and –H4 on the p21 promoter at the sp1/3 binding site (Mateen *et al.*, 2012). Another

HDAC inhibitor, depsipeptide, also induced p21 expression by p53 acetylation, allowing recruitment of p300 to the p53-responsive p21 promoter (Zhao *et al.*, 2006). p300 transcriptionally activates p21 by acetylating histones/nucleosomes at proximal and distal p53 binding sites of the p21 promoter. Transcriptional coactivator p300 requires bound p53 for this activation (Espinosa *et al.*, 2001), hence classifying this regulation as an indirect p53-dependent mechanism. The monocytic leukemia zinc finger, another HAT, interacts with p53 and acetylates histones on the p21 promoter to induce its transcription (Rokudai *et al.*, 2009). Another study shows an increase in histone acetylation in p53 induced p21 transactivation upon DNA damage by UVC (Espinosa *et al.*, 2003).

Various studies including our own reported similar upregulation of p21 by HDAC inhibitors like TSA (Scholpa *et al.*, 2014), azelaic bishydroxamic acid (Burgess *et al.*, 2001), n-butyrate (Dagtas *et al.*, 2009), suberoylanilide hydroxamic acid (Richon *et al.*, 2000, Gui *et al.*, 2004) and statins (Lin *et al.*, 2008). Further review revealed similar mechanisms of p21 regulation (Delcuve *et al.*, 2012). Induction of p21 by histone hyperacetylation may be the mechanism for dietary prevention of carcinogenesis (Archer *et al.*, 1999).

### 5.2. Ecological Risk Assessment

Ecological risk assessment evaluates the likelihood that the environment or the ecosystem is impacted by a stressor. The US-EPA performs environmental risk assessment in three phases that includes problem formulation to identify the components of the ecosystem at risk, analysis of the degree of exposure, and risk characterization to estimate exposure effects and describe the risk results (US-EPA, 2017a). Though the concerns for protection of the wild populations and ecosystems are of high importance in

the National Environmental Policy Act (NEPA), ecological risk assessments are typically anthropocentric (NEPA, 2007, Simon, 2014). Societal consent plays a key role in the assessment and adds a human perspective in protecting the ecosystems and exposures of value. For instance, $BrO_3^-$ is a water pollutant and does effect aquatic organisms, but was regulated based on probable toxicity to humans (Hutchinson *et al.*, 1997). A group of water contaminants that is often in the spotlight for both human health aspects and ecological significance are endocrine disrupting chemicals (EDCs).

### 5.2.1. Endocrine-disrupting Chemicals

EDCs are substances that interfere with the function of the endocrine system resulting in deviation from normal homeostasis and hence causing adverse health effects (Colborn *et al.*, 1993). This deviation rises from mimicking naturally occurring hormones like estrogens, androgens and thyroid hormones. These substances are prevalent in our food, environment, everyday consumer products and compounds like pharmaceuticals, pesticides, flame-retardants and plasticizers (Gore, 2007). Disruption of endocrine homeostasis leads to physiological impairments including decreased fertility, feminization/masculinization, cancer formations and developmental disorders (Parrott *et al.*, 2005, Lange *et al.*, 2001, Ramamoorthy *et al.*, 1997, Colborn *et al.*, 1993). In 1996 the US-EPA commissioned the endocrine disruptor screening program (EDSP) to make regulatory decisions about endocrine effects of environmental pollutants (US-EPA, 2017b, Harding *et al.*, 2006). It is a two-tiered approach for identifying endocrine-related adverse effects and determining dose-response for better screening of potential EDCs.

There has been a bloom in epigenetic data on endocrine disruption in recent years. The well-known pesticide permethrin and insect repellent N, N-diethyl-meta-toluamide

(DEET) have been shown to decrease DNA methylation and cause epimutations or differentially methylated regions in over 300 sperm-related gene promoters in rats leading to early onset puberty (Manikkam *et al.*, 2012). BPA was shown to induce apoptosis of reproductive follicles in zebrafish via aberrant histone trimethylation (Santangeli *et al.*, 2016). The phytoestrogen genistein and fungicide vinclozolin reduced the insecticide sensitivity transgenerationally in tiger mosquitos by general hypomethylation and hypermethylation, respectively (Oppold *et al.*, 2015).

One irrefutable compound with eminent ecological effects and excessive human usage is 17α-ethynylestradiol (EE2), an orally active synthetic estradiol used in contraceptives as well as estrogen replacement therapy. EE2 is found in the ng/L-range in effluent waters from sewage treatment plants in the USA and other countries (Huang *et al.*, 2001, Larsson, 1999). EE2 along with other estrogens in the treated effluent waters are shown to have feminizing effects in male fish (Purdom, 2006, Adeel *et al.*, 2017, Sumpter *et al.*, 1995). In zebrafish, a concentration of EE2 as low as 3 ng/L had been shown to arrest male gonad development (Fenske *et al.*, 2005).

### 5.2.2. Vitellogenin: a Biomarker for Estrogenic Exposure

Vitellogenin (Vtg) is an egg yolk precursor protein expressed in livers of all female oviparous vertebrates (Wahli *et al.*, 1981). Vitellogenesis is a process where Vtg is synthesized in the liver, secreted to plasma and carried via the blood stream for oocyte uptake. Expression of Vtg in the liver is controlled by the binding of estrogen receptors to estrogen response elements (Green *et al.*, 1987). The natural estrogen 17β-estradiol (E2) is the trigger for normal Vtg expression. Once diffused into the cells, estrogen binds to the estrogen receptor (ER) in the cytosol or nucleus and this complex binds to specific

palindromic sequences called the estrogen response elements (ERE) in the promoters of estrogen responsive genes like the ones coding for Vtg (Gruber *et al.*, 2004). Two main ERs characterized in mammals, birds and fish are ERα and β, and the three subtypes in zebrafish are ERα, β1 and β2 (Menuet *et al.*, 2002).

Estrogenic EDCs can elevate Vtg levels unnaturally by mimicking the natural ligand E2. Male and juvenile fish normally do not produce Vtg or at least at nominal levels. Hence Vtg in male fish has been predominantly used as a molecular marker of exposure to estrogenic EDCs in ecological testing. Therefore, it is popularly studied as a marker for feminization in fish (Sumpter *et al.*, 1995). Surprisingly, the mechanism behind Vtg induction is yet to be fully understood in spite of it being such a frequently used biomarker.

Zebrafish (*Danio rerio*) are a genetically well-characterized and popularly used model organism in ecotoxicology, especially in endocrine disruption studies. Vtg is heterogenous (types I, II and III) and is coded by *vtg1-7* genes with *vtgs 1,2, 4, 5, 6* and *7* on chromosome 22 and *vtg3* on chromosome 11 in zebrafish (Wang *et al.*, 2005). *vtg1* is the most highly expressed mRNA (Wang *et al.*, 2005). *vtg1* gene is normally silent in male zebrafish liver and is known to be over-expressed after exposure to estrogenic EDCs (Meng *et al.*, 2010, Hoffmann *et al.*, 2001, Versonnen *et al.*, 2004).

Though the epigenetic landscape of zebrafish is broadly studied to assess human diseases like diabetes mellitus (Sarras *et al.*, 2015), cancer (Mudbhary *et al.*, 2011), muscular dystrophies (Berger *et al.*, 2012), mercury-induced neurotoxicity (Carvan *et al.*, 2017), it is less investigated for ecotoxicological applications. It has been reported as early as 1982 that estrogen induces demethylation of the *vtg* gene in chicken (Wilks *et al.*, 1982). However, the only other report in ecotoxicology, to our knowledge, is the study

by Stromqvist *et al.*, 2010 showing estrogen-induced promoter demethylation of *vtg1* in zebrafish. This implies a gap-in-knowledge and a huge gap-in-literature in the field.

## 6. Summary and Conclusion

Understanding the role of epigenetic mechanisms in toxicant-induced alterations of gene expression is key to estimating the risk of adverse and long-term effects of several toxicants. Epigenetic marks tend to accumulate and might not lead to adverse effects instantaneously. Hence, it is important to investigate environmentally relevant concentrations of toxicants at the sub-chronic and chronic levels of exposure for studying epigenetic endpoints. Epigenetic changes can be stable and transgenerationally inherited or be transient. Hence, it is important to follow epigenetic changes after withdrawal of the stressor. These concepts hold true for both human health and ecological risk assessment. Understanding the specific epigenetic alterations induced by environmentally eminent pollutants like DBPs and EDCs would fill the gaps-in-knowledge about their mechanisms of action and would aid on how to incorporate these data into their risk assessment.

## 7. Hypothesis and Specific Aims

Based on the above discussed literature, this study tested the hypothesis that $BrO_3^-$ induced alterations in p21 expression via epigenetic mechanisms in human and rat renal cells. We also tested the hypothesis that EE2 induced Vtg expression in male zebrafish via promoter hypomethylation. The proposed specific aims to test this hypothesis are as follows:

**7.1. Specific Aim 1:** Test the hypothesis that $BrO_3^-$ alters p21 expression via alterations in DNA methylation of the promoter and/or coding regions. Aim 1 determines the sub-

chronic effects of environmentally relevant concentrations of $BrO_3^-$ on DNA methylation of the p21 promoter and coding regions in human and rat renal cells.

**7.2. Specific Aim 2**: Test the hypothesis that $BrO_3^-$ alters p21 expression via alterations in histone acetylation of the promoter region and that these alterations correlate to changes in DNA methylation. Aim 2 also determines the sub-chronic effects of environmentally relevant concentrations of $BrO_3^-$ on histone acetylation of p21 promoter in human and rat renal cells.

**7.3. Specific Aim 3:** Test the hypothesis that EE2 induced Vtg expression in male zebrafish is via promoter hypomethylation. Aim 3 determines the effects of EE2 on *vtg1* promoter methylation and correlates these changes to mRNA expression in adult male zebrafish livers.

Successful completion of these studies will advance our knowledge on toxicant-induced epigenetic mechanisms and their relevance in environmental risk assessment. These data would fill the gaps-in-knowledge about mechanisms of $BrO_3^-$-induced nephrotoxicity and advance the field of mechanistic toxicology. Data from the zebrafish model would prompt further investigation into epigenetic mechanisms of other contaminants of ecological relevance. This cumulative study strengthens the transfer of molecular concepts and techniques from human health perspectives to the field of ecotoxicology.

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# CHAPTER 2

# TARGETED GENE BISULFITE SEQUENCING IDENTIFIES CHANGES IN p21 METHYLATION BY THE NEPHROTOXICANT BROMATE[*]

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**Abstract**

This study developed a targeted next-generation bisulfite sequencing approach using an Illumina MiSeq platform and Bismark bisulfite mapper called targeted gene bisulfite sequencing (TGBS) to assess site specific changes in the methylation of the nephro-protective gene p21 (CDKN1a) after exposure to nephrotoxicants. 5-aza-2'-deoxycytidine (5-Aza), a DNA methyltransferase inhibitor, was used as a positive control, and differences between human and rat p21 methylation were also determined. TGBS analysis of human embryonic kidney (HEK293) and freshly isolated human proximal tubular cells (hPT) demonstrated a methylation sensitive site in the p21 promoter region called sis-inducible element-1 (SIE-1), which regulates p21 expression via the binding of transcription factors. 5-Aza treatment altered the methylation of this site, but no change in methylation was seen when cells were exposed to the nephrotoxicants cisplatin or bromate ($BrO_3^-$), even though these toxicants increased p21 protein expression. A SIE-1 site was not identified in normal rat kidney cells (NRK) suggesting species variation in p21 regulation. Further, cisplatin and $BrO_3^-$ did not decrease DNA methylation in the promoter site in either HEK293 or NRK cells. These data demonstrate the novel finding that the human and rat p21 promoter sequence differs in their basal DNA methylation. Further, these data show that changes in p21 expression induced by nephrotoxicants do not correlate to changes in DNA methylation in the promoter region. These data also show the utility of a novel method (TGBS) for rapid analysis of DNA methylation of specific loci.

## 1. Introduction

DNA methylation can add methyl groups to the 5-carbon position of cytosine residues in the CpG dinucleotide context in multicellular eukaryotes. Being an epigenetic mark, the process doesn't alter the core DNA sequence; however, depending on the location, it can regulate gene expression (Siegfried *et al.*, 2010). In general, promoter hypermethylation downregulates or silences gene expression (Suzuki *et al.*, 2008). Aberrant DNA methylation patterns in many genes have also been identified and correlated to the phenotypes of many cancers (Fang *et al.*, 2004, Teramen *et al.*, 2011, Das *et al.*, 2004, Baylin *et al.*, 2006). One such gene is p21 (CDKN1a), which is a cyclin-dependent kinase (CDK) inhibitor observed to be silenced by aberrant DNA methylation in many cancers including metastatic prostate cancer, lung cancer and lymphomas (Bott *et al.*, 2005, Teramen *et al.*, 2011, Moreira *et al.*, 2009, Ying *et al.*, 2004).

Despite the significant amount of knowledge about DNA methylation of p21 in cancer cells, less is known about how p21 methylation mediates the toxicity of chemical agents in non-cancer cells. Even less is known on how DNA methylation of p21 alters the mechanisms of action of nephrotoxicants. Such information is important because p21 protein expression is increased in response to many renal acting agents, including the chemotherapeutic cisplatin and the water disinfection byproduct (DBP) bromate ($BrO_3^-$) (Scholpa *et al.*, 2014, 2016). Increases in p21 expression in the kidney are believed to be protective (Price *et al.*, 2009, di Pietro *et al.*, 2012), and may occur independently of the prominent gene regulator p53 (Kolisetty *et al.*, 2013a, 2013b, Scholpa *et al.*, 2014). Indeed, studies suggest that induction of p53 in tandem with p21 is more correlative to cell death, as opposed to protection. This hypothesis is supported by our own studies

(Scholpa *et al.*, 2014), which suggested that increases in p21 expression induced by low environmentally relevant doses of $BrO_3^-$ were protective and occurred in the absence of p53 expression. In contrast, increases in p21 expression at higher, toxic doses correlated to expression of p53 and cell death. Thus, understanding the alternative molecular mechanism mediating p21 expression is critical to our understanding of the cyto-protective role of this protein.

Our previous work used cDNA arrays and immunoblot analysis to show that $BrO_3^-$ induced p21 (Zhang *et al.*, 2010, 2011). We subsequently showed that p53-independent expression of p21 correlated to changes in DNA methylation in the coding region of this gene. This suggested a role for epigenetics in the regulation of p21. However, changes in the methylation of the promoter region of this gene were not studied. Further, these changes were determined using methylation-specific PCR that only assesses methylation at the primer binding sites. While techniques exist for analysis of site-specific DNA methylation changes, such as methylation-specific PCR followed by cloning and sanger sequencing, such techniques are laborious, time-intensive and some have lower degrees of accuracy (Ku *et al.*, 2011, Zhu *et al.*, 2003).

Large –omic based approaches do yield excellent data on DNA methylation, but tend to be hypothesis generating data describing hundreds, if not thousands of genes. Because we, and others, have already used omic-based approaches to identify p21 as target gene for renal protection against $BrO_3^-$ (Kolisetty *et al.*, 2013b), and because we already published data suggesting roles for DNA methylation in regulation of p21 (Scholpa *et al.*, 2014), we developed and validated a rapid and robust approach to assess gene targeted DNA methylation. This approach, termed targeted gene bisulfite

sequencing (TGBS) was designed to identify changes in methylation of targeted portions of genes and study the effects of toxicant exposure on this methylation.

## 2. Materials and Methods

### 2.1. Materials

Human embryonic kidney (HEK293) and normal rat kidney (NRK) cells and penicillin and streptomycin were purchased from American Type Culture Collection (Manassas, VA). The freshly isolated human proximal tubule (hPT) cells were generously provided by Dr. Lawrence H. Lash at the Wayne State University (Detroit, MI). Potassium bromate ($KBrO_3$), 5-aza-2'-deoxycytidine (5-Aza) and trypsin EDTA were purchased from Sigma-Aldrich (St. Louis, MO), DMEM media from HyClone technologies (Logan, UT), 5-Aza was dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, PA), DNeasy blood and tissue extraction kit were purchased from Qiagen (Valencia, CA). The EZ-DNA methylation lightning kit and the Zyppy plasmid miniprep kits were purchased from Zymo research (Irvine, CA). Nucleospin gel and PCR clean-up kits were purchased from Macherey-Nagel (Düren, Germany). The MiSeq reagent v3 kit was purchased from Illumina Inc (San Diego, CA), the Strataclone PCR cloning kit from Agilent technologies (Santa Clara, CA), the Kapa HiFi PCR kit from Kapa Biosystems (Wilmington, MA), and the Maxima hot-start Taq polymerase and Sera-Mag magnetic speedbeads were purchased from Thermo Scientific (Waltham, MA).

### 2.2. Cell culture and treatment

5-aza-2'-deoxycytidine (5-Aza) is a DNA methyltransferase inhibitor (Christman, 2002) and is used in many studies for its demethylating properties (Broday *et al.*, 1999, Bott *et al.*, 2005, Shin *et al.*, 2000). HEK293 cells ($3 \times 10^6$) were seeded in T-175 tissue

culture flasks and grown at $37^0$C in a 5% $CO_2$ incubator for 24 hrs. Cells were then treated with 0-100 ppm bromate ($BrO_3^-$), 40 µM 5-Aza, DMSO (vehicle control for 5-Aza) or 1 µM cisplatin for 72 hrs. The total amount of DMSO was never above 0.5% of the total volume per flask. The rationale for these doses are explained in our recent studies (Zhang *et al.*, 2010, 2011 and Scholpa *et al.*, 2014, 2016). Cells were released from the plate following treatment using trypsin/EDTA and $5x10^6$ cells were collected for DNA isolation.

### 2.3. DNA extraction and bisulfite conversion

Cells ($5x10^6$) were pelleted at 1000 rpm for 5 min and the supernatant was discarded. Genomic DNA was extracted using the Qiagen's DNeasy blood and tissue kit following the manufacturer's protocol. DNA was eluted in two successive steps to obtain a maximum yield, using 120 µl followed by 40 µl of elusion buffer. Following quantification using a Nanodrop spectrophotometer, 2 µg of the extracted DNA was bisulfite treated using the Zymo Research's EZ-DNA methylation lightning kit following the manufacturer's protocol, and re-quantified.

### 2.4. Target amplification and purification

Bisulfite converted DNA (350 ng) was used to amplify different regions of the p21 promoter. The locus specific primers were designed using Methprimer (Li *et al.*, 2002) and were synthesized by Integrated DNA Technologies Inc. (IDT, Coralville, IA). Partial TruSeqHT sequences corresponding to part of the Illumina Read1 (R1) and Illumina Read2 (R2) sequencing primer-binding sites (Faircloth *et al.*, 2012, Glenn *et al.*, 2016, BadDNA, 2017) were added 5' to the locus specific primers during primer synthesis. The locus specific primers and the partial TruSeqHT sequences are as given in **Table 2.1**. Fusion primers were synthesized by IDT where R1 was fused to forward primers and R2

was fused to reverse primers. For example, the primer pair for hp21-TSS site was, forward: “iTru R1 + hp21-TSS F” (ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATAGTGTTGTGTTTTTTTGGAGAGTG) and reverse: “iTru R2 + hp21-TSS R” (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACAACTACTCACACCTCAACTA AC).

The first locus amplified was a 350 bp fragment of the human p21 promoter region adjacent to the transcription start site (TSS) termed as hp21-TSS. The second locus was a 335 bp fragment including the transcription factor binding site approximately 700 bp upstream of the TSS called the sis-inducible element (SIE-1) termed hp21-SIE1. The third site was the 250 bp fragment of the rat p21 promoter region near the TSS termed as rp21-TSS, and the fourth locus was the rat p21 coding region approximately 9 Kb downstream of the TSS, termed rp21-coding (**Figure 2.1**).

The 25 µl PCR amplification reaction mix contained 3 mM $MgCl_2$, 1X hot start buffer (Thermo Scientific), 0.2 mM of each deoxynucleoside 5’-triphosphate (dNTP), 0.4 µM each of the forward and reverse primers, 1.5 units HotStart Taq DNA polymerase (Thermo Scientific) and the 350 ng DNA template. PCR was performed under the following conditions: $95^0$C for 5 min, 40 cycles of $95^0$C for 30 sec, $61.8^0$C (hp21-TSS, hp21-SIE1) or $54.2^0$C (rp21-TSS or rp21-coding) for 45 sec followed by, $72^0$C for 45 sec and a final $72^0$C for 10 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel and visualized with ethidium bromide under a UV trans-illuminator and the amplicons corresponding to the loci were extracted from the gel using Nucleospin gel and PCR clean-up kit (Macherey-Nagel) following the manufacturer’s instructions. The sequences of the purified PCR products were confirmed using Sanger

sequencing at the Georgia Genomics Facility (GGF) at the University of Georgia. All sequences obtained were verified for locus-specificity using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

### 2.5. Sanger sequencing of bacterial cloes

StrataClone PCR cloning kits (Agilent) were used for Sanger bisulfite sequencing. Briefly, 50 ng of gel extracted PCR products were cloned into *Escherichia coli* (*E. coli*) following the manufacturer's instructions. After cloning and plating, about 3 bacterial colonies (white or light blue) were picked and suspension cultures were prepared for plasmid minipreps. Plasmids from the cultures were isolated using Zyppy plasmid miniprep kit (Zymo Research) following the manufacturer's instructions. The plasmid inserts were sequenced at the GGF by Sanger sequencing. The sequences were analyzed using BiQ Analyzer DNA methylation analysis software following software instructions (Bock *et al.*, 2005).

### 2.6. Library preparation and next-generation sequencing

Purified PCR amplicons from agarose gel extraction were normalized to 5 ng/µl. A limited cycle PCR was performed to attach the iTru5 and iTru7 primers with eight nucleotide indexes. The 25 µl limited cycle reaction contained 1X Kapa buffer, 0.3 mM of each dNTP, 0.3 µM of each primer, 25 ng template DNA and 0.5 U of HiFi hotstart DNA polymerase (Kapa Biosciences). The reaction conditions were: $98^0$C for 5 min, 11 cycles of $98^0$C for 15 sec, $60^0$C for 30 sec and $72^0$C for 30 sec and a final $72^0$C for 1 min. Aliquots (10 µl) from each reaction were pooled together and cleaned up using Thermo Scientific's Sera-Mag magnetic speedbeads. An equal ratio of speedbeads to the sample pool were vortexed and placed on the magnet and incubated at room temperature for 10 min. Once

the beads were drawn to the magnet, the supernatant was discarded. The beads were washed with 80% ethyl alcohol twice and the residual liquid was removed by absorption using a wooden toothpick. DNA was then eluted using TLE buffer (10 mM Tris pH 8 & 0.1 mM EDTA) and supernatant collected. The pooled and cleaned sample was then processed for sequencing on an Illumina MiSeq platform as described by Glenn *et al* 2016 using the Illumina's MiSeq 600 cycle v3 kit.

### 2.7. Sequence analysis

#### 2.7.1. Read quality and trimming

The paired end 250-350 bp reads obtained from Illumina MiSeq were demultiplexed using Illumina software bcl2fastq (bcl2fastq2, 2013). The sequence reads in fastq format were trimmed for better alignment using Babraham Bioinformatics' free software Trim Galore (TrimGalore, 2012) or Geneious (Geneious, 2017, Kearse *et al.*, 2012). However, since the sequencing templates included mostly the uniformly sized PCR products, trimming did not affect read alignment (data not shown).

#### 2.7.2. DNA methylation analysis using Bismark

Bismark bisulfite mapper is a Linux based free software from Babraham Bioinformatics Institute (Krueger *et al.*, 2011). Methylation analysis using Bismark was carried out in three steps listed below.

##### 2.7.2.1. Genome preparation

A reference genome was prepared where an NCBI genome sequence for the target locus (p21 promoter or coding region) was downloaded as a fasta file. The reference genome was prepared using the following command from the software guide:

“/bismark/bismark_genome_preparation --path_to_bowtie /usr/bin/bowtie2/ --verbose /data/genomes/homo_sapiens/GRCh37/”. For example: “bismark_genome_preparation --/home/user/DNA/bowtie2-2.3.0/ --verbose /home/user/DNA/bowtie2-2.3.0/bismark_v0.17.0/REF/”, where the reference fasta file was saved in a directory or folder REF in the home folder of the user within the bismark folder. This created two folders within the genome folder REF, one with C ->T genome index and another with G ->A for the reverse reads.

#### 2.7.2.2. Read alignment

The second step was running Bismark using the command from the guide: “bismark –bowtie2 -n 1 -l 50 /data/genomes/homo_sapiens/GRCh37/ test_dataset.fastq”. For example, read alignment for sequences in the folder named SampleSeq_R1 with a single-end approach was performed using: “bismark --bowtie2 /home/user/DNA/bowtie2-2.3.0/bismark_v0.17.0/REF/ SampleSeq_R1.fastq.gz”. This aligned the sequence reads to the reference genome and created a combined alignment or methylation call output in a binary representation of sequence alignment map called BAM format, and yielded a run statistics report. Output files included a bam file and report.txt file (**Appendix Datafile 1**). The BAM file can only be opened in Bismark.

#### 2.7.2.3. Methylation extraction

The third and final step was methylation extraction of the bam file generated in the second step. The command used was “bismark_methylation_extractor --gzip test_dataset.fastq_bismark.bam”. For example: “bismark_methylation_extractor -s –comprehensive SampleSeq_R1.fastq_bismark_bt2.bam”. This generated output files that included M-bias.txt file, M-bias_R1.png file, CpG (**Appendix Datafile 2-4**), CHH and CHG

context bt2.txt files, which contain information on strand specific methylation. The key information on CpG site specific percent methylation was obtained from the M-bias.txt file (**Appendix Datafile 2**). The targeted bisulfite sequencing with short products allowed for manual extraction of methylation values for comparison across samples and treatments. A processing report was generated using the command “bismark2report” that summarized the process with a read alignment chart, methylation extraction report and an M-bias plot.

#### 2.7.2.4. VirtualBox with ready-to-run Bismark package

VirtualBox is an open source software that runs on various operating systems and supports various guest operating systems (VirtualBox, 2017). The path to download a ready-to-run VirtualBox package containing all the tools and installations required for DNA methylation analysis of a given fastq sequence file is indicated below. The package includes step-by-step instructions for running Bismark, which is a Linux software, in a VirtualBox on Windows host system. This can be found at “https://drive.google.com/file/d/0B8YCoq3MYnhKN3RJMllQcUh3Y0k/view?usp=sharing” for the VirtualMachine named “TGBS” that can be accessed with the username “user” and password “TGBSKolli”. The instructions are detailed in the **Appendix**.

### 2.8. Statistics

Samples isolated from a distinct cell passage represented one experiment (n=1). Data are represented as mean ± SEM (standard error of the mean) from at least three separate experiments (n=3). An unpaired Student’s t-test was used to compare two groups using Graphpad PRISM considering $p<0.05$ indicative of a statistically significant difference between the mean values.

## 3. Results

### 3.1. Sanger's vs next-generation bisulfite sequencing

NRK cells were treated with acutely toxic concentrations of the nephrotoxicant $BrO_3^-$, 5-Aza as a positive control, or its vehicle control DMSO for 72 hrs as previously described (Scholpa *et al.*, 2014). The extracted DNA was subjected to bisulfite conversion and the rp21-coding region was amplified using the primers described in **Table 2.1**. The PCR products were separated by electrophoresis and then gel purified. The 350 bp purified products were cloned into competent bacteria for further target enrichment. Plasmids from three clones per treatment were individually sequenced by the Sanger's tube method. The sequences obtained were either manually aligned, or aligned using BiQ Analyzer software (**Figure 2.2A**). In general, at least one out of three clones showed different methylation pattern leading to inconsistency and resulting in need for more clones to reach an acceptable power of analysis, irrespective of the treatment. Typically, this would require an average of 8-10 clones per treatment group.

To address the inconsistency of Sanger's Sequencing, we sequenced the same rp21-coding region on the Illumina MiSeq next-generation sequencing (NGS) platform using TGBS. The gel purified 350 bp products were subjected to limited cycle PCR to attach the iTru5 and iTru7 primers with unique index combinations for each sample. All the samples were pooled, purified and then sequenced using the 600 cycle v3 kits. An average of about 10,000 reads were obtained per sample, hence increasing the statistical power of analysis (Morrill *et al.*, 2013) as compared to the Sanger's sequencing. The data from Bismark's text file outputs were compiled together and the percent DNA methylation changes were displayed using heat-maps (**Figure 2.2B**). The methylation status of this

coding region did not change either with the $BrO_3^-$, cisplatin or the 5-Aza treatment, suggesting that the coding region is not one of the key components in epigenetic regulation of p21 expression. Nevertheless, in addition to being derived from about 10,000 reads per sample, the data showed consistency across three independent experiments.

### 3.2. Differential methylation analysis

#### 3.2.1. Difference in basal DNA methylation of the p21 promoter region between human and rat kidney cells

We used TGBS to assess differences in basal DNA methylation between human and rat p21 promoters isolated from HEK293 and NRK cells. This included analysis of a 350 bp upstream fragment of the human p21 promoter adjacent to the transcription start site (hp21-TSS) and a 250 bp upstream fragment of the rat p21 promoter near the transcription start site (rp21-TSS). The data showed differences in methylation between the two cell lines with an average of 16.4% at the rp21-TSS site and 0.8% at the hp21-TSS site (**Figure 2.3A-B**), suggesting species-dependent differences in basal methylation of the p21 promoter region at the TSS.

#### 3.2.2. Regional differences in basal DNA methylation of p21

A differential methylation analysis was performed on p21 DNA isolated from untreated cells. The regions analyzed are as shown in **Figure 2.1**. Across the two species, we observed differential methylation across these regions with an average of 0.8% at the hp21-TSS, 57.9% at the transcription factor binding site (SIE-1), 16.1% at the rat-TSS and 95.8% at the rat p21-coding region (**Figure 2.3C**).

### 3.3. Effect of 5-Aza

We used 5-Aza as a positive control to verify the ability of TGBS to detect changes in DNA methylation. The basal level of methylation in the CpG sites spanning 350 bp adjacent to the human p21 transcription start site (hp21-TSS) showed a low level of total percent methylation (0.9%) in the presence of DMSO, which was similar to the 5-Aza treated cells (**Figure 2.4A**). Total percent methylation in this context is the sum of the average methylation of all the CpG sites in the region analyzed. In contrast, treatment of cells with 5-Aza caused about a 35% decrease in total methylation of the SIE-1, as compared to DMSO treated cells (**Figure 2.4B**). This correlated to increases in the protein expression of p21 as shown in our previously published study (Scholpa *et al.*, 2014).

### 3.4. Difference in basal DNA methylation of the p21 promoter region between HEK293 cells and freshly isolated human proximal tubule cells

As we observed decreases in the percent DNA methylation at the hp21-SIE1 site after treatment with the demethylating agent 5-Aza, we wanted to investigate the differences in basal level methylation between HEK293 cells and freshly isolated human proximal tubule (hPT) cells at the TSS and sites. The average methylation of hp21-TSS in hPT cells was 1.4% and not significantly different from that in HEK293 cells **(Figure 2.5A)**. In contrast, the average methylation of all three CpG sties in hPT cells were lower than that measured in HEK293 cells at the hp21-SIE1 site **(Figure 2.5B)**.

### 3.5. Effects of nephrotoxicants on HEK293 and NRK cells

The above studies assessed the effect of acutely toxic concentrations of $BrO_3^-$. Our recent studies (Scholpa *et al.*, 2014) demonstrated changes in p21 DNA methylation in the coding region in the presence of low environmentally relevant concentrations of $BrO_3^-$ after sub-chronic exposures. These previous studies did not determine changes in methylation in the promoter region of p21 or differences between rat and human p21 methylation. We addressed this gap-in-knowledge by exposing both NRK and HEK293 cells to $BrO_3^-$ at concentrations we previously demonstrated not to induce cell death (Scholpa *et al.*, 2014). In agreement with this recent study treatment of HEK293 cells with doses of $BrO_3^-$ below 100 ppm did not significantly alter cell morphology or number after 72 hrs of treatment (**Figure 2.6A-F, L**). Cells treated with 100 ppm $BrO_3^-$ showed initial signs of cell rounding, detachment and small decreases in cell number compared to the control cells (**Figure 2.6G and L**). In contrast, cisplatin (1 µM), used a positive control, significantly altered cell morphology and cell number (**Figure 2.6K-L**). Exposure of cells to 5-Aza, as well as to the histone deacetylase inhibitor trichostatin A (TSA), also did not alter cell morphology or number compared to the vehicle control DMSO (**Figure 2.6H-J, L**). Similar results were observed in NRK cells, with the exception that concentrations of 5-Aza of 40 µM did slightly decrease the cell number (**Figure 2.7A-L**).

### 3.6. Effects of nephrotoxicants on DNA methylation

Treatment of HEK293 cells with environmentally relevant concentrations of $BrO_3^-$ for 72 hrs did not significantly alter the DNA methylation in the hp21-S1E1 site at any position assessed, which include the CpG cytosines at -691, -855 and -895 bp upstream of the TSS (**Figure 2.8**). In contrast, 5-Aza significantly decreased DNA methylation, as

compared to its DMSO control, at the CpG sites -895 and -855. We also used TGBS to assess changes in methylation in the CpG sites located in the human p21 promoter region adjacent to the transcription start site (hp21-TSS) (**Appendix Figure 2**) and did not detect any changes in methylation induced by $BrO_3^-$ or 5-Aza. It should be noted that we have shown that these same concentrations and exposure periods of $BrO_3^-$ and 5-Aza do increase the protein expression of p21 (Scholpa *et al.*, 2014).

**Table 2.1.** TruSeqHT fusion stubs and locus-specific primers. The iTru R1 fusion sequence was synthesized on the 5’ end of each of the four forward primers and the iTru R2 fusion sequence was synthesized on the 5’ end of each of the four reverse primers.

| Locus | Forward primer (5'→3') | Reverse primer (5'→3') | $T_m$ ($^0$C) | Product size ( bp) |
|---|---|---|---|---|
| TruSeqHT fusion | iTru R1: ACACTCTTTCCCTACACGACGCTCTTCCGATCT | iTru R2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT | | |
| hp21-TSS | ATAGTGTTGTGTTTTTTTGGAGAGTG | ACAACTACTCACACCTCAACTAAC | 61.8 | 350 |
| hp21-SIE1 | TTTTTTGAGTTTTAGTTTTTTTAGTAGTGT | AACCAAAATAATTTTTCAATCCC | 61.8 | 335 |
| rp21-coding | TGTAATTAGTTATAGGTATTATGTTCGA | ACCCCTACAACAAAACCGAA | 54.2 | 326 |
| rp21-TSS | TTTTTTATTTTTGGTTGTTTTTTTT | ACAAACAATTAACTCTCCTCAAATC | 54.2 | 208 |

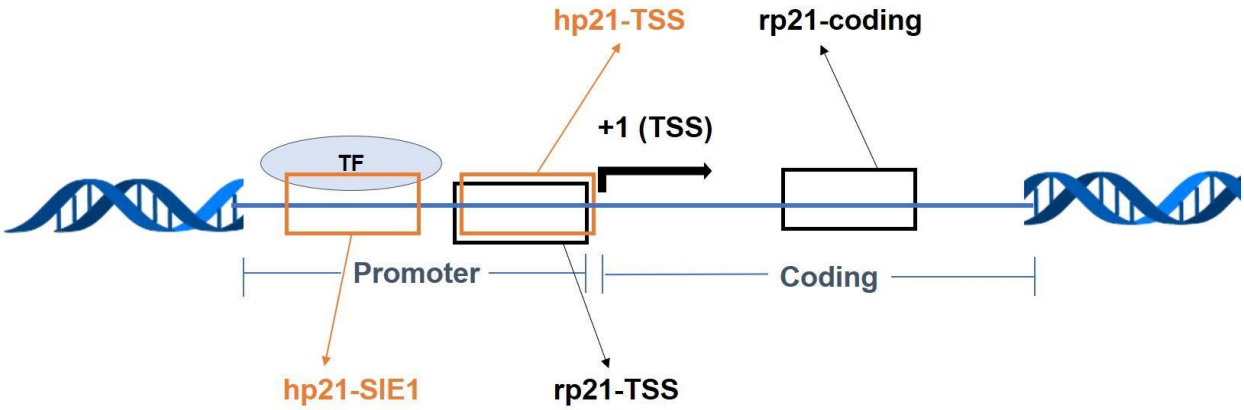


**Figure 2.1**. Schematic of p21 gene organization highlighting the loci of interest for DNA methylation analysis. This includes the human p21 promoter region adjacent to the transcription start site (hp21-TSS), the human transcription factor binding site called the sis-inducible element (hp21-SIE1), the rat p21 promoter region starting near the start site (rp21-TSS) and the rat p21 coding region (rp21-coding).

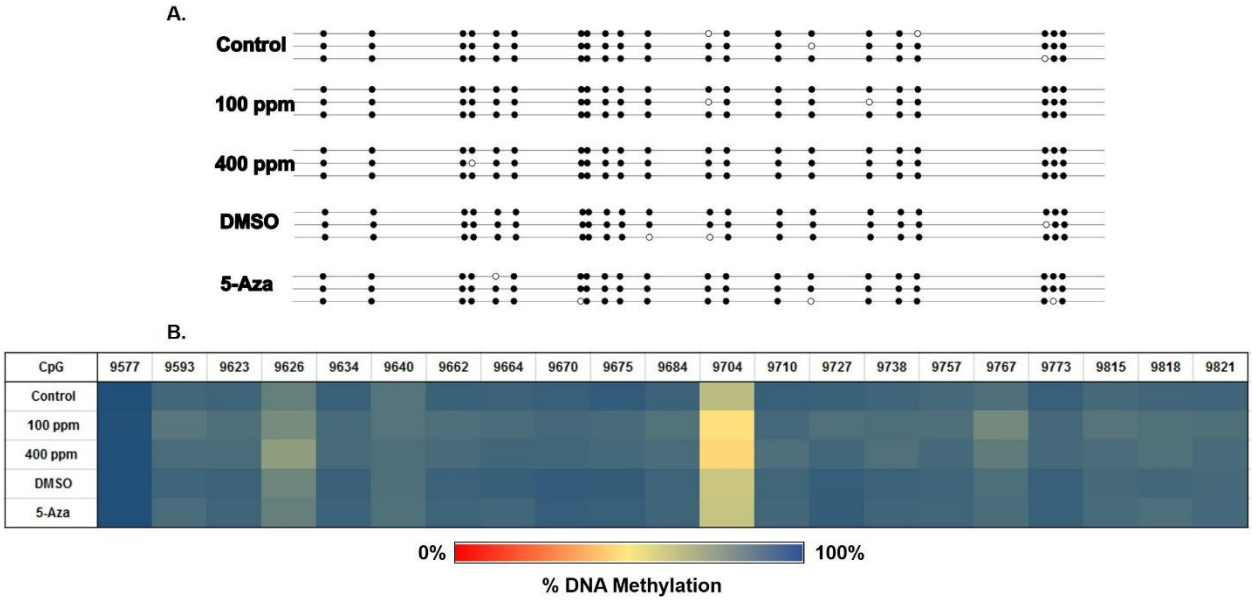


**Figure 2.2.** DNA methylation status of the rat p21 coding region. **A)** Methylation of the rat p21 coding region as determined using Sanger's bisulfite sequencing: Data are shown as a lollipop plot generated using BiQ Analyzer. Each treated group includes three random clones and each line represents sequence from a clone. Black indicates methylated CpGs and the white represents unmethylated CpGs. **B)** Methylation of the rat p21 coding region as determined by TGBS using Illumina next-generation sequencing: Data are represented as a heat-map with average DNA methylation increasing from red (0%) to blue (100%). The position indicates the CpG dinucleotide site in the sequenced fragment.

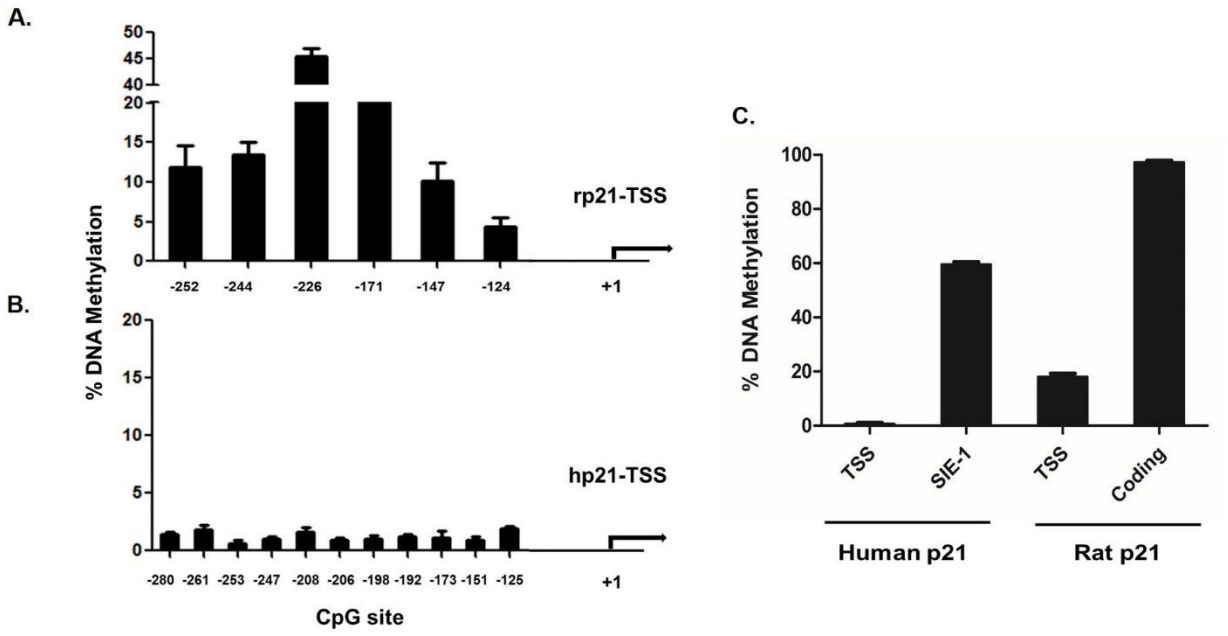


**Figure 2.3.** Differential methylation analysis: Comparison of methylation between rat (**A**) and human (**B**) p21 transcription start sites. DNA methylation data are represented as percent methylation of each CpG site in the analyzed fragments of the human and rat p21 promoter regions near the respective transcription start sites (rp21-TSS and hp21-TSS). **C)** Comparison of methylation in different regions of the rat and human p21 gene. Differential methylation data are represented as percent DNA methylation of the transcription start site (TSS), sis-inducible element (SIE-1) and gene coding regions of human and rat p21. Next-generation sequencing data was analyzed using Bismark bisulfite mapper. Data are represented as the mean ± standard error of the mean (SEM) of three independent experiments (n=3).

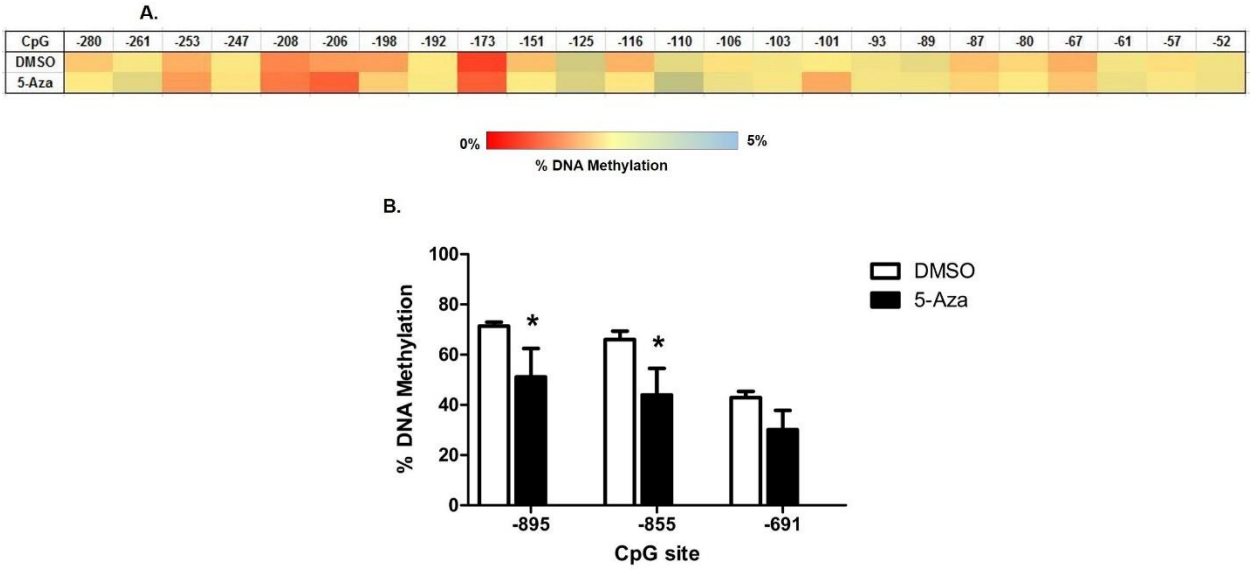


**Figure 2.4.** Effect of 5-Aza on DNA methylation of the promoter region of human p21. **A)** Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the human p21 promoter region at the transcription start site (hp21-TSS) after 3 days of exposure to DMSO (vehicle control) or 40 µM 5-Aza (positive control). The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%. **B)** Effect of 5-Aza on DNA methylation of cytosine residues in the SIE-1 site in human p21 promoter region (hp21-SIE1). Data are represented as the mean ± SEM of three independent experiments (n=3). *P<0.05 compared with 0 ppm $BrO_3^-$.

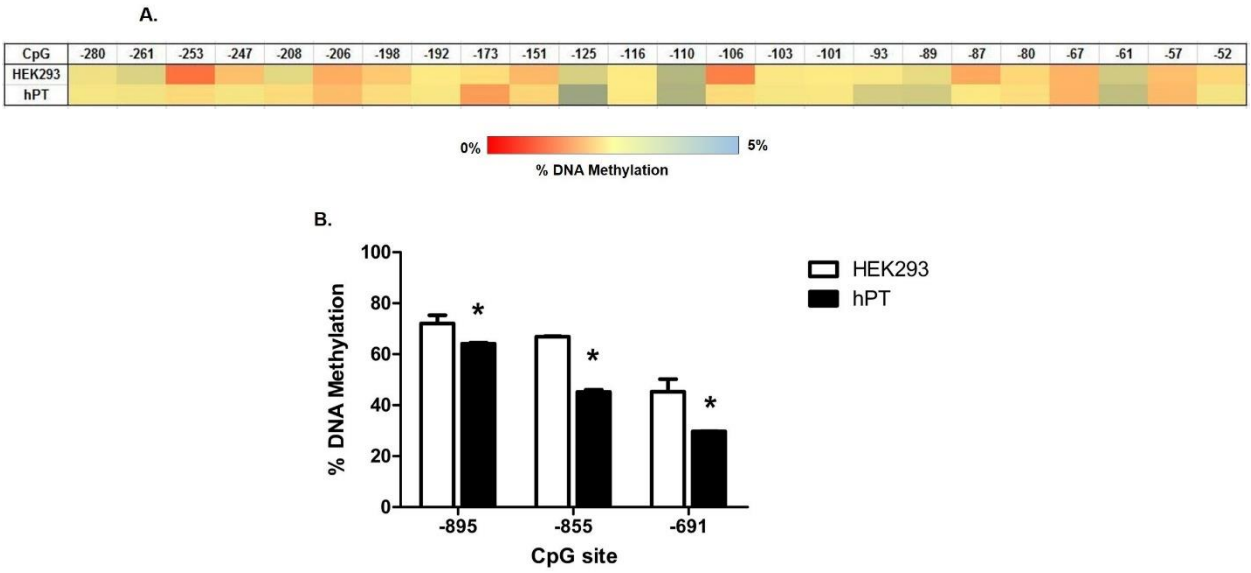


**Figure 2.5.** Comparison of basal DNA methylation of the p21 promoter region between HEK293 cells and freshly isolated human proximal tubule (hPT) cells. **(A)** Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the human p21 promoter region at the transcription start site (hp21-TSS). Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%. **(B)** Comparison of methylation of human p21 promoter at the transcription factor binding site SIE-1 between HEK293 and hPT cells. Data are represented as the mean ± SEM of three different passages of HEK293 cells and three different pools of the hPT isolated cells (n=3). *P<0.05 compared with HEK293.

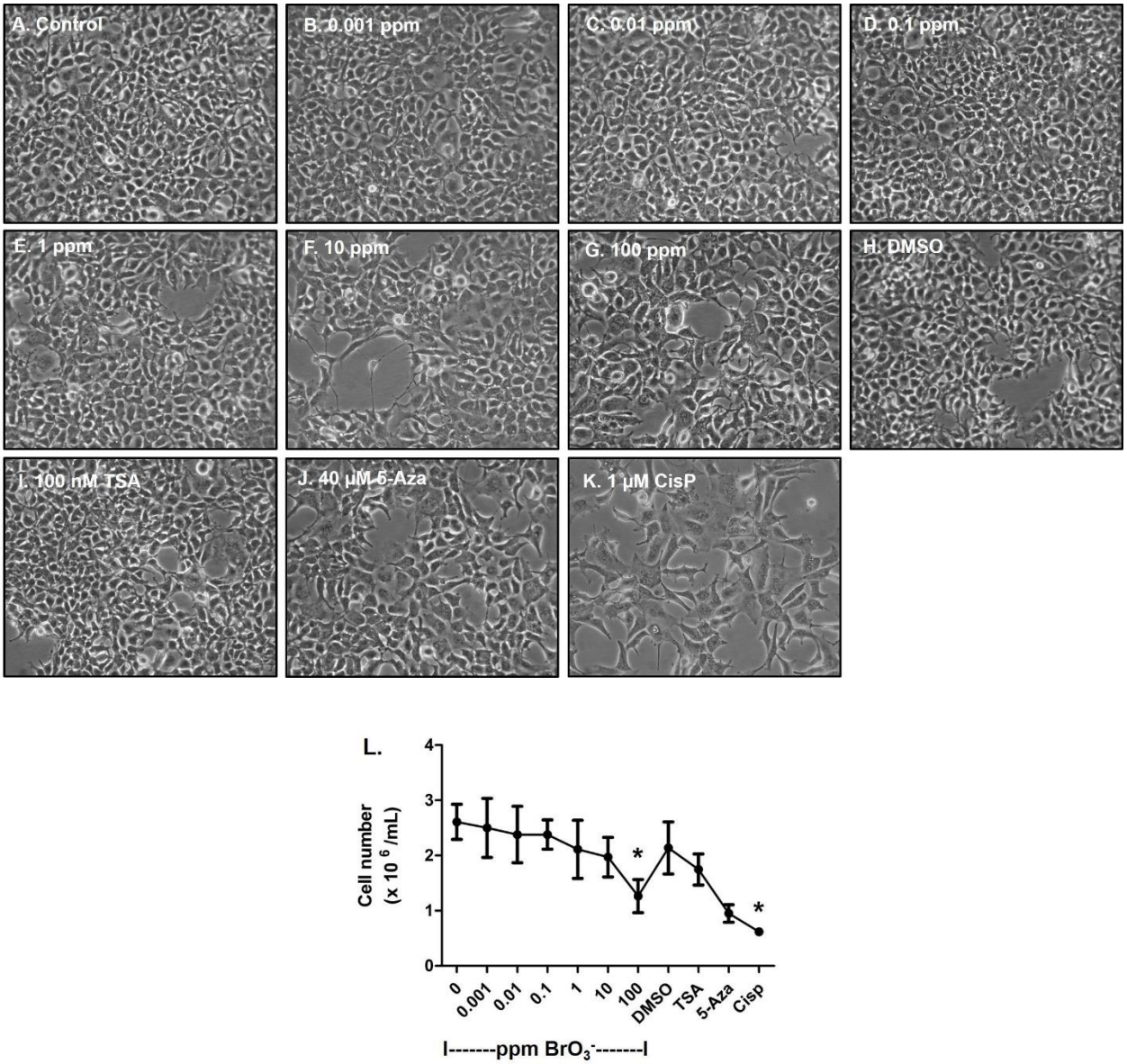


**Figure 2.6.** Effect of nephrotoxicants and epigenetic inhibitors on HEK293 cell morphology and number. HEK293 cells were exposed to 0-100 ppm $BrO_3^-$ (**A-G**), vehicle control DMSO (**H**), 40 µM 5-Aza (**I**), 100 nM TSA (**J**) or 1 µM cisplatin (**K**) for 72 hrs. The cell number data in **L** are represented as mean ± SEM of three separate passages (n=3). *P<0.05 compared with 0 ppm $BrO_3^-$.

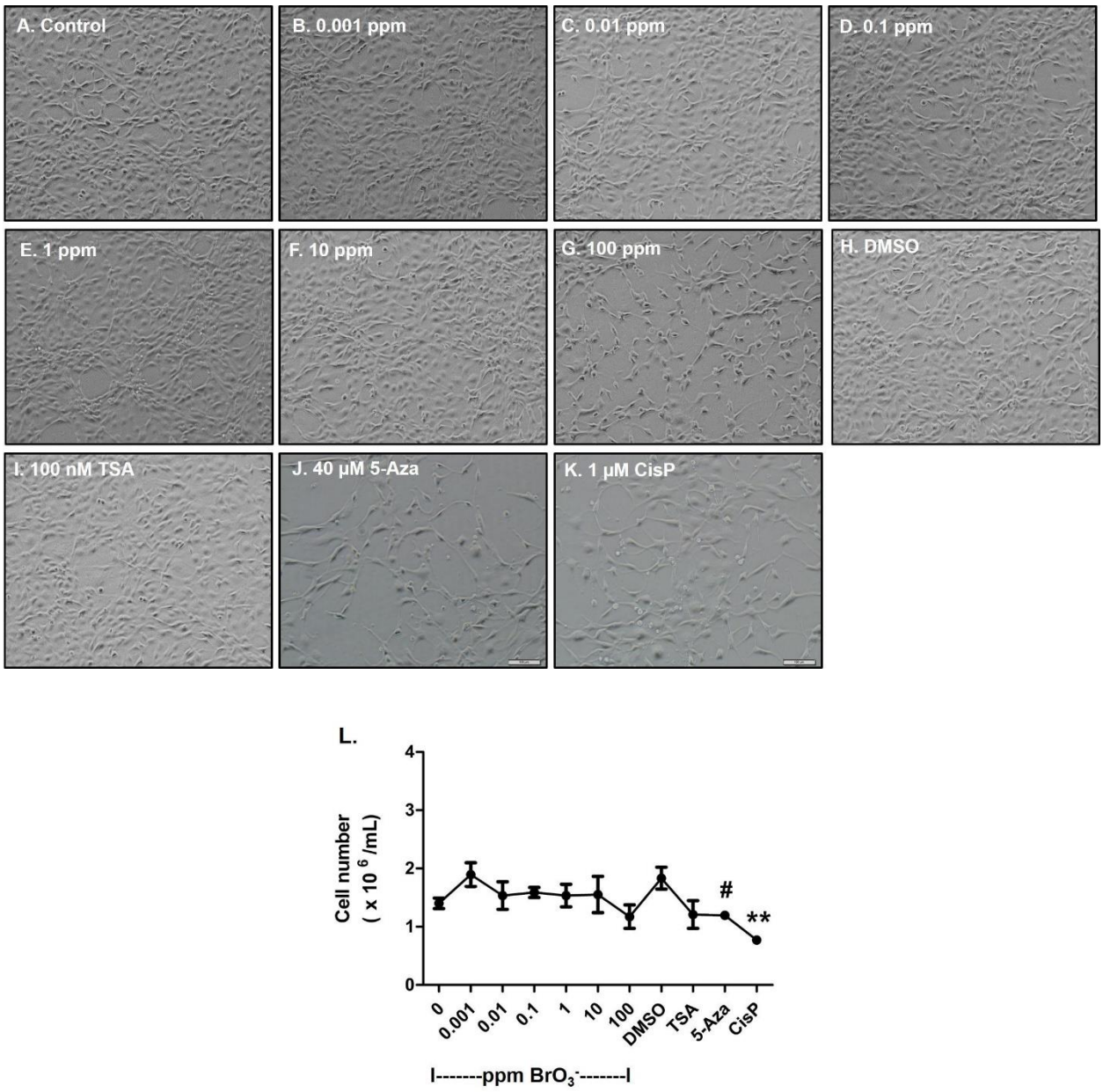


**Figure 2.7.** Effect of nephrotoxicants and epigenetic inhibitors on NRK cell morphology and number. NRK cells were exposed to 0-100 ppm $BrO_3^-$ (**A-G**), vehicle control DMSO (**H**), 40 µM 5-Aza (**I**), 100 nM TSA (**J**) and 1 µM cisplatin (**K**) for 72 hrs. The cell number data in **L** are represented as mean ± SEM of three separate passages (n=3). *P<0.05 compared with 0 ppm $BrO_3^-$ and $^{\#}$P<0.05 compared with DMSO.

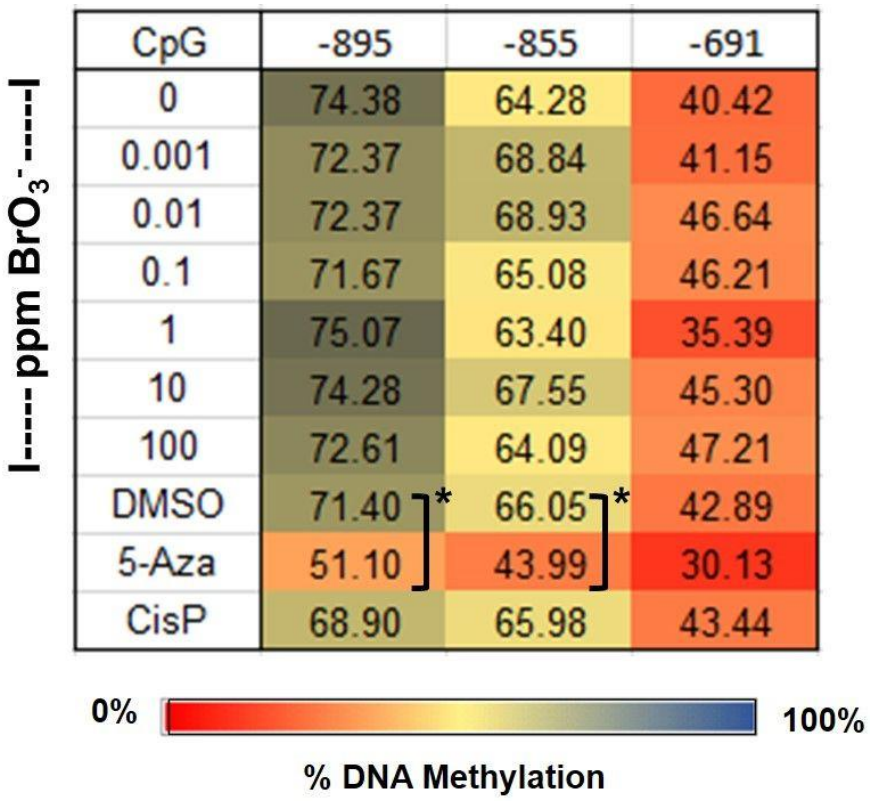


**Figure 2.8.** Effects of the nephrotoxicants $BrO_3^-$ and cisplatin on the percent DNA methylation of cytosines in the SIE-1 site in human p21 promoter. HEK293 cells were treated with water (vehicle control for $BrO_3^-$ and cisplatin), 0.001 to 100 ppm $BrO_3^-$, 1 µM cisplatin, DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for 72 hrs. The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and deep blue indicating towards 100%. Data are presented as the average percent DNA methylation of three separate passages (n=3). *P<0.05 compared with compared with DMSO.

## 4. Discussion

This study tested the hypothesis that nephrotoxicants induce the expression of the renal protective protein p21 by epigenetic mechanisms, specifically by altering the DNA methylation of CpG sites in the promoter region of this gene. A secondary goal was to determine how epigenetic regulation is different between rat and human p21. These questions are important because p21 is reported to protect against nephrotoxicity induced by numerous drugs and environmental agents, including anti-cancer drugs, such as cisplatin, and environmental oxidants such as $BrO_3^-$. Our previous studies already demonstrated that p21 expression was increased after exposure to $BrO_3^-$ and that increases in expression were altered by the DNA methyltransferase inhibitor 5-Aza (Scholpa *et al.*, 2014). The current study confirms our previous data but also identifies key regulatory sites within the p21 gene that may mediate its expression. The current study also demonstrates the novel finding that rat and human p21 differ significantly in their DNA methylation within their respective promoter regions. Identification of these differences supports the hypothesis that the basal epigenetic landscape of rat and human significantly differ, at least for p21, and suggest that caution should be used when comparing epigenetic data for this gene between the two species, and possibly even between different cell lines. This impacts several fields beyond nephrotoxicity, since p21 is also known to be involved in several cancers (Chlapek *et al.*, 2014; Gauger *et al.*, 2014; Irshad *et al.*, 2013; Mateen *et al.*, 2012; Warfel *et al.*, 2013), including renal cancers (Bull *et al.*, 2006; Moore *et al.*, 2006).

An innovation of the current study is the combination of bisulfite sequencing PCR, with Illumina sequencing and subsequent Bismark bisulfite mapping. This approach,

TGBS allowed us to assess site specific changes in p21 (CDKN1a). Such an approach requires that the gene of interest already be identified. For example, our previous studies used omic-based approaches to identify genes whose expression changed in both male and females after $BrO_3^-$ exposure, which identified roles for CDKN1a (Kolisetty *et al.*, 2013a). While we could use NGS studies to understand transcriptome changes, these would be incremental. As such, we used TGBS to identify the specific sites in p21 where these changes are occurring. TGBS has some advantages over existing approaches for identification of DNA methylation. For example, we used it to assess differences in methylation of 200-350 bp of DNA, which is considerably more than pyrosequencing, which typically can only assess 100 bp per read (Mashayekhi *et al.*, 2007). TGBS is also less expensive than methylome sequencing (hundreds per sample per gene Vs. thousands) (Glenn, 2011), can be easily adapted to any gene, and is compatible with both *in vitro* and *in vivo* models. Further, while methylome analysis using whole-genome bisulfite sequencing (WGBS) is excellent at identifying whole scale changes in gene methylation, it is not as practical for identifying the specific CpG sites targeted in specific genes.

The use of TGBS, as opposed to Sanger or pyrosequencing, provides major advantages over the other existing technologies in general (Glenn, 2011). Recent studies, including our own (Scholpa *et al.*, 2014) have used methylation-specific PCR to analyze changes in DNA methylation. This only allowed us to assess change in methylation of 2 CpG sites within the primer binding region. In contrast, the use of TGBS let us assess ~10,000 reads of each p21 locus from hundreds of samples in a single run, at costs similar to cloning and sequencing a few copies of each gene from a few samples using traditional

cloning and Sanger sequencing. The differences in the work flow between first-generation Sanger's bisulfite sequencing and next-generation TGBS is described in **Appendix Figure1**. By combining the TGBS approach with standard statistical approaches (multiple passages and samples) a more robust approach is evident. We also used the MiSeq platform which allows the longest reads possible among Illumina instruments. Targeted sequencing of 200-350 bp products also required very low depth, as low as 10,000 reads. This is significantly below the millions needed per sample for whole-genome sequencing. The low depth allowed for more rapid data analysis, and far lower costs per sample.

A limitation to TGBS is the post run analysis, which includes transforming library sequences to fully bisulfite converted forward C→T and reverse read G→A sequences. Further, a reference genome must also be obtained from GenBank, in FASTA file format, prior to aligning the sequence reads to it. The sequence reads that produce unique alignments are compared to the normal genomic sequence and the methylation state of each cytosine is inferred. The post run analysis is performed using the Bismark bisulfite mapper by the Babraham Bioinformatics Institute (Bismark, 2017; Krueger *et al.*, 2011), which is a free Linux-based software. The instructions and commands codes involved are available in **Appendix**. This information includes an explanation of the software system and step-by-step guide to DNA methylation analysis of the Illumina MiSeq-generated sequence.

The above discussed analysis was performed on four different regions of the p21 gene across two species and three cell lines. The regions include human and rat transcription start sites (hp21-TSS and rp21-TSS), the transcription factor binding site SIE1 in human p21 promoter (hp21-SIE1) and the rat p21 coding region (rp21-coding).

TGBS demonstrated minimal changes to methylation in the rp21-coding region after exposure to acutely toxic doses of $BrO_3^-$. While we previously used methylation specific PCR to demonstrate decreased methylation in this region (Scholpa *et al.*, 2014), such data was obtained after sub-chronic dosing at concentrations that did not result in cell death. The minimal changes in CpG methylation induced by $BrO_3^-$ suggest that changes in p21 protein expression induced by $BrO_3^-$ are not mediated by methylation in this region.

Assessment of methylation in the TSS of p21 isolated from HEK293 and hPT cells showed a very low percent total methylation of all the CpG cytosine's (~0.9%). In general, promoter regions near the start sites are rich in CpG islands (GC% > 50), which are known to be typically unmethylated in normal cells for active transcription to persist (Cross *et al.*, 1995). The low level of methylation detected in the human p21 promoter region most likely explains the relative infectiveness of 5-Aza, as a two-fold change would only result in decrease of 0.45%. To address this, we also assessed methylation upstream of the TSS at a transcription factor binding site about 700 bp from the start site CpG islands, called the sis-inducible element (SIE-1). This site is recognized by members of the signal transducer and activator of transcription family (STAT). The binding of STAT1 protein to SIE-1 has also been shown to upregulate p21 expression (Chin *et al.*, 1996). In contrast to the TSS site, treatment with 5-Aza decreased methylation of the SIE-1 site by about 35% as compared to its vehicle control DMSO. However, exposure of cells to $BrO_3^-$ and cisplatin did not yield similar results. The positive results with 5-Aza suggest that the SIE-1 is an important region for epigenetic regulation of p21 expression. This assertion is strengthened by the fact that we have already shown that 5-Aza alone induced p21 expression in these cells (Scholpa *et al.*, 2016). To our knowledge this is the first report

demonstrating the methylation of specific DNA bases within p21 promoter that are altered by 5-Aza exposure. These data also suggest that the mechanism by which cisplatin and $BrO_3^-$ increase p21 protein expression in renal cells does not involve this SIE-1 site. These data also allowed us to compare HEK293 cells to freshly isolated human proximal tubule cells. A significant difference in basal DNA methylation was observed between the two cell types. However, the toxicological manifestation of this difference in basal methylation is yet to be investigated.

In addition to determining changes in DNA methylation induced by cisplatin, $BrO_3^-$ and 5-Aza, TGBS also demonstrated the novel finding that the basal methylation of the p21 promoter region differs between rat and human p21. As mentioned above, the basal methylation in the promoter regions in human p21 was folds lower than corresponding regions analyzed in the promoter region of rat p21. It supports the previously mentioned hypothesis that the overall methylation status in specific rat genes may not be directly comparable to corresponding human genes. This is a critical point as it further suggests that epigenetic changes in rat genes cannot be used as a surrogate for human genes, a key consideration in using epigenetics in rats to assess the risk of toxicants in humans.

A limitation of this study was that we only focused on about 1,000 bp of DNA upstream of the TSS of both rat and human p21 (i.e. the promoter region). As such we did not assess the entire upstream non-coding regions of p21. Thus, it is still possible that changes in methylation in CpG sites would regulate changes in p21 expression in response to 5-Aza or cisplatin and $BrO_3^-$. Thus, even though TGBS allows for a greater amount of bp to be analyzed compared to pyrosequencing or Sanger sequencing, it is still limited in that stretches under 1,000 bp works better than larger fragments. We

focused on the promoter regions of p21 for obvious reasons including the fact that these sites are previously suggested to be involved in transcription. Future studies will focus on using TGBS to assess changes in methylation of these sites.

In conclusion, we combined two different existing technologies to develop a novel approach to rapidly identify specific CpG sites whose methylation is altered in the nephron-protective gene p21. We also used this technique to demonstrate differences in the basal promoter methylation between rat and human p21, and to further show that methylation at these CpG sites does not appear to regulate p21 expression induced by the nephrotoxicants cisplatin and $BrO_3^-$. These data suggest that methylation in the promoter region assessed are not involved in the epigenetic regulation of human and rat renal p21. Such data will be important into understanding the molecular mechanisms mediating the renal protective effect of p21 against toxicants as well as understanding difference in these mechanisms between rats and humans.

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# CHAPTER 3

# BROMATE-INDUCED CHANGES IN p21 DNA METHYLATION AND HISTONE ACETYLATION IN RENAL CELLS

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**Abstract**

Bromate ($BrO_3^-$) is a water disinfection byproduct we have previously shown to induce nephrotoxicity *in vitro* and *in vivo*. We also showed that the epigenetic inhibitors 5-aza-2-deoxycytidine (5-Aza) and trichostatin A (TSA) increased $BrO_3^-$ nephrotoxicity while altering the expression of the cyclin-dependent kinase inhibitor p21 expression. In this study we used a novel approach called targeted gene bisulfite sequencing (TGBS) to determine the percent methylation at the p21 promoter in human embryonic kidney (HEK293) and rat normal kidney (NRK) cells. Treatment of the cells with 5-Aza decreased DNA methylation by 35% at the sis-inducible element (SIE-1), a transcription factor binding site in human p21 promoter and also altered methylation at the rat p21 promoter. We also observed differences in the basal promoter methylation between rat and human p21, suggesting that the rat and human p21 are differentially regulated by DNA methyltransferases. In contrast, sub-chronic $BrO_3^-$ exposure failed to alter this methylation in both human and rat renal cells. In contrast, sub-chronic exposure of NRK cells to similar concentrations of $BrO_3^-$ altered histone acetylation, as determined by chromatin immunoprecipitation (ChIP) assays. Changes in histone acetylation correlated to changes in p21 protein expression. Changes in histone acetylation were not observed in HEK293 cells. The persistence of these epigenetic changes was assessed by discontinuing exposure to $BrO_3^-$ or the epigenetic inhibitors. 5-Aza-induced promoter demethylation remained stable after the withdrawal; however, TSA- and $BrO_3^-$ -induced histone hyperacetylation recovered back to basal levels after 3 days of withdrawal. These data suggest that $BrO_3^-$ regulates the renal expression of p21 using mechanisms that alter histone acetylation, and not by inducing de-methylation of its promoter. The data also

show species- and time-dependent difference in the epigenetic regulation of p21. These data suggest that epigenetic changes in rat p21 expression cannot be directly extrapolated to human p21, especially when assessing the risk of renal toxicants in humans.

## 1. Introduction

Ozonation is an extensively used method for disinfection of source water (US-EPA, 1998a). Bromate ($BrO_3^-$) is a disinfection byproduct (DBP) formed by the reaction of ozone with naturally occurring bromide ($Br^-$) in water and is designated as a probable human carcinogen by the International Agency for Research on Cancer (IARC, 1999). The regulated level for $BrO_3^-$ established by the United State Environmental Protection Agency (US EPA) is 0.01 ppm (US-EPA, 1998b, US-EPA, 2012) which is usually less than what is formed after ozonation of fresh water. The kidney is the major target organ for $BrO_3^-$-induced toxicity (Kurokawa *et al.*, 1982), as $BrO_3^-$ has been shown to induce renal cell tumors in rats after chronic exposures (Kurokawa *et al.*, 1990). $BrO_3^-$ also induces DNA damage characterized by 8-OHdG (8-hydroxyguanosine) production, which is also a measure of oxidative stress *in vitro* and *in vivo* (Kawanishi *et al.*, 2006). Our laboratory also previously reported that $BrO_3^-$ induced G2/M cell cycle arrest prior to the occurrence of cell death, and increased the expression of stress response kinases and DNA damage response proteins such as p38 and mitogen-activated protein kinase (MAPK) (Zhang *et al.*, 2010 and 2011). We have also demonstrated that $BrO_3^-$ increased the expression of a cyclin-dependent kinase (CDK) inhibitor p21, both *in vitro* and *in vivo* (Zhang *et al.*, 2010, Kolisetty *et al.*, 2013a).

p21 regulates cell cycle progression at the G1 and S phase (Gartel *et al.*, 2005). p21 activation has been shown to be protective against various nephrotoxicants such as cisplatin (Jiang *et al.*, 2008, Nowak *et al.*, 2003, Price *et al.*, 2009, di Pietro *et al.*, 2012). Several studies have shown that p21 can be activated by the tumor suppressor p53, and that such activation correlates to cell death (el-Deiry *et al.*, 1994). However, our recent

studies demonstrate that exposure of both cells and animals to lower, more environmentally relevant dose of $BrO_3^-$ resulted in p21 activation independent of p53 (Scholpa *et al.*, 2014). Hence, the current manuscript was focused on understanding the alternative mechanism mediating how $BrO_3^-$ activates p21 independently of p53.

As mentioned above, we previously demonstrated that exposure of renal cells to doses of $BrO_3^-$ as low as 0.01 ppm (the MCL) increased p21 expression (Scholpa *et al.*, 2014). To our knowledge these were some of the lowest doses ever shown for $BrO_3^-$ to induce molecular changes. This same study also showed that $Bro_3^-$ exposure altered methylation of the coding region of p21 in correlation with increases in its expression. Although p21 is known to be regulated by epigenetic mechanisms (Yoon *et al.*, 2012, Bott *et al.*, 2005), including DNA methylation and histone modifications, almost all of these data have focused on epigenetic changes of p21 in cancer cells (Bott *et al.*, 2005, Teramen *et al.*, 2011, Moreira *et al.*, 2009, Ying *et al.*, 2004). In contrast, a gap-in-knowledge exists with regards to the epigenetic regulation of p21 by environmental oxidants like $BrO_3^-$ or any other DBPs. Such knowledge is important to understanding the mechanism of $BrO_3^-$ -induced toxicity and to understanding how epigenetic changes in gene expression determine the risk of these compounds to humans (Moore *et al.*, 2006). Further, evidence supporting a role for epigenetics in the toxicity of $BrO_3^-$ is the fact that both the HDAC inhibitor trichostatin A (TSA) and the DNA methyltransferase (DNMT) inhibitor 5-aza-2-deoxycytidine (5-Aza) increased $BrO_3^-$-induced cytotoxicity when compared to $BrO_3^-$ or TSA and 5-Aza alone (Scholpa *et al.*, 2014). The increase in toxicity correlated to decreased p21. While change in p21 expression correlated to changes in p21 DNA methylation, the specific CpG sites altered were not identified. We recently

addressed this limitation using a novel methylation analysis approach called targeted gene bisulfite sequencing (TGBS), but we only assessed DNA methylation after short term exposure and we did not assess changes in histone acetylation.

Histone acetylation/deacetylation is believed to play a key role in p21 regulation in cancer cells (Shin *et al.*, 2000, Sowa *et al.*, 1997). For example, treatment with TSA or sodium butyrate up-regulated the transcription of p21 by inducing the acetylation of histones H4 and H3 in human colon cancer cells (Fang *et al.*, 2004). We recently showed that TSA increases p21 expression in renal cells (Scholpa *et al.*, 2014) by increasing the acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) (Scholpa *et al.*, 2016). However, the effect of $BrO_3^-$ on H3K9/14 Ac levels at the p21 promoter are not known. This study investigated the sub-chronic effects of low dose $BrO_3^-$ on epigenetic regulation of p21 in human and rat renal cells. Finally, while we have identified that $BrO_3^-$ can induce epigenetic changes in p21, we did not determine the persistence of these changes. To address this issue, we introduced withdrawal groups where the exposures to $BrO_3^-$ and the epigenetics inhibitors were discontinued. This is critical to understanding the role of epigenetic in the toxicity of this compound as the stability of these events must be determined.

## 2. Materials and Methods

### 2.1. Materials

Normal rat kidney (NRK) cells, human embryonic kidney cells (HEK293) and penicillin and streptomycin solution were purchased from American Type Culture Collection (Manassas, VA). Potassium bromate ($KBrO_3$), 5-aza-2'-deoxycytidine (5-Aza), trichostatin A (TSA), trypsin EDTA, glycine and 37 wt. % formaldehyde were purchased

from Sigma-Aldrich (St. Louis, MO), Dulbecco's modified Eagle medium (DMEM) was purchased from HyClone technologies (Logan, UT), 5-Aza was dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, PA). DNeasy blood and tissue extraction kit was purchased from Qiagen (Valencia, CA). The EZ-DNA methylation lightning kit and the Zyppy plasmid miniprep kits were purchased from Zymo research (Irvine, CA). Nucleospin gel and PCR clean-up kit was purchased from Macherey-Nagel (Düren, Germany). EpiQuik acetyl-histone H3 ChIP kit was purchased from Epigentek (Farmingdale, NY). The PCR master mix was purchased from Promega (Madison, WI) for ChIP-PCR. The MiSeq reagent v3 kit was purchased from Illumina Inc (San Diego, CA), the Strataclone PCR cloning kit was purchased from Agilent technologies (Santa Clara, CA), the Kapa HiFi PCR kit from Kapa Biosystems (Wilmington, MA), and the Maxima hot-start taq DNA polymerase for bisulfite-PCR and the Sera-Mag magnetic speedbeads were purchased from Thermo Scientific (Waltham, MA).

**2.2. Cell Culture and Sub-chronic Treatment**

5-aza-2'-deoxycytidine (5-Aza) and trichostatin A (TSA) were used as positive controls. 5-Aza is a DNA methyltransferase inhibitor and is used in many studies for its demethylating properties (Christman, 2002, Broday *et al.*, 1999*,* Bott *et al.*, 2005, Shin *et al.*, 2000). TSA is a histone deacetylase inhibitor and is studied widely for its potential for anti-cancer and anti-inflammatory mechanisms (Vanhaecke *et al.*, 2004, Drummond *et al.*, 2005, Adcock, 2007). HEK293 cells ($3 \times 10^6$) were seeded in T-175 tissue culture flasks and NRK cells ($1.5 \times 10^6$) were seeded in T-75 flasks and grown at $37^0$C in a 5% $CO_2$ incubator. Cells were treated with 0-100 ppm bromate ($BrO_3^-$), 40 µM 5-Aza, 100 nM TSA or DMSO (vehicle control for TSA and 5-Aza) for 72 hrs at log phase (after 24 hrs of

seeding). A portion of the cells were used for reseeding and the rest for sample collected for targeted gene bisulfite sequencing (TGBS), chromatin immunoprecipitation (ChIP) and immunoblot analysis. The first set of samples were collected from passage 1 (P1) of the sub-chronic regime and it continued till P6, i.e., the cells were treated for 18 days and passaged every 3 days for sample collection. The rationale for this regime is explained in our previous studies (Zhang *et al.*, 2010, Zhang *et al.*, 2011, Scholpa *et al.*, 2014 and 2016). Unlike our previous studies, this was followed by the withdrawal studies where the exposures to $BrO_3^-$ or the epigenetic inhibitors were discontinued for 9 days (P7- P9). This sub-chronic regime is illustrated in **Figure 3.1**.

### 2.3. Targeted Gene Bisulfite Sequencing (TGBS)

The DNA methylation of p21 promoter and coding regions in HEK293 and NRK cells was analyzed using targeted gene bisulfite sequencing (TGBS). The process and analysis details along with a ready-to-run VirtualMachine access are detailed in Chapter 2. In short, DNA was extracted from HEK293 or NRK cells ($5x10^6$). The extracted DNA (2 µg) was bisulfite converted, 350 ng of which was used for target amplification. The targets amplified included the human p21 transcription start site (hp21-TSS:: sense: 5'-ATAGTGTTGTGTTTTTTTGGAGAGTG -3' and anti-sense: 5'-ACAACTACTCACACCTCAACTAAC-3'), human p21 sis-inducible element 1 (hp21-SIE1:: sense: 5'-TTTTTTGAGTTTTAGTTTTTTTAGTAGTGT-3' and anti-sense: 5'-AACCAAAATAATTTTTCAATCCC -3'), rat p21 transcription start site (rp21-TSS:: sense: 5'-TTTTTTATTTTTGGTTGTTTTTTTT-3' and anti-sense: 5'-ACAAACAATTAACTCTCCTCAAATC-3') and rat p21 coding region (rp21-coding:: sense: 5'-TGTAATTAGTTATAGGTATTATGTTCGA-3' and anti-sense: 5'-

ACAAACAATTAACTCTCCTCAAATC-3'). The target primers were designed using MethPrimer (Li *et al.*, 2002) and synthesized with the TruSeqHT fusion stubs, iTru R1: ACACTCTTTCCCTACACGACGCTCTTCCGATCT and iTru R2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT on the 5' ends of forward and reverse primers, respectively (**Chapter 2, Table 2.1**) by Integrated DNA technologies Inc. (IDT, Coralville, IA). After amplification by polymerase chain reaction (PCR), the products were separated by electrophoresis in a 1% (w/v) agarose gel and visualized with ethidium bromide under a UV trans-illuminator. The single-sized amplicons were purified from the gel using Macharey-Nagel's Nucleospin gel and PCR clean-up kit following manufacturer's protocol.

Products containing the target and the TruSeqHT fusion primer sequences were further amplified to attach iTru5 and iTru7 indexing primers by limited-cycle PCR. This allowed for pooling of hundreds of sub-chronic samples and sequence in a single run. The pool was then purified and analyzed using an Illumina MiSeq platform. The sequences obtained were demultiplexed to get individual products from the pool. The sequence reads were then analyzed using Bismark bisulfite mapper available with instructions (**Appendix**) and a sample Illumina sequence file in downloadable VirtualMachine are available at https://drive.google.com/file/d/0B8YCoq3MYnhKN3RJMllQcUh3Y0k/view?usp=sharing.

### 2.4. Chromatin Immunoprecipitation (ChIP)

The effect of $BrO_3^-$ on p21 histone acetylation was investigated using Epigentek's EpiQuik acetyl-histone H3 chromatin immunoprecipitation (ChIP) kit based on manufacturer's protocol with conditions optimized for HEK293 and NRK cells.

### 2.4.1. Preparation of Assay Plate and Cells

Prior to the assay, strip wells were washed with 150 µl of wash buffer, followed by the antibody buffer (100 µl) and then the Anti-Acetyl-Histone H3 antibody (1 µl). The wells were coverered with Parafilm M and incubated at room temperature for 2 hrs. Cell extracts were than prepared by washing HEK293 or NRK cells ($3x10^6$) cells with 10 ml of phosphate buffered saline (PBS), followed by centrifugation at 1,000 rpm for 5 min. The resulting cell pellet was resuspended in fresh culture medium (9 ml) containing 1% formaldehyde (final concentration) and incubated at room temperature for 10 min on an orbital shaker (50-100 rpm).

### 2.4.2. Cell lysis and DNA shearing

1.25 M Glycine solution (1 ml) was added to fixed cells, mixed and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the cells washed with ice-cold PBS (10 ml). The cell pellet was lysed using the pre-lysis buffer (600 µl). The cell suspension was transferred to a 1.5 ml vial and incubated for 10 min on ice, vortexed vigorously for 10 sec and centrifuged at 5,000 rpm for 5 min. The supernatant was carefully removed and the lysis buffer (300 µl containing 3 µl protease inhibitor cocktail) added. The sample was incubated on ice for 10 min and vortexed occasionally. DNA was sheared by sonication using a Branson Microtip probe sonicator, with 8 sets of 10 sonication pulses for 1 sec each were performed at level 3, followed by 1 set of a 10 sec pulse. The samples were placed on ice between each pulse. Cell debris was pelleted by centrifugation at 14,000 rpm for 10 min.

### 2.4.3. Protein/DNA Immunoprecipitation

Clear supernatant was transferred to a new 1.5 ml vial (often supernatant was stored at –80ºC at this step). The supernatant (60 µl) was diluted at a 1:1 ratio with ChIP dilution buffer. The incubated antibody solution was aspirated, and the strip wells washed three times with the antibody buffer (150 µl) by pipetting. The diluted supernatant (100 µl) was transferred to each strip well, covered and incubated at room temperature for 2.5 hrs on a rocking platform (50-100 rpm). The supernatant was aspirated, and the wells washed six times with the wash buffer (150 µl) at 100 rpm for 2 min. The wells were then washed once with 1X Tris-EDTA buffer (150 µl).

### 2.4.4. Reverse Cross-link and DNA Purification

DNA release buffer (40 µl containing 1 µl proteinase K) was added to each well and the sample wells were covered with strip caps and incubated at 65ºC in a water bath for 15 min. Reverse buffer (40 µl) was added to the samples, which were mixed, re-covered and incubated in a65ºC water bath for 1.5 hr. Binding buffer (150 µl) was added to the samples and the solution transferred to a spin column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 20 sec. The column was washed with 70% ethanol (200 µl) followed by 95% ethanol (200 µl) twice. The column was placed in a new 1.5 ml vial and purified DNA was eluted using the elusion buffer (15 µl).

### 2.4.5. Polymerase Chain Reaction

Purified DNA linked to acetylated lysines 9 and 14 on H3 histones Were subjected to PCR using primers describe in **Table 3.1**. These include primers for the human and rat p21 promoter region, as schematically shown in **Figure 3.2**, and the housekeeping gene,

glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The 25 µl of PCR reaction mix contained 1X Promega master mix, 0.4 µM each of the forward and reverse primers and 40 ng of the immunoprecipitated DNA template. PCR was performed under the following conditions: $95^0$C for 5 min, 35 cycles of $95^0$C for 30 sec, respective $T_m$ (**Table 3.1**) for 45 sec for the regions rp21-ChIP, rGAPDH, hp21-ChIP and hGAPDH, $72^0$C for 45 sec and a final $72^0$C for 10 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel and visualized with ethidium bromide under a UV trans-illuminator. The band intensities were then quantified by densitometry using an Alpha Innotech FluorChem HD2 system (ProteinSimple, Santa Clara, CA) and normalized to the respective GAPDH.

### 2.5. Statistics

Samples isolated from a distinct cell passage represented one experiment (n=1). Data are represented as mean ± SEM (standard error of the mean) from at least three separate experiments (n=3). An unpaired Student's t-test was used to compare two groups using Graphpad PRISM considering $p<0.05$ indicative of a statistically significant difference between the mean values.

## 3. Results

### 3.1. Effect of $BrO_3^-$ on Cell Number and Morphology

Our studies assessed the effects of sub-chronic exposure of renal cells to environmentally relevant concentrations of $BrO_3^-$ on p21 DNA methylation and histone acetylation. Our previous studies Scholpa *et al.*, 2014 demonstrated that these low concentrations of $BrO_3^-$ induced p21 expression as early as 3 days of exposure at all doses tested including 0.01 ppm which is the recommended maximum contaminant level (MCL) by the US EPA (US-EPA, 2012). In this sub-chronic study, we hypothesized that

these changes in p21 expression are a result of epigenetic mechanisms. To test the general premise of stability of these epigenetic changes, we introduced an extra 9 days of withdrawal studies where the cells did not receive doses of $BrO_3^-$ or epigenetic inhibitors.

In agreement with our previous study (Scholpa *et al.*, 2014), treatment of HEK293 cells with doses of $BrO_3^-$ below 100 ppm did not significantly alter cell morphology or number after 18 days of sub-chronic treatment (**Figure 3.3A-F, J**). We also did not observe significant alterations in cell number or morphology of the cells after 9 days of withdrawal (**Figure 3.4A-F, J**). As observed in our acute study, cells treated with 100 ppm $BrO_3^-$ showed initial signs of cell rounding, detachment and small decrease in cell number compared to the control cells after 3 days (**Chapter 2, Figure 2.6G, L**). We could not study the sub-chronic effect of 100 ppm $BrO_3^-$ past 6 days (P2) in HEK293 cells and past 9 days (P3) in NRK cells due to further toxicity with immense decrease in cell number.

Exposure of HEK293 cells to 40 µM 5-Aza altered cell morphology and cell number after 3 days, a trend that continued through the 18 days of exposure (**Figure 3.3I-J**). This morphology and number recovered after 9 days of withdrawal, but were still significantly different from the DMSO withdrawal cells (**Figure 3.4I-J**). Exposure of cells to 100 nM TSA did not alter cell morphology or number after 18 days of sub-chronic treatment. Similar results were observed in NRK cells with $BrO_3^-$ and the epigenetic inhibitors.

### 3.2. Effect of $BrO_3^-$ on p21 Promoter Methylation

Our previous studies (Scholpa *et al.*, 2014) demonstrated changes in p21 methylation in the coding regions that correlated to sub-chronic exposure to environmentally relevant concentrations of $BrO_3^-$. Despite these data, the changes in

methylation in the promoter region of p21 were not assessed nor were differences between rat and human p21. Further, the exact CpG sites targeted was not identified. We addressed this gap-in-knowledge by analyzing DNA methylation of p21 in both rat and human renal cells exposed to $BrO_3^-$ at concentrations that did not induce detectable cell death. We analyzed different regions of the p21 promoter in HEK293 and NRK cells using targeted gene bisulfite sequencing (TGBS). The regions analyzed were: a 350 bp fragment of the human p21 promoter region adjacent to the transcription start site (hp21-TSS), a 335 bp fragment including the transcription factor binding site approximately 700 bp upstream of the TSS called the sis-inducible element (hp21-SIE1), a 250 bp fragment of the rat p21 promoter region near the TSS (rp21-TSS) and the rat p21 coding region approximately 9 Kb downstream of the TSS (rp21-coding). We previously used TGBS to identify basal differences in basal DNA methylation at different sites of the p21 gene, and also demonstrated species variability (**Chapter 2, Figure 2.3**). We also used this method to assess the effects of acute exposure of HEK293 and NRK cells to $BrO_3^-$ and observed that these exposures did not significantly alter the percent methylation in neither hp21-TSS nor rp21-TSS regions. In the current study, we assessed the same regions after sub-chronic exposure of the renal cells to $BrO_3^-$ and 5-Aza. In addition, we assessed the persistence of any changes by instituting a withdrawal protocol for 9 days after the initial 18 days of exposure. Neither 5-Aza nor $BrO_3^-$ (0.001 – 10 ppm) altered DNA methylation at hp21-TSS in HEK293 cells (**Appendix Figure 3**) and rp21-coding region in NRK cells (**Appendix Figure 4**) after 9 days (P3) and 18 days (P6) of exposure. These concentrations and exposure times had been shown to induce p21 protein expression in these renal cells (Scholpa *et al.*, 2014). However, 5-Aza decreased DNA methylation at

hp21-SIE1 site after 9 days of exposure (**Figure 3.5A-B**). In contrast, $BrO_3^-$ did not alter the percent DNA methylation at hp21-SIE1 site in HEK293 cells at any dose. The decrease in DNA methylation induced by 5-Aza was greater after 18 days of exposure, as compared to 9 days ((**Figure 3.5C-D**). Similar decrease was observed in NRK cells. 5-Aza decreased DNA methylation at the rp21-TSS site after 9 days of exposure and $BrO_3^-$ did not (**Figure 3.6A-B**). However, DNA methylation was not analyzed after 18 days of exposure due to scarcity of the sample resulting from toxicity of 5-Aza in NRK cells and to obtain enough cells for the withdrawal studies. Withdrawal of 5-Aza for 9 days did not result in return of DNA methylation to control levels at CpG site -855 in hp21-SIE1 site in HEK cells (**Figure 3.5E-F**) and at CpG sites -252, -244, 226, -171 and -147 in rp21-TSS in NRK cells (**Figure, 3.6C-D**), suggesting that this change had some permanence.

### 3.3. Effect of $BrO_3^-$ on p21 Promoter Histone Acetylation

Our previous studies showed that treatment of HEK293 and NRK cells with the HDAC inhibitor TSA upregulated p21 protein expression (Scholpa *et al.*, 2014), suggesting a role for histone acetylation in the regulation of this protein. We recently demonstrated that TSA-induced p21 expression correlated to the increased acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) in the p21 promoter region (Scholpa *et al.*, 2016). However, we do not know the effect of long-term exposure of $BrO_3^-$ on these cells, nor do we know the persistence of the changes.

Acute exposure (P1) of the renal cells with 0.001 – 100 ppm $BrO_3^-$ and 100 nM TSA did not affect the H3K9/14 Ac levels of the human p21 promoter but did alter acetylation in the rat p21 promoter at concentrations of 0.001 and 1ppm $BrO_3^-$ in a concentration-independent manner (**Figure 3.7A**). The sub-chronic effects of $BrO_3^-$ and

the positive control TSA were assessed at P3, P6 and P9 passages. Treatment of NRK cells with 0.001 – 10 ppm $BrO_3^-$ or 100 nM TSA significantly increased H3K9/14 Ac levels of p21 promoter after 9 days (P3) of sub-chronic treatment (**Figure 3.7B**). Similar increases were observed after 18 days (P6) of treatment with 0.01 – 10 ppm $BrO_3^-$ treatment (**Figure 3.7C**). Withdrawal of $BrO_3^-$ for 9 days resulted in a return of H3K9/14 acetylation to control levels (**Figure 3.7D**). This same reversion was seen in cells exposed to TSA. To understand the time-dependence on this reversion, we assessed the recovery of the acetylation mark after 3 days of withdrawal (P7), which was long enough to result in a decrease in acetylation comparable to controls (**Appendix Figure 5**). In contrast to NRK cells, H3K9/14 acetylation in HEK293 cells was not altered by $BrO_3^-$ or TSA at any concentration and time point measured (**Figure 3.8A-B**).

**Table 3.1.** Primer sequences for ChIP

| Locus | Forward primer (5'→3') | Reverse primer (5'→3') | $T_m$ ($^0$C) | Product size ( bp) | Reference |
|---|---|---|---|---|---|
| rp21-ChIP | GTTCAGCCCTGG AACCGAAG | GTACCAAACACCCTTCAC CTGGTAC | 59 | 227 | Yuan H *et al* 2013 |
| rGAPDH | CACGGCAAGTTCAACGG CACAGTCA | GTGAAGACGCCAGTAGA CTCCAGGAC | 54.2 | 150 | |
| hp21-ChIP | GGGGCTTTTCTGGAAATT GC | CTGGCAGGCAAGGATTT ACC | 54.2 | 116 | Mitani Y *et al* 2005 |
| hGAPDH | AAGGTCGGAGTCAACGG AT | TGGAAGATGGTGATGGG ATT | 54.2 | 221 | |

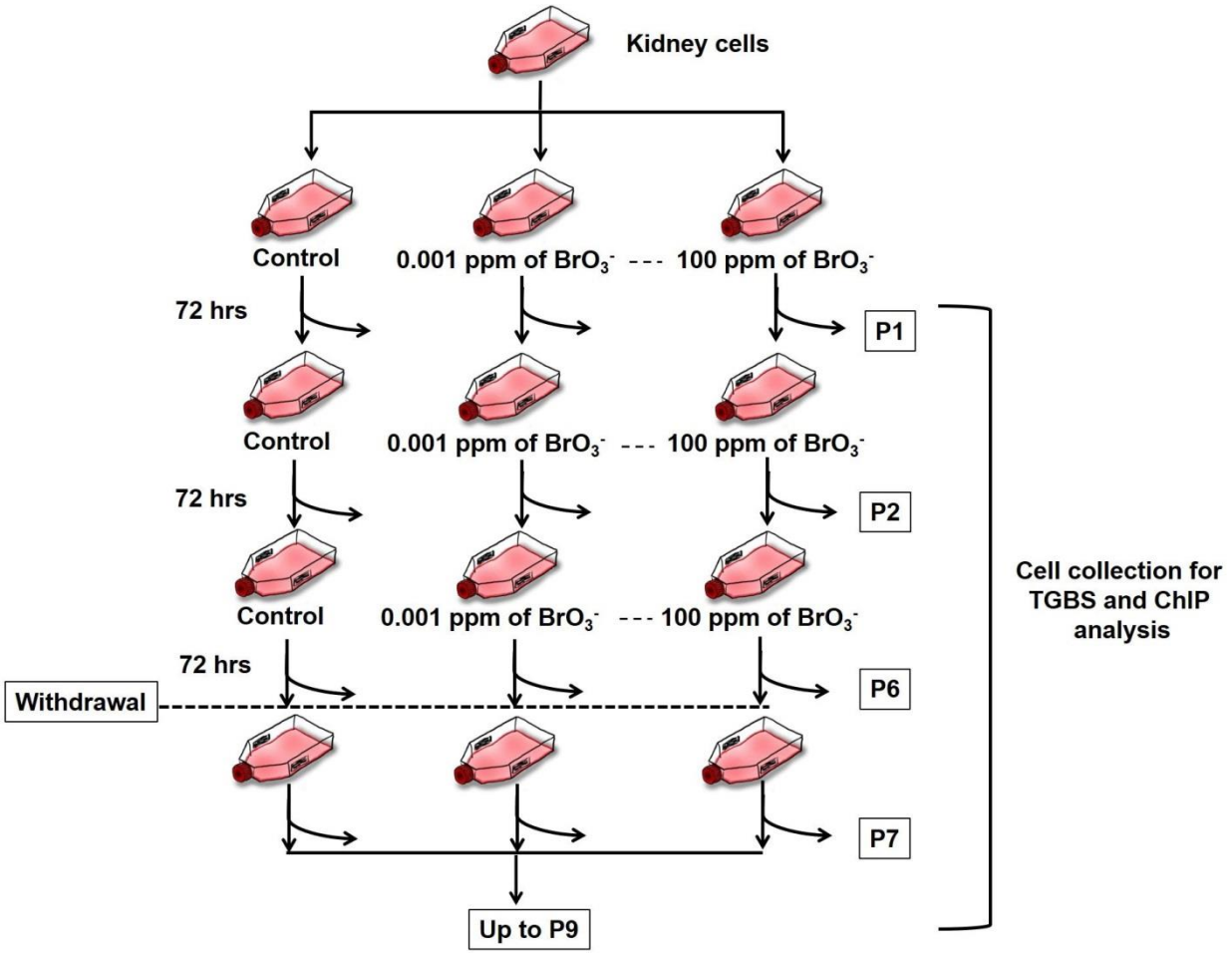



**Figure 3.1.** Sub-chronic dosing regime for $BrO_3^-$ and epigenetic inhibitors. Cells were exposed to various concentrations of $BrO_3^-$ at log phase (after 24 hrs of seeding) for 72 hrs. A portion of the cells were used for reseeding and the rest for sample collection for targeted gene bisulfite sequencing (TGBS) and chromatin immunoprecipitation (ChIP). This was called passage 1 (P1) and the regime continued till P6. Cells were recovered by discontinuing the exposures for the next three passages (P7-P9) to obtain withdrawal samples.

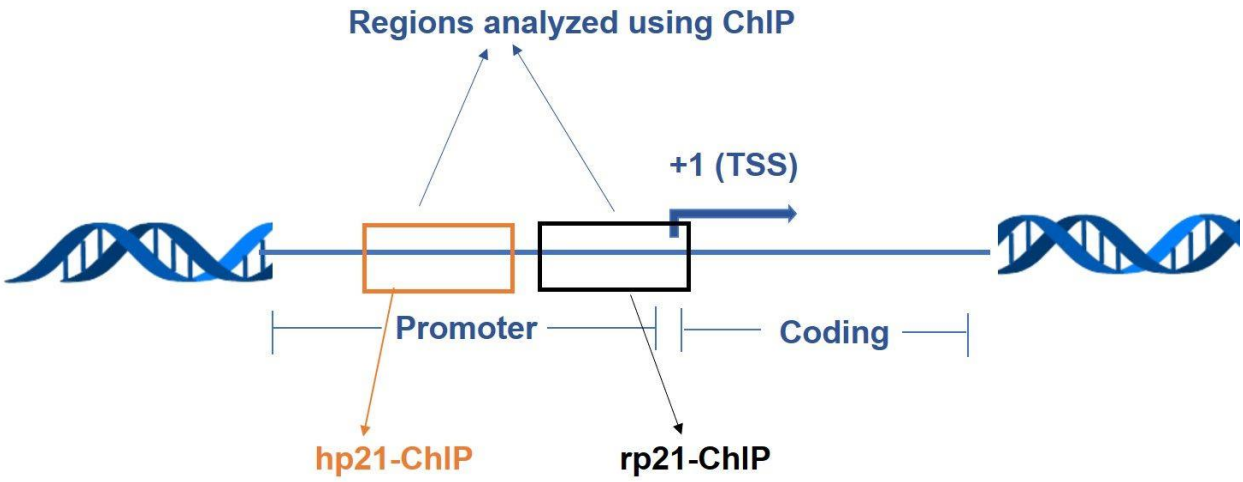
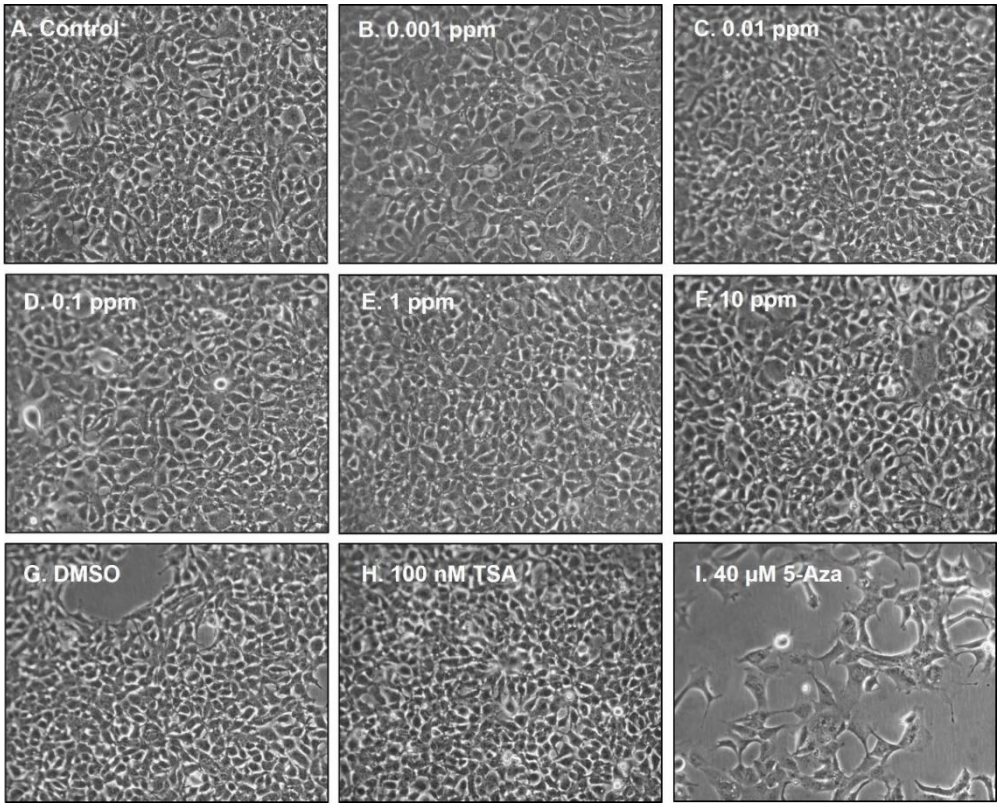



**Figure 3.2.** Schematic of p21 gene organization highlighting the loci of interest for histone acetylation analysis. This includes the human p21 promoter region (hp21-ChIP) about 1000 bp upstream of the transcription start site (TSS) and rat p21 promoter region (rp21-ChIP) at the TSS.



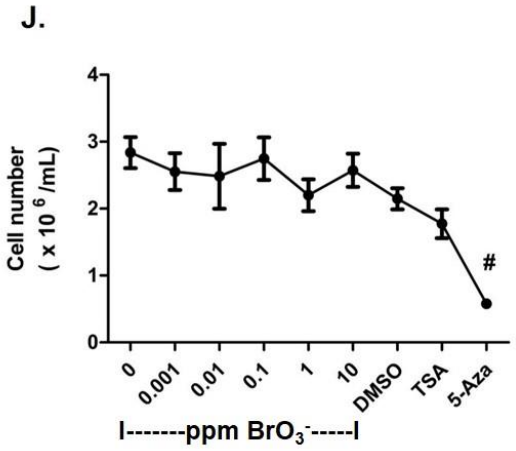




**Figure 3.3.** Sub-chronic Effect of $BrO_3^-$ and epigenetic inhibitors on HEK293 cell morphology and number. HEK293 cells were sub-chronically exposed to 0-10 ppm $BrO_3^-$ (**A-F**), vehicle control DMSO (**G**), 100 nM TSA (**H**) or 40 µM 5-Aza (**I**) for 18 days (P6). The cell number data in **J** are represented as mean ± SEM of three separate passages (n=3). $^{\#}P<0.05$ compared with 0 ppm $BrO_3^-$.

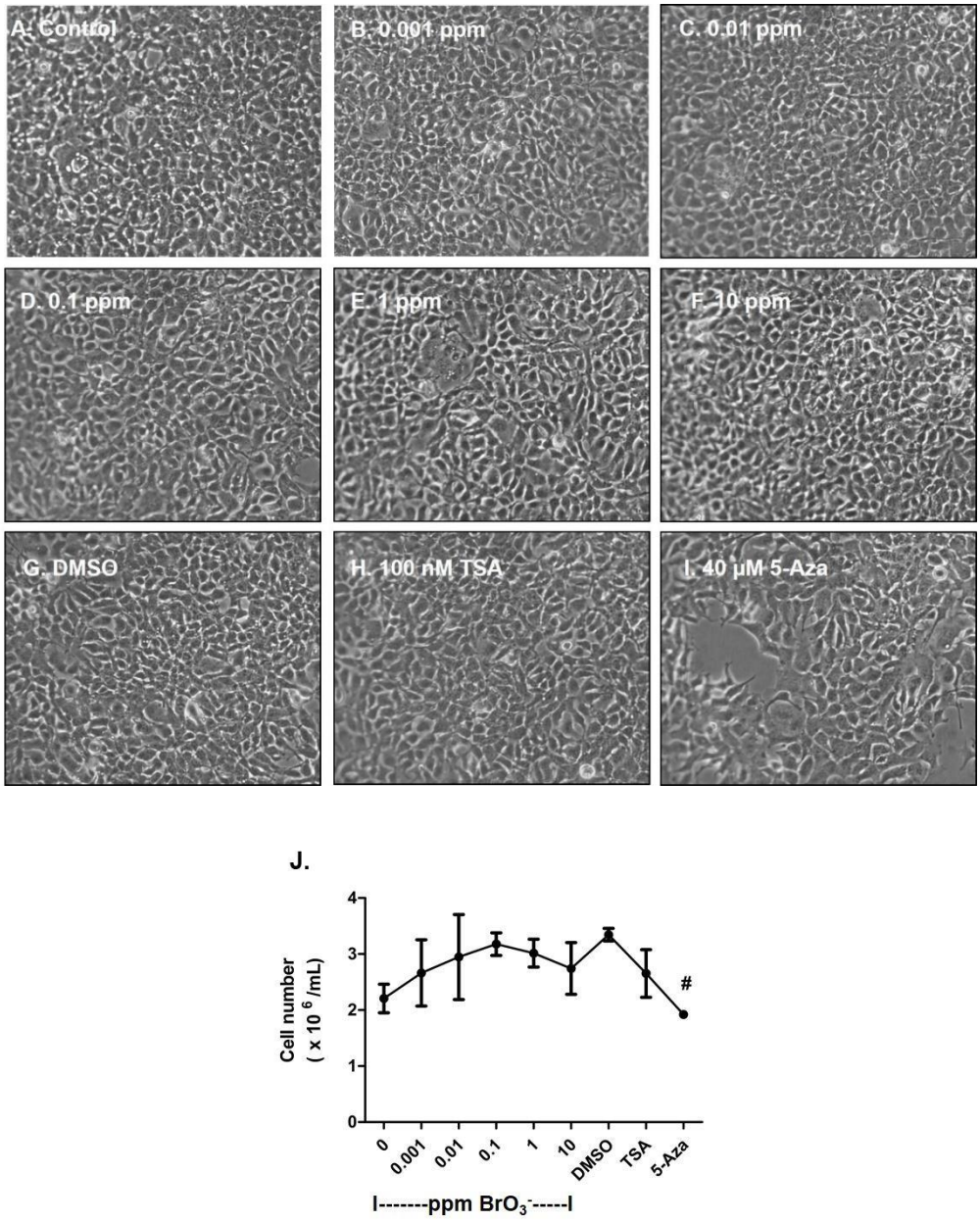



**Figure 3.4.** Effect of $BrO_3^-$ and epigenetic inhibitors on HEK293 cell morphology and number after withdrawal. HEK293 cells were sub-chronically exposed to $BrO_3^-$ for 18 days and then allowed to recover (**A-F**), vehicle control DMSO (**G**), 100 nM TSA (**H**) or 40 µM 5-Aza (**I**) for 9 days (P9). The cell number data in **J** are represented as mean ± SEM of three separate passages (n=3). $^{\#}P<0.05$ compared with 0 ppm $BrO_3^-$.

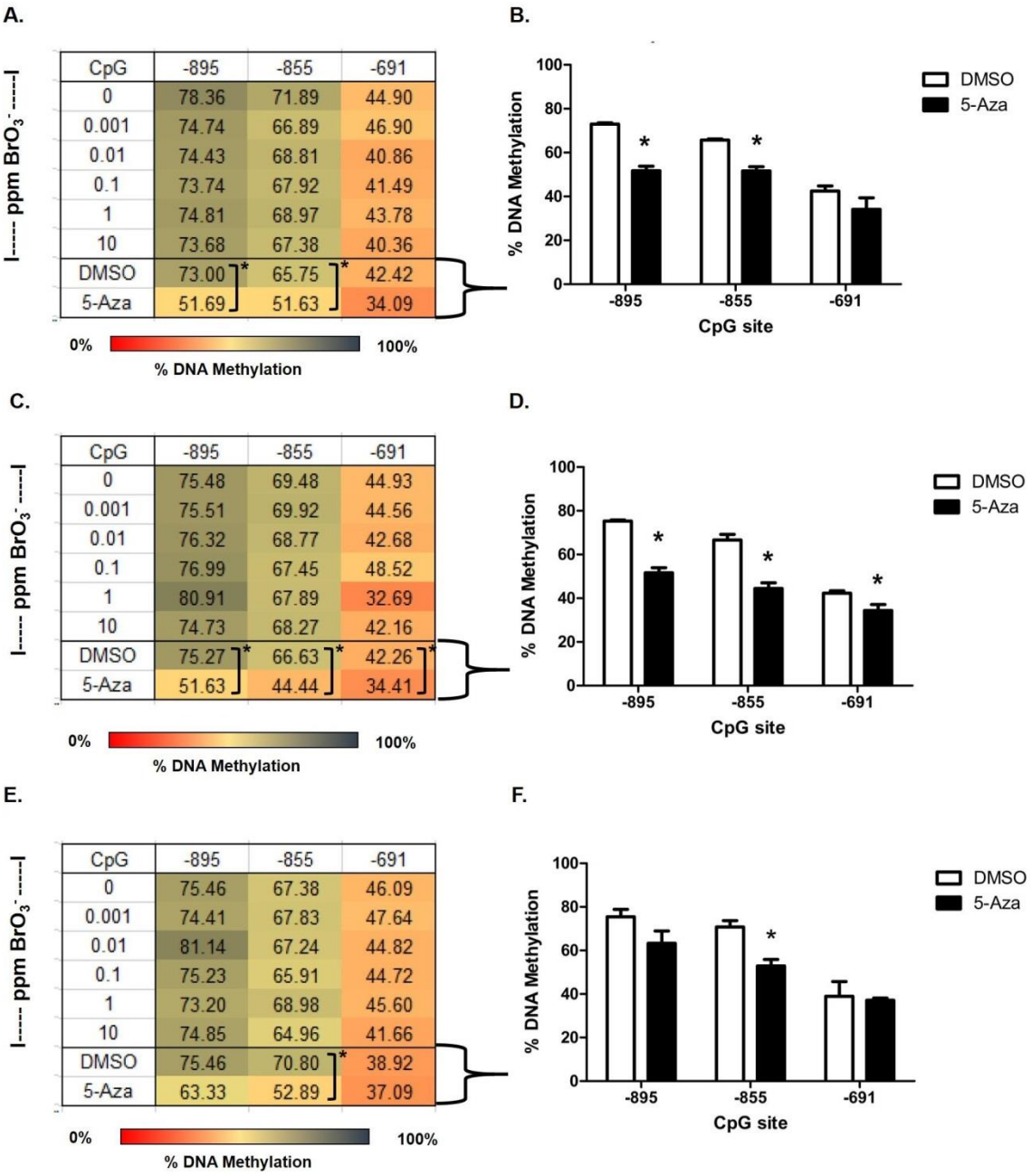



**Figure 3.5.** Sub-chronic effects of $BrO_3^-$ on the percent DNA methylation of cytosines in the SIE-1 site in human p21 promoter. HEK293 cells were treated with water (vehicle control for $BrO_3^-$ ), 0.001 to 100 ppm $BrO_3^-$ , DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for 9 days or P3 (**A-B**), 18 days or P6 (**C-D**). The cells were allowed to recover by discontinuing the exposures for 9 days or P9 (**E-F**). The first row in

the heat-map represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and deep blue indicating towards 100%. Data are represented as the average percent DNA methylation of three separate passages (n=3) as determined by TGBS analysis. The effects of 5-Aza are emphasized in the bar graphs for the respective exposure times. Data are represented as the mean ± SEM of three different passages (n=3). *P<0.05 compared with compared with DMSO.

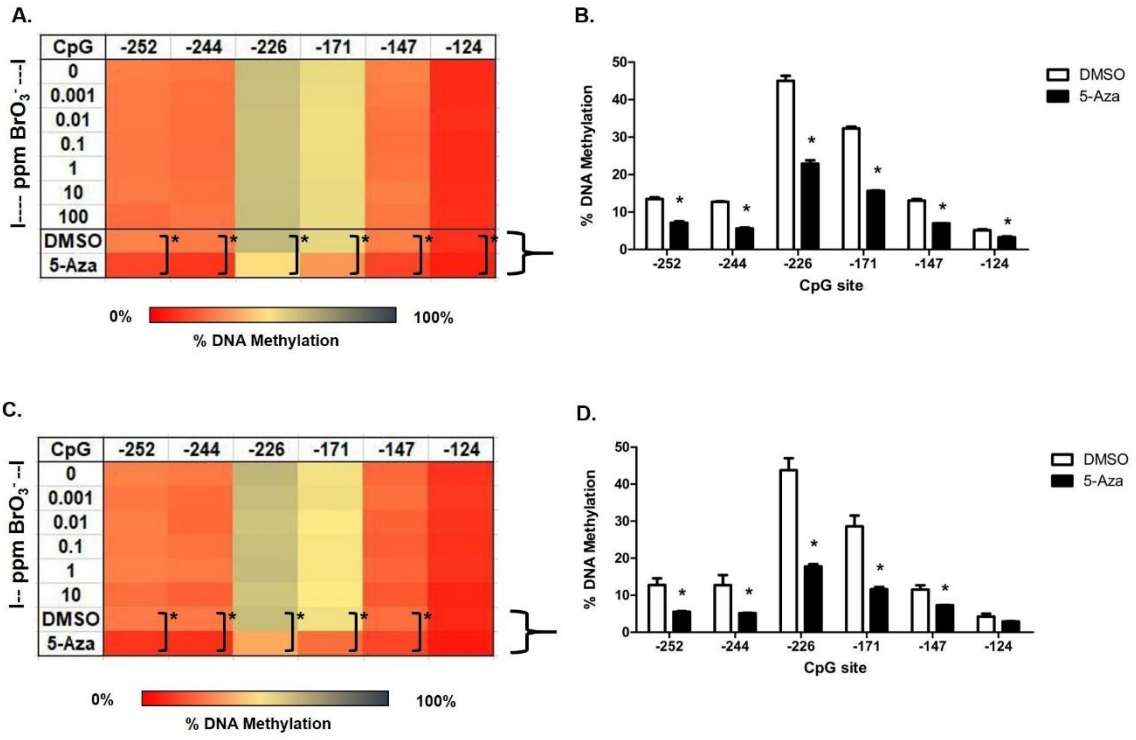



**Figure 3.6.** Sub-chronic effects of $BrO_3^-$ on the percent DNA methylation of cytosines in the TSS site in rat p21 promoter. NRK cells were treated with water (vehicle control for $BrO_3^-$ ), 0.001 to 100 ppm $BrO_3^-$ , DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for 9 days or P3 (**A-B**). The cells were further treated for 9 days and recovered by discontinuing the exposures for 9 days or P9 (**C-D**). The first row in the heat-map represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and deep blue indicating towards 100%. Data are represented as the average percent DNA methylation of three separate passages (n=3) as determined by TGBS analysis. The effects of 5-Aza are emphasized in the bar graphs for the respective exposure times. Data are represented as mean ± SEM of three different passages (n=3). *P<0.05 compared with with DMSO.

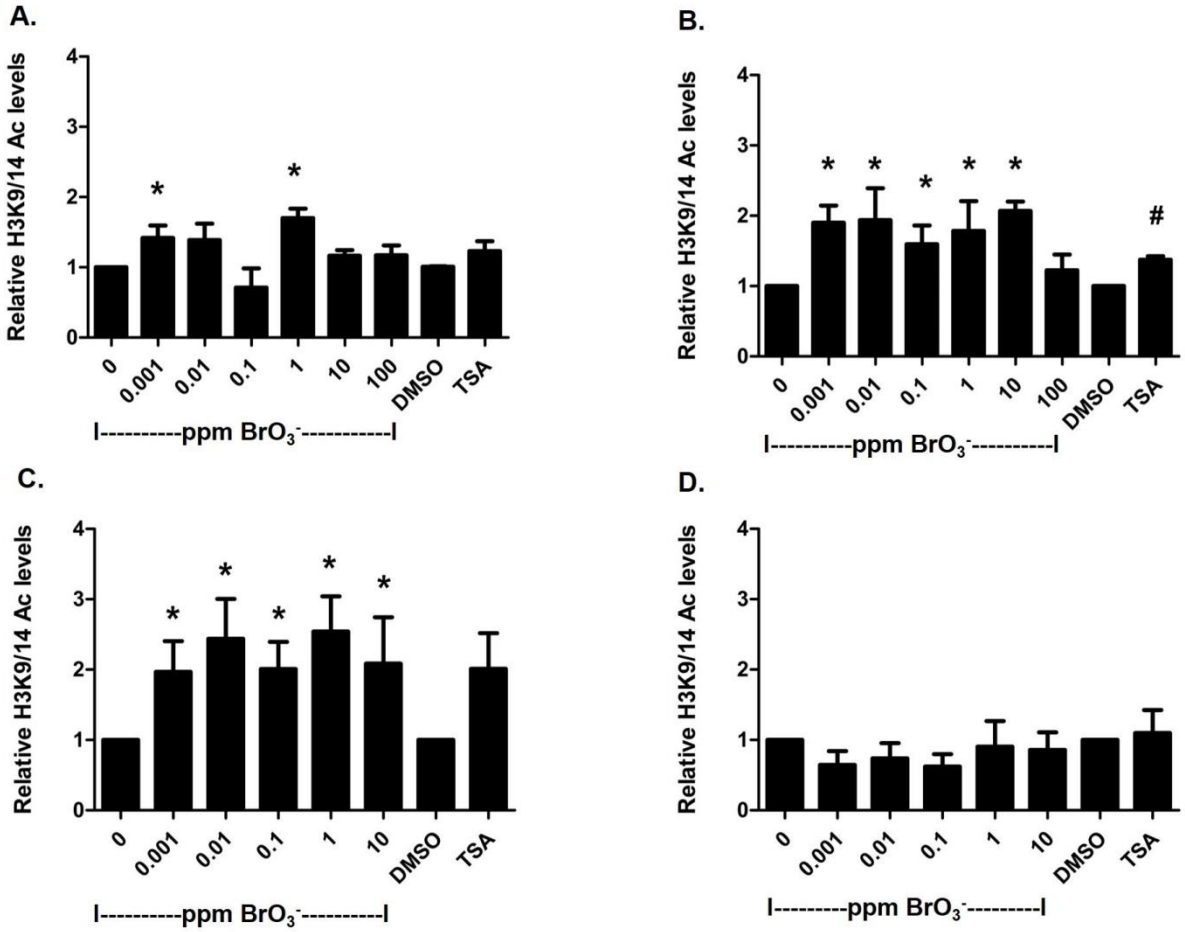



**Figure 3.7.** Sub-chronic effects of $BrO_3^-$ on acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) of the rat p21 promoter region. NRK cells to were exposed to 0.001 – 100 ppm $BrO_3^-$ for 3 days or P1 (**A**), 9 days or P3 (**B**) and 18 days or P6 (**C**). The cells were allowed to recover by discontinuing the exposures for 9 days or P9 (**D**). Data are represented as the relative fold increase in H3K9/14 Ac levels as normalized to GAPDH as determined using the ChIP assay. Data are represented as the mean ± SEM of three different passages (n=3). *P<0.05 compared with water control and $^{\#}$P<0.05 compared with DMSO.

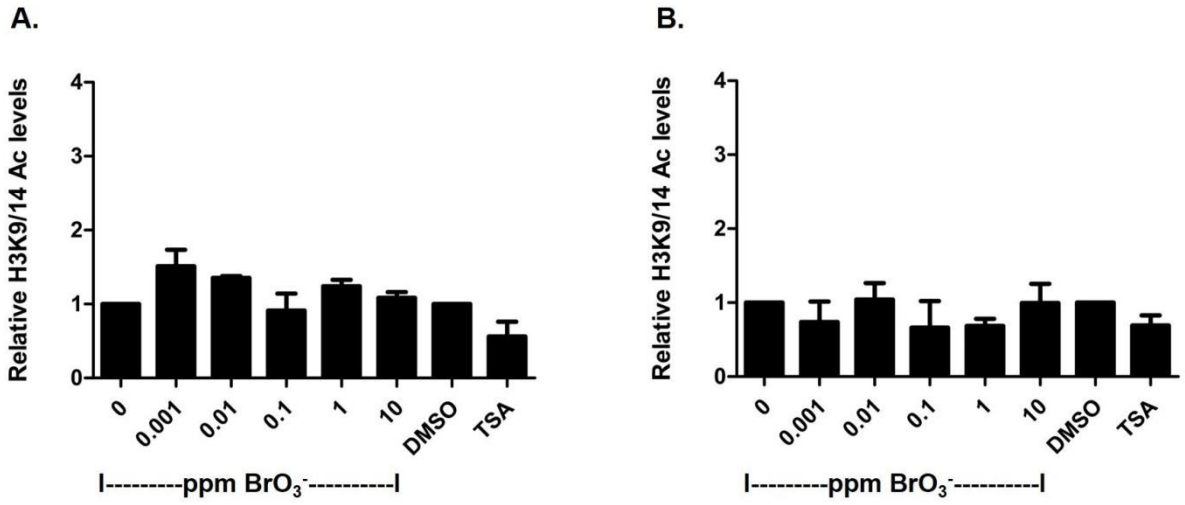



**Figure 3.8.** Sub-chronic effects of $BrO_3^-$ on the acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) of the human p21 promoter region. HEK293 cells were exposed to 0.001 – 100 ppm $BrO_3^-$ for 9 days or P3 (**A**) and 18 days or P6 (**B**). Data represented as the relative fold increase in H3K9/14 Ac levels as normalized to GAPDH as determined using the ChIP assay. Data are represented as mean ± SEM of three different passages (n=3).

## 4. Discussion

Our recently published work showed that non-cytotoxic concentrations of $BrO_3^-$ induced p21 expression (Scholpa *et al.*, 2014). In fact, sub-chronic exposure of cells to $BrO_3^-$ at concentrations as low as 0.01 ppm, the EPA suggested maximum contaminant level (or the MCL) increased p21 expression. Interestingly, higher concentrations of $BrO_3^-$ (10 ppm) did not induce p21, but did induce cell damage. Even more interesting is that changes in p21 expression correlated to changes in methylation within the DNA coding region (Scholpa *et al.*, 2014). Further, inhibition of DNA methylation or histone deacetylation increased p21 expression and altered cell death. These data strongly support the hypothesis that $BrO_3^-$ mediated p21 by epigenetic mechanisms. This hypothesis was supported by the fact that 5-Aza and TSA, inhibitors of DNA methyltransferase and histone deacetylases, respectively, induced p21 expression. Unfortunately, our previous studies did not identify the exact sites that were targeted by $BrO_3^-$, differences between rat and human p21 epigenetic regulation, or the persistence of these changes. Our previous studies using acute doses of 5-Aza showed that this compound significantly decreased percent methylation at the hp21-SIE1 site in HEK293 cells after 72 hrs of exposure. The current study verified these data and further showed that decreases in DNA methylation were enhanced in HEK293 cells after further exposures. Additionally, the data also showed that changes in methylation of at least one CpG site was maintained after withdrawal of 5-Aza. The responsiveness to 5-Aza suggests that the SIE1 is an important region for epigenetic regulation of p21 expression given that we have already shown 5-Aza to induced p21 expression in these cells (Scholpa *et al.*, 2014).

Exposure of cells to $BrO_3^-$ (0.001 – 10 ppm) did not alter DNA methylation at the human p21 promoter (hp21-TSS and hp21-SIE1), rat p21 promoter (rp21-TSS) and rat p21 coding (rp21-coding) region, even after 18 days of exposure (P6). In contrast, 5-Aza significantly decreased percent methylation at the hp21-SIE1 site in HEK293 cells after 9 days (P3) and 18 days (P6) of exposure and at the rp21-TSS site in NRK cells after 9 days of exposure. This data again confirms that, in addition to hp21-SIE1, rp21-TSS is an epigenetic regulatory site for p21 expression and suggests that the mechanism by which $BrO_3^-$ induced p21 in renal cells does not involve either of the sites. It is known that DNA methylation can be a stable epigenetic mark (Cedar *et al.*, 2009). The stability of the mark in our sub-chronic exposure regime was investigated by introducing withdrawal studies. The demethylating effect of 5-Aza on hp21-SIE1 and rp21-TSS sites was persistent.

Our sub-chronic data further suggests that DNA methylation within the promoter region of either HEK293 or NRK p21 is not altered by $BrO_3^-$. This does not suggest that epigenetic mechanisms are not involved in the regulation of $BrO_3^-$-induced expression of p21 in renal cells. Rather, it suggests that change in DNA methylation at these sites are not involved in the regulation of this gene. It's quite possible that changes in methylation at sites distant from the promoter region mediate p21 expression. Our choice for the site to be analyzed with TGBS are obvious, as these sites are within the promoter region. Future studies are needed to address this hypothesis and identify these sites.

In contrast to methylation, $BrO_3^-$ clearly altered histone acetylation. The fact that p21 expression is mediated by histone acetylation is not novel as Fang *et al.*, 2004 has previously reported that histone acetylation can regulate p21 expression in many cell

lines. Shin *et al* 2000 also showed that p21 promoter region was not methylated in gastric cancer cells, but was more regulated by histone deacetylation 1. Various studies, including ours (Scholpa *et al.*, 2014), reported that inhibition of HDAC increases p21 expression, including that induced by azelaic bishydroxamic acid (Burgess *et al.*, 2001), n-butyrate (Dagtas *et al.*, 2009), suberoylanilide hydroxamic acid (Richon *et al.*, 2000, Gui *et al.*, 2004) and statins (Lin *et al.*, 2008). The novelty in the current study is that this is one of the first report to demonstrate that $BrO_3^-$ exposure increases H3K9/14 acetylation. Further, the current study is one of first to identify the location of this acetylation. Of further interest is that changes in acetylation occurred at doses as low the EPA limit (0.01 ppm). The fact that changes in acetylation occurred in the absence of cell death, and that these changes were not persistent supports the hypothesis that induction of p21 after exposure to $BrO_3^-$ is a protective rather than a toxic response.

Another interesting finding from these studies is that $BrO_3^-$ did not alter H3K9/14 acetylation in HEK293 cells at similar sties in NRK cells. As mentioned above, we previously demonstrated significant differences in the methylation of rat and human p21 with in the promoter region of untreated cells (**Chapter 2, Figure 2.3**). Data on the effect of both TSA and $BrO_3^-$ on acetylation of H3K9/14 further support the hypothesis that epigenetic regulation of p21 is species-dependent.

It should be noted that histones are modified by marks like lysine methylation, arginine methylation and citrullination, serine, tyrosine or threonine phosphorylation, and we have only assessed lysine acetylation on histone H3. It is possible that the lack of histone acetylation in human p21 in response to $BrO_3^-$ may be a result of acetylation at different histones within the promoter region, or a difference in the location of histone in

the non-coding region. Nevertheless, such data would still support the premise that epigenetic regulation of p21 is species-dependent.

In conclusion, our data demonstrate that the expression of rat p21 is regulated by histone acetylation and not DNA methylation of the regions analyzed, after sub-chronic exposure of rat renal cells to $BrO_3^-$. In contrast, it appears that while sites for epigenetic regulation do exist within the promoter regions of human and rat p21, these sites exist at different regions of the respective promoters. These data also demonstrate that DNA methylation within the promoter sites does not appear to mediate changes in expression of either rat or human p21 in response to $BrO_3^-$. Finally, these data demonstrate the species-specific differences in the epigenetic regulation of p21 and suggest an uncertainty in extrapolating rat epigenomic data for assessing the risk of toxicants in humans.

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# CHAPTER 4

# CHANGES IN CpG-METHYLATION OF THE *VITELLOGENIN 1* PROMOTER IN ADULT MALE ZEBRAFISH AFTER EXPOSURE TO 17α-ETHYNYLESTRADIOL (EE2)*

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**Abstract**

Numerous pharmaceutical and industrial chemicals are classified as endocrine disrupting chemicals (EDCs) that interfere with hormonal homeostasis leading to developmental disorders and other pathologies. 17α-ethynylestradiol (EE2) is a EDC used in oral contraceptives and other hormone therapies that are inadvertently introduced into aquatic environments through several avenues primarily municipal wastewater effluent. Exposure of male fish to EE2 increases the expression of the egg yolk precursor protein vitellogenin (Vtg), which has been used as a molecular marker of exposure to estrogenic EDCs and feminization. The mechanisms behind Vtg induction are not fully known and we hypothesized that it is regulated via DNA methylation. DNA methylation at the *vtg1* promoter was assessed using targeted gene bisulfite sequencing (TGBS), in the livers of adult male zebrafish exposed to 20 ng/L EE2. A significant increase in mRNA was observed in the EE2-exposed fish as early as 6 hrs. A decrease in DNA methylation at the CpG sites, however, was not observed until after 4 days. Decreases in the overall methylation of v*tg1* promoter in male zebrafish resulted in comparable levels as that seen in female controls, suggesting feminization. Importantly, DNA methylation levels in males remained significantly decreased after 7 days post EE2 removal, unlike mRNA levels. These data suggest a role for DNA methylation in Vtg induction and identify a novel epigenetic mark of feminization that may serve as an indicator of previous exposure to EE2.

## 1. Introduction

The concern about the adverse effects of endocrine disrupting chemicals (EDCs) on animals and humans has encouraged the development of various screening methods for their presence, as well as mechanistic studies determining their ability to alter gene expression. EDCs ability to alter gene expression results in interference with the normal function of the endocrine system, resulting in deviation from normal homeostasis and hence causing adverse health effects (Colborn *et al.*, 1993). This deviation could rise from pro-estrogenic, anti-androgenic effects, thyroid disruption, etc., and the known effects include, but are not limited to, developmental and reproductive malformations, neurodevelopmental disorders and increased cancer risk (Swan *et al.*, 2005, Colborn *et al.*, 2007, O'Connor *et al.*, 2009). Broad categories of putative EDCs include pesticides, polychlorinated biphenyls, dioxins, and polybrominated diphenyl ethers (Gore, 2007).

Vitellogenin (Vtg) is an egg yolk precursor protein expressed in livers of all female oviparous vertebrates (Wahli *et al.*, 1981). Vitellogenesis is a process where Vtg is synthesized in the liver, secreted to plasma and carried via the blood stream for oocyte uptake. Expression of *vtg* mRNA is controlled by the binding of estrogen receptors to estrogen response elements (Green *et al.*, 1987). The natural estrogen 17β-estradiol (E2) is a trigger for *vtg* expression. Once diffused into cells, estrogen binds to the estrogen receptor (ER) in the cytosol or nucleus, and this complex binds to specific palindromic sequences called the estrogen response elements (ERE) in the promoters of estrogen responsive genes like vitellogenin (Gruber *et al.*, 2004).

Estrogenic EDCs can elevate Vtg levels unnaturally by mimicking the natural ligand 17β-estradiol (E2) (Bjornstrom *et al.*, 2005, Wang *et al.*, 2005). Male and juvenile

fish normally do not produce Vtg. Hence Vtg in male fish has been predominantly used as a molecular marker of exposure to estrogenic EDCs in ecological testing and is popularly studied as a marker for feminization in fish (Sumpter *et al.*, 1995). Surprisingly, the mechanism behind this Vtg induction is yet to be fully understood. In this study, we investigated the most common positive control for estrogenic activity of 17α-ethynylestradiol (EE2). EE2 is an orally active synthetic estradiol used in contraceptives as well as other hormone replacement therapies with eminent ecological effects used excessively in humans. EE2 is found in the ng/L-range in effluent waters from sewage treatment plants in the USA and other countries (Huang *et al.*, 2001, Larsson, 1999). EE2, along with other EDCs in treated effluent waters, has shown to have feminizing effects in male fish (Purdom, 2006, Adeel *et al.*, 2017, Sumpter *et al.*, 1995). In zebrafish, a concentration of EE2 as low as 3 ng/L is reported to arrest male gonad development (Fenske *et al.*, 2005). We hypothesized that the induction of Vtg in male zebrafish after exposure to EE2 is regulated by epigenetic mechanisms, specifically the demethylation of the 5'-flanking region of the *vtg1* gene promoter. We further studied the time-dependency of this demethylation.

Zebrafish (*Danio rerio*), is a genetically well-characterized and popularly used model organism in ecotoxicology. Vtg protein is heterogenous (types I, II and III) and is coded by *vtg1-7* genes with *vtgs 1,2, 4, 5, 6* and *7* on chromosome 22 and *vtg3* on chromosome 11 in zebrafish (Wang *et al.*, 2005). Of these, *vtg1* mRNA is the most highly expressed (Wang *et al.*, 2005). Further, the *vtg1* gene is normally silent in male zebrafish liver and is known to be over-expressed after exposure to estrogenic EDCs (Meng *et al.*, 2010, Hoffmann *et al.*, 2001, Versonnen *et al.*, 2004).

Epigenetics is the study of heritable changes in gene activity without any changes in the DNA sequence (Waddington, 1942, Russo *et al.*, 1996). DNA methylation is an epigenetic mechanism catalyzed by DNA methyltransferases (DNMT) where methyl groups are added to the cytosine nucleotides forming 5-methylcytosine residues (Holliday *et al.*, 1975). This is seen predominantly with cytosine residues in the CpG dinucleotides in multicellular eukaryotes. DNA methylation decreases gene transcription by impeding the access of transcription factors to their binding sites (Choy *et al.*, 2010). It is known that promoter demethylation plays a crucial role in activating gene expression (Phillips, 2008). Though the methylome of zebrafish is broadly studied to assess human diseases like cancer (Mudbhary *et al.*, 2011) and diabetes mellitus (Sarras *et al.*, 2015), it is rarely investigated for ecotoxicological applications. It has been reported as early as 1982 that estrogen induces demethylation of vitellogenin gene in chickens (Wilks *et al.*, 1982). However, the only other report in ecotoxicology, to our knowledge, is the study by Stromqvist *et al.*, 2010 showing estrogen-induced promoter demethylation of *vtg1* in zebrafish. The time-dependent changes and the permanence of the changes is not known. This later point is critical for the changes to be considered as epigenetic marks.

Epigenetic changes typically need to be mitotically stable through cell division, transgenerationally inherited and persist through biological memory even after removal of the stressors (Richards, 2006). DNA methylation is known to be a stable epigenetic mark leading to long-term gene repression (Cedar *et al.*, 2009), but the ability of EE2 to induce such permanent changes is not known. With this in mind, we tested the persistence of EE2-induced DNA methylation changes of *vtg1* promoter in zebrafish after discontinuing EE2 exposure.

## 2. Materials and Methods

### 2.1. Materials

17α-ethynylestradiol (EE2) was purchased from Sigma-Aldrich (St. Louis, MO), EE2 was dissolved in dimethyl sulfoxide (DMSO) from American Type Culture Collection (Manassas, VA), Zeigler adult zebrafish diet and Tricaine-S were purchased from Pentair (Minneapolis, MN). The DNeasy blood and tissue extraction kits were purchased from Qiagen (Valencia, CA). The ZR-Duet DNA/RNA miniprep plus kit, the genomic DNA clean and concentrator kit and the EZ-DNA methylation lightning kits were purchased from Zymo Research (Irvine, CA), Nucleospin gel and PCR clean-up kit was purchased from Macherey-Nagel (Düren, Germany). The MiSeq reagent v3 kit was purchased from Illumina Inc (San Diego, CA), the Kapa HiFi PCR kit from Kapa Biosystems (Wilmington, MA), the Maxima hot-start Taq polymerase and Sera-Mag magnetic speedbeads were purchased from Thermo Scientific (Waltham, MA) and the iTaq universal SYBR green one-step kit was purchased from Biorad (Hercules, CA).

### 2.2. Zebrafish Maintenance

Adult zebrafish (*Danio rerio*), 8-12 months of age, were generously provided by Dr. Scott Dougan at the department of Cellular Biology at University of Georgia (Athens, GA). The fish were kept in aerated 20 L glass aquaria filled with dechlorinated tap water at $28^0$C under 14 hr light/10 hr dark cycle. Ziegler adult zebrafish diet was provided *ad libitum*. All activities involving fish were covered by the University of Georgia Animal Use Protocol # A2016 06-030-Y2-A2.

### 2.3. Zebrafish Exposure

The automated renewal system consisted of 10 head tanks, each feeding into two 20 L glass aquaria in the reservoir tub maintained at $28^0$C. Treatment tanks were pre-conditioned with 20 ng/L EE2 for 3 days prior to adding fish. Five or six fish were transferred to each aquarium after a 100% water renewal. The system contained five treatment and five control aquaria. The treatment aquaria received a nominal concentration of 20 ng/L EE2 and the controls received DMSO (0.001%). About 95% of the water was renewed every second day with a fresh dose of EE2 or DMSO. Using a stratified assignment system, fish were sampled from each of the tanks at each time point. To test the persistence of any changes, we included a recovery group that received EE2 for 7 days and did not receive any for the following 7 days, in the total 14-day study. Fish were euthanized at different time points with an overdose of 0.1% solution of Tricaine-S (tricaine methanesulfonate). and the livers dissected and stored at $-20^0$C until further processing.

### 2.4. DNA Methylation Analysis

The DNA methylation of *vitellogenin 1* (*vtg1*) promoter in zebrafish liver was analyzed using targeted gene bisulfite sequencing (TGBS). TGBS is a combination of bisulfite conversion, target amplification and next-generation sequencing. The process and analysis details along with access to a ready-to-run VirtualMachine are elaborately explained in Chapter 2. The key steps in the analysis are described below.

#### 2.4.1. DNA extraction and Bisulfite Conversion

Frozen livers (~2 mg) were homogenized in DNA/RNA shield buffer using a micro-grinder pestle mixer from Research Products International (RPI, Mt Prospect, IL). The

pestle was rinsed with 70% ethanol followed by nuclease-free water between samples. Genomic DNA and total RNA were extracted using the ZR-Duet DNA/RNA miniprep plus kit, following the manufacturer's protocol. The eluted DNA was further purified and concentrated using a DNA clean and concentrator kit, following the manufacturer's instructions. DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Template DNA (1 µg) was bisulfite treated using the EZ-DNA methylation lightning kit, following manufacturer's protocol.

### 2.4.2. Target Amplification and Purification

Bisulfite converted DNA (350 ng) was used as a template for amplification of a 248 bp promoter fragment that corresponded to the 1802 bp upstream of exon 1 of *vtg1* (Stromqvist *et al.*, 2010). The fragment contains three CpG sites at 1828, 1846 and 1873 bp upstream of the exon 1 of *vtg1*, that are considered to be located between two hypothetical estrogen responsive elements (ERE) in zebrafish (**Figure 4.1**). The primers were synthesized by Integrated DNA Technologies Inc. (IDT, Coralville, IA). TruseqHT fusion primer sequences (Faircloth *et al.*, 2012) were added to the 5' ends of locus specific primers during primer synthesis (**Table 4.1**). Loci were amplified using Maxima hot-start Taq polymerase. The PCR amplification reaction mix (25 µl) contained 3 mM magnesium chloride ($MgCl_2$), 1X hot start buffer, 0.2 mM of each deoxynucleoside 5'-triphosphates (dNTPs), 0.6 µM each of the forward and reverse primers and the 300 ng bisulfite converted DNA template. PCR was performed under the following conditions: $95^0$C for 5 min, 40 cycles of $95^0$C for 30 sec, $53^0$C for 45 sec, $72^0$C for 45 sec and a final $72^0$C for 10 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel and visualized with ethidium bromide under a UV trans-illuminator. The

amplicons were extracted from the gel using Macherey-Nagel's Nucleospin gel and PCR clean-up kit following manufacturer's instructions.

### 2.4.3. Library preparation and Illumina sequencing

Libraries were prepared that corresponded to the above mentioned locus (*vtg1* promoter fragment) using the locus-specific primers (zf-vtg1) and the Universal 5' TruseqHT fusion primers. Library preparation was similar to that descried in Chapter 2. In short, the iTru5 and iTru7 indexing primers were added to the gel-extracted products by performing a limited-cycle PCR using the Kapa HiFi PCR kit. This was done to facilitate pooling a large number of samples to be sequenced in a single run. The pool was then purified using Sera-Mag magnetic speedbeads and run on an Illumina MiSeq platform using Illumina's MiSeq 600 cycle v3 kit.

### 2.4.4. Sequence Read Analysis

The sequences obtained above were demultiplexed to obtain individual products from the pool using Illumina software bcl2fastq (bcl2fastq2, 2013). The sequence reads were then analyzed using Bismark bisulfite mapper (Krueger *et al.*, 2011). The instructions for analysis are included in **Appendix** along with a sample Illumina sequence file available with a ready-to-run VirtualMachine at https://drive.google.com/file/d/0B8YCoq3MYnhKN3RJMllQcUh3Y0k/view?usp=sharing.

## 2.5. mRNA Analysis

Total RNA extracted from zebrafish liver using the ZR-Duet DNA/RNA miniprep plus kit was quantified using a Nanodrop spectrophotometer. The RNA template (500 ng) was reverse transcribed and the cDNA was amplified using the iTaq universal SYBR

green one-step kit. The reaction mix (20 µl) includes 1X iTaq universal SYBR green reaction mix, 1X iScript reverse transcriptase, 0.5 µM each of the forward and reverse qRT primers (zf-vtg1-qRT and zf-ef1α-qRT, **Table 4.1**) and 0.5 µg of RNA template. The reaction conditions were: $50^0$C for 10 min, $95^0$C for 1 min, 40 cycles of $95^0$C for 10 sec followed by $60^0$C for 30 sec and $60^0$C for 30 sec, and a final increment of $0.5^0$C/step from 65-95 $^0$C. The reactions were run in a 96 well PCR plate on Biorad CFX Connect real-time PCR system (Biorad, Hercules, CA).

### 2.6. Statistics

Samples isolated from the liver of a single fish represented one experiment (n=number of fish per treatment). Data are represented as mean ± SEM (standard error of the mean) from at least four different fish per treatment group (n≥4). An unpaired Student's t-test was used to compare two groups and one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison to compare multiple groups using Graphpad PRISM, considering $p<0.05$ indicative of a statistically significant difference between the mean values.

## 3. Results

### 3.1. Differences in Basal DNA Methylation and mRNA Expression of Vitellogenin in Male and Female Zebrafish

Before analyzing the effects of 17α-ethynylestradiol (EE2) on feminization in zebrafish we first assessed the basal differences in DNA methylation of *vtg1* promoter and the mRNA expression between adult male and female zebrafish livers. A significant difference in basal methylation was observed between male and female for *vtg1* at all three CpG sites assessed (-1873, -1846 and -1828). In general DNA methylation at these

sites in females was about 50% lower than that in males (**Figure 4.2A**). The decrease in methylation at these sites in females corresponded to increased *vtg1* mRNA. As expected, the mRNA expression of *vtg1* was thousands of folds higher in the female livers as compared to males (**Figure 4.2B**).

### 3.2. Effect of EE2 on DNA Methylation and mRNA Expression of Vitellogenin in Male Zebrafish

A time-dependent analysis of DNA methylation and mRNA expression of *vtg1* was performed in the adult male zebrafish exposed to 20 ng/L EE2 in a static-renewal system. The percent DNA methylation of all three CpG sites analyzed did not change until after 4 days of exposure (**Figure 4.3**). DNA methylation decreased by at least 35% after 7 days of exposure (**Figure 4.3A-C**). DNA methylation had decreased by 50% after 14 days of exposure at all CpG sties analyzed. Interestingly, the level of DNA methylation did not return to control levels even after 7 days of withdrawal from EE2 exposure by placement in clean water (**Figure 4.3A-C**). In contrast to DNA methylation, significant increases in *vtg1* mRNA expression were observed in the EE2-exposed male zebrafish as early as 0.25 days (**Figure 4.3D**). Unlike what was observed with DNA methylation, removal of EE2 at day 7 resulted in a decrease in *vtg1* mRNA back to controls levels after 7 days (**Figure 4.3D**).

DNA methylation was also assessed in adult female zebrafish exposed to 20 ng/L EE2 for 14 days. Exposure of female fish to EE2 did not result in changes in CpG methylation at the analyzed sites (**Figure 4.4A**). Interestingly, there was still an increase in *vtg1* mRNA expression, as compared to controls, but the magnitude of this increase was thousands of folds lower than that observed in exposed males (**Figure 4.4B**).

A comparison of the percent DNA methylation of the *vtg1* promoter of males exposed to EE2 for 14 days demonstrated levels of methylation similar to that detected in female controls (**Figure 4.5A**). While level of DNA methylation at these CpG sites was similar, the *vtg1* mRNA expression was significantly higher in the males exposed to EE2 for 14 days as compared to the female controls (**Figure 4.5B**).

**Table 4.1.** TruSeqHT fusion stubs and locus-specific primers for TGBS and qRT-PCR.

| Locus | Forward primer (5'→3') | Reverse primer (5'→3') | $T_m$ ($^0$C) | Product size ( bp) | Reference |
|---|---|---|---|---|---|
| TGBS | | | | | |
| TruSeqHT fusion | iTru R1: ACACTCTTTCCCTACACGACGCTCTTCCGATCT | iTru R2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT | | | |
| zf-vtg1 | AGAGGGAGGAGAGGAATTTA | CTCAACACCATAAAACTCCTCCTTATATCC | 53 | 248 | Stromqvist *et al* 2010 |
| qRT-PCR | | | | | |
| zf-vtg1-qRT | CATCTTATGCTGGTGATTTGTCA | CTTGAGCTTGAACTTGAACTTGA | 55.5 | 478 | Eide *et al* 2014 |
| zf-ef1α-qRT | CTGGAGGCCAGCTCAAACAT | ATCAAGAAGAGTAGTACCGCTAGCATTAC | 55.5 | 87 | Tang *et al* 2007 |

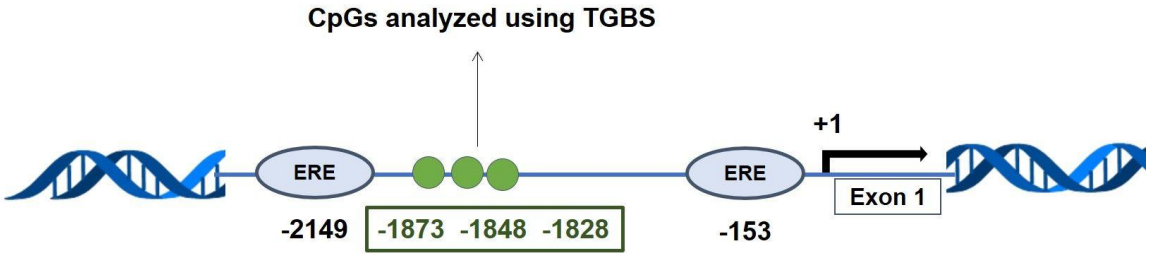


**Figure 4.1.** Schematic of vitellogenin (*vtg1*) gene organization highlighting the sites analyzed by targeted gene bisulfite sequencing (TGBS)**.** The CpG sites analyzed, at 1828, 1846 and 1873 bp upstream of the exon 1 of *vtg1*, are considered to be located between two hypothetical estrogen responsive elements (ERE) in the zebrafish *vtg1* promoter (Stromqvist *et al.*, 2010).

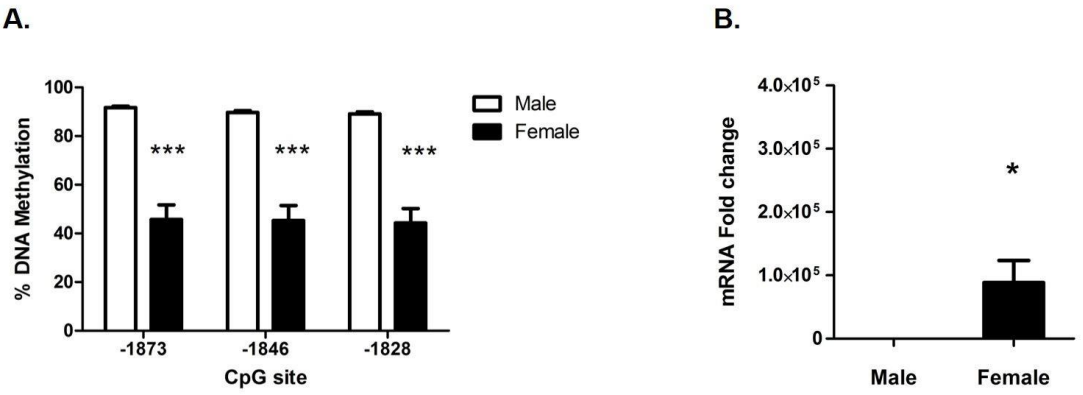


**Figure 4.2.** Comparison of *vtg1* DNA methylation and mRNA expression between male and female zebrafish livers. Differences in basal levels of **A**) DNA methylation at the CpG sites 1828, 1846 and 1873 bp upstream of exon 1 of the vitellogenin promoter (*vtg1*) represented as percent methylation in male and female zebrafish livers. Bisulfite converted DNA from the zebrafish livers were sequenced by TGBS on an Illumina MiSeq platform and analyzed using Bismark bisulfite mapper. **B**) Basal mRNA expression of *vtg1* represented as $2^{-\Delta\Delta Ct}$ fold change between male and female zebrafish livers normalized to the housekeeping gene, elongation factor 1 alpha (ef1α). The number of fish was greater than or equal to 4 for each treatment group (n≥4). Data are represented as the mean ± standard error of the mean (SEM). *P<0.05 and ***P≤0.0005 as compared with male counterparts.

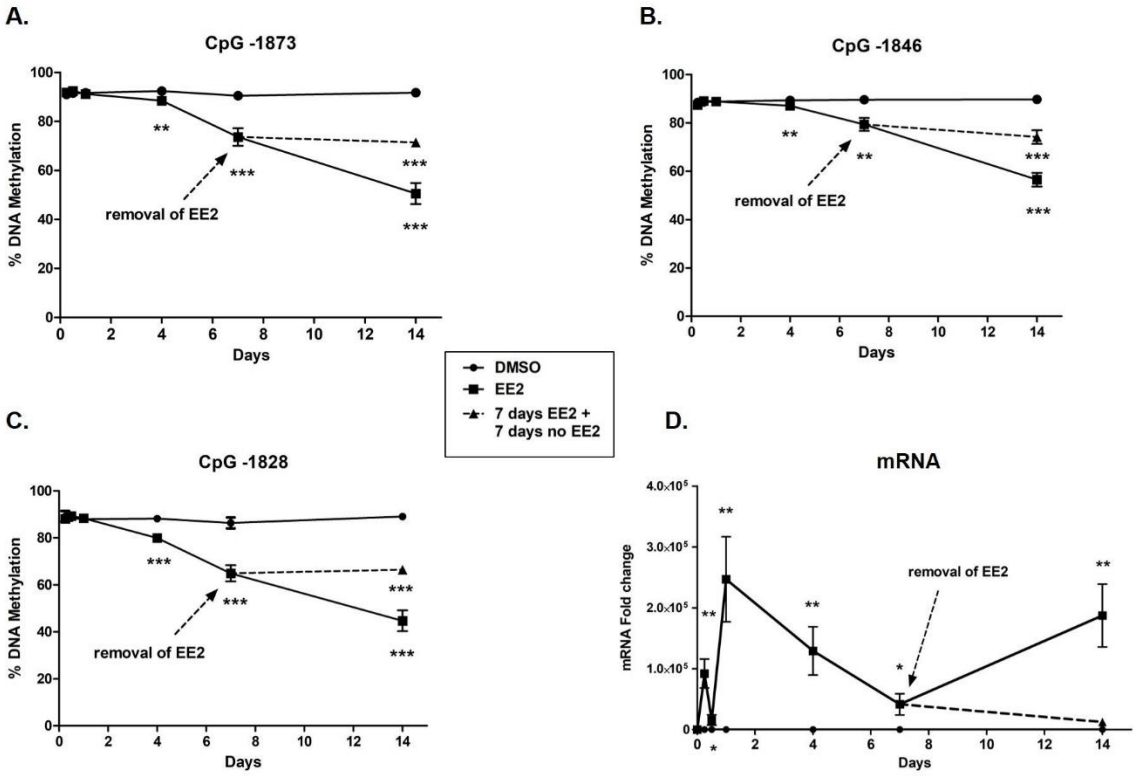


**Figure 4.3.** Time-dependent changes in DNA methylation and mRNA expression of vitellogenin in adult male zebrafish exposed to 20 ng/L EE2. The number of fish was greater than or equal to 4 for each treatment group (n≥4). Fish were euthanized and the livers dissected at 0.25, 0.5, 1, 4, 7 and 14 days. Percent DNA methylation at the **A**) CpG site 1873 bp, **B**) CpG site 1846 bp and **C**) CpG site 1828 bp upstream of exon 1. **D**) mRNA fold change compared to DMSO control fish normalized to elf1α. *P<0.05, **P≤0.008 and ***P≤0.0005 as compared with DMSO control of the respective time points.

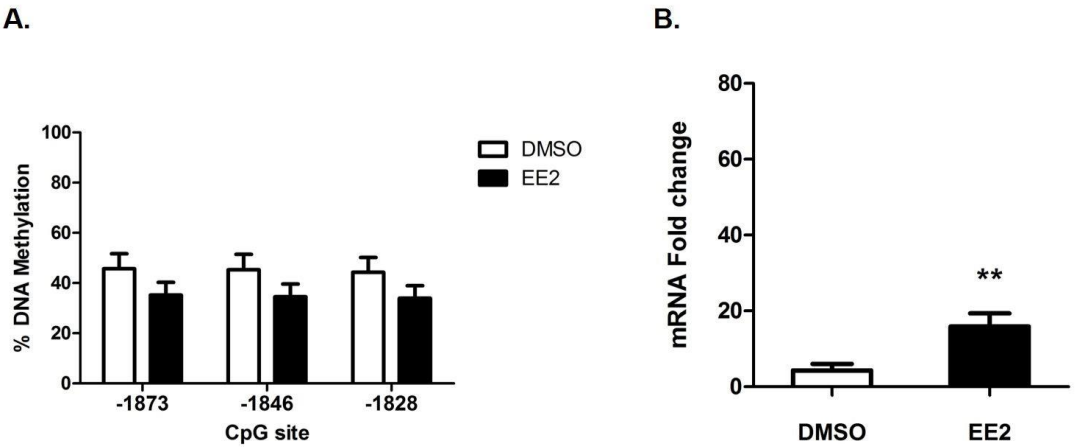


**Figure 4.4.** Effect of 20ng/L EE2 on adult female zebrafish after 14 days of exposure. **A)** Percent methylation changes of CpG sites located at -1873, -1846 and -1828 bp upstream of exon1 of *vtg1* promoter. **B)** changes in mRNA expression of *vtg1* in the same conditions as normalized to ef1α. The number of fish was greater than or equal to 4 for each treatment group (n≥4). **P<0.008 compared with DMSO control.

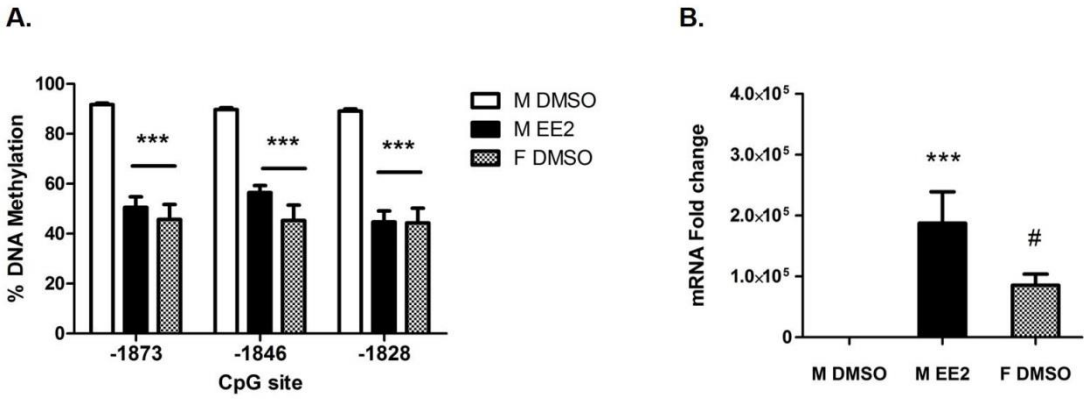


**Figure 4.5.** Comparison of *vtg1* DNA methylation and mRNA expression between EE2-exposed male and control female zebrafish livers: **A**) DNA methylation at three CpG sites in the *vtg1* promoter 1802 bp upstream of exon 1 and **B**) mRNA expression of *vtg1* between control males (white bar), EE2-exposed males (black bar) and control female zebrafish (grey bar) after 14 days. The number of fish was greater than or equal to 4 for each treatment group ($n \geq 4$). ***$P \leq 0.0005$ compared with male control and $^{\#}P < 0.05$ compared with EE2-exposed male.

## 4. Discussion

Aberrant expression of vitellogenin (Vtg) in male fish has been predominantly used as a molecular marker of exposure to estrogenic EDCs, and is also a marker for feminization in fish (Sumpter *et al.*, 1995). However, the mechanisms mediating Vtg induction are yet to be fully understood. This study tested the hypothesis that induction of Vtg upon exposure to estrogenic compounds corresponds to demethylation of the *vtg1* gene. The CpG sites analyzed in this region were mapped to two hypothetical estrogen response elements (EREs) at 1828, 1846 and 1873 bp upstream of exon 1 in *vtg1* gene (Stromqvist *et al.*, 2010). Further, these data also showed that differences in DNA methylation exist between male and females in the absence of any EE2. The 50% lower levels of DNA methylation at all CpG sites analyzed in females from control fish correlated to differences in *vtg1* mRNA expression, as compared to unexposed males.

In general, promoter demethylation plays a crucial role in activating gene expression (Phillips, 2008). In agreement with this phenomenon, decreases in DNA methylation correlated to increases in *vtg1* mRNA expression. Further, the differences in the basal methylation at these CpG sites between male and female zebrafish, also agree with differences in basal expression of *vtg1* mRNA. The lower *vtg1* methylation in females might be facilitating the accessibility of the proximal EREs to the transcription factors like estrogen receptor (ER), further transcriptionally activating the gene. The opposite is true for males in that the increased methylation at these sites may block the access for transcription factors and inhibit mRNA expression.

Exposure of male fish, but not female fish, to EE2 significantly decreased *vtg1* DNA methylation at the analyzed CpG sites, and increased mRNA expression. The lack

of responses in female fish at these CpG sites may be due to the fact *vtg1* transcription is ongoing and that EE2 is simply activating responses in males that are already ongoing in females. It may also be the case that the transcriptional response is maximal, or saturated. Thus, additional stimuli may not result in a linear decrease in methylation. In contrast, despite no decrease in DNA methylation, EE2 still increased *vtg1* mRNA levels in female fish. These data suggest that vtg1 mRNA expression in both male and females may be mediated by multiple sites. Further, the time-dependent studies directly show that mRNA expression can be regulated by mechanisms independent of changes in DNA methylation at the CpG sites analyzed. This is supported by the fact that DNA methylation was not altered in males until after 4 days of EE2 exposure, while increases in *vtg1* mRNA occurred as early as 0.25 days after exposure.

The dissociation between changes in mRNA expression and DNA methylation during the early periods of exposure to EE2 is thought-provoking. Some effects of estrogens are known to be rapid enough to depend on non-genomic mechanisms instead of RNA and protein synthesis (Levi *et al.*, 2009). A review by Bjornstrom *et al.*, 2005 discusses the non-genomic mechanisms of gene regulation by ER. These include protein-protein interactions as well as other DNA-binding transcription factors. In yeast cells, ER binding lead to hypermethylation of the regions flanking EREs independent of transcription induction (Kladde *et al.*, 1996). It is quite possible that the same phenomenon is ongoing in zebrafish. Future studies analyzing a broader portion of *vtg1* promoter could help address this correlation. It would also be interesting to correlate EE2-induced *vtg1* expression to histone modifications at the *vtg1* promoter.

The fact that DNA methylation is not decreased by EE2 until later time points correlates to the fact that Vtg protein expression increases significantly later than mRNA expression. For example, Vtg protein expression was induced in male fathead minnows after 15 days of exposure to 50 ng/L EE2, whereas mRNA is induced only after 3 days (Schmid *et al.*, 2002). This suggest that changes in CpG methylation would correlate more closely to protein induction than mRNA changes. Future experiments assessing Vtg protein expression are needed to address this hypothesis. We did not conduct such experiments in this study as the livers isolated were used primarily for DNA methylation and mRNA analysis.

The fact that withdrawal of EE2 decreased *vtg1* mRNA levels almost immediately, but did not result in a return of DNA methylation to control levels further supports the hypothesis that these two events are not directly linked. The stable maintenance of decreases in DNA methylation supports the conclusion that this change is a possible epigenetic change, i.e. one that persist in the absence or removal of stimuli. The persistence of these changes may also facilitate long-term induction of Vtg in the absence of EE2. This hypothesis is supported by Schmid *et al.*, 2002 who reported a similar pattern that the Vtg protein induction stayed stable and the mRNA did not when EDC were removed. A multigenerational study is a mandate to further confirm stability of these changes.

Regardless if changes in DNA methylation at the CpG sites analyzed are mediating *vtg1* mRNA or protein expression, the major finding from this study is that EE2 changes the methylation profile of male zebrafish liver to that of female controls. This suggest that the EE2-induced changes in methylation of these CpGs could be novel markers for

feminization. Even more striking is the persistence of these changes, which suggests that they can be used to identify previous EE2 exposure. Thus, it possible that these changes in methylation can be used as a biomarker of environmental exposure. Such a marker would have benefits over mRNA due to the persistence, enabling screening for previous exposure. Further studies are needed to determine the stability of these changes in DNA methylation, including transgenerational studies. Studies are also needed determining the effect of repeated exposure on these epigenetic marks.

In conclusion, we used a novel approach to identify changes in DNA methylation of CpG sites located in the promoter of a gene known to induce feminization in zebrafish. These data identify specific molecular changes induced by a prominent EDC and demonstrate that these changes are persistent. Hence, these data identify a novel epigenetic change in male zebrafish livers that EE2 changed the epigenetic landscape of males comparable to that of the females. These novel findings give insights into the molecular mechanisms of action of estrogenic EDCs and suggest that epigenetic changes may have a prominent role in regulating *vtg1* mRNA expression.

**Disclaimer:** The opinions presented here are those of the author and do not represent official policy of the US EPA.

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# CHAPTER 5

## SUMMARY

The role of epigenetic mechanisms in gene expression has been under study since the mid-1900s (Waddington, 1942, Holliday *et al.*, 1975). Understanding the role of the same in toxicant-induced alterations of gene expression is key to estimating risk of adverse and long-term effects of toxicants. Epigenetic endpoints are abundantly studied in cancer biology in terms of chemical and non-chemical carcinogenesis. Various chemical-induced cancers have showed abnormalities in epigenetic landscapes (Phillips *et al.*, 2008, Hoffmann *et al.*, 2001). The non-cancer endpoints of epigenetic toxicants are gradually gaining emphasis due to advance in technology and decrease in analysis costs. The gaps-in-knowledge on toxicant-induced epigenetic effects on ecological systems is even larger than that in human health data.

Epigenetic changes could serve as surrogate markers where environmental exposure samples have short half-lives and low biological doses (Ladd-Acosta *et al.*, 2016). They accumulate gradually and hence provide for early detection strategies for risk prediction (Pashayan *et al.*, 2016). However, the plasticity of epigenome across cell types, developmental stage, age and environmental exposures makes it a challenge to incorporate epigenetic endpoints in risk assessment. Typically, unrealistically high concentrations of toxicants are already regulated and assessed by morphological endpoints that are cheaper to assess. Due to the cumulative nature of epigenetic

endpoints at lower concentrations, it is key to narrow down the dose spectrum and investigate environmentally relevant doses for both mechanistic and cost advantages.

With regards to human health, we investigated the epigenetic effects of the water disinfection byproduct bromate ($BrO_3^-$) on renal cells. Our previous findings showed that $BrO_3^-$ induced expression of the cyclin dependent kinase inhibitor p21 at environmentally relevant non-toxic concentrations after sub-chronic treatment of human and rat renal cells (Scholpa *et al.*, 2014). We also reported that p21 is induced by the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (5-Aza) and the histone deacetylase inhibitor trichostatin A (TSA) in these cells. Based on this groundwork, we hypothesized that $BrO_3^-$ -induced p21 expression is mediated via DNA methylation and histone acetylation.

To address this hypothesis, we first assessed differential CpG methylation of p21 across species. We observed that rat and human p21 differ significantly in their DNA methylation within their respective promoter regions indicating that the basal epigenetic landscape of rat and human significantly differ, at least for p21. These data suggest caution when comparing epigenetic data for this gene between the two species. The fact that sub-chronic exposure of cells to $BrO_3^-$ (0.001 – 10 ppm) did not alter DNA methylation at the human and rat p21 promoter and coding regions suggests that $BrO_3^-$ induced renal p21 expression is not mediated by promoter hypomethylation, at least at the sites studied. However, since 5-Aza decreased the percent methylation at the human p21 transcription factor binding site, sis-inducible element (SIE1), it is clear that SIE1 is an epigenetic regulatory site for p21 expression and $BrO_3^-$ induced p21 in renal cells does not involve SIE1. We also showed in our withdrawal studies that the hypomethylation induced by 5-Aza was a stable epigenetic mark. It is quite possible that changes in methylation at sites

distant from SIE1 or even from the promoter region mediate p21 expression. This is the basis for future studies to analyze a broader target region of the gene to identify probable regulatory CpGs.

Analysis of histone acetylation at p21 promoter showed that $BrO_3^-$ increased acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) in the rat p21 promoter region, but that similar results were not seen for human p21. Unlike promoter hypomethylation, the H3K9/14 Ac mark was not persistent. This transient increase in acetylation occurred at non-toxic concentrations of $BrO_3^-$, suggesting that p21 induction after exposure to $BrO_3^-$ is a protective rather than a toxic response. These data further supports the hypothesis that epigenetic regulation of p21 is species-dependent. The lack of H3K9/14 Ac in human p21 promoter in response to $BrO_3^-$ may be a result of alterations of other histone marks like lysine methylation, arginine methylation and citrullination, serine, tyrosine or threonine phosphorylation. We now know that, at least at the sites and marks analyzed, $BrO_3^-$ induces renal p21 expression via histone hyperacetylation and not promoter hypomethylation in rats and that these marks were not altered by $BrO_3^-$ in humans. Such data will be important into understanding the molecular mechanisms mediating the renal protective effect of p21 against toxicants and suggest an uncertainty in extrapolating rat epigenomic data for assessing the risk of toxicants in humans.

In the ecological perspective, we investigated the epigenetic effects of 17α-ethynylestradiol (EE2) in zebrafish. EE2 is an estrogenic endocrine disrupting chemical (EDC) used in oral contraceptives. It is known that EE2 induces the expression of the egg yolk precursor protein vitellogenin (Vtg), which is a well-known molecular marker for estrogenic exposure in male fish. In spite of this popularity, the mechanisms behind

estrogenic induction of Vtg are understudied. We hypothesized that this induction is mediated via DNA methylation. To address this hypothesis, we first assessed differences in the basal methylation of vitellogenin promoter (*vtg1*) between male and female zebrafish. We observed 50% lower DNA methylation in female livers compared to that of males, at the CpG sites analyzed. These levels correlated to *vtg1* mRNA levels. These data indicate that the lower *vtg1* methylation in females might be the regulatory mechanism behind expression of the gene. In general, promoter hypomethylation facilitates the accessibility of the transcription factors like estrogen receptor (ER) to the specific DNA recognition sequence like the estrogen receptor element (ERE) for transcriptionally activating the gene.

Exposure of male fish to EE2 significantly decreased in *vtg1* DNA methylation at the analyzed CpG sites, and increased mRNA expression. This demethylation was not observed in female fish. This might be due to the fact that *vtg1* in females is actively transcribing even before the exposure to EE2 and fewer fold increase in mRNA expression indicates a saturated transcriptional state of the gene. The fact that mRNA expression was induced as early as 0.25 days after EE2 exposure and promoter hypomethylation after 4 days, suggests that *vtg1* mRNA can be regulated by mechanisms independent of changes in DNA methylation at the CpG sites analyzed. This suggests methylation changes at other distal sites of the promoter and coding regions. It also suggests non-genomic mechanisms of ER pathway involving transcription factors other than the ER and other epigenetic mechanisms like histone modifications at the *vtg1* promoter. According to current literature on EE2, *vtg1* mRNA is induced significantly earlier than Vtg protein suggesting that changes in CpG methylation observed would

correlate more closely to protein induction than mRNA. Future studies are needed to assess Vtg protein levels to address this hypothesis.

The fact that decreases in DNA methylation were stably maintained after the recovery of zebrafish from EE2, supports the conclusion that this change is a true epigenetic change. The persistence of these changes may also facilitate long-term induction of Vtg in the absence of EE2. This persistence suggests that the CpG sites can be used to identify previous EE2 exposure of fish. This might also help establish if the ecosystem was previously polluted with EDCs. However, a multigenerational study is a mandate to further confirm stability of these changes. Shaw *et al.*, 2017 suggest at least a four-generation study to understand the stability of the marks. This would include a generation or more of non-exposed offspring (F3+). The lack of understanding of such transgenerational effects is a key limitation in applying epigenetic data in ecological risk assessment.

We also observed that EE2 exposure changed the methylation profile of male zebrafish liver to that of female controls. This identifies these CpG sites as novel markers for feminization. However, further studies are needed to determine if EE2 induces these epigenetic changes at lower doses after repeated exposure.

The mechanistic studies presented above aid in the understanding of the role of epigenetic data in environmental risk assessment. As discussed, epigenetic marks tend to accumulate and might not lead to adverse effects instantaneously. Hence, it is important to investigate environmentally relevant concentrations of toxicants at the sub-chronic and chronic levels of exposure for studying epigenetic endpoints. It is also important to follow epigenetic changes after withdrawal of the stressor to determine the

persistence of the toxicant-induced changes. These concepts hold true for both human health and ecological risk assessment. Epigenetic data in toxicological studies is at the early stages of identifying various toxicant-induced effects and building causal relationships. The above discussed limitations need to be addressed before incorporating epigenetic data from mechanistic studies into risk assessment.

APPENDIX

INSTRUCTION FOR RUNNING BISMARK IN VIRTUALBOX

**1. System requirements**

- A minimum of 2GB RAM for VirtualBox installation with a preferred system total RAM of 8GB.
- A minimum disk storage of 10GB.
- Make sure the “Virtualization Technology” is “enabled” in the system “bios”.

**2. VirtualBox installation**

- Download the available VirtualBox version from https://www.virtualbox.org/wiki/Downloads for Windows hosts or any suitable host operating system.
- Follow the instructions and launch VirtualBox.
- Once launched, go to the “settings” menu of the VirtualBox and select the options “type: Linux and version: Fedora 64”.

**3. Download image file containing Bismark tool-set**

- Open the “Mozilla Firefox” within the VirtualBox and go to https://drive.google.com/file/d/0B8YCoq3MYnhKN3RJMllQcUh3Y0k/view?usp=sharing.
- Download the “TGBS.ova” file and save it on the VirtualBox desktop.
- In the VirtualBox Manager, go to “File” and “Import Appliance” and browse for the location of the “TGBS.ova” file.

### 3. Run the VirtualMachine

- Start the “TGBS machine”. If error occurs, go to “Settings”, select “USB” and disable the “USB controller”. Then, start the “TGBS machine” again.
- Use password TGBSKolli for the User login.
- Click on the “Activities” tab and open “Files”.
- The home directory includes the Bismark software version 0.17.0 and the required tools like ActivPearl. It also includes a sample fastq sequence file “SampleSeq_R1.fastq.gz”.

### 4. Download Reference Genome:

- Select “Firefox” from the “Activities” tab and go to https://www.ncbi.nlm.nih.gov/
- Select “Nucleotide” from the drop-down menu and search for “U24170.1”
- Click on “FASTA” and select the option to “Send” the “Complete Record” to the “File” destination and select “Create File” and “Save File”.
- The “Sequence FASTA” is now in the “Downloads” folder.
- Move it to Home>>DNA>>bowtie2-2.3.0>>bismark_v0.17.0>>REF.

### 5. DNA Methylation analysis

Following are the command lines for a single-end analysis using R1 forward reads.

- Note: Click on the “Activities” tab to move between the folders.

### 5.1. Prepare Reference Genome:

- Select “Terminal” from the “Activities” tab.
- The reference genome folder “REF” now contains a fasta file from NCBI with the Accession# U24170.1 for the human p21 promoter. Use the following command to

prepare the reference genome before mapping the bisulfite converted reads from the sample datafile.

bismark_genome_preparation --/home/user/DNA/bowtie2-2.3.0/ --verbose /home/user/DNA/bowtie2-2.3.0/bismark_v0.17.0/REF/

- This creates two folders within the genome folder “REF”, one with C ->T genome index and another with G ->A.

**5.2. Run Bismark:**

- Read alignment step for sequences in the R1 read file “SampleSeq_R1.fastq.gz” with a single-end approach.

  bismark --bowtie2 /home/user/DNA/bowtie2-2.3.0/bismark_v0.17.0/REF/ SampleSeq_R1.fastq.gz

- This aligns the sequence reads to the reference genome and creates a combined alignment/methylation call output in BAM format, and gives a run statistics report.
- Output files:

  SampleSeq_R1_bismark_bt2.bam

  SampleSeq_R1_bismark_bt2_SE_report.txt

**5.3. Methylation extraction:**

- Extracting methylation information out of the “.bam” file created in step 2.

  bismark_methylation_extractor -s –comprehensive SampleSeq_R1.fastq_bismark_bt2.bam

- This extracts methylation information from the alignment output of the above NGS file.
- Output files:

    SampleSeq_R1_bismark_bt2.M-bias.txt

    SampleSeq_R1_bismark_bt2.M-bias_R1.png

    SampleSeq_R1_bismark_bt2_splitting_report.txt

    CHG, CpG and CHH contexts for the SampleSeq_R1_bismark_bt2.txt

**5.4. Generate report:**

- Generating Bismark processing report on read alignment and methylation extraction.

    **bismark2report***

- This gives an overall methylation report on the total number of reads and their alignment and methylation.
- Output file:

    SampleSeq_R1_bismark_bt2_SE_report.html

**Appendix Datafile 1.** SampleSeq_R1_bismark_bt2_SE_report.txt

Bismark report for: /home/user/SampleSeq_R1.fastq.gz (version: v0.17.0)

Option '--directional' specified (default mode): alignments to complementary strands (CTOT, CTOB) were ignored (i.e. not performed)

Bismark was run with Bowtie 2 against the bisulfite genome of /home/user/DNA/bowtie2-2.3.0/bismark_v0.17.0/hp21sie1ref/ with the specified options: -q --score-min L,0,-0.2 --ignore-quals

Final Alignment report

==========

Sequences analysed in total: 30135

Number of alignments with a unique best hit from the different alignments: 10571

Mapping efficiency: 35.1%

Sequences with no alignments under any condition: 19564

Sequences did not map uniquely: 0

Sequences which were discarded because genomic sequence could not be extracted: 0

Number of sequences with unique best (first) alignment came from the bowtie output:

CT/CT: 10571 ((converted) top strand)

CT/GA: 0 ((converted) bottom strand)

GA/CT: 0 (complementary to (converted) top strand)

GA/GA: 0 (complementary to (converted) bottom strand)

Number of alignments to (merely theoretical) complementary strands being rejected in total: 0

Final Cytosine Methylation Report

==========

Total number of C's analyzed: 829618

Total methylated C's in CpG context: 18365

Total methylated C's in CHG context: 647

Total methylated C's in CHH context: 2294

Total methylated C's in Unknown context: 0

Total unmethylated C's in CpG context: 13334

Total unmethylated C's in CHG context: 189351

Total unmethylated C's in CHH context: 605627

Total unmethylated C's in Unknown context: 0

C methylated in CpG context: 57.9%

C methylated in CHG context: 0.3%

C methylated in CHH context: 0.4%

Can't determine percentage of methylated Cs in Unknown context (CN or CHN) if value was 0

Bismark completed in 0d 0h 0m 19s

**Appendix Datafile 2.** SampleSeq_R1_bismark.bt2.M-bias.txt

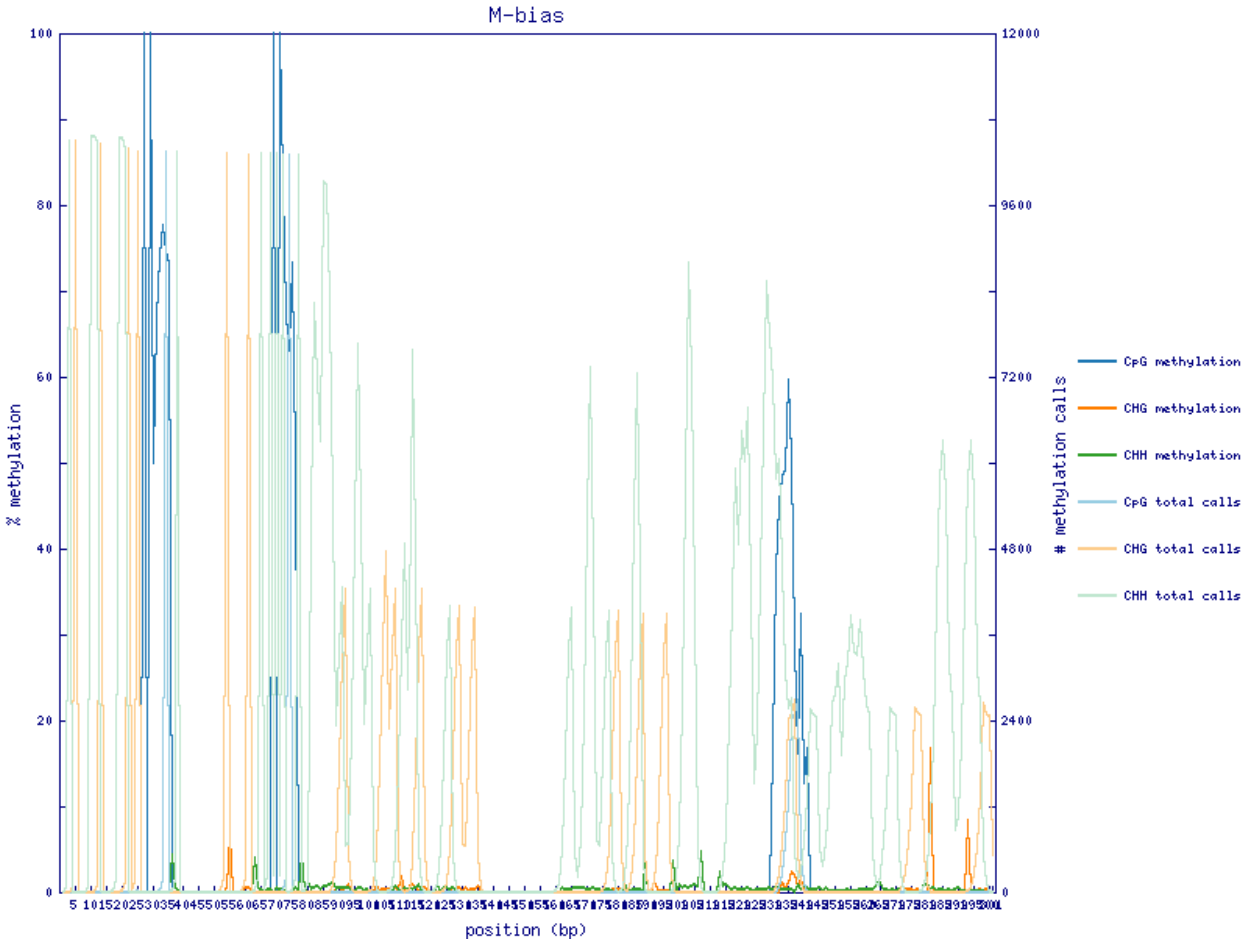
CpG context

==========

| position | | count methylated | | count unmethylated | % methylation | coverage |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | | 0 | | |
| 2 | 0 | 0 | | 0 | | |
| 3 | 0 | 0 | | 0 | | |
| 4 | 0 | 0 | | 0 | | |
| 5 | 0 | 0 | | 0 | | |
| 6 | 0 | 0 | | 0 | | |
| 7 | 0 | 0 | | 0 | | |
| 8 | 0 | 0 | | 0 | | |
| 9 | 0 | 0 | | 0 | | |
| 10 | 0 | 0 | | 0 | | |
| 11 | 0 | 0 | | 0 | | |
| 12 | 0 | 0 | | 0 | | |
| 13 | 0 | 3 | 0.00 | 3 | | |
| 14 | 0 | 0 | | 0 | | |
| 15 | 0 | 0 | | 0 | | |
| 16 | 0 | 0 | | 0 | | |
| 17 | 0 | 0 | | 0 | | |
| 18 | 0 | 0 | | 0 | | |
| 19 | 0 | 0 | | 0 | | |

| | | | | |
|---|---|---|---|---|
| 20 | 0 | 0 | 0 | |
| 21 | 0 | 0 | 0 | |
| 22 | 0 | 3 | 0.00 | 3 |
| 23 | 0 | 0 | 0 | |
| 24 | 0 | 0 | 0 | |
| 25 | 0 | 13 | 0.00 | 13 |
| 26 | 0 | 0 | 0 | |
| 27 | 1 | 0 | 100.00 | 1 |
| 28 | 0 | 0 | 0 | |
| 29 | 1 | 0 | 100.00 | 1 |
| 30 | 2 | 2 | 50.00 | 4 |
| 31 | 4 | 2 | 66.67 | 6 |
| 32 | 17 | 6 | 73.91 | 23 |
| 33 | 136 | 39 | 77.71 | 175 |
| 34 | 7696 | 2639 | 74.47 | 10335 |
| 35 | 11 | 4 | 73.33 | 15 |
| 36 | 0 | 2 | 0.00 | 2 |
| 37 | 0 | 0 | 0 | |

**Appendix Datafile 3.** SampleSeq_R1_bismark_bt2.M-bias_R1.png



**Appendix Datafile 4:** CpG_context_SampleSeq_R1_bismark_bt2.txt

Bismark methylation extractor version v0.17.0

M02849:171:000000000-ANHND:1:1101:12957:2144_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 499 Z

M02849:171:000000000-ANHND:1:1101:12957:2144_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 539 Z

M02849:171:000000000-ANHND:1:1101:12957:2144_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 703 Z

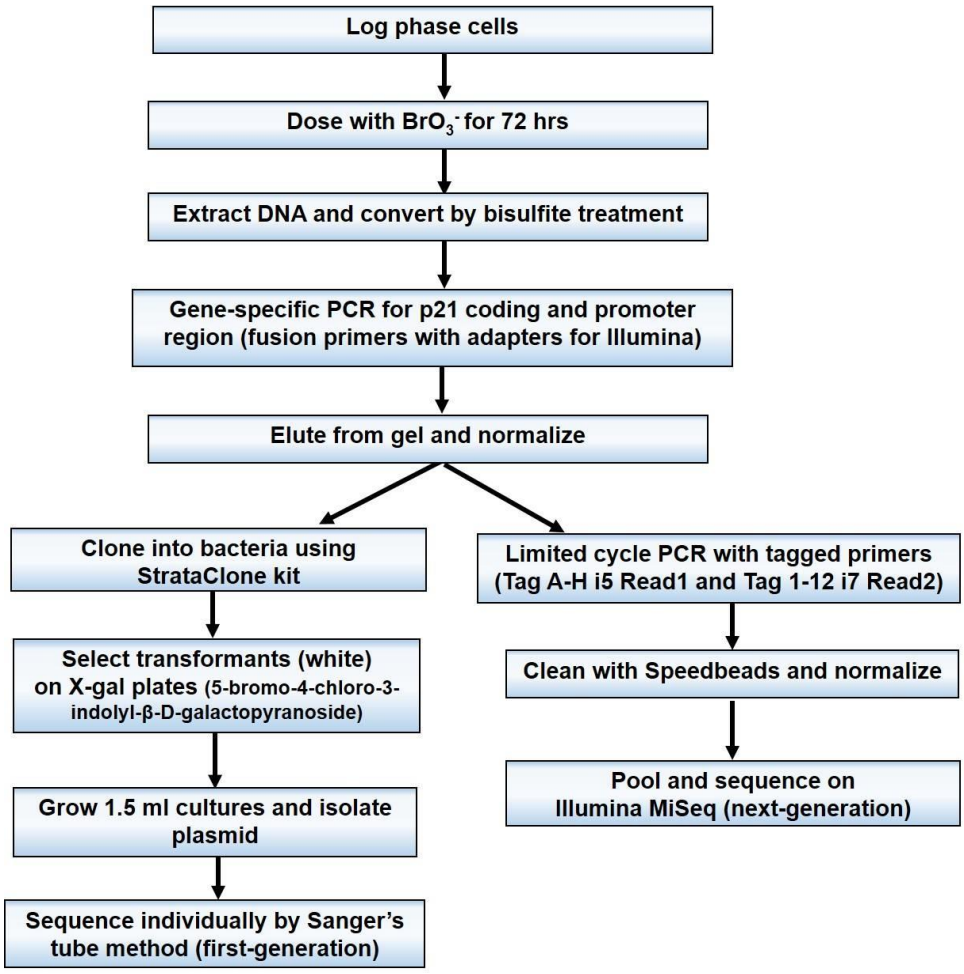
M02849:171:000000000-ANHND:1:1101:17290:2154_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 499 Z

M02849:171:000000000-ANHND:1:1101:17290:2154_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 539 Z

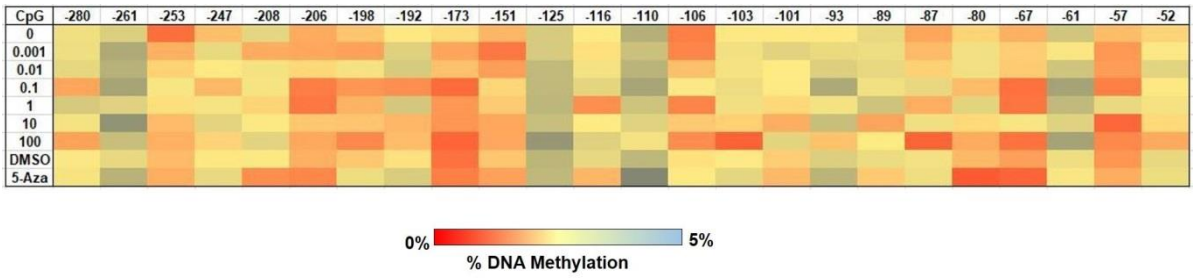
M02849:171:000000000-ANHND:1:1101:17290:2154_1:N:0:102 - gi|902576|gb|U24170.1|HSU24170 703 z

M02849:171:000000000-ANHND:1:1101:19751:2237_1:N:0:102 - gi|902576|gb|U24170.1|HSU24170 499 z

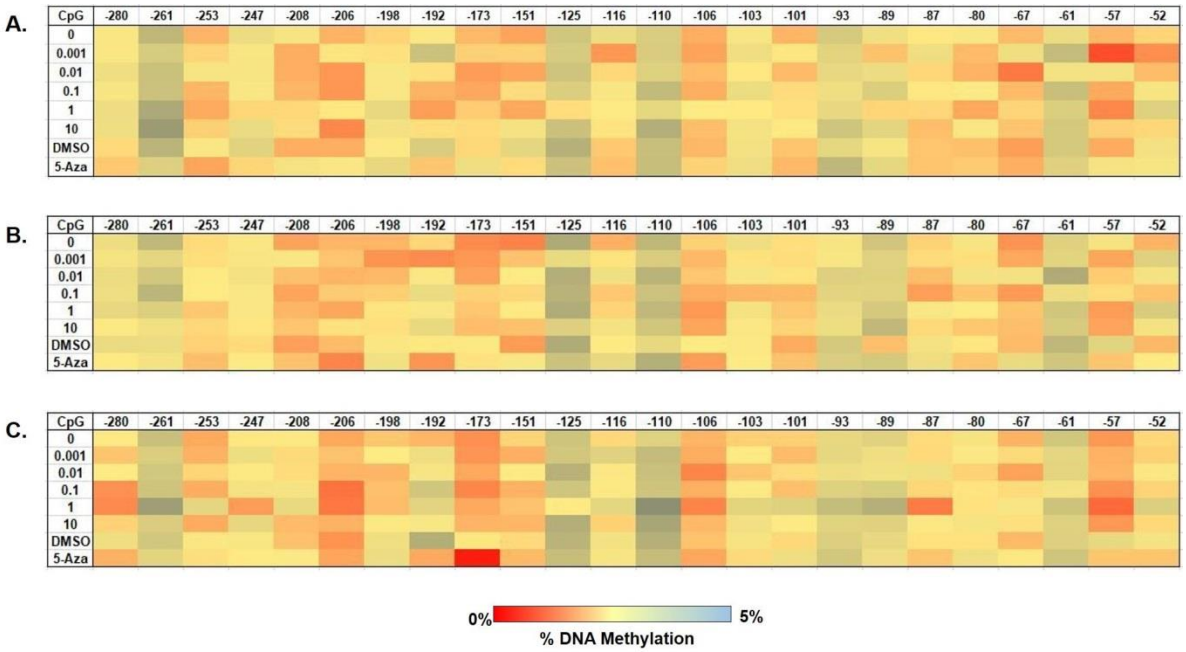
M02849:171:000000000-ANHND:1:1101:19751:2237_1:N:0:102 + gi|902576|gb|U24170.1|HSU24170 539 Z



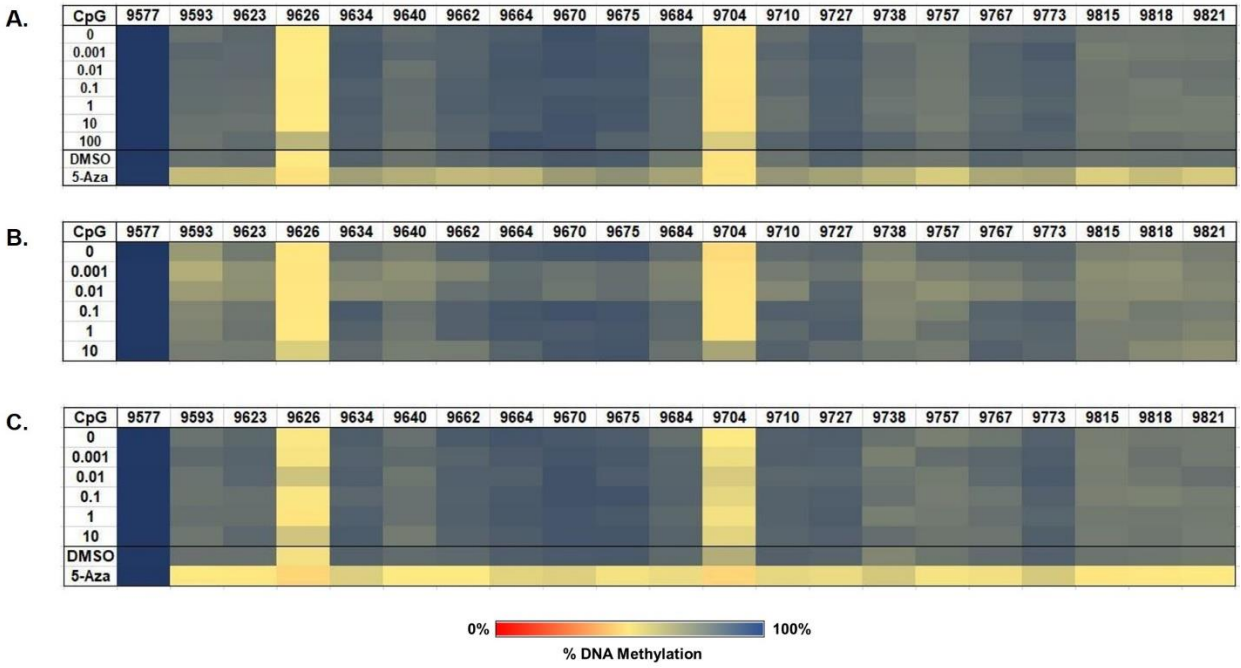

**Appendix Figure 1**: Schematic for comparing the workflows of first-generation Sanger's and next-generation targeted gene bisulfite sequencing (TGBS) methods.



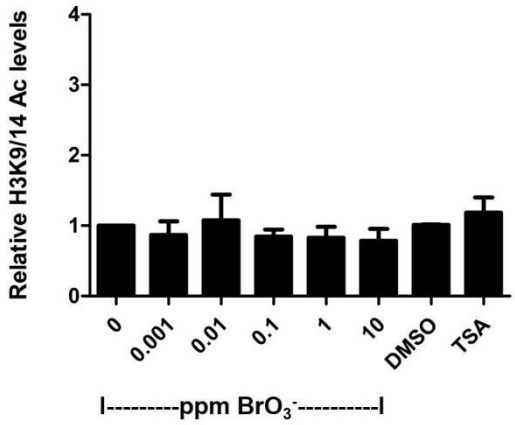

**Appendix Figure 2.** Effects of acute $BrO_3^-$ exposure on the percent DNA methylation of cytosine residues at the transcription start site of human p21 promoter (hp21-TSS). Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the hp21-TSS. The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. The first column shows the treatments of HEK293 cells water (0), 0.001 to 100 ppm $BrO_3^-$, DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for 72 hrs. Heat map intensity is shown in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%.




**Appendix Figure 3.** Effects of sub-chronic $BrO_3^-$ exposure on the percent DNA methylation of cytosine residues at the transcription start site of human p21 promoter (hp21-TSS). Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the hp21-TSS. The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. The first column shows the exposure of HEK293 cells to water (0), 0.001 to 10 ppm $BrO_3^-$, DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for **A)** 9 days, **B)** 18 days and **C)** 9 days of withdrawal. Heat map intensity is shown in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%.




**Appendix Figure 4.** Effects of sub-chronic $BrO_3^-$ exposure on the percent DNA methylation of cytosine residues at the coding region of rat p21 (rp21-coding). Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the rp21-coding. The first row represents the chromosomal position of the cytosine in the CpG dinucleotide context. The first column shows the exposure of NRK cells to water (0), 0.001 to 10 ppm $BrO_3^-$, DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for **A)** 9 days, **B)** 18 days and **C)** 9 days of withdrawal. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 100%. Data are represented as the mean ± SEM values of three independent experiments (n=3).




**Appendix Figure 5.** Recovery of $BrO_3^-$ -induced acetylation of histone H3 lysine 9 and 14 (H3K9/14 Ac) of the rat p21 promoter region after 3 days following 18 days of sub-chronic exposure of NRK cells to 0.001 – 10 ppm $BrO_3^-$. Data are represented as relative fold increase in H3K9/14 Ac levels as normalized to GAPDH as determined using ChIP assays. Data are represented as mean ± SEM of three different passages (n=3).