Table 4.5 Jak/Stat Mouse Gene IDs with Human Homologs that Interact with BPA

GeneID	Symbol	Description
11651	*Akt1*	thymoma viral proto-oncogene 1
12443	*Ccnd1*	cyclin D1
12983	*Csf2rb*	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
328572	*Ep300*	E1A binding protein p300
14784	*Grb2*	growth factor receptor bound protein 2
16451	*Jak1*	Janus kinase 1
15170	*Ptpn6*	protein tyrosine phosphatase, non-receptor type 6
20662	*Sos1*	son of sevenless homolog 1 (Drosophila)
20850	*Stat5a*	signal transducer and activator of transcription 5A
20851	*Stat5b*	signal transducer and activator of transcription 5B

Table 4.6 Mapk Mouse Gene IDs with Human Homologs that Interact with BPA

GeneID	Symbol	Description
11651	*Akt1*	thymoma viral proto-oncogene 1
12367	*Casp3*	caspase 3
12675	*Chuk*	conserved helix-loop-helix ubiquitous kinase
14103	*Fasl*	Fas ligand (TNF superfamily, member 6)
14673	*Gna12*	guanine nucleotide binding protein, alpha 12
14784	*Grb2*	growth factor receptor bound protein 2
15507	*Hspb1*	heat shock protein 1
16150	*Ikbkb*	inhibitor of kappaB kinase beta
16476	*Jun*	jun proto-oncogene
23938	*Map2k5*	mitogen-activated protein kinase kinase 5
26399	*Map2k6*	mitogen-activated protein kinase kinase 6
26401	*Map3k1*	mitogen-activated protein kinase kinase kinase 1
26409	*Map3k7*	mitogen-activated protein kinase kinase kinase 7
26412	*Map4k2*	mitogen-activated protein kinase kinase kinase kinase 2
26415	*Mapk13*	mitogen-activated protein kinase 13
50772	*Mapk6*	mitogen-activated protein kinase 6
26419	*Mapk8*	mitogen-activated protein kinase 8
26420	*Mapk9*	mitogen-activated protein kinase 9
17165	*Mapkapk5*	MAP kinase-activated protein kinase 5
17346	*Mknk1*	MAP kinase-interacting serine/threonine kinase 1
17869	*Myc*	myelocytomatosis oncogene
18479	*Pak1*	p21 protein (Cdc42/Rac)-activated kinase 1
18750	*Prkca*	protein kinase C, alpha
19353	*Rac1*	RAS-related C3 botulinum substrate 1
76089	*Rapgef2*	Rap guanine nucleotide exchange factor (GEF) 2
19697	*Rela*	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
20111	*Rps6ka1*	ribosomal protein S6 kinase polypeptide 1
20112	*Rps6ka2*	ribosomal protein S6 kinase, polypeptide 2
56613	*Rps6ka4*	ribosomal protein S6 kinase, polypeptide 4
20662	*Sos1*	son of sevenless homolog 1 (Drosophila)
21808	*Tgfb2*	transforming growth factor, beta 2
21813	*Tgfbr2*	transforming growth factor, beta receptor II

Figure 4.1 Mouse and Human Genes Altered by BPA Exposure in the Jak/Stat Signaling Pathway. Unique mouse microarray gene IDs involved in Jak/Stat signaling that overlap with human JAK/STAT gene IDs with known interactions with BPA in The Comparative Toxicogenomics Database

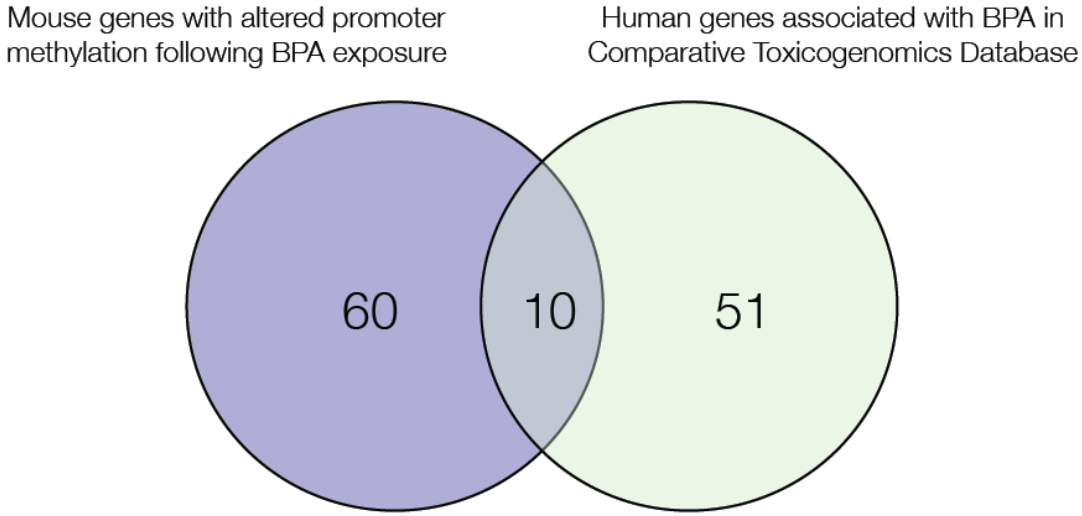


Figure 4.2 Mouse and Human Genes Altered by BPA Exposure in the Mapk Signaling Pathway. Unique mouse microarray gene IDs involved in Mapk signaling that overlap with human MAPK gene IDs with known interactions with BPA in The Comparative Toxicogenomics Database

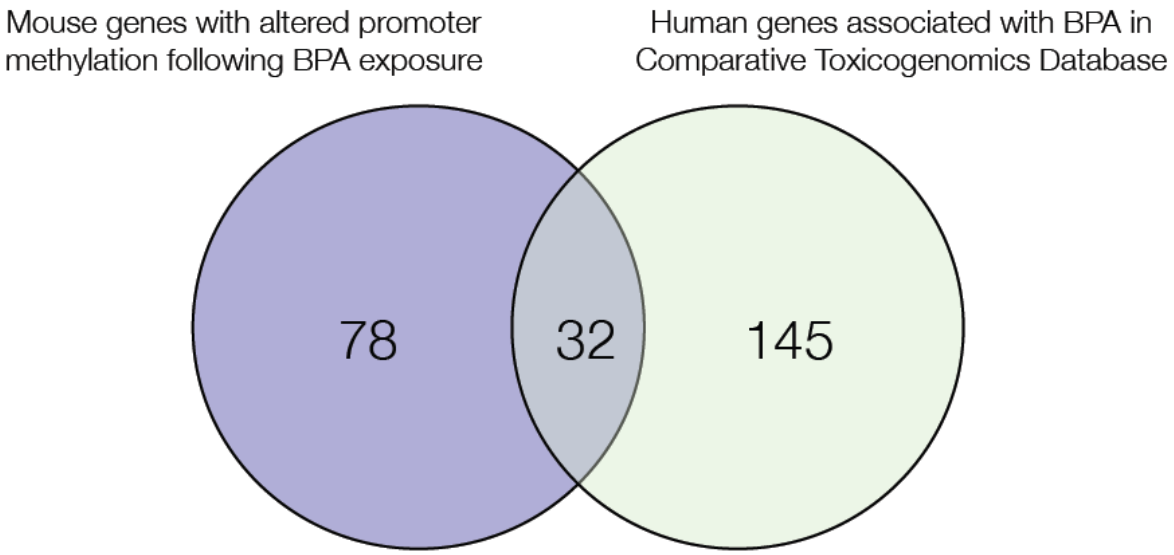
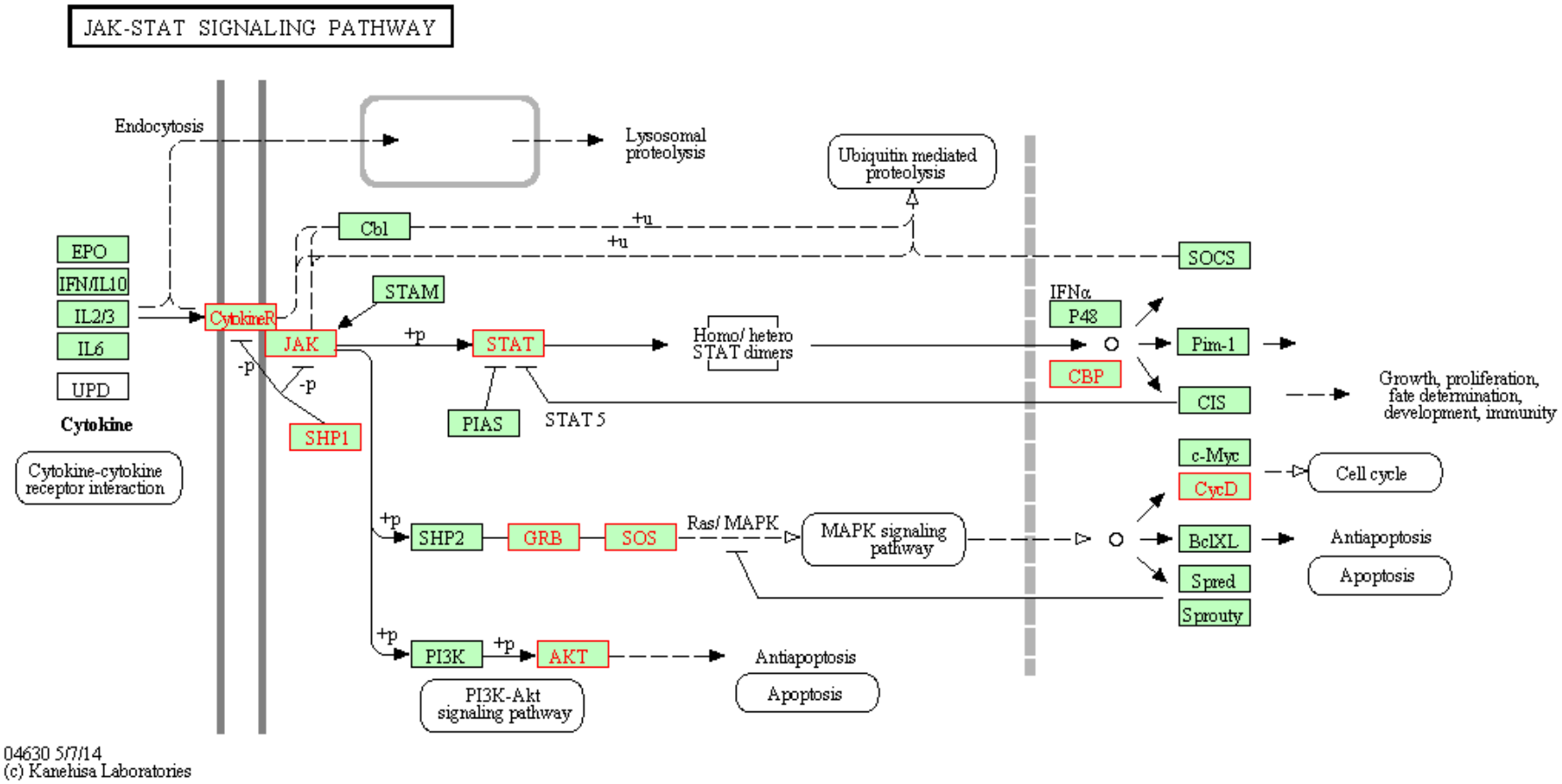
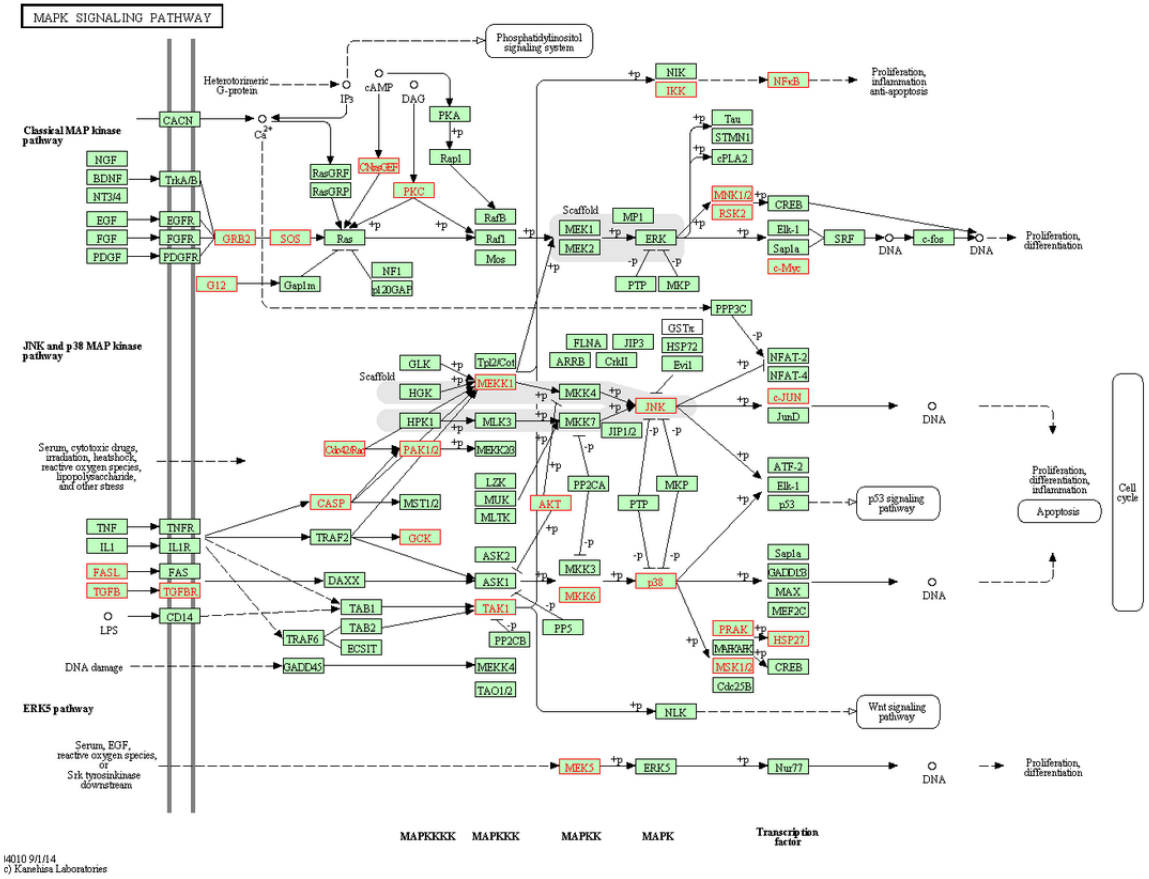


Figure 4.3 Overlapping Mouse and Human Genes Altered by BPA Exposure in the Jak-Stat Signaling Pathway



Unique mouse microarray gene IDs involved in Jak/Stat signaling that overlap with human JAK/STAT gene IDs with known interactions with BPA in The Comparative Toxicogenomics Database are shown in red.

Figure 4.4 Overlapping Mouse and Human Genes Altered by BPA Exposure in the Mapk Signaling Pathway



Unique mouse microarray gene IDs involved in Mapk signaling that overlap with human MAPK gene IDs with known interactions with BPA in The Comparative Toxicogenomics Database are shown in red.

CHAPTER 5

Conclusion

5.1 Review and Synthesis of Dissertation Findings

Potential human health effects, including cancer, resulting from exposure to bisphenol A (BPA), have been the subjects of much recent controversy. To our knowledge, this dissertation represents one of the first statistically significant reports of frank tumors, in addition to precancerous lesions, in any organ following perinatal or adult BPA exposure alone, in the absence of additional chemical or hormone administration. The overall goal of this dissertation was to test the central hypothesis that early life BPA exposure dysregulates the DNA methylome and thereby modifies risk for adult liver tumors.

In Chapter 2, I characterized the phenotype and note a lack of sexual dimorphism in incidence, as well as a lack of regenerative response to injury, suggesting a solely proliferative response to BPA. A criticism of many traditional rodent toxicology assays is that they typically use high doses of chemicals, in order to increase power to detect potential health effects (Paranjpe et al., 2014). However, high doses often incur tissue damage, which in turn stimulates inflammation and cellular proliferation in response to injury, in order to regenerate healthy tissue (Michalopoulos, 2013). In this study, no necrosis or fibrosis were observed, suggesting that the doses administered in our study did not incur tissue damage and the cellular proliferation observed occurred as an isolated response to BPA rather than as a cellular response to damage

inflicted by BPA exposure. This is notable in relation to human health, as well. As noted in Chapter 2, the majority of human HCC arises in cirrhotic, or fibrotic livers with regenerative responses to injury, often as a result of long-term alcohol use, obesity, or viral infection. HCC also presents more frequently in men, regardless of etiology (Bosetti et al., 2014). Here, no differences were seen in tumor rate in male mice as compared to female mice, which is uncommon, as there typically is also a male predisposition in mice (Hoenerhoff et al., 2011). To my knowledge, no human subtype of hepatocellular carcinoma exists that does not display male predisposition in incidence. However, long term use (>24 months) of first generation oral contraceptives, which contained up to 2-5 times more estrogen and 5-10 times more progestin than current formulations (Petitti, 2003), was estimated to increase the annual incidence of hepatic adenoma in women from 0.13 per 100,000 OCP non-users to 3-4 per 100,000 OCP users (Rooks et al., 1979). This suggests that hormonally active agents may contribute to disease risk.

The liver is a hormonally responsive organ, and both normal and HCC livers in males and females express estrogen receptor and androgen receptor (Giannitrapani et al., 2006). Both endogenous and exogenous estrogens, as well as anabolic steroid use, have been implicated in development of liver tumors (Giannitrapani et al., 2006), although the relative roles of sex steroid hormones in HCC are unclear. Male cirrhotics with HCC have been reported to be relatively hyperestrogenic (Giannitrapani et al., 2006). This may be due to an increased estrogen to testosterone ratio (ETR) due to decreased testosterone levels with no change in circulating estrogen levels (Giannitrapani et al., 2006), and only rarely due to an increase in mean estradiol levels (Farinati et al., 1995). Males with cirrhotic HCC commonly present with testicular atrophy and gynecomastia despite unaltered clearance of sex steroids (Giannitrapani et al., 2006). In contrast, elevated testosterone levels have been reported as a risk factor for HCC (Lukanova et

al., 2014), and anti-androgenic compounds have been tested in clinical treatment of HCC, although they have been ineffective (Di Maio et al., 2006). Similarly, anti-estrogenic drugs, including tamoxifen, have been shown to be ineffective in HCC therapy. Early studies in small numbers of patients reported reduced tumor growth and increased survival in patients receiving tamoxifen treatment compared to untreated patients (Cheng et al., 1998; Shin et al., 2003) but larger follow-up studies showed no effect of tamoxifen (Barbare et al., 2005; Perrone et al., 2002; Verset et al., 2007; Zeeneldin et al., 2014.) Interestingly, ineffectiveness of anti-estrogen therapy may be due to the predominance of a variant estrogen receptor lacking a hormone-binding domain in HCC patients (Giannitrapani et al., 2006), supporting the importance of identifying regulatory epigenetic alterations, including intronic DNA methylation, that may contribute to shifts in expression of gene variants, such as the work presented in Chapter 3.

The observation of decreased testosterone with no change in mean estrogen levels noted in men with cirrhotic HCC (Giannitrapani et al., 2006) suggests a role for aromatase in hormonal imbalances seen in HCC. Estrogen is commonly synthesized by aromatization of testosterone by the enzyme aromatase; therefore, estrogen levels are predominantly dependent on testosterone levels (van Thiel et al., 1985). Human HCC tissues showed elevated aromatase activity with higher estrogen formation rates than in nontumoral liver tissues, as well as in peritumoral tissue (Hata et al., 2013), suggesting the utility of aromatase inhibitors in HCC (Castagnetta et al., 2003). BPA has been shown to increase aromatase expression and subsequently increase ETR in adult male rat prostate (Castro et al., 2013), suggesting that hormonal imbalance in xenoestrogen-associated HCC may be due to aromatase expression or activity. In contrast, decreased expression of aromatase may be linked to increased risk for HCC. Knockout of the Cyp-19 gene that codes for aromatase leads to insufficient endogenous estrogen production in

mice, which subsequently contributes to the development of symptoms related the metabolic syndrome, including excess adiposity and insulin resistance associated with HCC (Bader et al., 2011). Further, near normal estrogen levels with lower testosterone levels reported in males with cirrhotic HCC (Giannitrapani et al., 2006) may be due to peripheral aromatization of steroidal estrogen precursors and overproduction of adrenal androgens that can be converted to estrogens, not due to altered gonadal production or altered aromatase expression or activity (van Thiel et al., 1985).

In addition to the conflicting evidence for the relative roles of steroid hormones in HCC, hormonal imbalances observed in HCC may be entirely unrelated to hepatic carcinogenesis. For example, alcohol abuse, which is thought to contribute to HCC risk directly as a genotoxicant and indirectly by inducing liver tissue damage and cirrhosis, has been shown to directly decrease testosterone levels in males, suggesting that altered hormonal status in men with alcohol-related HCC may be due to alcohol use rather than liver disease or dysfunction (van Thiel et al., 1985). Lowered testosterone in males has been linked to alcohol use and abuse alone, and alcohol may be linked independently to HCC development. Thus, males with HCC that abuse alcohol may show a hormonal imbalance unrelated to their liver disease (Farinati et al., 1995).

Therefore, although the literature suggests a role of sex steroid hormones and xenoestrogens in HCC and HA, the precise mechanism of action for estrogens and endocrine disrupting chemicals such as BPA is not well characterized. Experiments and analyses in Chapters 3 and 4 focused on elucidating possible explanations for two notable phenotypic characteristics of BPA-associated HCC and HA observed in Chapter 2: a lack of sexual dimorphism in incidence and a proliferative response in the context of no apparent liver injury. Since these tumors arose in isogenic animals, I chose to focus on potential epigenetic responses

to BPA exposure that may have played a role in hepatic tumorigenesis. However, I cannot rule out non-epigenetic drivers of tumorigenesis, including genotoxicity of BPA leading to accumulation of mutations and subsequent liver tumors. I chose to focus on DNA methylation as a representative epigenetic mark, as it is currently accepted to be the most stable epigenetic mark over the life-course, making it ideally suited to the study of epigenetic dysregulation by early life exposures that persists into adulthood and mediates risk for adult disease. I cannot rule out the possibility for epigenetic responses mediated by marks other than DNA methylation, including histone modifications and changes in chromatin architecture.

In Chapter 3, I report changes in DNA methylation patterns associated with tumor status in a dose-dependent manner at *Stat3* in 10-month mice, as well as in post-natal day 22 sibling mice from the same exposure study, which implicates *Stat3* as a potential early life biomarker of both exposure and disease. Further, DNA methylation profiles at the human homolog *STAT3* were associated with continuous total and free BPA levels in fetal liver tissue, providing early evidence for a link with human health. These data support *STAT3* as a candidate biomarker for further testing, but do not support any statement of biological role or direct mechanistic link to either exposure or disease within the context of this dissertation. However, *STAT3* has previously been implicated as a prognostic indicator of HCC progression in humans (Tai et al., 2014) and is currently being tested as a pharmacologic target in HCC therapy (Mohan et al., 2014; Tai et al., 2014), indicating that *STAT3* is plausibly a key player in HCC development and clinical therapy.

In an ideal study of biomarker identification, I would have been able to demonstrate mechanistic and biological relevance of clear and consistent gene-specific changes in DNA methylation with clear temporal links to early life exposure and later life disease. The field of environmental epigenetics is in a period of discovery, however, and clear methods for detection

of epigenetic changes associated with exposure and disease are not currently available. Therefore, I chose to focus this Chapter on careful distinction of DNA methylation changes associated with tumor status and BPA exposure across the life-course, by testing animals of two ages from the same exposure study, and across species, by extending the analysis to human fetal livers. The modified candidate gene approach that I devised successfully detected the type of candidate biomarker that I intended to detect at *Stat3* and *STAT3*, and Chapter 3 therefore represents validation for a novel method for detection of candidate life-course epigenetic biomarkers. This is a highly useful hypothesis-generating tool for future environmental epigenetic studies for identification of possible candidates for epigenetic change, and therefore represents a necessary step prior to testing potential biomarkers for biological relevance in mechanistic and epidemiologic studies. As such, Chapter 3 represents proof of principle for a novel method for identification of epigenetic biomarkers of exposure and outcome across the life-course and across species.

In Chapter 4, I demonstrate that epigenome-wide discovery experiments in animal models are effective tools for identification and understanding of paralagous epimutations in cell signaling pathways salient to human disease, in an attempt to provide a clearer picture of possible biological responses to BPA than the candidate gene-focused chapter (Chapter 3). Few studies have been published to date using epigenome-wide platforms to assess DNA methylation patterns across the genome, and analysis methods vary widely. In addition, the direct biological implication of DNA methylation changes in 5' gene promoters assessed in this microarray experiment are as yet unclear and may be gene-specific. Therefore, I made analysis decisions with the express intention of generating the most clarity in biological response possible from 5' gene promoter DNA methylation microarray analysis in BPA-exposed animals with hepatic

tumors. As described in the Methods text in Chapter 4, steps for the analysis of the raw data were carefully considered for appropriate application to data generated by this platform. As such, this analysis pipeline represents a novel addition to the literature on analysis of DNA methylation microarrays based on enrichment protocols.

Following analysis of the raw data, I assessed GO term and pathway enrichment data first agnostically, by evaluating terms with significant FDR values (<0.1). GO terms are often the terms with the most significant FDR values in microarray experiments, because these terms contain a large number of gene IDs related to a large concept, such as *metabolism* or *cancer pathways*. However, broadly general terms commonly found in GO databases may be significant in an analysis and yet not meaningfully inform a specific biological response of interest in a given study. Therefore, I also ran an initial enrichment limiting the analysis to databases containing pathway terms, since alterations in specific pathways have clearer interpretations and more meaningfully inform potential biological responses.

Agnostic pathway analyses revealed hypermethylation of neuronal signaling pathways with links to metabolic dysfunction and cellular proliferation, suggesting that BPA disrupts neuronal signaling with metabolic consequences in liver. Metabolic dysfunction has been associated with both exposure to environmental estrogens and liver disease states. Elevated IGF-1 is a risk factor for HCC development, possibly due to altered metabolism secondary to liver damage (Lukanova et al., 2014). Type II diabetes confers a 2.3 fold increase in risk for HCC, regardless of other risk factors, including cirrhosis (Lukanova et al., 2014). Diabetes and obesity may have synergistic effects on hepatic carcinogenesis in other etiologic settings. In a study of 23,820 Taiwanese individuals with viral hepatitis (HBV or HCV), the risk for HCC was increased more than 100-fold for those with both diabetes and obesity (Giannitrapani et al.,

2006). Some hormonal contraceptives have been linked to altered metabolism, as well. Depot medroxyprogesterone acetate increased glucose and insulin levels in the first 18-30 months of use (Berenson et al., 2011), and African-American women using oral contraceptives were more insulin resistant than controls (Frempong et al., 2008.) Hepatic adenomas (HA), a rare form of benign liver tumor historically associated nearly exclusively with long term use of high dose oral contraceptives, have been increasingly linked to hormonal or metabolic imbalances that stimulate hepatocyte proliferation (Farges & Dokmak, 2010; González-Lara, et al., 2013). HAs are linked with obesity and metabolic syndrome in men (Farges & Dokmak, 2010). The rate of incidence of malignancies in men with HA has increased over a 15 year period but has largely remained stable for women (Farges & Dokmak, 2010). In addition, HAs account for 2-4% of liver tumors in children (Lautz et al., 2008; Resnick et al., 1995; Wheeler et al., 1986), and are largely associated with metabolic dysfunction in the absence of cirrhosis, including glycogen storage disorders, diabetes mellitus, androgen therapy, and sex hormone disturbances (Resnick et al., 1995; Vaithianathan et al., 2013). Spontaneous HA with multinucleate giant cell formation has been reported in five children (Wheeler et al., 1986), similar to the multinucleated hepatocytes reported in Chapter 2, further supporting the relevance of the HA phenotype to the liver tumors observed this study. Previously, we have reported an increase in insulin sensitivity and increased adiponectin levels in 22-day-old mice perinatally exposed to BPA (Anderson et al., 2013; Kim et al., 2014). Altered adipokine release may influence insulin resistance and inflammation, and lipotoxicity secondary to increased lipogenesis may influence signaling pathways and increased oxidative stress and DNA damage, increasing risk for HCC development (Lukanova et al., 2014).

BPA has previously been shown to alter metabolic function by disrupting neuronal signaling, specifically gonadotropin-release hormone (GnRH) signaling, a component of the hypothalamic-pituitary-gonadal (HPG) axis (Cao et al., 2012). This endocrine axis comprises the hypothalamic gonadotropin-releasing hormone (GnRH), pituitary gonadotropins, including luteinizing hormone (LH), which stimulates production of gonadal testosterone, and indirectly, estrogen (Dickerson & Gore, 2007). Relative levels of estrogen and testosterone during perinatal period have a defining role in morphological and functional development of male or female brain (Dickerson & Gore, 2007). Neonatal life is a critical window for the sexually dimorphic organization of the HPG axis, and exposure to endocrine active compounds during this period could underlie compromised adult reproductive physiology (Cao et al., 2012). Estrogen is masculinizing in the murine brain during perinatal devleopment and even exposure to low levels of estrogen or xenoestrogens during this time can permanently alter neuroendocrine pathways critical for mediating gonadotropin release, energy homeostasis, and sexual behavior (Amateau et al., 2004; Bader et al., 2011; Faulds et al., 2012). BPA can alter sex specific organization of hypothalamic regions in the murine brain known to be important for coordinating gonadotropin release and sexual behavior, most notably a portion of the anterior hypothalamus (Patisaul et al., 2006, Patisaul et al. 2007, Rubin et al., 2006) and arcuate nucleus (Patisaul et al., 2009.) CD-1 mice exposed perinatally to BPA showed upregulated expression of GnRH mRNA in both male and female pups (Xi et al., 2011). Downstream LH, which directly stimulates testosterone and indirectly estrogen levels in the gonads, is reportedly altered following early life BPA exposure, as well. Female Sprague-Dawley rats exposed to 0.1 mg or 1.2 mg BPA/kg BW/day during gestational day (GD) 6 through lactation exhibited decreased levels of plasma LH in adulthood (Rubin et al., 2001). Exposure to rats on postnatal day (PND) 21-35 to 2.4 μg/kg/day suppressed

serum LH and testosterone levels. However, results should be interpreted carefully, as BPA may act directly on the gonads, rather than indirectly on the hypothalamus, yielding different phenotypic outcomes. Exposure to 1 mg daily via subcutaneous injection in adult male rats yielded decreased plasma concentrations of testosterone, and, counterintuitively, to increased plasma LH, possibly due to direct inhibition of testicular function by BPA and reduction in negative feedback by testosterone that would ordinarily normalize LH levels (Tohei et al., 2001). Exposure of male mice from GD6-PND20 to either 0, 4, 40, or 400 mg BPA/kg BW via oral gavage led to an increase in plasma testosterone at 9 weeks which normalized by 36 weeks, with no change in plasma LH at 9 weeks (Watanabe et al., 2003). Female rats injected with 50μg or 500 μg daily from PND1-PND10 exhibited lowered basal and GnRH-induced LH and increasing GnRH pulsatility in adulthood (Watanabe et al., 2003). Lastly, sheep exposed to 5mg/kg BW/day BPA from GD30-90 exhibited lower GnRH expression (Mahoney & Padmanabhan, 2010).

Collectively, these observations support the hypothesis that disruption of hypothalamic organization during critical window of development could underlie direct neuroendocrine effects and indirect reproductive and metabolic effects of BPA exposure (Cao et al., 2012.) Hypermethylation of neuronal signaling pathways reported in Chapter 4 may be interprted cautiously within the context of this literature. Norepinephrine acts to increase GnRH secretion (Xi et al., 2011); histamine, serotonin, and muscarinic acetylcholine receptor signaling pathways have been shown to inhibit norepineprhine release (Dickerson & Gore, 2007). These neuronal signaling pathways were hypermethylated on agnostic microarray analysis; if downregulated, these signaling pathways would fail to inhibit norepinephrine release and could conceivably increase GnRH signaling and downstream testosterone and estrogen production stimulated by LH. If BPA exposure during neonatal development mitigates or eliminates sexual dimorphism in

morphology and function of the hypothalamus, perhaps alteration in GnRH signaling with BPA exposure in both sexes would represent aberrantly similar hypothalamic function in brains of male and female mice permanently induced by transient BPA exposure during this critical developmental window.

In addition to the agnostic microarray analysis, I subsequently chose to pursue a hypothesis-driven approach in which I identified two pathways *a priori*, Jak/Stat and Mapk signaling, that were linked to a unique characteristic of observed tumors, the lack of male: female dimorphism in incidence. I chose this characteristic over cellular proliferation, as tumors are inherently proliferative lesions, and pathways involved in injury-induced proliferation would not be expected to differ from those involved in non-injury-induced proliferation. As outlined in Chapter 4, I demonstrated overlap of genes empirically shown to be responsive to BPA exposure in our mouse experiments with genes altered following human BPA exposure in JAK/STAT and MAPK signaling pathways, both of which are linked to downstream inflammatory responses. Inflammatory signaling and metabolic implications of neuronal signaling pathways identified in hypothesis-driven and agnostic analyses of microarray data, respectively, may be linked phenotypically; the inflammatory subtype of hepatic adenoma commonly presents with increased activation of the IL-6 signaling pathway and is associated with obesity and steatosis (Farges & Dokmak, 2010).

Large, networked pathways such as Jak/Stat and Mapk signaling cannot themselves serve as biomarkers, but perhaps alterations in specific key players within those pathways at specific times in certain cell types might more clearly inform the cellular processes that lead to development of hepatic tumors following BPA-exposure. In the context of other microarray analyses, some of which do not incorporate any pathway enrichment analysis at all, many of

which limit their analyses to GO terms or broad statements of change, the hypothesis-driven approach presented in Chapter 4 allows for the most biological relevant interpretation possible within the limitations of this tool and the current unknowns in the field of environmental epigenetics.

As a whole, this dissertation has generated hypotheses that BPA may lead to metabolic dysfunction via alterations in neuronal signaling pathways and that Jak/Stat and Mapk signaling may mediate the link between early life BPA exposure and later life hepatic tumors in mice, although further experiments are needed to fully characterize whether or how these pathways act in the context of this phenotype. Candidate genes of interest from pathways identified in Chapter 4 may be further analyzed with the novel tool presented in Chapter 3. As such this dissertation represents a tool kit for moving forward with follow-up investigation of altered DNA methylation. Given the opportunity to perform follow-up experiments, I would choose to characterize activity of these pathways at several time points following perinatal BPA exposure in mice, including a number of closely timed time points during gestational exposure in mouse hepatocytes to determine early cellular signaling changes, and adult time points in whole animals, to assess the temporality and first occurrence of many of the lesions described in Chapter 2. In addition, I would isolate oval cells, or hepatobiliary stem cells, from animals with hepatic tumors and inject them into SCID mice, to determine whether these stem cells are sufficient to drive tumorigenesis, which would indicate a stem cell origin of disease. A stem cell origin is suggested in the data, both by a monotonic increase in oval cell hyperplasia with increasing BPA exposure that mimics the pattern exhibited by hepatic tumors, and by a predominance of hepatocellular carcinomas over hepatic adenomas or pre-neoplastic lesions. Assuming equal susceptibility of all mice in the study, a stepwise model of carcinogenesis would

predict greater prevalence of precursor lesions as compared to advanced lesions. Given the model that pre-neoplastic lesions give rise to hepatic adenomas, which then progress to carcinomas, we would expect a larger number of hepatic adenomas than hepatocellular carcinomas, and yet more pre-neoplastic lesions than adenomas. Instead, we observed a large number of carcinomas, with fewer adenomas, and even fewer pre-neoplastic lesions, supporting a model other than a stepwise progression towards carcinogenesis and further indicating the likelihood of a stem cell origin of disease.

5.2 Study Strengths and Limitations

The experimental approaches taken in this dissertation were thoughtful and innovative methods of testing the DOHaD hypothesis in the context of *in vivo* mouse data, with a noted focus at each stage on relevance to human exposure and health outcomes.

First, many environmental epigenetics studies are cross-sectional in nature, in that they test epigenetic changes associated with either exposure or disease, but do not link health outcomes to exposure. Our study is one of few that allow for testing of both exposure and disease in animals from the same exposure study across the life-course. We used both mouse and human liver samples in parallel to allow for empirical demonstration of translational relevance at each stage of this study.

Second, our statistical data analyses across all Chapters were carefully chosen for appropriateness to the research question of interest. For example, hepatic tumors and associated lesions were assessed with both exact tests to account for small cell sizes and logistic regression models to cluster data by litter, with a focus on results robust to both methodologies. Clustering data by litter allowed for experimental capture of population variance within, in addition to among, litters. DNA methylation models were designed to account for lack of independence of

neighboring CpG sites, by modeling them as spatially repeated measures of average methylation, while still allowing for site-specific analyses to detect differences in basal methylation and epigenetic lability within an epigenetic locus.

Third, our approach in Chapters 3 and 4 combined high resolution, quantitative candidate gene approaches with epigenome-wide experiments to develop a complementary framework for interrogating relationships between exposure and disease in these and future studies. In Chapter 3, we presented a novel method for identification of epigenetic biomarkers of exposure and outcome across the life-course and across species by combining traditional quantitative candidate gene sequencing with data mining of epigenome-wide studies. In Chapter 4, we demonstrated the utility of epigenome-wide promoter methylation tiling microarrays as discovery tools for identification of large scale, pathway level changes linked to exposure and disease that demonstrate translational relevance of our observed liver tumor phenotype. Future studies may funnel candidate genes within pathways identified in Chapter 4 through the pipeline proposed in Chapter 3 for quantitative testing of methylation change linked to exposure and disease and the relevance of detected changes to mice and humans.

Limitations of this dissertation including methodological constraints, analyses of heterogenous cell populations in Chapters 3 and 4, a focus on DNA methylation over other epigenetic and non-epigenetic changes, and difficulties involved in linking epigenetic changes to functional outcomes, particularly in a causal manner.

Evidence of absent sexual dimorphism in incidence noted in Chapter 2 and followed up on in Chapters 3 and 4 would have been stronger if an additional statistical test had been performed. In Chapter 2, I tested for the presence of a statistically significant interaction between sex and dose of BPA and found that the p-value did not reach the cutoff of <0.05, supporting the

null hypothesis of no interaction, and therefore no difference by sex in association between liver tumor rate and BPA exposure. However, this result may simply indicate a lack of statistical power to detect the presence of a true interaction, particularly in the context of small sample sizes used in Chapter 2. If I had performed an additional test with a null hypothesis stating that an interaction were present (using an interaction effect estimate derived from the literature) and demonstrated a lack of evidence for this null hypothesis with a p-value <0.05, this result would have strengthened the evidence for a true lack of sex-specific effect.

The Sequenom MassARRAY EpiTYPER platform is a quantitative approach to candidate gene sequencing, but is limited to sequencing short reads of approximately 400 base pairs in length. This limitation requires careful assay placement in candidate gene experiments, and, in the absence of several consecutive assays, does not allow for full interrogation of the gene of interest or of its regulatory regions. Microarray platforms are broader in scope, but, unlike deep sequencing methods, are still limited by the range of probes included on the arrays, which in this case, is limited to tiling of 5' gene promoter regions across the mouse genome. Epigenome-wide approaches can be costly, however, and microarray platforms may represent experimental approaches that allow for partially unbiased analyses that are affordable in a meaningful number of samples.

In addition, DNA samples for experiments in Chapters 3 and 4 were extracted from liver tumor tissue that was dissected and classified by a board-certified veterinary pathologist (ILB.) Epithelial liver tumors are primarily composed of hepatocytes, but may also contain vascular, nervous, and fat tissue. As cell types were not identified and separated via Fluorescence-Assisted Cell Sorting (FACS) or Laser Capture Microdissection (LCM), we cannot rule out the possibility

that differences in percent DNA methylation across DNA molecules in a sample may be due wholly or in part to differences in relative proportions of cell types within tissue samples.

Last, this dissertation was designed as a series of association studies, investigating potential links between DNA methylation profiles and exposure or disease to identify putative epigenetic biomarkers of exposure-mediated disease risk and to characterize relevant pathways in order to elucidate potential biological responses to BPA exposure for future study. However, association studies do not indicate temporality of change, and it is unclear if epigenetic changes reported here are causes or consequences of exposure and disease, or, alternatively, bystander changes with no biological link to exposure or disease. Similarly, evidence of epigenetic or other biological changes in humans that mimic mouse data presented in this dissertation that suggest translational relevance to human health do not represent definitive evidence of applicability of results to humans. Validation with both *in vitro* and *in vivo* mechanistic studies to determine causality and the relative role of DNA methylation changes, as well as epidemiological cohort studies to better describe biological processes following exposure that may mediate human disease risk are important to provide additional information not evident from association studies.

5.3 Implications of Dissertation Findings

The data presented in this dissertation suggest that early life exposure to BPA alters the epigenome and increases risk of adult hepatocellular carcinoma (HCC), a globally widespread disease with poor therapeutic options and prognosis (Shaw and Shah, 2011). Therefore, there is a critical need for identification of predictive biomarkers for epigenotoxic developmental programs induced by BPA using complementary candidate gene and epigenome-wide discovery methods, as demonstrated in Chapters 3 and 4. This dissertation represents a first step toward identifying potentially modifiable epigenetic risk factors associated with multiple, environmentally-relevant

BPA exposures and hepatocellular carcinoma, a disease with high global burden and few effective treatments. Characterization of epigenetic biomarkers indicative of early BPA epigenotoxicity and predictive of HCC development will allow at-risk individuals to be identified long before they develop disease, opening new avenues for potential disease prevention strategies, such as dietary supplementation or pharmaceutical intervention (Dolinoy et al., 2007; Kalra et al., 2008). Further, therapeutic modification of these epialleles in individuals with existing HCC may facilitate reversal of disease progression, due to the plasticity of the epigenome (Waterland and Jirtle, 2003).

The mechanism by which BPA may increase risk for hepatic tumors, via epigenetic dysregulation or other means, has not been fully characterized. As the experiments in this dissertation focused on DNA methylation, as noted in the beginning of this Chapter, the subsequent discussion in this section will elucidate what is known about alterations to this mark in the setting of this study. In Chapter 1, I noted that BPA has been linked to increased levels of reactive oxygen species, and therefore might plausibly directly oxidize methylcytosine to hydroxymethylcytosine, formyl-methylcytosine, or carboxymethylcytosine, triggering demethylation by *tet* enzymatic activity. Alternatively, as indicated in Chapter 1, BPA may dysregulate the methylome indirectly by altering levels of epigenomic regulators, including DNA methyltransferases or methyl binding proteins, or may induce depletion of pools of methyl donors, such as S-adenosylmethionine, although the mechanisms detailing BPA's induction of these effects have not been described. All aforementioned hypotheses predict a combination of global and stochastic changes to the epigenome (**Figure 5.1**). Global hypomethylation following toxicant exposure is a plausible driver of tumorigenesis. Fischer-344 (Chandar et al., 1987; Lombardi et al., 1991) and B6C3F_1 (Newberne PM et al., 1982) mice fed choline-deficient diets

that presumably decrease available methyl donors and lead to global hypomethylation develop liver tumors in absence of exposure to chemical carcinogens. Global hypomethylation may lead to increased cellular proliferation, due to genomic instability and hypomethylation of oncogenes, leading to increased mutation rates and further proliferative responses (Goodman and Counts, 1993.) However, toxicant-linked activation of cell signaling pathways that stimulate cellular proliferation may directly induce a proliferative response, leading to subsequent hypomethylation and increased mutation rate due to the inability of DNA repair pathways to repair mutations or of DNA methyltransferases to accurately reproduce DNA methylation patterns on hemimethylated DNA prior to cell division (Goodman and Counts, 1993). This second model indicates specific cell pathway induction by toxicant exposure, followed by epigenetic responses, which may plausibly be functionally relevant to the toxicant of interest. Indeed, altered DNA methylation at estrogen receptor α (*ESR1*) following exposure to BPA (Doshi et al., 2011) and phthalate BBP (Kang and Lee, 2005) supports epigenetic changes at non-random target genes (**Figure 5.1**). The data presented in this dissertation indicate altered DNA methylation profiles at candidate genes and intracellular signaling pathways implicated in endocrine activity and mediation of hepatocellular carcinoma risk, further supporting a hypothesis of non-random, gene-specific dysregulation, likely downstream of cell signaling events triggered by initial BPA exposure in early life, but not in adulthood.

5.4 Future Work in Environmental Epigenetics

Past studies linking early environmental exposure to altered epigenetic profiles have cited the period of DNA methylation reprogramming in early embryogenesis as a critical time window for potential dysregulation of the epigenome, implying direct alteration of the methylome by toxicant activity (Dolinoy et al., 2007; Prins et al., 2008; Vandenberg et al., 2007). However, recent evidence suggests that altered DNA methylation may not be a stable first responder to

exposure but rather a downstream consequence of toxicant-specific cell signaling events, leading to altered transcription of candidate genes, which in turn alters epigenetic profiles, including DNA methylation patterns. This hypothesis is supported by studies demonstrating that a transient decrease in gene expression triggers a pathway for gene silencing that includes histone deacetylation and subsequently increased promoter DNA methylation (Kangaspeska et al., 2008; Métivier et al., 2008; Oyer et al., 2009.) Work by Abarrategui and Krangel demonstrated that insertion of a transcription stop sequence altered downstream histone acetylation at the murine *Tcra* locus (Abarrategui and Krangel, 2007.)

Notably, models that posit altered DNA methylation as a consequence, rather than, or perhaps in addition to, a cause of functional change provide testable mechanisms for targeted, gene-specific DNA methylation changes following toxicant exposures. Although candidate gene approaches assume gene-specific changes following environmental exposures, hypotheses that consider DNA methylation changes to be causative have not linked toxicant exposure to DNA methylation at specific candidate genes. If BPA exposure, for example, resulted in direct alteration of DNA methylation via oxidative damage of DNA or changes in activity of epigenetic regulators, such as DNA methyltransferases, global or stochastic epigenome-wide changes would be expected to result. Designing mechanistic studies to empirically determine if methylation changes are causally linked to subsequent functional outcomes is limited by the inability to experimentally manipulate DNA methylation levels in a tightly controlled manner at specific targets, either *in vitro* or *in vivo*. Recently, a transgenic model for targeted promoter hypermethylation has been described (Yu et al., 2014), representing a novel and effective method for mechanistic DNA methylation studies; however, the model does not allow for controlled manipulation of methylation profiles to mimic effect sizes commonly reported in environmental

epigenetics studies. In contrast, targeted gene knockdowns via RNAi or use of well-established transgenic model organisms might be used to test the alternate hypothesis that early life gene expression changes, due to alterations in cell signaling, may lead to transient or persistent changes in DNA methylation. Following this hypothesis, later life methylation profiles would represent residual effects of earlier life biological changes, rather than persistent changes that play causal roles in health outcome risk. The temporality of expression and methylation changes may be tested with experimental outcomes at multiple time points in *in vitro* studies of human liver cells exposed to BPA, with the caveat that these changes may depend on the developmental environment *in vivo*. In addition, thorough characterization of timing of gene-specific changes following toxicant exposure would allow researchers to better distinguish targeted changes from global or stochastic alterations to the epigenome. Bachman *et al*. demonstrated early target-specific changes in DNA methylation profiles in a SENCAR two-stage mouse skin tumorigenesis model, followed by later global hypomethylation, perhaps as a result of, as well subsequent driver of, increased cellular proliferation (Bachman et al., 2006). Perhaps early and persistent regions of altered methylation are more likely to play a role in tumorigenesis, underscoring the importance of distinguishing early, exposure-related changes from later global and stochastic alterations, using a method similar to the one employed in Chapter 3.

In addition, the relative biological role that DNA methylation plays following environmental exposure to BPA and in mediating risk for adult disease is unknown. DNA methylation is the focus of many epigenetic studies in mice and humans, because high quality, sensitive tools are available to measure DNA methylation profiles quantitatively, and because DNA methylation is widely accepted to be the most stable epigenetic mark over time, a characteristic that recommends it as a source for potential biomarkers of early life exposure that

persist over the life-course. However, recent evidence suggests that DNA methylation patterns are cyclical (Kangaspeska et al., 2008; Métivier et al., 2008; Rose et al., 2014), likely due to consistent oxidation of methylcytosine and possible removal and repair via thymine DNA glycosylase and base excision repair (Rose et al., 2014), followed by re-methylation of repaired cytosines by maintenance DNA methyltransferases (Kangaspeska et al., 2008). Therefore, cross-sectional measurements of DNA methylation profiles may represent a snapshot of a cyclical profile, perhaps partly explaining differences in basal DNA methylation at CpG sites within a genetic locus. Cyclical DNA methylation profiles do not necessarily indicate instability of profiles over time, assuming high fidelity of maintenance DNA methyltransferase activity. However, other biological factors, including fidelity and activity of DNA repair pathways, may play a role in persistent message stability.

5.5 Conclusion

In this dissertation, characterization of a frank liver tumor phenotype in isogenic mice perinatally exposed to BPA, coupled with complementary candidate gene and epigenome-wide methods for identification of putative early life epigenetic biomarkers linked to exposure and disease, implicate BPA as a potential liver carcinogen associated with altered DNA methylation profiles in genetically identical animals. These findings warrant further study of BPA as a potential carcinogen in estrogen-target organs, including non-reproductive organs, such as the liver. In particular, this dissertation highlights a mitogenic response following BPA exposure, as indicated by proliferative lesions in the absence of evidence of regenerative response to injury (Maronpot, 2009). DNA methylome dysregulation has been suggested as a possible non-genotoxic mechanism of cellular proliferation that drives carcinogenesis in the absence of additional initiating or promoting factors (Goodman and Counts, 1993.) No prior study to our

knowledge has demonstrated a statistically significant link between frank tumors in any organ and BPA exposure alone, with no additional chemical or hormonal promoters. Thus, these data represent an important contribution to the body of literature on BPA, with possible extension to re-examination of carcinogenicity risk of other known chemical mitogens.

5.6 References

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Figure 5.1 Overview of models of epigenetic change in response to bisphenol A exposure.
BPA exposure may induce global epigenetic changes directly via direct oxidation of 5-methylcytosine and altered levels of epigenomic mediators or gene-specific epigenetic changes indirectly by altering gene expression in specific intracellular signaling pathways