The influence of intrinsic and extrinsic factors on epigenetic modulation in response to chemical exposures

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Abstract:

The epigenome, a dynamic interface between the genome and the environment, is shaped by a combination of intrinsic and extrinsic factors that can modulate DNA methylation and histone modifications. This essay explores how genetic variation, age, sex, and tissue type contribute to individual variability in epigenetic landscapes, and how external influences such as diet, reproductive status, and chemical exposures—particularly endocrine-disrupting chemicals like bisphenol A (BPA) and triclocarban—further alter these profiles. Through a detailed examination of studies across multiple species and tissues, this work underscores the context-specific nature of epigenetic responses and highlights the interconnectedness between different epigenetic marks. It further illustrates how these factors can confound or mediate the impact of environmental chemical exposures, emphasizing the importance of accounting for biological and environmental variability in epigenetic toxicology. An understanding of these complex and interconnected processes is critical to improving risk assessment and identifying truly adverse epigenetic alterations in response to chemical exposures, as well as identifying potential therapeutic pathways.

1.1 Overview of Epigenetics

Epigenetic regulation of gene expression involves modifications to DNA and chromosomal structure, without changing the underlying DNA sequence. It is important to acknowledge that the definition of "epigenetics" has evolved over time, and epigenetic mechanisms have also been implicated in tagging cells for apoptosis and for sites of DNA repair (Rogakou et al., 1998; Fernandez-Capetillo et al., 2004; Gao et al., 2013). Epigenetics encompass a broad category of mechanisms that modify DNA bases, histone proteins or involve non-coding RNAs. All somatic cells within an organism are comprised of the same genome, as has been demonstrated that cell differentiation does not result in any irreversible change in genomic DNA (Gurdon et al., 1975). However, different cell types can have different transcriptomes, leading to different structures and functions which have been attributed to epigenetic regulation of the genome. Transcriptome differences exist even between similar cell types, such as in the case of visceral and subcutaneous adipocytes subtypes, which has been found to be dependent on epigenetic regulation by DNA methylation (Bradford et al., 2019). Epigenetic profiles can be influenced by both intrinsic factors such as genetic, age, sex, tissue type, as well as extrinsic factors, such as environmental exposures, diet and mating status.

Within this essay, I will be discussing how specific intrinsic and extrinsic factors have been shown influence DNA methylation and histone methylation profiles. Given the complexity and breadth of this subject, only select topics within each sub-section will be discussed. I acknowledge that there remain many studies and topics, as well as much nuance that cannot be captured within the confines of this essay. Additionally, I will discuss scenarios in which these factors may be considered regarding epigenetic responses to chemical exposures. I focus attention on endocrine disrupting chemicals, and specifically I discuss exposure to the chemical Bisphenol A (BPA) in several contexts. BPA is commonly found in many consumer plastic products and has endocrine disrupting properties through its interaction with estrogen receptors (Ohore & Zhang, 2019).

1.2 DNA Methylation

DNA methylation commonly involves the covalent addition of a methyl group (-CH_3) to the 5' carbon of cytosine residues. Non-cytosine residues, such as N^6-methyldeoxyadenosine, can be methylated in vertebrates as well (Koziol et al. 2016). Three major DNA methyltransferase enzymes (DNMTs) are involved in DNA methylation: DNMT1, DNMT3A, and DNMT3B (Davletgildeeva & Kuznetsov, 2024). DNMTs catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to nucleotide residues (Froese et al., 2018; Mentch & Locasale, 2016; Dhar et al., 2021). DNA methylation occurs predominately at CpG sites (cytosine and guanine dinucleotides) in mammals, however cytosine DNA methylation has also been observed on non-CpG sites in mammals (Arand et al., 2012) and other organisms such as plants (Zabet et al., 2017). CpA sites (cytosine and adenine dinucleotides) were the predominant form of observed non-CpG methylation in mouse embryonic stem cells and appeared to be exclusively catalyzed by DNMT3A and DNMT3B (Arand et al., 2012). While DNA methylation is considered a relatively stable mark, both active and passive demethylation mechanisms exist. Active demethylation can be TET (ten-eleven translocation) enzyme-mediated (Onodera et al., 2021), while passive demethylation can also occur during DNA replication if DNMT activity is reduced (Kraus et al., 2016).

Levels of CpG methylation have been shown to vary between 72-85% in human embryonic stem cell lines (Chen et al., 2011). CpG islands (CGIs) are often found in or near promoter regions, are largely unmethylated and the methylation state was found to be evolutionarily conserved across certain vertebrate species such as humans, mice and zebrafish (Weber et al., 2007; Long et al., 2016). DNA methylation has been shown to result in transcriptional repression in certain genes by preventing certain transcription factors from binding promoter or regulatory regions (Gaston & Fried, 1995; Watt & Malloy, 1988; Ben-Hattar & Jiricny, 1988). However, CpG methylation impact on transcription factor binding or gene expression appears to be site, gene and transcription factor-dependant (Keshet

et al., 1986; Watts & Malloy, 1988; Höller et al., 1988; Yin et al., 2017). In fact, CpG site methylation may have no impact or increase transcription factor binding (Höller et al., 1988; Yin et al., 2017). Therefore, there is a context-dependant relationship between DNA methylation and gene expression regulation, including instances of gene activation (Yang et al., 2018).

1.3 Histone Modifications

Histones are proteins that form nucleosomes, around which DNA wraps to create the structural unit of chromatin. Both the N- and C- terminals of histone tails undergo numerous post-translational chemical modifications, such as acetylation, methylation, phosphorylation, and ubiquination, which can influence chromatin conformation (Rogakou et al., 1998; Fernandez-Capetillo et al., 2004; Liu & Duan, 2008; Yin et al., 2008; Furukawa et al., 2020). It has been shown that removal of the H3 or H4 N-terminal tails in particular results in a 70% release of the DNA supercoiling structure around the histone proteins (Morales & Richard-Foy, 2000), indicating the importance of these tails for chromatin stability. The C-terminal tail on Histone H2AX has additionally been shown to be a substrate for phosphorylation in mice and acts as a mark for DNA damage (Rogakou et al., 1998; Fernandez-Capetillo et al., 2004), indicating the many roles of histone modifications. There is an extensive number of histone-modifying enzymes, as reviewed by Kooistra & Helin (2012) and Morgan & Shilatifard (2020). In this essay, I will focus my discussion on histone acetylation and methylation and some of their associated enzymes.

Histone acetylation is primarily regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation has been shown to both increase and decrease gene expression in a variety of models (Richon et al., 2000; Lusic et al., 2003; Kurdistani et al., 2004; Ma & Schulz, 2008; Aizawa & Yamamuro et al., 2010). Kurdistanti et al. (2004) determined that acetylation of histone H3K9/18/27 were found to be positively correlated with transcription, while H4K16 and H2BK11/16 were found to be negatively correlated with transcription in yeast (Kurdistani

et al., 2004). In a human cancer cell line, it has been demonstrated that inhibition of HDAC activity resulted in the accumulation of H3 and H4 acetylation at the promoter site of tumor suppressors gene *p21*, which correlated with a 9-fold increase in transcription (Richon et al., 2000). In mouse embryos experiencing an RNAi-mediated silencing of *HDAC1*, global H4 acetylation was found to be significantly increased, though only 44% of genes assessed saw a significant increase in transcription (Ma & Schultz, 2008), further indicating that increased histone acetylation can have gene-specific outcomes on transcription. These examples indicate how histone acetylation can result in both increases and decreases in gene expression, depending on the site of acetylation and gene.

Histone methylation is primarily regulated by the activity of histone methyltransferases and histone demethylases. Histone methylation has been shown to both activate or repress gene expression, and highly depends on the specific residues modified, including on lysine and arginine residues (Xu et al., 2010; Barski et al., 2007). Xu et al. (2010) investigated 20 different histone lysine and arginine sites using a knock-out mouse model, and determined that H3R3me3 was the most transcriptionally repressive mark, while H3K36me3 was the most activating. A study by Barski et al. (2007) assessed all three states (mono-, di-, tri-methylation) of H3K4 around the Transcriptional Start Sites (TSS) of in relation to gene expression. In particular, elevated H3K4me3 around the TSS was positively correlated with gene expression, while H3K4me2 was less concentrated around TSS in comparison, while still being positively correlated with gene expression. Interestingly, in this same study H3K27me3 was found to be higher at silenced promoters, while H3K27me1 was found to be higher at active promoters (Barski et al., 2007). Taken together, these findings indicate that histone methylation can be site and state-dependant in its effects on gene expression.

DNA methylation and histone modifications have been shown to influence one another, suggesting interconnectedness in epigenetic regulation of gene expression. For example, the two chromatin-modifying machineries G9a/GLP and Polycomb Repressive Complex 2 (PRC2) which are

involved with H3K9 and H3K27 methylation, respectively, have been shown be co-recruited in mouse embryonic stem cells (Mozzetta et al., 2014). It was also found that H3K27me3 and DNA methylation were mutually exclusive in regions of high CpG density, while H3K9me3 and DNA methylation were co-localized in mouse embryonic stem cells (Brinkman et al., 2012). Finally, Fuks et al (2003) found that a specific methyl-CpG-binding protein (MeCp2) which was shown to bind methylated CpG sites on the *H19* gene is also involved with an increase of H3K9 methylation at the same locations in mouse fibroblast cells. Taken together, there is much evidence to support that there is interconnection between different epigenetic modifications. It is possible that chemical exposures which affect a particular modification may also have an impact on the profiles of other interconnected modifications.

2. Intrinsic Factors Affecting the Epigenome

2.1 Genetics

There are several ways in which genetic sequences influence epigenetic landscapes. Monozygotic twin studies have been used to demonstrate greater similarity in DNA methylation profiles within human adipose tissue and blood samples than dizygotic twins (Bell et al., 2012; Grundberg et al., 2013), which indicates a genetic heritability component of epigenetic profiles. Methylation quantitative trait loci (mQTLs) are one specific way by which genetic sequences can influence DNA methylation. mQTLs are specific gene loci where methylation states are influenced due to Single Nucleotide Polymorphisms (SNPs) (Rakyan et al., 2011; Gao et al., 2017). SNPs that lead to sequence variations where a cytosine or guanine in a dinucleotide is altered for a different base can lead to a loss of CpG site available for methylation. For example, if a cytosine in a cytosine-guanine dinucleotide is altered for a thymine, this would lead to the loss of a CpG site. Vice versa, a methylation site can also be gained due to the inclusion of a cytosine or guanine dinucleotide. Therefore, these SNPs provide a mechanism where DNA methylation patterns between individuals can differ (Gao et al., 2017; Volkov et al., 2016; Grundberg et al., 2013). For example, Grundberg et

al. (2013) investigated mQTLs in human adipose tissue and concluded that 37% of DNA methylation variability in their samples could be attributed to genetic variation and 15% of the total SNPs identified contributed to more than 50% of the genetic variability component. Volkov et al., (2016) state that they were able to replicate many of the same mQTLs found by Grundberg et al. (2013), though they do not mention which specific sites were replicated. To highlight the impacts of mQTLs a gene-specific level, one study investigated mQTLs on the *IGF2* and *H19* genes using a twin study and determined that 75-80% of the total observed variability in DNA methylation profiles on the *IGF2* gene could be attributed to mQTLs, while only 35% could be attributed to mQTLs in the *H19* gene (Heijmans et al., 2007). The discrepancies in the genetic influence on DNA methylation patterns found in Grundberg et al. (2013) and Heijmanns et al. (2007) may be due different gene-specific genetic influence on epigenetic regulation. Nonetheless, these findings indicate that certain SNPs in genetic sequences can have impacts on DNA methylation profiles, overall demonstrating a genetic influence on the epigenome.

Another genetic factor to consider is that genetic polymorphisms may be located in genes encoding for epigenetic modifying enzymes, such as DNMTs or HDACs. These polymorphisms may directly cause changes to the efficiency and activity of the encoded enzymes, leading to changes in the epigenetic landscape. For example, there are particular polymorphisms of the EZH2 enzyme, which catalyzes H3K27me3 (Margeuron et al., 2009; Yap et al., 2011), that are denoted as the rs3757441 variants. The *EZH2* rs3757441 variants have been compared for their correlations with H3K27me3 levels and EZH2 protein expression in humans (Fornaro et al., 2015). Fornaro et al. (2015) determined that there were up to 60% differences in H3K27me3 levels and 52% differences in EZH2 protein expression between three genotypes of this variant, with carriers of the C/C genotype exhibiting the highest levels/expression (Fornaro et al., 2015). These findings indicate how genetic

polymorphisms in epigenetic modifying enzymes, such as in the *EZH2* gene, can influence epigenetic profiles and lead to variability between individuals.

When assessing differentially methylated regions (DMRs) in response to chemical exposures, genetic variability is an important factor to keep in mind during interpretation of results. For example, Mao et al. (2017) assessed whether *in utero* BPA exposure affected DNA methylation profiles of specific CpG sites within the *IGF2* gene in rats. They concluded that in male rats, 60 % of assessed CpG sites were differentially methylated, while in female rats 25% of sites were (Mao et al., 2017). However, this study did not perform genetic sequencing to characterize SNPs within the *IGF2* gene in their model which might contribute to mQTLs. As previously mentioned, mQTLs in the *IGF2* gene in humans was shown to influence DNA methylation profiles, specifically resulting in 75-80% of the variability seen at this gene (Heijmans et al., 2007). Therefore, it is possible that to some extent, genetic variability in the rats used in the Mao et al. (2017) study may contribute to the DNA methylation differences seen, rather than entirely being due to BPA exposure. The existence of mQTLs on the gene of interest represent a confounding factor when interpreting DNA methylation following chemical exposure. The influence of genetic variability on epigenetic profiles may become even more crucial when studying chemical exposures in humans, who likely have greater genetic heterogeneity than lab animals.

2.2 Age

Ageing is a factor that influences the epigenome through a process referred to as epigenetic drift, characterized by gradual genome-wide alterations in DNA methylation and histone modification profiles (Issa, 2014). In human blood samples, 14.5% of genome-wide methylation sites were found to have increased deviation in association with increased age across an 82-year age-range, which were also associated with increased deviation in gene expression in the 326 genes tested (Hannum et al., 2013). Interestingly, another study reported far lower (< 5%) total DNA methylation site change with

age, however the study took place across a 33-year age range and only assessed in males (Steegenga et al., 2014). Despite this discrepancy, even if <5% of methylation sites saw changes, this still represents hundreds of thousands of sites across the human genome seeing alterations to epigenetic regulation. Overall trends towards global hypomethylation have been observed with ageing in humans (Jintaridth and Mutirangura, 2010; Bollati et al., 2009), however studies have found both increases and decreases of DNA methylation at specific sites. For example, Horvath (2013) analyzed 7844 samples from publicly available data sets encompassing 51 different healthy human cell types and determined that out of 353 CpG sites found to be strongly correlated with age, 54.7% were hypermethylated while 45.3% were hypomethylated. This study divided age categories into individuals younger than 35, and individuals older than 55 (Horvath, 2013). In terms of gene-specific DNA methylation changes with age, Issa et al. (1994) showed that DNA methylation of the estrogen receptor in human colon mucosa increases with age, potentially indicating an epigenetically mediated decline in cell growth and proliferation with age. West et al. (2013) used a network-analysis algorithm to elucidate areas of differential methylation in association with age in humans, encompassing over 1000 different samples from a variety of human tissue types, found that the gene *UTF1* was one of the most consistently differentially methylated age-related genes. *UTF1* is involved in stem cell differentiation, and therefore promoter hypermethylation of this gene suggests a reduction of stem cell differentiation with increasing age.

It is clear that an ageing epigenome exhibits different DNA methylation profiles over time, which can be gene specific. Therefore, age may play a role in variable epigenetic responses to chemical exposures. Broadly, changes to DNA methylation patterns may cause older individuals to have greater susceptibility to toxic outcomes. Many studies have explored the effects of various chemical exposures on gene-specific DNA methylation, including the *p16* gene. For example, *p16* promoter methylation was found to be over 20% higher in the stomach tissue of humans over 40 years

of age compared to under (Waki et al., 2003). Though *p16* gene expression was not measured in this study, decreased promoter methylation in humans may lead to increased expression of this gene. Herrera-Moreno et al. (2019) found that occupational organophosphate pesticide exposures resulted in a significant increase in methylation by ~2% across several CpG sites on the *p16* gene from human blood samples. A 2% change may not seem like a significant increase at face value; however this study did not control for different ages in this methylation analysis and therefore these findings must be interpreted with caution. Considering the possibility that methylation in the promoter of this gene increases with age, perhaps even differing up to 20% between older and younger individuals (Waki et al., 2003), the extent to which organophosphate pesticide exposures may further methylate CpG sites on gene could have greater implications on the expression of *p16* in older individuals. If an older individual with a more highly methylated *p16* gene promoter encounters a chemical which results in additional 2% increase in methylation across this gene, this exposure could potentially result in lower overall *p16* gene expression than a younger individual experiencing a 2% increase. Therefore, the influence of age on the epigenome may lead to variable epigenetic responses following chemical exposures.

Histone modifications profiles can also be influenced by ageing. Several studies have found genome-wide changes in histone modifications in older organisms compared to younger ones. For example, H3K9me3 was found to be reduced by nearly half, while H3K9Ac was found to be increased by over 1.5-fold in the cortexes from old (55-65 weeks old) compared to young (4-8 weeks old) female mice (Rodrigues et al., 2014). However, another study found 30-month-old rats saw an ~10% decrease in H3K9Ac in their liver compared to 6-month-old rats (Kawakami et al., 2009). These discrepancies in H3K9Ac reductions observed could potentially be due to the age comparisons, with the Kawakami et al. (2009) study comparing rats that were twice as old as the Rodrigues et al (2014) study or due to tissue type. Nonetheless, it can be concluded that some site-specific histone modifications profiles,

such as H3K9me3 or H3K9ac, likely differ in association with age. Ageing is also associated with increased cellular senescence. Ageing has been shown to increase cellular senescence in mouse models (Biran et al., 2017; Farr et al., 2016). Ageing and cellular senescence is partly mediated by histone modifications. Senescence-associated heterochromatin foci (SAHF) are specific chromatin patterns that have been found to regulate genes signalling for cellular senescence and growth arrest, such as decreased *PCNA* gene expression (Narita et al., 2003). Narita et al. (2003) first described SAHF following the observation of 30-50 specific histone modification loci in senescent human fibroblasts that were distinguishable from chromatin patterns in healthy human fibroblasts. Specifically, it was found that methylated H3K9 was enriched in SAHF while acetylated H3K9 was largely lacking via immunofluorescence, however this study did not state the extent to which these modifications differed to normal cells (Narita et al., 2003). Several factors have been implicated in the induction of SAHF formation, including expression of the tumor suppressor gene *p16* (Narita et al., 2003). However, it is important to mention that several studies have also found cellular senescence to occur in without the formation of SAHF in cell lines and mouse models, as reviewed by Aird & Zhang (2012). Nonetheless, SAHF formation is a particular histone modification pattern that differs between senescent cells and healthy cells, and therefore is another way that age may influence the epigenome.

Differences in histone modifications profiles between individuals due to ageing may influence how chemical exposures affect the epigenome. Specific chemical exposures may have impacts on histone modifications profiles via activation of tumor-suppressors genes. Exposure to 5 μM of BPA in murine endothelial cells for 5 days has been shown to nearly double the protein expression of p16, as well as increase cellular senescence by 30% (Moreno-Gómez-Toledano et al., 2021). As mentioned above, gene expression of *p16* is one factor shown to contribute to SAHF formation (Narita et al., 2003). Therefore, exposure to environmental chemicals such as BPA that potentially induce

expression of *p16*, could have implications for cellular senescence via SAHF-mediated epigenetic regulation. Future studies that consider the effects of BPA exposure on H3K9 acetylation/methylation levels should consider how age is an intrinsic factor which may contribute to higher expression of both cellular senescence and SAHF. BPA exposure could potentially cause differences in H3K9Ac and H3K9me3, which may already be variably expressed depending on age (Narita et al., 2003; Kawakami et al., 2009; Rodrigues et al., 2013). These exposures could have variable effects on histone modification profiles of an ageing epigenome, therefore should be considered when designing a study to investigate BPA-mediated epigenetic alterations.

2.3 Sex

Sex is another intrinsic factor which can have an impact on DNA methylation and histone modification profiles. In terms of DNA methylation, 179 DMRs between sexes were identified on autosomal chromosomes across 3795 adult human whole blood samples (Gatev et al., 2021). In this study, it was found that 85% of the DMRs had higher methylation levels in females than males. Another study found 3604 DMRs between sexes from cord blood samples at birth, with 75.8% having higher methylation in females compared to males, though this analysis did not mention being specifically restricted to autosomes (Yousefi et al., 2015). Histone modifications profiles have also been shown to have sex-specific patterns. One particular histone modification, H3K27me3, has been shown to have sexually dimorphic expression due to its involvement with X-chromosome inactivation and imprinting (Rouguelle et al., 2004; Li et al., 2012; Casciaro et al., 2021). Li et al. (2012) determined the expression of H3K27me3 was highly localized to the inactivated X chromosome in female human embryonic lung cells. Rouguelle et al. (2004) also showed a 4-7x higher association of H3K27me3 with several X-linked genes including *chic1*, *g6pd* and *hprt* in female cells compared to male cells. Finally, a study investigating H3K27me3 distributions in the brains of mice found that the intensity of the H3K27me3 signal around TSS of X-linked genes was nearly doubled in females compared to

males, though no differences were identified when assessing TSS on autosomal chromosomes only (Casciaro et al., 2021). In humans only 3.75% of genes are located on the X-chromosome however nearly a third of these genes are involved with cognition, as reviewed by Skuse (2005). This indicates the potential downstream significance of chemically induced epigenetic dysregulation of H3K27me3 on the X-chromosome.

When considering the impact of specific chemical exposures on the epigenome, there may be sex-specific implications to H3K27me3 patterns. For example, Bhan et al. (2014) demonstrated that exposure to BPA increased the expression of the *ezh2* gene, which as previously mentioned is a methyltransferase for H3K27me3*,* by nearly 4-fold in mammary glands of female rats. However, these results were not compared to male rats. Exposure to BPA may alter the epigenetic landscape by increasing the expression of *EZH2* and thus H3K27me3, potentially leading to sex-specific outcomes given the importance of this histone modification for X-inactivation and imprinting. Indeed, using ChIP-Seq, Weng et al. (2024) have determined that out of 10,581 identified gene loci associated with H3K27me3 in mouse brains exposed to BPA *in utero*, 125 (1.2%) saw a significant increase in H3K27me3 occupancy and were highly located near TSS. However, this study did not state whether the genes associated with H3K27me3 were X-linked and did not stratify the sexes, therefore not capturing possible sex-specific implications. BPA exposure *in utero* has also been shown to result in sex-specific effects on anxiety behaviour and cognition tests in mice (Kundakovic et al., 2013), indicating BPA alters brain development in a sexually dimorphic way. Future research investigating BPA exposure on H3K37me3 patterns should consider how innate sexually dimorphic distributions, especially with higher distributions on X-linked genes in females, might confound global measurements of H3K37me3. Overall, there are sex-specific distributions of DNA methylation and histone modification and there may also be sexually differential responses in the epigenome when exposed to chemicals such as BPA.

2.4 Tissue Type

Tissue type is another factor which can influence DNA methylation and histone modification profiles, with different tissue types exhibiting variable levels of various modifications. It has been demonstrated that both H3K9 methylation levels, as well as the methylation state (mono-, di-, tri-) can vary widely between tissue types (Garcia et al., 2009). Garcia et al (2009) investigated levels of several histone modifications in various rat tissue types and found H3K9me2 levels were 3-fold lower in the testes compared to the liver. Furthermore, H3K9me2 to H3K9me3 levels were found to be nearly identical in the lungs, while H3K9me2 was nearly 3-fold lower than H3K9me3 in the liver (Garcia et al., 2009). In terms of DNA methylation changes, it has been shown that human embryonic stem cells, adult blood, adult breast and fetal brains exhibit many DMRs in relation to each other. Human embryonic stem cells were found to have a total of 721 DMRs in comparison to the other three tissue types, with 82% being hypermethylated, adult blood was found to have 725 total DMRs (31.5% hypermethylated), adult breast had 578 DMRs (19.9% hypermethylated) and fetal brains had a total of 751 DMRs (0% hypermethylated, 100% hypomethylated) (Zhang et al., 2013). These differences likely reflect tissue-specific functions, with fetal brains likely exhibiting high genome-wide levels of transcription. These findings underscore the degree to which DNA methylation and histone modification profiles depend on tissue-type.

While epigenetic marks can be differentially expressed between tissues, so can the enzymes which regulate them. Chemicals can interact with epigenetic regulating enzymes, and if the affected epigenetic enzymes are tissue-specific, chemical exposures can cause variable consequences to the epigenome depending on tissue type. Wang et al. (2022) assessed the tissue-specific expression of hundreds of different epigenetic regulators (ERs) across 30 different human tissues. This study stated that the majority of the differentially expressed ERs were either testis or brain specific. For instance, *PRDM9*, which has histone methyltransferase activity on H3K4 (Wu et al., 2013), was found in this

study to be exclusively expressed in the testis out of the 30 tissues tested (Wang et al., 2022), however other studies have shown it to be expressed in human placenta and lung cells as well (Winterbottom et al., 2019; Ladias et a., 2023). Winterbottom et al. (2019) investigated the expression of a variety of ERs in human placentae in association with prenatal arsenic exposure. This study found that *PRDM9* expression was negatively correlated with arsenic exposure in female offspring placentae but not in male offspring placentae following arsenic exposure. This finding suggests that exposure to arsenic could have differential epigenetic effects depending on tissue type, with greater implications perhaps in tissues which highly express *PRDM9*. When interpreting the epigenetic effects of a specific chemical, it is important to have an understanding of innate differences in tissue-specific epigenetic profiles. Given that DNA methylation profiles can differ significantly between human embryonic stem cells, adult blood, adult breast and fetal brain (Zhang et al., 2013), it would likely be incorrect to assume that a chemical that decreases DNMT activity, for example, by 20% would impact all tissue types equally. Furthermore, in the case of investigating arsenic exposure on H3K4 methylation, very different results could plausibly be found in tissues expressing *PRDM9* like the testis, placenta or lungs vs. non-expressing tissues. For example, one study did not find any changes to H3K4 methylation in human blood samples following arsenic exposure (Chervona et al., 2012), while two other studies did find significant decreases in human lung cells (Zhou et al., 2009; Tu et al., 2018). It is possible that tissue-specific expression of *PRDM9* led to discrepancies in findings, underscoring how different tissue-types may have variable epigenetic responses to chemical exposures.

3. Extrinsic Factors Affecting the Epigenome

3.1 Diet

Diet is an external factor that can influence the epigenetic profiles in several different ways, namely by affecting the availability of substrates and co-factors for epigenetic modifications. The extent to which diet can impact the epigenome is likely highly dependent upon the specific dietary variable,

therefore I discuss diet in regard to a specific co-factor. The essential Vitamin B_{12} is a rate-limiting co-factor for an enzyme involved the process of regenerating methionine from homocysteine in one-carbon metabolism (Guéant et al., 2020), and is acquired through the diet. Methionine is converted to SAM, which is a methyl donor for many biological processes. Amongst them, for methylation-related epigenetic modifying enzymes, such as supplying DNMTs and histone methyltransferases (Froese et al., 2018; Mentch & Locasale, 2016; Yu et al., 2019).

In terms of impacts on histone modifications, a B_{12} deficient diet (0 mg/kg) in pregnant mice reduced H3K9me3 levels by 2.5-fold in placental tissue compared to a normal B_{12} -level diet group (0.025 mg/kg) (Mahajan et al., 2021). Furthermore, H3K4me2 was also found to be increased by 5 to 6-fold in the same B_{12} deficient versus normal diet groups. Taken together, these findings indicate that a B_{12} deficient diet may impact the expression of certain histone methylation modifications. To investigate the impacts of B_{12} supplementation on DNA methylation, Yadav et al. (2018) identified genome wide differentially methylated CpG sites from blood samples from B_{12} supplemented children compared to a placebo receiving group. They found a total of 589 differentially methylated sites out of ~483,000 loci measured (Yadav et al., 2018). Though this represents a 0.1% genome-wide change, many of these sites were found to be associated with genes involved with type 2 diabetes, insulin resistance and obesity, indicating that there were targeted methylation changes (Yadav et al., 2018). Interestingly, the 60% of these differentially methylated sites following supplementation were found to be hypomethylated rather than hypermethylated (Yadav et al., 2018). However, there has been conflicting evidence. A study by Monasso et al. (2023) reports maternal B_{12} blood concentrations were associated with 109 differentially methylated CpG sites in offspring blood samples, with even distribution of hypo- and hypermethylated sites. Furthermore, a study by Tanwar et al. (2020) found that there were a greater number of hypermethylated sites than hypomethylated sites in the offspring of rats that were B_{12}-deficient during pregnancy. This study found that differentially methylated sites

were enriched at genes involved with fatty acid metabolism. Taken together, these findings indicate that B_{12} levels can impact DNA methylation profiles, however an increase or decrease in methylation status can be site-specific, and perhaps also dependant on the study design.

Overall, these studies suggest that when investigating histone modification levels and genome-wide methylation profiles in association with chemical exposures in humans, it would be wise to control for diet. Especially for dietary factors such as B_{12} which have evidence in the literature of altering methylation profiles. For example, the gene *acaa2*, which encodes an enzyme for fatty acid oxidation, was found to be hypermethylated in the B_{12}-deficient group of rats (Tanwar et al., 2020). One study investigated the effect of exposure to a mixture of perfluorooctane sulfonic acid, perfluorohexanoic acid and 3,3′,4,4′,5-pentachlorobiphenyl in zebrafish embryos on the expression of *acaa2*, and found decreased expression, along with increased expression of *dnmt1* and *dnmt3b* (Blanc et al., 2017). While this study by Blanc et al. (2017) does not confirm whether this chemical mixture results in hypermethylation of the *acaa2* gene, future studies investigating this link should consider how diet is a confounding variable, especially the implication of B_{12} deficiency on the hypermethylation state of this gene. More generally, variability in diet in human epidemiological studies can be a major confounding factor when investigating epigenetic effects of certain chemicals due to the involvement with providing substrates and co-factors for epigenetic processes, such as B_{12}.

3.2 Mating Status

Mating status can have a variety of different meanings in different species; here, I discuss it through the lens of cyclical reproductive states in female humans. DNA methylation and histone modification profiles can be influenced by the menstrual cycle. In humans, DNA methylation profiles have been shown to change between the pre-receptive phase of the menstrual cycle to the receptive phase (Kukushkina et al., 2017). Genome-wide analysis revealed 2026 DMRs, of which 81% were hypermethylated and 19% were hypomethylated between the two phases (Kukushkina et al., 2017).

Saare et al. (2016) performed a similar study but divided the menstrual cycle up into more phases than Kukushkina et al (2017). It was found that DMRs differed significantly between certain phases, for example there were 2781 DMRs, associated with 1698 different genes, identified between the menstrual and proliferative phases (Saare et al., 2016). These findings indicate that DNA methylation patterns may significantly differ between menstrual phases in humans.

Histone modifications are also involved with menstrual cycle regulation, and experience changes throughout the cycle. As ovarian follicles mature, they produce estradiol-17β (E_2) (Drummond & Findlay, 1999). Estrogen receptors are transcription factors for a number of genes with Estrogen Response Elements, including the H3K27 histone methyltransferase gene *EZH2* (Bhan et al., 2014). In humans, E_2 levels fluctuate throughout the menstrual cycle and are highest during the mid-follicular phase, which may shape histone methylation profiles via EZH2. A review conducted by Retis-Resendiz et al. (2021) compiled evidence from several studies and concluded that both *EZH2* gene expression and H3K27me3 levels are higher during the proliferative phase compared to the secretory phase of the endometrial cycle, which tracks with estrogen levels. For example, Grimaldi et al. (2011) determined that the differentiation of human endometrial stromal cells into decidual cells (which occurs during the shift between the proliferative and secretory phases of the endometrial cycle as estrogen levels drop and progesterone levels rise) leads to a nearly 75% reduction in *EZH2* gene expression and H3K27me3 levels. Thus, specific epigenetic marks such as H3K27 methylation may be influenced by menstrual cycle phases.

As mentioned, certain endocrine disrupting chemicals such as BPA may alter EZH2 activity (Bhan et al., 2014). Bredfeldt et al. (2010) demonstrated that exposure to another estrogenic endocrine disrupting chemical diethylstilbestrol (DES) in rats led to a 0.5x reduction of H3K37me3 in their uterine tissue. Furthermore, using human breast cancer cell lines, the same study also determined that DES phosphorylates the EZH2 enzyme, activating it. Therefore, it is possible that variable estrogen

levels in reproductive tissues throughout the menstrual cycle could lead to differences in EZH2 activity and H3K27 methylation. When investigating the impacts of chemical exposures, such as to DES, which are also capable of altering *EZH2* and H3K27me3 in humans, the phases of the menstrual cycle should be considered when interpreting results as they may have a role in shaping the epigenome.

3.3 Chemical Exposures

Quantifying the extent to which chemicals affect the epigenome is highly dependent on the specific chemical and its interactions with epigenetic machinery. In real world settings, chemical exposures often occur in undefined mixtures, which carries the potential of chemicals acting synergistically or antagonistically on the epigenome. Separating the individual effects of chemicals on the epigenome in human studies is one limitation often faced in toxicological and epidemiological studies. Therefore, here I discuss how two chemicals, triclocarban and BPA, may interact with the epigenome individually, and with one another in their cumulative effects.

Firstly, it was demonstrated that female mice exposed to 5 mg/kg of triclocarban, an antibiotic commonly found in hand soaps, had a 32% decrease in nanograms of globally methylated DNA per nanogram of total DNA in brain tissue, while males had a 20% increase (Wnuk et al., 2021). The effects of the triclocarban on histone modifications has also been investigated. It was determined that exposure to 6 μM triclocarban in human placenta cells increased the histological fluorescent intensity of H3K4me3 by ~25% and H3K27me3 by ~50%, while H3K9 and H3K27 acetylation were both found to be decreased by ~20% (Ding et al., 2023). In terms of BPA, exposure to 15 μg/L of BPA for 7 days in zebrafish embryos resulted in ~1% decrease in global DNA methylation levels in both ovaries and testes (Liu et al., 2016). Song et al. (2021) investigated DNA methylation in human placental tissue in association with environmental BPA exposures as measured through serum levels in humans. This study identified 155 hypomethylated differentially methylated sites and 127

hypermethylated sites, which could potentially amount to an overall global decrease in methylation. In terms of the effects of BPA on histone modifications, H3K4me3 was significantly reduced following 10nM of BPA, while H3K27me3 was significantly increased at the same concentration, determined with immunofluorescence in human embryonic stem cells (Xiong et al., 2020). Taken together, both triclocarban and BPA have been shown to influence DNA methylation and histone modification profiles when exposed in isolation.

However, exposure to both triclocarban and BPA in undefined mixtures may occur in humans, so individual contributions to epigenetic changes may not provide an accurate picture. For example, Chung et al. (2011) explored how the combined exposure to triclocarban and BPA interact with aromatase (*AroB*) gene expression in zebrafish embryos. BPA exposure alone resulted in a significant increase of *AroB* in the brain, while triclocarban exposure alone resulted in a slight increase in *AroB* in the brain. When exposed together however, *AroB* expression was suppressed compared to the response of BPA exposure alone, demonstrating a form of antagonism. Given that the aromatase enzyme converts testosterone into estrogen, it is possible that these two chemicals together alter estrogen production in a more attenuated way than would be expected. Based on the studies of the two individual chemical exposures, it may be hypothesized that exposing female embryos to triclocarban and BPA together would result in a more significant decrease in DNA methylation than if either chemical were exposed alone (Wnuk et al., 2021; Liu et al., 2016; Song et al., 2021). However, given the findings of Chung et al. (2011), it is possible that these two chemicals may act antagonistically to a certain extent, leading to a surprising attenuation of the DNA methylation response. Both chemicals have been identified as environmental pollutants (Amigun Taiwo et al., 2022, Huang et al., 2012), but have not yet been studied in combination to specifically investigate their effects on the epigenome. Therefore, any future studies investigating the impacts of these chemicals on the epigenome in humans may want to consider how inadvertent environmental

exposures to both chemicals may act as confounding factors when attempting to elucidate the impact of each individual chemical on DNA methylation profiles. For example, the study mentioned in the previous paragraph by Song et al. (2021) did not consider nor control for triclocarban exposure, unsurprisingly. This is just one specific example of how two different environmental pollutants may interact with DNA methylation profiles, but given the wide variety of different chemicals in the environment and various possibilities for interactions with the epigenome, this remains a large limitation to consider in human studies.

5. Conclusion

In sum, there is evidence to support that the epigenome is shaped by various intrinsic and extrinsic factors such as genetic polymorphisms, age, sex, tissue type, diet, reproductive state and chemical exposures. These factors can influence DNA methylation and histone modifications, often in interconnected ways. These intrinsic and extrinsic factors which already influence the epigenome may result in variable responses when individuals are exposed to chemicals. A key consideration when considering how chemical exposures shape the epigenome is recognizing the inherent variability in how different individuals and tissues respond to chemical exposures due to their unique epigenetic landscape. Understanding and quantifying what constitutes normal epigenetic fluctuations is a major consideration for future toxicological assessment of chemicals in order to identify chemically-induced changes as adverse outcomes.

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