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Establishing a role for environmental toxicant exposure induced epigenetic remodeling in malignant transformation.

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Abstract

Humans are exposed to a wide variety of environmental exposures throughout their lifespan. These include both naturally occurring toxins and chemical toxicants like pesticides, herbicides, and industrial chemicals, many of which have been implicated as possible contributors to human disease susceptibility [1–3]. We, and others, have hypothesized that environmental exposures may cause adaptive epigenetic changes in regenerative cell populations and developing organisms, leading to abnormal gene expression and increased disease susceptibility later in life [3]. Common epigenetic changes include changes in miRNA expression, covalent histone modifications, and methylation of DNA. Importantly, due to their heritable nature, abnormal epigenetic modifications which occur within stem cells may be particularly deleterious. Abnormal epigenetic changes in regenerative cell linages can be passed onto a large population of daughter cells and can persist for long periods of time. It is well established that an accumulation of epigenetic changes can lead to many human diseases including cancer [4–6]. Subsequently, it is imperative that we increase our understanding of how common environmental toxins and toxicants can induce epigenetic changes, particularly in stem cell populations. In this review, we will discuss how common environmental exposures in the United States and around the world may lead to epigenetic changes and discuss potential links to human disease, including cancer.

Introduction

In an industrialized world with many rapidly expanding economies, the requirement for, and usage of pesticides and chemical byproducts is constantly expanding. This increased generation and usage of pesticides and chemical byproducts magnifies the worldwide issue of environmental toxicant accumulation and subsequent human exposure. It has been established that exposure to environmental toxicants may be associated with a negative

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impact on human health in vulnerable populations [7]. However, these epidemiological links have been challenging to expand to broader human populations, largely due to variability in exposures and the incomplete penetrance of observable phenotypes. Studies are confounded by the impact of genetic variability and lifestyle and the observation that in virtually all cases, individuals are exposed to toxicants as mixtures. Additionally, the majority of these chemical compounds undergo biotransformation and/or metabolic processing in the human body, adding complexity to experimental design. We, and others, have hypothesized that epigenetic remodeling is a critical mechanism by which environmental toxicants alter a cell or an organism. The multigenerational effect of toxicant exposure infers that embryonic and somatic stem cells are experiencing the damaging effects of environmental toxicant exposures [8]. Research also suggests that fluctuations in epigenetic regulation of gene expression secondary to toxicant exposure can be heritable and potentially passed down to future generations, helping to explain the variability in penetrance seen in epidemiological studies. In this manuscript, we review common environmental toxicants and their impact on the genome and epigenome, and discuss how these changes may contribute to human cancer incidence.

Environmental Toxicant Exposures and Cancer Etiology

Many biological and molecular processes which are potentially altered by exposure to environmental toxicants have been shown to independently contribute to malignant transformation. However, it is very difficult to confirm a direct causative effect between exposure to a single environmental toxicant and the onset of any specific cancer. This is due to the complexity and variability of toxicant exposures, a wide range of factors that may affect the potency of these exposures, and the often long latency periods between known exposures and the onset of human disease. Despite these and other challenges with regard to these complicated epidemiological studies, multiple research studies have shown a significant association between environmental toxicant exposure and cancer incidence [9, 10], most notably with heavy metal and pesticide exposures. Heavy metal exposures, including exposure to arsenic (As-), cadmium (Cd-), chromium (Cr-), and nickle (Ni-) have all been shown to induce cell transformation and have been linked to human malignancy. Heavy metal carcinogenesis has been reviewed extensively elsewhere [11, 12], and this review will focus primarily on non-metal exposures except to note where toxicant mechanisms converge [13].

Pesticides are collections of chemicals which are spread throughout the environment with the sole purpose of eliminating pests. They are designed to eradicate bacteria, fungi, plants, insects or small animals, and as such, are intrinsically toxic to these targeted organisms. Worldwide between 2008 and 2012, herbicides were the most commonly used pesticide, followed by insecticides and fungicides respectively[14]. In the United States, the agriculture industry has been the largest consumer of pesticides and herbicides in recent years. The EPA estimated that as recently as 2011 and 2012, the United States used over 1.1 billion pounds of pesticides annually [14]. This high agricultural usage of pesticides is of particular concern as it increases the likelihood of human exposure and the risk of bioaccumulation [15]. Bioaccumulation of a substance occurs when the rate of intake exceeds the ability of the organism to excrete or metabolically transform that substance. The

result is an accumulation of a toxic chemical in the various tissues of a living organism. Dozens of pesticides are currently approved for both personal and commercial use in the United States. Subsequently, pesticide usage varies by geographic location, crops, and targeted organisms. Accordingly, individuals from different environments are exposed to these chemicals as mixtures, and the risk associated with human exposure and bioaccumulation of pesticides is exacerbated. While safety information is available for these chemicals individually, little is known about the potential toxic effects of pesticide mixtures and industrial chemical by-products or about the effects of these chemicals on the genome and the epigenome of humans.

Perhaps the most studied and best understood association between pesticide exposure and cancer incidence involves the link between exposure to Agent Orange and cancer incidence during the Vietnam War. It is estimated that 74 million liters of herbicides were used during the Vietnam War with the most commonly used herbicide being Agent Orange [16]. Agent Orange is a 1:1 mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) herbicides. A chemical byproduct in the synthesis of many pesticides, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is a known carcinogenic persistent organic pollutant (POP) which has also been determined to be a heavy contaminant in Agent Orange [17]. The National Academies of Science has acknowledged sufficient evidence to link thirteen different cancer types to Agent Orange exposures [18, 19]. One group of these cancers, soft tissue sarcomas, has also been linked to other environmental toxicant exposures independently from wartime pesticide exposure including both organochlorines and organophosphates.

Organochlorine chemicals are historically the most widely used class of insecticide. They are defined by having a chlorinated hydrocarbon structure. Subsequently, they have low water solubility and are highly lipophilic. Furthermore, organochlorines are very stable molecules which have been shown to persist in the environment for extended periods of time and have the capability of bioaccumulation.

Organophosphates (OPs) are derivatives of phosphoric acid. Generally, they are produced by the esterification of phosphoric acid. This class of chemicals is widely used as pesticides and in the production of plastics or solvents. Most OPs are highly lipid-soluble and subsequently readily absorbed through the skin and mucus membranes. The chemical conformation of these compounds contributes to their ability to function as neurotoxins. A 2009 Brazilian study observed an increased incident of soft tissue sarcoma in individuals who reside in areas with a high accumulation of pesticides [20]. Similarly, a notable 2011 Canadian population-based study reported a statistically significant increase in risk of developing soft tissue sarcoma in individuals exposed to organochloride and organophosphate pesticides [21]. While many reports suggest a positive correlation between pesticide exposure and cancer, these epidemiological studies remain controversial as other studies report more modest, limited causative effects of toxicant exposure [22, 23]. Given these recent contradictory reports on the strength of association between environmental exposures and cancer incidence, further studies are warranted to examine the involvement of chemical mixtures in cancer etiology and to increase our understanding of the molecular mechanisms by which environmental exposures may contribute to tumorigenesis.

Environmental toxicant induced DNA damage

DNA damage and genomic instability have been widely studied as a result of oxidative stress. Exposure to environmental toxicants is known to result in the accumulation of DNA damage [24–28]. These exposures tend to happen over long periods of time and with varying chemical mixtures. Many environmental toxicants, including both metals and pesticides, have been shown to generate reactive oxygen species (ROS), leading to oxidative DNA damage including single and double strand breaks [24]. Low doses of pesticides have been shown to inhibit the activity of topoisomerase II, an enzyme responsible for relieving positive supercoiling during replication and transcription [25, 26]. Specifically, OP pesticides inhibit topoisomerase II and consequently induce DNA damage in human cells [29]. These findings suggest that exposure to environmental toxicants can induce genomic instability ancillary to topoisomerase inhibition.

Interestingly, lymphocytes isolated from agricultural workers exposed to organophosphate pesticides showed elevated levels of oxidative stress and DNA damage [30]. Furthermore, DNA damage in mammalian peripheral blood lymphocytes has been specifically shown to be a result of oxidative stress induced lesions after exposure to organophosphates [27]. Several studies have shown a dose dependent relationship between exposure to pesticides and oxidative stress [28, 31]. The dose dependent relationship between toxicant exposure and oxidative stress has also been correlated with a dose dependent decrease in cell viability [31]. This suggests that at lower levels of exposure, cells will experience less than lethal amounts of DNA damage. Common low level exposure experienced by individuals throughout their lifetimes do not induce overt toxicity; therefore, it is important to consider that for the majority of the population, less than lethal levels of DNA damage can contribute to genomic and epigenomic instability and leave cells poised for deleterious transformation.

Several other toxicants have been implicated in the modulation of and response to DNA damage. Synthetic auxin herbicides are a class of chemicals that have been shown to render cells vulnerable to accumulation of DNA damage[32]. Synthetic auxins have been designed to mimic the structure and function of naturally occurring plant hormones, auxins, which are required for normal plant growth and development. Auxinic herbicides are widely used as selective herbicides because they target dicot weeds and spare monocots. Interestingly, Indole 3 acetic acid (IAA), the most common auxin found in plants, has also been identified in the tissues of mammals [33]. A recent study showed that auxins can induce increased proliferation in mammalian epithelial cells [34]. Furthermore, IAA was shown to attenuate p53 mediated apoptosis secondary to hydrogen peroxide induced oxidative toxicity [35]. Attenuation of p53 mediated apoptosis could potentially allow for cellular proliferation despite the presence of deleterious DNA damage. Cellular proliferation in the presence of induced DNA damage and acquired mutations may be particularly detrimental in a population of regenerative cells such as somatic stem cells. Considering that many herbicide mixtures contain both synthetic auxins and additional compounds which are known to induce oxidative stress and DNA damage, further investigation into the possible synergy between compounds within these mixtures and their potential role in malignant transformation and oncogenesis is warranted.

Examples of synthetic auxins that may be implicated in tumorigenesis are Picloram and 2,4-Dichlorophenoxyacetic acid (2,4-D). Picloram is a selective herbicide which targets woody plants and broad-leaved weeds, while leaving most grasses unaffected. The selective herbicidal qualities of Picloram make it appealing in both the residential and agricultural settings. Picloram functions as an herbicide by replacing the plant growth regulator, indoleacetic acid, and subsequently inhibiting protein synthesis, ultimately leading to plant death [36]. Many studies have shown that exposure to Picloram correlates with an increased incidence of benign neoplasm in mammals [37]. Furthermore, Picloram, along with 2,4-D (discussed below), and triisopropanolamine were major components of Agent White, an herbicide commonly used during the Vietnam war. Exposure to Agent White has been speculated to correlate with an increased incidence of neoplasms in exposed populations, though data is limited. The U.S. Environmental Protection Agency (EPA) and the World Health Organization both list Pilcloram as “unlikely to be hazardous”. As a result, it is still commercially available and widely used both in residential and commercial settings. 2,4-Dichlorophenoxyacetic acid (2,4-D), like Picloram, selectively targets broad-leaved weeds without affecting most grasses. Specifically, 2,4-D functions by mimicking the plant hormone auxin which stimulates uncontrolled growth. The uncontrolled growth initiated by 2,4-D is unsustainable and eventually leads to plant death, making it an effective herbicide. While reviews have not found statistically significant correlations between exposure to 2,4-D and any chronic adverse effect in humans [38], the World Health Organization classified 2,4-D as a possible carcinogen. Interestingly, studies have shown 2,4-D to induce oxidative stress as well as hepatotoxicity [39]. Despite the suggested carcinogenic properties of 2,4-D, it is also still commonly used in residential and commercial settings.

DNA Damage Response in Stem Cells and Cellular Regeneration

Stem cells, being regenerative and multipotent, experience an increased demand to repair damaged DNA. This leaves them particularly vulnerable to an accumulation of repair-induced gene expression changes and epigenetic alterations. These alterations could drastically impact the normal functions of an organism over time. Many groups have suggested that stem cells or early progenitor cells may be the precursors from which cancer cells are derived. This hypothesis is based on the observation that tumors often express stem/precursor cell population markers, and have heterogeneous (non-uniform, mixed) cell populations among which only a small percentage of cells have tumor regeneration capacity. Tumor cell growth is also driven by the up-regulation of many pathways important in the maintenance of normal stem/precursor cells including the Wnt, Hedgehog, and Notch pathways. This cancer stem cell hypothesis may be partially responsible for why cures for many types of cancer have remained so elusive, and it is thought that acquiring stem cell like properties may help cancer cells to metastasize. Chemotherapeutic drugs, which target the rapidly proliferating bulk tumor population, may leave the relatively quiescent cancer stem cells un-touched and allow for tumor re-growth at a distant point in the future. Both normal and malignant stem cells are also known to be comparatively resistant to chemotherapy and retain some degree of differentiation potential. We, and others, have hypothesized that it is this differentiation capacity, and the degree of epigenetic plasticity that is associated with that potential, that may leave stem cells particularly vulnerable to environmental insults and

malignant transformation. Therefore, it is important to consider the effects these toxicants may have on stem cell biology and how this may contribute to pathogenesis.

Within a normal cell, significant DNA damage in the absence of efficient repair would most likely result in cell death. However, stem cells harbor the ability to manage larger amounts of these side effects due to their increased ability to repair DNA. In order to protect the genome from building up harmful mutations, cells have evolved elaborate mechanisms that allow for the detection and proper removal of deleterious lesions. There are several different types of damage, and consequently there also exists several different types of repair processes, together termed the DNA Damage Response (DDR) [40, 41]. The DDR is controlled by a large group of enzymes that are capable of recognizing genomic lesions, halting the cell cycle, and repairing damaged DNA. Interestingly, stem cells, especially embryonic stem cells, have an increased capacity to repair their DNA [42]. Stem cell's increased capacity to handle genomic insults has obvious benefits considering their crucial role during development and tissue repair. However, recent studies suggest that the repair of DNA may result in lingering effects on the genome, particularly via modifications to the epigenome and subsequent changes in gene expression [43–46]. Specifically, recruitment of the histone modifying complexes Polycomb, NuRD [46], and DNMTs to genetic lesions has been observed secondary to DNA damage [43]. Also, upon DNA damage, a transient, global, downregulation of gene expression due to DNA damage-induced degradation of RNA Pol II has been shown [44]. With repeat acute exposures, or long term chronic exposures, these affects may linger, ultimately disrupting normal cellular function and highlighting the key role that epigenetic remodeling is likely to play in mediating environmental toxicant exposures.

Epigenetic Remodeling and Toxicant Exposures

Epigenetics is defined as the study of heritable changes in gene expression that occur independently of alterations in the primary DNA sequence. Gene expression is controlled via cell and tissue type dependent spatial and temporal patterns, and epigenetic remodeling plays a key role in both the conversion of adult stem/progenitor cells into terminally differentiated cells and the reprogramming of differentiated cells for tissue repair and regeneration. Variation in gene expression not only underlies the phenotypic differences amongst humans, but also an individual's susceptibility to develop diseases and response to environmental insults. Increasingly, epigenetics has been recognized as a key component to the onset and progression of many devastating human diseases, including cancer. There are several epigenetic mechanisms which can alter gene expression including DNA methylation, histone modifications, and miRNA expression; all of which have been shown to be induced by toxicant exposure [47]. While environmental toxicant exposures are known to induce DNA damage at higher concentrations and exposure levels, there is also ample emerging evidence that lower level exposures are associated with abnormal epigenetic changes within cells.

In order to understand how epigenetic reprogramming contributes to tumorigenesis, and to investigate how environmental exposures may contribute to this reprogramming, we first need an in depth understanding of how the epigenome is regulated in normal cells compared

to a disease state. In human cell nuclei, genomic DNA is wrapped around a protein scaffold, forming nucleosomes, which is the basic unit of chromatin. While nucleosome formation allows the tight packaging of genomic DNA into a relatively small nucleus, the physiological consequence of this is the tightly regulated accessibility of DNA to transcription factors. Transcription factors bind to specific sequences on genomic DNA and regulate gene expression. However, epigenetic changes, such as DNA methylation, post-translational modifications of histone proteins, and non-coding RNA, may positively or negatively regulate gene expression, by altering the DNA packaging in the nucleosomes and ultimately regulating the binding of transcription factors. Interestingly, this epigenetic information is heritable, and failure to maintain correct epigenetic information leads to drastically altered gene expression, and in some cases, apoptosis [48–50]. The enzymes and accessory factors mediating the epigenetic changes may access the DNA during chromatin replication during S phase of the cell cycle, which allows these epigenetic marks to be robustly propagated into the future generations of cells and their subsequent tissue [51].

Importantly, stem cells go through a cascade of developmental processes involving transition in transcriptional controls that determine a given cell's fate, and this is integrated into the overall physiology and phenotype of an organism. While the genetic control of these developmental processes is stable, the associated epigenetic cascade of events is subject to change with the environment the cell is exposed to. This cascade of epigenetic modifications can be severely impacted by environmental toxicant exposure. Specifically, many studies have shown a correlation between developmental exposure to environmental toxicants and epigenetic regulation of biological processes including endocrine dysregulation [52–54]. During early periods of development, such as the fetal or early postnatal periods, subtle shifts in the epigenome can profoundly affect the transcriptome and developmental stages of a cell. These early periods are defined as "critical windows of susceptibility" where an environmental toxicant may permanently modify the epigenome, which then continues through development to result in a modified adult epigenome and transcriptome. These epigenetic changes can increase susceptibility to development of diseases in adult life, or enhance biological variation among individuals. These epigenetic changes, introduced during critical windows of susceptibility, can also be passed on between generations through male germ line (sperm) mediated transmission of epigenetic information and disease phenotypes [55].

Environmental toxicants have been shown to induce transgenerational disease phenotypes. One such class of environmental toxicants is the Pyrethroids. Pyrethroids are synthetic chemicals designed to mimic the structure and function of naturally occurring plant pyrethrins. Pyrethroid insecticides, similar to organochlorides, act on sodium channels within neurons. They delay the closing of sodium channels, resulting in overstimulation and acute neurotoxicity [56]. Unlike organochlorides, pyrethroids decay rapidly and are generally photosensitive [57]. While pyrethroids are not known to bioaccumulate, they do still pose the threat of acute toxicity in humans and other small mammals at high doses and in chemical mixtures [58]. Transgenerational inheritance of adult disease phenotypes has been demonstrated for pyrethroid pesticides (permethrin) and insecticide (DEET) mixtures [59]. Epigenetic transgenerational inheritance of adult onset disease and sperm epimutations has also been described for dioxin (2,3,7,8-tetrachlorodibenzo[p]dioxin, TCDD) [60],

mixtures of plastic derived endocrine disruptor compounds bisphenol-A (BPA), bis(2-ethylhexyl)phthalate (DEHP) and dibutyl phthalate (DBP) [61], and a hydrocarbon mixture involving jet fuel (JP-8) [62].

The chemical attributes of organochlorines led the Stockholm Convention on Persistent Organic Pollutants (POPs) to call for the elimination of the “dirty dozen” POP in 2004, and numerous other Organochlorides since. Remarkably, nine of the original “dirty dozen” chemicals (Aldrin, Chlordane, DDT, Dieldrin, Endrin, Heptachlor, Hexachlorobenzene, Mirex, and Toxaphene) are pesticides. The mechanism of action for organochlorines is linked to their lipophilic nature, which allows them to penetrate the insect cuticle and nerve sheath [63, 64]. Unfortunately, the chemical characteristics which make organochlorines effective pesticides also contribute to their neurotoxicity in mammals, including humans (Ref.). Many organochlorides have also been shown to induce epigenetic changes upon exposure [65, 66]. Epigenetic changes secondary to organochloride exposure suggest that the deleterious effects of these chemicals are not only sustained within the host, but could also be transgenerational.

Epigenetic modifications are modulated by environmental toxicant exposures

The three major events which define epigenetic changes include 1) alterations in DNA methylation, 2) changes to covalent histone modifications and associated chromatin remodeling, and 3) non-coding RNA mediated regulation of gene expression, and all three have been shown to be potentially disrupted with environmental exposures.

DNA methylation

DNA methylation involves formation of a modified DNA base called 5-methylcytosine (5mC), and is characterized by enzymatic, covalent addition of a methyl group to the cytosine-5 position. In 1948, Rollin Hotchkiss first hypothesized that 5mC is naturally found in DNA [67]. However, it was not until the 1980s, that several studies demonstrated the involvement of DNA methylation in gene regulation and differentiation of stem and progenitor cells during development [68, 69]. DNA methylation is now the most widely studied and best understood epigenetic modification, and it has been shown to be essential for many key regulatory functions in the genome including silencing retroviral elements in the genome [70], regulating tissue-specific gene expression, genomic imprinting, and X-chromosome inactivation.

DNA methylation is catalyzed by a group of enzymes called DNA methyltransferases (Dnmts). Dnmt1 is highly expressed in mammalian tissues, and is considered to be the primary maintenance methyltransferase as it has been shown to repair and/or copy the methylation pattern from parent DNA strand onto the daughter strand during DNA replication. This guarantees transfer of methylation marks through the cell cycle in eukaryotic cells and in stem cell maintenance and differentiation. While the biological function of Dnmt2 remains poorly understood, Dnmt 3a and 3b are considered to be *de novo* methyltransferases, as these proteins have a demonstrated ability to establish methylation

patterns at previously unmethylated CpG dinucleotides. Dnmts are extensively involved in the development of an embryo, but their expression is significantly reduced in terminally differentiated cells. This is particularly true for DNMT3b, which is primarily expressed in stem and progenitor cells. One additional atypical member of the Dnmt family is Dnmt 3L, which lacks the catalytic domain itself, but associates with Dnmt3a and 3b to enhance their methyltransferase activity. DNMT3L is also primarily expressed during early development, including developing brain, but is generally restricted to germ cells and thymus in adults.

DNA methylation mostly occurs at CpG dinucleotides, and can be associated with altered gene expression depending on the location and extent of the methylation change. While evidence exists for methylated non-CpG sites in embryonic stem cells [71, 72] and in mouse frontal cortex [73], the biological significance of these sites remain unclear. At CpG dinucleotides, methylation is normally observed at many locations across the genome, with the exception of dense regions of CpG dinucleotides called CpG islands, which are generally protected from DNA methylation in normal cells [74]. Functional DNA methylation changes are also reported to be present in CpG island shores, stretches of DNA up to 2 kb beyond a defined CpG island [75].

DNA methylation was historically considered to be a “permanent” epigenetic mark, but in recent years, research has shown that DNA methylation is a stable, but reversible epigenetic phenomenon. Demethylation involves a series of chemical reactions that modify 5mC, by deamination and/or oxidation to a modified base, which is then excised by base excision repair (BER) pathway. The enzyme responsible for deaminating 5mC is AID/APOBEC (activation induced cytidine deaminase/apolipoprotein B RNA editing enzyme complex). Oxidation of 5mC to 5hmC is achieved by a group of enzymes called ten-eleven translocation (Tet) enzymes. The BER pathway utilizes thymine DNA glycosylase to remove the modified base [76]. Hypomethylation of non-coding regions of DNA has been associated with genomic instability and increased mutation rates [77], and exposure to POPs from both industrial chemicals and their by-products has been associated with global DNA hypomethylation [78, 79].

Numerous chemicals which are produced inadvertently by industrial processes or as byproducts of combustion have been shown to disrupt normal DNA methylation patterns [80]. Many are generally classified as organochlorides and demonstrate the same bioaccumulation and toxicity as organochloride pesticides, potentially posing a serious risk to both plants and animals. Hexachlorobenzene (HXB) is one of the original “dirty dozen” POPs identified by the Stockholm Convention in 2004. It was initially synthesized as a fungicide to treat crop seeds. However, it is also synthesized as a byproduct in the manufacturing of other chemicals. This is of particular interest because it is found as a contaminating impurity in several pesticide formulations. Recent studies have suggested that endocrine disrupting chemicals (EDCs) such as HXB may form a risk factor for obesity by altering energy metabolism through epigenetic gene regulation[81]. Numerous EDCs including tributyltin (TBT), diethylstilbestrol (DES), bisphenol A (BPA), 2,2’,4,4’,5,5’-hexachlorobiphenyl (PCB-153), hexachlorobenzene (HCB), hexabromocyclododecane (HBCD), 2,2’,4,4’-tetrabrominated diphenyl ether (BDE-47), perfluorinated octyl acid

(PFOA) and perfluorinated octyl sulfonate (PFOS) have been shown to induce a significant, but modest decrease in global DNA methylation in some models [82].

Polychlorinated dibenzo-p-dioxins are generated as by-products in the synthesis of many chemicals, including pesticides. Furthermore, dioxins are also generated as a result of combustion reactions such as incineration of plastic waste, and hot burning wildfires. While there is no commercial use for dioxins, they have been extensively studied due to their classification as a class I carcinogen by the EPA [83]. Dioxins have been associated with the development of endometriosis, immune effects, developmental delays, and cancer [84]. The lipophilic nature of dioxins allows them to readily enter the cytoplasm of a cell where they have been shown to bind the aryl hydrocarbon receptor (AhR). AhR is a transcription factor which drives the expression of genes important for drug metabolism, cell growth, and differentiation [85]. Dioxin binding to AhR causes a release of AhR chaperone proteins and its subsequent activation and nuclear translocation [86]. Within the nucleus, the AhR nuclear translocator protein (Arnt) complex binds DNA and drives the expression of several genes including the phase I enzyme CYP1A1 [86]. Breakdown of chemical compounds by phase I enzymes is known to generate ROS and subsequent oxidative stress [87]. Interestingly, exposure to dioxins has been shown to result in epigenetic changes in cells, altering DNA methylation in small mammals and humans [88–91].

Oxidative stress has been implicated in abnormal DNA methylation. Shutoh et.al. report low levels of oxidative stress secondary to organochlorine dichlorodiphenyltrichloroethane (DDT) exposure lead to an overall malfunction in the DNA methylation machinery and subsequent hypomethylation [65]. Additionally, DDT-induced changes in DNA methylation exhibit a dose dependent correlation with gene expression [92]. As previously discussed, POP exposure can generate significant oxidative stress, and oxidative stress has been associated with global hypomethylation and increased dense methylation at gene specific regions [93]. The same is true for heavy metals, which have also been shown to significantly disrupt DNA methylation [94]. While oxidative stress is most well-known for its role in generating DNA damage, its role in altering DNA methylation is a potentially important mechanism by which stem cells or other differentiating cells alter gene expression, ultimately contributing to malignant transformation.

Histone modifications

Histone proteins are the major constituent of DNA complex nucleosomes and have important roles in regulating gene expression at the epigenetic level. Histone proteins forming a nucleosome have a globular C-terminal domain and an unstructured N-terminal tail, which allows for several post-translational modifications [95]. Post-translational modifications on histone tails can remodel the chromatin, thereby altering the accessibility of DNA for the transcription factors, and regulating gene expression. These modifications include acetylation, methylation and ubiquitination on lysine, methylation and citrullination on arginine, and phosphorylation on serine, threonine and tyrosine. The extent, type and site of the histone modification ultimately regulate gene expression. For example, while acetylation of H3K9, H3K14, H3K18, H3K23 and H3K27 or trimethylation of H3K4 is associated with transcriptional activation, trimethylation of H3K9 is linked with

transcriptional repression [96]. Indeed, deciphering the histone code is one of the major focuses of epigenetic research, but, this is complicated by the fact that histone modifications do not always serve as codes for switching genes on and off. Rather histone modifications may be added as a consequence of gene activation and RNA polymerase elongation [97]. Some of these modifications, such as acetylation and phosphorylation, are reversible and associated with inducible gene expression, while others, such as methylation, are more stable and associated with long-term maintenance of gene expression states [98]. Histone modifications have been shown to be dynamically regulated during an inflammatory response (84–86), suggesting that they may also be altered in response to environmental toxicant exposures. Much less is known about the ability of environmental toxicant exposures to regulate chromatin, but this is a rapidly expanding area of research. In one interesting example, exposure to the organochlorine insecticide Dieldrin has been shown to increase histone acetylation in a time dependent fashion, and increased histone acetylation plays a pivotal role in the neurotoxic effects of Dieldrin exposure [99]. Heavy metals and other xenobiotic stressors have also been shown to alter numerous histone modifications [100], with H3Me3K4 and H3Me2K9 being the most frequently reported. An increased understanding of toxicant exposure and alterations in covalent histone modifications is expected to contribute significantly to our knowledge of environmental reprogramming of the epigenome.

MicroRNA

Another mechanism for regulating gene expression is microRNA (miRNA). miRNAs are a class of small non-coding RNAs which target specific mRNA for degradation or translational repression. Because miRNA generally have multiple transcript targets, even minor alterations in the abundance of one or more miRNA can have profound effects on global gene expression [101]. Wahlang et.al. found that exposure to polychlorinated biphenyls significantly altered the expression profile of miRNAs in human endothelial cells [102]. More recently, POP exposure was shown to alter expression of miRNAs which regulate the Wnt and p53 signaling pathways [103]. This is of particular importance due to the implications of Wnt and p53 in the malignant transformation of cells. miRNAs present in urine samples have been proposed as potential biomarkers of pesticide exposure. In a recent study by Weldon et. al., six miRNAs were found to be predictive of occupational status (farm-worker vs. non-farm worker) during the harvest season, and expression of five of these miRNAs trended towards a positive dose response relationship with organophosphate pesticide metabolites in farmworkers [104]. Our knowledge of the ability of certain environmental chemicals to alter miRNA expression is expanding rapidly, and we now have evidence that metals, organic pollutants, cigarette smoke, pesticides and carcinogenic drugs can all impact miRNA [105]. Researchers have hypothesized that environmentally altered, cancer-related miRNAs could serve as potential markers for chemically induced cancers. Additional research is needed to understand how miRNAs are altered in response to the environment and to further establish a direct link to carcinogenesis.

Challenges for Environmental Epigenomics Research

While our knowledge of how environmental exposures regulate the epigenome is increasing dramatically, challenges remain in closing the gap between epidemiological links and our understanding of the direct molecular mechanisms of transformation. Two specific challenges stand out for ongoing environmental epigenetic research as we attempt to link exposures to malignancy. First, humans are exposed to most chemicals as mixtures in the environment, but most current environmental epigenomic experiments are conducted using single toxicants. Second, toxicants undergo biotransformation and metabolic processing *in vivo* which complicates direct exposure studies, particularly when using *in vitro* model systems.

Experiencing Environmental Toxicants as Mixtures

Chemicals in the environment are virtually always experienced as mixtures. For example, pesticides, exhaust from combustion, and environmental toxicants present in the air and food, are all frequently being experienced simultaneously. Predicting the toxic effect of chemical mixtures is intrinsically difficult due to a number of variables, including variations in routes of administration, dosing, and concurrent chemical interactions. The difficult nature of predicting the toxic effect of chemical mixtures infers the importance of evaluating health-related outcomes using a multi-pollutant exposure strategy [106–108]. In addition to the individual actions of chemicals, the combined effects of exposure to chemical mixtures can be additive, potentiated, synergistic, or antagonistic [109]. For instance, chemical exposures may be antagonistic in instances where the interaction of two or more chemicals within a mixture is capable of reducing the effect predicted for any single compound. This may occur when one chemical in a mixture is able to induce the metabolism of another toxic chemical within the mixture.

The cumulative risk from exposure to chemicals with similar mechanisms of action can be estimated based on the relative potency of each chemical. When similar chemicals work simultaneously as part of a mixture, their effects are likely to be dose additive [110]. Biologically relevant examples of chemicals which exhibit a dose-addition effect are organophosphates and carbamates. Both chemical groups work by inhibiting the lysis of acetylcholine leading to neuronal dysfunction, and as a result, their combined toxic effect can be estimated based on the relative exposure of each chemical [111]. Both potentiation and synergism enhance the toxic effect of exposure to chemical mixtures. Potentiation is described as an enhancement of toxic effect secondary to the ability of additional chemicals within the mixture to increase the length of time a host organism requires to metabolize and clear the chemicals. For example, one chemical within the mixture could inhibit the clearance of another toxic chemical. Synergism is the toxic effect of exposure to chemical mixtures which is greater than additive. Synergistic effects of organophosphate pesticides have been shown in mammals.

The ability of chemicals to cause distinct effects when experienced as mixtures is particularly relevant when it comes to carcinogenesis. In a recent study examining 85 environmental toxicants for their ability to influence key pathways in carcinogenesis, the research team concluded that each chemical tested was able to disrupt at least one key

pathway with a known role in promoting carcinogenesis. Furthermore, when the chemicals were experienced as a mixture, all carcinogenic pathways examined were impacted [112]. Many environmental toxicants are lipophilic and therefore bioaccumulate in adipose tissues and other lipophilic regions of cells. The exposure and bioaccumulation of toxicants suggest that individuals may experience these chemical mixtures on a chronic basis. The outcomes from chronic and acute exposure to chemical mixtures resulting from bioaccumulation will be discussed below.

Biotransformation and metabolic processing of environmental toxicants

Further complicating these studies is the recognition that many of these compounds undergo biotransformation *in vivo*, as a series of enzyme catalyzed reactions increase the hydrophilic nature of compounds. This is generally required for the elimination of lipophilic toxicants [113]. This process is generally broken into two phases. In phase I, enzymes such as the CYP450 family introduce reactive or polar moieties to the toxicant [113]. Phase II reactions further conjugate the toxicants with polar moieties, enhancing their hydrophilicity [114]. It is important to note that while biotransformation is necessary for the clearance of toxic xenobiotics, a consequence of this process is the accumulation of reactive oxygen species and subsequent oxidative stress. The majority of the environmental toxicants discussed in this review are highly lipophilic. There are three general mechanisms for lipophilic toxicant absorption: inhalation and entrance into the respiratory tract, ingestion and absorption by the gastrointestinal tract, and cutaneous penetration through the skin [115]. Regardless of the route of entry, toxicants will eventually reach the bloodstream where they will be circulated and transported throughout the body. As a result, highly perfused internal organs experience the highest concentration of toxicants in the shortest amount of time. However, in tissues with a relatively low level of perfusion, toxicants are likely to persist for longer periods of time. Toxicants which persist due to low perfusion tend to accumulate, resulting from long retention times in the absence of metabolic breakdown or excretion [115]. The most lipid rich tissues in the human body are found in the central nervous system and adipose tissue. Accumulation of toxicants within the central nervous system leads to a potential for neurotoxicity. However, adipose tissue is unique in that it has a very low level of perfusion, and also contains many lipid droplets, facilitating the accumulation of lipophilic toxicants. Accumulation of toxicants within adipose tissue represents a pseudo clearance or temporary neutralization for the host, due to the lack of targets for toxic effect [116, 117]. However, disruption of adipose tissue via trauma, medication effects or weight loss can elicit the re-entry of toxicants into circulation where they again pose a toxic threat [118]. As with many other tissues, adipose tissue harbors a pool of undifferentiated, regenerative cells with enhanced replicative ability, known as somatic stem cells. A unique protective mechanism exploited by stem cells is a heightened ability to repair damaged DNA. Due to the regenerative, multipotent nature of stem cells, it is imperative to shield this population of cells from mutations which could be deleterious to a specific tissue and ultimately, the host. Considering the lipophilic nature of many environmental toxicants, and their ability to induce cellular mutations, adipose specific stem cells may be a particularly vulnerable population of regenerative cells.

Largely due to their lipophilicity and slow clearance, POPs have been shown to accumulate in adipose tissue [119, 120]. Accumulation of POPs have been linked to multiple comorbidities including obesity, dysglycemia, hypertension, and cardiovascular risk [121–124]. An often overlooked risk factor of POP accumulation in adipose tissue is release of and subsequent re-exposure to these chemicals upon lipolysis [125].Metabolically-healthy individuals have normally functioning adipose tissue and seldom experience lipolysis. Barring dramatic weight loss or injury, metabolically-healthy individuals have a low chance of experiencing disruption of adipose tissue and subsequent re-exposure to adipocyte accumulated POPs [126]. This is in contrast to metabolically-unhealthy individuals, who may commonly experience dysfunctional adipose tissue and subsequent lipolysis [127]. Interestingly, exposure to POPs can contribute to insulin resistance and other associated metabolic disorders, ultimately leading to dysregulation of adipose tissue [128–133]. This information taken together suggests that adipose tissue plays a dynamic role in comorbidities associated with exposure to environmental toxicants. While acute exposure to some chemicals may be experienced on a single chemical basis, the ability of lipophilic chemicals to concentrate in adipose tissue creates an environment where individuals may be exposed to novel chemical mixtures reintroduced to the bloodstream and internal organs, essentially undergoing a secondary exposure. Further investigation is required to elucidate the downstream health effects of multiple exposures of toxicant mixtures.

Discussion

As discussed above, emerging data suggests that repeated or consistent long term exposure to low doses of toxins and environmental toxicants may lead to additional detrimental changes to the epigenome and gene expression profile of stem cells, resulting in pathogenesis. Subsequently, it is important to elucidate the impact of environmental toxicant exposure on the epigenome of stem cells and how these changes could be contributing to disease progression. Whole genome approaches to understand DNA methylation, histone modifications, miRNA expression, and three dimensional (3D) chromatin architecture are now a mainstream part of environmental toxicant studies on normal and cancerous stem cell populations. We hope that these studies will yield important mechanistic insights into how environmental exposures may lead to epigenetic remodeling in stem cells and position these cells for malignant transformation.

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