

# The Promises and Challenges of Toxico-Epigenomics: Environmental Chemicals and Their Impacts on the Epigenome

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**BACKGROUND:** It has been estimated that a substantial portion of chronic and noncommunicable diseases can be caused or exacerbated by exposure to environmental chemicals. Multiple lines of evidence indicate that early life exposure to environmental chemicals at relatively low concentrations could have lasting effects on individual and population health. Although the potential adverse effects of environmental chemicals are known to the scientific community, regulatory agencies, and the public, little is known about the mechanistic basis by which these chemicals can induce long-term or transgenerational effects. To address this question, epigenetic mechanisms have emerged as the potential link between genetic and environmental factors of health and disease.

**OBJECTIVES:** We present an overview of epigenetic regulation and a summary of reported evidence of environmental toxicants as epigenetic disruptors. We also discuss the advantages and challenges of using epigenetic biomarkers as an indicator of toxicant exposure, using measures that can be taken to improve risk assessment, and our perspectives on the future role of epigenetics in toxicology.

**DISCUSSION:** Until recently, efforts to apply epigenomic data in toxicology and risk assessment were restricted by an incomplete understanding of epigenomic variability across tissue types and populations. This is poised to change with the development of new tools and concerted efforts by researchers across disciplines that have led to a better understanding of epigenetic mechanisms and comprehensive maps of epigenomic variation. With the foundations now in place, we foresee that unprecedented advancements will take place in the field in the coming years. <https://doi.org/10.1289/EHP6104>

## Introduction

Exposure to environmental chemicals in the air, water, soil, food, and consumer products, whether intentional or not, is an everyday occurrence ([ACOG Committee Opinion No. 575 2013](#)). Because global chemical production and usage are projected to increase in the coming decades ([OECD 2012](#)), there is a growing recognition that, although many comforts of modern life are due in part to chemicals that are on the market today, their sound management is critical for the preservation of human health and the environment ([Massy and Jacobs 2013](#)).

A recent assessment by an expert panel convened by the World Health Organization estimated that as many as 20% of cancers, 31% of cardiovascular diseases, 42% of stroke cases, and 35% of chronic obstructive pulmonary disease cases are attributable to environmental factors ([Prüss-Ustün et al. 2016](#)). Chronic illnesses that may be caused or exacerbated by environmental chemicals include asthma, obesity, Alzheimer's disease, diabetes, infertility, and ovarian dysgenesis syndrome (reviewed by [Bijlsma and Cohen 2016](#)). Taken together, it has been estimated that the health and socioeconomic costs associated with environmental chemical exposure are likely to exceed 10% of the global gross domestic product, amounting to approximately \$6.23 trillion ([Grandjean and Bellanger 2017](#)).

These reports indicate that there are major inadequacies in current risk assessment and hazard management practices. First, toxicity studies are generally conducted with individual chemicals with the objective of providing an estimated point of departure ([Goodson](#)

[et al. 2015](#)). Such assessments, although undoubtedly useful, are ineffective at predicting the hazardous effects of chemicals that exhibit nonmonotonic dose responses and low dose relationships (reviewed by [Vandenberg et al. 2012](#)) and fail to account for mixture effects (reviewed by [Bijlsma and Cohen 2016](#)). An additional challenge is that a subgroup of these chemicals, endocrine-disrupting chemicals (EDCs), can mimic or alter endogenous endocrine processes ([Alofe et al. 2019](#)). Therefore, it is often challenging to disentangle the effects of environmental hazards (such as EDCs) from those of inherent differences within and between populations ([Nadal et al. 2005](#)). Indeed, multiple reports have shown that the effects of an exposure could differ in individuals depending on the individuals' age or sex ([ACOG Committee Opinion No. 575 2013](#)). The burden of environmental hazards is reported to be inequitably distributed according to socioeconomic status, disproportionately affecting vulnerable populations ([Brown 1995](#)). Moreover, efforts to pinpoint associations between environmental chemicals and health effects in longitudinal studies are often complicated by the fact that chemical environments change relatively rapidly over time ([LaKind et al. 2016](#)).

To date, several major classes of hazardous or potentially hazardous environmental chemicals have been identified. These include toxic elements (e.g., arsenic, cadmium, lead, mercury); naturally occurring animal, plant, and food allergens; pesticides, persistent organic pollutants (e.g., perfluorooctanoic acid, polychlorinated biphenyls); volatile organic compounds (e.g., benzene, formaldehyde, gasoline); plastic components (e.g., bisphenol A, phthalates); and air pollutants (e.g., asbestos, radon, tobacco smoke) (reviewed by [Bijlsma and Cohen 2016](#); [Sears and Genuis 2012](#)). Biochemical effects commonly reported to be triggered by environmental toxicants include oxidative stress, endocrine disruption, genotoxicity, enzyme inhibition, epigenetic changes, and dysbiosis (reviewed by [Herceg et al. 2018](#); [Sears and Genuis 2012](#)). Evidently, there is a need for a concerted effort across all levels to understand and mitigate the impact of toxic exposures on human health and the environment.

A compelling puzzle in the field is the observation that a subgroup of environmental toxicants can induce persistent phenotypic effects after transient exposures, generating adverse effects that can be manifested years or even generations later ([Gore et al. 2011](#); [Skinner et al. 2008](#); [Uzumcu et al. 2004](#); [Vandenberg 2013](#)). Although the mechanisms by which these long-lasting or transgenerational adverse effects occur have yet to be fully elucidated, one

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explanation for this phenomenon is that environmental toxicants could perturb epigenetic mechanisms (Verma 2015). Described as the interface between the environment and the genome, epigenetic marks are responsive to environmental stimuli and transmissible and are, therefore, promising mechanisms by which the effects of environmental toxicants are perpetuated (Herceg and Vaissière 2011).

In this commentary, we discuss the impact that environmental chemicals can have on epigenetic regulation, the impacts of chemical-induced epigenetic changes on health outcomes, current risk assessment strategies for detecting epigenetic modifiers, and the unique challenges of and strategies for incorporating epigenomic analyses in toxicology.

### **Epigenetics and Its Growing Role in Environmental Epidemiology**

Epigenetic mechanisms refer collectively to mitotically or meiotically heritable alterations in gene expression (and associated phenotypic traits) that do not arise from changes in DNA nucleotide sequences (reviewed by Bird 2002; Feil and Fraga 2012). The definition of epigenetics encompasses chromatin modifications that directly influence chromatin conformation (i.e., DNA methylation and histone posttranslational modifications), as well as the activity of noncoding RNAs (ncRNAs), in view of accumulating evidence that ncRNAs can influence chromatin conformation and induce heritable effects (Ashe et al. 2012; Braconi et al. 2010; Buckley et al. 2012; Burton et al. 2011; Esteller 2011; Fabbri et al. 2007; Garzon et al. 2009; Lei et al. 2016) (Figure 1). Although these three modes of epigenetic regulation converge on a common cellular process (i.e., regulating gene expression), the mechanisms may act with different degrees of dynamic effects, strengths, and reversibility (Bintu et al. 2016). Moreover, although they are often studied independently, it is becoming increasingly clear that there is extensive cross-talk between DNA methylation, histone modifications, and ncRNAs (Hanly et al. 2018; Rose and Klose 2014; Vaissière et al. 2008).

Epigenetic mechanisms regulate transcriptional activity across the genome and can thereby determine temporal and spatial gene expression patterns (Baylin and Jones 2011; Jaenisch and Bird 2003). The heritability of epigenetic marks allows for faithful maintenance of cell identity (Lee et al. 2014), whereas their reversibility allows for developmental plasticity, enabling the manifestation of different phenotypes in response to developmental cues and environmentally induced changes from a single unchanged genotype (Low and Gluckman 2018). Although some degree of stochastic epigenetic alterations, or epigenetic drift, can be observed in aging or as asymptomatic occurrences (Bjornsson et al. 2008; Feil and Fraga 2012; Fraga 2009; Wong et al. 2010), epigenetic aberrations have been described as important drivers of neurological diseases and cancer (Herceg et al. 2018; Zoghbi and Beaudet 2016). Therefore, external stimuli that can cause shifts away from baseline rates of age-related epigenetic drift have been described as elements that cause “environmental deflection” (Kochmanski et al. 2017).

Moreover, epigenetics can provide key mechanistic evidence for the developmental origins of health and disease (DOHaD) hypothesis (Goyal et al. 2019). The DOHaD concept was proposed as a means to explain epidemiological evidence linking early life exposure to environmental factors with the onset of adverse health effects later in life (Barouki et al. 2018; Barrett 2017; Bianco-Miotto et al. 2017). DOHaD proposes that early developmental stages are windows of vulnerability to psycho-social or chemical stressors. Hence, relatively small alterations at this stage of life could have impacts on risk of diseases across the life course into adulthood. This is in line with the notion that profound epigenetic

reprogramming, characterized by a sequential genome-wide erasure and *de novo* lineage-specific establishment of epigenetic marks, underlies pluripotency and lineage commitment, which occur early in embryonic development (Lee et al. 2014). Thus, perturbations during this window of development could enable the propagation of aberrant epigenetic patterns and associated gene activity states at doses of exposure that would otherwise be biologically inconsequential (Anway et al. 2005; Breton et al. 2017).

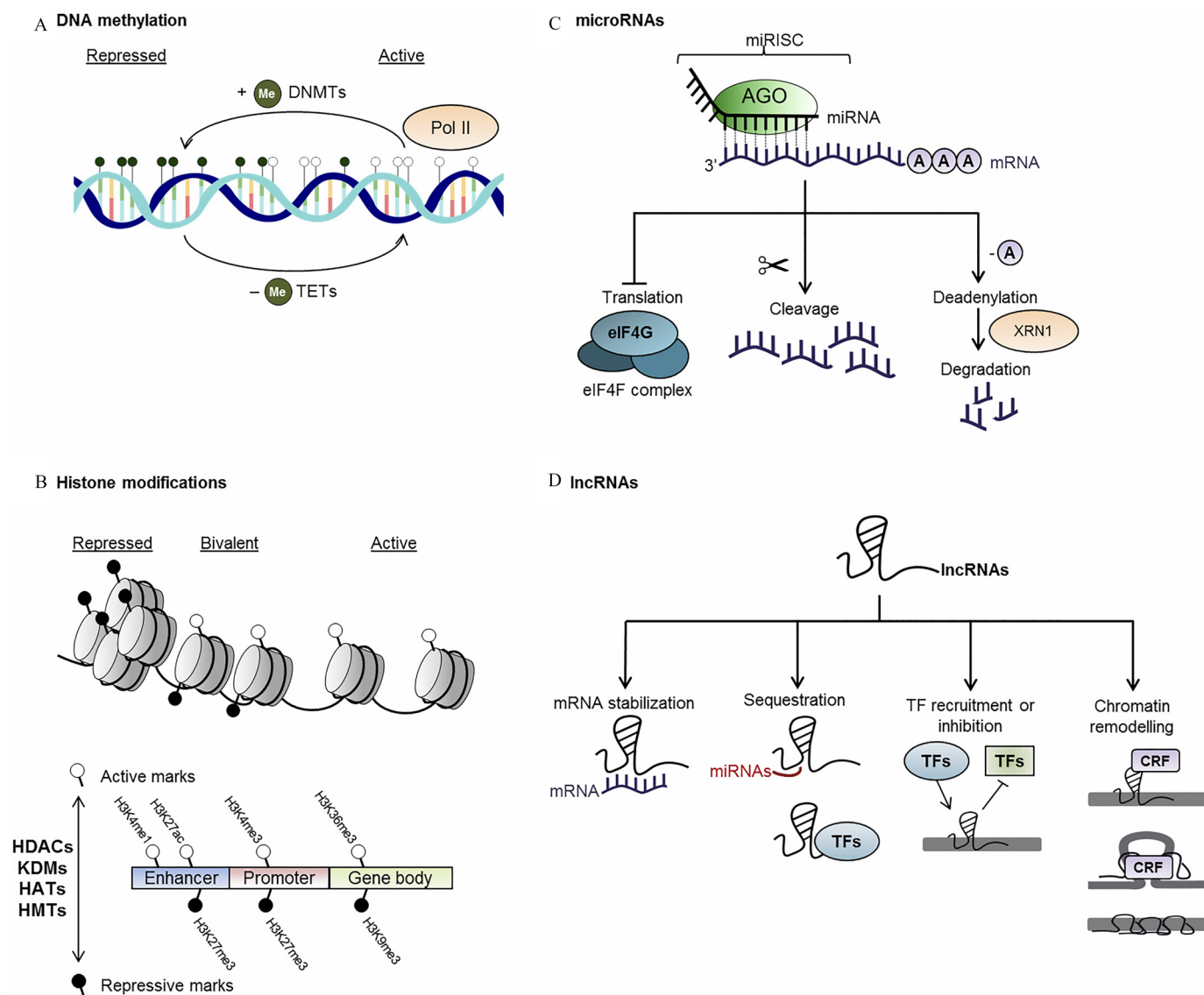
### **Environmental Toxicants as Disruptors of Epigenetic Regulation**

Following the frequent inclusion of epigenetics as a parameter in environmental epidemiology studies, numerous reports have been made in recent years associating alterations in DNA methylation with various environmental factors, including biological agents, dietary habits, and air pollution (Ambatipudi et al. 2016; Barouki et al. 2018; de FC Lichtenfels et al. 2018; Degli Esposti et al. 2017; Fasanelli et al. 2019; Feil and Fraga 2012; Hattori and Ushijima 2016; Herceg et al. 2018; Martin and Fry 2018; Perrier et al. 2019; Woo et al. 2018). Similarly, many reports indicate that microRNA (miRNA) profiles are responsive to various environmental exposures, including air pollution [R Chen et al. (2018); Espín-Pérez et al. (2018), both epidemiological studies], nanoparticles [Brzóška et al. (2019), utilizing human liver cells], endocrine disruptors, such as bisphenol A (BPA) [Chou et al. (2017), using human endometrial cells, and Martínez-Ibarra et al. (2019), using human blood samples], and dichlorodiphenyltrichloroethane (DDT) [Krauskopf et al. (2017), using human blood]. Long noncoding RNAs (lncRNAs), although still a relatively new area of research, have been reported to be associated with levels of phthalates in first trimester urine of pregnant women (LaRocca et al. 2014), benzene in seven exposed individuals (Bai et al. 2014b), and occupational exposure to cadmium (Zhou et al. 2015) and to be responsive to developmental exposure to BPA in mice (Kumamoto and Oshio 2013) and to certain heavy metals in *in vitro* (Tani et al. 2014; Zhou et al. 2015) and *in vivo* experimental systems (Zhou et al. 2015). Alterations to histone marks have been reported in response to various substances including arsenic in mice (Cronican et al. 2013) and in cell lines (Jo et al. 2009; Li et al. 2002; Zhou et al. 2009), BPA [Senyildiz et al. (2017), using human neuroblastoma cells], phthalates [Sonkar et al. (2016), in mesenchymal stem cells], and dichlorodiphenyltrichloroethane [Ben Maamar et al. (2018), in male rats descended from exposed gestating female rats].

Table 1 provides a non-exhaustive summary of environmental chemicals with demonstrated epigenome-altering properties. Here, we discuss three salient examples, namely, benzene, vinclozolin, and BPA, in greater detail.

### **Benzene—Epigenome Perturbations at Low-Level Exposures**

Benzene, a common aromatic compound in crude oils, is a human carcinogen (IARC 2018) and may be one of the most potent epigenetic modifiers in the environment. Airborne, occupational exposure to benzene has been consistently associated with hypomethylation in repetitive elements in peripheral blood DNA obtained from occupationally exposed individuals (Bollati et al. 2007; Fustinoni et al. 2012; Seow et al. 2012) and is associated with lower levels of *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) promoter methylation in whole blood DNA (Li et al. 2017). Of note, significant differential methylation was reported between a group of individuals with a median personal benzene exposure of 22  $\mu\text{g}/\text{m}^3$  (Bollati et al. 2007) and in individuals exposed to a median 8-h time-weighted average benzene exposure of 110  $\mu\text{g}/\text{m}^3$  (Li et al. 2017), both of which are below



**Figure 1.** Diagrammatical summary of epigenetic modes on action. (A) The deposition of methyl groups is mediated by the DNA methyltransferases (DNMTs), and their removal is mediated by the ten-eleven translocation (TET) family proteins (reviewed by [Greenberg and Bourc'his 2019](#); [Wu and Zhang 2010](#)). Increased deposition of methyl groups promotes the condensation of chromatin, whereas reduced DNA methylation is associated with increased accessibility to transcription machinery [represented by RNA polymerase II (Pol II)]. DNA hypermethylation generally leads to the silencing of gene expression when it occurs at gene promoters (reviewed by [Greenberg and Bourc'his 2019](#); [Wu and Zhang 2010](#)). (B) Histone modifications are deposited by multiple classes of enzymes [histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs), and histone lysine demethylases (KDMs)] (reviewed by [Chen et al. 2017](#)). Examples of histone modifications illustrated here are histone H3 lysine 27 trimethylation (H3K27me3) and H3 lysine 9 trimethylation (H3K9me3), which are usually associated with repression of gene transcription, and H3 lysine 4 monomethylation (H3K4me1), H3 lysine 4 trimethylation (H3K4me3), H3 lysine 36 trimethylation (H3K36me3), and H3 lysine 27 acetylation (H3K27ac) marks that are typically associated with activation of transcription. Simultaneous deposition of opposing histone marks is associated with poised or bivalent chromatin, which is in a transitional state poised to be resolved into active or repressed states (reviewed by [Chen et al. 2017](#)). (C) microRNAs (miRNAs) bound to the Argonaute (AGO) protein make up the miRNA-induced silencing complex (miRISC). miRNAs direct the miRISC to target mRNAs base-pairing partially to complementary binding sites, which will be cleaved by catalytically active AGO proteins ([O'Brien et al. 2018](#)). Alternatively, AGO proteins can recruit additional protein partners, initiating the process of deadenylation, decapping and 5'-to-3' mRNA degradation by 5'-to-3' exoribonuclease 1 (XRN1) (reviewed by [Jonas and Izaurralde 2015](#)). There has also been evidence that miRNAs inhibit translation by inhibiting the eukaryotic initiation factor 4F (eIF4F) complex, although this process has yet to be fully elucidated (reviewed by [Jonas and Izaurralde 2015](#)). 5' polyA tails are denoted by circles labeled A. (D) Long noncoding RNAs (lncRNAs) may influence gene expression by increasing or decreasing target mRNA stability, by acting as decoys to miRNA and transcription factors (TFs), thus sequestering them from their cognate promoters, or they may recruit or inhibit TF binding to their target sites on the chromatin (reviewed by [Angrand et al. 2015](#)). lncRNAs may also recruit chromatin remodeling factors (CRFs) such as the polycomb repressive complexes or cohesin proteins that recruit histone modifier complexes or initiate long-range chromatin looping (reviewed by [Angrand et al. 2015](#)). The Xist lncRNA triggers stable repression of the presumptive inactive X-chromosome by physically coating the X-chromosome (reviewed by [Lee and Bartolomei 2013](#)).

the National Institute of Occupational Safety and Health's recommended exposure limit for benzene ( $320 \mu\text{g}/\text{m}^3$ ) ([NIOSH 2019](#)). Similarly, individuals exposed to benzene at median concentrations of  $120 \mu\text{g}/\text{m}^3$  had significantly higher global levels of H3 lysine 4

trimethylation (H3K4me3) marks compared with a control group ([J Li et al. 2018](#)). Perturbations to miRNA ([Bai et al. 2014a](#); [Yang Liu et al. 2016](#)) and lncRNA ([Bai et al. 2014b](#)) expression have likewise been detected in pooled plasma ([Yang Liu et al. 2016](#)) and peripheral

**Table 1.** Summary of evidence of environmental chemicals and their effect on epigenetic regulation.

Environmental chemicals	Experimental evidence			Observational evidence		Epigenetic impact	References
	Human	Animal	<i>In vitro</i>	Human	Animal		
Organic pollutants							
2,3,7,8-tetrachloro-dibenzo- <i>p</i> -dioxin	—	+	+	—	—	DNA methylation	Bastos Sales et al. 2013; Ding et al. 2018
Benzene	—	+	+	+	—	DNA methylation	Bollati et al. 2007; Coulter et al. 2013; Fustinoni et al. 2012; Hu et al. 2014; Ji et al. 2010; Li et al. 2017; Seow et al. 2012; Yang et al. 2014
						Histone marks	J Li et al. 2018
						Noncoding RNA	Bai et al. 2014a; Yang Liu et al. 2016; Wei et al. 2015
BPA	—	+	+	+	—	DNA methylation	Anderson et al. 2012, 2017; Bansal et al. 2017; Basak et al. 2018; Bastos Sales et al. 2013; Bromer et al. 2010; Cheong et al. 2018; Dolinoy et al. 2007; Doshi et al. 2011, 2012; Drobná et al. 2018; González-Rojo et al. 2019; Goodrich et al. 2016; Ho et al. 2006; Itoh et al. 2012; Jadhav et al. 2017; Jorgensen et al. 2016; Kitraki et al. 2015; Kumar and Thakur 2017; Kundakovic et al. 2013; Laing et al. 2016; Lee et al. 2018; G Li et al. 2014; Y Li et al. 2018; Yan Liu et al. 2016; Mao et al. 2015; Miao et al. 2014; Novo et al. 2018; Patel et al. 2013; Prins et al. 2008; Senyildiz et al. 2017; Susiarjo et al. 2013; Tang et al. 2012; Taylor et al. 2018; T Wang et al. 2016; Weinhouse et al. 2015; Ye et al. 2019
						Histone marks	Doherty et al. 2010; González-Rojo et al. 2019; Ho et al. 2015; Hussain et al. 2015; Jiang et al. 2016; Kumar and Thakur 2017; Lee et al. 2018; Y Li et al. 2018; Lombó et al. 2019; Perrot-Appanat et al. 2018; Santangeli et al. 2016; Senyildiz et al. 2017; Verbanck et al. 2017; Q Wang et al. 2016; T Wang et al. 2016
						Noncoding RNAs	Bhan et al. 2014; Chou et al. 2017; Ho et al. 2015; Kumamoto and Oshio 2013; Perrot-Appanat et al. 2018; Renaud et al. 2017
BPA substitutes (bisphenol AF, F, and S)	—	+	+	—	—	DNA methylation	Huang et al. 2019
						Histone marks	Ding et al. 2017
						Noncoding RNAs	Verbanck et al. 2017
Endosulfan	—	+	—	—	—	DNA methylation	Milesi et al. 2017
Glyphosate	—	+	—	—	—	DNA methylation	Smith et al. 2019
Hexachlorobenzene	—	—	+	—	—	DNA methylation	Bastos Sales et al. 2013
Methoxychlor	—	+	—	—	—	DNA methylation	Anway et al. 2005; Stouder and Paoloni-Giacobino 2011; Gore et al. 2011; Manikkam et al. 2014; Zama and Uzumcu 2009
<i>n</i> -Butylparaben	—	+	—	—	—	DNA methylation	Zhang et al. 2016
Organophosphate flame-retardant (e.g., BDE-47, tetrabromobisphenol A)	—	+	+	+	—	DNA methylation	Bastos Sales et al. 2013; Byun et al. 2015; Soubry et al. 2017
						Histone marks	Otsuka et al. 2014
Phenols (e.g., 4-nonylphenol, 4-octylphenol)	—	—	+	—	—	Histone marks	Ghosh et al. 2019; Hung et al. 2010
Phthalates	—	+	+	+	—	DNA methylation	C-H Chen et al. 2018; J Chen et al. 2015; Grindler et al. 2018; LaRocca et al. 2014; Martínez-Arguelles and Papadopoulos 2015; Solomon et al. 2017; Stenz et al. 2017; Tindula et al. 2018; Wu et al. 2017; Xia et al. 2018; PJ Yang et al. 2018
						Histone marks	Gutiérrez García et al. 2019; Sonkar et al. 2016
						Noncoding RNAs	LaRocca et al. 2016; Machtinger et al. 2018; Martínez-Ibarra et al. 2019; Meruvu et al. 2016; Stenz et al. 2017; Zhong et al. 2019
Polyhalogenated biphenyls (e.g., polybrominated biphenyl and polychlorinated biphenyls)	—	+	+	+	—	DNA methylation	Bastos Sales et al. 2013; Curtis et al. 2019; Matsumoto et al. 2014; Priya et al. 2018
						Histone marks	Casati et al. 2013
<i>p,p'</i> -DDE	—	+	—	—	—	DNA methylation	Song et al. 2014; Song and Yang 2018

**Table 1.** (Continued.)

Environmental chemicals	Experimental evidence			Observational evidence		Epigenetic impact	References
	Human	Animal	<i>In vitro</i>	Human	Animal		
<b>Pharmaceutical compounds</b>							
Cyproterone acetate	—	—	+	—	—	Nucleosome occupancy	Ankolkar et al. 2018
Diethylstilbestrol	—	+	+	—	—	DNA methylation	Bastos Sales et al. 2013; Bromer et al. 2009; Chen et al. 2015; Singh et al. 2018
						Histone marks	Jefferson et al. 2013; Sato et al. 2006, 2009; Singh et al. 2018
						Noncoding RNA	Bhan et al. 2014; Padmanabhan et al. 2017; Singh et al. 2018; Stenz et al. 2017
Exogenous estrogen (e.g., ethinyl estradiol, estradiol benzoate)	—	+	+	—	—	DNA methylation	Cheng et al. 2008; Cheong et al. 2018; de Assis et al. 2012; Gore et al. 2011; Ho et al. 2006; Meunier et al. 2012; van der Weijden et al. 2018; Y-M Yang et al. 2018
Tamoxifen	—	+	+	—	—	Noncoding RNA	Maillot et al. 2009; Meunier et al. 2012
						DNA methylation	Stone et al. 2012; Tryndyak et al. 2007
						Nucleosome occupancy	Ankolkar et al. 2018
						Histone marks	Tryndyak et al. 2006
<b>Dietary compounds</b>							
Epigallocatechin gallate	—	+	—	—	—	Histone marks	Lombó et al. 2019
Phytoestrogens (e.g., genistein, resveratrol, and daidzein)	+	+	+	—	—	DNA methylation	Adjakly et al. 2011; Bosviel et al. 2012; Dolinoy et al. 2007; Fudhaili et al. 2019; Jadhav et al. 2017; Kala and Tollefsbol 2016; Lou et al. 2014; Majid et al. 2010; Qin et al. 2009; Tang et al. 2008; Vanhees et al. 2011; Xie et al. 2014
						Histone marks	Kala and Tollefsbol 2016; Majid et al. 2008, 2010; Venturelli et al. 2013
<b>Mixtures</b>							
Mixed contaminants (industrial chemicals, e.g., <i>p,p'</i> -DDE, Li, Fe, Ni, Sb, Pb, Bi, V, As, S)	—	—	—	—	+	DNA methylation	Guillette et al. 2016
Mixture—plastic derived EDCs (BPA, DEHP, DBP)	—	+	—	—	—	DNA methylation	Manikkam et al. 2013
Total xenoestrogens	—	—	—	+	—	DNA methylation	Vilahrur et al. 2014
<b>Others</b>							
Metals (e.g., inorganic As, Cd, Cr, Pb)	—	+	+	+	—	DNA methylation	Castillo et al. 2012; Doi et al. 2011; Meakin et al. 2019; Pilsner et al. 2009; Ronco et al. 2010; Sen et al. 2015; Smeester et al. 2011; Winterbottom et al. 2019
						Histone marks	Cronican et al. 2013; Jo et al. 2009; Li et al. 2002; Winterbottom et al. 2019; Zhou et al. 2009; Zhu et al. 2018
						Chromatin accessibility	VonHandorf et al. 2018
						Noncoding RNA	Cheng et al. 2018
Tetrahydrofuran diols	—	+	—	—	—	Histone marks	Shoulers et al. 2008
Vinclozolin	—	+	—	+	—	DNA methylation	Anway et al. 2005; Beck et al. 2017; Goodrich et al. 2019; Guerrero-Bosagna et al. 2010, 2012; Iqbal et al. 2015; Lee and Oh 2012; McCarrey et al. 2016; Pietryk et al. 2018
						Histone retention sites	Ben Maamar et al. 2018
						Noncoding RNAs	Briño-Enríquez et al. 2015; Schuster et al. 2016
Zearalenone	—	+	+	—	—	DNA methylation	Gao et al. 2019; Karaman and Ozden 2019; Men et al. 2019
						Histone marks	Gao et al. 2019; Men et al. 2019

Note: Animal studies include findings in both vertebrate and invertebrate models. Plus signs (+) indicate that there is evidence that the compound(s) in question exhibits epigenetic-modulating properties in the systems specified. —, Not applicable; As, arsenic; BDE 47, tetrabromodiphenyl ether; Bi, bismuth; BPA, bisphenol A; Cd, cadmium; Cr, chromium; DBP, dibutyl phthalate; DEHP, di-2-ethylhexyl phthalate; EDC, endocrine-disrupting chemical; Fe, iron; Li, lithium; Ni, nickel; Pb, lead; *p,p'*-DDE, *p,p'*-dichlorodiphenoxydichloroethylene; S, sulfur; Sb, antimony; V, vanadium.

blood mononuclear cell (Bai et al. 2014a, 2014b) samples of benzene-exposed individuals. Although it has been reported that environmental exposures to benzene also affect the transcriptome in blood mononuclear cells (McHale et al. 2009,

2011), the direct contribution of benzene-induced epigenetic alterations to the transcriptome remains to be established.

Benzene and some of its metabolites have also been shown to induce global hypomethylation in *in vitro* (Coulter et al. 2013; Hu

et al. 2014; Ji et al. 2010) and *in vivo* (Philbrook and Winn 2015) experimental systems, recapitulating the effects observed in exposed humans. Cells subjected to multiple rounds of exposure to hydroquinone, a benzene metabolite, over a period of 5 weeks exhibited a gradual increase in the levels of H3K4me3, an active histone mark (Mancini et al. 2017). In mice, exposure to benzene was associated with lower levels of peripheral blood cells and hematopoietic progenitor cells in the bone marrow and alterations to the miRNAs mmu-miR-34a-5p; mmu-miR-342-3p; mmu-miR-100-5p, mmu-miR-181a-5p, and mmu-miR-196b-5p, many of which are dysregulated in hematological malignancies (Wei et al. 2015).

### Vinclozolin—Transgenerational Impacts on the Epigenome

Vinclozolin is a dicarboximide fungicide used on fruits, vegetables, ornamental plants, and turf grass and is a prominent example of an environmental toxicant capable of triggering adverse health effects long after the initial exposure (Uzumcu et al. 2004). Mice exposed to vinclozolin *in utero* displayed significantly higher numbers of apoptotic germ cells at puberty [postnatal day (PND) 20], an effect that became more pronounced in adulthood (PND60) (Uzumcu et al. 2004). Moreover, maternal exposure to vinclozolin negatively affected fertility in male mice in the descendent F1 generation and epigenetic alterations in more than 1,000 targets (primarily hypomethylation events) compared with controls (Lee and Oh 2012). Guerrero-Bosagna et al. (2012) further observed that differential DNA methylation patterns could be observed up to the F3 generation of vinclozolin-lineage mice (Guerrero-Bosagna et al. 2012). Interestingly, administration of vinclozolin to pregnant mice led to higher levels of DNA methylation in the promoters of two paternally imprinted genes [imprinted maternally expressed transcript (*H19*) and maternally expressed 3 (*Gtl2*) also known as *Meg3*] and lower levels of DNA methylation in regions regulating three maternally imprinted genes [small nuclear ribonucleoprotein polypeptide N (*Snrpn*), paternally-expressed Gene 1 (*Peg1*) also known as *Mest*, and paternally expressed 3 (*Peg3*)] (Stouder and Paoloni-Giacobino 2010). In Sprague-Dawley rats, alterations in more than 200 small ncRNAs (Schuster et al. 2016) and 99 histone retention sites (Ben Maamar et al. 2018) were observed in the sperm from the F3 generation of vinclozolin-lineage rats compared with the control group. The F3 generation of vinclozolin-lineage male rats had higher incidence of prostate histopathology and abnormalities and displayed altered gene expression, ncRNA expression, and DNA methylation patterns compared with the untreated controls (Klukovich et al. 2019).

Because the above-mentioned studies suggest that vinclozolin can induce transgenerational epigenetic effects, it stands to reason that differential epigenetic marks observed in the F3 generation should also be present in the F1 and F2 generations of the vinclozolin-lineage rats or mice. Intriguingly, however, analyses of F1 and F3 vinclozolin-lineage rats revealed that although there were a total of 290 differentially methylated regions (DMRs) ( $p < 10^{-4}$ ) in the F1 generation (control vs. vinclozolin-lineage animals) and a total of 916 total DMRs in the F3 generation, there was no overlap between the F1 and F3 DMRs (Beck et al. 2017). This indicates that the epigenetic alterations accumulated in vinclozolin-lineage rats or mice are not directly inherited by subsequent generations. Instead, exposure could result in alterations to developmental programming and epigenetic mechanisms in early generations that are transmitted to subsequent generations, amplifying differences in epigenetic patterns between control and exposure-lineage mice.

One caveat to these findings, however, is that the transgenerational epigenetic effects of vinclozolin were reported in studies using intraperitoneal administration of vinclozolin at 100 mg/kg body weight per day (Ben Maamar et al. 2018; Lee and Oh 2012; Nilsson et al. 2018; Stouder and Paoloni-Giacobino 2010; Uzumcu et al. 2004). This is orders of magnitude above the calculated

chronic population adjusted dose of 0.0012 mg/kg per day (U.S. EPA 2000) or the estimated occupational exposure in production workers (0.012 mg/kg per day) (Zober et al. 1995). To our knowledge, there is currently no evidence that vinclozolin induces transgenerational effects when administered at lower doses and via dermal and/or oral routes, which are arguably closer to the likely routes of vinclozolin exposure in humans.

### Bisphenol A—a Stealthy Hazard

No summary on environmental toxicants could be complete without the mention of BPA. BPA is now a household name, and it is not uncommon to see “BPA-free” statements on plastic products or printed material. Despite efforts to limit the usage of BPA, BPA production is predicted to increase in the coming decades (reviewed by Almeida et al. 2018). BPA is a known endocrine disruptor, exhibiting agonistic effects on both estrogen receptors alpha and beta ( $ER\alpha$  and  $ER\beta$ ), and antagonistic effects on the androgen receptor (Delfosse et al. 2014). A wide range of health effects have been associated with BPA exposure, including obesity, reduced reproductive capacity, and metabolic disease (Manikkam et al. 2013). Traces of BPA were detected in 92.6% of sampled participants from the National Health and Nutrition Examination Survey (NHANES) (Calafat et al. 2008), suggesting that exposure to BPA is nearly ubiquitous.

BPA exposure has been consistently associated with hypomethylation in repetitive elements in humans (Faulk et al. 2015; Miao et al. 2014; Zheng et al. 2017). In a cross-sectional human study, multivariate analysis indicated that workplace BPA exposure was associated with significantly lower long interspersed nuclear element 1 (LINE-1) methylation levels relative to an unexposed control group, and that urine BPA levels were significantly inversely associated with LINE-1 methylation in sperm (Miao et al. 2014). In corroboration with this, a similar study reported that sperm collected from BPA-exposed men exhibited 19.6% higher levels of global 5-hydroxymethylcytosine, an intermediate of the demethylating process by ten-eleven translocation (TET) enzymes, compared with men without occupational exposure to BPA (Zheng et al. 2017). Interestingly, an epigenome-wide study on human fetal liver tissue reported that although exposure was generally associated with global hypomethylation, increasing BPA levels were positively correlated with hypermethylation of CpG islands, suggesting that the effects of BPA on DNA methylation and gene expression are genomic region-dependent (Faulk et al. 2015). *In utero* exposure to BPA was also found to induce persistent epigenetic effects. Using data from a longitudinal birth cohort, Goodrich et al. (2016) reported that blood leukocyte DNA methylation in participating peri-adolescents was positively correlated with increasing BPA levels both in archived maternal urinary samples collected during the third trimester of pregnancy and in the urinary samples obtained from the peri-adolescents themselves. Although this positive correlation was observed at all analyzed loci, namely, the LINE-1 repetitive elements, and imprinted genes *H19*, *IGF2*, and *HSD11B2*, statistical significance was observed only when associating maternal urinary BPA levels with *IGF2* hypermethylation (Goodrich et al. 2016). However, these findings are not completely concordant with the previously described results in human subjects whereby BPA exposure was associated with decreased LINE-1 methylation. Nevertheless, taken together, these reports suggest that environmentally relevant concentrations of BPA can affect the epigenome.

The effect of BPA on the epigenome, including persistent and early life effects, has been demonstrated in numerous experimental models (Table 1). For example, prenatal exposure to BPA resulted in dysregulated expression of the lncRNAs *Xist* and its antisense *Tsix* in the mouse brain, suggesting that *in utero* BPA exposure during the critical period of brain development may affect neurodevelopment through the deregulation of epigenetic mechanisms involved in X-

chromosome inactivation (Kumamoto and Oshio 2013). Epigenetic alterations were detected in mice exposed to environmentally relevant doses (as low as 50 ng/kg) of BPA during development (Anderson et al. 2012). Similarly, rats exposed to transient, environmentally relevant doses of BPA exhibited greater prostate gland susceptibility to adult-onset precancerous lesions and persistent alterations in DNA methylation patterns (Ho et al. 2006).

However, it is worth noting that studies on BPA have not been immune to flaws in design and execution. An expert opinion on the design and execution of the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) study has highlighted that the use of gavage as a route of exposure, without a non-gavaged negative control, could complicate the interpretation of the results and lead to erroneous conclusions (vom Saal 2019). This argument was previously made by Vandenberg et al. (2014) in the context of EDCs (Vandenberg et al. 2014), indicating that further review and reconsideration of supposedly established methods may be in order.

## Discussion

### *Epigenetic Marks in Risk Assessment*

As described in the previous section, stable epigenetic patterns can be observed in response to a wide range of environmental toxicants. Thus, we hold the opinion that there are clear benefits to using epigenetic marks as biomarkers of exposure, for reasons which will be discussed in this section. For instance, DNA methylation events are particularly attractive as biomarkers because of their relative robustness in different storage conditions (Ghantous et al. 2014; Thirlwell et al. 2010; Wong et al. 2008).

Moreover, the properties of DNA methylation biomarkers as both stable and responsive to environmental cues make them attractive candidates as markers of exposure and predictive markers of disease (Ladd-Acosta 2015). DNA methylation-based predictors of health and lifestyle factors such as smoking habits (Ambatipudi et al. 2016; Bojesen et al. 2017; McCartney et al. 2018; Philibert et al. 2012, 2016), alcohol consumption (McCartney et al. 2018), and body mass index (McCartney et al. 2018) have been described and have been proposed as molecular predictors of disease and morbidity or as an alternative to self-reported measurements. Because epigenetic marks can persist even after cessation of the exposure, using epigenetic marks as biomarkers of exposure could be particularly useful in situations where the exposures tested have short half-lives *in vivo* (Ladd-Acosta 2015).

As biomarkers of disease, epigenetic disease signatures, or epigenatures, have been described in cancers (Crujeiras et al. 2019), neurological diseases (Bend et al. 2019; De Jager et al. 2014; Henderson-Smith et al. 2019), and heart diseases (Roetker et al. 2018) and may be applied as molecular predictors of disease or indicators of disease progression. Epigenetics-based diagnostic tests are already commercially available; examples include the DNA methylation-based Epi proColon<sup>®</sup> 2.0 CE (Epigenomics AG) for colorectal cancer (Lamb and Dhillon 2017) and the miRNA-based RosettaGX Reveal<sup>™</sup> (Rosetta Genomics) for thyroid malignancies (Lithwick-Yanai et al. 2017).

Episignatures as biomarkers have, in theory, the potential of capturing information on current tissue states, predisposition to disease, and the cumulative effects of environmental stressors and exposures. However, although carcinogenicity testing is an important component of testing for regulatory approval, the role of toxico-epigenomics in the risk assessment process has yet to be established (Herceg et al. 2013; Ray et al. 2014).

### *Challenges of Applying Epigenetics in Toxicology*

A major impediment to the application of epigenomic analysis in toxicology is the fact that consensus normal epigenomes have yet

to be fully defined for all tissue and cell types (Stueve et al. 2016; Tonge and Gant 2016). Given the large epigenetic variability within and between tissue types, age groups, and populations, reference epigenomes are particularly critical (Marczylo et al. 2016; Marsit 2015) because comparing epigenetic patterns across heterogeneous sample groups without suitable adjustment will, in our experience, substantially reduce statistical power.

These challenges could be overcome in the near future with the introduction of correction methods for cell-composition effects (Breeze et al. 2016; Houseman et al. 2016; McGregor et al. 2016; Zou et al. 2014) and the generation of cell-type-specific epigenomic maps by large-scale initiatives of the International Human Epigenome Consortium (Bujold et al. 2016), namely, Encyclopedia of DNA Elements (ENCODE) (Djebali et al. 2012; ENCODE Project Consortium 2012), the National Institutes of Health Roadmap Epigenomics Program (Roadmap Epigenomics Consortium et al. 2015), the BLUEPRINT projects (Fernández et al. 2016), and the 4D Nucleome Project (Dekker et al. 2017). The Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET) program provides an invaluable resource of high-quality transcriptomic, epigenomic, and chromatin accessibility data from experimental *in vivo* systems that not only enables an improved understanding of the potential impacts of environmental exposures on the epigenome but also provides a means to better assess the utility of surrogate tissues (Wang et al. 2018). However, although these resources have brought unprecedented progress to the field, gaps remain in the representation of different human populations and phenotypes that need to be addressed.

Another challenge that especially affects the testing of EDCs is the fact that the health outcomes associated with exposure and epigenetic alterations can occur years or generations after the initial exposure. As discussed in previous sections, epigenetic impacts are often amplified when exposure occurs in early life (Barouki et al. 2018)—a vulnerable period that is not generally amenable to human testing. A further complication is the fact that epigenetic mechanisms act in concert to have a net functional effect on chromatin accessibility and gene regulation (Kelly et al. 2010). Therefore, a single end point measure is often insufficient to gain a thorough understanding of the epigenotoxicity of a compound.

A potential solution for studying environmental chemicals (such as EDCs) with unknown or persistent effects is to conduct real-time observations of epigenetic states. In recent years, applications of reporter systems have enabled real-time observation of DNA methylation, enhancing our understanding of the dynamics of epigenetic states in development. For instance, *in vitro* reporters (Bintu et al. 2016; Stelzer et al. 2015), fluorescent probes specific for DNA methylation (Kumar et al. 2018), the MethylRO mouse (Ueda et al. 2014), and the mCherry-MBD transgenic zebrafish line (Zhang et al. 2017) enable users to observe spatial and temporal changes to epigenetic states. An application of fluorescence lifetime imaging-based Förster resonance energy transfer (FLIM-FRET) has demonstrated the interactions between epigenetic marks and the ER $\alpha$  and the disruption of these interactions by the antiestrogen tamoxifen (Liu et al. 2019). High-throughput applications of this approach could enable the screening of other endocrine regulators in response to a wide array of environmental chemicals. Other live-cell methods include bimolecular anchor detector (BiAD) sensors, which have been applied for the detection of DNA methylation and H3K9me3 levels in living cells (Lungu et al. 2017). This system gives a fluorescence readout dependent on co-occurrent binding of epigenetic mark-specific detector proteins and site-specific anchor proteins, which are targeted to sites of interest by zinc-finger, transcription activator-like (TAL)-effector or clustered regularly interspaced

short palindromic repeats (CRISPR)-dCas9 systems (Lungu et al. 2017).

The third challenge is the limitations of current epigenomic assays. Current assays can be broadly divided into three categories: measurements of global epigenetic effects, locus-specific assays, and epigenome-wide methods. Commonly used measurements of global epigenetic effects include techniques such as high-performance liquid chromatography (Roberts et al. 2018), mass spectrometry (Chen et al. 2013; Molden and Garcia 2014; Yuan et al. 2014), dot blots (Jia et al. 2017), enzyme-linked immunosorbent assay (reviewed by Andersen et al. 2015), lumino-metric methylation assays (Karimi et al. 2006), and bisulfite pyrosequencing of repetitive elements (Yang et al. 2004). Cell-based reporter assays for global methylation have also been described (Baba et al. 2019; Yoshida et al. 2016). Although these techniques have undoubtedly produced valuable findings, potentially epigenotoxic compounds may induce locus-specific effects that are too subtle to be detected by these methods. Moreover, whereas DNA methylation levels in repetitive elements are often used as surrogates for global DNA methylation, these readings do not always correlate well with true global DNA methylation (BLUEPRINT Consortium 2016).

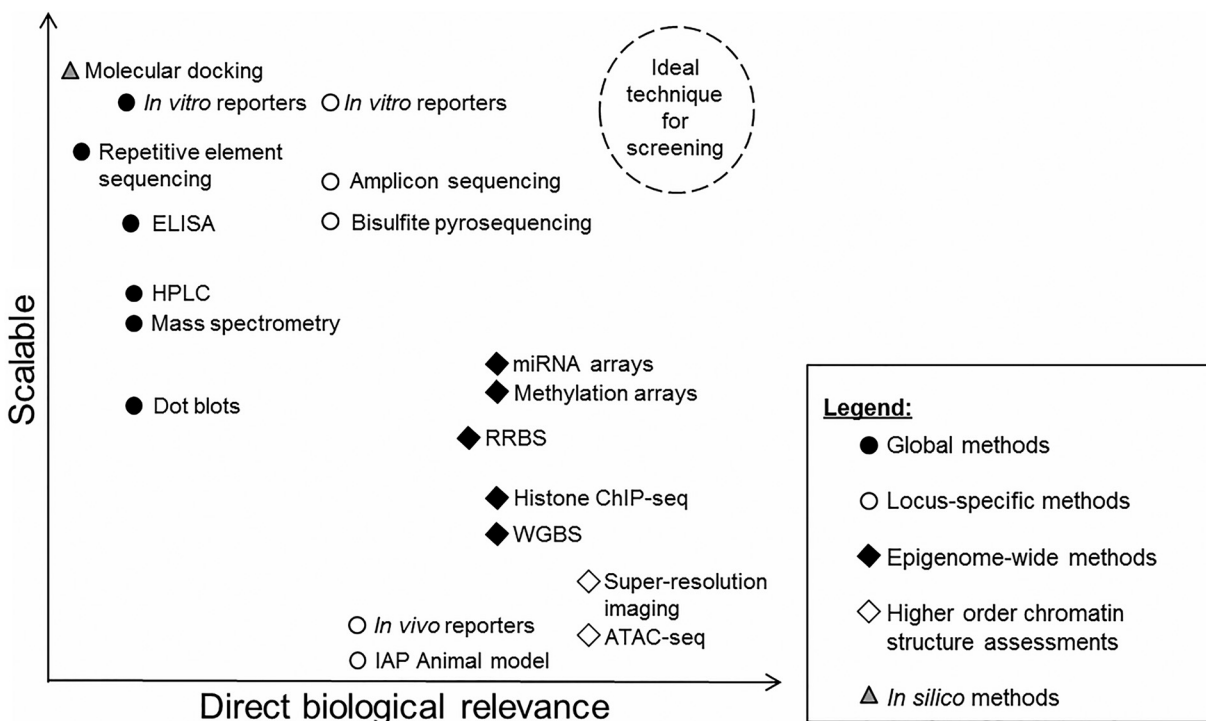
Techniques that enable locus-specific assays include bisulfite pyrosequencing, amplicon bisulfite sequencing, and cell-based reporter assays (Han et al. 2013; Stelzer et al. 2015). *In vivo* models include intracisternal A-particle (IAP) mouse models, based on IAP retrotransposon reporter-type systems (Blewitt and Whitelaw 2013). IAP mouse models display a range of coat color and tail morphology changes that reflect epigenetic states in response to test materials administered to the mice. One limitation of these models is that changes in tail kinks and coat color may be subtle and could be subject to observer bias. Moreover, a limitation that affects all locus-specific assays is that *a priori* knowledge of the

gene to be analyzed is required, making such assays less useful in screening for new epigenetic modifiers with unknown targets.

Epigenome-wide methods, as the name suggests, provides high resolution and coverage for the epigenetic mark analyzed. Requiring no *a priori* knowledge of potential targets, epigenome-wide methods enable the discovery of new biomarkers and target regions. Examples include DNA methylation arrays, reduced-representation bisulfite sequencing (RRBS) (Meissner et al. 2005), histone chromatin immunoprecipitation sequencing (ChIP-seq) (Schmidl et al. 2015), and whole-genome bisulfite sequencing (Urich et al. 2015). These methods, although highly informative, are to our knowledge not currently amenable to miniaturization or cost effective for screening large libraries of compounds at a time. Nevertheless, this could change in the near future as more resources are dedicated to this field of study and the costs of sequencing and analysis are reduced.

Evidently, none of the discussed methods would be universally applicable. In a review by Rasoulopour et al. (2011), the authors suggested that the ideal toxicology screen should be scalable to medium or high throughput, be *in vitro* based, be cost effective, have robust end points, and have good false-positive/negative rates (Rasoulopour et al. 2011). Although none of the methods discussed match such an ideal screening method (Figure 2), future technological advances could make this a reality.

One particularly exciting avenue of development is the rapid advance of genome editing technology. The relative efficiency and adaptability of CRISPR/Cas9/Cas12a (Campa et al. 2019; Ding et al. 2019) techniques could soon give rise to multi-end point, high-throughput, *in vitro* reporter assays for epigenetic modifiers. In our view, the first challenge to reach this goal is to identify robust and biologically relevant end points for which reporter assays could be generated. Future assays could reflect higher-order chromatin structures in biologically important regions of the chromatin,



**Figure 2.** Comparison of major techniques for epigenetics analyses on subjective scales for scalability and direct biological relevance. Axes are in arbitrary scales, with scalability denoting how amenable a given technique is to be implemented in a high-throughput screening setting. Direct biological relevance denotes how information-rich the results of a given technique are. Note: ATAC-seq, assay for transposase-accessible chromatin using sequencing; ChIP, chromatin immunoprecipitation sequencing; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; RRBS, reduced-representation bisulfite sequencing; WGBS, whole-genome bisulfite sequencing.



as opposed to being locus-specific. One of the most exciting new developments in the field has been the development and refinement of techniques that profile chromatin accessibility, whether by sequencing (Buenrostro et al. 2015; Corces et al. 2018) or super-resolution imaging (Bintu et al. 2018). As discussed previously, examining a single aspect of epigenetic regulation may often be insufficient, because epigenetic mechanisms typically work in concert. By assessing higher-order chromatin states, these techniques take into account the summation of these mechanisms. Although these methods are currently not as easily implemented in a high-throughput setting, further research in this field is certainly warranted.

Finally, it may also be argued that *in vitro* screening measures would be limited in identifying biologically relevant epigenetic toxicants. However, we hold the opinion that the implementation of screening programs is still useful because they could lead to the identification of highly epigenotoxic compounds, thus halting their use or introduction into the environment until further analyses are carried out. Moreover, it is worth noting that each class of method—whether *in vitro*, *in vivo*, *in silico*, or based on populations—will

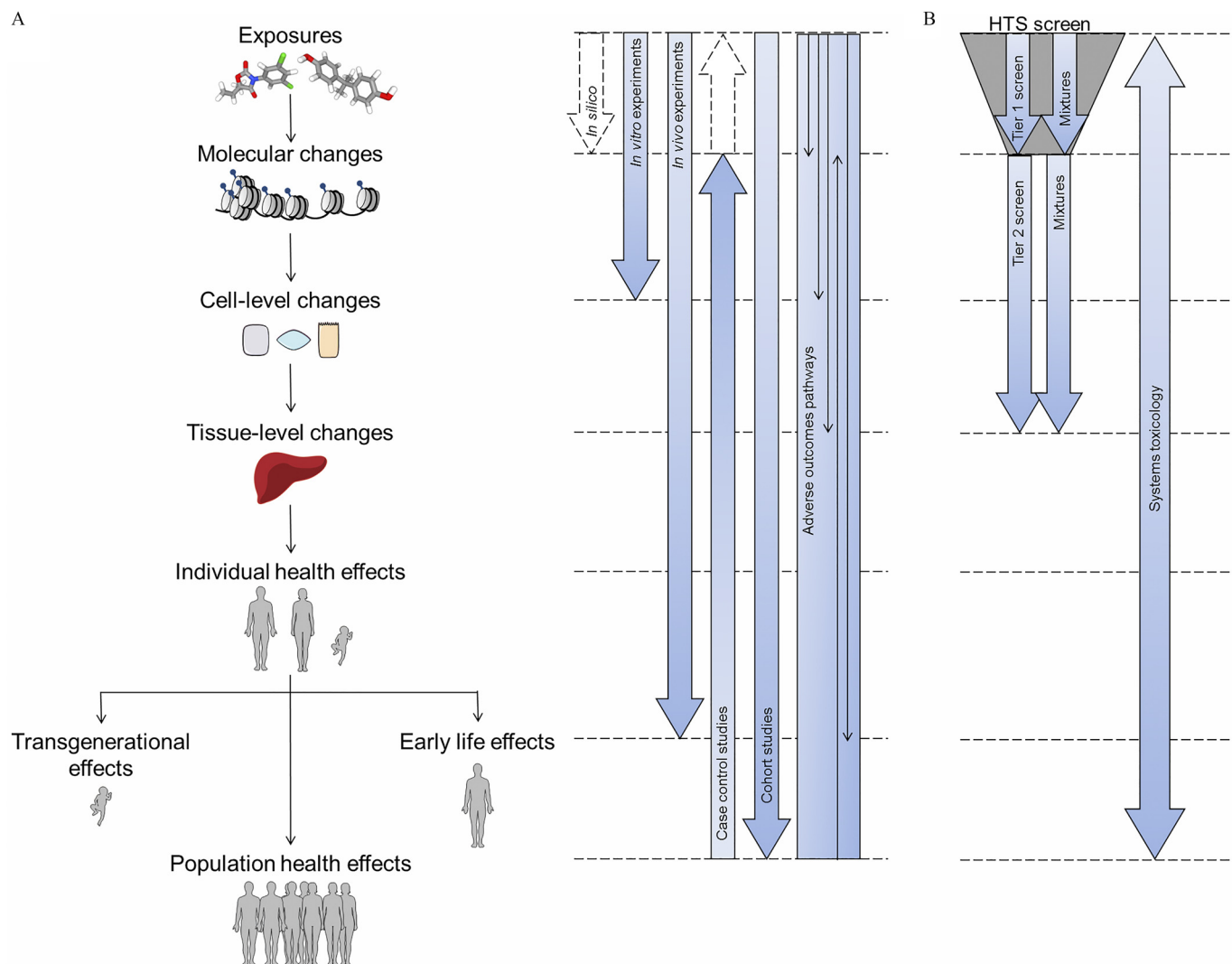
have a different capacity to provide different levels of evidence, as well as different strengths and weaknesses (Figure 3A).

Even assuming that all the technical challenges can be overcome, interpreting epigenetic results from the perspective of classical toxicology could still be complicated. For instance, if a change in epigenetic state is identified, is it truly an adverse event, or is it an early adaptive response? Is there a no-effect threshold when it comes to epigenetic modification?

### Strategies for Toxico-Epigenomic Screening

In addition to technical improvements, screening can be improved by reassessing current strategies for evaluating environmental toxicants as epigenetic modifiers. In this section, we discuss potential strategies for conducting epigenomic screening in the face of current technical limitations.

**Applying drug discovery pipelines for identifying epigenetic modifiers.** Perspectives from the drug discovery pipeline could prove useful for developing similar approaches for toxicological screening. The burgeoning fields of computer-aided drug design



**Figure 3.** An integrative method for investigating the impact of environmental chemicals on the epigenome and a proposed approach for toxico-epigenomic screening. (A) Comparison of *in silico*, *in vitro*, *in vivo*, population-based, and integrative methods in fully understanding the potential effects of environmental chemicals on the epigenome. Dotted arrows indicate situations where evidence can be inferred but not directly proved by the described methods. (B) An illustration of a proposed approach for toxicoepigenomic screening. A high-throughput screen (HTS) using *in vitro* and *in silico* methods can be conducted using single compounds and mixtures. Hits identified from the Tier 1 screen can be characterized more extensively using relevant *in vitro* and *in vivo* experiments. Finally, a systems toxicology approach could be used to integrate all data, including human data, to generate a complete profile of epigenotoxicology.

and molecular docking have been successfully implemented for drug discovery processes (Macalino et al. 2015). By using homology models of the DNMTs or, in theory, any of the epigenetic enzymes, it is possible to perform virtual screens of thousands of compounds at relatively little cost. As reviewed by Lu et al. (2018), this approach has already led to the discovery of the DNMT inhibitors RG108, NSC14778, nanaomycin A, SET7 inhibitor DC\_S100, and EZH2 inhibitor DCE\_254, among others (Lu et al. 2018). This approach could also be applied in toxicology to screen for potential epigenetic disruptors. However, although *in silico* approaches offer invaluable opportunities for streamlining target discovery processes, this does not preclude the usual standards of *in vivo* and *in vitro* testing. It does, however, enable the prioritization of potential targets.

On a related point, another approach for identifying lead chemicals is to pinpoint molecular features that enable the identification of epigenetic perturbagens. To date, common features have been identified in a subset of epigenetic modifiers, for instance phenoxyacetic acid moieties in fibrates, and the presence of halogenated polycyclic features in DDT, polychlorinated biphenyls, and chlordane (Kobets et al. 2019). This approach has been challenging because nongenotoxic epigenetic agents typically lack easily identifiable DNA-reactive chemical features. Moreover, as far as we know, the features identified in fibrates and polychlorinated compounds are not direct evidence of epigenetic-modifying ability. However, we expect that improvements in computational technology, artificial intelligence, and our understanding of the chemical processes underlying epigenetic modifications could bring new advances in this area of study.

Advances in high-content screening or high-content analysis (HCS/HCA) can also be adapted for toxico-epigenomics. HCS/HCA has been applied in drug discovery and has been driven by advances in automated microscopy and image analysis (Esner et al. 2018). An example of this is the recently described microscopic imaging of the epigenetic landscape (MIEL) system (Farhy et al. 2019). In this approach, the authors applied HCS approaches for multiparametric staining and imaging of multiple epigenetic marks. They demonstrated that the MIEL machine-learning algorithm could accurately rank and classify epigenetically active drugs, proving that the imaging of epigenetic marks and chromatin states can be added to the battery of possible HCA phenotypic screens (Farhy et al. 2019).

**Emulating the endocrine disruptor screening program.** One overarching approach may be to emulate and extend the Endocrine Disruptor Screening Program (EDSP), which was implemented by the U.S. Environmental Protection Agency (EPA) in response to mounting concerns over EDCs (Fenner-Crisp et al. 2000). The EDSP screens for chemicals that could perturb estrogen, androgen, and thyroid systems in humans, fish, and wildlife and uses a pathway-based approach in which potential lead compounds are identified based on their interactions with endocrine pathways without relying on the observation of an adverse response or phenotypic end point (Laws et al. 2011).

The EDSP uses a two-tier framework. Tier 1 was designed as a screening tier, comprising multiple *in vitro* and *in vivo* test systems, which screen for a compound's potential to interact with endocrine pathways without considering potential adverse effects. Tier 2 was designed to test established adverse effects and the dose-response relationships of hits identified in Tier 1 of the program (Fenner-Crisp et al. 2000). Using a similar approach, epigenetic modifiers may first be identified based on their impact on epigenetic enzyme activity or chromatin states, using techniques that are readily implemented in a high-throughput setting. The idea is that given the importance of epigenetic machinery in determining cell fate and function, evidence that a compound is

capable of influencing epigenetic enzyme activity sufficiently warrants additional research and testing. This approach is particularly useful when applied to epigenetic modifiers because of the breadth of possible health outcomes they could inflict.

Some limitations to this approach are that it is uncertain whether it allows for adequately sensitive detection (Coady et al. 2017). Moreover, without prior knowledge of apical or adverse outcomes, it is uncertain whether Tier 2 testing would adequately capture the toxicity profile of the test compounds and whether it accounts for differences in species and life stages (Coady et al. 2017). Some measures can be taken to at least in part mitigate the issue of sensitivity. For example, the U.S. EPA suggested in a report that assay sensitivity could be maximized while permitting an acceptable level of false positives, based on the assumption that false positives could be detected and eliminated in the second tier of the screen (U.S. EPA 2006). Other approaches are to use multiple assays for each end point, from which weighted data can be determined (Rotroff et al. 2013); to use a screening strategy based on concentration-response curves as opposed to single-dose assays (Inglese et al. 2006); and to use a compound set enrichment approach, whereby potential active chemicals are identified in sets rather than as individual compounds (Varin et al. 2010).

### **Adverse Outcome Pathways and Systems Toxicology—Filling in the Gaps**

A potential solution to the limitations discussed above is to implement the adverse outcome pathway (AOP) paradigm (Ankley et al. 2010). An AOP is a collection of data that connects an exposure to an adverse effect through a series of separate but overlapping pieces of evidence. In an AOP, a molecular initiating event (exposure to a toxicant) must be shown to induce a series of key events (e.g., DNA methylation change, transcriptional changes, sequence changes, cell proliferation), which in turn are shown to result in an adverse outcome (e.g., cancer, a disease state).

Although an AOP is itself a collection of correlative evidence, it provides a framework for organizing data across multiple disciplines in a biologically plausible and evidence-based manner. The AOP approach enables the integration of findings from epidemiology studies, which are generally observational associations between an exposure and adverse health outcomes, and *in vitro* and *in vivo* studies. The totality of this evidence can be used to establish a causative link between the exposure and the adverse outcome.

An application of AOPs is the systems toxicology strategy, which is based on the idea that any functional perturbation is a result of changes in genomic, proteomic, and metabolic states (Sturla et al. 2014). Systems toxicology integrates classical toxicology with multiple levels of molecular data, with the aim of establishing causality via a chain of molecular events. Systems toxicology aims to describe adverse biological effects as perturbed networks, as opposed to empirical end points (Hartung et al. 2017). This strategy has been made possible with advances in computational biology and molecular biology techniques.

However, the successful implementation and practice of systems toxicology requires characterization of exposures with regard to composition, dose, and duration, as well as extensive phenotypic and molecular profiling of standard biological model systems. Many of the building blocks for the widespread implementation of systems toxicology are already in place. For instance, the L1000 and Connectivity Map resources developed through the Library of Integrated Network-Based Cellular Signatures (LINCS) project enable the prediction of mechanisms of action based on the gene expression profile induced by a particular exposure (Li et al. 2019). The connectivity framework has also been applied to define and compare proteomic and histone mark signatures (Litichevskiy et al. 2018). Other valuable resources include the Toxin and Toxin-

Target Database (T3DB), which makes gene expression data on more than 3,600 compounds publicly available (Wishart et al. 2015), and the ENCODE project, which provides systematic maps of regions of transcription, transcription factor association, chromatin structure, and histone modification (ENCODE Project Consortium 2012).

### **Chemical Mixtures—Something from Nothing?**

An additional consideration is the fact that real-life exposures typically occur as mixtures, as opposed to single agents (Kienzler et al. 2016). There have thus been concerns that the risks of complex chemical mixtures are not adequately assessed (European Commission 2012; Kortenkamp 2014). A systematic review aimed at quantifying synergetic toxicity in mixtures identified evidence of synergistic interactions in a small subset of pesticides, metals, and antifoulant mixtures (Cedergreen 2014). Cedergreen (2014) defined synergetic mixtures as those which elicited an observed effect that was at least 2-fold greater than the predicted effect, using concentration addition as a reference model.

As reviewed extensively by Bopp et al. (2019), the challenge in realistically assessing exposure to mixtures is taking into account the target population(s), the timing of exposure, or whether individuals or organisms are exposed to mixtures simultaneously or sequentially (Bopp et al. 2019). Kienzler et al. (2016) described current and potential future tools for modeling mixture effects but indicated that further guidance, data, and expertise are required before they can be fully applied (Kienzler et al. 2016). However, the authors did note that there is still merit to assessing single substances because a thorough characterization of single agents could minimize uncertainties and the requirement for assumptions in the risk assessment of chemical mixtures (Kienzler et al. 2016).

An interesting proposal by Bornehag et al. (2019) suggests that exposure mixtures should first be identified and reconstructed based on evidence from epidemiological and biomonitoring data (Bornehag et al. 2019). These mixtures can then be tested with *in vivo* experiments to determine a point of departure associated with the studied adverse health outcome, and the final step is to compare the similarity of the experimental data with outcomes measured in the human population (Bornehag et al. 2019). In addition to taking into account mixtures in the risk assessment process, this approach could enable the systematic integration of epidemiological and experimental evidence (Bornehag et al. 2019). We note that with this proposed notion, the mechanistic effects of combinations of chemicals—whether independent, additive, or synergistic—are not a primary concern; the main concern is that these mixtures are relevant based on epidemiological and biomonitoring data.

We also note that there could be merit to the screening of mixtures at the preliminary *in vitro* screening stage. This could, at least in part, enable the detection of synergetic xenobiotic combinations that might have been missed if the constituent chemicals were analyzed individually.

### **Bringing It All Together**

Applying a combination of these approaches could prove useful in screening for epigenetic modifiers. For instance, we envision that assays for global changes in epigenetic or chromatin states could be used in a manner analogous to the EDSP Tier 1 screening method. This screen may include mixtures of chemicals that have been identified and reconstructed through epidemiological observations or biomonitoring data, as described by Bornehag et al. (2019). Hits identified in Tier 1 screens can be further examined with more sensitive epigenome-wide techniques, followed by the identification of phenotypic and molecular perturbations incurred by the exposure. Tier 2 screens may also include time-

dependent, early life experiments in order to establish the full epigenotoxicity profile of the exposures in question. Pathway-enrichment technologies may then be applied to predict for epigenotoxicity, followed by AOP to organize preliminary data into coherent chains of evidence, and to identify areas in which further research is warranted. Finally, hit compounds may be characterized using systems toxicology approaches, ultimately resulting in comprehensive portraits of exposures and disease states (Figure 3B).

### **Perspectives for the Future of the Field**

As with many aspects of molecular science, the field of epigenetics has benefited greatly from computational and analytical advances (Cazaly et al. 2019; Lim et al. 2010; Mensaert et al. 2014). Our view is that the future iterations of toxicology are unlikely to consider genetic, epigenetic, or proteomic factors individually, with more calls to consider the totality of these mechanisms using multi-omics analyses. This new horizon of possibilities allows for more optimistic and ambitious concepts for the future of toxicology.

Another interesting concept is to generate fingerprints of exposure to environmental toxicants using evolving -omics technologies (Messerlian et al. 2017) and apply these signatures as biomarkers and predictors of response. An important step toward this is to establish robust and universally recognized epimutation signatures, analogous to the mutational signatures established by genome sequencing (Kucab et al. 2019). Our view is that a compendium of epigenomic signatures will enable systematic comparisons between test compounds. An educated guess of an unknown compound's mechanism of action could be made, for example, based on the epimutation signature it induces in reference to known entries. With additional information on toxicity and adverse outcomes, it may be possible to make calls on how hazardous a compound is based on its epigenomic and mutational signature. Libraries of phosphoproteomics and chromatin profiles are already in development, using the connectivity framework developed with the LINCS Connectivity Map in the context of epigenetics (Creech et al. 2015; Litichevskiy et al. 2018). The availability and growth of these resources represent exciting opportunities for the future of epigenomic research.

In an extension of -omics analyses, one of the most ambitious concepts is that of the exposome, which is defined as a summation of an individual's experiences over the course of his or her lifetime, and the biological response to these exposures (Wild 2005). The exposome is represented by molecular measurements influenced by a lifetime of exposure to a variety of factors such as diet, lifestyle, infections, and psychosocial factors (Wild 2005). The Human Exposome Project, the environmental analog of the Human Genome Project, is a mammoth undertaking, because the possible range of exposures and individual response is immensely complex (Niedzwiecki and Miller 2017). Exposome research projects include the Human Early Life Exposome (HELIX) (Vrijheid et al. 2014), enhanced exposure assessment and omic profiling for high priority environmental exposures in Europe (EXPOsOMICS) (Vineis et al. 2017), Health and Exposome Research Center: Understanding Lifetime Exposures (HERCULES) (Niedzwiecki and Miller 2017), and Health and Environment-wide Associations based on Large population Surveys (HEALS) (<http://www.heals-eu.eu/>; Sarigiannis 2014) projects. Although many of the proposed ideas are not feasible at present, the concept of the exposome has important implications for the assessment of exposure in human health research; for instance, considerations that environmental exposures do not exist in isolation and that individual response to exposures can be influenced by a multitude of inherent or acquired factors.

In our view, another major issue to consider in the future is how well current assays are adapted for measuring the environmental impact of chemical agents resulting from human activity. Currently, most high-throughput assays are based on end point

measures relevant to humans or mammals. Although this is of clear importance, a holistic assessment of the impact of chemical agents on the environment would require the development of robust nonmammalian or cross-species assays (Coady et al. 2017).

## Conclusion

Significant advancements in analytical technologies are now enabling more robust testing and risk assessment approaches in evaluating the hazards of environmental chemical exposure. With accumulating evidence that environmental chemicals are at least partially responsible for the onset of various noncommunicable diseases, there is an urgent need to understand the mechanisms underlying chemical-induced adverse outcomes. Considering epigenetic modifications in the risk assessment process could lead to the development of more sensitive and predictive assays for detecting chemical-induced changes. In view of the impact that environmental chemicals could have on the environment, and on current and future generations, increased efforts in research and regulation are certainly warranted.

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