Review

Importance of investigating epigenetic alterations for industry and regulators: An appraisal of current efforts by the Health and Environmental Sciences Institute

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ABSTRACT

Recent technological advances have led to rapid progress in the characterization of epigenetic modifications that control gene expression in a generally heritable way, and are likely involved in defining cellular phenotypes, developmental stages and disease status from one generation to the next. On November 18, 2013, the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) held a symposium entitled "Advances in Assessing Adverse Epigenetic Effects of Drugs and Chemicals" in Washington, D.C. The goal of the symposium was to identify gaps in knowledge and highlight promising areas of progress that represent opportunities to utilize epigenomic profiling for risk assessment of drugs and chemicals. Epigenomic profiling has the potential to provide mechanistic information in toxicological safety assessments; this is especially relevant for the evaluation of carcinogenic or teratogenic potential and also for drugs that directly target epigenetic modifiers, like DNA methyltransferases or histone modifying enzymes. Furthermore, it can serve as an endpoint or marker for hazard characterization in chemical safety assessment. The assessment of epigenetic effects may also be approached with new model systems that could directly assess transgenerational effects or potentially sensitive stem cell populations. These would enhance the range of safety assessment tools for evaluating xenobiotics that perturb the epigenome. Here we provide a brief synopsis of the symposium, update findings since that time and then highlight potential directions for future collaborative efforts to incorporate epigenetic profiling into risk assessment.

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Contents

1. Introduction

The term "epigenetics" has undergone much reinterpretation since first coined by Conrad Waddington, who proposed that development and evolution can be viewed as a succession of relatively stable states, separated by stages of instability and change (Stern, 2000; Waddington, 1940). A modern molecular view of epigenetics refers to the induction of stable changes in gene expression and chromatin organization that are independent of changes in the DNA sequence and propagated through cell division (Herceg et al., 2013). Key components involved in epigenetic mechanisms include DNA methylation, modifications of histone proteins and expression of non-coding RNA species, as well as X-chromosome inactivation and genomic imprinting which might be considered as secondary effects (Ferguson-Smith, 2011).

The fundamental structural unit within the chromatin structure is the nucleosome, which consists of 146 bp of DNA wrapped around a nucleosome core that is composed of evolutionary conserved histone proteins. The subsequent compaction of nucleosomes packages the DNA in the cell nucleus into characteristic cytological structures that include heterochromatin (Kouzarides, 2007). The higher order structure of chromatin is important in the regulation of gene expression with an "open" or euchromatic state able to facilitate transcription while a compact or heterochromatic state is associated with silenced regions of the genome (Sproul et al., 2005). Core histones (H2A, H2B, H3, and H4) have flexible N-terminal domains that contain residues that can be post-translationally modified at specific sites by methylation, acetylation, phosphorylation, ubiquitination, and sumoylation (Kouzarides, 2007). Sets of modifications are associated with transcriptionally active and silent states. Complex interactions between histones, modifying enzymes, DNA sequences, and other partner proteins contribute to gene expression regulation in specific contexts.

Epigenetic modifications can in general be grouped into those which are either deposited directly onto the DNA or those which mark the N-terminal tails of histone proteins. They are part of a complex network of interactions that fine-tune cells to their environmental conditions. These changes modify the DNA landscape to qualitatively and quantitatively determine how proteins interact with DNA segments, and thereby regulate gene expression globally and locally. Methylation catalyzed by DNA methyltransferases at the 5th position of cytosine residues in the context of the CpG dinucleotide results in formation of 5-methylcytosine (5-mC), which decorates the DNA landscape of mammalian somatic cells (Cruickshanks et al., 2013; Ehrlich et al., 1982; Jeltsch and Jurkowska, 2014). Methylation of cytosine at regulatory regions (promoters and enhancers) is associated with transcriptional repression and is considered to be rather stable in the genome. Although the majority of CpG dinucleotides are methylated in somatic DNA, a significant fraction of them, termed CpG islands (CGIs), are non-modified and are typically promoter associated (Illingworth et al., 2010).

The pathways governing active removal of 5-mC are only now beginning to be understood due to the recent discovery of further modified forms of cytosine nucleotides (Ito et al., 2011; Kriaucionis and Heintz, 2009). In 2009 the dioxygenase enzymes that convert 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formyl cytosine (5-fC) and 5-carboxylcytosine (5-caC) were described (Tahiliani et al., 2009). In terms of abundance 5-hmC is characteristically present at 10% or less relative to 5-mC, with 5-fC and 5-caC many orders of magnitude lower (Pfaffeneder et al., 2014). The attraction of studying 5-hmC in conjunction with 5-mC is that it is associated with reprogramming of DNA methylation patterns and is correlated with active genic regions in multiple tissues (Ficz et al., 2011; Nestor et al., 2012; Szulwach et al., 2011; Wu and Zhang, 2014). As such, 5-hmC profiles are indicative of cellular state (Laird et al., 2013).

In recent decades, the potential for xenobiotics to alter expression of genes has been well-studied. Termed toxicogenomics, this field endeavors to elucidate molecular mechanisms that underlie adverse responses to toxic agents by measuring alterations in messenger RNA (mRNA) expression. Such perturbations may then lead to altered protein expression and activity, thereby propagating the intracellular response to the agent. Recently attention has focused on understanding alterations of epigenetic modification in gene regulatory regions that control expression of genes. In that context, large epigenomic data sets for multiple tissues and disease states have been generated over the last decade that identify characteristic epigenetic alterations in cellular state, development, disease and cancer (Sproul and Meehan, 2013) (Fig. 1). Accumulating evidence suggests that epigenetic markers and/or the molecular machinery regulating them may be perturbed by exposure to various environmental, chemical, and

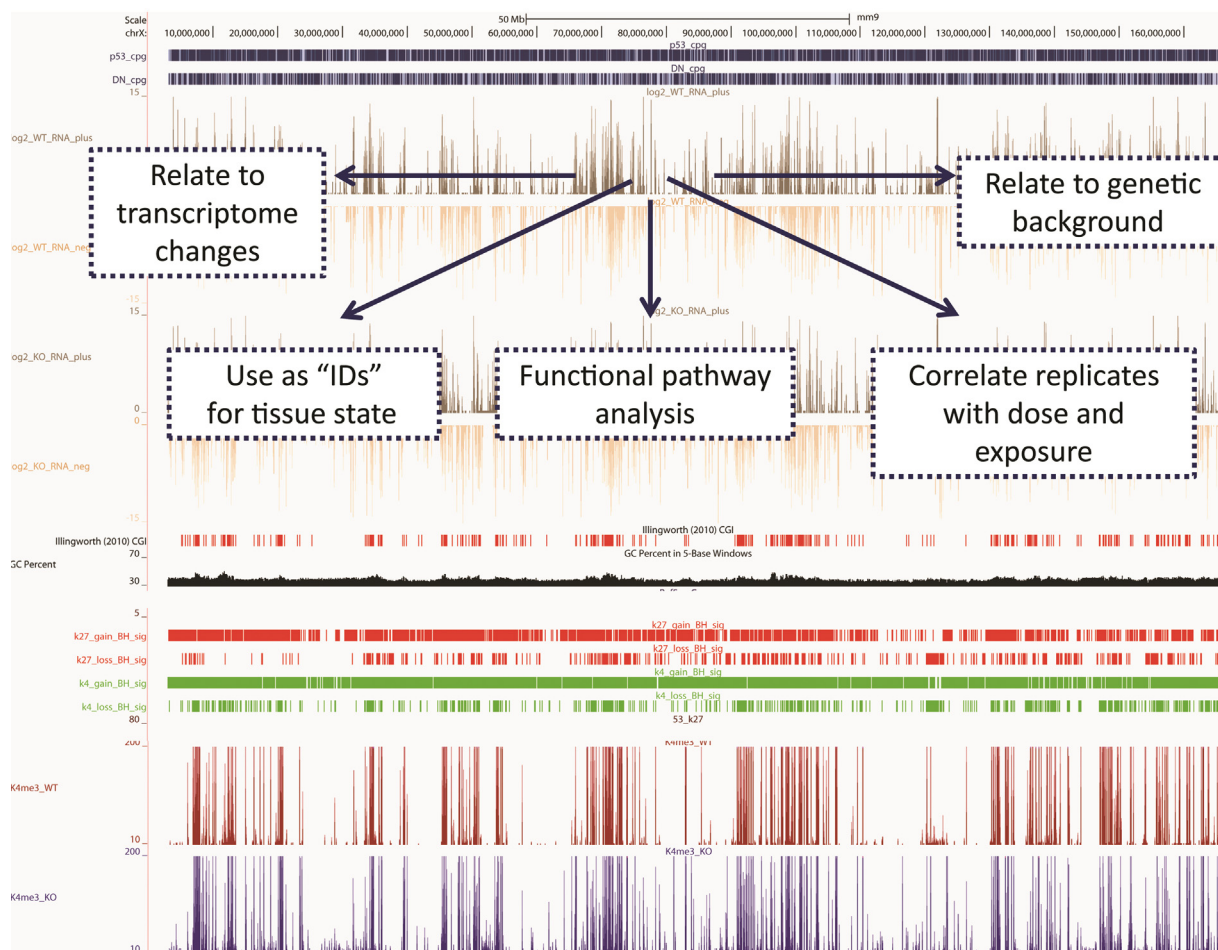## Potential toxicological applications of epigenetic datasets

Fig. 1. Exploitation of epigenetic datasets. Representative schematic indicating DNA methylation, gene expression and chromatin modification states on a region of mouse chromosome X (data from (Reddington et al., 2013)). Superimposed are potential avenues for genome wide analysis of epigenome datasets in relation to environmental exposure studies.

biological stressors (Thomson et al., 2014). From a chemical toxicity viewpoint, this is a possible mechanism of action that links transient exposure to an inducer to potentially stable cellular changes including alteration of gene expression. Therefore, epigenetic parameters have been proposed as biomarkers of exposure to environmental toxicants and carcinogens (Alyea et al., 2014; Goodman et al., 2010; Herceg et al., 2013; Koturbash et al., 2011; Laird et al., 2013). However, significant knowledge gaps exist that prevent the inclusion of epigenomics data into xenobiotic hazard characterizations. One ongoing question regards the determination of whether observed epigenetic alterations are an outcome of exposure or a direct facilitator of change. In addition, there is a lack of data agreement regarding which epigenomic changes are adverse versus those that are merely reactive to a chemical stimulus. With increasing interest in assessing hazard potential by measuring subtle, molecular changes in adverse outcome pathways (AOPs) within both the environmental and pharmaceutical regulatory agencies, it is critical to identify which epigenomic changes will be useful to assess and in which contexts.

On November 18, 2013, the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) held a symposium entitled "Advances in Assessing Adverse Epigenetic Effects of Drugs and Chemicals" in Washington, D.C. which was

sponsored by the Technical Committee for the Application of Genomics to Mechanism-Based Risk Assessment. The goal of the symposium was to identify gaps in knowledge and promising areas of progress that represent opportunities where collaborative efforts could further the utilization of epigenomic profiling in drug and chemical safety assessment programs. In this review, we summarize topics discussed during the workshop that are supplemented by a review of the current literature.

2. Implications of epigenetics in various sectors involved in safety assessments

2.1. Pharmaceutical safety

Past attention on the role of genetics in cancer has focused on the accumulation of changes in DNA sequence. However, recent investigations using global proteomic and genomic approaches have suggested that alterations in both genetic and epigenetic factors contribute to cancer development (Di Costanzo et al., 2014). In contrast to changes in genetic sequence, epigenetic modifications can potentially be reversed or restored by therapies that target over expressed or mutated chromatin regulatory proteins. As a result, the pharmaceutical industry has directed efforts to develop targeted therapies against epigenetic-modifying enzymes,

including histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone acetyltransferases (HATs), and DNA methyltransferases (DNMTs). Significant debate surrounds the potential risks that might be associated with long-lasting drug-induced epigenetic perturbations. Factors to consider include the duration of toxicology studies, reversibility, gene locus-, tissue- and species-specificity, and the importance of distinguishing between adaptive and adverse epigenetic perturbations. Furthermore, differences in risk-benefit for oncology versus non-oncology therapeutic indications need to be considered. The *innovative medicines initiative* (IMI) bioMARKers and molecular tumor classification for non-genotoxic CARcinogenesis (MARCAR) consortium have made significant progress in the characterization of long-lasting drug-induced epigenetic perturbations associated with rodent non-genotoxic hepatocarcinogenesis via integrated epigenomic and transcriptomic profiling (Lempiainen et al., 2011, 2013; Luisier et al., 2014; Thomson et al., 2012, 2013). A similar strategy may provide valuable insights into additional long-lasting toxicities such as allergen-induced T cell responses (Chapman et al., 2014; Moggs et al., 2012).

Several epigenetic-modifying drugs have gained FDA approval or are being utilized in ongoing clinical trial investigations. Inhibitors of DNMTs and HDACs already received FDA approvals. The DNMT inhibitors, 5-azacitidine and 5-aza-2′-deoxycytidine, are approved for the treatment of acute myelogenous leukemia and myelodysplastic syndrome, and the HDAC inhibitors, romidepsin and vorinostat, are approved for treatment of cutaneous and peripheral T cell lymphoma (CTCL and PTCL). Despite their efficacy for treating cancers, the reported and common side effects for each class included thrombocytopenia, neutropenia, anemia, and gastrointestinal adverse events (among them are nausea, vomiting, and diarrhea). With some HDAC inhibitors, clinically insignificant, transient and reversible QTc prolongations have also been previously reported. The cumulative effect of antiemetics (serotonin receptor antagonists associated with QTc prolongation (Fraczek et al., 2013) used during HDAC treatments might contribute to these electrocardiographic changes. However, recent analyses of QTc interval data obtained from CTCL and PTCL patients that received antiemetics prior to romidepsin treatment demonstrated that treatment with romidepsin did not markedly prolong the QTc interval despite the use of the QTc-prolonging antiemetics (Sager et al., 2015). For another HDAC inhibitor, belinostat, ECG analysis did not identify any QTc prolongation (Spectrum Pharmaceuticals, 2014).

Epigenetic-modifying drugs have the potential to promote viral reactivation via the phenomenon of activating latent viral DNA in the host genome. In vitro experiments indicated the role of histone acetylation and CpG methylation in maintaining viral latency of Epstein bar virus (EBV), cytomegalovirus (CMV), human immunodeficiency virus-1(HIV-1) and hepatitis B virus (HBV) (Belloni et al., 2009; Blazkova et al., 2009; Liu et al., 2013a; Pollicino et al., 2006). HDAC inhibitors (sodium butyrate and trichostatin A), a DNMT inhibitor (azacitidine), or a combination treatment of HDAC and DNMT inhibitors have been shown to activate the lytic cycles of both EBV (Li et al., 2012) and Kaposi's sarcoma associated herpesvirus (KSHV) (Gorres et al., 2014) via activation of transactivator proteins that drive the lytic cycle. While such viral reactivation has been shown in in vitro models, the challenge is to determine whether viral activation due to drug-induced loss of viral latency could present a safety risk to patients. Recent analyses have suggested an apparent relationship between romidepsin treatment and viral reactivation (i.e., high frequency of reactivation of the latent EBV) in South Korean patients with a rare type of EBV-induced T-cell lymphoma (15-day increased frequency report, Celgene). EBV reactivation due to romidepsin in lymphoma patients is rarely reported in Western patient populations,

probably because this type of EBV-induced T cell lymphoma is extremely rare in the West. Viral reactivation may be of therapeutic benefit in certain instances such as the reactivation of HIV-1 from dormancy in infected T-cells may allow anti-retroviral therapy to become effective. Future research aimed at better understanding transcriptional control of HIV-1 may, in theory, enable development of combinatorial therapies that include drugs designed for viral reactivation (Mbonye and Karn, 2014).

2.2. Environmental risk assessment in the context of transgenerational considerations

Transgenerational epidemiology is a field that entails investigation of exposure effects beyond a single generation. While biological inheritance of a phenotype was long regarded as inheritance of genetic sequence variation, there is increasing appreciation for the role of exposure-induced epigenetic changes. According to investigators participating in the Network in Epigenetic Epidemiology, the field seeks to identify "what exposure at which life stage in parents, grandparents, or distant ancestors is associated with measurable phenotypic outcome in the offspring or subsequent generations" (Pembrey et al., 2014). Importantly, the parents that experience the epigenetic modifications may not exhibit the adverse phenotype.

Transgenerational inheritance of epigenetic modifications begins with exposure in the F0 generation that results in phenotypes not only through simple fetal exposures (F1 generation) but also in the F2 or F3 generations. Current models indicate that the majority of epigenetic modifications are reprogrammed in developing primordial germ cells (PGCs) and during embryogenesis (Hajkova et al., 2002; Lee et al., 2002). However, some modifications are consistently maintained at specific regions in developing PGCs and are delivered to subsequent generations (Lane et al., 2003). The study of embryogenesis in model organisms and humans is essential to define transgenerational phenomena and propagation, since there are still controversies whether supposed transgenerational epigenetic effects are truly transgenerational at least in animals and humans, and the mechanisms of inheritance are poorly understood (Heard and Martienssen, 2014).

Animal studies have been used to gain a broader understanding of transgenerational inheritance of epigenetic modifications due to environmental exposures. In addition, to separate the contribution of the maternal uterine environment and that of the epigenome both maternal and paternal transmission as well as embryo transfer have been investigated (Padmanabhan et al., 2013). In the folate cycle hypomorphic model, for example, congenital malformations were due to the epigenetic makeup, while growth retardation was related to the uterine environment. The appearance of congenital malformations independent of maternal environment persisted for five generations, which is suggested to be due to transgenerational epigenetic inheritance by a modality that is yet to be identified.

In addition to transgenerational inheritance of adverse effects, studies have also reported adaptive transgenerational responses. One example is the adaptive wound-healing response in rat liver. In a landmark study, it was demonstrated that F1 and F2 offspring born to male rats that had sustained liver damage due to carbon tetrachloride (CCl$_4$) had inherited protective epigenetic changes. The heritable epigenetic signatures were found to be associated with increased hepatic expression of antifibrogenic PPAR-γ and decreased expression of profibrogenic TGF-β. Importantly, the transgenerational mode of transmission was found to be through the male germline, eliminating confounding factors from the uterine environment (Zeybel et al., 2012).

2.3. Human health risk assessment of agrochemicals

Given recent datasets that suggest that chemically-induced epigenetic changes may adversely impact current and future generations, consideration has been given toward the role of epigenetics in product safety assessment for industrial and agricultural compounds as well. Although not generally required by current safety assessment guidelines, chemical manufacturers have begun to evaluate transgenerational case studies with integration of epigenetic dose–response curves into experimental design. These studies are intended to evaluate whether the dose-dependency of potential transgenerational epigenetic phenomena occurs at or below "no adverse effect levels (NOAELs)" of traditional apical endpoints that are currently assessed in regulatory studies. These data may be useful to eventually determine the adequacy of the current regulatory toxicity testing guidelines to detect adverse epigenetic effects. A case study conducted by Dow Chemical using the fungicide vinclozolin was recently published (Alyea et al., 2014). In utero exposure of vinclozolin and its major metabolites act as antiandrogens, causing male rat offspring to be demasculinized as typified by reduced anogenital distance, retained nipples, a vaginal pouch, cleft phallus with hypospadias, suprainguinal ectopic scrota and testes, and altered sex accessory glands in male offspring. While transgenerational research studies in rats exposed gestationally to vinclozolin did result in adverse reproductive effects to offspring, these occurred at doses that are 40–80 fold higher than the NOAEL and LOAEL (lowest adverse effect level). Detailed analysis showed that effective doses for epigenetic endpoints were covered by apical endpoint NOAELs. It is important to note that studies done by other investigators using the same dose of vinclozolin found no effects on the same reproductive endpoints over four generations in rats (Schneider et al., 2013). Reasons for the differences between studies may include sources of variability, such as genetic stock of rats, the role of gut microbiota, and other factors. However, taken together, the data indicated that the risk assessment performed for vinclozolin using apical endpoints was conservative enough to prevent the observed epigenetic changes.

3. Model systems and biomarkers to assess epigenetic effects

Several promising model systems and biomarkers for assessing epigenetic effects were discussed during the symposium. Selected topics are presented in this section.

3.1. Natural genetic variation and interaction with the epigenome at the population level

A significant knowledge gap in the field is in determination of the natural variability in the epigenome within populations. As one goes forward in associating epigenetic patterns with a disease, it is essential to trace the limits of what epigenetic marks may be considered "normal". A substantial hurdle to the integration of epigenetics for toxicological assessment is the current uncertainty around what constitutes a normal, adaptive response to an exogenous stimulus and what is an adverse, disease-associated alteration. Investigation of dynamic variations in the epigenome between animal strains, sexes, and ages are thus a starting point to identify natural variability. While the studies conducted in a single strain of inbred mice are tremendously useful in identifying basic mechanisms, they present significant limitations in terms of representing the breadth of variability found in the human population. Current efforts to overcome these limitations include the mouse Methylome Project, directed by the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP). The scope of this project is a comparative

analysis of the methylomes in the livers of males and females in three different inbred mouse strains with the goal of producing a better understanding of the relationships between allele-specific methylation patterns, regulation, and altered expression in response to environmental stimuli. Data collected via this cross-disciplinary effort will include information on the methylome, genome, and transcriptome of paternal strains and F1 progeny. These data will provide insights on epigenomic inheritance to offspring as well as how the epigenetic landscape might differ among genders, siblings, and strains (i.e., genetic background).

Population-based rodent models provide a means to study the interaction between genetic sequence variation and epigenetic modifications in the context of a xenobiotic insult. One such model is the diversity outbred, a mouse population with genetic sequence variation that is more extensive than comparable human populations. Because of a low minor allele frequency (12.5% on average), a well-powered study will require far fewer mice than humans (Churchill et al., 2012). A recent study by French and coauthors explored the use of diversity outbred mice as a tool to set a benchmark dose for benzene-induced micronucleus formation in genetically diverse populations (French et al., 2015). The authors demonstrated that the inclusion of genetically sensitive individuals would result in a more conservative benchmark dose than that suggested by genetically homogeneous B6C3F1/J mice, which are the standard mouse model for the National Toxicology Program. While the model provides a promising paradigm for assessing the adverse effects of chemicals in a diverse population, to date there have been no studies linking genetic variation to epigenetic modifications using the diversity outbred mice. Similar efforts are being pursued by several other public/private consortia or partnerships, such as a recent European Chemical Industry Council (CEFIC) Long-range Research Initiative (LRI) to define "normality of the rodent epigenome" including outbred strains (http://cefic-lri.org/projects/c3-ed-a-comprehensive-epigenomic-profile-of-liver-tissue-from-rat-and-mouse/). Even in plant research, efforts in mapping epigenomes are underway to understand plant growth, development, and adaptation to the environment (Consortium, 2012).

With the advent of comprehensive human tissue-specific epigenome maps (Kundaje et al., 2015), Epigenome-Wide Association Studies (EWAS) of human populations and patient cohorts are likely to provide additional novel insights into mechanisms and biomarkers underlying xenobiotic responses. For example, cigarette smoking has been linked with specific CpG methylation alterations in the aryl hydrocarbon receptor repressor gene in blood cells derived from both adult smokers and newborn babies exposed to maternal smoking in utero (Joubert et al., 2012; Monick et al., 2012; Shenker et al., 2013). EWAS studies are already being extensively used to characterize human disease states and identify potential epigenetic risk factors (Liu et al., 2013b; Rakyan et al., 2011). Whether or not peripheral epigenetic biomarkers (e.g., blood- or skin-derived) can help predict patient susceptibility to drug-induced toxicities remains to be established. EWAS might ultimately assist in designing transgenerational studies in humans depending on the mode of inheritance (Heijmans et al., 2009).

3.2. Non-rodent models for epigenetic assessment

As summarized in Section 2, there has been considerable concern and controversy surrounding the potential for xenobiotics to cause adverse effects in subsequent generations. Similar concerns could be applicable to targeted therapies for epigenetic modifiers. Therefore, it is becoming increasingly important to have improved methods for assessing the likelihood that specific classes of xenobiotics might produce delayed effects that impact

future generations. As mentioned previously, multigeneration studies up to the F3 generation are required to reveal transgenerational effects. However, such studies are long and therefore expensive to conduct in mammalian species, and fit poorly into safety assessment development paradigms. The duration of these studies could be shortened by the use of non-mammalian models like zebrafish that recapitulate the cell migration and pattern formation inherent in the mammalian conceptus in an intact organism.

Zebrafish are frequently used in toxicological studies and the species has recently gained attention as an attractive model for transgenerational effects of xenobiotics owing to strong conservation of gene and protein structure and function between zebrafish and humans (Mudbhary and Sadler, 2011). Additional advantages are ease of genetic manipulation, utility for high content imaging, and the relatively short time frame of breeding and development. In addition, chemical exposure to zebrafish embryos can be monitored and standardized more effectively than rodent embryos that are subject to maternal metabolism and differences in parity. Increasingly, zebrafish are employed in high-throughput assays aimed at assessing developmental morphology endpoints (Truong et al., 2014). In addition, zebrafish are an ideal model for RNAi studies and offer the advantage of providing a more rapid screen when compared to generation of knockout rodent models (Gaytan and Vulpe, 2014). Several publications have shown that transgenerational adverse effects can be demonstrated in zebrafish using different chemical treatments, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Baker et al., 2014), androgens (Xu et al., 2014), and perfluorononoate (Liu et al., 2011).

3.3. Stem cells and reprogramming

Stem cell models may be employed to assess the effects of chemicals on the differentiation process, as proteins functioning in epigenetic pathways are very often expressed in embryonic and adult stem cells and are involved in regulating key events during stem cell self-renewal, differentiation, and tissue senescence (Gifford et al., 2013; Hemberger et al., 2009; Paige et al., 2012; Raveh-Amit et al., 2013; Xie et al., 2014). In embryonic stem cells, modulation of such proteins is known to be associated with embryonic and developmental toxicity. Adult stem cells are distributed in most adult mammalian tissues as partially differentiated progenitor cells and are involved in maintaining tissue homeostasis and help with regeneration and repair during tissue damage (Iglesias-Bartolome and Gutkind, 2011). Because stem cells in each tissue rely on a different suite of transcription and epigenetic factors to maintain their own particular state of differentiation (Chen et al., 2012), modulation of epigenetic protein targets could lead to stem cell exhaustion, bone marrow suppression, immune suppression, gastrointestinal toxicity, and impaired tissue regeneration and repair. Although the effect of modulation of epigenetic targets on adult stem cell function can be modeled in vitro, it is not practically feasible to develop assays that would comprehensively cover multiple adult tissues. Therefore, a more general screen based on human (or rodent) embryonic or induced pluripotent stem cells (hESC/hiPSC) may serve as a surrogate for adult progenitor/stem cells. Undifferentiated hESC/iPSC could be dosed with compounds and evaluated for a variety of differentiation and also epigenetic endpoints. A requirement for the latter is the availability of robust differentiation protocols.

3.4. Novel epigenetic biomarkers for safety assessment

The incorporation of modern molecular and "omics" technologies into regulatory decision-making has great potential to improve risk and product safety assessment methodologies. One promising avenue is the identification of epigenetic markers of chronic toxicities like carcinogenesis in rodent studies that might ultimately be translated into surrogate markers of an adverse response in humans (Laird et al., 2013; Thomson et al., 2014). Epigenetic marks could serve as potential biomarkers of prior exposure of chemicals that affect the epigenome in a reliable and dose dependent manner. There are, however, significant gaps in the knowledge of epigenetic effects caused by xenobiotics that need to be bridged before epigenetic signatures and biomarkers can be introduced into risk assessment.

4. Knowledge gaps and future directions

Based on symposium presentations and discussions, several data gaps were identified that should be addressed in order to advance the use of epigenetics in drug and agrochemical development and environmental risk assessment.

4.1. Dose–response analysis

There is a lack of sufficient studies that evaluate the epigenetic effects of exposure to multiple doses of the same compound. Given that not all toxicological responses follow a linear model, carefully designed studies that would utilize a range of doses of the test article are clearly needed to explore the dose–response potential of epigenetic parameters. Multiple examples exist where testing at high doses are not predictive of biological effect at environmentally relevant doses, as is the case for numerous endocrine-disrupting chemicals (Almstrup et al., 2002). In addition, assessment of epigenetic changes at multiple dose levels may provide key data that would enable identification of a benchmark dose for adverse epigenetic modifications caused by xenobiotic agents.

4.2. Non-cancer endpoints

Since carcinogenesis involves epigenetic alterations, there is a strong emphasis of current epigenetic studies on cancer causation or therapy. However, it is becoming increasingly evident that epigenetic changes are also associated with the pathogenesis of an increasing number of other common disorders, such as type 2 diabetes, cardiovascular disease (Kim et al., 2010; Chen et al., 2011), and autism, to name a few. Furthermore, associations have been made between environmental exposures and increased risk for certain non-cancer diseases. Examples of potential relationships that have been investigated include pesticides and Parkinson's disease (Pezzoli and Cereda, 2013) and persistent organic pollutants and type 2 diabetes (Everett et al., 2007; Lee et al., 2006). It remains a challenge to identify epigenetic alterations associated with a specific disease and to determine if they are causative or casual changes.

4.3. Drug development

Questions remain on how or whether safety assessment of drugs targeting the epigenetic machinery will differ from other classes of drugs. Potential unique risks for such drugs could include aberrant adult stem cell differentiation including reduction of cells available for self-renewal, reactivation of latent viruses considering the role for H3K9 methylation in maintenance of HIV virus latency, or transgenerational effects resulting from perturbation of imprinted gene loci. Investigation of such potential risks are expected to be associated with specific challenges concerning the required length of studies, inter-species and inter-individual variability, distinction of adaptive versus adverse effects, reversibility, and tissue specificity.

Furthermore, guidelines to inform risk-benefit assessment for oncological as compared to non-oncological indications will require additional considerations.

4.4. Transgenerational safety assessment

Since new approaches for the treatment of cancer and other disease areas are increasingly targeting epigenetic mechanisms, concerns are rising about the potential of such drugs to cause effects in subsequent generations. As the first epigenetics treatments for cancer were shown to be effective and tolerated, these approaches are now moving into less life-threatening indications. Therefore, it becomes increasingly important to develop an assessment of the likelihood, or not, that these treatments might produce delayed effects on future generations. To reveal transgenerational effects, multigeneration studies up to F3 are required. Such studies are long and therefore expensive, and fit poorly into drug development paradigms. Zebrafish may be able to help shorten the duration of such studies, but they are not yet a widely used model for this application. Thus one potential research area is the development of the zebrafish model for epigenetic research with the ultimate goal of using this model to study the potential transgenerational effects of pharmaceuticals and chemicals. A potential limitation of studies in zebrafish is the relevance of findings to mammals. There are important differences in both the mechanisms and phasing of genomic DNA methylation in mammals and zebrafish during early development (Jiang et al., 2013; Potok et al., 2013) and differences concerning demethylation waves after fertilization during early development that will need to be taken into account (Hackett and Surani, 2013).

4.5. Stem cell models

The effects of drugs and chemicals on human safety and efficacy are ideally evaluated in humans; however, tissue availability and suitability of current in vitro models are generally a limiting factor. Stably transformed cell lines have multiple mutations and epigenetic defects that could indirectly modulate or confound epigenetic effects of drugs. In addition to supply issues, primary-derived differentiated cells do offer the opportunity to develop testing models which are more physiologically relevant for humans, and which better model human disease if derived from cells of patients via a induced pluripotent stem cell (iPSC) state (Engle and Puppala, 2013; Pistollato et al., 2012).

Concerning chemical effects on the epigenetic network in cells, stem cell models may be used either in their differentiated state or during differentiation. With respect to the former, cells differentiated from human iPSC may be better suited for assessment of chemical effects on epigenetic parameters since iPSC from different individual genotypes can be derived and then differentiated into non-transformed cell types such as cardiomyocytes, hepatocytes and neurons under defined conditions. Thus this model system offers a unique opportunity to define intra-individual and intra-cell type effects under non-confounding experimental conditions. Since chromatin within different cell types exists in different conformations, chemicals may induce different epigenetic changes in, for example, hepatocytes or cardiomyocytes, which may then be much better assessed in these more coherently differentiated cells.

4.6. Determination of which epigenetic alterations are adverse

One of the most promising avenues is the identification of epigenetic markers of toxicity, especially of chronic toxicities like carcinogenesis, that can be further translated into surrogate markers of an adverse response in humans (Laird et al., 2013; Thomson et al., 2014). However, one of the significant gaps in the knowledge of epigenetic effects caused by xenobiotics that need to be bridged before epigenetics can be introduced into risk assessment is the determination of which epigenetic changes are "adverse" versus "adaptive" and in what contexts do the changes exert a deleterious effect on an individual. To fulfill this need, more data are needed on epigenetic alterations that occur in target tissues and blood in the context of exposure to a wide variety of xenobiotic agents that span multiple modes of action. Such studies would include several doses and time points to derive a comprehensive picture of the time course of appearance (and possibly resolution) of epigenetic alterations.

5. Conclusions

Significant progress has been made in epigenetic research and in our understanding of the epigenetic effects associated with exposure to drugs and chemicals in the last several years. Technological developments, including novel methodologies and approaches for evaluation of existing epigenetic endpoints, as well as establishment of new epigenetic parameters, promise to significantly increase the robustness of these methods to allow effective application in safety assessment. The HESI symposium on advances in assessing adverse epigenetic effects of drugs and chemicals provided a forum to review the primary advances in epigenetic research and to discuss major concerns and knowledge gaps that need to be addressed before epigenetic endpoints may be used in toxicological evaluations. Natural variability and dynamic variation in the epigenome between strains, sexes, and ages present challenges in defining the line between "healthy" and "diseased" epigenomes. Specific epigenetic end-points that can be utilized in safety assessment as well as assays and model systems for assessment of these parameters need to be further identified and evaluated for consistent implementation. These gaps could be bridged by consortial efforts that generate the necessary comprehensive data sets.

Declaration of interest

Acknowledgements

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