ENDOCRINE DISRUPTORS AND THE EPIGENOME

John M. Greally

1.0 Introduction

1. The mechanism by which the group of chemicals referred to as 'endocrine disruptors' exert their phenotypic effects remains unknown, but there is emerging evidence that dysregulation of the cell's epigenome is involved. In the last decade, it has become clear that the emerging field of epigenetics is of significant relevance for both the study and practice of toxicology and safety assessment. At the research level, these efforts currently aim to elucidate the involvement of chemical-induced epigenetic changes in adverse health effects, as well as to the exploitation of epigenetics particularly in the area of *in vitro* and *in vivo* modeling. This chapter reviews our current understanding of the intersection of these two fields of research and proposes avenues of exploration encompassing epigenetic information that will form the foundation for definitive testing of this relationship and provide a basis for future practical applications for regulatory safety assessment.

2. How to eventually incorporate the understanding of these mechanisms into the OECD chemical safety assessment regulatory activities, in the ED conceptual framework screening, priority and definitive testing levels, is the major challenge and objective that this chapter will begin to explore and address.

2.0 Definitions.

2.1 The epigenome.

3. The word epigenome is derived from epigenetics, a term attributed to Waddington (Waddington, 1942) who defined it as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being". Waddington was looking for an explanation of how the same genome could be used to generate different cell types in multicellular organisms, suggesting a higher level of regulation acting on non-autonomous genes. The term epigenetic was resurrected more recently as a broad description of heritable processes that do not depend on changes in DNA sequence, to include phenomena such as genomic imprinting and X chromosome inactivation. In each of these examples, a locus on one of two chromosomes identical in terms of DNA sequence is silenced, with the other active, a state that remains stable from parent to daughter cells, thus the heritability component.

4. Some of the molecular mechanisms implicated in allelic silencing included methylation of DNA (Sapienza et al., 1989), histone modifications and variant deposition (Delaval and Feil, 2004), DNA replication timing (LaSalle and Lalande, 1996), antisense and non-coding RNA transcription (Whitehead et al., 2009) among others (see below). Of these, only DNA

methylation had a demonstrable biochemical mechanism for parent to daughter cell propagation of its regulatory message (maintenance DNA methyltransferase, DNMT1 (Goyal et al., 2006)), making DNA methylation the standard bearer for an epigenetic regulator, but this is mostly because of a current dearth of knowledge about how other mechanisms may be heritable, which may in time be revealed but at present prove elusive.

5. "Epigenome" represents the collective noun to describe the sum of the epigenetic modifications throughout the genome. This is where the common use of the term deviates from the strict definition, as the term describes molecular mediators and not heritable influences on cellular properties. As such, the term describes a broad group of transcriptional regulatory processes, of which only DNA methylation is demonstrably heritable. This incorrect use of the term is, however, useful, as there is no other obvious term that describes the broad group of transcriptional regulatory processes including chromatin and DNA properties that gets across the idea that some of these properties may mediate a cellular memory.

6. Acknowledging that the use of the term epigenome or epigenomic is inherently flawed in terms of its origins, this chapter will utilise the term as commonly and incorrectly used to describe the full spectrum of transcriptional regulatory processes that appear to mediate environmental influences and change a cellular state to reflect past exposures.

2.2 Epigenomic regulatory mechanisms

7. The molecular mechanisms believed to mediate epigenetic and transcriptional regulation are diverse (summarized in **Table 1**).

Table 1: Examples of molecular regulators of the epigenome.

Molecular mediator	Example	References
Histone post-translational modifications	Histone H3 lysine 9 trimethylation (H3K9me3), a repressive mark	(Hiragami-Hamada et al., 2009)
Histone variants	Histone Macro H2A.1	(Bernstein et al., 2008)
Nucleosome positioning	Nucleosome-free regions at gene promoters	(Hartley and Madhani, 2009)
Chromatin looping	*Kit* regulation by Gata1/Gata2	(Jing et al., 2008)
DNA modifications	Cytosine methylation	(Klose and Bird, 2006)
DNA structural variation	R-loop formation	(Roy et al., 2008)
RNA-mediated	Antisense RNA transcription	(Beiter et al., 2009)

8. What these regulators have in common is a lack of innate DNA sequence specificity (with the possible exception of certain DNA methyltransferases which may preferentially target certain CG dinucleotide periodicities (Jia et al., 2007)). To exert sequence-specific events, it is likely that transcription factors and other DNA-binding proteins with sequence preferences help to recruit modifying enzyme complexes (Beckerman and Prives, 2010), one of the ways that the boundary between transcriptional and epigenetic regulators blurs in terms of functions. Another

source of sequence-specificity may be the endogeneous short interfering RNAs (siRNAs) that have been found to induce heterochromatinisation in plants and yeast (Pikaard, 2006; Zofall and Grewal, 2006), although there is little evidence for such mechanisms in mammalian cells at present (Kim et al., 2006; Morris et al., 2004).

9. As mentioned earlier, only DNA methylation has a molecular mechanism defined that allows it to act in a heritable manner from parent to daughter cells. The DNA methyltransferase 1 enzyme (DNMT1) has the ability to recognize (with UHRF1) loci where a symmetrically-methylated CG dinucleotide (methylation on both the Watson and Crick strands) becomes hemi-methylated following DNA replication (which introduces an unmethylated cytosine when creating the new complementary strands of DNA) and restores the locus to symmetrical methylation. This maintenance methyltransferase function allows a methylation mark in a parent cell to be maintained in both daughter cells.

10. The stability of other putative epigenetic regulators in populations of growing cells suggests that they can also maintain themselves in a site-specific manner through DNA replication, potentially through the association of enzymes with chromatin through DNA replication, as demonstrated using an *in vitro* system (Francis et al., 2009). RNA-mediated effects such as paramutation, best described in plants (Chandler, 2007), have been observed in mice (Rassoulzadegan et al., 2006), although it is unclear how RNA molecules can self-replicate in mammals which appear to lack the RNA-dependent RNA polymerase needed for paramutation in plants (Alleman et al., 2006).

11. While the molecular basis for the maintenance of epigenomic marks at a locus in dividing or post-mitotic cells remains largely unknown, the stability of these marks is well-recognised and suggests that the failure to find maintenance mechanisms does not mean that they do not exist but that they are eluding our scrutiny.

2.3 Influences exerted by epigenomic regulatory mechanisms.

12. The primary means by which the genome communicates its information is through transcription, so it should not be surprising that the major outcome of epigenomic regulators is usually viewed as gene expression. When histone post-translational modifications are referred to as active or repressive marks, it is in terms of gene expression locally.

13. It becomes more complicated – the relationship of a chromatin mark with a gene activity is also dependent on the genomic context of that mark. For example, the histone H3 lysine 9 trimethylation (H3K9me3) mark is recognized as a repressive mark, associated with heterochromatin on a cytological scale (Peters et al., 2002) and with gene silencing when present at a promoter. However, the same modification is found to be enriched in the bodies of actively-transcribed genes (Vakoc et al., 2005), the opposite correlation. The same has been found for DNA methylation, increased at promoters of silent genes but also increased in the transcribed bodies of highly-expressed genes (Ball et al., 2009). This contextual information is important when defining relationships of epigenomic regulatory marks and transcription.

14. Epigenomic regulation has also been associated with other genomic properties. DNA replication occurs at different times in the cell cycle in different genomic regions, with specific patterns of timing defining some regions as early and others late-replicating. Even at the cytological scale it is apparent that silencing marks are enriched at later-replicating regions and *vice versa*. Meiotic recombination in humans has been linked to germline DNA methylation

patterns (Sigurdsson et al., 2009), and has been more precisely mapped to areas of open chromatin in yeast (Kauppi et al., 2004). Decreased global DNA methylation in mammalian cells has been linked causally to chromosomal instability (Karpf and Matsui, 2005), while mutations of the *DNMT3B* maintenance DNA methyltransferase causes distinctive chromosomal morphological abnormalities (Hansen et al., 1999). The highly abnormal nuclear morphology of B lymphocytes infected with Epstein-Barr virus reflects a profound disturbance of DNA methylation globally in these cells (Grafodatskaya et al., 2010), indicating that even cytological-scale morphology has regulatory input by these epigenomic mediators.

15. Recently there has been an unexpected relationship revealed between chromatin organization (Vakoc et al., 2005) or DNA methylation (Laurent et al., 2010) and the exonic organization of genes. This is unexpected because at the stage of generation of the primary transcript the gene might be expected to be agnostic regarding where splicing is occurring, an event that occurs distantly from the gene within the nucleus, an assumption being refined in recent years (Schwartz and Ast, 2010). In spite of this, the patterns of nucleosomal positioning (Tilgner et al., 2009) and DNA methylation observed at intron/exon boundaries have been shown to be distinctive (Laurent et al., 2010). This raises the possibility that epigenomic regulators could be influencing splice isoform choices made in a cell type, which could have significant functional consequences for the cell. This relationship has yet to be proven rigourously, but represents an intriguing avenue of exploration.

2.4 Large-scale studies of the epigenome.

16. With the large number of regulators involved, each causing potentially different organization not only in the several hundred cell types within the body but also in the same cell types over time and in different sexes (Fraga et al., 2005; Thompson et al., 2010a), it is clear that there is a very large number of potential epigenomes for each organism. As a further complicating factor, we do not understand how to interpret many of the regulatory marks in different genomic contexts, so that even if we could catalogue epigenomes, understanding their meaning would remain difficult.

17. With these issues in mind, there are several large-scale initiatives to study epigenomic organization. The ENCyclopedia Of DNA Elements (ENCODE) is a project focused on understanding the function of non-coding DNA sequences in the genome, starting originally with transformed human cell lines and expanding through the modENCODE project to include primary cells from model organisms. This project has involved technology development, a lot of mapping, and insights through the development of new, sophisticated analytical approaches. This created a foundation for the Roadmap in Epigenomics, which was set up to differ in terms of a focus on primary, non-diseased human cell types, but also includes technology development and analytical aspects. The Cancer Genome Atlas (TCGA) represents another substantial project that includes an epigenomic component, but the focus in this case is not solely the epigenome. Finally the International Human Epigenome Consortium (IHEC) is in an early stage of development but plans to bring the Roadmap in Epigenomics concept a step further by looking specifically at human diseases. **Table 2** lists these initiatives and web-based resources for the reader to explore further.

Table 2: Large-scale studies studying epigenomic organization.

Project	Abbreviation	Web resources
ENCyclopedia Of DNA Elements	ENCODE, modENCODE	http://www.genome.gov/10005107 http://genome.ucsc.edu/ENCODE/ http://www.modencode.org/ http://www.genome.gov/modencode/
Roadmap in Epigenomics		http://www.roadmapepigenomics.org/ http://www.epigenomebrowser.org/ http://www.ncbi.nlm.nih.gov/epigenomics
The Cancer Genome Atlas	TCGA	http://www.genome.gov/17516564 http://cancergenome.nih.gov/ http://tcga-data.nci.nih.gov/tcga
International Human Epigenome Consortium	IHEC	http://www.ihec-epigenomes.org/

18. These projects are now productive and provide insights into how epigenomes are organized, and how epigenomic information interacts with genetic polymorphism (Kasowski et al., 2010; McDaniell et al., 2010). While insights into the epigenomic organisation of a specific human cell type can be gained from the Roadmap project, it should be stressed that these studies tend to be deep (many assays performed on a single cell sample) rather than broad (testing many cell samples), and there are relatively few metadata captured about the donors, making these data unsuitable for most human disease or exposure studies.

2.5 Genome-wide assays: the transition from microarrays to massively-parallel sequencing.

19. When performing a genome-wide study, in essence they involve enriching a fraction of the nucleic acid in the cell and determining where in the genome the nucleic acid came from. Gene expression microarrays represent a well-known paradigm for genome-wide assays. To perform these studies, RNA from the cell is isolated and hybridized to short DNA sequences immobilized on a glass slide. These short DNA sequences are designed to represent each gene in the genome. The RNA is converted to DNA and labeled with fluorescent molecules, so that the presence of a specific gene's RNA in the pool isolated from the cell sample will cause the short DNA sequence on the glass slide to acquire a fluorescent signal proportional to the amount of RNA labeled. As the location of the DNA sequences on the slide are pre-defined, scanning the slide to look at relative fluorescence of each DNA sequence location can be converted to a gene expression measure for each gene represented. Chromatin immunoprecipitation (ChIP) can likewise be performed with microarrays, but the starting material differs (immunoprecipitated DNA) and the DNA sequences on the slide also differ (representing regions of interest like gene promoters, for example),. Massively-parallel sequencing differs by taking the RNA or immunoprecipitated DNA and performing sequencing of the molecules, so that relative enrichment of a certain gene's expression or a chromatin component is measured not by fluorescence intensity but the relative amount of sequence mapped to a specific gene or location.

20. Microarray technology matured years before massively-parallel sequencing (MPS) and remains a significant means of investigation of the epigenome and transcriptome. Microarrays have some problems, both technical and financial. From a technical perspective, we have

noted that the signal/noise discrimination and dynamic range of signal associated with MPS-based detection greatly exceeds that of identical assays performed using microarrays (Suzuki et al., 2010). From a cost perspective, the price per unit of DNA sequence length is now much less for MPS, but an advantage still retained by microarrays is the ability to study only a limited subset of the genome, which still makes such studies more affordable for microarrays, although the rationale for such limited studies is decreasingly warranted. Furthermore, the data characteristics from MPS-based assays are substantially different from those generated by microarrays – the sequence information allows allelic discrimination, alternative splicing detection, nucleotide resolution DNA methylation studies, and information from as yet unsequenced regions of the genome, making MPS data potentially of even greater value with time.

21. All massively-parallel sequencing technologies to date involve the sequential addition of nucleotides to immobilised target DNA sequences, detecting the events usually through distinctive fluorescence signals and light microscopy but more recently also through hydrogen ion release (http://www.iontorrent.com/). The technologies thus far involve a tradeoff between shorter (≤200 bp) sequences but more of them (hundreds of millions) per machine run, or the opposite, longer (≥500 bp) but fewer (≤1 million) sequences per run. The trend of sequencing technologies is towards continued rapidly growing capacity, with decreasing costs per unit length of DNA sequence, with the oft-stated benchmark goal of a $1,000 genome (Mardis, 2006). The use of MPS is likely to continue to expand beyond even that of today, leading to profound new insights but also the data challenges summarized later in **section 9**.

2.6 The problem of choice in epigenomic assays.

22. When cells with an identical genome are compared for epigenomic differences following exposure to different toxins, it is apparent even from **Table 1** that there are many potential mediators of epigenomic organization, and frequently no indication which one that can be assumed to be informative *a priori*. If anything, **Table 1** vastly oversimplifies the problem – histone H3 lysine 9 trimethylation (H3K9me3) is only one of hundreds of post-translational modifications of the canonical core histones (H2A, H2B, H3 and H4) (Bannister and Kouzarides, 2011), before the many on histone variants are considered, the positioning of the nucleosomes they assemble, the influence DNA methylation of the DNA they package, and so on. It is therefore extremely difficult to choose the most appropriate assay for a given question, and the cost and cell quantities required for these studies remain sufficiently substantial that a scattershot approach is not an option.

23. In practice, the choice is often constrained to a focus on DNA methylation studies for a number of reasons. There is a more general familiarity with the assays involved, the sample requirements are generally less onerous (in terms of quantity and preparation) than for RNA or chromatin-focused studies, and the assays are demonstrably quantitative, something that has yet to be shown for ChIP followed by MPS (ChIP-seq), an important issue discussed later in **section 5**.

24. A significant problem with DNA methylation is that we don't really know how to interpret many of the observed non-promoter changes, and the correlation of DNA methylation with local gene expression changes is far from straightforward. This kind of consideration has kept ChIP-seq of major interest to researchers of human disease, prompting attempts to miniaturise the assay in terms of sample requirements (Adli et al., 2010). Furthermore, as many chromatin components with regulatory associations appear to have redundancy in terms of genomic

location and transcriptional function, it appears that it may not be necessary to survey all possible chromatin marks. This hypothesis was tested as part of the ENCODE project (Ernst and Kellis, 2010). They found that certain combinations of chromatin marks or constituents were able to predict regulatory function, and that much of this information could be captured by a subset of the 41 that they tested. This indicates a means by which we may be able to make some informed choices about how to study this large number of regulators when performing epigenomic studies.

3.0 Potential effects.

25. The obvious pathogenetic mechanism for endocrine disruptors is through their action on their cognate sex steroid receptors. This is enough to account for many of the effects of these agents on animal phenotypes, including male fertility (Guerrero-Bosagna and Skinner, 2009), anatomical structures and histological organization of male reproductive organs (Svechnikov et al., 2010), ovarian development (reviewed in (Uzumcu and Zachow, 2007)) and female reproductive tract cancer (Ma, 2009). This accounts for much of the recognized phenotypic spectrum of endocrine disruptors, prompting the question whether the epigenome is worth studying at all?

26. The reason for interest in the role of the epigenome is based on several observations. Firstly, as will be described next (**section 4**), there is emerging evidence that sex steroid receptor activity exerts consequential effects by means of some of the epigenomic and transcriptional regulatory processes outlined earlier. Secondly, the field of endocrine disruptor effects is notable for transgenerational consequences – a risk of disease in the unexposed progeny of exposed parents. When this kind of cellular memory event occurs, the obvious question is how such a memory is mediated at the molecular level. The epigenome has properties as described earlier (**section 2**) that allow it to be considered as a candidate for mediating such long-term memory mechanisms. Thirdly, there are now several studies that link known endocrine disruptors to effects on the epigenome (**section 4**) that offer more direct evidence for mechanistic associations.

27. A model for conceptualising the mechanism by which endocrine disruptors exert their effects might be proposed as follows. Sex steroid hormones have their effects mediated in part through epigenomic and transcriptional regulators. This induces long-lasting changes in cellular states that we recognize to be due to normal sex hormone exposure. The long-term maintenance of these new cellular states relies in part upon epigenomic reorganization. The exposure to endocrine disruptors causes similar or distinct effects on cellular states, again mediated in part by epigenomic reorganization. This epigenomic reorganization is not the same as that mediated by endogeneous sex steroids, in terms of timing and perhaps the type of epigenomic changes themselves. There is furthermore a possibility that the epigenomic changes induced by endocrine disruptors are unusually stable, long-lived, and widespread enough in terms of target cell types that gametes become involved and mediate transgenerational inheritance of these changes, with phenotypic consequences.

28. This hypothesis is based upon the observations of the next two sections, but it should be stressed that direct evidence, especially in humans, remains only partial. As a consequence, while we have sufficient evidence to be concerned about the epigenome mediating pathogenic effects of endocrine disruptors, we lack definitive proof that this is the sole or even major means by which these environmental agents cause human disease consequences.

4.0 Evidence for endocrine disruption being mediated by epigenomic processes.

29. The relationship between the epigenome and epigenetic regulation has mostly been studied in terms of how genes involved in endocrine signaling are themselves regulated by epigenetic processes such as DNA methylation. This has been reviewed comprehensively (Zhang and Ho, 2011) and allows the data of **Table 3** to be presented as a summary of the state of this field.

TABLE 3: Endocrine genes regulated by DNA methylation (from (Zhang and Ho, 2011)).

Gene name	**Gene symbol**
P450scc	*CYP11A1*
3ß-hydroxysteroid dehydrogenase	*HSD3B1/2*
17α-hydroxylase	*CYP17A1*
17ß-hydroxylase	*HSD17B3*
Vitamin D synthesis	*CYP27A1/B1*
Androgen receptor	*AR*
Oestrogen receptor 1	*ESR1*
Oestrogen receptor 2	*ESR2*
Progesterone receptor	*PGR*
Glucocorticoid receptor	*NR3C1*
Mineralocorticoid receptor	*NR3C2*
Retinoic acid receptor α	*RARA*
Retinoic acid receptor ß	*RARB*
Somatostatin	*SST*
Vasopressin	*VAP*
Melanocyte-stimulating hormone	*POMC*
Secretin	*SCT*
Insulin	*INS*
Leptin	*LEP/OB*
Oxytocin	*OXTR*
Follicle stimulating hormone	*FSHR*
Thyroid stimulating hormone	*TSHR*
Insulin-like growth factor 2	*IGF1R/IGF2R*

30. The more relevant process from the perspective of this review is the effect that endogenous hormones have on the epigenome, so that we can understand how endocrine disruptors may exert their effects. This has also been studied reasonably comprehensively and has been reviewed in detail recently (LeBaron et al., 2010; Vandegehuchte and Janssen, 2011; Zhang and Ho, 2011). A major reason for considering a link between endocrine disruptors and the epigenome is because the action of certain hormones is mediated in part through epigenetic regulators. Retinoic acid, steroid hormones, calcitriol and thyroid hormone bind to nuclear receptors within the cell and enter the nucleus to bind as a complex to hormone-responsive elements (Evans, 1988). For example, when thyroid hormone binds to a response element, the nuclear receptor can do so on its own, in which case it appears to act as a transcriptional repressor, recruiting a multi-protein complex that includes histone deacetylase and SIN3A, whereas if the thyroid hormone receptor binds as a heterodimer with another nuclear receptors such as retinoid X receptors it activates transcription by recruiting the histone acetyltransferases

PCAF and CBP (Zhang and Lazar, 2000). This example represents a much broader picture, with almost 300 nuclear receptor co-regulators now documented (O'Malley et al., 2008) and multiple examples of ligand-dependent effects to activate or repress transcription (Kato et al., 2011). Genes that represent specific targets of oestrogen receptor alpha have been identified using the chromatin immunoprecipitation approach (Jin et al., 2004; Lin et al., 2007), allowing insight into the downstream effectors of hormonal signaling.

31. While the regulation of chromatin organization is part of the mechanism for rapid activation or silencing of gene expression, it was described earlier (**section 2.2**) how the same mediators can propagate their patterns of activity to daughter cells, allowing them to play a role in mediating cellular memory and permanent changes in cellular states such as differentiation or reprogramming. Why chromatin organization proceeds from a dynamic, reversible state to one that is stable and irreversible is not known, although it is likely that this is a common decision within differentiating cells during development. The epigenetic changes we observe associated with diseases may represent these decisions being made in an abnormal manner. One paradigm of note is intrauterine growth restriction (IUGR), which has been observed in humans and mammalian model organisms to increase the risk of the affected individual to develop obesity and type 2 diabetes mellitus in adulthood, which in the case of humans is decades subsequent to the causative environmental event (Simmons, 2008). When our group studied a rat model of IUGR and quantified cytosine methylation throughout the genome in beta islet cells from the pancreas of young adult rats, we found a distinct pattern of methylation discriminating the animals that had undergone IUGR, at loci already implicated in glucose metabolism or type 2 diabetes mellitus (Thompson et al., 2010b). It has been proposed that IUGR induces an adaptive response to the scarcity of calories *in utero*, causing the foetus to reprogram its metabolism during development to hoard calories, which becomes a maladaptive behavior postnatally in the presence of adequate nutrition (Gluckman and Hanson, 2004). This represents a paradigm for a remote event causing epigenetic changes that confer a cellular memory of phenotypic consequence. Such a model of epigenetically-mediated changes conferring cellular memory appears to be worth considering for normal endocrine processes and for abnormal hormonal exposures such as those from endocrine disruptors.

32. There are examples of endogenous hormones and endocrine disruptors having effects mediated by different epigenetic and transcriptional regulatory processes. The endocrine disruptor bisphenol A has been a major focus of investigation for some time, given its broad exposure within the population (Calafat et al., 2008) and the observed effects in animal models on the development of breast and prostate (reviewed in (Weng et al., 2010)). Interestingly, there is little published to demonstrate epigenome-wide effects of bisphenol A, with several reports focusing on individual loci (Bromer et al., 2010; Dolinoy et al., 2007; Weng et al., 2010), and two relatively limited genome-wide studies of cytosine methylation in mice, one using Restriction Landmark Genomic Scanning (RLGS) technique on DNA from mouse forebrain (Yaoi et al., 2008), the other testing prostate tissue using Methylation-Sensitive Restriction Fingerprinting (MSRF) (Ho et al., 2006), both of which are based on gel electrophoresis and are relatively limited in their genomic comprehensiveness. Despite this, these studies all showed changes in cytosine methylation associated with exposure, some changes occurring at loci that were found to be transcriptionally altered. While these studies have established a foundation for more detailed and sensitive investigation of effects on cytosine methylation, despite the availability of genome-wide methylation assays for some time (Zilberman and Henikoff, 2007) these studies have yet to be published.

33. Exposure to endocrine disruptors other than bisphenol A has also been found to be associated with epigenetic changes. Pregnant rats were exposed to high doses (100-200

mg/kg/day) of the oestrogenic methoxychlor or the androgenic vinclozolin endocrine disruptors between embryonic days 8-15, and spermatogenesis was observed to be abnormal and compromised in several generations of males subsequently in the absence of subsequent exposures (Anway et al., 2005). This transgenerational inheritance of the phenotype suggested an epigenetic mechanism, tested by performing cytosine methylation analyses on testes, again using methylation-sensitive restriction enzymes and a gel electrophoresis detection step. Changes in methylation were indicated by these studies, and while sodium bisulphite validation (see **section 11.3**) was described the primary data were not presented in that original study (Anway et al., 2005). Another study of methoxychlor exposure in rats used a methylation-sensitive restriction enzyme and gel electrophoresis approach with bisulphite PCR or sequencing to assess methylation at a few loci, finding modest changes in methylation levels (Zama and Uzumcu, 2009). Vinclozolin administered to mice allowed testing of the methylation status of several loci undergoing genomic imprinting (at which the paternal and maternal chromosomes have different epigenetic organization that results in parent of origin-dependent gene expression (Kacem and Feil, 2009). Bisulphite pyrosequencing at these loci showed very modest degrees of change of cytosine methylation (at most ~20%) associated with exposure (Stouder and Paoloni-Giacobino, 2010). The anti-androgenic di-2-(ethylhexyl) phthalate administered to gravid mice results in testicular function abnormalities in offspring, prompting testing of cytosine methylation for overall cytosine methylation levels using high-performance liquid chromatography and DNA methyltransferase expression studies. A global increase in cytosine methylation was observed in the exposed animals, with increases in DNA methyltransferase gene expression and protein levels (Wu et al., 2010). No locus-specific studies were performed in this project.

34. There has recently been published a report that links exposure to diethylstilbestrol or bisphenol A with increased expression of *EZH2*, a histone methyltransferase that generates the H3K27me3 repressive modification, when tested in MCF7 breast cancer cells *in vitro* (Doherty et al., 2010). The same study tested *in utero* exposure to these agents with the outcome of EZH2 expression in adult mammary gland in a mouse model, finding that both chemicals increased EZH2 protein levels and activity. No locus-specific studies were performed as part of this project, but the next logical step will be to perform H3K27me3 ChIP-seq to see whether this increased EZH2 activity results in new sites of repressive chromatin modifications.

35. As stressed earlier (**section 2.2**), epigenetic regulatory mechanisms are very numerous, and a focus solely on cytosine methylation is unlikely to be sensitive to all changes occurring in response to endocrine disruptor exposure. *In vitro* exposure of mammary epithelial cells to diethylstilbestrol was associated with changes in expression of microRNAs (Hsu et al., 2009). There have yet to be published any studies using genome-wide chromatin immunoprecipitation approaches, and while chromatin looping studies have been employed to test how oestrogen mediates its effects using cultured cells (Hsu et al., 2009), no comparable experiments have been described for endocrine disruptors.

5.0 Assay methods

5.1 Issues to address when considering an epigenomics study.

36. The studies described above include several approaches towards assessing the role of epigenetic dysregulation. The simplest approach is to perform a candidate gene study, in which one or more genes are chosen based on prior suspicion that they may be involved in the cellular phenotype, and epigenetic studies are performed usually targeting the transcriptional start site

(promoter) of the gene. Candidate genes are frequently chosen based on their functional properties or because they were found to change transcriptional levels by using gene expression microarrays. The advantages of this kind of approach are those of time and cost, and usually allow highly-quantitative approaches to be performed, at the expense of comprehensiveness and unbiased discovery.

37. As the comprehensiveness and quantitative capabilities of genome-wide assays improved while costs decreased, the focus has shifted towards what can be described as epigenome-wide association studies. Just as genome-wide association studies look for polymorphisms of DNA sequence that are non-randomly associated with disease phenotypes, epigenome-wide association studies aim to discover loci with changes in epigenetic regulation that occur preferentially in subjects with disease.

38. Technical approaches used for epigenome-wide association studies currently include those based on microarrays or on massively-parallel sequencing (reviewed in (Boyle and Furey, 2009)), largely superseding the gel-based detection systems described in the prior section. The field of endocrine disruptor biology could benefit from carefully-designed analyses of the epigenome using these updated approaches, especially in human subjects, so it is worth describing some of the challenges involved in performing these studies stringently.

39. First of all, epigenetic dysregulation events are believed to be somatic rather than constitutional, requiring that the cell type mediating the phenotype be sampled. It is possible that with an exposure event the epigenetic effects may be more widespread, allowing easily-accessible cell types to be sampled as a surrogate, but in general if there is a disease phenotype affecting a specific organ, it is presumed that cells from that organ should be sampled. This becomes a problem in human studies when the cell type is relatively inaccessible, and serves as a justification for the use of rodent or other animal models (**section 7**).

40. A further issue has to do with cell purity. Admixture of other cell types presents a challenge because the epigenotypes of histologically-distinctive cell types generally appear to be markedly different. If the proportion or type of cell admixture differs systematically between test and control groups, this may exert enough of an influence to confound the experiment, as the effect sizes (discussed below) may be small. Purifying the cells is not without problems either, as it reduces the sample amount to the point that we may not have sufficient starting material for the epigenomic assay.

41. We have also stressed that there are numerous possible regulators of the epigenome, which creates the problem of choice referred to earlier in **section 2.6**, which may be addressed by using an informative subset of chromatin marks (Ernst and Kellis, 2010). In practice, studies usually focus on cytosine methylation, largely because the samples are easily prepared as DNA compared with the more complex sample preparation required for chromatin immunoprecipitation-based assays and because of the relative stability of DNA compared with RNA. Cytosine methylation and transcriptional assays are also reasonably quantitative (Suzuki et al., 2010), whereas genome-wide chromatin immunoprecipitation assays have been described to be able to call the presence or absence of peaks but have not been shown to be able to discriminate intermediate values. This is a major concern limiting the use of chromatin immunoprecipitation, as the emerging literature indicates that in non-cancer disease states the differences in methylation at a locus tend to be moderate, our IUGR study finding values differing by as little as 10-20% (Thompson et al., 2010b), and a recent paper testing liver epigenomes of mice whose fathers were fed different diets showed a comparable value (Carone et al., 2010). Mechanistically, this is of interest, as cytosine methylation values in an individual

cell can be 0% (neither allele), 100% (both alleles) or 50% (one allele methylated), so the only way that there can be a 20% difference in methylation is when a subset of cells in the population changes its methylation status. This highlights how even modest proportions of contaminating cells can cause problems, as mentioned above, and imposes a requirement for assays to be quantitative as well as comprehensive when performing genome-wide studies.

42. If the effect size is limited, and the assay has a defined quantitative discriminatory capacity, the cohort sizes required for genuinely comprehensive studies can be modeled. We have determined that the use of the MSCC (Ball et al., 2009) or our HELP-tagging (Suzuki et al., 2010) assays will require 100 subjects in each of the test and control groups to be fully powered (unpublished data). While this represents substantially fewer subjects than generally required for genome-wide association studies, it greatly exceeds the numbers described in the studies of **section 4**. When amassing the samples, the cohorts should be chosen with care. It is now recognized that DNA sequence polymorphism can influence chromatin organization, causing it to be polymorphic between individuals (Birney et al., 2010; Kasowski et al., 2010; McDaniell et al., 2010). It is also notable that cytosine methylation appears to be influenced by age (Fraga et al., 2005; Thompson et al., 2010a) and sex (Sarter et al., 2005), combining to require that cohorts should be matched in terms of self-reported ethnicity, age and sex in order to reduce these potential sources of variability.

43. The need for comprehensively genome-wide assays arises because of the emerging evidence that epigenetic regulation of gene expression may not be occurring at predictable locations. There is now a substantial amount of information to suggest that *cis*-regulatory sequences in the genome are frequently located far from promoters (Heintzman et al., 2007), and that these loci may be preferentially involved in mediating disease states, as we found in our IUGR study (Thompson et al., 2010b). While microarray-based approaches have had to compromise to focus on pre-defined loci such as promoters or CpG islands (Hoque et al., 2008; Yamashita et al., 2009), massively-parallel sequencing-based approaches have no such constraints and can survey the entire genome. This gives rise to a problem of interpretability – while changes at a promoter are relatively easy to interpret in terms of likely effect on that gene's expression, the non-promoter changes may not even be regulating the nearest gene. It is hoped that the functional annotation of mammalian and model organism genomes being undertaken by the ENCODE and Roadmap in Epigenomics projects will provide some insights that will increase the interpretability of many of these loci, but in the interim many studies will generate significant loci in terms of disease associations without insight into how they may be having mechanistic effects. It is for this reason that concurrent transcriptional studies performed on the same samples offer a means of interpreting how an epigenetic regulatory change may be having functional consequences.

44. An impediment to these genome-wide studies has been costs, especially when the cohort sizes of several hundred individuals are required, and massively-parallel sequencing is employed. What is making these assays more affordable at present is the huge amount of sequence now being generated by massively-parallel sequencing, allowing many samples to be combined following barcoding of the individual samples using short sequence tags introduced during library preparation. This multiplexing of samples is driving costs down significantly, to the point that library preparation costs represent the major financial obstacle. With continued increases in sequencing performance, it should be anticipated that these massively-parallel sequencing-based assays will become increasingly cost-effective and will allow their widespread adoption for epigenome-wide association studies.

5.2 Designing an epigenome-wide association study of endocrine disruptors.

45. The discussions of **section 5** give us some guidelines about how we might go about searching for the effects of endocrine disruptors on the epigenome. As a first step we would need to determine which cell type to study. The options are more plentiful in animal models, whereas the cell types that represent hormonally-responsive tissues in humans tend to be difficult to acquire with the exception of spermatozoa, which may allow the effects of anti-androgenic endocrine disruptors to be evaluated. A comparable cell type that could be easily sampled in females to test the effect of anti-oestrogenic agents is not as obvious. Model organisms such as rodents would not have the same constraints, but have other problems with regard to how they reflect human toxin exposure (**section 7**).

46. The next question is which assay to choose. The effects of steroid hormones on the epigenome were summarized in **section 4** and point to numerous chromatin components (nuclear receptors, ligands, enzyme complexes) and modifications (histone acetylation) that are functionally linked to hormonal signaling and would be prime targets for analysis. The drawback of the genome-wide chromatin immunoprecipitation-based assays is their non-quantitative properties, which could be reflected by insensitivity of detection of changes at many loci in the genome where subpopulations of cells alter their epigenetic regulatory patterns. Cytosine methylation has been shown in many of the rodent models to be relatively informative, and genome-wide assays designed to test it are reasonably quantitative, making these a first choice system at present.

47. In addition to the quantitative analysis of the epigenetic regulators themselves, it is valuable to add a transcriptional study of the same cells, so that epigenetic changes can be interpreted in part by presumed effects on gene expression, an especially problematic issue for loci of unknown function.

48. If a preliminary evaluation of the possibility of epigenomic abnormalities is being sought, there are global molecular approaches that could be attempted. Genome-wide cytosine methylation can be tested a number of ways, using high-performance liquid chromatography as described earlier (Zhang et al., 2011), testing transposable elements like long or short interspersed nuclear elements (LINEs, SINEs) with bisulphite sequencing (Yang et al., 2004), or performing luminometric methylation analysis (LUMA) (Karimi et al., 2006), to name a few. If a more functional test is required, the viable yellow (A^{vy}) mouse model has characteristics that have caused it to be described as an ‘epigenetic biosensor (Dolinoy, 2008). The IAP transposable element that alters the coat colour phenotype in these animals appears to be unusually susceptible to influences that alter the epigenome, such as dietary influences in mice exposed to endocrine disruptors (Dolinoy et al., 2007), generating a readout in terms of coat colour which is easily recognizable, and allowing direct analyses of the IAP element in terms of its cytosine methylation as a more quantitative readout (Waterland and Jirtle, 2003).

49. Cell culture systems represent the mainstay of many of the current studies of endocrine disruptors effects upon the epigenome. A problem with cultured (Meissner et al., 2008) and transformed (Wild et al., 2010) cells is that they tend to be substantially modified in terms of their epigenetic organization compared with primary cells, making them poorly comparable with cells sampled from *in vivo* sources, but potentially useful if the culture conditions are kept identical between conditions being compared.

5.3 Is the use of model organisms necessary?

50. The decision-making process above has the effect of directing us towards the use of model organisms, primarily because of sample acquisition issues, but there are other factors to consider. The ability to control and monitor exposures with animal colonies kept in controlled conditions should exceed that possible for human subjects. The potentially confounding effect of genotypic polymorphism can be avoided by using inbred strains, and specific genetic backgrounds can be introduced experimentally. Live animals allow metabolism of agents to other active byproducts that is difficult to achieve using cultured cells. Cells *in vitro* are also prone to changes in their epigenetic patterns with culture (Allegrucci et al., 2007; Meissner et al., 2008). There are thus numerous advantages to the use of animal models.

51. One especially advantageous reason for using animal models is the ability to pursue the transgenerational effects of endocrine disruptors (Anway et al., 2005; Anway and Skinner, 2006; Crews et al., 2007; Guerrero-Bosagna and Skinner, 2009). This has already proven interesting in terms of studies of epigenetic organization in testes (Anway et al., 2005), and appears worth pursuing further. It is interesting that cytosine methylation changes have been observed in rat testes occurring sufficiently markedly and reproducibly that they could be detected as a specific effect of vinclozolin (Anway et al., 2005). This is a counter-intuitive result given what is known about cytosine methylation during spermatogenesis and development. The vinclozolin-induced changes in methylation would have to survive two massive waves of demethylation of DNA genome-wide, one occurring early during spermatogenesis (at the foetal stage, following the differentiation of primordial germ cells into early spermatogenic cells), and a second demethylation wave early in embryogenesis that affects the paternally (sperm)-derived haploid genome prior to the maternally-derived contribution, with two phases of remethylation during later spermatogenesis and at the time of implantation (Reik et al., 2001). There is precedent for epigenetic marks surviving these waves of global cytosine methylation changes, as imprinted loci appear to retain the memory of their gametic origin despite the early post-fertilisation demethylation event (Reik et al., 2001).

52. So while rodent models have limitations in how they represent human exposures (Stokes, 2004), due to many common mammalian factors on which current usage in chemical hazard and safety assessment is based, the information that can be obtained from them can perhaps be more extensive and better utilised to provide a practical and short-term means of obtaining additional insights into whether and how endocrine disruptors influence epigenomic organization *in vivo,* whilst not increasing animal usage. Ultimately the information yielded on epigenetic pathways should allow reductions in the use of animals, particularly as epigenetic endocrine pathways are clearly identified such that *in vitro* building block predictive assays can be constructed for chemical safety assessment purposes, on the basis of clearly identified transgenerational pathways *in vivo*.

5.4 What are the potential future advances facilitating new approaches?

53. There is reason for optimism regarding our ability to use technology more effectively to gain insights to the epigenomic effects of endocrine disruptors. This is largely based on the phenomenal pace at which massively-parallel sequencing is advancing in terms of increasing throughput and reducing costs, probably exceeding the Moore's law paradigm for the number of transistors that can be placed on an integrated circuit doubling every 2 years – recent experience suggests that sequencing costs per basepair are dropping at a substantially faster rate. This is going to make cytosine methylation assays more cost-effective, as mentioned

earlier, but also more quantitative, as we can move from limited sampling techniques based on restriction enzymes or reduced genomic representations and instead use shotgun bisulphite sequencing (BS-seq, MethylC-seq (Harris et al., 2010)), a substantially more powerful approach. Chromatin immunoprecipitation-based assays will not change in terms of resolution but it is possible that for transcription factor studies the extra depth of sequencing may allow more comprehensive data to be generated, whereas histone modification studies do not appear to benefit in the same way from greater depth.

54. The second area of advance will be in terms of interpretability of findings, highlighted earlier in terms of the non-promoter findings that we will uncover. We are already beginning to understand that transcribed sequences in the genome behave differently in terms of their epigenetic organization compared with non-transcribed sequences, with specific histone modifications (Vakoc et al., 2005) and paradoxically increased cytosine methylation (Ball et al., 2009), requiring that we treat these functionally-defined genomic contexts separately in order to be able to interpret results. A goal for many ongoing studies is to be able to define optimal methods for integrating different types of genome-wide data in order to be able to understand epigenomic and transcriptional regulation as a system, including the influences of DNA sequence polymorphism, advances that will greatly facilitate studies addressing specific questions such as the effect of endocrine disruptors upon the epigenome.

6.0 Challenges.

55. These advances do not come without cost. The amount of information generated by these increasingly comprehensive genome-wide assays is becoming the single biggest impediment to gaining insights into the underlying biology. The data need to be managed and secured as a first step, as all digital information can be easily lost through hardware failures unless steps are taken to maintain the system and store copies remotely. This amplifies the magnitude of the datasets, but allows data derived from precious samples to be maintained for subsequent analysis.

56. The analysis challenge is also substantial. Many analyses cannot be performed using standard desktop computing resources because the processing and storage requirements greatly exceed what they can provide. The analyses of these data require multiple steps, for each of which there are competing analytical approaches rather than universally-accepted standard algorithms. Likewise, quality assessment and control metrics are also heterogeneous and require substantial computational processing to generate meaningful results. The transformation of raw data (microarray fluorescence intensities, massively-parallel sequencing DNA sequence reads) into biological information allows very large initial datasets to be shrunk to relatively smaller and more manageable formats, but then the next challenge emerges of making sense of this information. Whether this involves comparing the results from that sample with those in a similar and a comparison cohort, or comparing the results against other genomic annotations, the end result is similar in that there need to be multiple datasets assembled in a single analysis. Again, this represents a computational challenge that is usually addressed by high-performance computing resources, with cloud computing as an emerging alternative or complement.

57. When integrating datasets, it becomes necessary to remove nuances about the data and transform information into genomic ‘objects’ (a categorization of epigenetic events by locus). It is not always apparent that the decisions made about how these kinds of transformations are performed reflect relatively subjective decisions, and these may not always be transparent or

well-founded. For example, it was earlier described that increased sequencing of chromatin immunoprecipitation of STAT1 defines increasing numbers of binding sites (Rozowsky et al., 2009), so the definition of the genomic objects of STAT1 binding sites is dependent upon the depth of sequencing performed, which may not be consistent from sample to sample or lab to lab. This issue reinforces the need for not only breadth in epigenomic studies (genome-wide comprehensiveness) but also depth (at specific loci). Another problem has to do with the assumption that different epigenomic events occurring at the same locus must mean that they are present in the same cell. This can only be confidently concluded when all or the vast majority of the cells have the epigenomic event, requiring in turn a quantitative capability for the assay used. If 50% of the cells are found to have cytosine methylation and 50% of the cells are found to have a specific histone modification at the same locus, it could be interpreted that the same cells have both events occurring, but it is also possible that none of the cells have the same event and that they are mutually exclusive. These are challenges inherent to epigenomic data integration that remain largely unsolved and will need to be a focus for the near future.

7.0 Conclusions and testing recommendations

7.1 Conclusions.

58. In conclusion, it is possible to state that the evidence thus far is highly-suggestive of a role for epigenomic dysregulation mediating the effects of exposures to endocrine disruptors. Mechanistically, it is plausible that the epigenome is responsible for some of the phenotypic consequences of these exposures. These conclusions need to be weighed against the relative weakness of the studies performed to date, which have neither been comprehensive nor quantitative, have frequently used *in vitro* tissue culture systems or have used mixed cell types from rodent models. There is a major paucity of human subject data at present, another reason for concern.

59. There are reasons for optimism regarding our abilities to perform well-designed, comprehensive and sensitive studies to test for epigenomic dysregulation following endocrine disruptor exposure. The absence of standardized assays and analytical approaches coupled with the challenges of managing and analyzing data represent impediments to progress, while we also recognize that there are drawbacks to *in vitro* cell culture systems, animal models and human studies, making no system ideal for these studies, although rodent models offer a lot of advantages in the short-term.

7.2 Testing recommendations.

60. In defining some testing recommendations, we are taking into account a number of influences. Firstly, we want to minimize animal use to the greatest extent possible, so we include cell culture systems that may be useful. Secondly, we want to be guided by prior experience in this field, including dosage regimens for *in vitro* and *in vivo* systems. We therefore summarise a wide range of studies of endocrine disruptor use with epigenetic consequences, in which details were provided that could be used to guide formal testing. The *in vitro* cell systems are listed in **Table 4**.

TABLE 4: Prior cell culture systems for testing the epigenetic effects of endocrine disruptors.

[More information on the specific endpoints studied (e.g. ER, AR, TR) will be included in the next version of the chapter]

Cell type	Agent	Dosage and schedule	Vehicle	Reference
MCF-7	DES	$5x10^{-6}$ - $5x10^{-8}$ for 48 hours	DMSO	(Doherty et al., 2010)
	BPA	$2.5x10^{-5}$ - $2.5x10^{-6}$ for 48 hours		
CV-1	DHT	10^{-8} M for 24 hours	DMSO:ethanol 1:1	(Chaturvedi et al., 2010)
	BCH DDT 2,4'-DDT 4,4'-DDT 2,4'-DDE 4,4'-DDE Procymidone Fenitrothion Vinclozolin Nitrofen Linuron Methoxychlor Difenoconazole Chlozolinate Metribuzin Tetramethrin	10^{-6} M for 24 hours		
Human mammospheres	17ß-estradiol	70 nM for 3 weeks	DMSO	(Hsu et al., 2009; Hsu et al., 2010)

DES: Diethylstilbestrol
DMSO: Dimethylsulfoxide
BPA: Bisphenol A
DHT: Dihydrotestosterone
BCH: Brominated flame retardant
DDT: Dichlorodiphenyltrichloroethane
DDE: Dichlorodiphenyldichloroethylene

TABLE 5: Prior animal systems for testing the epigenetic effects of endocrine disruptors.

Animal species and strain	Agent	Dosage and schedule	Vehicle	Tissue sampled, timing	Reference
Rat (Sprague-Dawley)	DEX	50 mg/kg PO PND 25, 60 or 65	Corn oil	Liver Up to 4 days following exposure	(Ronis et al., 2011)

Mouse (Swiss albino)	DDT	50 mg/kg/day in olive oil x 7 days PND 28-42, male	Olive oil	Liver Testis 1 day following last exposure	(Chaturvedi et al., 2010)
Rat (Fisher)	Methoxychlor Vinclozolin	100 or 200 mg/kg/day IP GD 8-15	DMSO	Testis Sperm PND 60 (F1-F4)	(Anway and Skinner, 2006)
Mouse (*a/a*)	BPA	50 mg/kg PO 2 weeks pre-mating, then throughout gestation/lactation Adult female	None	Tail Brain Liver Kidney PND 22	(Dolinoy et al., 2007)
Rat (Sprague-Dawley)	BPA 17ß-estradiol 3-benzoate	10 µg/kg 0.1 and 2500 µg/kg PND 1, 3, 5 SQ injections	Corn oil	Prostate 28 weeks	(Ho et al., 2006)
Mouse (FVB)	Vinclozolin	50 mg/kg/day IP GD 10-18	Corn oil	Motile sperm Tail Liver Skeletal muscle PND 60 (F1 - F2)	(Stouder and Paoloni-Giacobino, 2010)
Mouse (Kunming)	Di-2-(ethylhexyl) phthalate	500 mg/kg/day PO GD 12.5 –19	Corn oil	Testes GD19	(Wu et al., 2010)
Mouse (CD-1)	DES BPA	5 mg/kg/day IP GD 9-26 10 µg/kg/day IP GD 9-26	Sesame oil	Mammary 6 weeks	(Doherty et al., 2010)

DEX: Dexamethasone
PO: *Per orem*, by mouth
PND: Postnatal day
GD: Gestational day
IP: Intraperitoneally
BPA: Bisphenol A
DES: Diethylstilbestrol

61. Note that only one animal study performed pharmacokinetic analyses, Doherty *et al.* measuring BPA levels in mice on gestational day 13, at 1, 6, 12 and 18 hours after the last dose of BPA. They found peak levels of BPA of 24.69 ng/mL at 1 hour following administration, when control (vehicle-only) levels were 1.70ng/mL. At 6 hours the BPA levels were 3 ng/mL, and subsequently indistinguishable from background (Doherty et al., 2010). It will be necessary to incorporate information from other studies that do not have epigenetic components for their more detailed pharmacokinetic data, such as the recent report from Prins *et al.* (Prins et al., 2011) testing BPA doses in Sprague-Dawley rats.

62. We therefore have some recommendations for testing, based on existing test systems used by the OECD. We list them by broad category below. A point worth mentioning is that the essence of epigenomic dysregulation is the potential for longer-term memory of exposure, making a delay between exposure and effect testing desirable, in contrast with many other outcomes that may be sought.

TABLE 6: OECD tests that could potentially be adapted for epigenomic studies of effects of endocrine disruptors.

Type of study	*Test Guidelines (TG)*	*Description*
***In vitro* systems**	• TG 473	• *In vitro* Mammalian Chromosome Aberration Test
General exposure studies	• TG 451 • TG 452 • TG 453	• Carcinogenicity Studies • Chronic Toxicity Studies • Combined Chronic Toxicity/Carcinogenicity Studies
Post-mitotic cell studies	• TG 424	• Neurotoxicity Study in Rodents
Pre-natal effects	• TG 414 • TG 426	• Prenatal Development Toxicity Study • Developmental Neurotoxicity Study
Transgenerational effects	• TGs 415, 416 • TG 421 • TG 422 • TG 443	• One- and Two-Generation Reproduction Toxicity • Reproduction/Developmental Toxicity Screening Test • Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test • Extended One-Generation Test

7.2.1 Choice of tests.

63. Cultured cells should be the first focus for potential tests, as they will allow animal use to be limited. A modification of TG 473 (*In vitro* Mammalian Chromosome Aberration Test) that left out the use of metaphase-arresting substances in exposed cells could be used to screen for epigenetic effects. We also describe in **section 7.3** how human embryonic stem cells may represent a new reagent for *in vitro* cultured cell studies of chemical toxicity. Where definitive animal tests are necessary, there are three tests that appear suitable for testing epigenetic effects of exposures throughout the body. TGs 451 (Carcinogenicity Studies), 452 (Chronic Toxicity Studies) and 453 (Combined Chronic Toxicity/Carcinogenicity Studies) all involve animal exposures by different routes, with a necropsy subsequent to the exposure schedule that would allow the opportunity for tissue harvesting. Correlative histopathology and clinical chemistry studies will allow some epigenetic findings to be interpreted. The cell types to be tested should meet the criteria of **section 5** in terms of purity and phenotypic relevance, in the current case choosing cells that are hormonally-responsive. TG 424 (Neurotoxicity Study in Rodents) focuses more specifically on the central nervous system, composed mostly of post-mitotic cells, with studies of brain function to complement histopathology and epigenetic studies. Prenatal effects are potentially studied using TG 414 (Prenatal Development Toxicity Study) which involves the exposure to animals of agents during pregnancy, testing the foetus at term for abnormalities, while TG 426 (Developmental Neurotoxicity Study) allows the offspring to be

born and to develop, testing specifically for neurological consequences. Tissues harvested at both timepoints could shed light on epigenetic effects of agents used for exposure.

64. One concern about endocrine disruptors is that they may have lingering transgenerational effects mediated by the epigenome. A genuinely transgenerational study requires looking as far as the F3 generation (Skinner, 2008). Current test systems only proceed to the F2 generation, so available test systems are not going to be definitive in testing for transgenerational effects, and will at best generate indicative, preliminary insights. There exist five tests that may allow such preliminary testing for transgenerational effects mediated by epigenetic dysregulation. TGs 415 and 416 (One- and Two-Generation Reproduction Toxicity), TG 421 (Reproduction/Developmental Toxicity Screening Test) and TG 422 (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) all involve exposures followed by assessments of reproductive capacity and/or gonadal histology and function. Of these, the two-generation TG (416) could allow multiple tissues to be sampled in offspring of parents exposed to the agent of interest, allowing screening for inherited epimutations. The new Extended One-Generation Reproductive Toxicity Study (TG 443) has the potential for the necropsy and neurological studies of the tests described in the previous paragraph, and therefore represents our recommended mainstay for animal testing.

7.2.2 Specific examples of tests.

65. Cultured cells will be exposed to chemicals to look for toxicological effects on those cells. Epigenomic studies can use the same test parameters (*e.g.* dose levels) as those that led to toxicological effects in previous studies, with the caveat that the cells should be assessed for viability, as the presence of dead cells in substantial proportions (>10%) in the material assayed could cause artefacts, requiring that viable cells be sorted from the dead cells (*e.g.* using propidium iodide and flow sorting). The cells will need to be fixed with formaldehyde soon after harvesting if chromatin immunoprecipitation is a planned assay, whereas flash freezing and/or the use of RNAlater (Qiagen) can be used to preserve RNA for later expression studies. Flash freezing is sufficient for preservation of DNA for later cytosine methylation analyses. Samples should be stored at -20^{o}C until ready for use.

66. Epigenomic assays should initially be performed on at least 10 exposed and 10 non-exposed samples, allowing the presence and degree of epigenomic dysregulation to be assessed, allowing a decision to be made about whether to (a) proceed with the number of further samples defined by the effect sizes and power calculations estimated on the basis of the first groups, (b) perform single-locus validation on loci appearing to be non-randomly altering their epigenetic regulatory patterns in response to chemical exposure, expanding the numbers beyond the initial limited groups.

67. Should animal systems be required, the exposures should be those that (a) reproducibly induce the associated phenotypic effect in that animal and, ideally, (b) are comparable to any exposure described for humans. It may be necessary to perform pharmacokinetic profiling in the animal system if human exposures are described in terms of measured concentrations of the chemicals in blood or other body samples. For example, serum levels of bisphenol A were measured in cohorts of women with and without histories of recurrent miscarriages, revealing the former group to have mean±SD values of 2.59±5.23ng/mL with the controls averaging 0.77±0.38ng/mL, levels comparable to those that can be generated in mice (Doherty et al., 2010). A bisphenol A study could therefore be guided by prior studies (Doherty et al., 2010) with a dosage schedule of 10 µg/kg/day IP in sesame oil administered to gravid mice between gestational days 9-26, testing cells from offspring at 6 weeks of age.

68. Cell samples should be collected from (a) the phenotypically-affected organ(s) (b) to a reasonable degree of purity, as discussed in **section 5.1**. For example, motile sperm collected from mice following vinclozolin exposure (Stouder and Paoloni-Giacobino, 2010) represents an homogenous cell population manifesting the phenotypic effect of the chemical, meeting these criteria. It is probably worthwhile sampling more cell types than are obviously necessary at the outset, as this will allow future studies to be performed without the need to use more experimental animals.

7.3 Potential new test systems.

69. While there are many ways that current OECD test systems can be adapted quite easily for analysis of epigenetic dysregulation, it is worth considering a couple more possibilities that may add utility. The viable yellow (A^{vy}) mouse model was described earlier (**section 6**), allowing screening for effects of exposures during pregnancy by the use of coat colour or cytosine methylation analysis of the IAP element (Waterland and Jirtle, 2003). This is not the only useful mouse resource, however. The Axin 1 fused ($Axin1^{Fu}$) mice also have a visible phenotype that is responsive to influences perturbing the epigenome (Waterland et al., 2006). Mice have been described that variegate transgenes expressed in peripheral blood, allowing genetic screens to look for mediators of the variegation phenotype (Ashe et al., 2008), a system that may be amenable to testing for epigenetic regulatory polymorphism. In each case the animals could be used as a means of screening for epigenetic disturbances, without the need for genome-wide molecular assays at the outset. Set against this is the lack of insight into how sensitive each mouse system is in reporting diverse influences on the epigenome, making it uncertain how valuable these experimental animals are for screening purposes.

70. The other possible avenue involves the use of embryonic stem (ES) cells that are *in vitro*-differentiated to the germ cell lineage, which is now technically feasible (Rohwedel et al., 2001), allowing a cell culture model that may be able to recapitulate the effects of *in vivo* exposures. This is potentially a very interesting means of generating human cell types that are normally very difficult to obtain, and is not restricted to the use of germ cells, as many lineages can now be generated from pluripotent ES and induced pluripotent stem (iPS) cells should different cell types be potentially informative. The Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS) initiative in Europe (http://www.esnats.eu/) represents a formal attempt to use ES cells for toxicity testing, potentially providing a new system that can be widely adopted and allow more limited use of animal systems.

7.4 Validation of tests.

71. The epigenomic tests of greatest current value are those that study cytosine methylation, for reasons described earlier, and will represent the cornerstone of epigenomic testing for some time to come. Other valuable tests will include transcriptional profiling (of RNA and of small processed RNAs) and chromatin immunoprecipitation-based techniques.

72. The validation of each requires a different type of assay. For cytosine methylation, the gold standard is the chemical mutagenesis of DNA with sodium bisulphite to create uracil where there existed an unmethylated cytosine in the original DNA, whereas methylcytosine remains unconverted. Quantitative single locus studies of PCR amplicons that compare the proportion of cytosine to thymine (to which the uracil is converted during PCR) measures the methylation

at that locus. Platforms such as Sequenom's MassArray (Ehrich et al., 2005) or Qiagen's Pyrosequencer (Fakhrai-Rad et al., 2002) can perform this measurement highly quantitatively.

73. For transcriptional profiling and for chromatin immunoprecipitation, validation is performed by quantitative PCR using primers directed at specific loci. The relative enrichment of one locus compared with another is compared with that predicted from the genome-wide approach as a means of quantitative validation.

74. These validation steps are appropriate for testing how individual experiments perform, but a second avenue of validation is to test how variable are the individual experiments themselves. Validation should seek to capture not only experimental variability but also the variability of the biological system. The former can be assessed by performing replicate experiments repeatedly on the same sample, while the latter is best assessed by testing multiple separate samples. The goal is to determine how much of an influence experimental variability has on biological variability, and how much influence biological variability has on the test system, combining to generate a measure of confidence in the results as a whole.

75. Any identification of epigenomic effects with endogeneous hormones (dihydrotestosterone, 17ß-estradiol) as used previously (Chaturvedi et al., 2010; Ho et al., 2006). would provide a very useful benchmark for candidate endocrine disruptors, which can be substantially less potent in inducing cellular proliferation but comparable in other respects such as inducing calcium influx when compared with endogeneous hormones (vom Saal and Hughes, 2005).

76. What is not yet possible is the ability to influence epigenetic regulation at specific loci to make them reflect those observed associated with the phenotype of interest. For example, it is impossible to turn a locus from an unmethylated to a methylated state, although global methylation can be driven in different directions by drugs (Claus and Lubbert, 2003) or diet (Niculescu and Zeisel, 2002). Functional validation remains an elusive component of current studies of epigenomic dysregulation.

Recommendations *[will be expanded in the next version to address short term, medium term and long term]***.**

- To gain insights into the epigenomic dysregulatory effects of endocrine disruptors, creating a foundation for understanding how these chemicals may mediate their phenotypic effects, by using as a starting point the endocrine disruptors and dosage schedules described in **Tables 4-5**, with careful cell isolation and preparation and epigenome-wide assays (for e.g. *in vitro* cell culture such as TG 473, *In vitro* Mammalian Chromosome Aberration Test or rodent models such as TG 443, Extended One-Generation Reproductive Toxicity Study)

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