The Role of Epigenetics in Reproductive Toxicity

12-13 November 2015, Brussels

Co-organised by ECETOC and Cefic-LRI

Workshop Report No. 30

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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Brussels, August 2016

ISSN-2078-7219-30 (online)

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European Centre for Ecotoxicology and Toxicology of Chemicals
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CONTENTS

1. EXECUTIVE SUMMARY

This workshop explored the current state of the science on epigenetics and its role in reproductive toxicity. Experts from a range of scientific disciplines met over two days to share knowledge and brainstorm research needs in the field. The objectives and conclusions of the workshop were as follows:

Objective 1: Define epigenetics and understand its potential value for reproductive toxicology

Outcome:

Participants emphasised the need to clarify definitions and semantics. Four concordant definitions of epigenetics were cited:

- "Heritable modifications, superimposed on DNA base sequence that regulate gene expression" (Jessica LaRocca).
- "Heritable information governing a cell state unrelated to DNA sequence variability, or information that can be inherited from a parent cell that is not encoded in the DNA sequence" (John Greally).
- "Chemical modifications of DNA that control expression of genes" (Daniele Fallin).
- "Chemical modifications of chromatin (histone PTMs, ncRNAs) which affect gene expression and may be heritable, and play a role in reproductive toxicology" (Peter Alestrøm).

Discussions regarding the potential value for reproductive toxicology concluded that:

1) Our understanding of epigenetically-mediated toxicity is still underdeveloped. There is evidence to suggest that exposures to toxicants early in life (e.g. *in utero* or childhood, intergenerational effects) may result in epigenetic mechanisms that contribute to adverse health outcomes later in life.
2) The next step in furthering our understanding of the relationship between epigenetic change and adverse effects is to identify strong, reproducible, apical endpoints for use in models to investigate mechanisms of toxicity *in vivo*.
3) The ultimate goal would be to identify early predictive epigenetic markers causally linked with adverse apical endpoints that could help guide chemicals management decisions.

Objective 2: Understand the relationship between epigenetic change and adverse endpoints

Outcome:

It is not possible to distinguish or predetermine adaptive epigenetic effects from adverse epigenetic effects, especially when the link between epigenetic measurements and apical endpoints is not fully established. Too many epigenetic studies to date - including those reporting toxicant-induced transgenerational effects in mammals - have lacked causality determination; are underpowered; and have used only a single high dose level exposure.

Transgenerational effects should not be the focus of study at this time. Efforts to understand somatic effects and the potential consequences of environmentally-induced epigenetic effects within a single generation or between parent and child would be more helpful. This should include the following:

- Ensure studies are reproducible and high confidence with interpretable results.

- Determine which epigenetic alterations represent adverse changes, adaptive changes or are 'biological noise' (i.e. not alterations).
- Identify and examine reproducible epigenetic endpoints within a mechanism/mode of action/adverse outcome pathway framework, to establish a robust correlation between an epigenetic change and an *in vivo* adverse outcome. These relationships must be examined across time and the dose-response *continuum*.
- Epigenetic endpoint measurements could include one or more of the following: DNA methylation (including DNA hydroxymethylation), histone modifications, and miRNAs.
- Model Systems: choices for whole organisms must be based on a thorough understanding of their advantages and limitations with regard to risk assessment in humans. Biological relevance to the human and mechanistic understanding to underpin regulatory utility is the primary driver. But cost, time and throughput criteria are also important. Validated *in vitro* model systems using well characterised cell lines should be included where appropriate because they can elucidate and validate important mechanistic understanding and address questions around causality. They can also provide epigenetic markers to complement *in vivo* studies.
- Model compounds should be selected on the basis of a strong understanding of known phenotypic (apical endpoint) effects that are relevant to the hypothesis. The following were suggested: dexamethasone; phthalates; dioxin; oestrogen; copper; DES; and/or valproate as a control compound.

Objective 3: Develop a roadmap for the practical use of epigenetic studies in regulatory applications

Outcome:

Some participants felt the preparatory work to assess utility could be included in test guidelines now — by collecting relevant tissues on a contingency basis for later retrospective analysis and comparison to apical endpoints. This could contribute to a better understanding of epigenetics (its potential role in human toxicology and the development of appropriate test methods); reduce animal use in the future; and make better use of those already involved in experimentation and regulatory safety assessments. It would also allow the collection of data on chemicals of concern and thus, in the longer term, enhance chemical safety. However, other participants agreed that the current understanding of toxicant-induced epigenetic change is still too limited for epigenetic endpoints to be formally incorporated into current test guidelines and that more research is required to demonstrate that examining epigenetic endpoints provides value in a regulatory context.

The following elements should be incorporated into the Roadmap:

- Define the range of normality for epigenetic endpoints, particularly normality at the time of analysis and normality within the system.
- Establish transparent guidelines to ensure that study designs include consistent and standardised data generation and management processes.
- Consider *in vitro* systems, which have the potential for development as mechanistic test methods, but ensure that the *in vitro* system reliably models *in vivo* toxicity.
- Establish whether epigenetic endpoints will provide added value (mechanistic understanding and insights and/or improved predictive capacity) to existing regulatory studies.

Objective 4: Develop a prioritised research agenda:

Outcome:

The following three research proposals were developed:

1) Develop *in vivo* intergenerational (not transgenerational) exposure models that will provide reproducible apical and epigenetic endpoints that can be used for correlative studies. Where appropriate, complement with validated *in vitro* studies to further elucidate and validate mechanistic understanding and markers.[1]
2) Define epigenetic normality across different laboratories and across different species, taking into consideration confounding issues such as age.
3) Develop a "Centre of Enabling Resources in Data Analysis and Coordination" for data management and analysis standardisation.

Conclusion

Participants outlined a roadmap for the practical use of epigenetic investigations in the regulatory context.

The next step in furthering our understanding of the relationship between epigenetic change and adverse effects is to identify strong, reproducible, apical endpoints for use in models to investigate mechanisms of toxicity *in vivo*. Too many existing studies demonstrating epigenetic effects are of limited value because they are not reproducible and confidence in their findings is low (see talks by LaRocca, Greally, Gray, and Buesen).

Focus should be on investigating somatic effects, effects in single generations or between parent and child (i.e. intergenerational). There is little value in studying transgenerational effects at this time. These studies should be complemented by validated *in vitro* models to provide mechanistic information, elucidate questions around causality, and possibly lead to the development of epigenetic markers. Further, best practice guidance should be produced and disseminated so that epigenetic studies yield high confidence, high interpretability and high reproducibility.

The voluntary augmentation of current regulatory guideline studies could enable the better use of animal tissues for retrospective analysis of apical endpoints potentially associated with epigenetic mechanisms, however this will be difficult to put into practice as the benefit of epigenetics measurements is not yet known.

Three concrete research proposals, including possible model systems, were outlined for future action.

[1] This research proposal is a result of two similar proposals developed during the course of brainstorm discussions and combines the outcomes of breakout group 1 (see page 25) and breakout group 3, RfP 2 (see page 27).

2. AIM OF THE WORKSHOP

Building on the success of an earlier ECETOC workshop in December 2011: Epigenetics and Chemical Safety, this 2015 workshop explored the most current understanding of epigenetics, and its potential role in understanding early life exposures resulting in later adverse effects. Experts from a range of disciplines including epidemiology, toxicology, epigenomics and regulatory science met over two days — first to share knowledge and then to brainstorm research needs in the field.

The objectives of the workshop were to:

- Define epigenetics and understand its potential value for reproductive toxicology.
- Understand the relationship between epigenetic change and adverse endpoints.
- Develop a roadmap for the practical use of epigenetic studies in regulatory applications.
- Generate a prioritised research agenda.

Day 1 of the workshop was open to all ECETOC member companies and invited guests as a capacity-building activity. Around 50 people from industry, academia and the regulatory community world worldwide, came together. Discussions centred around what epigenetics is, its potential value for reproductive toxicology and the relationship between epigenetic change and adverse end points.

Day 2, a smaller group of around 30 experts got together to discuss the practical use of epigenetic studies in regulatory applications and generate a prioritised research agenda.

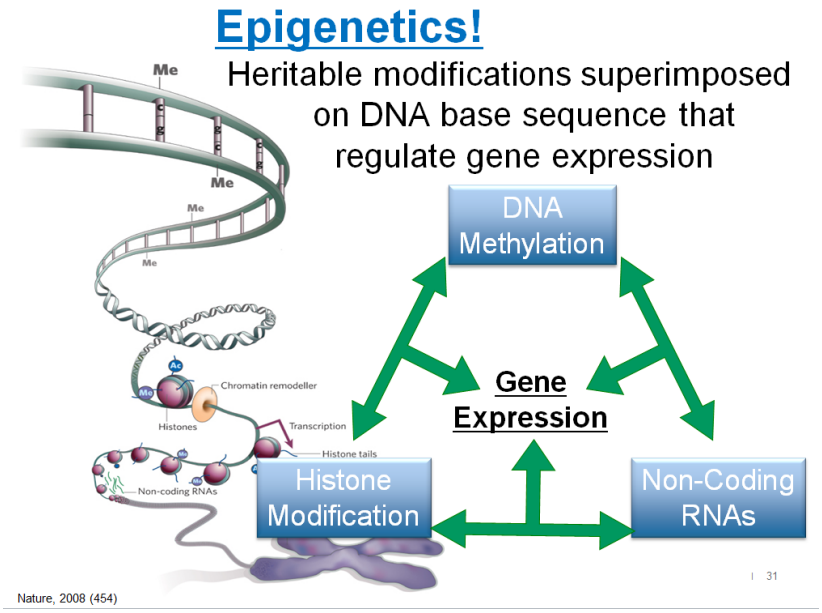
3. PLENARY LECTURES

3.1 Level Setting: Reproductive Toxicity Studies and Epigenetic Studies

3.1.1 What are reproductive toxicity guideline studies? Why are they conducted? What can they achieve? Where are the gaps?

Jessica LaRocca

Dow AgroSciences, USA



The presentation outlined the Product Safety Assessment process from hazard identification through dose-response, exposure assessment and finally risk characterisation. She described the regulatory drivers for toxicity testing and the guidelines for developmental and reproductive toxicity studies for industrial and agricultural chemicals. In this talk, epigenetics was defined as "heritable modifications superimposed on DNA base sequence that regulate gene expression."

Dr LaRocca discussed the Anway *et al* papers on Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility and the usefulness of Vinclozolin as a possible model compound to help understand epigenetics (Anway *et al*, 2005; 2008). Different groups have tried unsuccessfully to reproduce this study's findings (Schneider *et al*, 2008).

Several questions remain unanswered regarding epigenetics:

- What are the dose-response relationships for these epigenetic effects as compared to apical endpoints?
- Is identification of causal biomarkers necessary to demonstrate which epigenetic changes would be predictive of apical effects?
- How do we identify which changes are adaptive and which are adverse?

3.1.2 What are epigenetic studies? Technical aspects and data interpretation to achieve confidence in epigenetic studies (human epidemiology and animal-based studies)

John Greally
Clinical Genomicist and Professor of Genetics, Paediatrics and Medicine,
Albert Einstein College of Medicine, New York, USA

The presentation by Professor Greally explored epigenetic heritability, the epigenome-wide association study, cellular models of epigenetic perturbations and study design recommendations. He defined the modern use of the term epigenetics as "heritable information governing a cell state unrelated to DNA sequence variability, or information that can be inherited from a parent cell that is not encoded in the DNA sequence". He went on to discuss how and why, although numerous studies have been attempting to test how toxic exposures during pregnancy affect the epigenome of the offspring, these epigenome-wide association studies (EWAS) are now appreciated to be poorly interpretable (Greally *et al*, 2013). Indeed, the 2012 OECD review concluded that studies to date were not informative and recommended the need for definitive studies.

Given that EWAS are rapidly increasing in number and scope, it makes sense to develop guidance to ensure that future EWAS are rigorous and allow high confidence in their findings. Professor Greally went on to discuss the elements that should be included in future EWAS in order to increase understanding into the epigenetic effects of exposure to toxins during pregnancy: they should be designed in a manner that enables increased understanding about the biological processes that occur as a response to toxic exposure, the resulting cellular events and the primary regulatory effect of transcription factors.

3.1.3 Effects on rat reproductive development produced by antiandrogens: upstream indicators of downstream effects

Paul Foster
Chief of Toxicology, NIEHS, USA

Dr Foster briefly described the normal development of the male reproductive system in the rat and the phenotypic changes produced in the developing Wolffian duct (WD)/ foetal epididymis, following *in utero* exposure to two antiandrogens with different modes of action: di-n-butyl phthalate (DBP) which produces effects on the concentration of the androgen ligand and Linuron (Lin) which is predominantly a competitive inhibitor of the androgen receptor.

Both agents produced very similar phenotypic effects on the failure of the developing WD to undergo coiling — an essential component of its normal development. Exposure only during the period of sexual differentiation *in utero* produced profound effects in the offspring as they reach adulthood, with the occurrence of a high incidence of epididymal maldevelopment and consequent infertility.

A number of approaches have been taken to explore the early molecular events in the developing WD that resulted in the endocrine disruption of epididymal development. In the main these focused on the role of the androgen receptor, since androgens are required to prevent degeneration of the WD and that we know testosterone (T) induces coiling of the WD *in vitro*. Further, we know that T can act directly and indirectly via mesenchymal and epithelial interactions involving paracrine factors including EGF, IGF-1, and FGF (and/or their receptors) that have been shown to be altered following either DBP or Lin exposure.

However, Dr Foster emphasised that there is a 'disconnect' in the critical windows of exposure and development for the induction of epididymal malformations in rats (GD 15-17, the male programming window) — and when we can see changes in either WD phenotype, or in WD gene expression for the above growth factors (at GD 21 and not noted at earlier times). One could speculate that the adverse events in the WD/ epididymis are 'programmed' to occur later in development, and that a potential mechanism that could be explored for such programming could be via epigenetic changes induced by chemical exposure. However, it seems unlikely that such effects could be inherited, since the phenotype of interest leads to infertility and that this would argue that selection would be against this trait / phenotype of interest.

3.1.4 Effects on rat reproductive development produced by antiandrogens: AOPs and transgenerational effects

Earl Gray
Senior Reproductive Toxicologist, EPA, USA

Dr Gray discussed different examples of adverse effects of phthalates. He went on to focus on vinclozolin, stating that studies show that exposure with vinclozolin during androgen-dependent sexual differentiation causes adverse effects in male F_1 rats (Gray *et al* 1994; 1999).

However, he echoed earlier reference to the findings of the 2005 Anway *et al* study (gestational vinclozolin exposure was found to induce epigenetic alterations that were transmitted from F_1 to F_2, F_3 and F_4 generations), stating that these findings have not been found in other laboratories and three published studies could not replicate the effects of vinclozolin on the testis or fertility in any generation. The EPA conducted its own study to determine if vinclozolin treatment induced epigenetic effects in male rat offspring. It found that vinclozolin treatment did not reduce F_1 or F_2 fertility, nor did it induce the epigenetic changes described in the Anway study. The effects that were found were not transmitted to F_2 or F_3 generations.

Dr Gray concluded that his concern about many published TGE studies is that they cannot or have not been reproduced. He summarised other issues with studies:

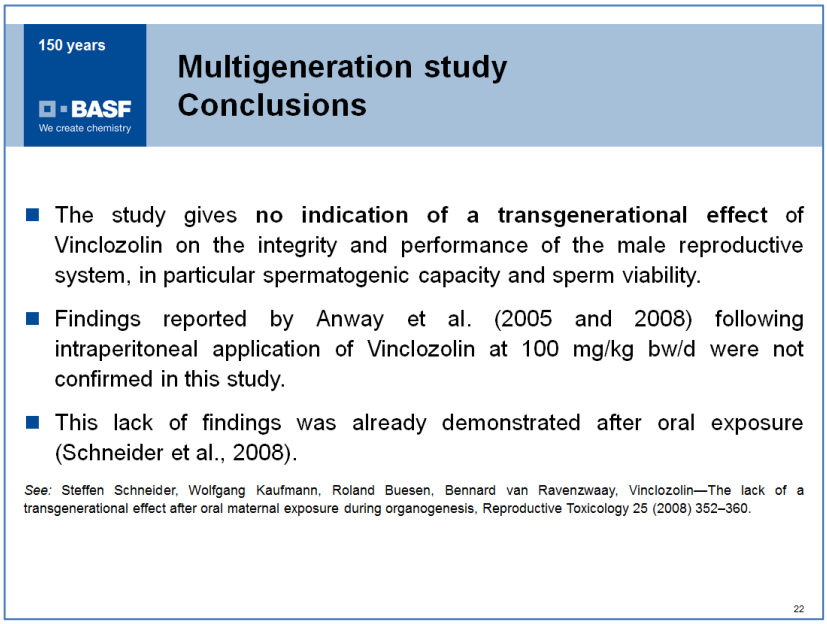
- Statistical issues.
- Litter effects from P_0 to F_3 generations – need to track the lineages.
- Small sample sizes.
- Questionable statistical analyses.
- Effects not always consistent from generation to generation.
- Are the effects truly adverse?
- Biologically plausible epigenetic mechanisms linking *in utero* epigenetic effects to an adverse developmental effect.
- Inter-animal variability in epigenetic measurements.
- Are we measuring the 'right' epigenetic events?

3.1.5 The lack of Transgenerational Inheritance of Anti-androgenic effects after Vinclozolin Treatment

Roland Buesen

Toxicologist and study director, BASF, Germany

Dr Buesen built on the previous presenter's discussions about the difficulty in reproducing epigenetics studies, exemplified by BASF's research project to reproduce the results of the Anway *et al* studies (2005; 2008). These studies reported that Vinclozolin administered to pregnant rats induced an adult phenotype in the F_1 generation of decreased spermatogenic capacity (cell number and variability); increased spermatogenic cell apoptosis and decreased male fertility – and that these effects were transferred to males of F_2 and subsequent generations. The BASF study (Schneider *et al*, 2013) examined the potential transgenerational inheritance of anti-androgenic effects induced by Vinclozolin administered intraperitoneally to pregnant Wistar rats (Crl:WI[Han]). Dams were dosed with Vinclozolin at 0, 4 or 100 mg/kg bw/d on gestation days 6-15. Male offspring of F_1-F_3 generations were bred with untreated females to yield F_2-F_4 offspring. No evident anti-androgenic effects were observed at 4 mg/kg bw/d, but a case of hypospadias as well as delayed sexual maturation in F_1 male offspring were observed as signs of anti-androgenicity at 100 mg/kg bw/d. However, F_1-F_3 males of the high-dose group developed normally to sexual maturity (slight delay in pubertal onset in F_1 males) and were able to mate and to generate healthy progeny. Sperm count, morphology and motility were not affected in F_1-F_4 generation male offspring. In conclusion, transgenerational inheritance of Vinclozolin's anti-androgenic effects was not evident in outbred Wistar rats.

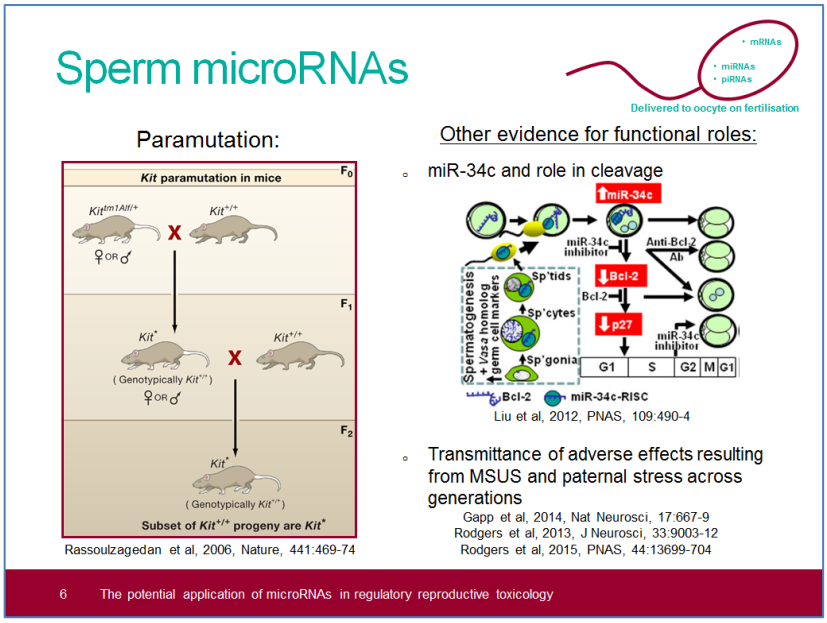


3.1.6 The potential application of microRNAs in regulatory reproductive toxicology

Emma Marczylo

Senior Toxicologist, Public Health, England

Dr Marczylo began with a description of microRNAs (miRNAs) — a family of endogenous short (18-22 nt), single-stranded, non-coding RNA species that regulate gene expression at the post-transcriptional level (predominantly via mRNA degradation or the inhibition of mRNA translation). They are important in fine tuning gene expression in a wide variety of cellular functions including proliferation, differentiation and development, and have been shown to be involved in both toxicity and disease. Recent evidence has also implicated miRNAs in the transmission of altered phenotypes across generations through the male germline. As such miRNAs can be classed as epigenetic mediators that play critical roles in developmental and reproductive toxicology.



Dr Marczylo then went on to introduce miRNAs as epigenetic regulators of the mammalian life cycle and potential environmentally-induced toxicity. She described ongoing work in Public Health England on human spermatozoa and primordial germ cell models investigating the roles of miRNAs in epigenetic toxicity, indicating applicability to regulatory assessment.

Finally, she discussed some of the issues and next steps, including initiatives currently in development within Public Health England, to help guide and drive the integration of epigenetics into regulatory toxicology. For example, next steps for moving forward with alternatives to *in vivo* models include: understanding / characterising variance in epigenetic responses to changes in the normal environment (what is normal?); assessment of stability and reproducibility; development of cost-effective assays with appropriate quality controls, possible potential for higher throughput and extrapolation to humans and human phenotypic endpoints. With regards to the bigger picture and the relevance of epigenetics to public health, Dr Marczylo explained the need to relate molecular initiating events (including epigenetic endpoints) to actual human disease outcomes and help identify knowledge gaps to guide epigenetic technical guideline development.

3.1.7 Introduction of Percellome Project with special reference to the concept of "signal toxicity" Case study: Single exposure adult studies with developmental cases, at signal dose levels, i.e. at the level of no overt cytotoxicity or organ toxicity monitorable by histopathology

Jun Kanno

Head of Cellular and Molecular Toxicology Division, NIHS, Japan

Dr Kanno explained the Percellome Toxicogenomics Project, with a focus on the concept of 'signal toxicity'. The Percellome project was initiated in 2001 and uses fewer animals, exposed to lower doses, at single exposures or over short time periods. The objective is to mechanistically reinforce the 'safety (uncertainty) factor' used for the extrapolation of animal data to humans — and eventually make the process *in silico*.

The project was designed to capture unpredicted toxicity resulting in the development of a normalisation method 'Percellome (Aisaki *et al*, 2011; Kanno *et al*, 2006)' for microarrays and Q-PCR in order to generate absolute copy numbers of mRNAs per single cell (on average). Quantified mRNA data of mouse liver (4 time points x 4 dose levels, n=3, 48 microarrays per organ per chemical) were obtained on over 100 chemicals. The project now includes studies on multi-organ relationships, low concentration inhalation, repeated dosing, etc. Data are visualised in 3D surface graphs (time x dose x mRNA copy number per cell) of each probe set of Affymetrix MOE430 2.0 GeneChip and subjected to comprehensive analysis by a series of in-house software.

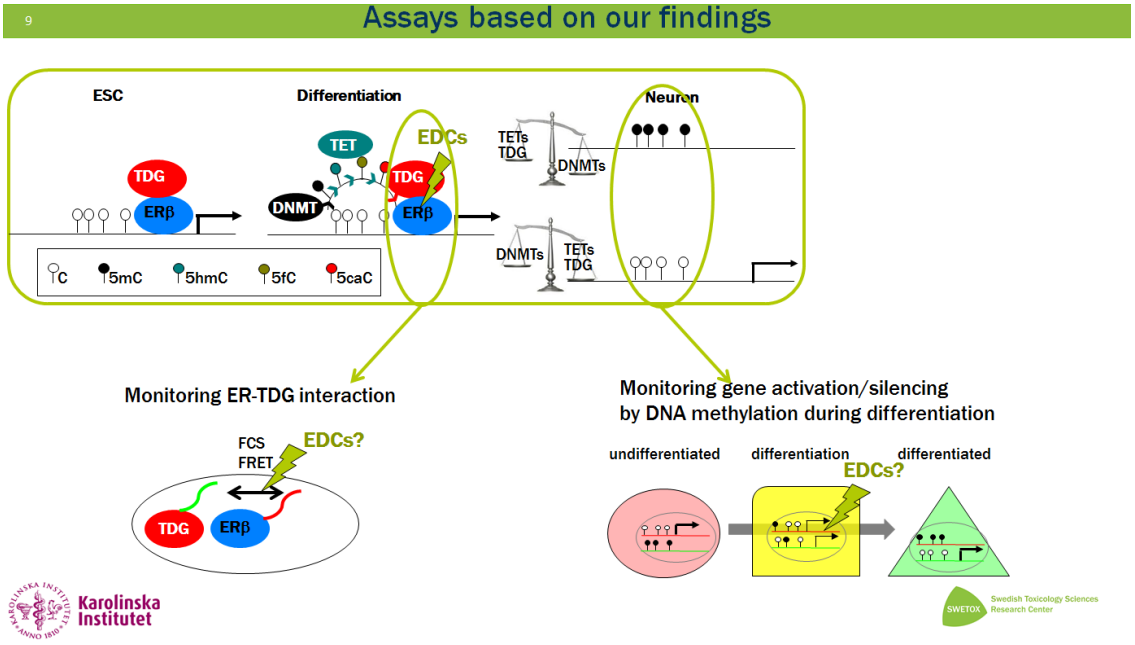
Dr Kanno reported on the case studies on estragole (Kanno *et al*, 2012) and pentachlorophenol (Kanno *et al*, 2013); these were single-exposure adult studies, cases at signal does levels (i.e. at the level of no overt cytotoxicity or organ toxicity by histopathology). Findings showed networks of PPAR-alpha and interferon signalling networks respectively. Next steps, including systems biology (Garuda Project5) were also briefly presented.

3.1.8 Mechanisms underlying epigenetic effects of EDCs

Joëlle Rüegg

Senior research fellow at the Swedish Toxicology Science Research Center Swetox and Karolinska Institutet, Stockholm

Joëlle Rüegg pointed out that there is a lack of understanding mechanisms underlying epigenetic effects of EDCs. She described her findings that the EDC target oestrogen-receptor beta is involved in regulating DNA methylation at specific genomic regions by interacting with thymine DNA glycosylase, an enzyme involved in DNA demethylation (Duong *et al*, in revision). That oestrogen-receptor beta is directly involved in epigenetic effects of EDCs was shown a study in which BPA exposure during differentiation and development induced DNA methylation changes in a murine hippocampal cell model and in rat hippocampus, respectively (Kitraki *et al*, 2015). The affected gene, Fkbp5, is an important regulator of the stress axis, and the BPA-exposed rats showed indeed changes in their stress response, thus linking epigenetic changes to a phenotypic outcome (Panagiotidou *et al*, 2014; Kitraki *et al*, 2015). The goal is now to study whether it is the interaction between TDG and oestrogen-receptor beta (and other nuclear receptors) that is disturbed by EDCs, and develop simple *in vitro* assays based on these findings.



Joëlle concluded with a slide about epigenetic transgenerational inheritance and emphasised that also in this case, lack of mechanistic understanding impedes the assessment of its importance. The hypothesis that DNA methylation marks are erased after fertilisation speaks against an epigenetic mechanism for transgenerational inheritance of a phenotype. However, there are exceptions, marks that are either not erased or immediately re-established. How this is achieved and how many exceptions there are is still unknown. Mechanistic understanding would elucidate the role of epigenetic regulation in transgenerational inheritance of phenotypes and hence would clarify our need for test methods for transgenerational effects.

3.2 Understand the Relationship Between Epigenetic Change and Adverse Endpoints

3.2.1 What are the general principles for indicating whether the epigenetic change is causal (adverse) or adaptive?

Bob Chapin
Senior Research Fellow, Pfizer Inc., USA

Toxicology has decided that to become a modern discipline, it must focus more on molecular biology and molecular toxicology. The US Government's Toxicology in the 21st Century program is the epitome of this approach, focusing on molecular changes in model cell systems as representative of the critical response in whole animals or populations. This is toxicology's equivalent of high-throughput drug discovery, having a reductionist focus on representative target gene or protein changes in model systems. However, there is a growing awareness in the drug discovery community that this approach is less successful than using a phenotype-based approach. Indeed, few or no drugs have been found and approved using the high-throughput, rational-design approach. The emerging appreciation that many gene-disease associations were really 'noise discovery' (Ioannidis, 2005) provides additional caution about a gene-focused approach to understanding mechanisms of toxicity.

An additional layer of complexity comes from recognising that environmental compounds (and many medicines, too) do not act as a single place in the cell. They never have just one target, and with higher doses they hit more targets. Phenotype aggregates all those differing molecular changes into one set of histologic presentations.

If we believe that looking at phenotype will better describe what sorts of changes are adverse, and which is instead, biological noise, then the way forward becomes clear: we must create a database of exposures of whole animals to epigenetic-modifying compounds and examine the galaxy of results. If a new pattern emerges which is distinct from that pattern created by other reproductive toxicants, then that provides a focus for future work, and we can consider whether a refined focus will save time or money. It may be that all epigenetic modifiers, regardless of specific mechanism, will produce effects similar to other reproductive toxicants, so that simply by knowing their mechanism allows a prediction of their *in vivo* activity. Science is never that simple, though, so we can suspect it is much more likely that some epigenetic compounds will be reproductive toxicants and others will be without reproductive toxicity except at significant multiples of exposure (which will thus likely be the result of undetermined off-target effects). In this case, simply knowing the mechanism will not be enough to allow us to predict their toxicity.

- So given our aptitudinal imbalance (we are much better at finding things to measure in biology than in understanding how they all fit together, or how they sum to a response), I suggest that the best way forward will be a period of studied observation. Specifically,
- We need a research program wherein we treat our animals of choice with a variety of compounds with known epigenetic MOAs. Take special note of unusual findings, and link mechanistic data with phenotype whenever you can.
- A broad accumulated training set will best put us in a position to know what the best next step is.

Only by accumulating a database correlating known (or intended) mechanism with *in vivo* effects in a model species or two will allow us to test for any association between mechanism and outcome. Critically, this will need to contain enough different exposures to allow the conclusion to be robust……or at least not to be misleading.

3.2.2 Practical experience with rodent species used in toxicology studies: what is a normal epigenetic background?

Richard Meehan

Group Leader, Chromosome Biology, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK

Modification of DNA resulting in 5-methylcytosine (5-mC) or 5-hydroxymethylcytosine (5hmC) has been shown to influence the local chromatin environment and affect transcription. MRC has developed a rapid and cost effective method of generating genome wide DNA modification maps utilising commercially available semiconductor based technology (DNA immunoprecipitation semiconductor sequencing; "DIP-SC-seq") on the Ion Proton sequencer. Focusing on the 5hmC and 5mC marks we can demonstrate, by directly comparing with alternative sequencing strategies, that this platform can successfully generate genome wide 5hmC patterns from as little as 500 ng of genomic DNA in less than four days. Such a method can therefore facilitate the rapid generation of multiple genome wide epigenetic datasets. The MRC wishes to apply this method to characterise the 'ground state' of liver epigenomes from commonly utilised rodent models. Dr Meehan presented his group's initial analysis of male and female 5hmC epigenomes from Wistar and SD rat liver. There was essentially little variation within strain samples but appreciable differences between strains. Gender specific differences can also be detected that are linked to expression patterns. This information will be useful in planning future experimental strategies.

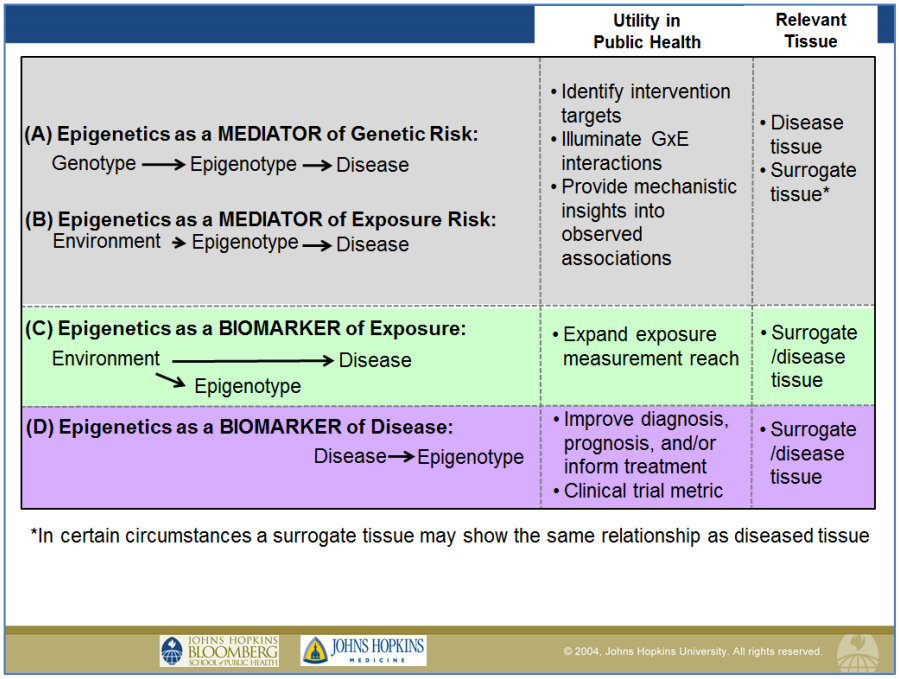
Epigenetic perturbations have been associated with exposure to a range of drugs and toxicants, including non-genotoxic carcinogens (NGCs). Although a variety of epigenetic modifications induced by NGCs have been studied previously, the MRC's recent genome-wide integrated epigenomic and transcriptomic studies using targeted array platforms, revealed for the first time the extent and dynamic nature of the epigenetic perturbations resulting from xenobiotic exposure. The interrogation and integration of genome wide 5hmC modification states, with other epigenetic modification and expression profiling studies, has the potential to identify unique epigenetic signatures for diverse drug exposure studies. These studies and methodologies can enhance mechanistic understanding of xenobiotic exposure and provide for the identification of novel safety biomarkers that will be of benefit in multiple clinical and safety studies.

3.2.3 Determining clear and robust biomarkers of epigenetic change and adversity

Daniele Fallin

Chair of Department, Johns Hopkins University, USA

Dr Fallin's talk provided examples of the exposure biomarker opportunities for epigenetics, as well as the challenges that must be addressed to realise these opportunities.



	Utility in Public Health	Relevant Tissue
(A) Epigenetics as a MEDIATOR of Genetic Risk: Genotype → Epigenotype → Disease **(B) Epigenetics as a MEDIATOR of Exposure Risk:** Environment → Epigenotype → Disease	• Identify intervention targets • Illuminate GxE interactions • Provide mechanistic insights into observed associations	• Disease tissue • Surrogate tissue*
(C) Epigenetics as a BIOMARKER of Exposure: Environment → Disease; Environment → Epigenotype	• Expand exposure measurement reach	• Surrogate /disease tissue
(D) Epigenetics as a BIOMARKER of Disease: Disease → Epigenotype	• Improve diagnosis, prognosis, and/or inform treatment • Clinical trial metric	• Surrogate /disease tissue

*In certain circumstances a surrogate tissue may show the same relationship as diseased tissue

Epigenetic marks can be useful for epidemiology and clinical medicine under multiple scenarios. Epigenetic machinery, such as DNA methylation, histone modifications and chromatin structure, controls regulation of when and where in the body particular genes are expressed. Thus, epigenetic marks can provide mechanistic insights about how genetic variation affects phenotype. Importantly, epigenetic marks are responsive to environmental changes, and thus may also be a mechanism for how exposures manifest disease, or how genes and environment work in concert towards a phenotype. Epigenetic measurement may also be useful as a biomarker of past exposure, even when the epigenetic changes are not directly causal of downstream phenotype. In this case, epigenetic biomarkers may be an attractive opportunity to estimate cumulative or specific prior exposure in situations where direct measurement of the exposure is not feasible. The question remains: Can we start to identify key biomarkers and potential mechanisms associated with the phenotypic adverse change? (Sound techniques, robust data interpretation procedures). The concept of 'repeated exposure' and possible links to epigenetic regulations — with repeated dose studies introducing baseline responses and transient responses with possible link to epigenetics.

3.2.4 The concept of "repeated exposure" and possible links to epigenetic regulations. Repeated dose studies introducing baseline responses and transient responses with possible link to epigenetics

Jun Kanno

Head of Cellular and Molecular Toxicology, NIHS, Japan

The Percellome Project was primarily designed for the comprehensive drawing of gene network(s) in a time- and dose-dependent way after a single oral dosing of a chemical. The dose of each test chemical was determined by the intensive dose-finding study, and the highest dose was set to a level ('signal dose') that does not induce morphological changes (macro and micro) and clinical symptoms at 24 or during the first 24 hours post administration. Consequently, 'phenotypic anchoring' was not considered as a tool for the transcriptomic data analysis. Along with the adoption of 'Per cell' normalisation strategy, use of gene knockout mice were considered for objective analysis of the gene network. It was expected that the gene network located downstream of the knocked-out gene will be highlighted as its 'shadow'. Indeed, for example, when aryl hydrocarbon receptor knock-out mouse (AhRKO) was challenged with 2,3,7,8 tetrachloro dibenzo-p-dioxin (TCDD) or 3-methyl cholanthrene (3-MC) and compared with wild type mice data, a group of genes including those known to be located downstream of AhR are silenced. The gene list was larger than that of known downstream genes.

During this analysis, Dr Kanno and his team came up with an idea of a new concept of repeated dosing: the 'chemically-induced transgenic state'. This concept allows us to compare the repeated-dose mice with the KO mice by challenging with a same test chemical. A series of trial studies were performed with a protocol as follows; all 48 mice were given a same amount of chemical A for up to 14 days by oral gavage, and then given, on the next day, a single gavage of the same chemical A or different chemical B at a dose of 0 (vehicle control), low, middle or high dose (in the range of 'signal dose') and sampled at 2, 4, 8 and 24 hours thereafter for transcriptomic analysis (designated as [14+1] study).

As a result, compared with mice only receiving single gavage (designated as [0+1] study), we found that repeated dosing induces two types of responses on gene expression, i.e. baseline response and transient response. In general, when the baseline (vehicle control group) goes down (up), the transient response is attenuated (exaggerated). Further analysis on the data, including those from [4+1], [2+1] and [1+1] studies, and using *in silico* method on the upstream events was discussed to help understanding of the molecular basis of repeated exposure including possible epigenetic mechanisms.

3.2.5 Zebrafish Model applications to address what is relevant for multiple generational studies

Peter Alestrøm
Professor of Biochemistry, Norwegian University of Life Sciences,

The understanding of how toxicants, alone and in combinations, affect human health is a complex issue and demands the use of both *in vitro* and *in vivo* model systems. Among *in vivo* models, mice have dominated much of the research, with zebrafish (*Danio rerio*) coming up as the second most used laboratory animal model. Among several advantages of zebrafish as vertebrate model organisms is the option for automatic high-throughput screening of compound effects in 96-384 well plate formats. A Zebrafish Embryo Acute Toxicity (FET) Test has been established (OECD Guideline 236, 2013) for screening standardisation. There is increased understanding of how epigenetics influences gene expression and as such can lead to adverse health outcomes without changes in the genetic code. It appears that much of the epigenetic control mechanisms of DNA methylation, histone modifications and non-coding RNAs are highly conserved among the vertebrates. Results from two ongoing projects, which aim at mapping transgenerational effects from induced epigenetic changes and the cause of inherited changed phenotypes, were presented. The Centre of Excellence for Environmental Radioactivity (CoE CERAD) at the Norwegian University of Life Sciences (http://cerad.nmbu.no) addresses how low dose gamma radiation in combination with secondary environmental stressors can affect ecosystems and human health. In this project the zebrafish model will be used in a multi-generational set-up up to F_4. The Institute for Environmental Studies at VU Amsterdam (J. Legler/J. Kamstra) assesses trans-generational effects of the phthalate metabolite mono(2-ethylhexyl)phthalate (MEHP) and the DNA methylation inhibitor 5-azacytidine (5AC) from early embryonic exposed zebrafish up to F_2. In both projects, global methylation will be measured with LC-MS. Site specific methylation will be assessed with reduced representation bisulphite sequencing (RRBS). Additionally in the CERAD project, transgenerational effect on histone modifications and transcriptome, including the miRNAome will be assessed with NGS methods, in order to provide a thorough mapping of the epigenetic landscape with and without exposure to radiation.

In summary, epigenetic landscapes can be subdivided into three levels: (i) DNAmethylation/hydroxymethylation, (ii) histone PTM and variants (histone code), and (iii) ncRNAs (short and long ncRNAs). Histone and ncRNA levels each have high complexity. Cross-talk between the epigenetic marker levels suggests higher sum complexity and is more difficult to predict outcome. Transgenerational epigenetic inheritance is still not well proven. Therefore, more basic research is needed and zebrafish is a good vertebrate model for epigenetic transgenerational studies.

3.2.6 How can the Zebrafish Model be applied to current regulatory test paradigms?

Ioanna Katsiadaki
CEFAS Weymouth Laboratory, UK

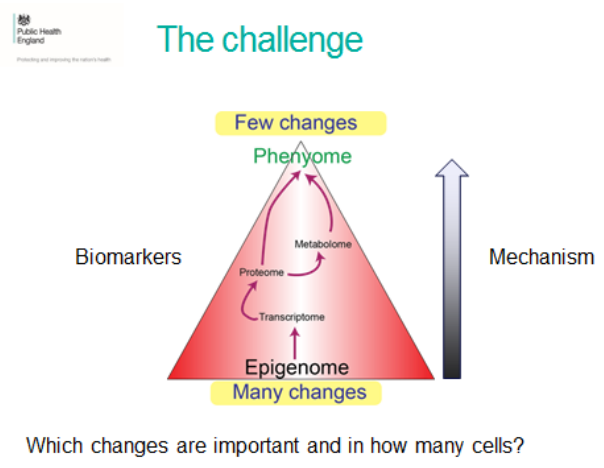
Dr Katsiadaki described some basic mechanisms of chemically-induced epigenomic effects that could be detected using existing OECD test guidelines involving fish. She also emphasised the need for validation studies that link epigenetic markers and adverse outcome — a critical step for regulation. Dr Katsiadaki said that adversity can be assigned within relatively few multigenerational studies as part of the validation process and that sampling at multiple time points and tissues is essential. The ultimate aim is to discover early predictive markers (FET). Future challenges include answering the following questions:

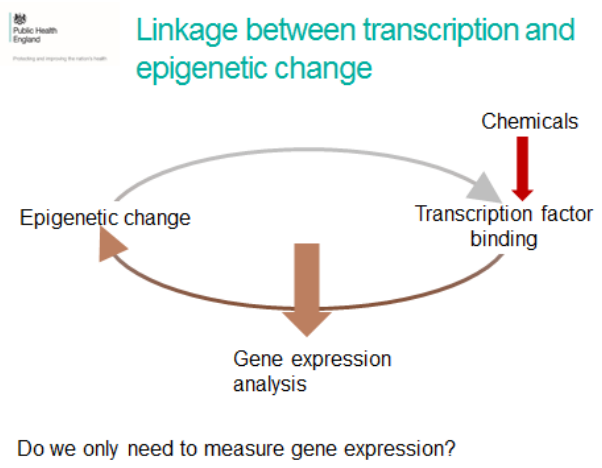
- Where do we start in terms of fish line?
- How do we confirm epigenetic nature-need functional assays?
- Permanent vs. transient changes; heritable vs. non-heritable changes?
- What are the fundamental differences between amniotic and non-amniotic species on epigenetic marking (role of female versus male in fish and mammals; sex determination mechanisms)?

3.2.7 Consensus on what and how?

Tim Gant
Head of the Department of Toxicology, Centre for Radiation, Environmental and Chemical Hazards (CRCE) Public Health England

Professor Gant summarised Day 1 discussions into a number of steps that should be taken together (in no specific order), and a number of questions that should be the basis of the brainstorm discussions of Day 2:

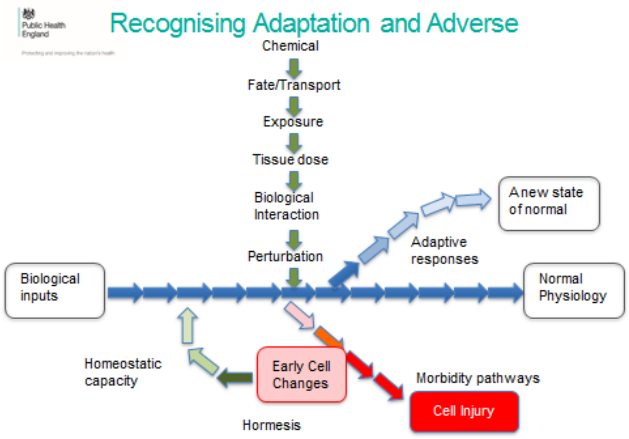




Step 1: Semantics and Definitions: Four definitions of 'Epigenetics' were heard today; all four were similar and concordant. Therefore, there is optimism that the scientific community can agree on a single reference definition of 'Epigenetics'.

Step 2: Decide what to measure: miRNA; methylation; hydroxymethylation; histone modifications – or gene transcription. (Whilst gene transcription measurements are not epigenetic measurements, they are necessary to put the epigenetic change in the context of mechanism/MoA.) Which of these are going to be the least variant, most informative and related to an adverse outcome? Do we measure the whole genome or part of a genome? Should we make molecular measures or concentrate on the phenotype?

Step 3: Incorporate Epidemiology: How do we determine epigenetic change in tissues for epidemiological studies? For DNA sequence, change determination of the genome sequence in one tissue will be the same as that in the tissue of interest. This is not true for epigenetic change. Can PBMLs act as a sentinel? Are sperms useful in epidemiology? What about females? What about mosaicism? Ancestry can confound exposure associations. All cells are unlikely to be epigenetically altered in the same manner throughout all cells in the tissue.



Step 4: Understand which epigenetics changes are adverse and which are adaptive. Can we identify epigenetic markers which are predictive of adverse change?

Step 5: Decide on the appropriate Model Systems: Relationship to humans, cost, throughput, which organs/tissue, measurements – specific or global? Cells/Fish/Mammals?

Step 6: Reproducibility: To ensure regulators, the scientific community and industry can have confidence in the findings of epigenetic studies – and that they can be reproduced: Strains and species differ in their epigenetic profiles; do we need to decide on appropriate strains for epigenetic studies? In reproducing studies, it is likely to be essential to ensure that the same species and strain is used? This will be essential information of any TG amendments.

Step 7: Address Hazard vs Risk: Are the effects we are seeing in model systems at such high doses that they are irrelevant for public health and only of academic interest? How do we examine long-term, low-dose exposure? Are early life stages of more importance? How do we take these into account? What about gametes?

Step 8: Proof of Principal and Test Guideline Discussions: Where he suggested that more research to identify epigenetic endpoints and markers is needed before these can be incorporated into testing. This was further developed by Miriam Jacobs who had prepared an overview of all the current TGs and where they might be adapted to start incorporating epigenetic measurements/assessment, which would augment and improve endpoint interpretations (See Room Document, Appendix 4). This would start to address regulators' need for understanding the link between molecular epigenetic changes and apical endpoints of concern in *in vivo* assays, but also address development of relevant *in vitro* assays that can help elucidate the mechanisms involved. One suggestion to support this will be to include the option of collection of relevant tissues in test guidelines for later retrospective analysis to develop an augmented TG.

Furthermore, for regulatory purposes, the discussion about transgenerational effects was considered not relevant. Miriam Jacobs emphasised that the bottom line is that the regulatory community and the wider public need test guidelines and integrated approaches to testing and assessment protective of early life chemical exposure and later life diseases such as cancers, obesity, diabetes etc, in the current generation, as well as subsequent generations. The intention is not to create new *in vivo* test methods solely for epigenetics. Rather it is to understand the normal range of epigenetic marks and which epigenetic markers are key events in disease progression. Then, this information would be used to further improve the current battery of regulatory tests and so further improve public health protection. This is the key challenge to be met.

4. BREAKOUT GROUP SESSIONS

On Day 2 of the workshop, a smaller group of experts were divided into 3 groups of 11 participants each.

Session 1 (morning) consisted of three separate questions for brainstorming related to "Develop a Roadmap for the practical use of epigenetic studies to underpin regulatory applications". Each brainstorm group discussed each of the three questions in rotation, spending 45 minutes on each question. At the end of Session 1, participants regrouped into plenary where the moderators and rapporteurs responsible for each of the three questions summarised the discussions.

Professor Gant gave the following guidance, summarised from Day 1 discussions, to provide context for the brainstorm:

- Definitions – multigenerational / transgenerational / epigenetics?
- What do we need to measure?
- Relationship of markers to phenotypic endpoints?
- Why do we need markers? Why not just measure phenotypic change as in current one-generation and two-generation studies?
- What are the likely adverse outcomes from epigenetic events?
- Where would it be realistic to consider application now?

4.1 Session 1: Develop a Roadmap for the Practical Use of Epigenetic Studies to Underpin Regulatory Applications

4.1.1 Question 1: Epigenetic Design Studies: Relevance to Regulatory Applications?

Moderator: Miriam Jacobs
Rapporteur: Jessica LaRocca

The underlying question is: can epigenetics elucidate if/where early life exposures could potentially lead to the onset of disease later in life? Many participants with an experimental *in vivo* background emphasised that there is a great need to have reliable, reproducible *in vivo* experimental model substances. The following were suggested as appropriate models where the adverse phenotypic effect of exposure was well documented and the relevant tissues to analyse were well known:

1. Dexamethasone – known phenotypic effects on both the male and female germ line on fertility and cardiovascular disease.
2. Phthalates – known male reproductive effects.
3. Oestrogen exposure – known female only effects on pubertal onset later in life and reproductive effects.
4. Dioxin – male infertility.

5. Valproate as a control compound for its known epigenetic modifications: histone deacetylase inhibitor and neural tube defects.

All breakout groups agreed that analysing the methylome will be important to assess persistent changes that could potentially lead to adversity. However, discussion on the benefits of analysing the transcriptome as opposed to focusing on DNA methylation was left without agreement – and a suggestion for further discussion.

Other suggestions included:

- Start with a thorough literature analysis to guide study design.
- In addition to specified tissues, take blood samples because this is the tissue that epidemiologists are most likely to have access to.
- Assess whether there is an epigenetic component that is driving a memory effect.
- Include temperature when undertaking comparative analysis between fish species.
- Exposure route: oral exposure was considered the primary route.

It was suggested that in parallel to *in vivo* models, *in vitro* assays (e.g. cell-type specific) are necessary to add value by elucidating and validating mechanistic information (cf. Jun Kanno's and Joelle Ruegg's presentations). These would also provide insight into secondary mechanisms that have been found in different tissues (for example lung, liver) that were not present in e.g. brain tissue. Teasing these out by using a co-culture format may help extrapolate those different mechanisms.

4.1.2 Topic 2: How to Design Epigenetic Studies?

Moderator: John Greally
Rapporteur: Madeleine Laffont

This question is linked with how you design the system that you want to study in the first place: what organism, what exposure etc. Discussions on this topic covered dose-response issues, developmental timing of how you do the exposure and how you sample the cells. The following points were agreed as necessary when designing epigenetic studies:

Multidisciplinary / consortium projects, which include a cross section of disciplines in the design phase as well as the implementation phase (experts from the fields of regulatory science to ensure the project is relevant to regulatory toxicology; developmental biology, epidemiology, computational and bioinformatics, toxicology, epigenetics, biochemistry, medicine, pharmacology etc). For example, developmental biologists will be needed since the cells that mediate the particular phenotype in question may disappear during development as a result of the toxicant exposure and not be available for study. The epigenome and transcriptome of these developmental cells should be assessed by performing testing at the appropriate developmental time point.

Detailed histology and full understanding of the cell sub-types present are needed in order to understand the molecular assays.

Reproducibility: the system set up in terms of exposure and outcome will work equally well in multiple different labs because we are studying variability – and if the variability is dependent upon the lab, then interpretation of results is impossible.

The experimental organism choice: consider organism specific issues. For example, zebrafish do not have a uterus; in rat some of the reference genomes needed for particular strains of rat is very poor – therefore a rat genome sequencing approach to upgrade the genomes of some of the rat strains consistently may be required).

Two phased approach to Study design: the first phase will be expensive – but as soon as it is informative it can be transitioned into cost-effective second-phase approaches:

- Phase 1: Experimental animal studies will include: DNA methylation, hydroxymethylation, chromatin analysis (helps with interpretability of other data), transcriptional analysis, genotype of the experimental animal to create interpretable molecular data. Additional expenses, such as cell phenotyping (flow cytometry) etc, will also be needed to adequately power the study, ensure it is useful, and provide the molecular markers needed for the overall phenotype
- Phase 2: more cost effective approaches over scale, based on phase 1 findings.

Model compounds: the compounds mentioned by Group 1, but also suggested DES – because, although less relevant to human health today, it affects cells that persist and ultimately give rise to the adverse phenotype of cancer in humans. This has the advantage over some of the other suggested model compounds where the adverse outcome is related to phenotype and therefore the cells ultimately disappear.

Regulatory perspectives: the epigenetic study must have added value over and above other accepted tests in terms of insights or predictive capacity, and the model system must be applicable to humans.

Consistent and standardised data management: consistent analysis, and terminology necessary for reproducibility of results. This has impacts on the choices of software and parameters used and metadata are collected systematically (transparency in experiment design).

4.1.3 Topic 3: What is Necessary to Interpret Epigenetics in Light of Reprotoxicity Studies?

Moderator: Roland Buesen
Rapporteur: Kamin Johnson

The following needs were identified – and independent of the chosen model:

Bioinformatics: This technique is required and should be developed. Training will be required: for staff preparing the samples, and for data interpretation. Processes to share results (e.g. publications) and the transfer of data should be agreed up front.

Defining normality for the epigenetic endpoint(s): Including normality at the time of analysis and normality within the system (tissue, cell, etc). Chemical companies could be asked to provide both control

and experimental samples from ongoing technical guideline studies and in-house work in order to support this work.

Functional linkage of the epigenetic endpoint to the apical (adverse) endpoint: This can potentially be used in the AOP framework.

Dose response especially with regard to Risk Assessment and No-effect level.

Inheritance of the epigenetic change: Is the effect transient or persistent across lifetime? Is the epigenetic change seen in the germline? Is the compound metabolism in the model similar to the human? (Which model is relevant to interpret data when we think about risk assessment for humans?)

Model? In utero exposure with postnatal examination? Do we have an influence on susceptibility to additional stressors – and can this be measured?

4.2 Session 2: Generating a Prioritised Research Agenda

Session 2 (afternoon) consisted of one action: "Generate a prioritised research agenda". Each brainstorm group discussed this topic and were tasked with preparing an outline request for proposals on the priority topic/theme that resulted from their discussion. At plenary, participants again regrouped and the moderators summarised the outcomes of the discussion.

Professor Gant gave the following guidance, summarised from Day 1 discussions, to provide context for the brainstorm:

- Mechanistic understanding – what do we need to know that will assist regulatory activity and product development?
- Do we need to further repeat and verify former research findings, e.g. vinclozolin?
- How do we get to dose-response relationships and causation?
- Are there likely to be thresholds? Or are responses likely to be non-threshold vs. genotoxins?
- Epidemiology: is it necessary and if so, what are we looking for?
- What more do we need to know that will be of practical application?

The following research proposals were developed by the breakout groups:

Breakout Group 1

Objective:

Identify sensitive and predictive markers for latent adverse outcomes following early life exposure. Evaluate feasibility of these epigenetic markers for relevance of human health risk assessment.

Scope:

After sufficient literature review, the researchers will submit a proposal focused on identifying sensitive, reliable, and predictive markers for latent adverse reproductive outcomes following early-life chemical exposure. Protocols for analysis and dose-level selection will need to be scientifically justified. Dose levels

will need to incorporate a high-dose group which is known to elicit a specific adverse outcome, as well as a dose level that is expected to determine a phenotypic NOAEL. The researchers will identify dose-response curves and temporal relationships for epigenetic, transcriptomic, and apical outcomes. Temporal relationships will be used to assess persistence of the epigenomic effect and memory and relation to the adverse outcome. The researchers will need to compare the biomarkers for specific adverse outcomes across molecules that are known to be true positives and negatives for the phenotypic outcome. It is recommended that the highest level and most cost effective methods currently available are used and scientifically justified bioinformatics approaches. It is recommended to use 5+ animals per dose group.

Chemical Examples:

- Dexamethasone and fertility (male and female germline).
- Phthalates and male reproductive effects.
- Dioxin and fertility.
- Oestrogen and female puberty.

Deliverables:

Cost and Timing/Duration of Project: USD 500,000. 3 years.

Partnering/Co-funding: It is recommended to have a consortium application with several different scientists with relevant multidisciplinary expertise.

Breakout Group 2

Title: Epigenetic Normality across different laboratories and across different species.

Background:

There are reports of *in utero* exposure causing effects in the adult suggesting a role for an epigenetic-mediated toxicity. Up to now we do not know the normal epigenetic landscape and how this epigenetic landscape can be influenced by chemical exposure. Companies would be willing to provide samples according to GLP standards to facilitate this work.

Objectives:

- What is the normal epigenetic landscape in different species and in different laboratories?
- Does *in utero* exposure result in persistent changes in the adult?

Scope:

- Examine epigenomic changes (methylation and miRNA patterns), transcriptome (RNAseq), and histology in the target tissue at different stages in life (*in utero*, postnatal, prior to the adverse effect being observed, at time the adverse effect is observed).
- Priority target organs are chemical dependent but should focus on sex organs for reproductive biology.
- Reproducibility across different labs: Are there different epigenomic patterns among labs in the target tissue from control organisms?

Breakout Group 3

RfP 1:

Title: Enabling Resources in a Data Analysis and Coordination Centre.

Background:

In order to be able to perform toxicological studies in model organisms relevant to human health there is a need to have enabling resources that involve data management and analysis standardisation.

Scope:

This Data and Analysis Coordination Centre should have the potential to generate new genomic data that could facilitate consortium investigator directed required data sets to include (but not restricted to) the development of model system genomic sources (e.g. rat) and single cell genomics and transcriptomics to enable understanding of cell and sub-cell composition issues in the cell type in the organism of interest: i.e. we expect integrated analysis in order to interpret interactions between genome, transcriptome and epigenome and how they reflect cellular sub-type proportional and physiological events.

Deliverables:

The data and Analysis Coordination Centre should develop protocols (standard operating procedures and data interpretation principals) to help others develop reproducible studies. This activity should be accompanied with a dissemination plan to share such protocols. Manage data to publicly recognised resources where they will continue to be available and updated.

RfP 2:

Title: An *in vivo* exposure model that will give reproducible apical endpoints that can be used for correlative studies involving molecular and cellular assays.

Objective:

The study should be of high confidence, reproducible and highly interpretable in terms of underlying high-confidence epigenetic events; therefore information should be gathered on the potential genomic, cellular and transcriptomic influences upon the epigenome that will allow the high-confidence identification of cell-innate epigenetic changes associated with the phenotype.

Scope:

Focus on early life exposure resulting in later onset of adverse endpoints with emphasis on reproductive toxicity but not restricted to fertility. Identify and justify the choice model compounds and the preferred model system. The use of validation using *in vitro* systems is encouraged to complement the *in vivo* systems. Longitudinal studies that enable the identification of permanent epigenetic changes (and therefore higher certainty of being associated with phenotype) are also encouraged. These studies should build towards future follow-up studies including the detailed description of the test system so that it can be reproduced in other laboratories and geographies.

4.3 Discussion

***In vivo* vs. *in vitro* studies**

In vivo studies are the priority in a research programme related to epigenetics and reproductive toxicity, because in vivo studies are the means by which robust systems with known adverse outcomes can be developed, and the context for understanding is established. Nevertheless, it was agreed that in vitro studies will be needed in order to complement the findings of in vivo work because they:

- May provide epigenetic markers that could be used to complement the *in vivo* studies;
- can help address mechanistic questions around causality;
- can be used in the longer term to develop simple test systems for regulatory purposes that accommodate the 3R perspective;
- it is important to choose adequate cell models that are able to mimic epigenetic rearrangements occurring during development, e.g. differentiation models. In contrast, tumour cell lines should be avoided as they are already perturbed, both genetically and epigenetically.

Regulatory relevance

1. **Too early to augment test guidelines:**

   There are examples where test guidelines (e.g. OECD TG 421 and TG 422, 2015) have been augmented on the basis of a thorough literature review and data analysis.

   Some participants indicated it is important to include adequate apical endpoints in epigenetic studies. This would already be an important signal as inclusion of epigenetic markers without inclusion of relevant apical endpoints would make the hazard interpretation impossible. They suggested that tissues could be collected and stored now (this could be a non-compulsory option) to be used as a tissue resource for retrospective analysis once epigenetics science has progressed. Such an approach, they said, would make better use of studies already performed and reduce animal use. Retrospective analysis of data from TGs is an acceptable approach at the OECD and is being increasingly utilised as part of the preparation for updating TGs with additional relevant endpoints. However, other participants, including industry and scientists from the USA, agreed that current understanding of toxicant-induced epigenetic change is still too limited to be formally incorporated into current test guidelines as a default requirement, and that more research is needed to demonstrate that examining epigenetic endpoints provides value in a regulatory context. Earl Gray from the US Environmental Protection Agency said: “It is too early at this time to augment current test guidelines, which have been used extensively and their value is known. We do not know what the added value of epigenetics measurements is”.

2. **More data on chemicals of concern:**

   The participants from EU chemicals regulation emphasised that currently, there is insufficient information on the apical endpoint for a lot of chemicals. Regulators have poor data on chemicals of concern for generational effects. They advised that studies to collect these data should be conducted now in order to have the results in the future to more effectively regulate chemicals of concern.

Intergenerational vs. Transgenerational studies

Discussions around transgenerational effects (those observed in generations that are not directly exposed to the initial signal or environment that triggered the change), concluded that these effects should not be the focus of study at this time. This is because publications reporting toxicant-induced transgenerational effects in mammals were poorly reproducible and therefore the human health significance of reported toxicant-induced transgenerational effects has not been established. The consensus of opinion was that rather than focusing on transgenerational effects, a more appropriate starting point would be to investigate and understand the potential consequences of environmentally-induced epigenetic effects within a single generation, or between parent and child (intergenerational), and to understand somatic effects (epigenetically-mediated cellular memory of prior toxicant exposure within an individual). Particularly since these, or similar mechanisms, would also likely mediate hypothetical transgenerational effects.

Uncertainty Analysis: It was recommended that a systematic literature review with weight of evidence and uncertainty analysis would be required to underpin inter- and intra-lab variability, before designing the definitive models.

Determine which epigenetic alterations represent adverse changes, adaptive changes or are 'biological noise'. Discussion centred on the fact that at this stage, we are not able to distinguish and cannot predetermine adaptive effects from adverse effects, especially when the causal link between epigenetic measurements and apical endpoints is not established.

Establish causality as much as possible: Reproducible epigenetic endpoints must be identified and examined within a mechanism/mode of action/adverse outcome pathway framework (including, but not limited to gene expression and histology) to establish a robust, mechanistically viable association between an epigenetic change and an *in vivo* adverse outcome. These relationships must be examined across time and the dose-response continuum.

Study design: There is a real need for reproducible and high confidence study designs with interpretable results. Too many epigenetic studies to date have lacked causality determination, were underpowered, and have used only a single, high dose-level exposure. Multiple dose levels must be examined to determine the dose-response relationships between an epigenetic endpoint and other molecular or apical endpoints. Careful analysis of cell populations should be performed to identify a true epigenetic alteration rather than an 'epigenetic effect' being caused by a change in cell ratio within the sample. The annex of the OECD 2012 review concluded that epigenetics studies to date were not sufficiently informative and recommended the need for definitive studies to better inform regulatory developments.

What epigenetic endpoints to measure: Discussions and presentations revealed that the following epigenetic endpoints are relevant: DNA methylation (including DNA hydroxymethylation) and histone post-transcriptional modifications of genes and miRNAs.

5. CONCLUSIONS AND RECOMMENDATIONS

The aim at the outset of the workshop was four-fold; here the conclusions and recommendations arising from this workshop will be discussed under each of the four relevant headings:

1. **Define Epigenetics and understand its potential value for reproductive toxicology**

   During the workshop, speakers defined Epigenetics as follows:
   - "Heritable modifications, superimposed on DNA base sequence, that regulate gene expression." (*Jessica LaRocca*).
   - "Heritable information governing a cell state unrelated to DNA sequence variability, or information that can be inherited from a parent cell that is not encoded in the DNA sequence." (*John Greally*).
   - "Chemical modifications of DNA that control expression of genes." (Daniele Fallin).
   - "Chemical modifications of chromatin (histone PTMs, ncRNAs) which affect gene expression and may be heritable, and play a role in reproductive toxicology." (Peter Alestrøm).

   All four definitions seem concordant. The potential value for reproductive toxicology is that the study of epigenetics may elucidate important mechanisms that address why and how early life exposures can result in adverse health outcomes later in life (i.e. epigenetic analysis may contribute to understanding mechanism/mode of action). However, since the linkage of specific epigenetic alterations to adverse apical outcomes has not been established, it is too early to causally link epigenetic changes to altered health outcomes, and thus, too early to apply routine epigenetic assessments to regulatory applications.

2. **Understand the relationship between epigenetic change and adverse endpoints**

   Proof of principle has not yet been fully established; however methods to help achieve this were described during Day 1. Whilst there have been numerous studies attempting to test how toxicant exposures during pregnancy affect the epigenome of offspring, these studies are poorly interpretable and poorly reproducible (OECD, 2012). In order to move forward with our understanding of the relationship between epigenetic change and adverse endpoints, future studies should be carefully designed to yield high confidence, high interpretability and high reproducibility. As such, guidelines of best practice should be developed and disseminated. The next step in furthering our understanding of the relationship between epigenetic change and adverse effects is to identify strong apical endpoints for use in models to investigate mechanisms of toxicity and causal linkage between epigenetic and apical endpoint changes. Research proposals and model compounds to achieve this were identified during the two-day workshop.

   The workshop also identified several areas to be included in future epigenetic considerations regarding epigenetics:

   - *Agreement on definitions and semantics.*
   - *Decide and justify what to measure:* DNA methylation (including DNA hydroxymethylation) and histone post-transcriptional modifications of genes and miRNAs.

- *Decide and justify what model systems are relevant to human health* (rats, zebrafish, *in vitro* assays, etc).

Use a consortium approach: multi-disciplinary engagement in the design of epigenetic studies is a requirement. Necessary disciplines may include: toxicology, regulatory toxicology, epidemiology, molecular epigenetics, statistics, bioinformatics, and developmental / reproductive biology.

3. Develop a Roadmap for the practical use of epigenetic studies in regulatory applications

Regulators stated that they need more data on apical and epigenetic endpoints for chemicals of high concern and that this could be extracted from augmented TGs. However, proof of principal is needed before epigenetics can be incorporated into regulatory applications: models with strong apical endpoints are required to investigate epigenetic mechanisms of toxicity and validate a robust functional linkage between epigenetic and apical (adverse) endpoints. The following elements will be required to achieve this:

- ***Model compounds*** should be selected on the basis of a strong understanding of known phenotypic (apical endpoint) effects that are relevant to the hypothesis (e.g. if the endpoint is male infertility, then this is not an appropriate model to test for effects in F2 or F3 offspring because no offspring will be produced). Possible model compounds were suggested:

Model Compound	Known Phenotypic Endpoint
Dexamethasone	Male and female infertility
Phthalates	Male reproductive effects
Dioxin	Male reproductive effects
Oestrogen	Female puberty and reproductive effects
DES	Cancer
Valproate	As a control (known epigenetic modifier: histone deacetylase inhibitor; neural tube defects)

- ***The organism of choice:*** choices must be based upon a thorough understanding of their advantages and limitations with regard to risk assessment in humans. Biological relevance to the human and mechanistic understanding to underpin regulatory utility is the primary driver. Cost, time and throughput criteria should also be considered. For example, zebrafish are evolutionarily more distant from humans compared to mammalian models, and their eggs are pre-treated with chemical preparations. However, they have the advantage that F_2 is sufficient to study transgenerational inheritance as compared to F_3 in mammals (however, workshop participants agreed that investigating transgenerational effects would not add value at this time). Some of the commonly used rat strain reference genomes are poorly annotated and may require upgrading with additional genome sequencing to maximise interpretation of experimental data and make them more useful as research models. However, their ability to gestate offspring makes the rat model more relevant for some applications when extrapolating to humans.
- ***Consistent and standardised data management and transparency of experimental design:*** Critical to the practical use of epigenetic studies is that they be of high confidence, high interpretability and high reproducibility – which have not been the case to date. Therefore,

participants propose the need for developing and disseminating standard operating procedures and data interpretation principles.

- ***A consortium approach*** is required in both the design and implementation stage of epigenetic projects. Bioinformatics expertise will be necessary to ensure proper analysis of high content data, but also required will be experts in toxicology, regulatory toxicology, epidemiology, molecular epigenetics, histopathology, and developmental/reproductive biology.
- ***Defining normality*** for the epigenetic endpoint(s): including normality at the time of analysis and normality within the system (tissue, cell, etc).
- **In vitro *studies are needed to complement* in vivo *studies*** as they will help elucidate and validate mechanistic understanding, including secondary mechanisms (Kanno *et al*, 2013).
- ***Additional elements*** such as dose-response and No-Effect-Level determination, exposure route and stability vs. transience must also be addressed. Epigenetic study must add value over and above what is already available in terms of mechanistic insight or predictive capacity.

4. Generate a prioritised research agenda

A consensus should be reached on the study type, species and strain. The studies should be performed under standardised conditions as required for regulatory studies (e.g. OECD TG 421, 2015). It might also be useful to ask CROs or companies' experimental facilities to provide control tissue out of current studies. This would save animal usage as well as costs and would guarantee defined conditions. Three possible research proposals were outlined as shown below:

1. Develop *in vivo* exposure models that will provide reproducible apical and epigenetic endpoints that can be used for correlative studies involving molecular and cellular assays. Complement with *in vitro* studies to further elucidate and validate mechanistic understanding and markers.[2] Include the identification of sensitive and predictive early epigenetic markers for latent adverse outcomes following early life exposure. Evaluate feasibility of these epigenetic markers for relevance in human health risk assessment.
2. Define epigenetic normality across different laboratories, species and tissues.
3. Develop an "Enabling Resources in a Data Analysis and Coordination Centre" for data management and analysis standardisation.

[2] This research proposal is a result of two similar proposals developed during the course of brainstorm discussions and combines the outcomes of breakout group 1 (see page 25) and breakout group 3, RfP 2 (see page 27).

ABBREVIATIONS

3-MC	3-methyl cholanthrene
5AC	5-azacytidine
5-mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
AhR	Aryl hydrocarbon receptor
AhRKO	Aryl hydrocarbon receptor knock-out
AOP	Adverse outcome pathway
BPA	Bisphenol A
bw	Bodyweight
CoE CERAD	Centre of Excellence for Environmental Radioactivity
CRO	Contract research organisation
DBP	Di-n-butyl phthalate (Dibutyl phthalate)
DEHP	Diethylhexyl phthalate
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
EDC	Endocrine disrupting chemical
EGF	Epidermal growth factor
EPA	(US) Environmental Protection Agency
ER	Oestrogen receptor
EU	European Union
EWAS	Epigenome-wide association studies
F_1	First generation of offspring
F_2	Second generation of offspring
F_3	Third generation of offspring
F_4	Fourth generation of offspring
FET	Fish embryo toxicity
FGF	Fibroblast growth factor
GD	Gestation day
GLP	Good Laboratory Practice
IGF-1	Insulin-like growth factor I
LC-MS	Liquid chromatography - mass spectrometry
Lin	Linuron

MEHP	Mono(2 ethylhexyl)phthalate
miRNA	MicroRNA
MoA	Mode of action
MOE	Margin of exposure
MRC	Medical Research Council
ncRNA	Non-coding ribonucleic acid
NGC	Non-genotoxic carcinogen
NIHS	National Institute of Health Sciences
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-Operation and Development
P_0	Parental generation
PBML	Primary bone marrow lymphomas
nihsPPAR	Peroxisome proliferator-activated receptor
PTMs	Post-translational modifications
Q-PCR	Quantitative polymerase chain reaction
QSAR	Quantitative structure-activity relationship
RfP	Request for proposal
RNA	Ribonucleic acid
RRBS	Reduced representation bisulphite sequencing
SD	Sex determination
TCDD	2,3,7,8-tetrachlorinated dibenzo-p-dioxin / 2,3,7,8-tetrachlorodibenzo-p-dioxin
TDG	Thymine-DNA glycosylase
TG	Test Guideline
TGE	Transgenerational effects
WD	Wolffian duct

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APPENDIX 1: WORKSHOP PROGRAMME

PROGRAMME DAY 1: THURSDAY, 12TH NOVEMBER

Open to all ECETOC member companies and invited guests

10:30-11:00	Registration and coffee	
11:00-11;10	Welcome and Introduction	**Alan Poole** ECETOC SG, Belgium

Session 1

Level Setting: Reproductive Toxicity Studies and Epigenetic Studies

11:10-11:40	What are reproductive toxicity guideline studies? Why are they conducted? What can they achieve? Where are the gaps?	**Jessica LaRocca** Dow, USA
11:40-12:00	What are epigenetic studies? Technical aspects and data interpretation to achieve confidence in epigenetic studies (human epidemiology and animal-based studies)	**John Greally** Albert Einstein College of Medicine, Yeshiva University, USA
12:00-12:20	Effects on rat reproductive development produced by antiandrogens: upstream indicators of downstream effects	**Paul Foster** NIEHS, USA
12:20-12:40	Effects on rat reproductive development produced by antiandrogens: AOPs and transgenerational effects	**Earl Gray** EPA, USA
12:40-13:00	The lack of Transgenerational Inheritance of Anti-androgenic effects after Vinclozolin Treatment	**Roland Buesen** BASF, Germany
13:00-14:00	*Lunch*	
14:00-14:20	The potential application of microRNAs in regulatory toxicology	**Emma Marczylo** Public Health, England
14:20-14:40	Introduction of Percellome Project, highlighting the concept of "signal toxicity". Case study: Single exposure adult studies and developmental cases, at signal dose levels, i.e. at the level of No overt cytotoxicity or organ toxicity monitorable by histopathology	**Jun Kanno** NIHS, Japan

Session 2
Understand the Relationship between Epigenetic Change and Adverse Endpoints

14:40-15:00	What are the general principles for indicating whether the epigenetic change is causal (adverse) or adaptive?	**Bob Chapin** Pfizer Inc, USA
15:00-15:20	Practical experience with rodent species used in toxicology studies: what is a normal epigenetic background?	**Richard Meehan** Medical Research Council, UK
15:20-15:40	Determining clear and robust biomarkers of epigenetic change and adversity	**Daniele Fallin** Johns Hopkins University, USA
15:40-16:00	Scoping Discussion: Can we start to identify key biomarkers and potential mechanisms associated with the phenotypic adverse change? (sound techniques, robust data interpretation procedures)	**Tim Gant/Miriam Jacobs** CRCE, UK
16:00-16:20	The concept of "repeated exposure" and possible links to epigenetic regulations. Repeated dose studies introducing baseline responses and transient responses with possible link to epigenetics	**Jun Kanno** NIHS, Japan
16:20-17:00	Zebrafish Model – applications to address what is relevant for multiple generational studies	**Peter Aleström** Norwegian University of Life Sciences, Oslo, Norway
17:00-17:20	How can these be applied to current regulatory test paradigms?	**Ioanna Katsiadaki** CEFAS Weymouth Laboratory, UK
17:20-17:40	Consensus on what and how?	**Tim Gant/Miriam Jacobs** CRCE, UK
17:40-18:00	Closure and guidance for Day 2	**Alan Poole** ECETOC SG, Belgium
18:30-20:00	*Networking cocktail for Day 2 participants*	

PROGRAMME DAY 2: FRIDAY, 13TH NOVEMBER
INVITED EXPERTS ONLY

08:30-08:45	Registration and coffee	
11:00-11;10	Welcome and Objectives for the day Instructions for Breakouts	**Alan Poole** ECETOC SG, Belgium

09:10-11:15

Breakout Topic 1:
Develop a Roadmap for the Practical Use of Epigenetic Studies to Underpin Regulatory Applications

Topic 1 45 minutes	*Epigenetic Design Studies:* *Relevance to Regulatory Applications?*	Moderator: **Miram Jacobs** *Rapporteur:* **Jessica LaRocca**
Topic 2 45 minutes	*How to Design Epigenetic Studies?*	Moderator: **John Greally** *Rapporteur:* **Madeleine Laffont**
Topic 3 45 minutes	*What is necessary to interpret Epigenetics in light of Reprotoxocity Studies*	Moderator: **Roland Buesen** *Rapporteur:* **Kamin Johnson**
11:15-11:45	*Coffee Break*	
11:45-13:00	Plenary: Breakout Conclusions	**Moderators**
13:00-14:00	*Lunch*	

Breakout Topic 2:
Generating a Prioritised Research Agenda

14:00-15:30	Generate Prioritised Research Agenda	
15:30-16:30	Plenary: Breakout Conclusions	***Rapporteurs***
16:30-17:00	Summary and Closure	**Alan Poole** ECETOC SG, Belgium

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APPENDIX 4: Background document

Background document on epigenetics in relation to the workshop on 'The role of Epigenetics in reproductive Toxicity'

Authors: Miriam Jacobs and John Greally

Current OECD human health related TGs and potential for epigenetic additions in relation to reproductive / developmental chemical safety

All human health TGs cited are publically available from the OECD website (http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788).

Table 1: OECD Test Guidelines that could potentially be adapted for epigenomic studies of effects of endocrine disruptors (ED) and reproductive toxicity

Type of study	Test Guidelines (TG)	Description
Alternative models integrating multiple mechanisms of action	TG 236	Zebrafish Embryo Acute Toxicity Assay (see discussion in Table 2)
		Xenopus Embryo Thyroid Assay (XETA assay) in validation
General exposure studies	TG 451	Carcinogenicity Studies
	TG 452	Chronic Toxicity Studies
	TG 453	Combined Chronic Toxicity / Carcinogenicity Studies
Post-mitotic cell studies	TG 424	Neurotoxicity Study in Rodents
Prenatal effects	TG 414	Prenatal Development Toxicity Study
	TG 426	Developmental Neurotoxicity Study
Reproductive effects	TG 415, TG 416	One- and Two-Generation Reproduction Toxicity
	TG 421	(Revised) Reproduction / Developmental Toxicity Screening Test
	TG 422	(Revised) Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test
	TG 443	Extended One-Generation Reproductive Toxicity Study
Genotoxicity tests	TG 483	(Revised 2015) Mammalian Spermatogonial Chromosome Aberration Test
	TG 488	Transgenic Rodent Somatic and Germ Cell Gene Test
Potentially relevant tests to be used in combination	TG 473	*In vitro* Mammalian Chromosomal Aberration Test

Table 2: Updated OECD Endocrine Disruptor Testing Conceptual Framework combined with potential epigenetic tests and preliminary reference chemicals

Level	Mammalian and non-mammalian Toxicology	Epigenetic test information	Potential prototype chemicals to determine the sensitivity and specificity of model systems
1 Existing Data and Non-Test Information	Physical and chemical properties, e.g. MW reactivity, volatility, biodegradability	Epigenetic literature review information	
1	All available (eco)toxicological data from standardised or non-standardised tests	Epigenetic literature review information	
1	Read across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions	e.g. literature-derived information about DNA methylation, RNA and miRNA expression studies and chromatin structure and modification data, with analyses to identify biomarker s for detection of compounds with epigenetic ED activity	
2 *In vitro* assays providing data about selected endocrine mechanism(s) / pathways	Oestrogen or androgen receptor binding affinity	Combine with TG 473 but leave out the use of metaphase-arresting substances in exposed cells, this could then be used to screen for potential downstream epigenetic effects	**Positive:** for ER 17β-Oestradiol **Positives:** for ER and epigenetic effects Diethylstilbestrol (DES), BPA, Genistein, Equol (includes metabolism) **Positives:** for AR Testosterone **Positives:** for AR and epigenetic effects Vinclozolin, Flutamide, Hydroxyflutamide (metabolite) **Negatives:** for ER effects Corticosterone, Spironolactone, Atrazine, Linuron
2	Oestrogen receptor transcriptional activation (TG 455)	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Studies of chromatin components and structure	**Positives:** for ED and epigenetic effects DES, Bisphenol A (BPA), Genistein, Equol (includes metabolism), OH Tamoxifen
2	Androgen or *thyroid* transcriptional activation (if/when TGs are available) Androgen Receptor STTA TG (lead Japan) expected 2016, validation work completed	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Studies of chromatin components and structure	**Positives:** for ER and epigenetic effects DES, BPA, Genistein, Equol (includes metabolism) **Positives:** for AR and epigenetic effects Vinclozolin, flutamide, hydroxyflutamide (metabolite)

Level	Mammalian and non-mammalian Toxicology	Epigenetic test information	Potential prototype chemicals to determine the sensitivity and specificity of model systems
2	Steroidogenesis *in vitro* (TG 456)	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Studies of chromatin components and structure - Multivariate / systems analysis to identify key regulatory factors mediating variability of steroidogenesis on a chemical specific basis	**Positives:** for ED Prochloraz, Forskolin, Atrazine, Aminoglutethimide, BPA, Dibutyl phthalate (DBP) **Negative:** for ED Human chorionic gonadotropin (HCG)
2	*MCF-7 cell proliferation assays (ER ant/agonist)*	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Studies of chromatin components and structure - Multivariate / systems analysis to identify key what is mediating variability of cell proliferation	*As for ER / transactivation assays, plus substances acting through oestrogenic but not receptor pathways (e.g. through non genomic pathways, sulphotransferases etc)* *DBP*
2	*Zebrafish embryo epigenetic assay adaptation of zebrafish embryo toxicity test (zFET). TG 236 and EASZY assay (a zebrafish embryo-based assay for Endocrine Active Substances)*	**Relevant endpoints:** - DNA modifications (cytosine methylation) - Studies of chromatin components and structure EASZY assay (in validation): Epigenetic effects upon thyroid	
2	Xenopus Embryo Thyroid Assay (XETA assay) (in validation)	**Relevant endpoints:** Re: thyroid hormone pathway physiology and epigenetic modification particularly in trans- and multigenerational pathways - DNA modifications (cytosine methylation) - Studies of chromatin components and structure Key epigenetic targets identified in the Zebrafish screens above could be examined cross species on the same genes in Xenopus embryos	
2	*Possible additional examples* *1. Casa assay (sperm cell toxicant)* *2. Comet assay (sperm cell mutagen)*	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Luminometric methylation analysis (LUMA) for global methylation analyses - Studies of chromatin components and structure	1. Valproic acid, DES, lindane, carbenazim, nonylphenol 2. DES, lindane, carbenazim, nonylphenol di-2-(ethylhexyl) phthalate (DEHP), DBP
	3. Sertoli cell assay *4. Leydig cell assay (cross ref with steroidogenesis assay TG 456)* *5. Oogenesis, follicular culture* *6. Mouse embryonic stem D3 cell assay (Kleinstreuer et al, 2011)* *7. Human embryonic stem cells* *8. Rat whole embryo culture toxicity assay*	- Multivariate / systems biology / reverse engineering analyses for the identification of gene modules critically involved in transcript abundance during development, to elucidate relevant regulation factors and pathways	3. BPA and as above 4. DES, carbenazim, nonylphenol, taxol, ketoconazole 5. DES, genistein, carbenazim, nonylphenol, ketoconazole

Level	Mammalian and non-mammalian Toxicology	Epigenetic test information	Potential prototype chemicals to determine the sensitivity and specificity of model systems
3 *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)	Uterotrophic assay (TG 440)	**Less relevant endpoint:** correlation changes in uterine tissue with molecular changes (epigenomic assays)	
3	Hershberger assay (TG 441)	No end organ present, not appropriate for testing	
4 *In vivo* assays providing data on adverse effects on endocrine relevant end-points	Repeated dose 28-day study (TG 407) TG 422	**Relevant endpoints:** - DNA modifications (cytosine methylation) - miRNA and RNA expression studies - Studies of chromatin components and structure e.g. Testicular histopathology combined with epigenomic dysregulation assays With tissues of interest available, need to consider issues of sample collection and preservation, cellular heterogeneity etc, as discussed in Greally and Jacobs, 2013	
4	Repeated dose 90-day study (TG 408)		
4	1-generation assay (TG 415)	1. Combination with TGs 451 (Carcinogenicity Studies), 452 (Chronic Toxicity Studies) and 453 (Combined Chronic Toxicity / Carcinogenicity Studies) with focus on hormonally-responsive tissues: combination with epigenomic assays 2. The rat model of IUGR and quantified cytosine methylation throughout the genome in beta islet cells from the pancreas of young adult rats, results indicate 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, Fazzari *et al*, 2010). BPA studies all showed changes in cytosine methylation associated with exposure, some changes occurring at loci that were found to be transcriptionally altered.	Valproic acid (male: reduction of spermatogenesis, testicular atrophy, degeneration of seminiferous tubules; female: polycystic ovaries high serum testosterone and menstrual disorders. Teratogenic)
4	Prenatal Development Toxicity Study (TG 414)		
4	Chronic toxicity and carcinogenicity studies (TG 451-3)		
4	Reproductive screening test (TG 421 from July 2015: enhanced)	Oestrus cycles, follicle counts, oocyte maturation, ovarian integrity; thyroid integrity, spermatogenesis combination with epigenomic assays including RNA analysis, toxicogenomics and multivariate data analyses	

Level	Mammalian and non-mammalian Toxicology	Epigenetic test information	Potential prototype chemicals to determine the sensitivity and specificity of model systems
4	Combined 28-day / reproductive screening assay (TG 422 from July 2015: enhanced) Developmental Neurotoxicity (TG 426)	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 time-points could shed light on epigenetic effects of agents used for exposure	
5 *In vivo* assays providing *more comprehensive* data on adverse effects on endocrine relevant endpoints over *more extensive parts of the life cycle of the organism*	Extended one-generation reproductive Toxicity Study (TG 443)	Necropsy and neurological studies of the tests for TG 426, 414, 424 etc	Valproic acid, DES, lindane, carbenazim, nonylphenol BPA, DBP, DEHP Taxol, ketoconazole, genistein, vinclozolin, methoxychlor
5	2-generation assay (TG 416 most recent update)	TG (416) could allow multiple tissues to be sampled in offspring of parents exposed to the agent of interest, allowing screening for inherited epimutations	DES, lindane, carbenazim, nonylphenol BPA Taxol, ketoconazole, genistein, vinclozolin, methoxychlor and as above

Italicised tests are not in OECD TG workplan.

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D-2016-3001-242

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