Epigenetics and Chemical Safety
5-6 December 2011, Rome

Workshop Report No. 23

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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4 Avenue E. Van Nieuwenhuyse (Bte 6), B-1160 Brussels, Belgium.

Epigenetics and Chemical Safety

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1. SUMMARY

Epigenetics is a rapidly developing and expanding biological science. In order to increase our knowledge of how the science of epigenetics could have an impact in (eco)toxicology, a solid understanding of the biology and variation of the epigenome is essential to better assess concerns about possible adverse health effects related to epigenetic changes. In particular, little is known about which epigenetic alterations are part of normal variability and which could be considered adverse, hence posing a health risk. To obtain a better insight into the current state of the art of epigenetics and to discuss its potential applications in (eco)toxicology, ECETOC organised a workshop with expert participants in the field of epigenetics as well as (eco)toxicological risk assessment.

Epigenetic regulation of gene activity appears to be a general mechanism to maintain cell function, homeostasis, proliferation and differentiation. This indicates that epigenetic mechanisms are likely to be a key component in biology. Although epigenetics is still a very young science, some mechanisms appear to be well established. DNA (deoxyribonucleic acid) methylation and histone modification have been identified as important factors in epigenetic dependent regulation. DNA methylation involves the addition of a methyl group to the 5' carbon of cytosine in a CpG sequence (cytosine-phosphate-guanine). Methylated cytosines (5mC) are primarily found in CpG rich regions. Histone tails can also be covalently modified by a number of different processes, e.g. methylation, acetylation, phosphorylation. In terms of regulation, it is important to note that histone modifications require both writers and erasers of the histone code. An example of the rapid development of epigenetics is the fact that DNA methylation changes were considered to be permanent in the very recent past, while this process is now known to be reversible. It most likely involves several processes that are still not fully understood at the biochemical level.

Another fascinating aspect is that, in plants, it has been shown that DNA methylation epialleles can be transmitted over multiple generations and maintained in the population. The situation is thought to be different in mammals, where embryonic development is characterised by two waves of global DNA demethylation erasure (in preimplantation embryos and primordial germ cells) that theoretically prevent the transmission of DNA methylation patterns through generations. However, it is unclear whether this erasure completely erases all germ line DNA methylation patterns.

Finally, microRNAs (miRNAs), a large family of non-coding RNAs (ncRNAs) that are evolutionarily conserved, endogenous and 21-23 nucleotides in length, need to be taken into account. miRNAs regulate gene expression by targeting messenger RNAs (mRNAs) by binding to complementary regions of targeted transcripts to repress their translation or trigger mRNA degradation. miRNAs are encoded by the genome, and more than 1000 human miRNAs have

been identified so far. How miRNAs function in regulating cell responses to environmental chemical stimuli is an unexplored field of compound risk evaluation.

The outcome of the workshop indicates that there are major gaps in knowledge about the extent of background variability in epigenetic processes and their normal dynamic range. Moreover the magnitude of change necessary for a cellular effect (be it adverse or adaptive), windows of susceptibility, the extent of autoregulation and redundancy in the system are not known.

There is evidence however that toxicants are capable of affecting the epigenome in perinatal and adult periods. Several examples were discussed in this workshop and can be found in this report. What is not known is which of the changes are directly associated with chemical exposure and adverse effects and which changes are the result of the cell's attempt to maintain homeostasis, i.e. are in effect beneficial.

In addition to presenting the state of the art, the workshop focussed on a number of basic issues which need to be addressed when new scientific information becomes available that has potential value for enhancing the quality of the risk assessment process. In a nutshell, here are some of the conclusions from the debate. It is uncertain which endpoints of (eco)toxicology will be particularly affected by epigenetic changes. miRNAs appear to be a part of the regulatory mechanisms affecting gene expression, and it is a matter of debate whether these should be included under the term 'epigenetic'. Epigenetic changes are not adverse *per se*. One of the major challenges will be to examine the nature of an epigenetic change. Three possible types of interactions could be considered: 1) true adaptive responses (non-adverse, potentially beneficial), 2) a direct interaction with the epigenetic control machinery (most likely adverse) and 3) an inappropriate epigenetic change at an inappropriate stage (critical period) resulting in a maladaptation (adverse). It can be expected that epigenetic changes will occur at dose levels lower than the no observed effect level (NOEL), but that these changes do not necessarily need to be adverse. Epigenetic changes observed in rodents may be relevant for humans to obtain better understanding of the process in cells and organs at a mechanistic level. The epigenome appears to be rather dynamic and potentially reversible. It was agreed that the dose-response curve resulting in an epigenetic change should be distinguished from a dose-response curve for an adverse effect and that epigenetic change does not necessarily lead to an adverse outcome.

What can be expected of epigenetics? Certainly another layer of complexity and most likely another mechanism by which the cell is able to integrate information in cascades of feedback mechanisms in an attempt to provide the best response to changes in its environment. A revolution in biology is underway. The discovery of epigenetic-dependent regulations of cell functions is changing our understanding of cell biology in a profound way. We are starting to decipher how complex epigenetic regulations are. The significance of epigenetic changes for classical (eco)toxicological endpoints is not yet clear and some of the methodologies for

measuring such changes are also still developing. At this time, it is probably too early to use epigenetic information within standard risk assessment paradigms. Epigenetic information can be expected to contribute to the understanding of the basic processes of cellular responses to environmental stressors (be it chemical or physical in nature). This insight is expected to provide a better assessment of the consequences of exposure to such stressors.

2. WORKSHOP OVERVIEW

2.1 Introduction

With the increased interest of the scientific community in epigenomics, the field is rapidly evolving but is still at a relatively early stage with respect to its significance to (eco)toxicology. Currently, epigenetic testing is insufficiently validated to be included into the regulatory process of chemicals. For example, there is no single test available for determining epigenetic effects, and there is an incomplete understanding of the normal DNA-modification patterns and long-term effects such as those on public health. In addition, a screening scheme to prioritise chemicals through epigenetic analysis has not been developed. Epigenetic changes can be triggered by environmental factors, e.g. exposure to metals, persistent organic pollutants. Endocrine disrupting chemicals have been shown to modulate epigenetic markers, not only in mammalian cells and rodents, but also in environmentally relevant species such as fish and water fleas.

In order to increase our understanding of how the science of epigenetics is involved in (eco)toxicology and in turn, risk assessment, a solid understanding of the biology and variation in the epigenome is essential to eliminate concerns about possible adverse health effects related to epigenetic changes. In particular, little is known about which epigenetic alterations are part of normal variability and which could be considered adverse, hence posing a health risk. Still under debate is the extent to which the fundamental principles that guide toxicology, such as relevant doses, dose rates, routes of exposure and experimental models, should to be taken into consideration in the design and interpretation of epigenomic studies.

This workshop addressed the relevance of epigenetic changes to the evaluation of chemicals. In particular, it examined scientific and technological approaches to identifying and quantifying the epigenetic effects of chemicals. This helped to assess their potential effects on human health and the environment. A series of breakout groups addressed specific questions and their findings were discussed at a plenary session.

2.2 Workshop structure

- Series of 20-minute talks and case studies.
- Syndicate sessions addressing specific questions.
- Plenary feedback.
- Further discussions.
- Conclusions.

2.3 Workshop objectives

- The relevance of epigenetic changes to the evaluation of chemicals.
- Examine scientific and technological approaches to identify and quantify epigenetic effects of chemicals and to assess their potential effects on human health and ecology.

In particularly the workshop addressed the following points:

1. Definition of epigenetic changes and effects in the context of (eco) toxicology.
2. What are the consequences of epigenetic changes induced by exogenous substances on human and environmental health?
3. Is the current way of assessing the safety of chemicals able to detect adverse effects related to epigenetic changes?
4. Case studies: Implications of epigenetics in adverse effects in humans, in animal models and ecotoxicology.
5. Recommendation of research needs.

3. PRESENTATION SUMMARIES

3.1 The epigenome: The interface between the environment and the genome

Frederick L. Tyson
National Institute of Environmental Health Sciences, USA

In recent years considerable effort has been invested in supporting research in the field of epigenetics and human disease. Epigenetics refers to a set of processes that regulate gene expression without modifying DNA sequence. The best known and most frequently studied epigenetic processes involve DNA methylation, histone modifications and several classes of ncRNAs. An epigenome is the constellation of epigenetic modifications covering the entire genome of a given cell. Specific epigenomic signatures are associated with specific cell types. Epigenetic programming of gene expression affords the genome its remarkable plasticity, allowing for one compliment of DNA or a single genome to give rise to the multiple phenotypes associated with diverse array of unique cells and tissues found in our bodies. Driven by temporal, spatial and environmental stimuli that initiate and maintain epigenetic changes, sets of programmed gene expression are established during development and over the life course, conferring specific phenotypes on cells. Each cell in a single human has the same compliment of genomic DNA, yet different epigenomic programmes direct expression profiles such that the same compliment of DNA can code for muscle cells, neuronal cells, hematopoietic cells, skin cells, intestinal cells, etc. This goal of this presentation is to provide an overview of some of the best known epigenetic modifications and discuss how environmental exposures can alter epigenetic programmes and thereby influence disease outcomes.

DNA methylation is the most widely studied epigenetic modification. It involves the addition of a methyl group to the 5' carbon of cytosine in a CpG sequence. 5mC are primarily found in CpG rich regions, called CpG islands. CpG islands vary between 300 - 3000 bp in length and are found in and near promoters in mammalian genomes. A group of DNA methyltransferases, methylated DNA binding proteins and methylated binding domains are involved in DNA methylation processes. It is beyond the scope of this presentation, but worth noting that there is an additional DNA epigenetic modification, hydroxymethylation of the 5' carbon of cytosine that produces 5hmC. 5mC is frequently referred to as the fifth base and now 5hmC is known as the sixth base.

Another widely investigated set of epigenetic processes and marks are determined by histone modifications. Histone tails can be covalently modified by methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, and ADP-ribosylation. Histone modifications require writers and erasers of code. Writers include acetylases, methylases and phosophorylases. Erasers include deacetylases, demethylases and phosphatases. Readers of histone modifications are specialised protein domains that recognise patterns of histone

modifications and facilitate the recruitment of factors that respond to histone modifications to initiate or repress transcription. Epigenetic modifications serve to open chromatin and make it accessible to transcription factors, remodelling complexes, etc, which facilitate gene transcription. Alternatively, epigenetic modifications can also serve to close chromatin, reduce or remove access to the DNA or gene and repress or silence transcription. In general, combinations of epigenetic modifications, i.e. histone acetylation and DNA demethylation status are associated with open chromatin and active transcription. Histone deacetylation and DNA methylation are associated with repressed / closed chromatin and transcriptional silencing.

There are a number of types of ncRNAs that are transcribed but have no apparent protein product. These include many classes of ncRNAs such as microRNAs, small RNAs (ribonucleic acids) and long intergenic RNAs (lincRNAs) according to their size and function. lincRNAs have been associated with scaffolding, recruitment of histone remodelling complexes and epigenetic mechanisms in different cell types. This concludes the introduction of epigenetic mechanisms. The rest of the presentation will address reprogramming of epigenomes by environmental chemicals and diseases influenced by exposures and aberrant epigenetic programmes.

How do environmental toxicants / chemicals influence epigenetic processes and disease pathogenesis?

A broad range of developed mentally induced human pathologies have been linked to chemical exposures. Including reproductive / endocrine disorders, e.g. breast / prostate cancer, endometriosis; autoimmune disorders; cardiovascular / pulmonary disease, e.g. asthma, heart disease / hypertension, stroke; and brain / nervous system disorders, e.g. Alzheimer's disease, Parkinson's disease, attention deficit hyperactivity disorders (ADHD) which have been linked to endocrine disrupting chemicals such as bisphenol A (BPA), organochlorine pesticides, dioxins, polychlorinated biphenyls (PCBs), lead and tributyltin. A major component of epigenetics research supported by the National Institute of Environmental Health Sciences (NIEHS) focuses on disease outcomes subsequent to developmental exposures to environmental factors during a critical window of susceptibility during the development of target organ. It is postulated that developmental exposures during a critical window of susceptibility, alters the epigenetic programming of cells, leading to increased disease susceptibility. A new paradigm for understanding the origin and development of exposure-induced diseases has emerged in the last few years. In the past it was believed that there was a simple relationship between environmental exposures and disease – that is, an exposure happens, and the exposure causes disease – we now understand that the effects are more complex. The current trends in research addressing the developmental basis of disease from an environmental epigenetics perspective, suggests exposures can occur *in utero* or during the neonatal or infant stages and symptoms and disease development are manifested at various different stages of later life.

Examples by NIEHS supported investigators are discussed that show how exposures *in utero* can re-programme the epigenomes such that abnormal responses are made to normal intrinsic signals, resulting in disease symptoms. Additional discussions are made to indicate that the epigenomes directs genomic responses to environmental stimuli, making the point that there is a great deal of interaction between the environment and the genome, with the 'epigenome' as the interface.

Question posed to the presenter

In the experiments presented on estrogenic compounds and epigenetic effects, did the investigators look into dose-response relationships?

Tyson: I do not know, the experiments were performed at a colleague's laboratory.

3.2 The potential of chemicals to cause epigenetic alterations

John P Thomson[1,2], Harri Lempiäinen[2,3], Jamie Hackett[1,2], Colm E. Nestor[1,4], Arne Müller[3], Federico Bolognani[3], Edward J. Oakeley[6], Dirk Schübeler[5], Rémi Terranova[3], Diana Rheinhardt[1,2,4], Jonathan G. Moggs[2,3], **Richard R. Meehan**[1,2,4]

[1] MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Western General Hospital, Edinburgh, U.K.
[2] Member of MARCAR consortium.
[3] Investigative Toxicology, Preclinical Safety, Translational Sciences, Novartis Institutes for Biomedical Research, Basel, Switzerland.
[4] Breakthrough Breast Cancer Research Unit, University of Edinburgh, Western General Hospital, Edinburgh, U.K.
[5] Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland.
[6] Biomarker Development, Human Genetics and Genomics, Translational Sciences, Novartis Institutes for Biomedical Research, Basel, Switzerland.

Evidence suggests that epigenetic perturbations are involved in the adverse effects associated with some drugs and toxicants, including certain classes of non-genotoxic carcinogens (NGCs). Such epigenetic changes (altered DNA modification and covalent histone modifications) may take place at the earliest stages of carcinogenesis and their identification holds great promise for biomedical research. As part of the MARCAR consortium (http://www.imi-marcar.eu/), the sensitivity and specificity of genome-wide epigenomic and transcriptomic profiling in phenobarbital (PB)-treated B6C3F1 mice, a well-characterised rodent model for early events leading to non-genotoxic liver carcinogenesis have been evaluated. Modified DNA Immunoprecipitation (MeDIP and hDIP)-coupled microarray profiling using tiled promoter arrays was combined with genome-wide mRNA expression profiling to identify liver tissue-specific PB-mediated DNA modification and transcriptional alterations. Only a limited number of significant correlations were observed between PB-induced transcriptional and promoter-based DNA modification perturbations. However, the constitutive androstane receptor (CAR) target gene Cyp2b10 was found to be concomitantly hypomethylated and transcriptionally activated in a liver tissue-specific manner following PB treatment. Furthermore, analysis of active and repressive histone modifications using chromatin immunoprecipitation revealed a strong PB-mediated epigenetic switch at the Cyp2b10 promoter. Our data suggest that the drug-inducible CAR pathway regulates an epigenetic switch from repressive to active chromatin at target genes. This study demonstrates the utility of integrated epigenomic and transcriptomic profiling for elucidating early mechanisms and biomarkers of non-genotoxic carcinogenesis.

Questions posed to the presenter

What is the scale of the phenomenon? If the same tissue is measured in two animals, will it be stochastic? Can we extrapolate from cell to tissue?

Meehan: Epigenetic changes reflect the cell of origin in case of cancer.

If oxidation is the rate-limiting step for the hmC rate, how do we manage with non-physiological oxygen concentrations in in vitro *experiments?*

Meehan: We should stick to animal models for the time being.

Experiments on PB are mainly done in the liver, but there are many methylation changes in kidney, as confirmed by two independent labs. Should we compare methylation changes between target and non-target organs?

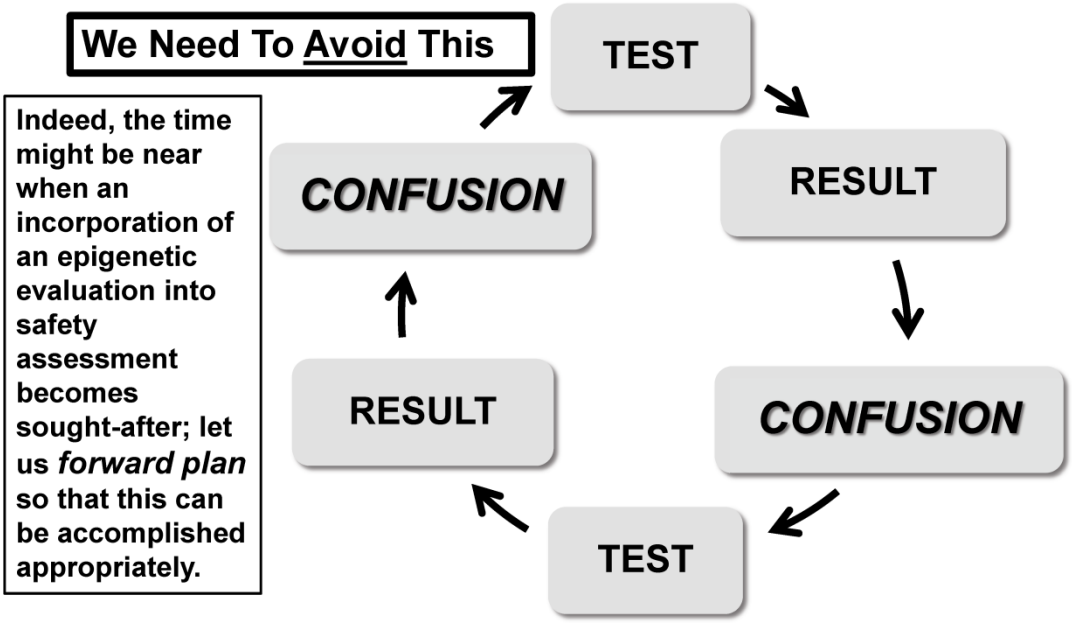
Meehan: Good point!

3.3 Epigenetics meets toxicology: Is it time to incorporate an epigenetic evaluation into risk assessment?

Jay I. Goodman
Michigan State University, Department of Pharmacology and Toxicology, East Lansing, MI, USA

The answer is 'no'. More basic information is required before contemplating the incorporation of an epigenetic evaluation into safety assessment in order to avoid testing that leads to confusion and more testing (Figure 1). The term epigenetics refers to heritable mechanisms superimposed on DNA base sequence that regulate gene expression (thus, the term epi- (Greek: over, above)-genetics). Methylation at the 5 position of DNA-cytosine is an epigenetic modification that can affect transcription. The histone code and ncRNAs also contribute to epigenetic regulation. Importantly, these parameters are interrelated. Epigenetics is a dynamic field that is expanding in new dimensions. Recent research indicates that components of the core promoter recognition complex change in a cell-specific fashion to turn on one transcriptional programme while turning off others, providing a novel epigenetic mechanism. Additionally, three new DNA bases have been identified recently (5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxycytosine) and their role(s) in epigenetic regulation of transcription is a focus of considerable attention. There is an overall extensive, and growing, amount of research in the area of epigenetics. This is especially true in toxicology, where there is considerable interest in understanding the extent to which epigenetic changes might underlie toxicity (as a causative or susceptibility factor), including adverse transgenerational effects. Understandably, this leads to discussions about epigenetics and risk assessment. However, there are numerous fundamental issues that must first be addressed before incorporating an epigenetic evaluation into risk assessment. For example, which aspect(s) of epigenetics (e.g. methylation, histone code, ncRNAs) will be evaluated; what genomic region(s) will be monitored; what methodology(ies) will be used; what biological endpoint(s) will be evaluated; what model system(s) and compounds might be employed? This presentation will address these basic, prerequisite issues within the context of the requirement that attention be paid to fundamental principles of toxicology, e.g. the need to understand the degree of normal variability (inter-individual, species-to-species and over time), dose-response, criteria for an appropriate maximum test dose, and what constitutes a change as compared to an adverse effect. In light of the issues discussed above, this is not the time to incorporate an epigenetic evaluation into risk assessment. However, it might be sought-after in the not too distant future. Therefore, let us forward plan so that this can be accomplished appropriately (Goodman *et al*, 2010).

Figure 1: Epigenetics meets toxicology: This is NOT the time to incorporate an epigenetic evaluation into risk assessment



Questions posed to the presenter

Remark: There are new publications available that mutations in key genes lead to changes in the methylation pattern. The genome does also seem to play a role in epigenetics.

With regard to risk assessment: should epigenetic results be ignored for the time being?

Goodman: We should not incorporate epigenetic data into the risk assessment for chemicals as long as we do not have apical endpoints, do not know the level of variability, and as long as the adversity of effects remains unknown. Scientific investigations are highly encouraged, but currently no inclusion of epigenetics into risk assessment is recommended.

The complexity of the field has to be stressed if one thinks e.g. of the large number of new histone modifications now known. But should we wait before taking epigenetics into account for toxicological assessments?

Goodman: We definitely cannot wait until we know everything.

If inbred strains are used for experiments in order to reduce variation, what is the impact on the epigenetic parameters?

Goodman: Emphasises that there is much variation already from experiment to experiment if the same sample is measured several times. So the use of inbred strains might be useful.

Which animal strain would be the recommended model for methylation?

Goodman: Recommends starting with two inbred strains to get a feeling for variability first.

3.4 Epigenetic marker as a component for systems toxicology useful for cancer prediction in addition to genetic toxicity

Sue-Nie Park
Korea Food & Drug Administration, Seoul, Republic of Korea

New regulatory requirements (e.g. the Registration, Evaluation, Authorisation and Restriction of Chemical substances recently implemented in Europe) have spurred the development of new technologies and testing strategies for chemical safety. OECD's (Organisation for Economic Co-operation and Development) molecular screening and toxicogenomics programme aims to facilitate regulatory acceptance of molecular screening and toxicogenomics as either alternative or complementary approaches. Its main purpose now is to develop a tool for prioritisation and categorisation of chemicals. At the moment, there are nine subgroups under the Molecular Screening Project. These include chemical nomination, databases and several pathway specific subgroups such as the Cancer epigenetics subgroup.

Evidence suggests that epigenetic perturbations are involved in the adverse effects associated with some drugs and toxicants, including certain classes of NGCs. Such epigenetic changes (altered DNA modification and covalent histone modifications) may take place at the earliest stages of carcinogenesis and the identification of related markers surely holds great promise for both risk assessments of chemical safety and biomedical research. Just recently, OECD distributed a letter proposing to develop a work plan for development of Adverse Outcome Pathways. This is directly associated with developing tools able to predict chemical toxicity focusing on MoA (mode of action) determination including the molecular level. MoA determination facilitates the building up of systems toxicology including toxicogenomics. I was mainly involved in the identification of biomarkers for genotoxic and/or non-genotoxic biomarkers. It was proposed to include epigenetics in systems toxicology for determining the possibility of identifying cancer prediction markers which could be useful for categorising chemicals in addition to genotoxicity test methods currently implemented. In this presentation, the goal statement and progress of the cancer epigenetics subgroup in the OECD molecular screening and toxicogenomics programme will be presented. In the future, depending on further development and evaluation, cancer epigenetics may be used more widely for carcinogenic potential assessments of chemicals for regulatory purposes not only for genotoxic but also for non-genotoxic carcinogens (NGCs).

3.5 The environment and the epigenome: Implications for environmental safety

Moshe Szyf
McGill University, Department of Pharmacology and Therapeutics, Montreal, Quebec, Canada

Identifying agents that have long-term deleterious impact on health but exhibit no immediate toxicity is of prime importance. It is well established that long-term toxicity of chemicals could be caused by their ability to generate changes in the DNA sequence through the process of mutagenesis. Several assays including the Ames test and its different modifications were developed to assess the mutagenic potential of chemicals (Ames *et al*, 1973a; 1973b). These tests have also been employed for assessing the carcinogenic potential of compounds. However, the DNA molecule contains within its chemical structure two layers of information. The DNA sequence that bears the ancestral genetic information and the pattern of distribution of covalently bound methyl groups to cytosines in the DNA. DNA methylation patterns are generated by an innate programme during gestation but are attuned to the environment *in utero* and throughout life including physical and social exposures. DNA function and health could be stably altered by exposure to environmental agents without changing the sequence, just by changing the state of DNA methylation. Our current screening tests do not detect agents that have long-range impact on the phenotype without altering the genotype. The realisation that long-range damage could be caused without changing the DNA sequence has important implications on the way we assess the safety of chemicals, drugs and food and broadens the scope of definition of toxic agents.

Several examples of environmental effects on DNA methylation patterns were discussed. Early life adversity results in stable changes in DNA methylation in rodents, non-human primates and humans that is hypothesised to lead to life-long changes in the phenotype. First, a candidate gene approach was used and a focus on target tissue such as the hippocampus. Weaver *et al* (2004) showed that variations in maternal care result in differences in DNA methylation and histone acetylation in the *GR*/*NR3C1* gene encoding the glucocorticoid receptor (GR exon 1_7 promoter) that emerge early in life and remain stable into adulthood. Cross fostering experiments showed a causal relationship between maternal care and the DNA methylation differences and reversal of the phenotypes with epigenetic drug treatments supported a causal relationship between DNA methylation differences and phenotypic variation. Although it is impossible to provide causal evidence for early life experience altering DNA methylation states in humans, as it is ethically unfeasible to randomise in humans early life abuse, it is possible to associate DNA variations with differences in early life environments. The state of methylation of *rRNA* gene promoters and *NR3C1* promoter in the hippocampus were examined in a cohort of suicide victims in Quebec who were abused as children and their control group. Site-specific differences in DNA methylation in the *NR3C1* exon 1f promoter and its expression were detected between suicide completers who had reported social adversity early in life and suicide completers who did not experience social adversity early in life.

Genes don't act independently but through functional gene circuitries. In addition, the phenotypic response to early life adversity involves multiple phenotypes suggesting a system-wide response. If indeed the response of DNA methylation states to early life adversity is an adaptation rather than a stochastic disruption, it should involve an organised change in DNA methylation across the genome. We tested this hypothesis in several studies. All studies point to the conclusion that the impact of early life adversity on the epigenome is broad and that it involves multiple systems and is not limited to the brain. This has important implications on the feasibility of assessment of DNA methylation changes induced by the environment in non-target tissue such as peripheral blood cells.

The changes in DNA methylation associated with differences in rearing in rhesus monkeys are widespread in the genome and are not limited to the brain and since they also occur in T cells . A study of the impact of socio-economic positioning on DNA methylation that examined blood DNA from the British birth cohort of 1958 has been initiated. This study detected a signature of DNA methylation that is associated with early life adversity supporting the hypothesis that social environment DNA methylation signatures are found system wide and can be examined in peripheral blood cells.

Implications:

1. First, environmentally derived epigenetic changes act as a memory of an exposure that lasts a life-time and is not detected by standard safety assays.
2. Second, the impact on phenotype could not be overt early on but nevertheless have profound impact on life. Many of the effects might be detected in adulthood by using sophisticated behavioural and physical phenotyping.
3. Changes in DNA methylation are genome wide and might require genome-wide methodologies to be detected.
4. DNA methylation changes that are triggered by environmental exposures could be measured in peripheral tissues and could provide information on the functional pathways involved.
5. There are high-throughput assays that could be utilised to measure the impact of environmental agents on the DNA methylation machinery.

Questions posed to the presenter

What is the paradigm to pick up the effects described in your talk?

Szyf: There can be good or detrimental effects. The main point is to pick them up in first instance measuring methylation changes. In-depth investigations will then help to identify further effects (example: valproic acid).

What is the impact of altered DNA methylation as measured in the nursing studies?

Szyf: Not only DNA methylation but also transcription was measured and found altered in these studies.

3.6 DNA methylation: Reprogramming during development and impact of endocrine disrupters

Michael Weber
Institut de Génétique Moléculaire de Montpellier (IGMM), France

DNA methylation is an epigenetic modification that plays essential roles in development and disease: it is required for embryo survival and perturbed in many pathologies such as cancer. In addition, alterations of DNA methylation by xenobiotic agents and its possible transmission through generations are a matter of great epidemiological concern. Studies in plant organisms have shown that DNA methylation epialleles can indeed be transmitted over multiple generations and maintained in the population. The situation is believed to be different in mammals where embryonic development is characterised by two waves of global DNA methylation erasure (in pre-implantation embryos and primordial germ cells) that theoretically prevent the transmission of DNA methylation patterns through generations. Very little is yet known about the targets and mechanisms of DNA methylation reprogramming during mammalian development, and more studies are required to get a complete picture of the dynamics of DNA methylation patterns during development at the genome level.

Our laboratory is using genome-wide approaches to better characterise the targets and mechanisms of regulation of DNA methylation during mammalian development, using mouse as a model. Recently, we used an optimised mapping strategy for low amounts of cells to identify the target sequences of DNA methylation during early mouse development. Our results show that DNA methylation is involved in the regulation of key developmental genes. We also show for the first time that certain non-imprinted genes resist global methylation reprogramming in pre-implantation embryos, which suggests that DNA methylation at certain targets can be transmitted from parental gametes to the next-generation. We applied a similar strategy in primordial germ cells and showed that the extent of DNA methylation erasure is more complete than in pre-implantation embryos. However we identified very rare sequences that consistently maintain high levels of DNA methylation in pre-implantation development and germ cells, which indicates that there is a potential, although minimal, for transgenerational transmission of DNA methylation over multiple generation in mice.

Currently, we are applying epigenome mapping to identify abnormal DNA methylation patterns in mouse models exposed to endocrine disrupters (EDs). Mice are exposed to EDs during pregnancy and samples are collected from the F_1 to the F_3 generation. The objectives of this project are to clarify whether EDs induce abnormal DNA methylation marks in germ cells of exposed embryos, and how persistent these epigenetic alterations are in the subsequent generations.

In order to consider introducing DNA methylation studies in the pipeline of toxicology, several aspects need to be taken into consideration. First, more studies are required to define the 'normal' epigenetic landscape in order to be able to evaluate potential effects of environmental chemicals. Second, the use of cultured cellular models needs to be rationalised because these cells accumulate abnormal patterns of DNA methylation in culture. Third, the field of epigenetics is moving very fast, in particular on the technological aspects, which makes it difficult to define standardised protocols. Finally, a key aspect that is very much debated in the field is whether epigenetic changes are a cause or a consequence of changes in gene expression. In my opinion, the most important question that needs to be answered is whether epigenetic approaches would help to identify toxic effects that are presently not identified by other methods. In that respect, one point that deserves more investigation is whether environmental compounds could induce small epigenetic changes that accumulate over time and generations in exposed populations.

Questions posed to the presenter

The three EDs chosen for your studies do all have different effects and modes of action. How will this impact your results?

Weber: This was done on purpose to see if there will be different methylation patterns.

May epigenetic effects be strain-specific?

Weber: We will only use a single strain C57BL/6 for our experiments.

What doses will be used?

Weber: The first dose will be equivalent to human exposure, the second dose 100-fold above.

Wouldn't it be better to use outbred rats since inbreds might produce artefacts?

Weber: As published by Skinner only outbred strains show certain effects.

Two experiments in Wistar rats (outbred) could not reproduce the "Anway data".

We use inbreds since they are easier for interpretation of the experiment; it is not the aim to prove Skinner is right or wrong.

3.7 Endocrine disrupting compounds and microRNAs

Mohamed Benahmed
INSERM, Nice, France

miRNAs are a large family of ncRNAs that are evolutionarily conserved, endogenous, and 21-23 nucleotides in length. miRNAs regulate gene expression by targeting messenger RNAs (mRNAs) by binding to complementary regions of transcripts to either repress their translation or trigger degradation. miRNAs are encoded by the genome, and more than 1000 human miRNAs have been identified so far. miRNAs are predicted to target as much as 60% of human mRNAs and are expressed in all animal cells and have fundamental roles in cellular responses to xenobiotic stresses, which affect a large range of physiological processes such as development, immune responses, metabolism, tumour formation as well as toxicological outcomes. How miRNAs function in regulating animal cell responses to environmental chemical stimuli is an unexplored field of compound risk evaluation. Data originating from different *in vivo* and *in vitro* models were presented to illustrate how environmental chemicals and particularly endocrine disrupting compounds may affect miRNAs expression and function machinery. These ncRNAs may link environmental chemicals and their related diseases in a new gene expression regulatory mechanism paradigm.

Questions posed to the presenter

Several speakers have shown that nutrition can influence the development. Do we have to be concerned about reduced food consumption in 2-generation studies leading to adverse effects on the offspring that cannot be distinguished from the effects produced by the chemical tested?

Benahmed: This is a difficult question. Parallel testing of a possible effect of food consumption might be useful.

Are increased levels of miRNA in human blood increased in parallel to increased levels in testes?

Benahmed: miRNAs tend to accumulate in blood.

Can miRNAs be seen as blood biomarkers of damaged cells similar to liver enzyme levels in blood?

Benahmed: This is not definitively sure. Some miRNAs are rapidly degraded in blood, others are not.

3.8 The identification of non-genotoxic carcinogens today

Romualdo Benigni
Istituto Superiore di Sanita', Environment and Health Department, Experimental and Computational Carcinogenesis Unit, Rome, Italy

For decades, traditional toxicology has been the ultimate source of information on the carcinogenic potential of chemicals: carcinogenesis results in rodents are a consistent and reliable indicator and predictor of human cancer risk (Huff *et al*, 1991; Huff, 1999a,b; Haseman *et al*, 2001; Tomatis *et al*, 2001). With increasing demand on regulation of chemicals and decreasing resources for testing, opportunities to accept 'alternative' approaches have dramatically expanded. However, the need for tools able to predict chemical carcinogens in shorter times and at a lower cost in terms of animal lives and money is still a research priority, and the present strategies and regulations for the prescreening of carcinogenicity do not adequately defend human health.

DNA-reactive carcinogens can be identified efficiently by the Ames test or by the Structural Alerts (SA), but other *in vitro* and *in vivo* mutagenicity tests do not have added value and rather impair the prediction ability of the Ames test alone (Tennant *et al*, 1987; Shelby *et al*, 1993; Zeiger, 1998; Benigni *et al*, 2010; 2012). In addition, alternative assays able to identify NGCs are not usually considered in regulations (Hernández *et al*, 2009).

However, there is evidence that the combination of the Ames test and the SAs for the DNA-reactive carcinogens, and *in vitro* cell transformation assays (more precisely, the Syrian Hamster Embryo (SHE) assay) for the non-genotoxic carcinogens (NGCs) permit the identification of a very large proportion of carcinogens (Benigni and Bossa, 2011a). From a mechanistic point of view, the successful combination of two assays that exemplify theories often presented as antagonistic (the somatic mutation theory, and the tissue organisation field theory respectively) may indicate that the distinction between genotoxic and NGCs is not so sharp, and that the theories on the early stages of carcinogenesis are not mutually exclusive. On the contrary, different pathways in the carcinogenesis process may co-exist and should be taken into account in testing strategies.

The success of the combination of the Ames test and the SHE assay in identifying carcinogens is in striking contrast with the fact that the majority of the reductionist approaches are weak predictors of *in vivo* biological phenomena. For example, constant patterns of chromosomal aberrations have been found in a number of tumours (Duesberg *et al*, 2005; Rowley, 2009). However, the *in vitro* assays for the induction of chromosomal aberrations are not good tools for the identification of chemical carcinogens (Zeiger, 1998; Benigni *et al*, 2010). Another example is the controversy on hypomethylation. The observation of hypomethylation of human tumours has been followed by the identification of hypermethylated tumour-suppressor genes and

inactivation of miRNAs genes by DNA methylation. However, contradictory evidence (i.e. experiments in which hypomethylation led to fewer tumours than expected) has been reported as well (Baker *et al*, 2010). Another example, which is also relevant to many modern trends in toxicology, is represented by the intensive use of genomics and proteomics technologies in the field of drug design. Wide panels of 'omics and high-throughput screening tools are used to identify *in vitro* promising compounds to be studied to a deeper extent in further steps of the drug development process as well as to predict undesirable toxic effects early in the design process. However, it appears that in recent years the number of new drugs entering e.g. the US market has declined sharply, while spending by the pharmaceutical industry on research and development has steadily increased (Young, 2007; Walters *et al*, 2011). The two single most important reasons for attrition in clinical development are (a) lack of efficacy and (b) clinical safety or toxicology, which each are estimated to account for 30% of failures. Failures have been largely ascribed to the lack of correlation between effects observed in isolated receptors *in vitro* and those observed in whole animals and in humans (Hopkins, 2008; MacDonald and Robertson, 2009). As a consequence, these new tools are considered to be able to permit hypothesis testing rather than definitive human hazard identification or risk assessment.

Thus, the Ames test and the SHE assays represent two success stories, and are quite unique in the landscape of the reductionist *in vitro* systems. It is interesting to speculate about the reasons for their success. The Ames test is sensitive to a very large family of carcinogens that are able to interact with DNA according to various molecular mechanisms (e.g. direct or indirect alkylation, acylation, intercalation, formation of aminoaryl DNA-adducts) (Benigni and Bossa, 2008b; 2011b). It should be added that chemicals able to react with DNA are usually also able to interact with proteins, thus they are able to interfere with the cellular processes in many different ways. The SHE cell transformation assay detects phenotypic alterations which are characteristic of tumorigenic cells. It should be emphasised that *in vitro* cell transformation can be produced by a plethora of different molecular mechanisms that do not include the induction of mutations (Bignami *et al*, 1984). Several suggestions have been made as to how the transformation assays may detect chemical carcinogens that operate by a range of mechanisms of action. It has been proposed that the assays may be detecting a basic carcinogenic change common to different modes of carcinogenesis. Within the theory on the four stages of cell transformation, SHE may be sensitive to a larger range of carcinogens types than other transformation assays because it detects more basic and non-specific mechanisms. An additional / alternative proposal is that the success of the SHE assay may be due to the use of primary cells containing a wide variety of cell types susceptible to a range of different transformation pathways (Mauthe *et al*, 2001; Haga *et al*, 2007). Thus both assay systems are characterised by a remarkable degree of non-specificity and very wide reach, and probably represent the 'optimal' level of investigation, between the microscopic and macroscopic levels of biological phenomena.

The contribution of SAs concepts to the identification and coding of the action mechanisms, and to the development of alternative strategies should be remarked as well. In this paper, the use of SAs for the rapid and inexpensive identification of DNA-reactive carcinogens has been presented. The availability of free, user-friendly implementations, e.g. the expert system Toxtree (free download: http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxtree) (Worth, 2010; Benigni and Bossa, 2011b), allows every scientist to easily apply the SAs to the query chemicals of interest. It should be emphasised as well that the SAs concepts have a much wider application (Benigni and Bossa, 2008a; 2011b; Woo, 2003; Woo and Lai, 2010), and are also at the basis of the Read-Across and Regulatory Category approaches aimed at filling gaps in experimental data by similarity with other, already tested chemicals (Van Leeuwen *et al*, 2009; Worth, 2010).

In conclusion, it appears that alternative approaches for the identification of carcinogens are more imminent than originally believed. The combination of the Ames test and the SAs for the DNA-reactive carcinogens, and the SHE assays for the NGCs permits the identification of a very large proportion of carcinogens. So, they constitute a solid ground for refinements by future research. However, the present, commonly accepted strategies for the pre-screening of carcinogenicity are quite inefficient, do not adequately protect public health and so need to be updated urgently.

Question posed to the presenter

How many human NGCs have been identified from the rodent bioassay?

Benigni: Some have been found. The number is going to rise due to the fact that more and more compounds are developed where genotoxic structures have been depleted.

3.9 Early exposure and late effects: The role of DNA methylation in prenatal programming

Juliette Legler
Institute for Environmental Studies, VU University Amsterdam, The Netherlands

Environmental factors *in utero*, including exposure to contaminants, may alter epigenetic dependent gene expression, with important consequences for development and susceptibility to disease. As part of the 'developmental origins of human health and disease' (DOHaD) hypothesis (Gluckman and Hanson, 2004), research groups worldwide are studying the implications of early life exposure to environmental chemicals on long-term human health via altered epigenetics. The DOHaD paradigm stems from the knowledge that environmental factors in early life can have profound influences on lifelong health. One of the most dramatic examples of this comes from the studies of the Dutch Hunger Winter cohort, in which prenatal famine has been associated with adult incidence of diseases such as cardiovascular disease, obesity and cognitive dysfunction (Schulz, 2010). In addition, permanent changes in methylation patterns have been found for these cohorts (Tobi *et al*, 2009). When focusing on obesity, evidence indicates that perturbations of central endocrine regulatory systems established in early gestation may contribute to the development of obesity in later life (Phillips and Young, 2000). Early life exposure to environmental chemicals has also been implicated in altering developmental programming, possibly resulting in higher susceptibility to obesity. One of the most convincing lines of evidence that chemicals may be obesogenic stems from animal studies performed with the synthetic oestrogen diethylstilbestrol (DES). Mice treated with very low concentrations of DES during neonatal life show a significant increase in body weight as adults (Newbold *et al*, 2007). Similarly, mice treated *in utero* with the environmental pollutant tributyltin, a compound used in anti-fouling paints and plastics, show elevated lipid accumulation at adult age (Grün *et al*, 2006). Epidemiological studies indicate an association between cord blood levels of environmental contaminants and elevated weight in children (Smink *et al*, 2008; Verhulst *et al*, 2009).

The Institute for Environmental Studies leads a European Commission-funded project called OBELIX ("OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life"), in which the hypothesis that prenatal exposure to endocrine disrupting chemicals plays a role in the development of obesity later in life (see also http://www.theobelixproject.org; Legler *et al*, 2011) is being examined. One of the main goals of this project is to determine whether epigenetic-dependent gene regulation is a mechanism underlying the potential 'obesogenic' effects of prenatal exposure to chemicals. OBELIX focuses on six major classes of EDCs found in food including dioxins and dioxin-like PCBs, non-dioxin-like PCBs, brominated flame retardants (BFRs), organochlorine pesticides, phthalates and perfluorinated alkyl acids (PFAAs), e.g. perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS). These chemicals have various endocrine disrupting properties

and have been implicated in affecting energy homeostasis and growth in epidemiological and/or animal studies. However, information on the causal relationships between early life exposure to these classes of EDCs and the development of obesity and related disorders later in life is scarce.

OBELIX takes a multidisciplinary approach that combines epidemiological studies in humans with animal and *in vitro* studies in the laboratory, in order to ultimately perform risk assessment of EDCs for their role in obesity development. The foundation of the exposure and health assessment research in OBELIX is a network of mother-child cohorts from four countries (Belgium, The Netherlands, Norway and Slovakia) (Legler *et al*, 2011). Initial studies have focused on the association between birth weight and cord blood PCB153 and p,p'-DDE levels using meta-regression analysis, including almost 8000 cases, making it by far the largest study ever performed on human reproductive effects of xenobiotic organochlorines. This study indicates that that low-level exposure to PCB (or correlated exposures) impairs foetal growth (Govarts *et al*, 2011). Impaired foetal growth, as shown in the Hunger Winter Studies, can be a risk factor for obesity later in life. Currently follow-up studies are ongoing in children at later ages in life (up to 8 years) as well as the analysis of other EDCs in OBELIX. In addition, a study is currently being set up to measure DNA methylation in cord blood samples in a case-control setting, in order to investigate possible epigenetic markers of obesity and chemical exposure.

Animal and *in vitro* mechanistic studies are ongoing in the OBELIX project, and indicate that perinatal exposure to EDCs such as BPA enhance body weight in male mice at adulthood (van Esterik *et al*, 2011) as well as stimulate adipocyte differentiation *in vitro* in a mouse 3T3-L1 adipocyte differentiation model (Bastos Sales *et al*, 2011). In the animal studies, DNA methylation analysis is currently ongoing using adult liver DNA samples from control and perinatally treated BPA male mice. Samples have been analysed with the "HpaII tiny fragment enrichment by ligation-mediated PCR" (HELP) assay (Suzuki and Greally, 2010) and have undergone massive parallel sequencing in the laboratory of John Greally, Albert Einstein College of Medicine, New York. Preliminary results indicate differential methylation in adult liver tissue following perinatal BPA exposure at levels below no adverse effect levels (NOAEL) for developmental toxicity. Results from *in vitro* studies indicate changes in global DNA methylation in 3T3-L1 cells exposed to a number of EDCs (Bastos Sales *et al*, 2011). In summary, our preliminary results in OBELIX indicate that early life exposure to certain EDCs may be risk factors for obesity later in life, and DNA methylation changes are concurrent with chemical exposure.

Questions posed to the presenter

Did you also do studies in frogs?

Legler: Studies have been performed also in *Xenopus*.

A large number of 2-Generation studies have been evaluated with regard to potential induction of obesity in offspring. Do you know the outcome?

Legler: There has been no correlation shown but this could be due to the fact that the doses tested were too high.

The compounds looked for in your study have similar lipid solubility. Is this accounted for in the cohorts with regard to nutritional intake?

Legler: Yes we ask for differences in diets and possible sources of the compounds.

So far, EPA (Environmental Protection Agency) has not identified a single endocrine disrupter. How will you do it?

Legler: EPA has failed to identify an EDC because they lack a proper definition. We look for chemicals that interact with hormonal regulation. For this we do not need an EPA definition.

3.10 Epigenetics in an ecotoxicological context

Michiel Vandegehuchte
U Gent, Belgium

Environmental exposure to various stressors has been shown to modulate epigenetic marks such as DNA methylation in several publications. DNA methylation at a locus in the insulin-like growth factor was reduced in adults who experienced the Dutch hunger winter of 1944-'45 *in utero*, which correlated with a higher incidence of coronary heart disease and diabetes (Heijmans *et al*, 2008). Weaver *et al* (2004) and Champagne *et al* (2006) demonstrated that maternal care in rats caused changes in DNA methylation in the pups, as well as the passing on of this licking and grooming behaviour to the next-generation. Also exposure to certain chemicals has been reported to affect DNA methylation. Long-term cadmium exposure resulted in increased DNA methylation in human cells (Jiang *et al*, 2008) and exposure to air pollution (PM_{10}) was associated with reduced DNA methylation in the leukocytes of steel plant workers (Tarantini *et al*, 2009).

Most studies in this context have been performed on human cells or on rodent models. In an ecotoxicological context, it would be interesting to assess the impact of stressors on epigenetic marks, and associated phenotypic effects, in environmentally relevant species. It should be noted, however, that the epigenetic machinery in other species may differ from that of humans and/or rodent models. Taking DNA methylation as an example, it is known that the amount of cytidine methylation differs largely between species, varying from 0% in some invertebrates to more than 30% in some plants (Field *et al*, 2004). In invertebrates, DNA methylation is mainly found intragenically and is hypothesised to repress the spurious initiation of transcription, opposed to the silencing function of promoter methylation in vertebrates.

A limited number of studies, as reviewed by Vandegehuchte and Janssen (2011), assessed epigenetic changes in species other than human, mouse or rat. Aniagu *et al* (2008) exposed three-spined stickleback *Gasterosteus aculeatus* to 30 and 300 ng/L hexabromocyclododecane (HBCD) for 30 days with no result on global DNA methylation, while exposure to 100 ng/L 17ß-oestradiol (E2) for 22-23 days resulted in increased global DNA methylation in male gonads. In a study with bluegill sunfish *Lepomis macrochirus* exposed to 1 µg/L benzo[*a*]pyrene for 40 days + 45 days post-exposure, there was a trend of global hypomethylation in liver DNA from day 2 to post-exposure (Shugart, 1990). Zebrafish showed reduced DNA methylation at three loci in the 5' flanking region of the vitellogenin gene in the liver after exposure to 100 ng/L 17α-ethinylestradiol (EE2) for 14 days (Strömqvist *et al*, 2010). This reduction in DNA methylation was not observed in the brain. One cause of alterations in DNA methylation may be a change in the s-adenosylmethionine: s-adenosylhomocysteine (SAM:SAH) ratio, as suggested in a study with false kelpfish *Sebasticus marmoratus* exposed to tributyl tin (TBT), triphenyl tin (TPT) and a mixture of both compounds in a concentration of 1 to 100 ng Sn

L-1 for 48 days (Wang *et al*, 2009). Several authors studied the epigenetic effects of exposure to metals. In a field study, Pilsner *et al* (2010) showed an inverse relationship between global DNA methylation and Pb content in the brain of male polar bears. When white clover *Trifolium repens* and industrial hemp *Cannabis sativa* were exposed to mixtures of Ni, Cr and Cd for two weeks, concentration-dependent global hypomethylation was observed, with most demethylation being sequence specific (Aina *et al*, 2004). On the contrary, an increase of global DNA methylation was observed in the liver of goldfish *Carassius auratus* exposed to Cu (100 µg/L), Zn (50 µg/L), Pb (20 µg/L), Cd (10 µg/L) and mixtures of these four metals in the same ratio at different concentrations (Zhou *et al*, 2001).

Epigenetic changes can in some cases be transferred to subsequent generations. A nice study was performed by Reinders *et al* (2009), who generated epigenetic recombinant inbred lines ('epiRILs') of *Arabidopsis* by crossing a wild type and a mutant with reduced methylation. These epiRILs had a mosaic DNA methylation pattern and the epigenetic variation of the different lines was reflected in phenotypic variation, e.g. in flowering time, biomass or tolerance to salinity stress. The aberrant DNA methylation at certain loci of these inbred lines could be stably transmitted over 8 generations, although a considerable fraction of these 'epialleles' was not stable over the generations. In a series of studies with the violet *Viola cazorlensis*, Herrera and Bazaga (2010, 2011) showed that epigenetic differentiation of DNA methylation in populations correlated with adaptive genetic divergence and that differential herbivory damage correlated to variation in epigenotypes. This indicated the interconnection between genotype and epigenotype and pointed to the possibility that epigenetic changes play an evolutionary role.

Transgenerational epigenetic changes can also be stress-induced, with effects observed in non-exposed progeny. Temperature or UV-B stress released the silencing of a GUS transgene in *Arabidopsis*, which was associated with a reduction in histone H3 and an increase in histone H3 acetylation (Lang-Mladek *et al*, 2010). The expression of this transgene was observed in small areas of non-stressed F_1 and F_2 progeny, but not in F_3. This could be counteracted by seed aging, pointing at the dynamic nature of epigenetic changes. Exposure of *Arabidopsis* to salt, drought, flood, heat, cold, UV-B stress induced increased / decreased global DNA methylation in non-exposed F_1 progeny (Boyko *et al*, 2010). This was associated with stress tolerance and dependent on ncRNA processing enzymes. Only for drought and salinity was this effect also observed in non-exposed F_2. Verhoeven *et al* (2010) exposed dandelion *Taraxacium officinale* to nutrient stress, salinity, jasmonic acid and salicylic acid, which resulted in DNA methylation variation throughout the genome. Many methylation changes were transmitted to the non-exposed nextgeneration. The highly debated study of Anway *et al* (2005) showed an altered pattern of DNA methylation, co-occurring with a reduced spermatogenic capacity in the non-exposed male progeny down to F_4 of female rats exposed to the pesticides vinclozolin or methoxychlor during pregnancy.

The above mentioned studies inspired us to study the transgenerational effects of Zn exposure on the water flea *Daphnia magna* (Vandegehuchte *et al*, 2009). No direct effect of Zn exposure on global DNA methylation in F_0 was found. However, a significant reduction of global DNA methylation was observed in the F_1 progeny of F_0 Zn exposed organisms. This was not passed on to the F_2 generation and was not reflected in reduced reproduction, whereas the F_0 Zn exposed daphnids did exhibit a decrease in reproduction. In a second study, *Daphnia magna* was exposed to 2.9 mg/L of 5-azacytidine for one generation (Vandegehuchte *et al*, 2010). A reduction in DNA methylation was observed in the exposed F_0, which was passed on to two non-exposed subsequent generations, coinciding with reduced juvenile growth.

This study indicates the possibility that populations can experience the effects of their ancestors' exposure to chemicals, which has implications for environmental risk assessment. In case of such transgenerational effects, risk assessment should incorporate the time interval between effects and exposure in previous generations. More basic research is needed to assess the potential phenotypic and population-level effects of epigenetic modifications in different species and to evaluate the persistence of chemical exposure-induced epigenetic effects in multiple subsequent generations. The potential impact of heritable epigenetic variation on evolution could potentially lead to local chemical stress adaptation (Flatscher *et al*, 2011). In this research, the basic ecotoxicological concepts such as bioavailability, exposure routes and relevant concentration-effect relations should not be neglected.

3.11 Recapitulation Day 1

Jos Kleinjans
University of Maastricht, the Netherlands

Toxicologists can think themselves lucky in being part of the revolution in biology which has been ongoing for a decade or so. In particular, the discovery of epigenetic regulations of cell functions is revolutionary, as biology is now advancing beyond Mendelian inheritability of gene function, and actually, is bringing Lamarck back into the spotlight. We now also get to understand how complex epigenetic regulations are, implying a range of mechanisms such as histone modifications, DNA methylation and microRNA expressions. With possibly more to come…

However, the critical issue for this workshop is: what does epigenetics actually bring to toxicology?

First of all, there is now evidence that toxicants are capable of affecting the epigenome. There are examples of epigenetic deregulation by xenoestrogens such as PCBs, by a range of metals, by particular agents such as valproic acid, etc. Furthermore it has been shown that such toxicants may interfere with the epigenome through multiple mechanisms, from disrupting the homocysteine pathway thus causing a decrease of methyl donation to the DNA and consequently hypomythylation, to deregulating the expression of methyltransferase or histone deacetylase. And, where NGCs are suggested to exert their activity by epigenomic interference, this certainly may complicate the understanding of their already complex range of their mechanisms of action.

Of course, toxicologists are keen to understand in-depth epigenomic mechanisms of action of harmful chemicals. It is important to keep in mind the ultimate goal of toxicology, namely to predict adverse health effects upon – hypothetical – exposure to such agents. Obviously, a complete insight in mechanisms of action would provide the best predictor of human health risks. But we have learned that achieving full understanding of toxic activities is not a realistic goal, and consequently we have learned to decide on human health risks in relation to chemical exposure, by simultaneously accepting a certain degree of uncertainty. So, the question really is: can novel biomarkers be derived from our epigenomics analyses, upon acute and/or chronic exposure, either from animal experimentation or from cellular systems *in vitro,* which strengthen current molecular profiles for predicting adverse human health risk, and can the plausibility of such markers by connecting them to a phenotype which is relevant for (the early stages of) human disease be demonstrated?

4. REPORTS FROM THE SYNDICATE SESSIONS

4.1 Syndicate 1: Definition of epigenetic changes and effects in the context of (eco)toxicology

Moderator: Jos Kleinjans
Rapporteur: Tim Gant

Nathalie Delrue
Juliette Legler
Krista Meurer
David Rouquié
Moshe Szyf
Mathieu Vinken
Michael Weber

1. Are epigenetic changes a mode of action?

Epigenetic changes are a mode of action rather than a mechanism of action. Epigenetic determinants include reversible histone modifications, DNA methylation and miRNA-related control.

- Chemicals are a means by which the phenotype may be altered by modifying of epigenetic marks.

- Chemicals change epigenetic marks on both DNA and histone, as abundantly documented for valproic acid and butyric acid. Evidence that chemicals can interfere with DNA methylation patterns mainly comes from cancer research where cancers have unstable DNA methylation patterns and these compounds can change the epigenetic marks in favour of the differentiated cellular phenotype.

- There are time windows where chemical exposure, altering DNA methylation pattern, may be more crucial, such as during embryogenesis and gamete formation, when DNA methylation patterns are (erased) and re-established.

- Many of the mechanisms by which DNA methylation profiles are changed in gamete formation are not understood, but it is known that these imprinted patterns can be affected by chemicals.

- Not all changes to epigenetic marks in the genome may be adverse. Indeed, epigenetic changes induced by cancer chemotherapy are generally beneficial and hence therapeutic. However, we don't necessarily understand which changes are/may be beneficial and which are/may be adverse. Discerning this difference is a challenge.

- There seems to be a link between endocrine disruption and epigenetics. However it is not known whether this interrelationship is causal or a consequence, i.e. do EDCs act through epigenetic means or do epigenetic marks change as a result of altered signalling from EDCs.

- There is a need to know more about time-response and dose-response effects in changes to epigenetic marks triggered by chemicals in order to establish causality.

- Relevance to humans is being demonstrated through the translation of some animal study results.

- Changes in the DNA methylome can be found in cells *in vitro* in response to chemicals, including valproic acid. However, these changes may not be the same as those produced in the *in vivo* situation. The baseline DNA methylation marks *in vitro* are likely different from those in cells *in vivo*. Therefore, showing relevance of *in vitro* to *in vivo* extrapolation is a major challenge.

- There is a need to consider the window of exposure and the cell type. For example, a compound that changes DNA methylation patterns *in vitro* may have adverse effects for the developing embryo or in gamete, formation but could be advantageous in some somatic cells, such as neuronal cells undergoing age-related DNA methylation changes.

- There is a need to know more about the specificity of chemicals for changing epigenetic patterns in individual cell types and genes.

- *S*-adenosyl methionine changes the methylation profiles in specific subsets of genes. The same may be true for 5-azacytosine that theoretically would be predicted to be relatively non-specific, but actually changes DNA methylation marks in specific gene sets. Glucocorticoids affect genes expression upon interaction with GREs (glucocorticoid-responsive elements), but may affect others through transcription factors and miRNAs that are affected in their expression and in turn can affect other genes. With good computational methods, these interactions can be predicted.

- Dose-response relationships are important for characterisation of epigenetic changes. Epigenetic dose-responses may not be classic because DNA methylation changes can have

beneficial and adverse effects. Cause and effect relationships on a dose-response basis are important.

2. Which endpoint of (eco)toxicology could be affected by epigenetic changes?

- Phenotypes due to epigenetic change can be difficult to detect, which for instance is the case for cognitive decline, though these have a huge impact on society.

- Is there a need to look for the phenotype? Could this be predicted by looking at the genes affected by the altered DNA methylation patterns and then predicting phenotype from the known functions of the genes? Epidemiology, prediction and phenotype testing at the moment need to go hand in hand to make and improve predictions.

- Another example is that by looking at the genes affected, it may be possible to design appropriate tests to look for the predicted phenotype at the appropriate age after exposure.

- An interesting historical point in toxicology has been reached because of the profound changes in technology and the ability of the public to access the information.

    - Starting point for a chemical epigenetic analysis could be to begin with an *in vitro* plasmid-based cell screen assay and to establish dose-response relationships.

    - For positive chemicals move to an *in vivo* assay and look at the tissue of interest using a whole genome screen, such as MeDIP or NGS (next-generation sequencing) to determine which genes are being methylated and demethylated.

    - Then look for differential expression (gene / protein) and from these data make phenotype predictions.

    - Undertake a phenotype assessment based on association with the genes.

- There will not be one paradigm for looking at epigenetic effects of chemicals.

- At this stage, it will be more efficient to focus on model chemicals in order to establish protocols.

- Epidemiology may establish relationships that should be followed up.

- One very important question is what is a normal DNA methylome? This is crucial to understanding an adverse effect.

- Phenotypes with the DNA methylome may not be obvious, these are subtle effects controlled by methylation effects.

- It may be easier to work with the DNA methylation patterns rather than the consequential gene transcription patterns for field studies. Indeed, DNA is easier and more stable to work with than RNA and the transcriptome pattern is constantly changing, which make it challenging. Though in an experimental situation there is no problem with working with the RNA though the plastic nature of the epigenome is still challenging.

- Example: Tributyltin causes obesity, which can be linked to changes in the DNA methylome. This example indicates that the principle of looking at the genes and pathways affected by the DNA methylome may direct towards the phenotype.

The Eco-environment.

- Environmental species may depend on DNA methylation marks for behavioural phenotypes and thus chemicals that may affect epigenetic patterns may be very important.

- This also not limited to animal species, but includes plant species.

CHALLENGING TIMES FOR TOXICOLOGISTS.

3. Should we consider including miRNA in the definition of epigenetics?

- There was disagreement about this question, as some of participants felt that it is part of the epigenetic machinery while others view miRNAs as a mechanism that can be affected by DNA methylation patterns and this in turn may affect their expression and therefore the translation of their target mRNAs. They may also affect epigenetic marks, both DNA methylation and histone modifications, and may be involved in the transmission of epigenetic marks. They are in themselves though not epigenetic marks.

- There is no doubt that miRNAs are important in toxicology and in epigenetics, but this does not make them part of the definition.

- miRNAs species are not persistent. They are expressed from the genome. A definition of epigenetics is that the effect should be persistent.

- However, this is not really a question for toxicologists and risk assessors. Leave this to the debates of the academic biologists.

Questions posed to the syndicate rapporteur

In your definition of epigenetics you have focused on DNA methylation. Is this the only valid marker?

No, this does not reflect the thoughts. Histone modifications and miRNA are also important epigenetic markers, but it remains to determine if they meet the definition of heritable changes.

Did you also talk about having a phenotypic outcome first and then look for epigenetic changes?

This would be done in an epidemiologic approach.

How do we look for epigenetic changes, in which organs, after which treatment time?

DNA methylation is the easiest epigenetic marker, since DNA is more stable than RNA. The data do not yet allow the identification of the most suitable organs to look at on a routine basis.

Is it feasible from a technical point of view to look at DNA methylation in several organs?

The technology is there but needs to be improved, for instance in terms of testing capacity, and to become cheaper.

At what life stage should we measure DNA methylation patterns?

Early embryo development is very important, but also during young and older age. Different life stages should be covered.

Shouldn´t we rethink the focus on early development? Aren´t there many other times in life where we might be affected by epigenetic changes?

In current study designs, early developmental effects are not well reflected.

Remark: As seen from human evidence, early development is one of the most sensitive stages for epigenetic changes.

Remark: Different life stages should be covered in our experiments.

4.2 Syndicate 2: What are the consequences of epigenetic changes induced by exogenous substances on human and environmental health?

Moderator: Ben van Ravenzwaay
Rapporteur: Robert Kavlock

Alan Boobis
Ross Brown
Neil Carmichael
Sarah Dutton
Alessandro Giuliani
Sean Milmo
Sue-Nie Park
Frederick L. Tyson
Maria Uhl

1. Are epigenetic changes adverse per se?

- Epigenetic changes could be key events within a mode of action depending on upstream events and downstream consequences
    - Important to keep focus on the phenotypic alteration.

- Interpretation hindered by lack of knowledge of reference status
    - The normal state of a particular cell type and life stage.
    - Including the extent of normal variability.

- An extraordinarily complex and dynamic process central to normal cell and tissue functioning
    - Autoregulatory mechanisms of function are important as there appear to be built in redundancies of control processes.

- A theoretical question was posed - If an individual with an epigenetic change caused by an environmental factor (e.g. famine or drought) would be exposed to the opposite environmental factor would the original epigenetic change be reversible for the subsequent generation(s)?

- Recall the definition of adverse – an intermediary process / system cannot be considered adverse without consideration of the downstream consequence on physiological function, including (in ecotoxicology) reproductive function linked to adverse population-level effects.

- It was postulated that epigenetic changes could be considered (generic) biomarkers of exposure rather than biomarkers of effect.

- In general, epigenetic responses were considered to be important in the adaptation to 'stress', the impact on survival and reproduction would be a key to the ultimate interpretation.

- Are the current methods of toxicology evaluation adequate to detect the consequence, or is it possible for subtle changes to go overlooked?

2. Would the detection of the epigenetic changes provide information useful for risk assessment?

- Perhaps – the data need to be judged on their quality and its relevance, just as all information is considered in the overall weight of the evidence during the risk assessment process
    - Are the targets-similar in test animals versus humans?
        - e.g. the case of an HDAC inhibitor.
    - Are there special life-stage consequences to be considered?
    - Aid in understanding cross-species responses for NGCs?

- For the near future, there is a need more basic knowledge about the epigenetic system before such information is likely to have an impact on the current state of the science of risk assessment.

- However, given the importance of epigenetic changes to biology, it will undoubtedly become more important to risk assessment as the science evolves.

- Currently there is a lack of information on dose-response relationships at the level of the epigenome and the dose-response relationships for the downstream events, but we could envisage this being considered a suitable alternative (e.g. cholinesterase inhibition and neurotoxicity) at some point in the future.

- The question of thresholds for epigenomic changes was briefly considered, and similar to other discussions, no unique considerations for this potential key event versus other key events (e.g. consider feedback loops, regulatory controls and propagation over levels of biological organisation) were identified.

3. Are epigenetic changes considered an adaptive response to environmental changes?

- Considered three types of interactions

  - A true adaptive response of an organism to a chemical exposure.
  - Two potentially ‘maladaptive’ responses
    - A direct interaction with the epigenetic control machinery.
    - Applying a signal at an inappropriate stage which gives an inappropriate response (critical period).
  - All three cases are dependent on the magnitude, duration, timing and downstream consequences on physiological and reproductive function being impacted.

- Perturbing the epigenetic apparatus may be considered undesirable (e.g. an Ames result), but still dependent on use of the mode of action framework for full interpretation.

Remark: The paradigm of dose-response-relationships should be reconsidered since the response to epigenetic changes might not work that way.

Remark: Dose-response of an epigenetic effect would most probably be bell-shaped. Since there are multiple interacting variables, a linear (or monotonic) dose-response is not to be expected.

Remark: If it is known that a specific chemical is able to induce epigenetic changes an additional uncertainty factor should be considered in the risk assessment.

Questions posed to the syndicate rapporteur

Did the group consider changes on the environmental toxicity level?

Plants and their adaptive response to e.g. climate changes were discussed. The wild-life population is considered to be also affected by epigenetic changes. If a plant species survives a change it would not be considered adverse.

Remark: The environmental context is important for the phenotypic expression. A methylome change e.g. leading to obesity will only appear in the phenotype if the individual has free access to fatty nutrition.

4.3 Syndicate 3: Is the current way of assessing the safety of chemicals able to detect adverse effects related to epigenetic changes?

Moderator: Saskia van der Vies
Rapporteur: Jay Goodman

Mohamed Benahmed
Romualdo Benigni
Mustafa Billur
Richard Currie
Malyka Galay Burgos
Marina Marinovich
Richard Meehan
Michiel Vandegehuchte

1. *Can epigenetic changes occur at doses levels traditional below NO(A)EL?*

   Given that gene expression changes have been shown to occur at doses at or below a traditional NO(A)EL, and epigenetic changes are known to be involved in the regulation of gene expression it is expected that epigenetic changes would indeed occur at dose levels traditionally lower than NO(A)EL. However, evidence for changes at dose levels traditionally below NO(A)EL is currently lacking.

2. *How relevant are rodent epigenetics changes to humans?*

   Epigenetic changes observed in rodents may be relevant to humans especially if the change occurs via a common mechanism in cells and organs. However, data to prove this relevance is currently absent. A clearer understanding of the mechanisms involved in epigenetics is needed, and for this, comparative studies using well characterised compounds will be very useful.

3. *What is the dose-response curve for epigenetic changes by chemical exposure?*

   In principle, the dose-response curve for epigenetic changes can be viewed as any other dose-response curve for a particular chemical including the concept of threshold. The dose-response curves for epigenetic changes may not differ from dose-response curves for other effects when looking at the level of signalling pathways. In a system with redundant pathways, it is questionable if and when an effect can be detected. The phenotypic change might not be related to the gene that showed a change in epigenetic markers. It might also be

that the dose-response curve varies across sites and so will depend on whether the entire genome is analysed or an individual gene.

It was further noted that the epigenome is a dynamic system and that epigenetic changes, in contrast to genetic changes, are potentially reversible. There is some evidence that epigenetic programming as a result of early life events is potentially reversible by certain pharmacological manipulations later in adulthood. In addition, permanent epigenetic changes that occur early in life may be more important than the epigenetic changes upon chemical exposure in later life. It was agreed amongst the group that the dose-response curve resulting in an epigenetic change should be distinguished from a dose-response curve for an adverse effect and that epigenetic change does not necessary lead to an adverse effect.

In addition, a phenotypic change might not be related to a single gene that showed a change in epigenetic markers. It was suggested to first analyse reference compounds that are well-known to be 'safe' i.e. a negative control and also well-known compounds that induce epigenetic changes i.e. positive control, to establish what kind of short and long-term effects can be determined. If 'safe' chemicals induce changes of epigenetic markers a rethink, about whether these markers are the right ones to use or not, will be necessary.

4. *Is it possible to link epigenetic changes at population level?*

In theory it will be possible to link epigenetic changes at a population level. However, in practice there is hardly any data available and at the moment this poses more questions than it answers. For example, what are the most significant parameters that need to be measured? Also, the normal variability in an individual and/or population as a whole is unknown. It should also be considered that different species in the ecosystem may have different mechanisms of epigenetic control.

Questions posed to the syndicate rapporteur

Is there any reason to assume that dose-response curves for epigenetic changes differ from dose-response curves for other effects?

They should not be different if we look at the level of signalling pathways. The dose-response depends on what is measured for the individual gene.

Remark: At the moment, the technology needs to be optimised and data generation and analysis expanded. Academic research alone is not sufficient and industry will need to start testing epigenetic changes so that regulators will be in a position to approve this new technology.

Considering the high costs arising from various types of diseases such diabetes, cancer and neurodegeneration, more effort and money should be invested to understand the role of epigenetic changes.

We need guidance on where to focus the investigations. Which genes, which endpoints for early warning? Where is the gap in research that needs to be covered?

Global technologies provide a great advantage to improve our comprehensive understanding of epigenetics changes. Unless there are specific questions regarding certain genes we should not focus our attention too soon, as we are likely to miss essential information. It is unclear, if one would focus on either one or a small set of genes, the observed epigenetic changes could be distinguished from an adverse effect?

It is much easier to study the whole genome than a single gene?

Preference was given to use genome-wide approaches rather than a single gene because it is technically easier. The 'low fruits', i.e. substances that are well known to induce epigenetic changes should be examined first, in order to see what effects they have on a short and long-term basis.

Should regulatory agencies push the science by demanding epigenetic research?

The science of epigenetics is developing rapidly and by understanding epigenetics a better understanding of the temporal relationships between key events in a mode of action leading to adverse events in (eco)toxicology may be gained. That is, it may allow us to understand things that we do not currently understand very well. Nevertheless, the existence of large knowledge gaps and the lack of consensus among experts as to the implications of epigenetic changes must be recognised. In spite of this, some consideration has being given to the inclusion of epigenetics screens for NGCs within regulatory testing frameworks e.g. OECD Molecular Screening and Toxicogenomics Programme. Some workshop participants called for caution to avoid rushing regulation and policy development before the science is understood and reliable methods have been proven via ring-testing (i.e. avoid what happened in the case of EDCs when policy was passed more than 10 years before robust guideline methods were available via EDSTAC).

5. CONCLUSIONS AND RECOMMENDATIONS

Concluding remarks from Alan R. Boobis

Professor Alan R. Boobis presented his views as a participant on the outcome of the workshop.

Whilst there is agreement that epigenetics is a rapidly developing field with potential importance in the adverse effects of chemicals, there are many areas where there are appreciable differences in opinion, largely due to current lack of knowledge.

There is agreement that DNA methylation and histone modification are key epigenetic mechanisms. There is less agreement as to whether ncRNAs comprise an additional epigenetic mechanism or some other regulatory process. Each of these key processes (DNA methylation, histone modification, ncRNAs) is complex and becoming more so as knowledge advances.

It is increasingly evident that epigenetic mechanisms are interdependent. Whilst they may regulate transcription or translation they in turn can be regulated by transcriptional or translational changes. For example, DNA methylation can alter transcription, but the levels of the enzymes responsible can be regulated by changes in expression of their coding genes. Similarly, miRNA species act post-transcriptionally, but are themselves the products of transcriptional activity.

As our knowledge of the epigenome increases, it is becoming apparent that epigenetic regulation is almost universal and that most if not all genes are subject to such regulation. Hence, it is important that scientists are able to distinguish between changes of potential toxicological consequence and those that reflect normal homeostatic adjustment.

Often, epigenetics (or the epigenome) is used as a catch-all term, but it is clear that not all epigenetic mechanisms are equal. Some DNA methylation reactions can change phenotype throughout life, and perhaps even in successive generations. In contrast, some histone modifications have a relatively short-lived impact on the cell.

It is important to distinguish between mode (of mechanism) of action and a toxicological response. Epigenetic changes can be key events in the mode of action for a response, but in themselves will not be adverse. Hence, if one starts with an adverse effect, understanding the epigenetic changes responsible may help in extrapolating from experimental observations to human (or environmental) risk, but is not essential in reaching a conclusion about acceptable levels of exposure. On the other hand, the potential paradigm shift in toxicology where chemicals are assessed *in vitro* and *in silico*, will require prediction of potential adverse effects based on systems approaches. This is a complex undertaking, and the present state of knowledge is such that such predictions are often not possible. Apical endpoints in general demonstrate monotonic

dose-response curves, and it is difficult to find robust evidence for exceptions to this. In contrast, some epigenetic processes exhibit complex dose-response relationships, complicating study design and interpretation.

There are some concerns that effects on epigenetic processes may result in subtle adverse effects that are difficult if not impossible to detect using current protocols. However, this is more a question of the adequacy of testing strategies than one on epigenetics. In terms of protecting human health (or the environment), regardless of the mechanism, it is important that subtle effects of potential impact are not overlooked. One possibility is to design an apical test to detect any such effects. Alternatively, if changes in epigenetic mechanism underlie some of these effects, understanding such mechanism would be a means to an end, helping in the prediction of any such potential.

There are differing views on how often interference with epigenetic mechanisms underlies chemical toxicity. Similarly, it is not clear how often chemically-induced changes in epigenetic processes translate into an adverse effect. There are major gaps in knowledge on the extent of background variability in epigenetic processes and their normal dynamic range, in the duration of persistence of changes and their reversibility (e.g. some DNA methylation changes once thought to be permanent are now known to be reversible), the magnitude of change necessary for an adverse outcome, windows of susceptibility, and the extent of autoregulation and redundancy in the system. Opinions vary as to how often epigenetic changes are a cause of toxicity or a consequence of toxicity.

Some have suggested that in the absence of detailed knowledge of epigenetic effects of chemicals the worst should be assumed and the precautionary principle adopted. However, this blurs the distinction between risk assessment and risk management, between science and policy. As indicated above, epigenetic changes may be key events in a mode of action for a toxic (or adverse) effect, but even before we were aware of the existence of the epigenome it was possible to identify levels of exposure to chemicals that were of negligible concern. Understanding epigenetic mechanisms may help improve risk assessment and reduce uncertainty in areas such as interspecies extrapolation, life stage sensitivity, human relevance and interindividual variability. In the absence of such knowledge uncertainty may be greater but can still be addressed, by using more conservative assumptions, as at present.

Simply demonstrating that a chemical causing toxicity also causes epigenetic changes does not establish cause and effect, any more than in a transcriptomics study. Hence, there needs to be an investigative strategy to confirm the relevance of any epigenetic changes observed in the toxicity of the compound. As knowledge increases, such epigenetic changes will eventually become more predictive. Many, but not all, are of the view that it is too early to routinely evaluate chemicals for their global impact on the epigenome, although this could be part of a focused MoA investigation.

ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorders
ADP	Adenosine diphosphate
BFR	Brominated flame retardant
BPA	Bisphenol A
CAR	Constitutive androstane receptor
CpG	Cytosine-phosphate-guanine
DDE	Dichlorodiphenyldichloroethylene
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of human health and disease
ED	Endocrine disrupter
EDC	Endocrine disrupting chemical
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EPA	Environmental Protection Agency
epiRILs	Epigenetic recombinant inbred lines
GR	Glucocorticoid receptor
GRE	Glucocorticoid-responsive elements
GUS	Beta-glucuronidase
HBCD	Hexabromocyclododecane
HDAC	Histone deacetylase
hDIP	Hydroxymethylated DNA immunoprecipitation
HELP	HpaII tiny fragment enrichment by ligation-mediated PCR
hmC	Hydroxymethylcytosine
HpaII	Haemophilus parainfluenzae II
lincRNAs	Long intergenic RNAs
mC	Methylcytosine
MeDIP	Methylated DNA immunoprecipitation
miRNA	MicroRNA
MoA	Mode of action
mRNA	Messenger RNA
ncRNA	Non-coding RNA
NGC	Non-genotoxic carcinogens
NGS	Next-generation sequencing
NIEHS	National Institute of Environmental Health Sciences
NO(A)EL	No observed (adverse) effect level

NOEL	No observed effect level
NR	Nuclear receptor
OECD	Organisation for Economic Co-operation and Development
PB	Phenobarbital
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PFAA	Perfluorinated alkyl acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonate
PM_{10}	Particulate matter of ~10 micrometers
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SA	Structural alert
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SHE	Syrian Hamster Embryo
TBT	Tributyltin
TPT	Triphenyltin
UV-B	Ultraviolet B

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APPENDIX A: LIST OF PARTICIPANTS

First Name	*Name*	*Affiliation*	*E-mail*
Rémi	Bars	Bayer CropScience, France	remi.bars@bayer.com
Mohamed	Benahmed	CHU Nice (Inserm), France	benahmed@unice.fr
Romualdo	Benigni	ISS, Italy	romualdo.benigni@iss.it
Mustafa	Billur	BfR, Germany	mustafa.billur@bfr.bund.de
Alan	Boobis	Imperial College London, UK	a.boobis@imperial.ac.uk
Ross	Brown	AstraZeneca, UK	ross.brown@astrazeneca.com
Neil	Carmichael	ECETOC, Belgium	neil.carmichael@ecetoc.org
Richard	Currie	Syngenta, UK	richard.currie@syngenta.com
Nathalie	Delrue	OECD, France	nathalie.delrue@oecd.org
Sarah	Dutton	HSE CRD, UK	sarah.dutton@hse.gsi.gov.uk
Malyka	Galay Burgos	ECETOC, Belgium	malyka.galay-burgos@ecetoc.org
Tim	Gant	Leicester University, UK	tim.gant@hpa.org.uk
Alessandro	Giuliani	ISS, Italy	alessandro.giuliani@iss.it
Jay	Goodman	Michigan State University, USA	goodman3@msu.edu
Robert	Kavlock	EPA, USA	kavlock.robert@epa.gov
Jos	Kleinjans	University of Maastricht, the Netherlands	j.kleinjans@grat.unimaas.nl
Juliette	Legler	VU University Amsterdam, the Netherlands	juliette.legler@ivm.vu.nl
Marina	Marinovich	University of Milan, Italy	marina.marinovich@unimi.it
Richard	Meehan	Medical Research Council, UK	richard.meehan@hgu.mrc.ac.uk
Krista	Meurer	BASF, Germany	krista.meurer@basf.com
Sean	Milmo	Chemical Watch, UK	smilmo@btconnect.com
Sue-Nie	Park	Korea Food & Drug Administration, South Korea	suenie@kfda.go.kr
David	Rouquié	Bayer, France	david.rouquie@bayer.com
Moshe	Szyf	McGill Faculty of Medicine, Canada	moshe.szyf@mcgill.ca
Frederick L.	Tyson	National Institute of Environmental Health Sciences, USA	tyson2@niehs.nih.gov
Maria	Uhl	Umweltbundesamt, Environment Agency Austria	maria.uhl@umweltbundesamt.at
Michiel	Vandegehuchte	UGent, Belgium	michiel.vandegehuchte@ugent.be
Saskia	van der Vies	VU University Medical Center, the Netherlands	vdvies@few.vu.nl
Bennard	van Ravenzwaay	BASF, Germany	bennard.ravenzwaay@basf.com
Mathieu	Vinken	Vrije Universiteit Brussel, Belgium	mvinken@vub.ac.be
Michael	Weber	IGMM, France	michael.weber@igmm.cnrs.fr

APPENDIX B: WORKSHOP PROGRAMME

Monday 5 December 2011

Chairman: Malyka Galay Burgos, ECETOC

12.30 - 13.30	Registration and lunch	
13.30 - 13.40	**Introduction to ECETOC**	Neil Carmichael ECETOC

EPIGENETIC CHANGES IN THE CONTEXT OF (ECO)TOXICOLOGY

13.40 - 14.05	**The epigenome: Interface between the environment and the genome – an overview**	Frederick L. Tyson National Institute of Environmental Health Sciences

WHAT ARE THE IMPLICATIONS OF EPIGENETIC CHANGES TO THE CURRENT REGULATORY (ECO)TOXICITY STUDIES?

14.05 - 14.30	**Epigenetics and chemical safety assessment**	Richard Meehan Medical Research Council
14.30 - 14.55	**Epigenetics meets toxicology: Is it time to incorporate an epigenetic evaluation into risk assessment?**	Jay Goodman Michigan State University

IS IT NECESSARY TO DETERMINE EPIGENETIC CHANGES IN REGULATORY (ECO)TOXICOLOGY STUDIES TO ADEQUATELY PROTECT HUMAN HEALTH?

14.55 - 15.20	**Epigenetic marker as a component for systems toxicology useful for cancer prediction in addition to genetic toxicity**	Sue-Nie Park Korea Food & Drug Administration
15.20 - 15.45	**The environment and the epigenome; implications for environmental safety**	Moshe Szyf McGill University
15.45 - 16.00	Coffee break	

CASE STUDIES: IMPLICATIONS OF EPIGENETICS IN ADVERSE EFFECTS IN HUMANS, IN ANIMAL MODELS AND ECOTOXICOLOGY

16.00 - 16.25	**DNA methylation: Reprogramming during development and impact of endocrine disrupters**	Michael Weber IGMM
16.25 - 16.50	**microRNAs and exposure to endocrine disrupting chemicals**	Mohamed Benahmed CHU de Nice
16.50 - 17.15	**The identification of non-genotoxic carcinogens today**	Romualdo Benigni ISS
17.15 - 17.40	**Early exposure and late effects: The role of DNA methylation in prenatal programming**	Juliette Legler Amsterdam Free University
17.40 - 18.05	**Epigenetics in an ecotoxicological context**	Michiel Vandegehuchte Ghent University
18.05	*End of Day 1*	
20.00	Dinner	

Tuesday 6 December 2011

08.30 - 08.45 **Recapitulation of Day 1 and introduction to syndicate sessions** — Jos Kleinjans, University of Maastricht

08.45 - 10.30 **Syndicates**

Syndicate 1 – Definition of epigenetic changes and effects in the context of (eco)toxicology

Moderator: Rémi Bars *Rapporteur: Tim Gant*

1. Are epigenetic changes a mode of action?
2. Which endpoint of toxicology could be affected by epigenetic changes?
3. Should we consider including µRNA in the definition of epigenetics?

Syndicate 2 – What are the consequences of epigenetic changes induced by exogenous substances on human and environmental health?

Moderator: Ben van Ravenzwaay *Rapporteur: Robert Kavlock*

1. Are epigenetic changes adverse per se?
2. Would the detection of the epigenetic changes provide information useful for RA?
3. Are epigenetic changes considered an adaptive response to environmental changes?

Syndicate 3 – Is the current way of assessing the safety of chemicals able to detect adverse effects related to epigenetic changes?

Moderator: Saskia van der Vies *Rapporteur: Jay Goodman*

1. Can epigenetic changes occur at doses levels traditional below NO(A)EL?
2. How relevant are rodent epigenetics changes to humans?
3. What is the dose-response curve for epigenetic changes by chemical exposure?
4. Is it possible to link epigenetic changes at population level?

10.30 - 11.00 Coffee break

Chairman: Neil Carmichael, ECETOC

11.00 - 12.00 **In plenary: Syndicate reports and discussion** — Audience and Speakers

12.00 - 12.30 **Summary / Conclusion** — Alan Boobis, Imperial College London

12.30 - 14.00 **Adjourn** and Lunch

APPENDIX C: ORGANISING COMMITTEE

Rémi Bars
Bayer CropScience
355, rue Dostoïevsky
F - 06903 Sophia Antipolis Cedex
France

Malyka Galay Burgos
ECETOC
Avenue Van Nieuwenhuyse, 4
B - 1160 Brussels
Belgium

Saskia van der Vies
VU University Medical Center
De Boelelaan 1117
NL - 1081 HV Amsterdam
The Netherlands

Ben van Ravenzwaay
BASF
Carl-Bosch-Strasse 38
D - 67056 Ludwigshafen
Germany

ECETOC Workshop Reports

No. Title

No. 1 Availability, Interpretation and Use of Environmental Monitoring Data. 20-21 March 2003, Brussels (Published December 2003)

No. 2 Strategy Report on Challenges, Opportunities and Research needs arising from the Definition, Assessment and Management of Ecological Quality Status as required by the EU Water Framework Directive based on the workshop EQS and WFD versus PNEC and REACH - are they doing the job? 27-28 November 2003, Budapest (Published March 2004)

No. 3 The Use of Human Data in Risk Assessment. 23-24 February 2004, Cardiff (Published November 2004)

No. 4 Influence of Maternal Toxicity in Studies on Developmental Toxicity. 2 March 2004, Berlin (Published October 2004)

No. 5 Alternative Testing Approaches in Environmental Risk Assessment. 7-9 July 2004, Paris (Published December 2004)

No. 6 Chemical Pollution, Respiratory Allergy and Asthma. 16-17 June 2005, Leuven (Published December 2005)

No. 7 Testing Strategies to Establish the Safety of Nanomaterials. 7-8 November 2005, Barcelona (Published August 2006)

No. 8 Societal Aspects of Nanotechnology. 7-8 November 2005, Barcelona (Published October 2006)

No. 9 The Refinement of Mutagenicity/Genotoxicity Testing. 23-24 April 2007, Malta (Published September 2007)

No. 10 Biodegradation and Persistence. 26-27 June 2007, Holmes Chapel (Published September 2007)

No. 11 The Application of 'Omics in Toxicology and Ecotoxicology: Case Studies and Risk Assessment. 6-7 December 2007, Malaga (Published July 2008)

No. 12 Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study. 14-15 April 2008, Barza d'Ispra (Published August 2008)

No. 13 Counting the Costs and Benefits of Chemical Controls: Role of Environmental Risk Assessment in Socio-Economic Analysis. 4 June 2008, Brussels (Published September 2008)

No. 14 Use of Markers for Improved Retrospective Exposure Assessment in Epidemiology Studies. 24-25 June 2008, Brussels (Published February 2009)

No. 15 The Probabilistic Approaches for Marine Hazard Assessment. 18-19 June 2008, Oslo (Published June 2009)

No. 16 Guidance on interpreting endocrine disrupting effects. 29-30 June 2009, Barcelona (Published October 2009)

No. 17 Significance of Bound Residues in Environmental Risk Assessment. 14-15 October 2009, Brussels (Published December 2009)

No. 18 The Enhancement of the Scientific Process and Transparency of Observational Epidemiology Studies. 24-25 September 2009, London (Published December 2009)

No. 19 'Omics in (Eco)toxicology: Case Studies and Risk Assessment. 22-23 February 2010, Málaga (Published June 2010)

No. 20 Guidance on Assessment Factors to Derive a DNEL. 25 March 2010, Barza d'Ispra (Published December 2010)

No. 21 Risk Assessment of Endocrine Disrupting Chemicals. 9-10 May 2011, Florence (Published November 2011)

No. 22 Combined Exposure to Chemicals. 11-12 July 2011, Berlin (Published October 2011)

No. 23 Epigenetics and Chemical Safety. 5-6 December 2011, Rome (Published May 2012)

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Responsible Editor:
Dr. Neil Carmichael
ECETOC AISBL
Av. E. Van Nieuwenhuyse 4 (bte. 6)
B-1160 Brussels, Belgium
VAT: BE 0418344469
www.ecetoc.org
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Established in 1978, ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) is Europe's leading industry association for developing and promoting top quality science in human and environmental risk assessment of chemicals. Members include the main companies with interests in the manufacture and use of chemicals, biomaterials and pharmaceuticals, and organisations active in these fields. ECETOC is the scientific forum where member company experts meet and co-operate with government and academic scientists, to evaluate and assess the available data, identify gaps in knowledge and recommend research, and publish critical reviews on the ecotoxicology and toxicology of chemicals, biomaterials and pharmaceuticals.