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DNA Methylation and Toxicogenomics

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3.1 Introduction

Exposure to toxic chemicals and environmental pollutants can result in acute or chronic effects in organisms which may range from mild irritation to life threatening conditions. The field of toxicology encompasses the study of exposures of living systems to chemicals. Toxicological analysis is very critical even in pharmacological investigations of drug efficacy and possible side effects. Since the advent of genomics and availability of DNA sequences and polymorphisms, it has become possible to examine and carry out genome wide association studies (GWASs) and analyse global changes in gene expression at the transcript or protein (functional genomics) level (van Hummelen and Sasaki, 2010). Toxicogenomics is a field that combines conventional toxicology with functional genomics (i.e. RNA, protein, metabolite profiling and polymorphisms/functional DNA mutations) (Nuwaysir *et al*., 1999). Such an analysis would help in unravelling the molecular, biochemical, cellular and physiological effects of chemicals in biological systems. It has been demonstrated that environmental and toxic exposures may lead to alterations in DNA methylation levels (Judson *et al*., 2009). DNA methylation is an epigenetic modification of DNA which has been shown to be involved in regulation of gene expression. The term Epigenetics describes the study and analysis of alteration in the function, expression, phenotype in an organism which is not modulated through genetic changes. Environmental parameters play an important role in establishing and modulating epigenetic marks. Epigenetic marks such as histone modifications, DNA methylation, micro RNA (miRNA) and small RNAs, and so on help in chromatin modelling/remodelling (Judson *et al*., 2009). Epigenetics plays an important role and can alter expression patterns and thereby phenotypes. It is becoming clear that the effects of exposure to chemicals and environmental changes may often result in epigenetic alterations. Variations in nutrition, diet, physiology, exposure to stress, etc. may also lead to changes in epigenetic

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marks such as DNA methylation, histone methylation, sumoylation, acetylation, etc. DNA methylation is a post replicative modification of DNA which adds an additional level of information over and above the sequence of DNA. 5-Methyl cytosine, the minor bases can influence DNA protein interactions and DNA conformation and work as an important signal/regulator in chromatin remodelling. DNA methylation levels change in a tissue and developmental stage specific manner and have been shown to be modulated by environment. DNA methylation is a DNA based modification which displays heritability and adaptability. It is expected that in addition to providing insights into the mechanism of toxicity, it would also lead to the identification of important biomarkers for toxicogenomic analysis. Such an analysis needs to be examined for consistency and once established it will provide a platform to develop indicators of exposure with high sensitivity, speed and accuracy. Early detection of stress and toxicity will provide possibilities of designing intervention strategies. It is expected that such an approach will also facilitate rapid screening of a large number of chemicals and toxins and will provide insights into the molecular mechanisms leading to toxicity.

3.2 Toxicology

Toxicology is the systematic study of the adverse effects of chemicals on living systems/organisms namely human, animals, plants and microbes. Toxicology is an inter-disciplinary science that integrates the principles and methods of many fields: namely, chemistry, biology, pharmacology, molecular biology, physiology and medicine. Science of toxicology encompasses both basic mechanistic component and a real-life practical application which leads to understanding and preventing life-threatening crises. Traditional toxicity testing approaches have resulted in identifying and defining effects of exposures in a systematic manner and are invaluable in pharmacological and drug regulatory affairs. 'Adverse effects' of an exposure can vary from a life threatening injury/lethality to a short term reversible effect or irritation. A mild irritant that causes watering of the eyes, skin discomfort, wheezing or allergy may not seem like a significant or real adverse effect and may even go unnoticed. However, such effect can be a potentially dangerous health hazard. For example, by combining the study of the physiological effects of certain chemical structures and the molecular mechanisms that correlate with those effects, toxicology can provide better understanding of the actions of a class of chemical substances. The elucidation of the rationale behind the mode of action of a drug or chemical can help the chemist and the pharmacist to design new less harmful chemicals and drugs with more activity (van Delft *et al*., 2004). Toxicology deals with effects of accidental exposures, deliberate poisoning, overdoses, suicide attempts, exposure to a mixture of toxins and toxicants, and so on. The effects of exposure can vary based on the species differences, genetic background, age, gender, developmental stage, etc. (Schrager, 2008). The concept of dose is the basic principle of toxicology. Dose is the amount of chemical that comes into contact with the body or enters the body. Even the most harmless chemicals and materials can have toxic effects upon exposure to high concentration or prolonged exposures. Exposure includes both the concentration of the chemical in the media and the length of time such chemical is in contact with the body (concentration $\times$ time). The effects of chemical or environmental exposures can show significant variations based on the dose and time of exposure. It can vary from lethal to mildly or highly beneficial effects. Most exposures will lead to toxic effects only at certain high doses, upon prolonged exposure or from a combination of genetic background and physiological effects. It is well known that many drugs and pharmaceuticals will show toxicity and adverse effects or side effects based on genetic variations, time and frequency of exposure and other physiological conditions. In case of some pharmaceuticals which require activation by metabolic enzyme like cytochrome P450 for functionality in the body of an individual, the genetic background can play an important role. If an individual harbours

inactive or less active enzyme, the drug will be ineffective and the chemical may therefore accumulate and lead to adverse effects. The form of cytochrome P450 that an individual harbours will be critical in such an evaluation. It has been reported that vinyl chloride is non-toxic at low doses, hepato-toxic in high doses and carcinogenic in intermediate doses. The study of toxicology involves observational and mechanistic aspects and risk assessment. Toxicology contributes to practice of medicine, clinical research, forensic science, pharmacy and pharmacology, drug regulatory affairs, marine, aquatic and environmental toxicity, public health and industrial hygiene and serves in a major way to protect the environment and human life. There are extensive methods and regulatory guidelines for toxicological analysis. However, due to the complexity of the effects which can vary based on dose, genetic background, the measurement and evaluation of toxicity is often a challenging task. Toxicological evaluation involves extensive animal experimentation and there have been several attempts to substitute this with use of cells in culture. With the advent of stem cell technology a lot of effort focuses on possibilities of developing alternatives to animal experimentation.

It is estimated that the recent European Community Regulation REACH (registration, evaluation, authorisation and restriction of chemicals) will require the registration of ~39 000 chemical substances over the next 11 years (van Hummelen and Sasaki, 2010), with a primary aim to progressively substitute the most dangerous chemicals with suitable alternatives. Current toxicity testing of chemical compounds is based on extensive animal testing, which is time-consuming, expensive and not possible for this large number of compounds (Gad, 1990). Alternative approaches which will provide large scale and intensive testing are clearly needed to meet this need. Animal toxicity tests are also limited in their ability to detect toxicity. Only a very limited subset of end points and potential modes of action is generally assessed. There are examples which demonstrate that long-term and fine modulatory effects on living systems are not identified in current toxicity analysis. Comprehensive mechanistic approaches are needed to assess the diversity of possible modes of toxicity. Traditional molecular analysis would investigate biochemical, histochemical parameters and expression or mutation in one or few genes.

3.3 Toxicogenomics

There is a need to develop and employ newly emerging techniques and strategies in order to meet the increasing challenge of current toxicity assessment requirements. Tens of thousands of chemicals are used annually in industry and in day to day life, for which no toxicological data is available, and this number is ever-increasing (Judson *et al*. 2009). In addition, recent advances such as nanotechnology, combinatorial drug chemistry, polymer science and synthetic chemistry have introduced new classes of compounds into general use that represent an additional challenge to risk assessment (North and Vulpe, 2010). It is often necessary to identify both the basic mechanisms of physical, chemical and biological agents that could induce any harmful effect on a target organism or ecosystem. Multiple end points of toxicity, at the molecular, biochemical, cellular, physiological, pathologies at the whole organism or ecosystem levels, morbidity, mortality, and so on have been used. Specific concepts and techniques (*in vitro*/*in vivo*, epidemiological, etc.) have been used to identify specific mechanisms of toxicities (e.g. histo-chemical and biochemical techniques, mutagenesis, cytotoxicity and altered gene expression or 'epigenetic toxicity') that might be responsible for the pathogenesis of diseases.

Toxicogenomics can provide insight into the mode of action of toxicants and allow for development of targeted cellular assays (Andersen and Krewski, 2009). Toxicogenomics was defined as 'the application of global mRNA, protein and metabolite analysis related-technologies to study the effects of hazards (chemical/physical) on organisms' (Hamadeh *et al*., 2002). Toxicogenomics will provide a link between

genetic variation and susceptibility to toxins. It will also have inputs in hormesis where adaptive effects and dose effects become predominant (Hayes and Bradfield, 2005). Toxicogenomics explores the interactions between the genome and adverse biological effects caused by exogenous agents such as environmental stressors, toxins, drugs and chemicals (Gatzidou, Zira and Theocharis, 2007). A large number of chemicals may have effects due to toxic, cytotoxic action at biochemical or physiological level leading to toxicity and teratogenic effects and gene mutations. It has also become apparent that the effects of exposures vary greatly depending on the genotype and genetic predisposition of an individual and may result in significant differences from no effects to mild and significant effects upon exposure to same dose.

Toxicogenomics initially arose through the use of microarrays to assess global gene regulation (measured by relative abundance of mRNA) following treatment with various stressors (Nuwaysir *et al*., 1999). Such an analysis is expected to generate valuable information about gene expression changes in response to treatment with different classes of known toxicants (oxidant stressors, polycyclic aromatic hydrocarbons, chemicals and environmental toxicants, nanomaterials, etc.). It has been suggested that this information can then be used to gain insight into the mode of action of unknown compounds and may provide 'fingerprints' of exposure. Expression profiling has been widely used (across many organisms) to discover biomarkers for a wide range of toxicants (reviewed in Hamadeh *et al*., 2002; Hayes and Bradfield, 2005; Gatzidou, Zira and Theocharis, 2007; Aardema and MacGregor, 2002 and Jayapal *et al*., 2010). The data obtained from expression profiling can be used in the selection of mechanism-based assays and lead to identification of toxicity endpoints for cellular assays. Such an analysis could have both genetic and epigenetic components.

Functional genomics has been defined as 'the development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of information and reagents provided by physical mapping and sequencing of genomes' (Waters and Fostel, 2004). Functional genomics directly measures phenotype, and thus provides a direct link between a specific gene, its expression and modulation by exposures (Hieter and Boguski, 1997). Studies can also be carried out by screening collections of cells/organisms that lack either genes (through deletion) or proteins (through blocking translation by using technologies such as RNA interference, RNAi) or by studying the response to varying dose and at different times after exposure. This leads to identification of gene ontologies, regulatory networks and pathways related to viability, stress and extent of treatment. It will lead to identification of genes involved in apoptotic or survival mechanisms and pathways which may lead to cell transformation and carcinogenesis at early time points. A toxicogenomic or functional genomic map of exposure and its effects and a comparative account of different toxicants and variations due to genotype, tissue, and so on can thus form a basis of identification of early signs of exposure and damage.

In recent years, toxicogenomics has been utilized extensively to identify and elucidate adverse biological effects resulting from exposures to environmental stressors, toxins, drugs and chemicals. Application of microarray technology provides a high throughput genome wide transcriptome analysis that is it identifies changes in gene expression patterns, as detected by alterations in mRNA levels and provides an understanding of alterations in mRNA stability or gene regulation. Other 'omics' technologies have been used too, notably proteomics and metabolomics where a systems-biology approach provides further fine-tuning in the collection and interpretation of toxicological data. In addition to changes in gene expression patterns or proteomics data, it has become apparent that epigenetic changes are extremely important and that they not only affect gene expression patterns and phenotypes but can provide an insight into effects of exposures. Thus epigenomics, a field which examines the heritable changes in gene expression without accompanying alterations in the DNA sequence, is being recognized as an important aspect of phenotype modulation (North and Vulpe 2010). These epigenetic changes are brought about by mechanisms such as DNA methylation, histone modifications and non-coding RNAs and play an extremely important role in the regulation of gene expression patterns and thereby lead to altered effects or phenotypes.

3.4 Epigenetics

Epigenetic mechanisms are essential in normal development and differentiation, but these can be misdirected leading to diseases, notably life style diseases, cancer, and so on. Indeed, there is now a mounting body of evidence that environmental exposures particularly in early development can induce epigenetic changes, which may be transmitted in subsequent generations or serve as basis of diseases developed later in life. In either way, epigenetic mechanisms will help interpret toxicological data or toxicogenomic approaches to identify epigenetic effects of environmental exposures (Figure 3.1). Thus, a full understanding of environmental interactions with the genome will require involvement of epigenetic analysis, that is routine analysis of epigenetic modifications as a part of elucidating the mechanism of actions of environmental exposure. A number of approaches are currently available to study epigenetic modifications in a gene-specific or genome-wide manner. Epigenetics investigates heritable changes in gene expression that occur without changes in DNA sequence. DNA methylation and histone modifications can lead to chromatin remodelling and modifying and establishing varied and heritable activity states. Several epigenetic mechanisms can change genome function under exogenous influence. Histone modifications include phosphorylation, acetylation, sumoylation, and so on. DNA base modifications occur in *cis* in DNA and include 5-methylcytosine and the recently discovered 5-hydroxymethyl cytosine. It was shown that even

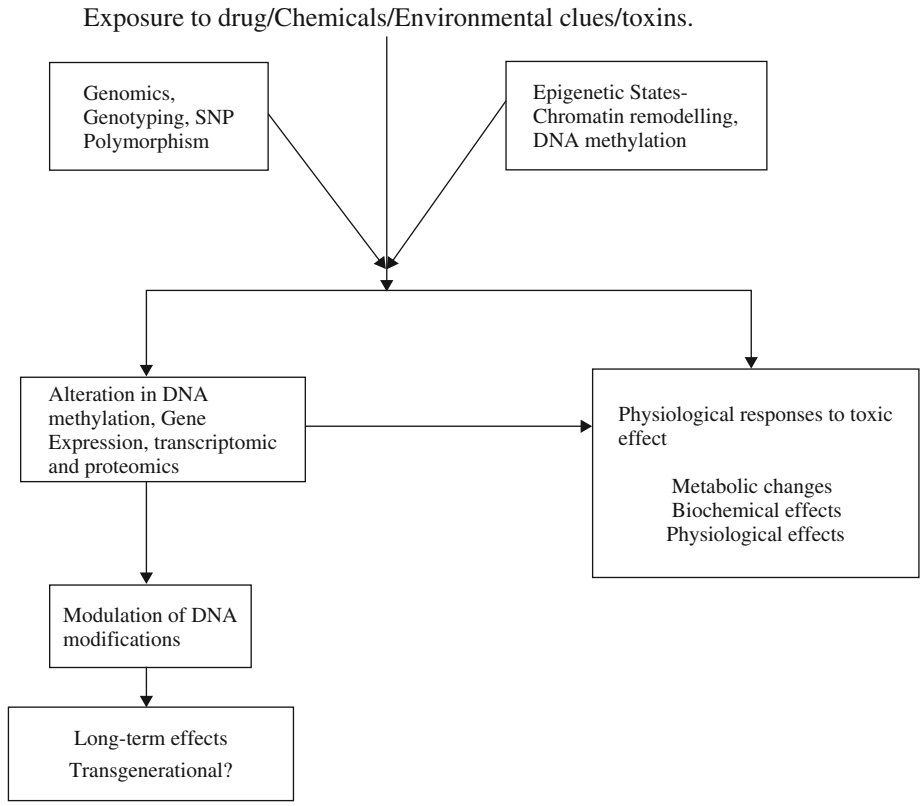

Figure 3.1 *Effects of chemical/toxicant exposure*

in embryonic and stem cells, a small proportion of 5-methylcytosine exists as 5-hydroxymethyl cytosine and this raises interesting questions about the possible role of this modification.

3.5 DNA methylation

DNA methylation is a stable epigenetic modification found in most of the eukaryotes and plays a crucial role in many biological processes, including gene expression regulation, gene imprinting and transposon silencing in mammals and plants (Bird, 2002; Goll and Bestor, 2005). This post replicative modification occurs in specific sequences and appears to demonstrate features of memory and heritability. DNA methylation is unevenly distributed in the genome. DNA methylation levels are known to change in a tissue and developmental stage and cell type specific manner. It has been reported that the heterochromatin region, transposons and repetitive sequences are usually hypermethylated, and the 5' and 3' flanking regions of genes are methylated at a relatively low level compared with the gene body regions (Zhang *et al*., 2006; Gehring, Bubb and Henikoff, 2009; Zilberman *et al*., 2006). The DNA methylation levels and patterns are important in several ways. Existing DNA methylation can lead to variation in response to exposures. DNA methylation patterns appear to change upon exposures and thus may play an important role in responses to exposures and this itself may serve as an early sign or biomarker. Treatment with toxins and chemicals or environmental exposures may alter DNA methylation patterns which could influence the short-term or long-term effects (Goll and Bestor, 2005). Many studies have pointed out that epigenetic alterations mediate effects caused by exposure to environmental toxicants. Results obtained from animal models indicate that *in utero* or early-life environmental exposures produce effects that can be inherited trans-generationally and are accompanied by epigenetic alterations. The methylation patterns can even change based on psychological traumas or infections. Recent investigations have identified a number of environmental toxicants that cause altered methylation of human repetitive elements or genes. Exposures can alter epigenetic states and similar epigenetic alterations can be found in patients with the diseases. There definitely is a close link between exposure to physical agents and chemicals/drugs and toxins and alterations in methylation pattern. The interplay between environmental exposures and the human epigenome emphasizes the importance of this regulatory signal. It has been demonstrated that although 50% of cancers have a genetic origin, several cancers arise due to stable epigenetic alterations which affect gene expression patterns. The relationship between exposure to environmental chemicals and epigenetics suggests that many toxicants can modify epigenetic states (Weitzman *et al*., 1994). Several studies indicate that environmental exposures have trans-generational epigenetic effects in humans. Although the consistency of such modifications and their utility as early biomarker needs to be further investigated, it is clear that epigenetics holds substantial potential for furthering our understanding of the molecular mechanisms of environmental toxicants, as well as for predicting health-related risks due to conditions of environmental exposure and individual susceptibility.

Identifying agents that have long-term deleterious impact on health but exhibit no immediate toxicity are also of importance (Figure 3.2). 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*., 1973; Ames, Lee and Durston, 1973). These tests have also been employed for assessing the carcinogenic potential of compounds. It has been documented that although some toxicants can have a mutagenic effect, several of them lead to changes in methylation of DNA (epimutations). The DNA sequence harbours genetic information in the sequence of DNA and the DNA methylation pattern superimposes an imprint about functionality and adaptability. DNA methylation patterns are generated by an innate program during gestation but are attuned to the environment *in utero* and throughout life including nutrition, physical and social exposures, and so on. DNA function and health could be stably

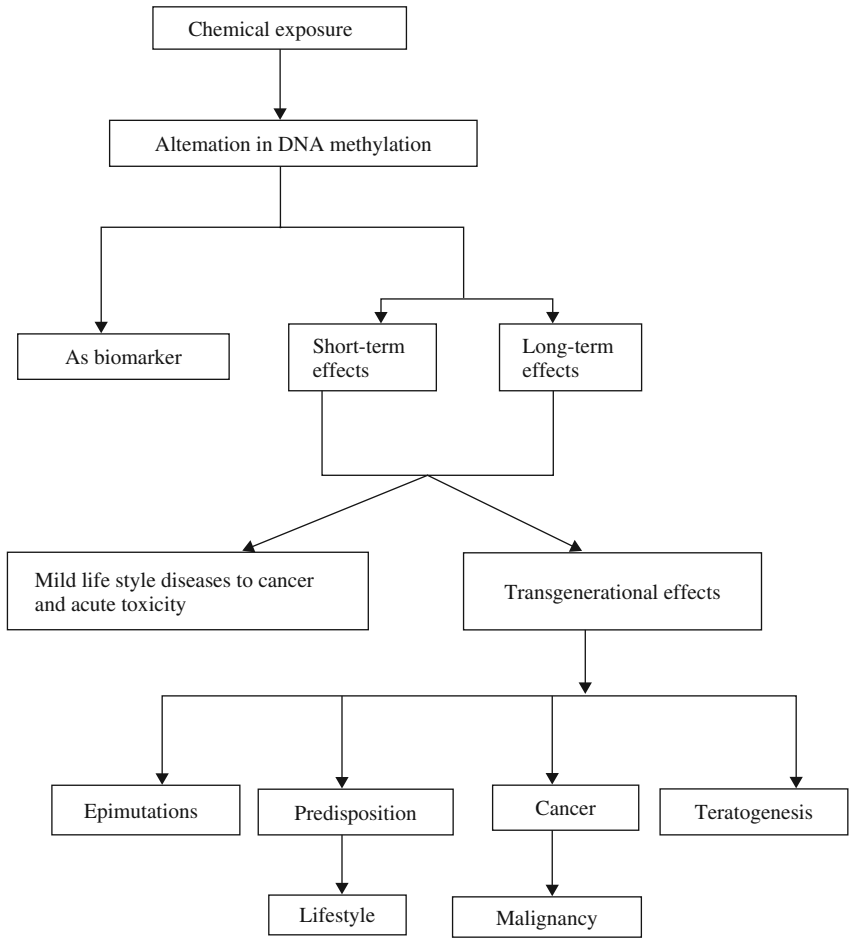

Figure 3.2 *DNA methylation and toxicogenomics*

altered by exposure to environmental agents without changing the sequence, just by changing the state of DNA methylation. Agents that have long-range impact on the phenotype without altering the genotype are also important. The fact that long-range damage could be caused without changing the DNA sequence has important implications on the way the safety of chemicals, drugs and food can be assessed (Patel and Butte, 2010).

The genomic revolution has resulted in determination of the sequence of the genetic material of humans and other organisms. It is commonly held that the DNA sequence is the blueprint which holds the secret for the phenotypic diversity of humans and their susceptibility to disease. Pharmacogenomics deals with differential responses to drugs which are due to differences in genotypes while toxicogenomics assesses differential response to toxicants. The basic principle is that the inter-individual variations in response to xenobiotics are defined by genetic differences and that the main deleterious effect from exposure to xenobiotics is mutagenesis or physical damage to DNA has been anticipated at the genomic level. The main focus of attention in pharmacogenetics has therefore been on identifying polymorphisms in genes

encoding drug metabolising enzymes and receptors. New xenobiotics were traditionally tested for their genotoxic effects (Skinner, Manikkam and Guerrero-Bosagna, 2011). It becomes important to identify highly susceptible and resistant individuals based on genetic screens. For example, polymorphism in drug metabolizing enzyme or receptors could lead to varied responses. This can be analysed in association with toxicogenomic analysis, since exposure to toxicants/chemicals can lead to changes in methylation patterns and methylation can alter gene expression patterns. DNA methylation analysis can provide valuable information about short term and long term responses to chemical exposures. Exposure to xenobiotic thus can modulate epigenetic states leading to epimutations. Non genotoxic agents could thus lead to stable and heritable changes in gene function. The prior methylation status and epigenetic states on the other hand could modify or influence the response of an organism to drug/chemical/toxicant (Szyf, 2007). However, it is becoming clear that epigenetic programming plays an equally important role in generating inter-individual phenotypic differences, which could affect drug response (Bernal and Jirtle, 2010). Moreover, the emerging notion of the dynamic nature of the epigenome and its responsivity to multiple cellular signalling pathways suggests that it is potentially vulnerable to the effects of xenobiotics not only during critical period in development but later in life as well. Thus, non-genotoxic agents might affect gene function through epigenetic mechanisms in a stable and long-term fashion with consequences, which might be indistinguishable from the effects of physical damage to the DNA. Epigenetic programming has the potential to persist and even being trans-generationally transmitted (Anway *et al*., 2005) and this possibility creates a special challenge for toxicological assessment of safety of xenobiotics/toxicants.

Epigenetic programs are dynamic and responsive to different environmental exposures during foetal development as well as early in life and even later. Thus, many of the phenotypic variations seen in human populations might be a result of modifications in long-term programming of gene function rather than the sequence per se. Any analysis of inter-individual phenotypic diversity should therefore take into account epigenetic variations in addition to genetic sequence polymorphisms (Meaney and Szyf, 2005b). For example, differences in expression between individuals in the level of activity of a P450 enzyme or a DNA repair enzyme might result from genetic polymorphism but might as well be derived from altered epigenetic programming which would result in differential functionality. Effects of imprinting and epigenetic gene silencing may lead to alternative states of functionality. Moreover, it is possible that epigenetic processes might override genetic polymorphisms (Bernal and Jirtle, 2010).

Epigenetic variations could potentially be established at distinct points in life and in specific tissues exclusively. This has implications on drug action and toxic effects of xenobiotics since cell-specific epigenetic variation could result in differential pharmacodynamics, pharmacokinetics and toxicity of drugs in different tissues. Thus, whereas a germ-line polymorphism is a static property of an individual and might be mapped in any tissue at any point in life, epigenetic differences must be examined at different time points and at diverse cell types (Figure 3.3).

Environmental exposure could alter the progression of epigenetic programming during development both *in utero* as well as postnatally (Yauk *et al*., 2008). Thus, variation in environmental exposures during these critical periods could result in epigenetic and therefore phenotypic differences later in life. As it has been already documented, exposure to nutritional deprivation and chemical toxins would affect the epigenetic machinery during development. Recent data suggest that in addition to these physical exposures, exposures to pesticides, endocrine disruptors, early in life could also impact on the epigenome resulting in differential epigenetic programming with physiological and behavioural consequences later in life (Hochberg *et al*., 2011; Meaney and Szyf, 2005a). Thus, early life environment exposures might have an impact on responsivity to drugs later in life through epigenetic programming of critical genes. Since these are important aspects, these need to be incorporated in the evaluation and assessment of effects of exposures and should be considered in future analyses of inter-individual variation in drug and toxin

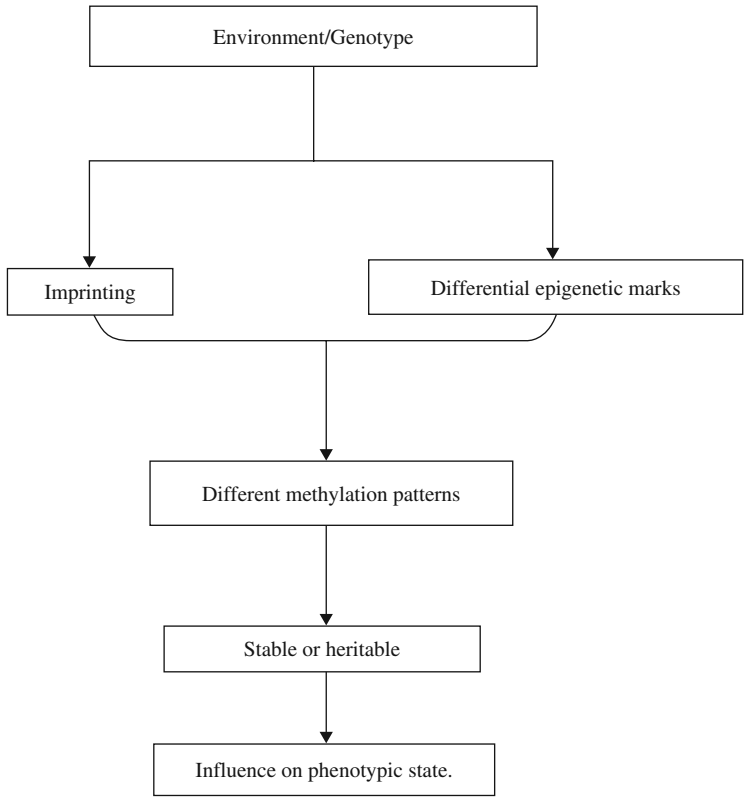

Figure 3.3 *Genotype, epimutations and phenotype*

response. It would be critical to demonstrate and establish consistency in order to validate these changes as biomarkers.

It is critical to understand the mechanisms driving variations in epigenetic programming in order to identify the exposures, which modulate inter-individual variations in response to drug and toxin actions. Epigenetic considerations should also be applied in drug development to identify potential toxic hazards to the epigenome as well as to discover agents, which modulate the epigenome in a therapeutically advantageous manner. Drugs, which target the epigenetic machinery, are currently tested in clinical trials in cancer (Kramer, Gottlicher and Heinzel, 2001; Weidle and Grossmann, 2000) and psychiatry disorders (Simonini *et al*., 2006).

DNA methylation is stable and can be propagated through cell division from mother to daughter cells. In addition, it has been already established that DNA methylation states in specific genes may change rapidly in response to environmental stressors and exposure to chemicals. Alterations in DNA methylation in tumour suppressor genes or oncogenes in response to short-term exposure to environmental chemicals needs to be determined. Tumour suppressor gene hypermethylation is widely proposed to represent one of the very early steps in human carcinogenesis (Yegnasubramanian *et al*., 2008). Even transient DNA methylation changes may reflect condition of cellular stress associated with altered apoptosis, cell cycle control and cell proliferation that may lead to the accumulation of persistent epigenetic and genetic damage after repeated exposures or in the presence of other pro-carcinogenic insults (Seaton *et al*., 1995).

3.6 DNA methyltransferases

DNA cytosine methyl transferases are enzymes which catalyse the transfer of methyl groups from S adenosyl methionine (SAM) to DNA. There appear to be multiple enzymes and forms (13 in human) and they are classified as '*de novo*' and maintenance methyl-transferases. '*De novo*' enzymes can transfer methyl group to unmethylated substrates and maintenance can use hemi methylated DNA as substrate. Although many methylated DNA binding proteins have been reported and mutations in these can lead to disease conditions in human (Rett Syndrome) the precise regulation of methylation machinery remains to be elucidated. The regulatory mechanisms related to sequence specificity, recognition of the site of methylation, signals which may govern the transfer of methyl groups, role of modulatory factors, and so on remain largely speculative. Although there is some evidence of role of chromatin remodelling machinery and miRNAs in setting and modifying DNA methylation patterns, the possible role of exposure to environmental and chemical agents in modulating the molecular genetic pathways of regulation of DNA methylation machinery has not yet been understood. There are several approaches to analyse the methyltransferase activity. In our analysis, we have designed and developed an assay for detection of DNA methyltransferase activity and can demonstrate that exposure to radiation leads to changes in methyltransferase activity.

DNA methylation is an essential modification of DNA in mammals that is involved in gene regulation, development, genome defence and disease. In mammals, three families of DNA methyltransferases (MTases) comprising (so far) four members have been found: Dnmt1, Dnmt2, Dnmt3A and Dnmt3B. In addition, Dnmt3L has been identified as a stimulator of the Dnmt3A and Dnmt3B enzymes. In order to lead to alterations in DNA methylation it is important to influence the activities of DNA methyltransferases in the context of site of action and levels of methylation. Recent evidence suggests roles for miRNAs and several regulatory proteins play an important role in guiding the methyltransferase machinery (Jeltsch, 2006). It is proposed that exposures to different environmental agents/metabolites, toxins could lead to inter-individual phenotypic diversity as well as differential susceptibility to disease and behavioural pathologies. Interindividual differences in the epigenetic state could also affect susceptibility to xenobiotics. Although our current understanding of how epigenetic mechanisms impact on the toxic action of xenobiotics is very limited, it is anticipated that in the future, epigenetics will be incorporated in the assessment of the safety of chemicals (Szyf, 2007).

3.6.1 Techniques of methylation detection

There are many approaches to decipher a genome-wide DNA methylation profile, including methylated DNA immunoprecipitation-sequencing/chip (meDIP-seq/chip), bisulfite-sequencing (bis-seq) and some enzyme digestion based techniques. MeDIP uses an antibody which can specifically recognizes methylated cytosines and pulls down the methylated fractions, MeDIP-chip was used to provide the first comprehensive DNA methylation map of an entire Arabidopsis thaliana genome (Zhang *et al*., 2006). The gold standard to determine the DNA methylome is genome-wide bisulfite sequencing, which converts all the unmethylated cytosines into uracil while leaving the methylated cytosines unchanged. These can be distinguished subsequently by sequence (Varley and Mitra, 2010). Despite its high resolution, genome-wide bis-seq remains a high cost and time-consuming method for DNA methylome study. Many studies showed that meDIP combined with high-throughput sequencing or chip could be considered as a method that can reflect the relative methylation state of a genome (Zhang *et al*., 2006; Li *et al*., 2010).

We have described a novel method using cDNA microarray which can be useful in determination of genome wide methylation landscape in a rapid, specific and sequence independent manner (Deobagkar *et al*., 2012; Kelkar *et al*., 2009). We have utilized it to examine the differential response of two strains of mice to radiation. There is a need for design and application of methodology which will lead to identification of targets and biomarkers for toxicity.

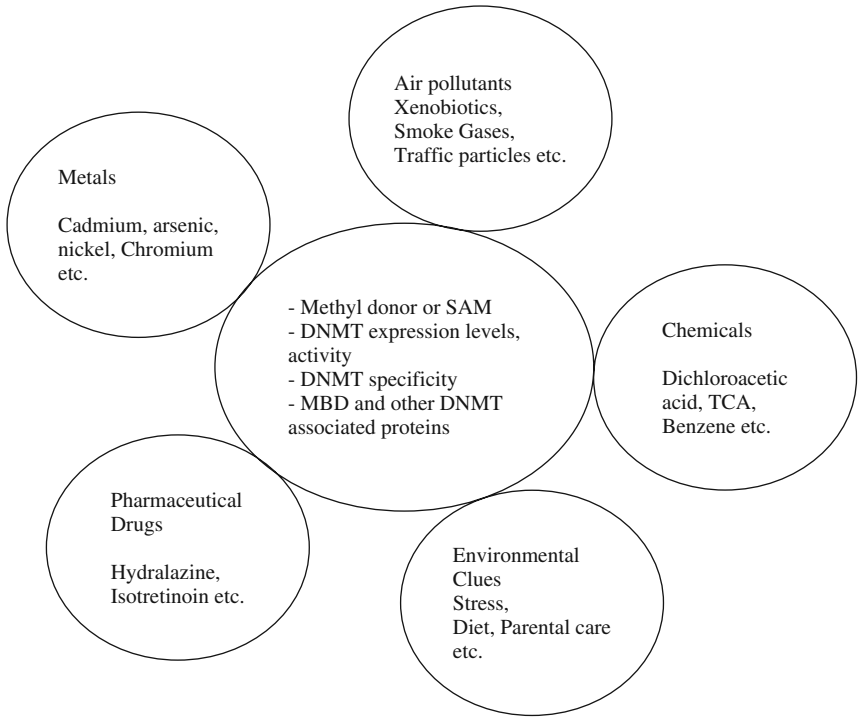

Figure 3.4 *Agents known to affect DNA methylation*

3.7 DNA methylation is alteres upon exposure to chemicals and toxins

Several classes of environmental chemicals/agents that modify epigenetic marks have been identified by *in vitro*, animal and human investigations. These include metals (cadmium, arsenic, nickel, chromium and methyl mercury), chemicals such as trichloroethylene (TCE), dichloroacetic acid (DCA) and trichloroacetic acid (TCA)), air pollutants (particulate matter (PM), black carbon (BC) and benzene) and endocrine-disrupting/reproductive toxicants (diethylstilbestrol (DES), bisphenol A (BPA), persistent organic pollutants and dioxin) (Figure 3.4). Although alterations in DNA methylation have been documented, the other epigenetic signals such as histone modifications and miRNA have not yet been extensively studied.

3.7.1 Drugs

The term pharmacoepigenetics, the study of the epigenetic basis for variations in drug response, relates to many genes encoding enzymes, drug transporters, nuclear receptors and drug targets all of which are under epigenetic control. It is important to unravel epigenetic regulation of drug-metabolizing enzymes and other proteins that might affect drug response by modifying epigenetic status. Drugs may alter epigenetic homeostasis by direct or indirect mechanisms. Some drugs are known to affect chromatin architecture or DNA methylation. For example, the antihypertensive hydralazine inhibits DNA methylation, while isotretinoin has transcription factor activity. With more chronic exposure, cells and organisms may adapt leading to drug induced epigenetic changes which may persist after the drug is discontinued.

3.7.2 Air pollution

Exposure to air pollution, particularly to PM, has been associated with increased morbidity and mortality from cardiorespiratory disease, as well as with lung cancer risk (Brook *et al*., 2004; Peters, 2005) In human, effects of PM exposure has been analysed on global (estimated through Alu and long interspersed nuclear element-1 or LINE-1 repeated elements) and gene-specific methylation in workers of a steel plant. These individuals had a well-characterized exposure to PM with defined aerodynamic diameters $<10\,\mu m$ (PM10). In this study it was reported that promoter methylation of iNOS (inducible Nitric Oxide Synthase) gene was significantly lower in post-exposure blood samples compared to baseline (Tarantini *et al*., 2009). Long-term exposure was negatively associated with methylation in both Alu and LINE-1. Exposure to BC, a marker of traffic particles, was also seen to associate with decreased DNA methylation in LINE-1. Patients with cancer (Ehrlich, 2002) or cardiovascular disease (Castro *et al*., 2003) show global DNA hypomethylation. In an animal study, sperm DNA of mice exposed to steel plant air was seen to be hypermethylated compared to control animals and this change persisted following removal from the environmental exposure (Yauk *et al*., 2008).

Ambient and occupational exposure to PM has been associated with increased risk of lung cancer (Pope *et al*., 2002). Foundry work, a specific condition of exposure to inhalable metal-rich particles, has been associated with increased risk of lung cancer (Chapman *et al*., 1997). Even in modern foundry facilities, workers are exposed to substantially higher levels of airborne PM compared to those found outdoors. Although the carcinogenic potential of several toxic metals in PM has been well-recognized, the molecular mechanisms underlying their association with cancer risks remain poorly understood. Experimental and epidemiologic studies suggest that PM mass and metal components may induce critical carcinogenesis-related biological changes, including oxidative stress, immune deficiency and chronic inflammation, which have recently been shown to alter gene expression via DNA methylation mechanism (Baccarelli and Bollati, 2009). Aberrant tumour suppressor gene promoter methylation has emerged as a promising biomarker for cancers, including lung cancer. It is of interest to speculate whether such methylation alterations may reflect processes related to PM-induced lung carcinogenesis. Even transient DNA methylation changes may reflect condition of cellular stress associated with altered apoptosis, cell cycle control and cell proliferation that may lead to the accumulation of persistent epigenetic and genetic damage after repeated exposures or in the presence of other pro-carcinogenic insults (Seaton *et al*., 1995).

3.7.3 Benzene

It has been reported that DNA methylation changes are induced by low-benzene exposure in peripheral blood DNA of gasoline station attendants and traffic police officers. High-level exposure to benzene has been associated with increased risk of acute myelogenous leukaemia (AML), which is characterised by aberrant global hypomethylation and gene-specific hypermethylation/hypomethylation (Bollati *et al*., 2007). Airborne benzene exposure was associated with a significant reduction in global methylation measured in LINE-1 and Alu. Airborne benzene was also associated with hypermethylation in p15 and hypomethylation of the MAGE-1 (melanoma antigen encoding gene) cancer-antigen gene (Bollati *et al*., 2007). This findings show that low-level benzene exposure may induce altered DNA methylation similar to the aberrant epigenetic patterns found in malignant cells. Preliminary epigenomics data showed effects of benzene on the DNA methylation of specific genes. Genomic screens for candidate genes involved in susceptibility to benzene toxicity are being undertaken in yeast, with subsequent confirmation by RNAi in human cells, leading to the identification of candidate genes. Data on these and future biomarkers will be useful. By employing toxicogenomics database, and bioinformatic approaches it would be possible to analyse the interactions among benzene toxicity, susceptibility genes, mRNA and DNA methylation through a systems biology approach.

Toxicogenomic studies, including genome-wide analyses of susceptibility genes (genomics), gene expression (transcriptomics), protein expression (proteomics) and epigenetic modifications (epigenomics), of human populations exposed to benzene are crucial to understanding gene-environment interactions, providing the ability to develop biomarkers of exposure, early effect and susceptibility. Comprehensive analysis of these toxicogenomic and epigenomic profiles by bioinformatics in the context of phenotypic endpoints comprises systems biology, which has the potential to comprehensively define the mechanisms by which benzene causes leukaemia. Preliminary epigenomics data showed effects of benzene on the DNA methylation of specific genes.

3.7.4 Endocrine-disrupting chemicals and reproductive toxicants

A large number of genes involved in epigenetic machinery are modulated by endocrine disruptive chemicals (EDCs). Developing organisms are extremely sensitive to perturbation by EDCs with hormone-like activity. Available evidence from animal models indicate that exposure to xenobiotics during critical periods of mammalian development may induce persistent and heritable changes of epigenetic states.

EDCs, such as antiandrogen vinclozolin (VCZ), have been reported to affect the male reproductive system. In this study, VCZ was administered to pregnant mice at the time of embryo sex determination, and its possible effects on the differentially methylated domains (DMDs) of two paternally (H19 and Gtl2) and three maternally (Peg1, Snrpn and Peg3) imprinted genes were tested in the male offspring. The CpGs methylation status within the five gene DMDs was analysed in the sperm, tail, liver and skeletal muscle DNAs by pyrosequencing (Stouder and Paoloni-Giacobino, 2010). EDCs are ubiquitous chemicals that interfere with growth and development. Several EDCs also interfere with epigenetic programming. The investigation of the epigenotoxic effects of BPA, an EDC used in the production of plastics and resins has further raised concern over the impact of EDCs on the epigenome. Using the Agouti viable yellow (A(vy)) mouse model, dietary BPA exposure was shown to hypomethylate both the A(vy) and the Cabp (IAP) (CDK 5 activator binding protein with IAP insertion and intracisternal A particle retroposon) metastable epialleles. This hypomethylating effect was counteracted with dietary supplementation of methyl donors or genistein. These results are consistent with reports of BPA and other EDCs causing epigenetic effects. Epigenotoxicity could lead to numerous developmental, metabolic and behavioural disorders in exposed populations. The heritable nature of epigenetic changes also increases the risk for transgenerational inheritance of phenotypes. Thus, epigenotoxicity must be considered when assessing these compounds for safety (Bernal and Jirtle, 2010).

3.7.5 Methylmercury

Methylmercury is an environmental contaminant and a potential neurotoxic agent that may be present at high levels in seafood. It has been shown that perinatal exposure to methylmercury causes persistent changes in learning and motivational behaviour in mice. Developmental exposure to low levels of methylmercury induces epigenetic suppression of BDNF (Brain Derived Neurotrophic Factor) gene expression in the hippocampus and predisposes mice to depression (Onishchenko *et al*., 2008). There have been reports which provide new insights into the control of methylation reactions by dopamine and by growth factors that increase PI3-kinase. By increasing methionine synthase activity and accelerating the conversion of homocysteine to methionine, they can lower SAH (S-adenosyl homocysteine) levels and promote methylation reactions. Neurodevelopmental toxins and thimerosal interfere with PI3 kinase-dependent methionine synthase, resulting in impaired methylation, including DNA methylation that is essential for normal development. D4 receptor-dependent PLM (phospholipid methylation) is an essential component of the molecular mechanism of attention, and reduced methionine synthase activity will therefore lead to impairments in

attention and in attention-related learning. Attention deficit hyperactivity disorder or ADHD may reflect a milder degree of impairment in these same mechanisms. The possible correlation of thiomersal and other agents and autism may lead to the discovery of new therapeutic approaches for the treatment of autism as well as new diagnostic tests that could identify individuals at high risk of developing autism in response to thimerosal or heavy metal exposure.

3.7.6 Trichloroethylene (TCE), dichloroacetic acid (DCA) and trichloroacetic acid (TCA) and persistant organics pollutants (POP)

TCE, DCA and TCA are environmental contaminants that are carcinogenic in mouse liver. Decreased methylation in the promoter regions of the c-jun and c-myc genes and increased levels of their mRNAs and proteins were found in livers of mice exposed to TCE, DCA and TCA. Methionine supplement prevented both the decreased methylation and the increased levels of the mRNAs and proteins of the two protooncogenes (Tao *et al*., 1999). This observation suggests that these carcinogens may act by depleting the availability of SAM, whereas methionine would prevent DNA hypomethylation by maintaining adequate SAM levels (Tao *et al*., 1999).

Rusiecki *et al*. evaluated the relationship between plasma persistant organics pollutant (POP) concentrations and blood global DNA methylation, estimated in Alu repeated elements, in 70 Greenlandic Inuit, a population presenting some of the highest reported levels of POPs worldwide. In this work, a significant inverse linear relationships was found for dichlorodiphenyltrichloroethane (DDT), DDE, β-BHC (benzene hexachloride), oxychlordane, α-chlordane, mirex, several PCBs (polychlorinated biphenyls) and the sum of all POPs (Rusiecki *et al*., 2008).

3.7.7 Metals

Several studies have established an association between DNA methylation and exposure to metals, including nickel, cadmium, lead and particularly arsenic (Takiguchi *et al*., 2003; Zhao *et al*., 1997). Metal-induced oxidative stress may represent one of the mechanisms accounting for these findings across different metals (Valko, Morris and Cronin, 2005). Metals are known to increase production of reactive oxygen species (ROS) in a catalytic fashion via redox cycling. Oxidative DNA damage can interfere with the ability of methyltransferases to interact with DNA (Valinluck *et al*., 2004) thus resulting in a generalized altered methylation of cytosine residues at CpG sites (Turk *et al*., 1995).

Chronic exposure to arsenite, nickel, chromium and cadmium increases cancer incidence in individuals, the molecular mechanisms underlying their ability to transform cells remain largely unknown. Carcinogenic metals are typically weak mutagens, suggesting that genetic mechanisms (mutations) may not be primarily responsible for metal-induced carcinogenesis. Growing evidence shows that environmental metal exposure involve changes in epigenetic marks (epimutations), which may lead to a possible link between heritable changes in gene expression and disease susceptibility and development.

3.7.7.1 Arsenic

Arsenic is an established agent that lacks carcinogenicity in animal models. Inorganic arsenic is enzymatically methylated for detoxication, using up SAM in the process. The observation that DNA methyltransferases also require SAM as their methyl donor suggested a role for DNA methylation in arsenic carcinogenesis and other arsenic-related effects (Mass and Wang, 1997). In rat-liver epithelial cell lines treated with chronic low arsenic doses, Zhao *et al*. showed malignant transformation associated with depressed SAM levels, global DNA hypomethylation and decreased DNA methyltrasferase activity (Zhao *et al*., 1997). Following this findings, several studies have shown that arsenic is associated with gene-specific

hypermethylation (Chanda *et al*., 2006; Zhong and Mass, 2001), as well as global DNA hypomethylation (Reichard, Schnekenburger and Puga, 2007; Sciandrello *et al*., 2004). An unexpected finding was recently reported *in vivo*, as a global dose-dependent hypermethylation of blood DNA was observed in Bangladeshi adults with chronic arsenic exposure (Pilsner *et al*., 2007). This effect was modified by folate, suggesting that arsenic-induced increases in DNA methylation were dependent from methyl availability. It has also been reported that lower blood DNA methylation was a risk factor for arsenic-induced skin lesions in a related Bangladeshi population (Pilsner *et al*., 2009).

In a human study from India, significant DNA hypermethylation of p53 and p16 promoter regions was observed in blood DNA of subjects exposed to toxic level of arsenic compared to controls (Chanda *et al*., 2006). In this study, hypermethylation showed a dose-response relationship with arsenic measured in drinking water.

Arsenic toxicity has been shown to be related to changes in miRNA expression. Marsit *et al*. showed alterations in miRNA profiles of human lymphoblastoid cells grown under sodium arsenite treatment (Marsit, Eddy and Kelsey, 2006) Interestingly, Arsenic altered expression of specific miRNAs that were involved in one-carbon metabolism (Marsit, Eddy and Kelsey, 2006). Arsenic is a non-mutagenic human carcinogen that induces tumours through unknown mechanisms. Although susceptibility to cancer can be inherited and a cancer phenotype is heritable, more than 50% of cancers are due to epigenetic changes, particularly alterations in DNA methylation. Changes in gene methylation status, mediated by arsenic, have been proposed to activate oncogene expression or silence tumour suppressor genes, leading to long-term changes in activity of genes controlling cell transformation. Interestingly, studies have demonstrated that arsenic exposure is associated with both hypo- and hypermethylation at various genetic loci *in vivo* or *in vitro*.

3.7.7.2 Cadmium

Cadmium is an established carcinogen that has very low mutagenicity (Filipic and Hei, 2004). Many possible mechanisms of cadmium carcinogenesis have been suggested and, among them, induction of ROS and alteration of DNA methylation seem to play a predominant biological role (Huang *et al*., 2008). Takiguchi *et al*. showed that cadmium reduces genome methylation, inhibiting DNA methyltransferases in a noncompetitive manner. This finding is suggestive of interference in enzyme-DNA interaction, possibly through an interaction of cadmium with the DNA binding domain of the methyltransferase enzyme (Eady *et al*., 1989; Takiguchi *et al*., 2003). Cadmium can also inhibit DNA methylation in proto-oncogenes inducing oncogene expression and resulting in cell proliferation (Huang *et al*., 2008).

3.7.7.3 Chromium

Kondo *et al*. (2009) investigated p16 methylation using a methylation-specific PCR method in 30 lung cancer cases associated with chromate exposure and 38 non-chromate lung cancers. A variety of genetic changes in lung cancers from chromate-exposed subjects is known, but the epigenetic effects of chromium are still poorly understood. Kondo *et al*. showed that chromate exposure influenced p16 hypermethylation measured in lung cancer tissues, compared to tissues from non-chromate lung cancer (Kondo *et al*., 2009). Chromium has been shown to reduce *in vitro* H3 phosphorylation and trimethylation, as well as various acetylation marks in H3 and H4 (Sun *et al*., 2009).

3.7.7.4 Nickel

The mechanisms underlying nickel health-related effects, including carcinogenicity and cardiorespiratory disease, are still largely unknown. It has been proposed that nickel may replace magnesium in

DNA interactions, enhance chromatin condensation, and trigger *de novo* DNA methylation (Lee *et al*., 1995). In Chinese hamster G12 cells transfected with *E.coli* gtp gene, Lee *et al*. demonstrated nickel-induced hypermethylation leading to the inactivation of the expression of the transfected gene (Lee *et al*., 1995).

Several studies have shown that nickel affects histone modifications. Exposure to soluble $NiCl_2$ has been shown to reduce histone acetylation, increase demethylation of H3K9 and increase monoubiquitination of H2A and H2B *in vitro* (Ke *et al*., 2006). Broday *et al*. studied nickel effects, at nontoxic levels, on yeast and mammalian cells and found a decrease in histone H4 acetylation, affecting only lysine 12 in mammalian cells and all of the four H4 lysines in yeasts (Broday *et al*., 2000).

Nickel ion exposure has been shown to increase global H3K9 mono- and dimethylation, both of which have been associated with increased DNA methylation and long-term gene silencing. Nickel ions also interfere with the removal of histone methylation *in vivo* and directly decrease the activity of a Fe(II)-2-oxoglutarate dependent histone H2K9 demethylase in nuclear extract *in vitro* (Chen *et al*., 2006). In human lung cells exposed to soluble nickel compounds, three major changes in histone modifications have been observed: (i) loss of acetylation of H2A, H2B, H3 and H4; (ii) increased H3K9 dimethylation and (iii) increased ubiquitinylation of H2A and H2B (Broday *et al*., 2000; Chen *et al*., 2006; Lee *et al*., 1995).

It has been proposed that the binding of Ni2+ is able to promote a secondary structure with organized side-chain orientation on the N-terminal tail of histone H4. Acetylation of lysine 12 and 16 in yeast exposed to nickel was more robustly affected than lysine 5 and 8. Nickel binding to histidine 18 in histone H4 may be accountable for this effect, acting as an anchoring binding site for metal ions (Zoroddu *et al*., 2002).

3.8 Toxicogenomics and epigenetics

It thus appears that the effects of toxins and chemicals on biological systems are complex. In animal studies, several chemicals including alloxan (Spergel, Levy and Goldner, 1971), cyclophosphamide (Hales, Crosman and Robaire, 1992), orthoaminoasotoluol (Popova, 1989), benzopyrene (Csaba and Inczefi-Gonda, 1998), DES (Newbold, Padilla-Banks and Jefferson, 2006) and VCZ (Anway *et al*., 2005) have been reported to induce transgenerational phenotypic effects. Transgenerational transmission of chemically-induced epigenetic changes have been suggested as a potential mechanisms for some of these effects. Anway *et al*. (2005) showed that gestational exposure of female rats to the endocrine disruptor VCZ at the time of gonadal sex determination caused a variety of abnormalities in the offspring that were then transmitted down the male line for at least three generations. The high incidence of the defects (approximately 90% of all males in all generations) and the absence of abnormalities when passed down the female line suggested gametic epigenetic inheritance. In this study, altered DNA methylation in two candidate genes was seen in sperm from VCZ-exposed males, and these abnormal methylation patterns were inherited. These results indicate that exposure of germ cells, possibly at a specific developmental stage, is necessary to produce heritable epigenetic changes. In addition, epigenetic mechanisms may underlie the effects of *in utero* and early life exposures on adult health, as *in utero*/early-life exposures to epigenetically-active chemicals may produce health effects later in life even independently of environmental risk factors in adults (Gluckman *et al*., 2008). As reported in the sections above, most of the studies on epigenetic effects of environmental chemicals have shown changes in DNA methylation, histone modifications or miRNA in somatic cells of adult individuals. Whether epigenetic changes observed in somatic cells are correlated with germline epigenetic changes is uncertain. Environmentally-induced epigenetic somatic alterations may be sufficient to cause anomalies in biological functions, but these changes are not heritable per se and may not be associated to any transgenerational risk.

Exposure to ionizing and ultraviolet light radiation can induce chromosome and point gene mutation while at low sub lethal doses, induce oxidative stress, which, in turn, can result in altered gene expression. Such exposure levels can have epigenetic effects (Trosko and Suzuki, 2009). It should also be pointed out that, at killing doses, any agent (radiation, chemicals, biological) can be an indirect epigenetic toxicant, in that the released substances from cell killing can act to stimulate the surviving cells to wound healing.

Toxicity must be viewed in a larger perspective. As the foreign agent (chemical or stem cell product) enters the body, it will directly or indirectly interact with the different types of cells. Such treatment would lead to intracellular signalling and in the immune system, various bioactive secreted factors are released that now can interact to incorporate a 'systems' aspect of how a physical, chemical or biological agent could affect a multicellular organism. At noncytotoxic concentrations or doses, an agent could simultaneously trigger oxidative stress in both the cells of the immune tissues and the epithelial/endothelial/stromal cells in various organs. Upon induction of ROS and of oxidative stress and induction of intracellular signalling in various cell types of the complex immune system, various cytokines would interact on tissues. It has been suggested that these cells would react to the agent differentially because of their different physiological/phenotypic state, the interaction could be very different (e.g. the normal stem cells might be induced to proliferate asymmetrically, any initiated pre-cancerous stem cell might proliferate symmetrically, the progenitor cells might be induced to proliferate symmetrically and to migrate, as in wound healing, and the terminally differentiated cell might adaptively respond or to apoptose). The role of microbial infections, the microbial diversity in the host will also play a role in such responses. In summary, each cell type of the immune system and of the various organ tissues, with their different expressed genes and cellular physiology, will respond differently to sublethal exposure to agents inducing oxidative stress-triggered intracellular signalling and epigenetic alterations. The role of inflammation, infection and immunity could then play a role in establishing wide range of diseases.

The epigenome plays the pivotal role as interface between genome and environment. True genome-wide assessments of epigenetic marks, such as DNA methylation (methylomes) or chromatin modifications could be evaluated through high-throughput arrays or high throughput second-generation DNA sequencing methods. The ability to collect these data at this level of resolution is expected to facilitate identification and analysis of changes that occur due to development, lineage and tissue-specificity, environmental influence, such as ageing, stress, diet, hormones or toxins. Common complex traits are under variable levels of genetic influence and additionally epigenetic effect.

We have described a novel method using cDNA microarray which can be useful in determination of genome wide methylation landscape in a rapid, specific and sequence independent manner (Kelkar and Deobagkar, 2009; Mukherjee, Sainis and Deobagkar, 2011). There is a need for design and application of methodology which will lead to identification of targets and biomarkers for toxicity.

Toxicogenomics combines toxicology with molecular profiling technologies, including genomics (DNA), transcriptomics (mRNA), proteomics (proteins) and metabolomics (chemical metabolites) to elucidate molecular mechanisms involved in chemically-induced toxicity. Chemically-induced alterations in the transcriptome, proteome and metabolome are analysed in the context of the stable, inherited genome, which is assessed by genomics. Toxicogenomic studies of human populations are crucial to understanding gene-environment interactions, and can provide the ability to develop novel biomarkers of exposure (exposome), early effect (responsome) and susceptibility (genome). Epigenomics is the study of epigenetic elements, including DNA methylation (methylomics), non-coding miRNA (miRNAomics) along with small interfering RNA (siRNA) and short hairpin RNA (shRNA) for RNAi and histone modification. Epigenetic modifications play an essential role in regulating gene expression and biological and molecular functions in living cells, without altering the genome.

3.9 Hydroxymethyl cytosine and toxicogenomics

Hydroxymethylcytosine has been described to occur as the sixth modified base and Tet proteins are involved in conversion of 5-methyl cytosine at a particular position to 5-hydoxymethyl cytosine. Environmental signals appear to be an important trigger in modulating conversion to hydroxymethyl cytosine. The Tet proteins (the 10–11 translocation family) have been shown to be crucial in the demethylation cycle where they convert 5-methyl cytosine to 5-hydroxymethyl cytosine. In embryonic stem cells both modified bases have been seen. It would be interseating to speculate and determine what role hydroxymethyl cytosine may play in response to toxic chemicals and drugs. This becomes particularly important since environmental perturbations are known to alter levels and patterns of hydroxymethyl cytosine. How the balance between the two modified bases is altered in response to exposures and stress needs to be established.

3.10 MicroRNAs

miRNAs are short single-stranded non-coding molecules that function as negative regulators to silence or suppress gene expression. Aberrant miRNA expression has been implicated in a several cellular processes and pathogenic pathways of a number of diseases. Evidence is rapidly growing that miRNA regulation of gene expression may be affected by environmental chemicals. Environmental exposures include those that have frequently been associated with chronic diseases, such as heavy metals, air pollution, BPA and cigarette smoking. Evidence has accumulated that miRNAs would play an important role in development of cancer and a number of diseases. In a few systems miRNA has been suggested to have an important role in establishing methylation patterns and influencing epigenetic regulation. Human miRNA microarrays (Agilent), containing probes for 470 human and 64 human viral miRNAs, were used to analyse the differential expression of miRNAs in the total PBMC (peripheral blood mononuclear cells) RNA from seven exposed-control matched pairs, in a pilot study. Preliminary analysis showed upregulation of four miRNAs (miR-154*; miR-487a; miR-493-3p and miR-668) by benzene exposure. Upregulation of miR-154* expression, possibly through a change in the methylation and acetylation status of the 14q32 region, has been reported in patients with acute promyelocytic leukaemia bearing the t(15;17) translocation (Dixon-McIver *et al*., 2008). It would be of interest to explore the role of miRNAs in DNA methylation changes on response to exposure to toxicants/chemicals.

3.11 DNA methylation in cancer

Global DNA methylation alterations in prostate and other cancer are correlated with adaptive changes in several signalling pathways that may be influenced by lifestyle changes. Dietary factors may influence the supply of methyl groups available for the formation of SAM, a coenzyme involved in methyl group transfer. Moreover, dietary factors may modify the utilisation of methyl groups by processes including shifts in DNMT1 activities. SAM (also known as AdoMet) is the methyl donor for the majority of methyl-transferases that modify DNA, RNA, histones and other proteins, dictating replicational, transcriptional and translational fidelity, mismatch repair, chromatin modelling, epigenetic modifications and imprinting. Fifteen superfamilies of SAM-binding proteins have been identified with multiple functions varying from methylation of phospholipids and small molecules such as arsenic to synthesis of polyamines or radical formation. SAM is regenerated from demethylated SAM via the methionine cycle, which involves folate. Modulation of this SAM biosynthesis cycle in humans, for example through folate shortage via dietary insufficiency, alcohol abuse, arsenic poisoning or hereditary factors, can lead to depletion of SAM and

human disease. DNA methylation patterns may influence the response to a bioactive food component. Several lines of evidence suggest that DNA hypomethylation and chromosome instability may result from insufficient dietary folate. Folate provides carbon units for a number of biochemical processes, including production of SAM, a universal methyl donor that also supplies the methyl group on cytosines in DNA. The effect of reduced dietary folate on hypomethylation is observed in dietary studies in humans, and the hypomethylation is reversible by controlled folate repletion. SAM is required for the biosynthesis of the polyamines spermidine and spermine, which are produced by normal prostate secretory cells. One of the possible explanations for a limitation in SAM is the increased requirement for folate biosynthesis in proliferating cancer cells. Insufficient concentrations of SAM for DNA methylation in cancers may be caused by an insufficient supply of metabolic precursors, for example methionine, folate, vitamin B12, zinc and choline, or increased demands from various other methylation reactions (Balaghi and Wagner, 1993; Rampersaud *et al*., 2000). Methionine deprivation stress induces apoptosis, which is mediated by downregulation of TP53 and increased production of TNF-related apoptosis-inducing ligand (TRAIL) and proinflammatory cytokines (Sun *et al*., 2009). Imbalances of nutrients and other bioactive food components have been shown to lead to global DNA hypomethylation, and gene-specific hypomethylation and/or hypermethylation.

Growing evidence indicates that epigenetic dysregulation of gene expression plays a primary role in cancer etiology. Methylation of 5'CpG islands in promoter region has emerged as one of the most important epigenetic mechanisms in the development of human cancer. Aberrant promoter methylation of a series of tumour suppressor genes has been detected in blood leukocyte DNA from lung cancer patients (Jarmalaite *et al*., 2003) and healthy subjects exposed to carcinogens. In our previous studies, we have reported blood leukocyte global hypomethylation in subjects exposed to PM and metal components (Bollati, Tarantini and Baccarelli, 2010) and benzene (Bollati *et al*., 2007). Global hypomethylation is frequently observed in cancer tissues, including lung cancer and blood leukocytes of cancer patients and it often coexists with gene-specific methylation alterations. However, whether ambient PM and its metal components can induce DNA methylation alterations in tumour suppressor genes, which may be involved in air pollution-related lung carcinogenesis, has not been examined.

1,3-Butadiene (BD) is a common environmental contaminant classified as 'carcinogenic to humans'. Formation of BD-induced DNA adducts plays a major role in its carcinogenicity. BD is also an epigenotoxic agent. BD-induced genotoxic and epigenotoxic events have been shown to be due to interstrain differences (Koturbash *et al*., 2011). Nickel-induced genotoxic effects such as DNA strand breaks, DNA-protein crosslinks and generation of ROS usually occur in heterochromatic DNA, which is not genetically active and thus this genotoxicity has little mutagenic consequence. This squares with evidence that nickel compounds are carcinogenic yet show no mutagenicity in mammalian cell assays. It is well established that chromosomally integrated transgenes in transfected cell lines and transgenic animals are susceptible to inactivation by changes in chromatin structure, thus revealing that chromatin structural change is a potential mechanism of transcriptional regulation (Fragou *et al*., 2011).

DNA methylation is a fundamental determinant of chromatin structure. In general, the extent of methylation at CpG islands, DNA sequence clusters rich in CG dinucleotides in the vicinity of gene promoters, correlates with the long-term silencing of gene transcription. By repressing gene expression, DNA methylation provides an essential level of control over genes regulating cell differentiation, proliferation and organism development, and these changes can be passed on to daughter cells during replication. Global disruption of DNA methylation is lethal to mammals, and even focal disruption at imprinted loci can produce serious developmental abnormalities. Changes in promoter CpG island methylation can significantly change cell behaviour. DNA hypermethylation has been implicated in the loss of tumour suppressor gene expression (Chimonidou *et al*., 2011). Evidence has accumulated in the last 20 years that oncogenic transformation is frequently associated with genome-wide hypomethylation. Hypomethylation appears to

contribute to malignant transformation by predisposing cells to chromosomal defects and rearrangements leading to genetic instability and spontaneous mutations (Dhayat *et al*., 2011). These observations suggest that genome hypomethylation precedes and likely predisposes to transformation. Thus, both DNA hypermethylation and hypomethylation appear to independently contribute to cancer development and progression.

3.12 Bioinformatics approach

Since the analysis of toxicology with systems biology approach generates complex datasets which need to be analysed with innovative and state of art approaches there have been various databases and bioinformatics tools which are specifically developed for this discovery process (Table 3.1). The comparative toxicogenomics database (CTD; http://ctd.mdibl.org) provides datasets and possibilities of examining interactions between environmental chemicals and genes/proteins. It helps in the understanding of the impact of environmental chemicals on human health (Mattingly, 2009). CTD curates and integrates toxicogenomic data from vertebrates and invertebrates, including 124 000 chemicals, 2.6 million gene and protein sequences and their associated Gene Ontology (GO) (Ashburner *et al*., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations (Kanehisa *et al*., 2008), 128 000 taxa and 6300 human diseases to produce a unique resource for the cross-species analysis of chemicals, genes and proteins, diseases and their complex interactions. Biocurators at CTD curate three types of data from the scientific literature: (i) chemical-gene/protein interactions, (ii) chemical-disease relationships and (iii) gene/protein-disease relationships. These data are curated in a structured format using controlled vocabularies and ontologies for chemicals, genes/proteins, diseases, molecular interactions and organisms. Curated gene-disease relationships in CTD are integrated with data from the Online Mendelian Inheritance in Man (OMIM) database. Over 110 000 molecular interactions involving 3700 chemicals and 13 200 genes from 260 species have been curated to date. CTD also contains curated data for more than 5700 and 2000 gene-disease and chemical-disease relationships, respectively. CTD provides a unique perspective about chemical-gene-disease relationships by integrating data curated from the scientific literature.

Table 3.1 *Databases and analytical tools to study relationship between DNA methylation and toxicology*

Database names	Web address	References
Comparative Toxicogenomic database (CTD)	http://ctd.mdibl.org/	Carolyn J. Mattingly *et al.* (2006)
Toxicology Network Dataset (TOXNET)	http://toxnet.nim.nih.gov/index.html	–
ChemIDplus	http://sis.nlm.nih.gov/chemical.html	–
Disease-Annotated Chromatin Epigenetics Resource (DAnCER)	http://wodaklab.org/dancer/.	Andrei L. Turinsky *et al.* (2011)
NCBI-epigenetics	http://www.ncbi.nlm.nih.gov/epigenomics	–

Using arsenic exposure as an example, it can be illustrated that CTD contains curated data from the published literature describing 2738 molecular interactions between 21 different arsenic compounds and 1456 genes and proteins and 516 putative diseases that interestingly parallel known arsenic-associated diseases categories. Analysis of these genes and proteins provide insight into the biological functions and molecular networks that are affected by exposure to arsenic, including stress response, apoptosis, cell cycle and specific protein signalling pathways. Integrating arsenic-gene data with gene-disease data yields a list of diseases that may be associated with arsenic exposure and genes that may explain this association.

Another database is TOXNET®: Toxicology Data Network which is a cluster of databases covering toxicology, hazardous chemicals, environmental health and related areas. It is managed by the Toxicology and Environmental Health Information Program (TEHIP) in the Division of Specialized Information Services (SIS) of the National Library of Medicine (NLM). TOXNET provides free access to and easy searching of the following databases.

In order to facilitate the identification of Biomarkers and early indicators of exposure to chemicals and toxins ChemIDplus®, has been developed. This database can provide access to structure and nomenclature authority databases used for the identification of chemical substances cited in NLM databases. ChemIDplus contains over 390 000 chemical records, of which over 299 000 include chemical structures. ChemIDplus is searchable by Name, Synonym, CAS Registry Number, Molecular Formula, Classification Code, Locator Code, Structure and/or Physical properties. Enhanced structure display is available in ChemIDplus Advanced.

Another database DAnCER: Disease-Annotated Chromatin Epigenetics Resource provides information of networks of chromatin modulators (Turinsky *et al*., 2011). Chromatin modification (van Delft *et al*., 2004) is a set of epigenetic processes that govern many aspects of DNA replication, transcription and repair. chromatin modification (CM) is carried out by groups of physically interacting proteins, and their disruption has been linked to a number of complex human diseases.

3.13 Summary

Toxicogenomics can provide a library of generic expression profiles for different classes of toxicity that allows the characterization of an unknown compound based upon the profiles with which it fits. It can be utilized on a large scale for pathway analysis and marker identification. In toxicological analysis use of *in vitro* systems will facilitate the analysis and identifications of molecular genetic mechanisms. As has been discussed, DNA methylation regulates gene expression and exposure to chemicals/toxins to modulate methylation levels and result in change in methylome. It will therefore be important to investigate the possibility of using such changes as biomarkers for early detection of exposure as well as design and development of interference strategies (Figure 3.5). The application of epigenomic profiling technologies within the field of drug safety sciences has great potential for providing novel insights into the molecular basis of a wide range of long-lasting cellular perturbations including increased susceptibility to disease and/or toxicity, memory of prior immune stimulation and/or drug exposure and transgenerational effects. As a new approach it will be essential to examine the consistency, reproducibility and specificity of methylomic changes in association with exposure. Although Epigenomics has significant potential to impact translational sciences in the coming years, it would be a daunting task to dissect out the meaningful changes which are associated with a particular exposure with associated changes. The paradigms and patterns will have to be established for meaningful use of toxicogenomics and epigenetics (in particular DNA methylation) in predicting and evaluating the application in toxicological analysis.

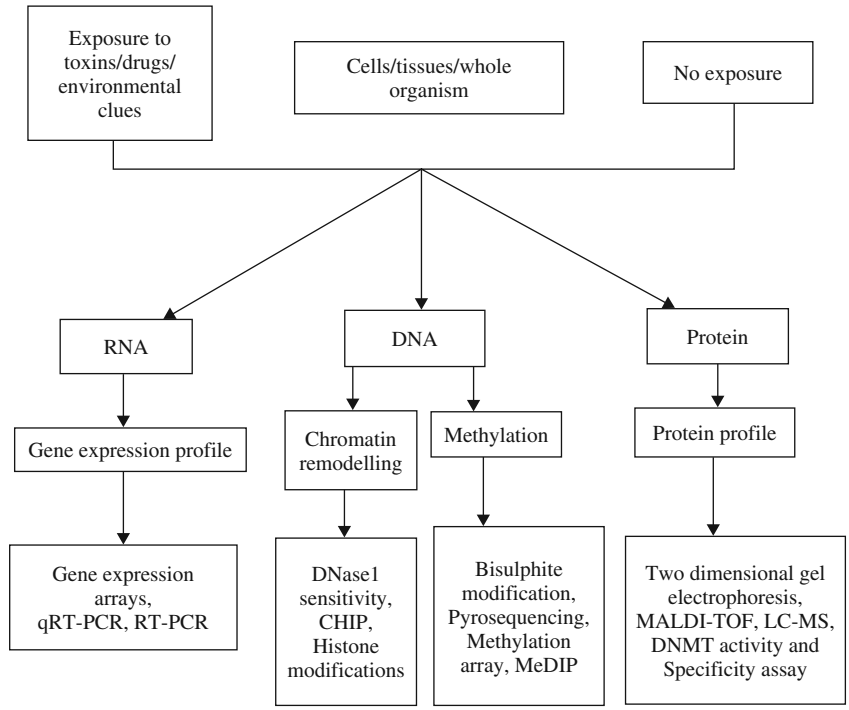

Figure 3.5 *An approach to discover link between toxicogenomics and DNA methylation*

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