Epigenetics in Toxicology: The Implications of Epigenetic Alterations Driven by External Exposures for Human Health

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Summary

The prospect that non-genotoxic environmental exposures of a physical, chemical, or social nature act on epigenetic mechanisms opens a new field in toxicology. Epigenetics refers to the stable changes in gene expression programming that could alter the phenotype in the absence of a change in DNA sequence. Epigenetic modifications, which include DNA methylation, covalent modifications of histone tails, and regulation by non-coding RNAs, among others, play a significant role in normal development and genome stability and constitute a mechanism of genome adaptation to external stimuli. However, the process of adaptation may bring about adverse effects resulting in the development of pathologies. Exogenous agents, therefore, could have long-term toxicity that lasts well after the initial insult has disappeared – without changing the DNA sequence. Such toxicities remain undetected by current screening methods. An increasing number of chemicals, referred to as epimutagens, are emerging. The current challenge in toxicology is to develop screening methods that would detect epigenetic alterations caused by this novel class of toxicants.

Keywords: epigenetics, environmental exposures, toxicology, the 3Rs principle

1 Introduction

Epigenetic modifications constitute a layer of information in addition to the genetic platform of information, comprising several components, such as DNA methylation, covalent histone modifications, particularly histone acetylation and methylation, and non-coding RNA-related mechanisms (Bergmann and Lane, 2003; Jenuwein and Allis, 2001; Razin and Riggs, 1980; Razin and Szyf, 1984; Strahl and Allis, 2000). Additional modifications of the methyl group on DNA have recently been highlighted, such as hydroxy-methylcytosine (Kriaucionis and Heintz, 2009) and formyl and carboxy-methyl cytosine (Ito et al., 2011). These mechanisms, although catalyzed by different enzymes and controlled by different protein complexes, mutually interact in the regulation of gene expression through changing chromatin configuration, degradation of RNA, and inhibition of translation (Bergmann and Lane, 2003; Razin and Riggs, 1980; Strahl and Allis, 2000). During gestation, the distribution of methyl groups in the genome is sculpted to generate cell-type specific patterns of DNA methylation. Similarly, histone modifications are rearranged to confer cell-type specific expression programs on the DNA (Gluckman et al., 2009; Hanson and Gluckman, 2008; Sinclair et al., 2007b).

For years it was a strongly held dogma that DNA methylation patterns generated during differentiation were fixed throughout life and strictly maintained by three enzymatic principles. First, in mitotic cells, a maintenance DNA methyltransferase (DNMT1) accurately copies the DNA methylation pattern and, second, in post-mitotic cells *de novo* DNA methylation or active demethylation do not occur (Razin and Riggs, 1980). This concept was challenged a decade ago by the suggestion that the DNA methylation reaction is reversible and therefore that DNA methylation could participate in physiological and pathological responses in mature organisms and in post-mitotic cells (Ramchandani et al., 1999). The dynamic nature of DNA methylation patterns has been confirmed by a long list of studies (Feng et al., 2010; Levenson et al., 2006; Miller and Sweatt, 2007; Sweatt, 2009; Weaver et al., 2004). This has extremely important implications for toxicomethylomics, as epimutagens could have an impact not only during pregnancy, as is widely suspected, but also throughout life in mitotic and post-mitotic tissues (Szyf, 2007, 2011). Epimutagens could possibly affect DNA methylation through interfering with chromatin and DNA methylation enzymatic reactions or through affecting signaling pathways that control the adaptation of the DNA methylation pattern to exogenous signals. In this review, we summarize recent data suggesting that environmental exposures affect epigenetic modifications, particularly DNA methylation. Furthermore, we highlight the unmet need of new approaches in toxicology to screen and detect adverse effects of epimutagens. We also discuss the possible role of epigenetics in the implementation of the 3Rs principle in biomedical research.

2 DNA methylation and its role in the regulation of gene expression

DNA methylation is a covalent modification of DNA that in mammalian cells occurs mainly at the 5th position of the cytosine pyrimidine ring located predominantly within CpG sequences (Gruenbaum et al., 1981; Hotchkiss, 1948; Wyatt, 1950). Approximately 30% of CpGs are found in CG-rich regions, called "CpG islands," which in normal cells are mostly unmethylated and located in promoter regions of housekeeping genes, tissue-specific genes, and tumor suppressor genes (Hermann et al., 2004). CpG islands are also present in promoters of some oncogenes, where their methylated state is associated with transcriptional silencing (Szyf et al., 2004). Apart from oncogene repression, DNA methylation in normal cells is implicated in the control of expression of genes crucial for cell proliferation, differentiation, and normal development as well as in parental imprinting, X chromosome inactivation, and preservation of chromosomal integrity by silencing of transposons and repetitive elements (Hermann et al., 2004; Szyf et al., 2004). An inverse correlation between gene expression and DNA methylation was confirmed by multiple studies and more recently by whole-genome approaches (Rauch et al., 2009; Razin and Szyf, 1984; Stefanska et al., 2011a). DNA hypermethylation was found to be a common mechanism of the silencing of tumor suppressor genes in different types of cancer (Baylin, 2005; Ren et al., 2011; Szyf et al., 2004). Reversal of the aberrant increase in DNA methylation restored gene expression (Alva et al., 2011; Stefanska et al., 2010b, 2011b; Szyf, 2005). Loss of DNA methylation was associated with the activation of genes of diverse biological functions implicated in multiple signaling pathways in cancer (Stefanska et al., 2011a).

Methylation of CpGs within gene promoters or enhancers effectively silences transcription by several mechanisms (Stein et al., 1982). First, methylation within a recognition element of transcription factors can block their binding to DNA, resulting in suppression of transcription (Comb and Goodman, 1990; Inamdar et al., 1991). Second, the access of transcription factors to regulatory regions of promoters can be impeded by methylated DNA-binding domain proteins (MBDs) that bind to methylated DNA and cover recognition elements (Nan et al., 1997). The third mechanism is associated with changes in chromatin structure. MBDs can recruit histone deacetylases and histone methyltransferases that set up an inactive chromatin state around the gene (Eden et al., 1998; Nan et al., 1997).

DNA methyltransferases catalyze the transfer of a methyl moiety from the ubiquitous methyl donor, S-adenosyl-L-methionine (SAM), to the 5th position on the cytosine ring (Fig. 1) (Gruenbaum et al., 1982; Razin and Cedar, 1977; Razin and Riggs, 1980). CpG is a palindromic sequence that serves as a

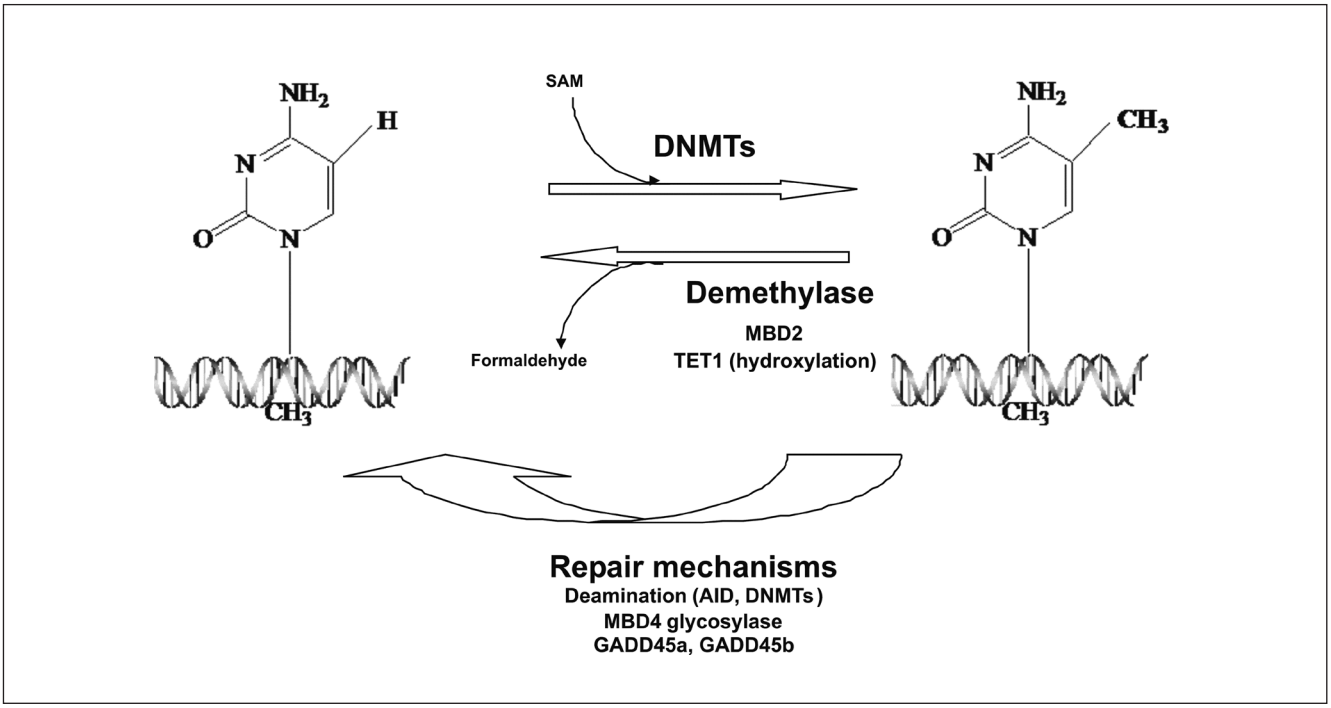

Fig. 1: DNA methylation is a reversible reaction involving methylating and demethylating enzymes
Methyltransferases (DNMTs) transfer a methyl group from S-adenosyl-L-methionine (SAM) to cytosine at the 5th position of the pyrimidine ring. Direct demethylases, with MBD2 as a putative candidate, reverse the methylation reaction. One of the proposed mechanisms involves oxidation and a release of a methyl group in the form of formaldehyde. Alternatively, 5-methylcytosine can be recognized by components of DNA repair machinery, cleaved and replaced with unmethylated cytosine. This replacement may be initiated by modifications of the methyl cytosine ring such as deamination that can be catalyzed by AID or DNMTs. Deamination results in a G:T mismatch that is recognized and repaired by glycosylase MBD4. GADD45a and GADD45b repair proteins also were shown to be associated with DNA demethylation.

template for copying DNA methylation from a parental methylated strand template to the unmethylated daughter strand during cell division by the maintenance DNA methyltransferase 1 (DNMT1). Other DNMTs identified in vertebrates include DNMT2, DNMT3A, DNMT3B, and DNMT3L. While DNMT1 is responsible for most of the methylation marks in the genome (Chen et al., 2007; Li et al., 1992), DNMT3A and DNMT3B methylate DNA *de novo*, which is especially crucial for the establishment of gene imprinting and silencing of retrotransposons (Okano et al., 1998). Although the DNA methyltransferase activity of DNMT2 is not clear, it is known to participate in the methylation of transfer RNA (Goll et al., 2006; Rai et al., 2007). DNMT3L, a DNMT that is missing a catalytic domain, was shown to regulate the activity and substrate recognition by DNMT3A and DNMT3B (Bourc'his and Bestor, 2004; Bourc'his et al., 2001). While DNMT1 shows preference to hemi-methylated DNA in accordance with its role as a maintenance DNA methyltransferase, it may also possess *de novo* methylation activity, since DNMT3A and DNMT3B are in charge of some, but not all, *de novo* methylation reactions during development (Okano et al., 1999).

Since the covalent bond between cytosine and a carbon in a pyrimidine ring is very strong, the common thinking in the field has been that a methyl group can be removed only in a passive way by blocking the activity of methyltransferases in dividing cells. However, active demethylation was demonstrated in several studies, for instance demethylation of the paternal genome during development, demethylation in post-mitotic neurons, or demethylation mediated by brain extracts (Dong et al., 2008; Feng and Fan, 2009; Feng et al., 2010; Levenson et al., 2006; Mastronardi et al., 2007; Miller and Sweatt, 2007; Weaver et al., 2004). Our group proposed that MBD2 is a demethylase reversing DNA methylation (Fig. 1) (Bhattacharya et al., 1999; Detich et al., 2002; Ramchandani et al., 1999). A mechanism for active enzymatic removal of a methyl group from 5-methylcytosine in DNA was proposed to involve oxidation of 5-methylcytosine to 5-hydroxymethylcytosine followed by release of a methyl group in formaldehyde (Hamm et al., 2008). Interestingly, 5-hydroxymethylcytosine was recently shown to be present in mammalian DNA (Kriaucionis and Heintz, 2009) and ten-eleven translocation-1 (TET1) enzyme converting methylcytosine to hydroxymethylcytosine was required for maintaining the demethylation of *nanog* in embryonic stem cells (Ito et al., 2010). It supports a possible role of 5-hydroxymethylcytosine and TET1 as an intermediary in the demethylation reaction. An alternative mechanism that has gained extensive experimental support is DNA demethylation by DNA repair-based mechanisms where methylated cytosine is replaced by the unmethylated base (Barreto et al., 2007; Jost, 1993; Rai et al., 2008; Razin et al., 1986). This replacement may be initiated by modifications of the methylcytosine ring, such as deamination. Demethylation in zebrafish embryos was shown to involve the activation-induced (cytosine) deaminase (AID), which deaminates 5-methylcytosine to thymine creating a G:T mismatch (Rai et al., 2008). The repair was carried out by a G:T mismatch-specific thymine glycosylase (Mbd4) and promoted by the growth arrest and DNA-damage inducible (Gadd45) protein. AID has been implicated in the global demethylation in mouse primordial germ cells (Popp et al., 2010). Additionally, bacterial DNA (cytosine-5)-methyltransferase was previously shown to catalyze deamination of 5 methylcytosine to thymine in the absence of SAM (Shen et al., 1992; Zingg et al., 1998). Subsequent studies demonstrated that vertebrate DNMTs could also participate in demethylation by deaminating the methylcytosine to thymine (Kangaspeska et al., 2008). GADD45a, a DNA repair protein, was also proposed to contribute to active DNA demethylation in mammals by an unknown DNA repair-based mechanism (Barreto et al., 2007).

Recent data on genomic sequencing indicate that methylation can occur equally in cytosines within dinucleotide sequences other than CpGs in undifferentiated cells (Fuso et al., 2010; Lister et al., 2009). This implies that not all methylation marks are automatically mitotically heritable by the classical semi-conservative model. Non-CpG DNA methylation may be maintained dynamically by the equilibrium between methylating and demethylating enzymes and may participate in the response to environmental exposures along with CpG methylation.

3 Histone modifications and non-coding RNA-related mechanisms

The nucleosome is the basic structural unit of the eukaryotic genome and is composed of an octamer of pairs of 4 core histones (H2A, H2B, H3, H4) around which 147 base pairs of DNA are wrapped (Bannister and Kouzarides, 2011; Choudhuri, 2011; Fullgrabe et al., 2011; Kouzarides, 2007; Reamon-Buettner et al., 2008; Szyf, 2007; Vandegehuchte and Janssen, 2011). The N-terminal tails of the core histones, protruding from the nucleosomes, contain a series of conserved amino acid residues, including lysine, arginine and serine. They interact with the negatively charged phosphate groups of the DNA, resulting in compaction of the chromatin (Bannister and Kouzarides, 2011; Ducasse and Brown, 2006; Fullgrabe et al., 2011; Gronbaek et al., 2007; Kouzarides, 2007; Reamon-Buettner and Borlak, 2007). Histone tails, especially those of histones H3 and H4, are subject to a number of posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, biotinylation and ADP ribosylation (Bannister and Kouzarides, 2011; Choudhuri, 2011; Fullgrabe et al., 2011; Kouzarides, 2007; Reamon-Buettner and Borlak, 2007; Reamon-Buettner et al., 2008). Most of the posttranslational histone modifications are dynamic, and the interplay between enzymes that introduce the modifications and those that remove them is believed to formulate a complex code that regulates gene transcription, mitotic condensation of chromatin, and DNA repair (Bannister and Kouzarides, 2011; Choudhuri, 2011; Costa, 2008; Kouzarides, 2007; Reamon-Buettner and Borlak, 2007; Reamon-Buettner et al., 2008; Vandegehuchte and Janssen, 2011). Acetylation is among the best understood components of the "histone code." The primary sites of histone acetylation are ε-amino groups in the positively charged lysine residues. A number of histone acetyltransferase complexes mediate the

addition of an acetyl group from acetyl-coenzyme A, resulting in the neutralization of the positive charge and thus the loosening of histone-DNA contacts. This process, in turn, promotes decondensation of the chromatin, thereby facilitating the accessibility of the transcriptional machinery to the DNA. The inverse reaction is catalyzed by histone deacetylase enzymes and is frequently associated with transcriptional repression (Bannister and Kouzarides, 2011; Choudhuri, 2011; Kouzarides, 2007; Reamon-Buettner and Borlak, 2007; Yang and Seto, 2007).

A major portion of the genome encodes non-coding RNA genes (Choudhuri, 2011; Ketting, 2011; Szymanski et al., 2005; Zhou et al., 2010). Increasing evidence indicates that these non-coding RNAs, and more specifically microRNAs (miRNAs), are implicated in a variety of epigenetic mechanisms (Costa, 2007, 2008). In the nucleus, miRNA genes are transcribed by RNA polymerase II, yielding long primary miRNA transcripts, which are subsequently cleaved by the nuclear microprocessor complex to 70-nucleotide hairpins, known as precursor miRNAs. The latter are transported to the cytoplasm by exportin-5, where they are processed by the endonuclease Dicer to 22-nucleotide duplexes of mature miRNA. These duplexes are loaded into the RNA-induced silencing (RISC) complex, where the Argonaute protein Ago2 mediates elimination of one of the miRNA strands. The remaining strand guides RISC to target messenger RNAs that have miRNA complementary sites in the 3'-untranslated region. RISC then suppresses translation, cleaves or degrades the mRNA, depending on the degree of mRNA-miRNA complementarity (Choudhuri, 2011; Chu and Rana, 2007; Filipowicz et al., 2008; Ketting, 2011; Saetrom et al., 2007; Zhou et al., 2010). The number of identified human miRNAs is rapidly increasing (Saetrom et al., 2007), and they were found to regulate about 30% of all protein-coding genes (Filipowicz et al., 2008; Zhou et al., 2010). Although a matter of debate, miRNAs are considered a component of the epigenome.

4 Bilateral relationship between DNA methylation and histone modifications: implications for toxicology

DNA methylation should be understood within the context of chromatin structure. Active chromatin is associated with DNA hypomethylation and active histone marks, whereas inactive chromatin contains hypermethylated DNA and histone modifications underlying inactive transcriptional state. Methylation of DNA results in the recruitment of histone modifying enzymes that reconfigure chromatin structure (Eden et al., 1998; Nan et al., 1997). On the other hand, studies showed that alterations in histone marks can trigger changes in DNA methylation patterns. For example, changes in chromatin modifications in patients with mutations in *ATRX* gene encoding SWI/SNF-like chromatin modifying protein resulted in diverse changes in DNA methylation patterns (Gibbons et al., 2000). Furthermore, an increase in histone acetylation was shown to cause DNA demethylation (Cervoni and Szyf, 2001; Fahrner et al., 2002; Selker, 1998), and histone H3 K27 methylation, along with EZH2 histone methyltransferase, was able to target DNMTs to specific sites in the genome (Vire et al., 2006). The bilateral relationship between DNA methylation and chromatin modifications (D'Alessio and Szyf, 2006), highlighting the dynamic aspect of DNA methylation, can have important implications for toxicology. Agents affecting histone modifying enzymes may indirectly drive changes in DNA methylation and subsequently the phenotype. For instance, valproic acid, an HDAC inhibitor, increases histone acetylation (Gottlicher et al., 2001) and causes concomitant DNA demethylation (Detich et al., 2003a; Ou et al., 2007). The third component of the epigenetic machinery, non-coding RNA, also seems to interact with DNA methylation and the "histone code." DNA methyltransferases and histone deacetylases were identified as targets for miRNAs (Rajewsky, 2006; Tuddenham et al., 2006; Zhou et al., 2010). This regulatory link seems to be mutual, as inhibitors of DNA methylation and histone deacetylation also affect miRNA expression (Lujambio and Esteller, 2007; Saito et al., 2006; Zhou et al., 2010).

5 Generation of DNA methylation patterns during embryogenesis and external exposures to toxic agents

Embryogenesis is a critical period of time when DNA methylation patterns are being generated (Monk et al., 1987; Reik, 2007) and are prone to aberrations by exogenous agents. DNMT3A and DNMT3B play an important role in establishing this pattern (Cedar and Bergman, 2009; Reik, 2007). Active demethylation of the paternal genome occurs at the zygote stage (Mayer et al., 2000; Oswald et al., 2000) followed by passive demethylation just before implantation (Reik, 2007). It erases marks created in the previous generation, and the pattern is re-established. Although DNMT3A and DNMT3L methylate imprinted loci and transposons (Cedar and Bergman, 2009; Reik, 2007), some specific loci are protected by DNA binding proteins, such as ZFP57 or MBD3, from demethylation during embryogenesis (Law and Jacobsen, 2010; Li et al., 2008; Reese et al., 2007). It was shown that lysine demethylase KDM1B, which is specific to oocytes, is required for methylation of differentially methylated regions within imprinted genes and protects from a loss of imprinting (Ciccone et al., 2009; Law and Jacobsen, 2010). Agents that interfere with enzymes involved in the generation of DNA methylation patterns can disrupt normal organogenesis, tissue-specificity, and gene imprinting. Their influence can have teratogenic outcomes when targeting crucial machineries or modulatory effects on DNA methylation at specific regions. The latter may be undetectable for a long time until they emerge later in life as late-onset diseases (Dolinoy and Jirtle, 2008; Gluckman et al., 2009; Hanson and Gluckman, 2008; Sinclair et al., 2007b). A pertinent example is pre-conceptional paternal exposure to cyclophosphamide, an anti-cancer and immunosuppressive agent, that was shown to increase the risk of miscarriages and malformations and lead to behavioral changes (Auroux et al., 1990). Its mechanism of action is linked to the induction of

hyperacetylation at lysine residue 5 of histone H4 of male and female pronuclei as well as to dramatic DNA hypomethylation of male pronuclei and DNA hypermethylation of female nuclei during zygotic development in rats (Barton et al., 2005). Another example of an epimutagen disrupting embryonal development is bisphenol A (BPA) that causes a decrease in DNA methylation. The exposure of embryos to BPA results in higher body weight, disrupted reproductive capacity, and increased risk for breast and prostate cancer in later life (Ho et al., 2006). These effects were overridden by a hypermethylating compound, genistein, which is the main isoflavone in soya (Dolinoy et al., 2007). Compounds present in our diet have a well-documented impact on DNA methylation patterns in adulthood and prenatal life (Selhub, 2002; Stefanska and Fabianowska-Majewska, 2010a). A wide range of diet components, such as vitamin B12, B6, folate, choline, and betaine, are important sources of methyl donors and regulate carbon metabolism affecting DNA methylation (Selhub, 2002). Nutritional restriction during pregnancy (Heijmans et al., 2008; Ke et al., 2006; MacLennan et al., 2004; Unterberger et al., 2009) and low folic acid content before conception (Sinclair et al., 2007a) were shown to leave permanent changes in DNA methylation linked to chronic diseases in adulthood, such as obesity. The critical role of maternal diet was demonstrated in Agouti mice where methyl donor-rich diet or high genistein content increased DNA methylation within a transposable element in the agouti gene causing changes in fur color and protecting the offspring from obesity (Dolinoy et al., 2006; Waterland and Jirtle, 2003). Increasing evidence demonstrates that some external agents that are added to food to improve flavor or extend storage duration may act as epigenetic toxicants. For instance, the flavoring agent dihydrocoumarin, added to food and cosmetics, triggers p53 acetylation and apoptosis in cultured human TK6 lymphoblastoid cells, inducing a phenotype associated with senescence and aging (Olaharski et al., 2005). Since natural food components with epigenetic activities appeared to have an impact on embryogenesis, compounds that are added to food and were proven to act as epigenetic toxicants in adults should be tested for potential effects on embryos. Generation of DNA methylation patterns during embryogenesis is a complex process that involves cooperation of multiple enzymes and regulatory proteins. Any external interruption may have detrimental outcomes that should be addressed in toxicology.

6 DNA methylation aberrations and other epigenetic alterations mediated by non-genotoxic environmental exposures and their impact on carcinogenesis

For decades, cancer development was perceived as a mutagenic process with mutagenesis as the principal characteristic of carcinogens. This concept has been modified in light of recent discoveries that cancer is also triggered by epigenetic modifications (Feinberg, 2001). Epigenetic mechanisms of carcinogenesis have been introduced into toxicology along with several compounds and factors that can lead to cancer development without changes in DNA sequence. An excellent example is a group of chemicals and other stimuli contributing to the development of hepatocellular carcinoma (HCC). HCC is the most prevalent type of liver cancer arising from hepatocytes and triggered by several environmental factors, including hepatitis B and C infection, alcohol, aflatoxin contaminated food, and chronic methyl-deficient diet (Asada et al., 2006; Park et al., 2007; Schafer and Sorrell, 1999). The example of HCC highlights the involvement of epigenetic mechanisms in the development of the disease. For instance, ethanol intake changes the metabolism of SAM leading to its depletion, which subsequently results in global DNA hypomethylation, liver cirrhosis, and increased risk of HCC (Lu and Mato, 2005; Purohit et al., 2007). Feeding rodents with methyl-donor deficient diet indeed caused hypomethylation and activation of oncogenes resulting in the development of HCC (Kanduc et al., 1988; Shen et al., 1998; Wilson et al., 1984; Zapisek et al., 1992). SAM as an inhibitor of DNA demethylation is important not only for keeping balance in the DNA methylation machinery but it also can prevent liver cancer evoked by carcinogens (Pascale et al., 1995; Pereira et al., 2004). Interestingly, known hepatic carcinogens such as aflatoxin B1 and N-nitrosodimethylamine also were shown to induce a prometastatic gene, *SYNUCLEIN GAMMA (SNCG)*, through its promoter hypomethylation in liver cancer (Zhao et al., 2006). Hepatitis B virus, which is considered a major etiological factor of chronic liver inflammation that could then deteriorate into HCC, encodes the HBx protein reported to be a key player in hepatocarcinogenesis (Kim et al., 1991), possibly through triggering DNA hypomethylation (Park et al., 2007; Yue et al., 2011). One of the proposed mechanisms for the demethylation induced by HBx is through direct interaction with DNMT3B on the cyclooxygenase-2 gene promoter (Yue et al., 2011). HBx decreases DNMT3B binding to the promoter which results in the induction of demethylation, increased binding of transcription factors and gene activation. Similarly, cigarette smoke, an undoubtedly carcinogenic agent, was reported to bring about hypomethylation of a prometastatic gene, *SNCG* in lung cancer cells through attenuation of DNMT3B activity (Liu et al., 2007). Hypomethylation of oncogenes and prometastatic genes associated with their activation is common in HCC, as was confirmed by recent studies (Stefanska et al., 2011a). Hypomethylated genes are involved in cell growth, cell adhesion and communication, signal transduction, mobility and invasion, which are all essential for cancer progression and metastasis. These hypomethylated genes are clustered together across the genome, indicating a high-level organization of hypomethylation in cancer (Stefanska et al., 2011a). Global DNA hypomethylation during liver tumorigenesis along with hypermethylation at specific sites also were observed to be triggered in response to exposure to phenobarbital (Counts et al., 1996; Kostka et al., 2007; Moggs et al., 2004; Phillips and Goodman, 2009; Watson and Goodman, 2002). Other non-genotoxic carcinogens, such as nickel and arsenic, are also known to cause changes in DNA methylation patterns (Lee et al., 1995; Zhao et al., 1997). Recent findings show that persistent organic pollutants, including the fungicide dichlorodiphenyltrichloroethane

(DDT), which increase the risk of several types of cancer in humans (Engel et al., 2007; Hoppin et al., 2000; Ritchie et al., 2003), may act through epigenetic mechanisms. It was reported that these compounds adversely affect DNA methylation patterns (Anway et al., 2006; Anway and Skinner, 2006; Chang et al., 2006; Shutoh et al., 2009) and lead to global hypomethylation in humans (Rusiecki et al., 2008). Furthermore, their effects are not limited to carcinogenesis. Transient exposure during gestation to the fungicides vinclozolin and methoxychlor, the latter being used in wine production, induced defects in spermatogenic capacity and sperm viability through alterations in DNA methylation patterns in the male germ cells (Anway et al., 2005). This exposure resulted in the increased risk of male infertility and caused transgenerational effects up to the F4 generation (Anway et al., 2005; Chang et al., 2006; MacPhee, 1998). Not surprisingly, non-coding RNA-related mechanisms, particularly miRNAs, are also frequently involved in carcinogenesis. In rat liver, for instance, miR-298 and miR-347 are downregulated by the hepatotoxicants acetaminophen and carbon tetrachloride, which are known to induce necrosis. Both miRNA species are essential regulators of genes that respond to oxidative stress (e.g., thioredoxin reductases), a process that ultimately burgeons into necrotic cell death (Fukushima et al., 2007). Wy-14,643, a non-genotoxic rat hepatocarcinogen, was found to trigger a miRNA-mediated signaling cascade that controls hepatocellular proliferation, eventually leading to liver tumorigenesis (Shah et al., 2007). Similarly, long-term exposure of female rats to tamoxifen, a widely used chemotherapeutic drug, induced miRNA deregulation prior to formation of hepatocellular carcinoma (Pogribny et al., 2007). Certain physical factors also were reported to have carcinogenic potential through inducing aberrations in epigenetic mechanisms. Thus, fractionated low-dose radiation exposure of mice leads to the loss of the trimethylated lysine 20 status in histone H4 in thymus tissue, which is a hallmark of carcinogenesis (Pogribny et al., 2005). In plants, such as *Arabidopsis*, low temperature induces histone deacetylation and dimethylation of lysine residues 9 and 27 of histone H3 (Sung and Amasino, 2004). Several lines of evidence support the opinion that cancer is not solely a genetic disease. Diverse environmental exposures could trigger changes in all three elements of epigenetics, i.e., DNA methylation, histone modifications, and regulation by non-coding RNAs (Fig. 2), which contribute to cell transformation. The presence and the consequences of such external factors need to be addressed in toxicology.

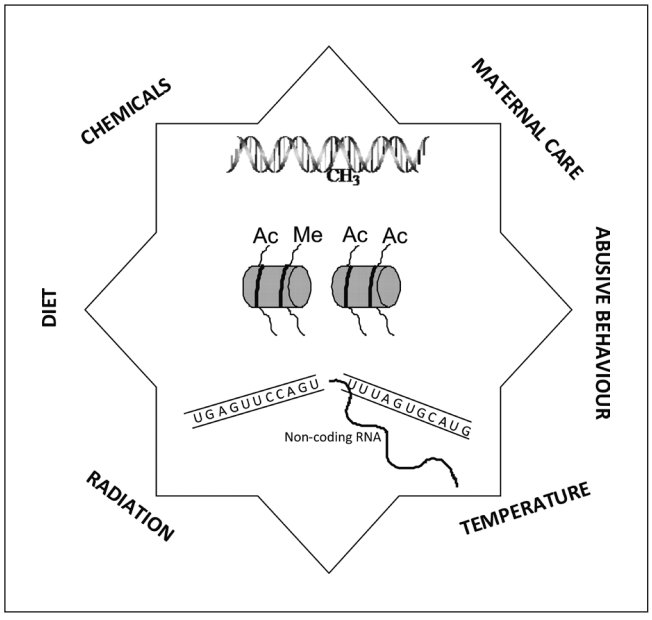

Fig. 2: Diverse exogenous exposures target all three elements of the epigenetic machinery: DNA methylation patterns, "histone code," and regulation by non-coding RNAs

7 DNA methylation and histone modifications as targets of social environmental stimuli

DNA methylation and chromatin modifications are dynamic and undergo changes during gestation, childhood, and possibly later in life as well. External exposures to chemical agents and diverse diet, as well as to abusive behavior and poor maternal care, leave traces through changes in the "histone code" and DNA methylation patterns (Fig. 2). DNA methylation serves as the memory that can have potential long-term adverse effects on human health and behavior. Differences in maternal care in rodents were shown to have an influence on the phenotype and stress response (Francis et al., 1999; Liu et al., 1997; Ruppenthal et al., 1976). Further studies in rats have provided an epigenetic explanation for these effects. The offspring of highly caring mothers displayed low levels of DNA methylation and high levels of histone H3 acetylated at lysine residue 9 at the glucocorticoid receptor (GR) gene promoter in comparison with the offspring of less caring rat mothers (Weaver et al., 2004, 2007). This early-life exposures trigger changes in signaling pathways that might result in recruitment of DNA and histone modifying enzymes to the GR promoter and change its transcriptional activity (Weaver et al., 2007). Interestingly, aberrations in GR promoter methylation in hippocampus also were linked to childhood abuse in humans (McGowan et al., 2009). Abusive parental behavior is a trigger that changes stress response through DNA methylation alterations. Numerous data indicate that the modifications in DNA methylation and the "histone code" by chemical, physical, or social exposures can have detrimental effects on human health and behavior. A challenge in toxicology is to identify agents that are able to directly or indirectly drive changes in DNA methylation and, more importantly, to distinguish adverse effects from mild changes with no influence on the phenotype.

8 New approaches in screening for agents with epigenetic toxicity

It is becoming clear that different agents that are routinely used by humans, as well as newly developed agents, could

potentially affect epigenetic systems and consequently cause stable alterations in the phenotype without changing the DNA sequence. These epigenetic alterations may have long-lasting effects and manifest themselves later in life in a variety of diseases (Baylin and Herman, 2000; Petronis et al., 2003; Yung and Richardson, 1994). It is therefore crucial to develop a methodical and comprehensive mechanism for screening agents that could directly or indirectly change DNA methylation patterns as an integral part of chemical safety assessment. DNA methylation changes that may contribute at least partially to the development of cancer (Baylin and Herman, 2000; Feinberg and Vogelstein, 1983), schizophrenia (Connor and Akbarian, 2008; Petronis et al., 2003), lupus (Cornacchia et al., 1988; Yung and Richardson, 1994), or other severe conditions (Mastroeni et al., 2010; Mastronardi et al., 2007; Qureshi and Mehler, 2010a; Qureshi and Mehler, 2010b) remain undetected by existing screens for toxic agents. Thus, we should consider including a DNA methylation screen in the process of safety assessment of drugs, foods, dietary supplements, and environmental exposures. This type of a screen test could be based on the methylated cytomegalovirus promoter-green fluorescence protein (GFP) reporter assay. Cervoni and Szyf have previously used *in vitro* methylated or unmethylated promoter-GFP reporters to examine compounds modulating DNA methylation patterns (Cervoni and Szyf, 2001). The test gave positive results for agents known to affect DNA methylating enzymes such as SAM (Detich et al., 2003b) directly and indirectly through histone modifying enzymes such as HDAC inhibitors (Cervoni and Szyf, 2001). Another approach is a screening assay developed by Olaharski et al. (2005) to detect environmental chemicals that disrupt heterochromatin silencing in *Saccharomyces cerevisiae*. A mating type in *S. cerevisiae* is regulated epigenetically by silencing one of the cryptic mating loci. If a compound affects chromatin structure, mixing the exposed cells with a strain of the same type will result in mating and subsequent growth due to disruption in heterochromatin silencing and switching of the mating type. It is a simple assay that could be used in the first stage of the screening process. An additional approach is to test for global changes in DNA methylation using an Luminometric Methylation Assay that can be automated (Karimi et al., 2006). The next phase of screening for epigenetic modulators could involve examining short- and long-term effects in rodents on phenotypes in the first generation as well as transgenerationally. Two excellent models have been developed for detecting epigenetic changes *in vivo*, the Agouti viable yellow (A^{vy}) mouse model (Dolinoy et al., 2007; Duhl et al., 1994) and Axin 1 fused ($Axin1^{Fu}$) mice (Rakyan et al., 2003). Although the existing assays and models might miss gene- and tissue-specific effects, genome-wide methylation mapping or multiple tissue testing could be applied if the evidence from clinical practice, epidemiology, or animal experiments indicates a possibility of epigenetic mechanisms mediating the effects of an environmental agent.

9 Future prospects and epigenetics in the context of the 3Rs principle

Numerous data have shown that environmental exposures that alter DNA methylation are able to produce latent long-term effects on the phenotype. Even factors that do not affect DNA methylation machinery directly can change DNA methylation patterns by targeting histone modifications due to the bilateral relationship between DNA methylation and the "histone code." Adding another layer of complexity, recent reports have demonstrated that aberrations in DNA methylation can be transgenerationally transmitted to future generations (Anway et al., 2005; Franklin and Mansuy, 2010; Guerrero-Bosagna et al., 2010). New approaches in the assessment of drug safety need to be developed taking into account possible effects on the phenotype through epigenetic modifications. A number of animal models are currently available to study the biological outcome of epimutagens. Among those, as mentioned before, a popular one is the A^{vy} mouse. When the agouti gene is fully expressed, the mice have yellow fur and suffer from obesity, diabetes, and increased tumor susceptibility. The expression of this gene, however, depends on the degree of DNA methylation in the A^{vy} locus. Thus, a hypermethylated A^{vy} locus results in brown fur, while a hypomethylated A^{vy} locus is associated with a yellow coat color. Another mouse model relevant to the field of epigenetics is the $Axin1^{Fu}$ mouse. Axin gene expression also is regulated by DNA methylation and is causally linked with the appearance of the tail, which may vary from a straight to a kinky shape (Rosenfeld, 2010; Vandegehuchte and Janssen, 2011). In both cases, it has been demonstrated that a number of chemical compounds, particularly dietary constituents (e.g., folate, vitamin B12 and vitamin B6), can affect DNA methylation of the genes involved and, as a result, their phenotypical expression. Indeed, when such DNA methylation-modifying compounds are administered to A^{vy} or $Axin1^{Fu}$ mice, the outcome is manifested in their offspring, which is another illustration of transgenerational epigenetic inheritance (Bruxner and Whitelaw, 2008; Rosenfeld, 2010; Sandovici et al., 2008; Vandegehuchte and Janssen, 2011).

At this point, a well-standardized animal-free approach to studying epimutagens is not yet available. Such assays are urgently needed, however, especially in light of the need to include epigenetic information in current risk assessment exercises and thereby to comply with current legislation, especially in Europe. Indeed, the European chemical and cosmetic industries are still facing drastic legislative changes. Thus, the novel European chemicals policy adopted in 2007, commonly known as REACH (i.e., Registration, Evaluation, Authorization and restriction of CHemicals) demands the safety assessment of thousands of chemical substances that are currently on the market (European Commission, 2006). On the other hand, for the safety evaluation of cosmetic ingredients European legislators expect a complete replacement of animal tests from 2013 onwards (European Commission, 2003), although the feasibility of this

ban is heavily debated (Adler et al., 2011). Collectively, these European legislative changes illustrate that the search for alternatives to animal testing should be prioritized, in line with the 3Rs principle of Russell and Burch, which calls for refinement, reduction and replacement of animal experimentation (Russell and Burch, 1959). Therefore, a major challenge now lies ahead in the establishment of *in vitro* methods, such as the methylated reporter assays (Cervoni and Szyf, 2001), to study epigenetics in a toxicological context.

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