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**REVIEW**

# Towards incorporating epigenetic mechanisms into carcinogen identification and evaluation

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**Remarkable progress in the field of epigenetics has turned academic, medical and public attention to the potential applications of these new advances in medicine and various fields of biomedical research. The result is a broader appreciation of epigenetic phenomena in the a etiology of common human diseases, most notably cancer. These advances also represent an exciting opportunity to incorporate epigenetics and epigenomics into carcinogen identification and safety assessment. Current epigenetic studies, including major international sequencing projects, are expected to generate information for establishing the 'normal' epigenome of tissues and cell types as well as the physiological variability of the epigenome against which carcinogen exposure can be assessed. Recently, epigenetic events have emerged as key mechanisms in cancer development, and while our search of the Monograph Volume 100 revealed that epigenetics have played a modest role in evaluating human carcinogens by the International Agency for Research on Cancer (IARC) Monographs so far, epigenetic data might play a pivotal role in the future. Here, we review (i) the current status of incorporation of epigenetics in carcinogen evaluation in the IARC Monographs Programme, (ii) potential modes of action for epigenetic carcinogens, (iii) current *in vivo* and *in vitro* technologies to detect epigenetic carcinogens, (iv) genomic regions and epigenetic modifications and their biological consequences and (v) critical technological and biological issues in assessment of epigenetic carcinogens. We also discuss the issues related to opportunities and challenges in the application of epigenetic testing in carcinogen identification and evaluation. Although the application of epigenetic assays in carcinogen evaluation is still in its infancy, important data are being generated and valuable scientific resources are being established that should catalyse future applications of epigenetic testing.**

## Introduction

Epigenetics is a rapidly expanding field of modern biology with a profound impact on our thinking and understanding of biological phenomena. The term 'epigenetic' refers to all stable changes in gene expression and chromatin organization that are independent of the DNA sequence itself and that can be mitotically inherited over cell divisions. Epigenetic phenomena, including genomic imprinting, X-chromosome inactivation and global reconfiguration of the DNA methylome, changes in chromatin compaction states and histone modification patterns, occur during development and contribute to the lineage-specific epigenome that is maintained over the lifetime of an organism.

Epigenetic mechanisms are essential for the stable propagation of gene activity states from one generation of cells to the next and thus the epigenome governs the establishment and maintenance of cell identity. DNA methylation, histone modifications and non-coding RNAs are the main epigenetic mechanisms that may act alone or in combination to govern the gene expression programme over the lifetime of an organism.

The remarkable progress in the field of epigenetics has turned academic and medical attention to the potential application of new conceptual advances in cancer research. The advent of novel technologies that allow cost-effective profiling of the epigenome with unprecedented resolution has dramatically accelerated cancer research and opened up new perspectives. Together, these advances have led to a broader appreciation of epigenetics in the a etiology of complex human diseases, including cancer. In addition to their application in mechanistic studies, cancer therapy and biomarker discovery, advances in epigenetics also need to be incorporated into carcinogen identification and safety assessment.

Unlike the genome, which is virtually identical among all cells within an organism, different tissues and cell types harbour a distinct epigenome, which may undergo substantial changes with ageing and in response to environmental factors (1,2). Epigenetic mechanisms can be viewed as an interface between the environment and the genome, the deregulation of which may disrupt key cellular processes, ultimately resulting in oncogenic transformation and tumour development (Figure 1). Exposure to environmental factors may leave a fingerprint on the epigenome that may be exploited in discovering new biomarkers for risk assessment and cancer prevention. Despite an increased interest in epigenetics and a better understanding of epigenetic mechanisms and their deregulation in human malignancies, relatively little is known about the potential application of epigenetics in carcinogen identification and evaluation. Although epigenetic assays have not yet been incorporated into the current carcinogen testing battery, recent evidence on the impact of environmental exposures on the epigenome argues that the time is ripe for established and suspected carcinogens to be specifically evaluated for their potential to deregulate epigenetic mechanisms. The gaps in our understanding of the normal variability of the epigenome in different cell types should be bridged by major international sequencing initiatives enabled by the genome/epigenome technology revolution. Once this is available, it will be possible to identify specific changes in the epigenome associated with exposures to a carcinogen. Several recent reviews on the impact of environmental/lifestyle factors on the epigenome have been published (3–7).

The advances in understanding the normal epigenome should also facilitate developing epigenetic assays in carcinogen testing and using epigenetic data in regulatory processes and policy-making. In this review, we will summarize current status of incorporation of epigenetics in carcinogen evaluation by the International Agency for Research on Cancer (IARC) Monographs Programme. We will also assess selected examples of studies demonstrating the epigenetic effects of specific agents in human carcinogenesis. Finally, we will discuss the issues related to opportunities and challenges in incorporating epigenetics into carcinogen evaluation and future perspectives.

### *Physiological and pathological changes of the epigenome*

An important feature of the epigenome is that it is susceptible to normal variations, that is to say that epigenetic modifications of DNA and histones exhibit variations across different cell types and over time.

**Abbreviations:** DES, diethylstilbestrol; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HBV, hepatitis B virus; HBx, HBV encoded protein X; HDAC, histone deacetylase; IARC, International Agency for Research on Cancer.

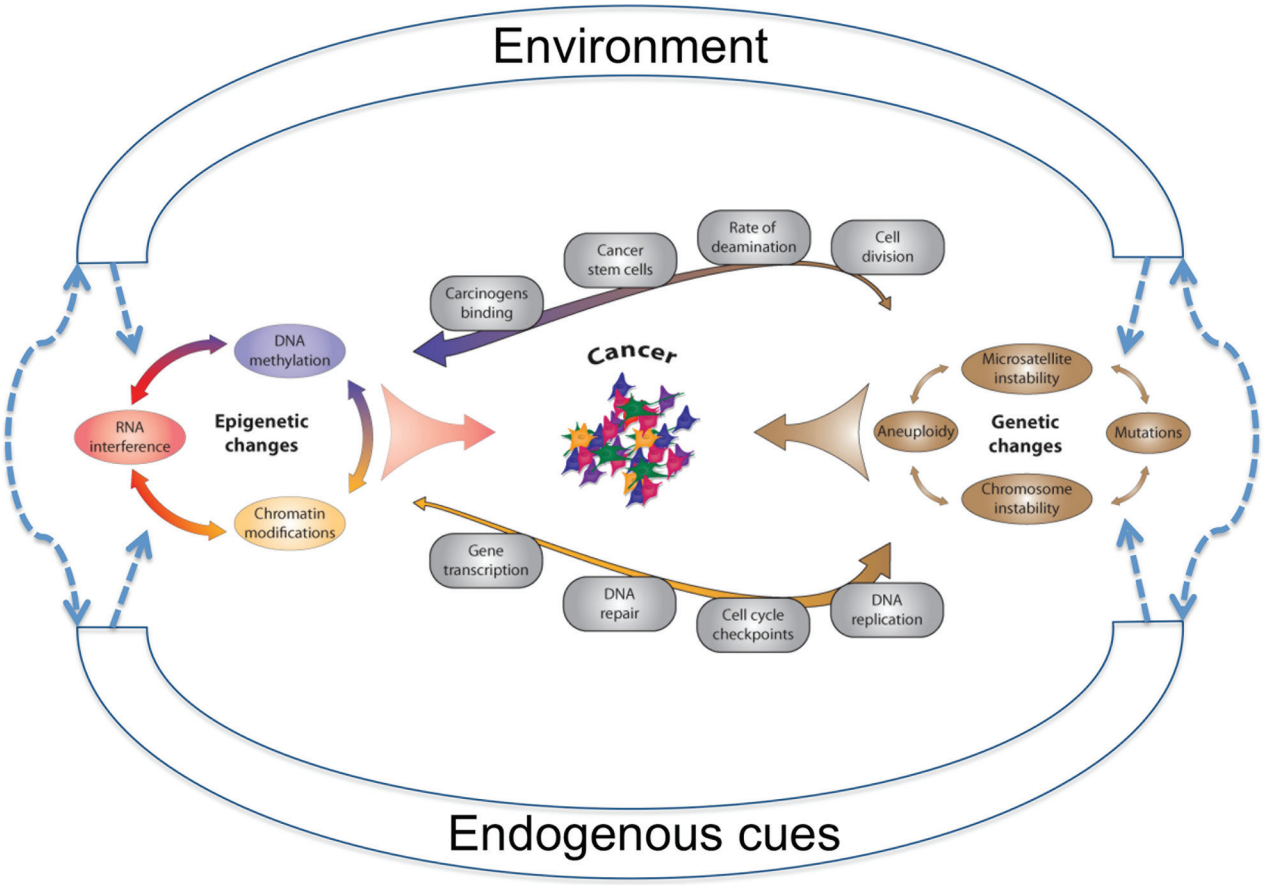


**Fig. 1.** Epigenetic mechanisms regulate key cellular processes (such as gene transcription, DNA repair and differentiation) and play critical roles in cellular responses to environmental exposures and endogenous stimuli. Deregulation of epigenetic mechanisms may promote the development of abnormal phenotypes and cancer. There is a crosstalk between epigenetic and genetic changes in the process of cancer development and progression. Given that epigenetic and genetic changes coexist in all cancers, it is often unclear what are the primary and secondary events pertinent to carcinogenesis.

It is thought that the genome needs to be plastic enough to respond to environmental stressors and endogenous cues (4,8). Such induced changes in the epigenome may be fixed and propagated over cell divisions, resulting in long-term or permanent changes in phenotype. Therefore, in contrast to genotype, modulation of the epigenotype is a physiological and essential process that controls an organism's response to environmental exposures.

The reconfiguration of the epigenome is particularly evident during embryonic development, cell differentiation and ageing (9–11). A complete erasure and re-establishment of the DNA methylation patterns in early embryonic development is a dynamic, but highly regulated, process that results in a profound reconfiguration of the epigenome (including the DNA methylome but also histone marks) (12) and is believed to be highly susceptible to environmental exposures. The reconfiguration of the methylome during early embryonic development provides a striking example of dynamic changes in the epigenome. In the fertilized egg before the fusion of the pronuclei, DNA methylation levels in the maternal and paternal genomes are heavily methylated and both pronuclei have roughly the same methylcytosine content (12,13). Soon after the fusion (within approximately an hour), the paternal genome is efficiently demethylated through an active mechanism (active demethylation), and its demethylation levels remain low during a few mitotic divisions. In contrast, the maternal genome undergoes a passive demethylation during several subsequent mitoses. The genome of the early embryo starts to be remethylated by *de novo* DNA methylases at implantation, and the establishment of DNA methylation patterns occurs in a tissue-specific manner. It is believed that this process of dramatic demethylation/remethylation represents a window of susceptibility to environmental stressors and that adverse changes in the epigenome during early embryonic development may be at the heart of intrauterine programming of childhood and adult diseases (14). Another stage during embryonic development that may be considered vulnerable is the primordial germ cells (15,16). At this stage, foetal germline is dynamically remodelled in a gender-specific manner and epigenetic modifications (such as DNA methylation marking of imprinted genes) are removed (12,17). However, primordial germ cells can contribute genetic material to the future offspring of the foetus; therefore, epigenetic changes induced by carcinogen exposure during this stage may contribute to transgenerational epigenetic inheritance. It is, therefore, essential to account for these events when evaluating carcinogens.

Despite a better understanding of epigenetic mechanisms and their deregulation in human cancer, much remains unknown about the normal dynamic variations of the epigenome and how to distinguish them from adverse epigenetic changes that pose a health risk. In general, the degree to which agents may promote carcinogenesis through epigenetic mechanisms depends on the amount and duration of exposure. The degree to which an adverse exposure alters the epigenome may also strongly depend on variation in susceptibility to the exposure. Individual susceptibility to a given exposure is likely to depend on the epigenetic make-up that dictates an individual's response and adaptation mechanisms. Differences in individual susceptibility may be attributed to patterns of DNA methylation, histone modifications and non-coding RNAs, as well as genetic make-up.

### *Genotoxic, non-genotoxic and epigenetic carcinogens*

Epigenetic mechanisms are thought to play important roles in the adaptation and response to environmental exposures, although a

clear-cut causal relationship between epigenetic change and specific exposure is often difficult to establish. The reason for this may be 3-fold. First, environmental agents may induce subtle changes and quantitative phenotypic manifestation may be evident only after repetitive or prolonged exposure. Second, environmental exposures are likely to induce global non-specific changes in one layer or multiple layers of the epigenome. Third, there is an important gap in our understanding of the 'normal' epigenome in tissues and cell types and the normal variability of the epigenome.

Recent studies have implicated epigenetic mechanisms in carcinogenesis linked to environmental exposures, although there is a paucity of evidence demonstrating molecular mechanisms by which the epigenome is deregulated in response to a specific carcinogen. A wide range of known and suspected carcinogens (including chemical, physical and biological agents) have been associated with changes in the epigenome, and it has been suggested that their mode of action may involve disruption of epigenetic mechanisms (Tables I–IV). The effects of these carcinogens on epigenetic states have been either demonstrated experimentally using different animal and cellular models or inferred from epidemiological studies (4,7). Environmental factors associated with epigenetic deregulation include tobacco smoke, arsenic, cadmium, nickel and ionizing and UV radiation. Different infectious agents such as human hepatitis B virus (HBV), hepatitis C virus, human papillomavirus and the bacterium *Helicobacter pylori* have been shown to deregulate proliferation, cell division and the gene expression pattern of the host cell via an epigenetic strategy. Among dietary factors, alcohol and fat consumption may act as epigenetic carcinogens.

It should be noted that many carcinogens may promote tumour development by inducing both epigenetic changes (aberrant DNA methylation and histone modifications) and genetic alterations (mutations). However, individual genetic polymorphisms and epigenetic make-up ('epigenetic polymorphisms') may also play pivotal roles in cellular response to environmental stress and thus may represent a part of an individual's predisposition to developing cancer. Therefore, individual cancer susceptibility is likely to depend not only on genetic but also on epigenetic make-up. These responses involve the action of diverse cellular machineries such as those involved in DNA repair, carcinogen detoxification, cell cycle control and cell death. The technological advances in epigenomics (high-throughput and genome-wide profiling) will soon allow the identification of entire epigenomes (genome-wide patterns of DNA methylation and histone modifications). This may provide critical information for testing the notion that differences in individual susceptibility may also be attributed to germline epigenetic make-up.

In the literature, the terms 'non-genotoxic' and 'epigenetic' are sometimes used interchangeably (203). However, not all non-genotoxic carcinogens act by altering epigenetic states (DNA methylation, histone modifications or non-coding RNAs). For example, some non-genotoxic agents, such as dioxin, can act via receptor-mediated

**Table I.** Epigenetic mechanisms and Volume 100A carcinogens

| Human carcinogen | Epigenetic mechanism | Model | | | References |
|---|---|---|---|---|---|
| | | Human samples | Cell lines | Animal model | |
| Part A: pharmaceuticals | | | | | |
| Tamoxifen | | | | | |
| | DNA methylation | * | | | (18,19) |
| | | | * | | (20) |
| | | | | * | (21,22) |
| | Histone marks | | | * | (22) |
| | miRNA | | | * | (23) |
| DES | | | | | |
| | DNA methylation | | * | | (24) |
| | | | | * | (24–30) |
| | Histone marks | | * | | (31) |
| | miRNA | * | | | (32) |
| | | | * | | (32) |
| Prostaglandin E2 | | | | | |
| | DNA methylation | | * | | (33,34) |
| | | | | * | (33) |
| | Histone marks | | * | | (35) |
| | miRNA | | * | | (36,37) |
| Hormone therapy | | | | | |
| | DNA methylation | * | | | (38–40) |
| Cyclophosphamide | | | | | |
| | miRNA | | | * | (41) |
| Chromium (VI) compounds | | | | | |
| | Histone marks | | * | | (42,43) |

miRNA, micro-RNA.
Asterisk (*) indicates the model used in a given study.

**Table II.** Epigenetic mechanisms and Volume 100B carcinogens

| Human carcinogen | Epigenetic mechanism | Model | | | References |
|---|---|---|---|---|---|
| | | Human samples | Cell lines | Animal model | |
| Part B: biological agents | | | | | |
| HBV | | | | | |
| | DNA methylation | * | | | (44–55) |
| | | | * | | (44,46,55–60) |
| | Histone marks | * | | | (61,62) |
| | | | * | | (61–65) |
| | | | | * | (61,62) |
| | miRNA | * | | | (66–71) |
| | | | * | | (64,66–68,72) |
| | | | | * | (72) |
| Hepatitis C virus | | | | | |
| | DNA methylation | * | | | (50,51,73–75) |
| | | | * | | (76,77) |
| | | | | * | (78) |
| | Histone marks | | * | | (79,80) |
| | miRNA | * | | | (71,81–84) |
| | | | * | | (83,85–89) |
| Human papillomaviruses | | | | | |
| | DNA methylation | * | | | (90,91) |
| | | | * | | (90,92) |
| | Histone marks | | * | | (93–98) |
| | miRNA | | * | | (99–101) |
| Human T-cell lymphotropic virus type 1 | | | | | |
| | DNA methylation | | * | | (102) |
| | Histone marks | | * | | (103–105) |
| | miRNA | * | | | (106,107) |
| | | | * | | (107,108) |
| Epstein–Barr virus | | | | | |
| | DNA methylation | * | | | (109–118) |
| | | | * | | (109–112,119–121) |
| | Histone marks | | * | | (119,122–126) |
| | miRNA | * | | | (127–129) |
| | | | * | | (70,127,129–140) |
| | | | | * | (129) |
| *Helicobacter pylori* | | | | | |
| | DNA methylation | * | | | (61,62,141–153) |
| | | | * | | (141–143,146,154–156) |
| | | | | * | (153,155) |
| | Histone marks | | * | | (154,157–160) |
| | miRNA | * | | | (161) |
| | | | * | | (161) |

Asterisk (*) indicates the model used in a given study.

**Table III.** Epigenetic mechanisms and Volume 100C–D carcinogens

| Human carcinogen | Epigenetic mechanism | Model | | | References |
|---|---|---|---|---|---|
| | | Human samples | Cell lines | Animal model | |
| Part C: arsenic, metals, fibres and dusts and Part D: radiation | | | | | |
| Arsenic | | | | | |
| | DNA | * | | | (124–130) |
| | methylation | | * | | (131–136) |
| | | | | * | (131,137,138) |
| | Histone marks | | * | | (139) |
| | miRNA | | * | | (140) |
| Cadmium | | | | | |
| | DNA | | * | | (141–144) |
| | methylation | | | * | (145–148) |
| | miRNA | * | | | (149) |
| Nickel | | | | | |
| | DNA | | * | | (150,151) |
| | methylation | | | * | (18) |
| | Histone marks | | * | | (152–163) |
| | | | | * | (163) |
| Beryllium | | | | | |
| | DNA methylation | | | * | (163) |
| Asbestos | | | | | |
| | DNA methylation | | | * | (164) |
| | miRNA | | | * | (165) |
| X-radiation | | | | | |
| | DNA | * | | | (166) |
| | methylation | | | * | (167–169) |
| | miRNA | * | | | (170) |
| | | | * | | (171) |
| | | | | * | (169) |
| Gamma radiation | | | | | |
| | DNA methylation | | * | | (172) |
| | miRNA | | | * | (170,173,174) |
| Smoky coal emissions (for cooking and heating) | | | | | |
| | DNA methylation | * | | | (175) |
| Chromium VI compounds | | | | | |
| | Histone marks | | * | | (176,177) |

Asterisk (*) indicates the model used in a given study.

**Table IV.** Epigenetic mechanisms and Volume 100E carcinogens

| Human carcinogen | Epigenetic mechanism | Model | | | References |
|---|---|---|---|---|---|
| | | Human samples | Cell lines | Animal model | |
| Lifestyle and diet | | | | | |
| Tobacco smoking | | | | | |
| | DNA | * | | | (55,176–185) |
| | methylation | | * | | (178,179,186–188) |
| | Histone marks | | * | | (189) |
| Alcohol consumption | | | | | |
| | DNA | * | | | (190–193) |
| | methylation | | * | | (194,195) |
| | | | | * | (196–198) |
| | Histone marks | | * | | (194,199) |
| | | | | * | (141,142,200) |
| | miRNA | * | | | (25,201,202) |

Asterisk (*) indicates the model used in a given study.

pathways (204), which cannot be considered as an epigenetic mechanism. This means that although some epigenetic agents are also genotoxic, not all non-genotoxic agents are epigenetic carcinogens and indeed, as discussed below, agents may act by multiple mechanisms. Therefore, oversimplified classification of carcinogens that does not consider potential contributing effects that are neither genetic nor epigenetic is confusing and should be avoided.

*The IARC Monographs Programme*

*IARC Monographs.* The IARC Monographs Programme identifies environmental and lifestyle factors that are human carcinogens (162). Interdisciplinary Working Groups of expert scientists review published studies and evaluate the strength of the evidence that an agent can increase the risk of human cancer. Since 1971, more than 950 agents have been evaluated, of which more than 400 have been identified as carcinogenic, probably carcinogenic, or possibly carcinogenic to humans. Recently, IARC completed a review of the more than 100 chemicals, occupations, physical agents, biological agents and other agents classified as carcinogenic to humans (205).

Volume 100 of the IARC Monographs reviews all agents classified previously by IARC as carcinogenic to humans (Group 1) and is divided into six parts (A, B, C, D, E and F), each of which describes a distinct class of carcinogens. Our search of the Monograph Volume 100 revealed that only a few chemical agents and nutritional or lifestyle factors evaluated by IARC Working Groups have been considered as epigenetic carcinogens. Diethylstilbestrol (DES), chromium (VI) compounds and ionizing radiation have been classified as carcinogens that may act through epigenomic deregulation. Curiously, for all of these, their impact on the epigenome is considered not as the major mechanism but rather as the secondary mechanism. The lack of focus on epigenetics in relation to the mechanistic data is perhaps surprising considering a wealth of studies demonstrating the impact of many of the Volume 100 carcinogens on epigenetic mechanisms (Tables I–IV). One explanation may lie in the fact that many studies failed to address whether changes in the epigenome were causal or associative to a carcinogenic exposure. In this section, we discuss the specific example of studies demonstrating the epigenetic effects of DES and infectious agents in human carcinogenesis.

*DES—an example of an epigenetic carcinogen from the IARC Monographs?* DES is a synthetic oestrogen that was widely used (from the 1940s to the 1970s in the USA) to prevent potential miscarriages (through its stimulation of placental synthesis of oestrogen and progesterone) and for the treatment of symptoms associated with menopause and ovariectomy as well as specific vaginal and vulvar conditions (such as inflammation and dystrophy). DES was also used as a postcoital emergency contraceptive ('morning-after pill') and to treat other conditions associated with dysfunctional menstrual cycles and female hypogonadism. It has been estimated that 5–10 million US citizens were treated with DES during pregnancy or were exposed to the drug *in utero* (206). Although nowadays DES is rarely used (e.g. to treat prostate cancer or specific forms of breast cancer), the consequences of its use are still felt among the treated individuals and their progeny.

DES was evaluated by IARC Working Groups in 1978 and 1987, and was evaluated again for Volume 100 (in 2008); IARC has classified this chemical as a Group 1 carcinogen (205). The IARC Monograph of 1987 states that there is sufficient evidence of a causal association between clear cell adenocarcinoma of the vagina or cervix and prenatal exposure to DES (207). It also states that there is sufficient evidence of a causal relationship between cancer of the breast and the use of DES during pregnancy. The carcinogenicity of DES was established or highly suspected in experimental animals before epidemiological studies confirmed its carcinogenicity in humans. Consistent with its oestrogenic properties, DES was shown to induce various effects on the reproductive system in both mice and humans (206,208,209). In a mouse model, it was shown that prenatal and perinatal exposure to DES produces multiple effects in uterine tissue, including uterine cancer (leiomyomas) (209).

Several studies have investigated the mechanism by which DES exposure promotes carcinogenesis. These include studies of gene expression and DNA methylation states in uterine tissue. Specific changes in gene expression in the uterus of young mice treated neonatally were detected after exposure to DES (208). These alterations

were detected in specific genes (fos and lactoferrin) and persisted for weeks, even after treatment cessation. Interestingly, gene expression changes were associated with specific epigenetic changes, namely changes in DNA methylation. For example, the genes that were differentially expressed in DES-treated animals also exhibited abnormal DNA methylation (208,210).

The above results strongly suggest that exposure to DES may have a significant and long-term effect on gene expression through epigenetic mechanisms. However, it should be noted that these studies focused on only a few genes and one epigenetic mechanism (DNA methylation). The impact of DES is unlikely to be limited to a small subset of genes, and epigenome-wide studies have yet to be performed. Indeed, microarray-based transcriptome analysis in both rats and mice has revealed DES-induced changes in expression of a wide range of genes, although whether these changes were accompanied by changes in DNA methylation states or other epigenetic alterations (histone modifications and noncoding RNAs) have been little studied (31,32,211).

Another interesting feature of DES exposure is its impact on cancer incidence in subsequent generations. It has been shown that in addition to an increased cancer susceptibility associated with epigenetic changes in DES-treated parents, an epigenetic mechanism may operate in subsequent generations of mice (the F2 generation) (208). These findings further support the notion that DES-induced carcinogenesis may operate through an epigenetic mechanism, although further studies extending to the F3 generation of exposed animals are needed in order to establish a true transgenerational epigenetic inheritance.

*Infectious agents and epigenetic mechanisms of carcinogenesis.* There is growing evidence that different infectious agents may promote carcinogenesis through epigenetic mechanisms. For example, evidence has emerged that infection by HBV, a major risk factor for developing liver cancer, promotes hepatocarcinogenesis by inducing epigenetic changes (212,213). HBV encoded protein X (HBx) acts as an oncogenic transcription factor by affecting the expression of important cellular genes. In HBV infection-associated cancer cells, HBx protein was found to up-regulate expression of the DNA methyltransferase (DNMT) genes and transcriptional silencing of key cellular genes has been attributed to promoter hyper-methylation mediated by DNMTs (214–217). This notion is further supported by the finding that HBx also directly interacts with the *de novo* methyltransferase DNMT3A, directing their recruitment to specific genes and thus affecting their methylation and silencing (56). Interestingly, HBx was also shown to mediate dissociation of DNMT3A from the promoter of a set of genes, thus resulting in their hypomethylation and transcriptional activation (56). However, chromatin immunoprecipitation experiments failed to find recruitment of HBx on the activated gene promoters suggesting that HBx could facilitate displacement of DNMT through an indirect mechanism.

In addition, a histone deacetylase (HDAC) was found to be a direct interacting partner of HBx protein, revealing a potential alternative epigenetic mechanism for its transcriptional suppressive activities (56). Furthermore, HBx was also shown to directly interact with histone acetyltransferase (HAT) complex CBP/P300 and HBx-mediated recruitment of CBP/P300 complex resulting in hyperacetylation of local chromatin and transactivation of the target cellular genes (44,56,63,218,219). HBx was also found to down-regulate the expression of E-cadherin, the gene frequently found silenced in liver cancer, by the recruitment of the mSin3A/HDAC complex to the E-cadherin gene (64). Therefore, in addition to DNA methylation changes, histone deacetylation of key cellular genes may be an important epigenetic mechanism contributing to HBV-related carcinogenesis. Interestingly, in infected cells, the HBV genome remains in minichromosomes (small chromatin-like structures composed of histones and non-histone proteins and additional genetic material that replicate autonomously) and HBx protein was shown to regulate transcription of both viral genes through epigenetic mechanisms (220–222). However, whether the HBx-mediated engagement of cellular epigenetic machineries in replication and transcription of the viral minichromosomes on critical processes of the host cell remains to be investigated. Although epigenetic mechanisms involved in carcinogenesis associated with viruses other than HBV are less understood, recent studies have suggested that human oncogenic viruses (including Epstein–Barr virus, simian virus 40, Kaposi's sarcoma-associated herpes virus and hepatitis C virus) may be involved in the deregulation of epigenetic modifiers, ultimately resulting in deregulation of the host genes (6,7,9). Therefore, human oncogenic viruses, in general, may promote carcinogenesis through different epigenetic mechanisms.

*Helicobacter pylori* is another example of an infectious agent that might promote carcinogenesis through epigenetic mechanisms (223). *Helicobacter pylori*-infected individuals with chronic gastritis have a significantly higher risk of developing gastric cancer (224) and studies on animal models demonstrated that infiltration of macrophages and expression of inflammation-related genes (proinflammatory cytokines) are associated with DNA methylation changes (225). Further mechanistic studies showed that DNA methylation changes associated with *H.pylori*-induced chronic inflammation could be suppressed when the infected animals are treated with an anti-inflammatory agent, despite the fact that the presence of *H.pylori* in gastric mucosae is unaffected (225). These findings argue that the mechanism by which some infectious agents promote carcinogenesis may involve DNA methylation changes induced by inflammation-induced mediators (223,226).

*Potential modes of actions for epigenetic carcinogens*

Generally, epigenetic carcinogens may promote carcinogenesis (i) through inducing direct changes in the epigenome or (ii) through an indirect deregulation of the epigenetic states. The agents that directly interact with and modulate methyl-cytosine or histone marks may be considered as direct epigenetic carcinogens. Nickel chloride, which was shown to induce changes in histone marks (227), may be considered as a direct epigenetic carcinogen.

The agents in the second group (indirect epigenetic carcinogens) include those that alter either the expression or the activity of enzymes involved in establishing and maintaining epigenetic patterns. The agents that deregulate the activity of *de novo* DNA methylation (DNMT3A and DNMT3B) or the DNA maintenance methyltransferase (DNMT1) can be considered as indirect epigenetic carcinogens. In addition, this group of agents should include the agents capable of altering the activity of proteins and protein complexes responsible for histone modifications, such as HATs and HDACs. Consistent with the evidence that HATs and histone acetylation are involved in the process of DNA repair (228), the agents that inhibit HAT and HDAC activities may compromise critical cellular processes and consequently compromise genomic stability. Reduced levels of histone acetylation or enhanced histone deacetylation may result in the compaction of chromatin, blocking access of DNA repair factors to DNA lesions. Therefore, epigenetic carcinogens may transiently alter chromatin-modifying/remodelling activities, thus impeding DNA repair and other chromatin-based processes. Consistent with this notion, regional mutation rates in cancer genomes were found to be largely influenced by chromatin organization (229).

Because there is intimate and mutually reinforcing crosstalk between the three epigenetic mechanisms in setting up and maintaining the genome-wide expression programme, epigenetic carcinogens that affect one of the interdependent epigenetic mechanisms are likely to also deregulate the other layers of the epigenome.

Our search of the Monograph Volume 100 revealed that epigenetic deregulation has been considered as a potential mechanism of carcinogenesis for only a handful of carcinogens (Table V). Yet, our comprehensive review of experimental evidence in the literature provided strong arguments for an important role of epigenetic deregulation in the mechanism by which many Group 1 carcinogens may contribute to carcinogenesis (Tables I–IV). This discrepancy may be explained by the fact that most mechanistic data on carcinogen-induced epigenome deregulation have been generated since the Volume 100 evaluation, but also by the need to resolve several issues before epigenetic testing can be fully incorporated into carcinogen evaluation. Less understanding of the relevance of epigenetic alterations and more familiarity with genetic changes as a prevailing mechanism of carcinogenesis may also account for epigenetic changes being overlooked as an important mechanism targeted by human carcinogens. Ongoing and future studies in environmental epigenetics and epigenetic toxicology may prove

**Table V.** Epigenetic mechanisms and Volume 100 carcinogens

| Agent | Major mechanism | Second mechanism | Human cancer | Comment |
|---|---|---|---|---|
| DES | Oestrogen receptor-dependent pathway | Epigenetic reprogramming | Cervix, vagina, breast, endometrium (limited evidence), testis (limited evidence) | |
| HBV | Integration into host DNA | | Hepatocellular carcinoma, cholangiocarcinoma (limited evidence), non-Hodgkin lymphoma (limited evidence) | Epigenetic silencing of tumour suppressor genes; interaction with aflatoxins |
| Chromium VI compounds | Genotoxicity | Epigenetic effects | Lung, nasal cavity and paranasal sinuses (limited evidence) | |
| Ionizing radiation | Energy transfer in clusters | Epigenetic effects | Many | |
| Coke production | Genotoxicity | Epigenetic effects | Lung | |

critical in providing crucial insights into the epigenetic mechanisms by which environmental carcinogens contribute to cancer development and progression.

*Current* in vivo *and* in vitro *technologies to detect epigenetic carcinogens*

Animal models represent an essential tool in carcinogen evaluation, and they are critical for epigenetic testing. However, in addition to the complex nature of the epigenome among species and across different tissues, the lack of appropriate animal models represents the major obstacle in studying the impact of environmental carcinogens on the epigenome. Several animal models have been used to show that exposure to environmental agents has an impact on epigenetic states. These include mouse, rat, rabbit, *Drosophila*, *Caenorhabditis elegans* and zebrafish. A few mouse models harbouring locus-specific reporters have been developed and exploited in studying environmentally induced epigenetic changes. For example, the yellow *agouti* mouse model has been used as a sensitive indicator of locus-specific epigenetic changes, and several studies have demonstrated its utility in studying the role of nutritional modulation on epigenetic states (230–232). The coat colour of the *agouti* ($A^{vy}$) mice may be used as a sensitive read-out of locus-specific DNA methylation states (Figure 2). This model proved to be instrumental in investigating the influence of maternal diet during pregnancy on the phenotype of the offspring (231,233). Specifically, it was found that feeding pregnant female mice a diet supplemented with folic acid, vitamin B12 or choline results in noticeable changes in the coat colour of their offspring (231). The loci responsible for the variable phenotype (coat colour) and which are susceptible to modulation by environmental exposures are known as metastable epialleles.

In addition to the *agouti* ($A^{vy}$) model, another murine metastable epiallele, *axin fused* [*Axin*(*Fu*)] (235), has been shown to exhibit epigenetic plasticity in response to changes in maternal nutrition (236). Furthermore, a model with a new metastable epiallele, CDK5 activator- binding protein intracisternal A particle ($Cabp^{IAP}$), has been characterized (237). Identification of new metastable epialleles suggests that this mechanism operates at multiple loci across the mouse genome. However, the locus activity and phenotype associated with metastable mouse models are dictated by the DNA methylation pattern at the specific regulatory elements (retrotransposons) of the locus. Therefore, phenotypes associated with modulation of epialleles are locus specific and cannot be easily extrapolated to the rest of the genome.

Despite a wide range of studies that have used these models, their unequivocal utility has been demonstrated only in the context of mother-to-offspring transmission of dietary/environmental cues. The models with metastable epialleles are also likely to contribute to characterizing the mechanism underlying transgenerational epigenetic inheritance. Current and future studies have yet to demonstrate the utility of these models in mechanistic studies aimed at identifying and evaluating carcinogens. More recently, metastable epialleles have been identified in humans (238), and with the completion of major epigenome profiling initiatives, many more metastable epialleles are likely to be identified. However, metastable epialleles are likely to represent a tiny fraction of the epigenome; therefore, should carcinogens modulate these alleles, these changes may not be considered as representative of the entire epigenome.

In addition to the mouse models discussed above, rats can be considered for epigenetic testing of carcinogens. The rat has been extensively characterized for transgenerational inheritance, and there is considerable knowledge of rat embryonic and postnatal development. Therefore, the rat may be a good model for assessing the role of environmentally induced epigenetic deregulation on development and teratogenicity as well as transgenerational epigenetic inheritance (239). However, several drawbacks associated with rat models outweigh these advantages. The genome and epigenome are far better characterized in mice than in rats, and tools are available for analyzing genome-wide changes in epigenetic states in the mouse. Therefore, the mouse represents a highly tractable model for epigenetic carcinogenicity testing. Among other *in vivo* model systems, *C.elegans*, *Drosophila*, zebrafish and honeybees offer the potential to be used in epigenetic evaluation of environmental carcinogens. For example, the fruit fly *Drosophila melanogaster*, a classic model for genetic research, has recently been suggested as a potential epigenetic model organism (240). In this regard, position effect variegation, the change in phenotype resulting from the change of a gene's position in the genome, was first discovered through observations of eye colour. Therefore, *Drosophila* eye colour may serve as an attractive read-out in studying epigenetic deregulation in response to carcinogenic exposure. However, a recent study using genome-scale sequencing at single-base resolution revealed that the genome of *Drosophila* lacks detectable DNA methylation patterns (241), consistent with the notion that DNA methylation is dispensable for some eukaryotic organisms. Similarly, the nematode worm *C.elegans* completely lacks genomic DNA methylation (242). Because both *C.elegans* and *Drosophila* have been used extensively in developmental biology, these organisms may represent suitable models in high-throughput screening approaches where epigenetic mechanisms (histone modifications and non-coding RNAs) can be studied independent of DNA methylation.

Finally, the use of mammalian cells (human and rodent) grown in culture should also be considered for epigenetic testing. Among these *in vitro* models, the use of stem cells (embryonic and tissue specific) may prove particularly informative. Cell lines may prove most valuable in untargeted screening and identification of potential epigenetic carcinogens. *In vitro* models may also be instrumental in a focused dissection of epigenetic events associated with carcinogen exposure, such as molecular pathways analysis and identification of gene targets. Two major shortcomings of cellular models are their susceptibility to epigenetic alterations during *in vitro* culture and their incompatibility with transgenerational assessment, neither of which are limitations of *in vivo* models.

*Genomic regions and epigenetic modifications and their biological consequences*

A distinguishing feature of epigenetic changes that needs to be considered in carcinogen evaluation is their intrinsic reversibility. Therefore, although epigenetic changes are generally stable and are usually transmitted with extreme fidelity over many cell generations, it is possible that adverse changes in DNA methylation, histone

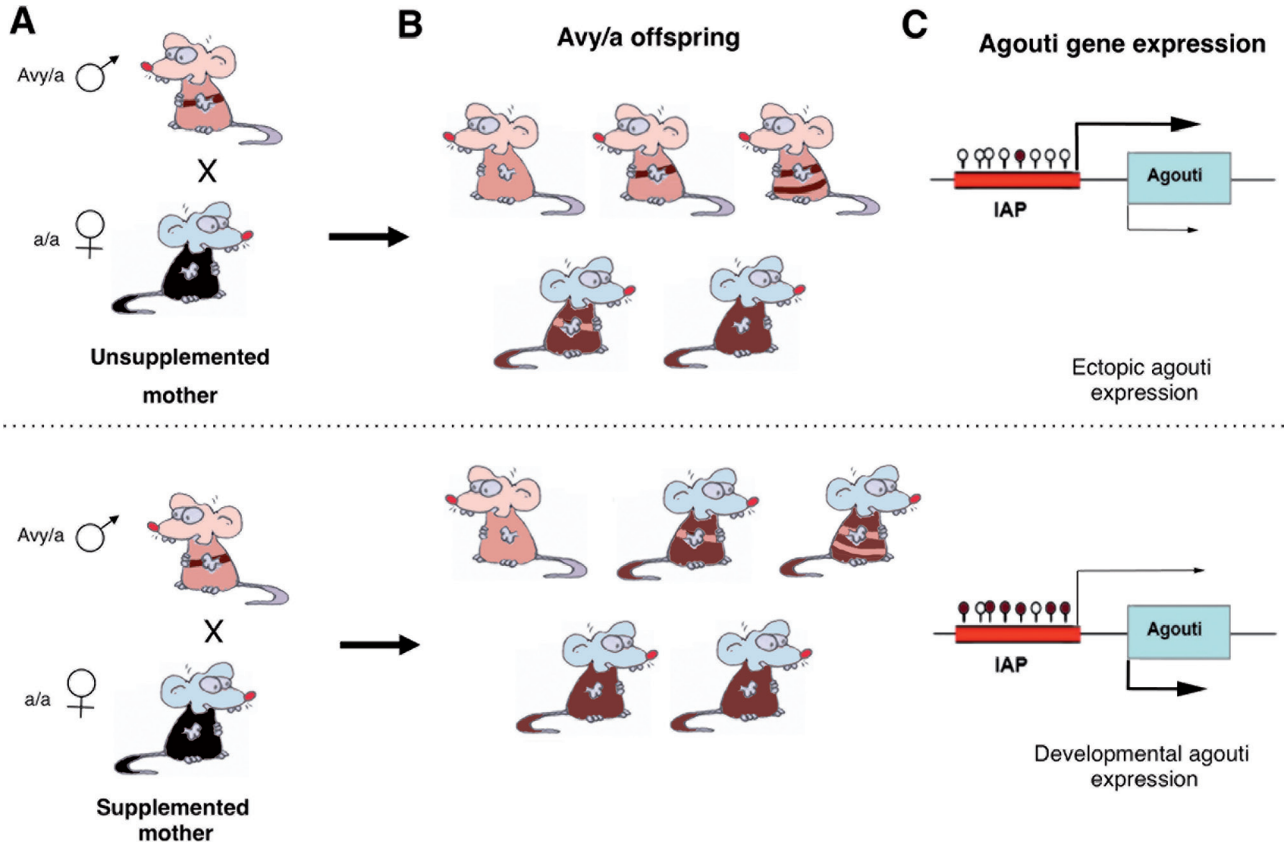


**Fig. 2.** Testing the effects of maternal diet on the phenotype of the offspring using the *Avy/a* mouse model. (**A**) The effect of dietary supplementation of female mice before or after mating with male Avy/a agouti mice can be tested. (**B**) The effect of maternal dietary supplementation on the epigenome can be 'read' by coat-colour distribution in Avy/a offspring. (**C**) The effect of DNA methylation at the intracisternal A particle (IAP) on agouti gene expression. Maternal dietary supplementation may shift the coat-colour distribution of the offspring. This shift is a result of change in methylation at IAP element upstream of the agouti gene. Adapted from ref. (234).

modifications and expression of non-coding RNAs associated with carcinogen exposure may be modulated by subsequent exposure to other epigenome-modulating agents.

Although intrinsic to all cells and absolutely essential, the plasticity and reversibility of the epigenome represents an important challenge in the development of methodologies and a battery of assays for epigenetic testing. In principle, epigenetic assays can be developed at two levels: (i) epigenetic patterns or marks and (ii) the expression and/or activity of epigenetic players (enzymes and other molecules involved in setting up and erasing epigenetic marks). Many environmental chemicals are likely to interfere with the activity of epigenetic players, some of which may contribute to carcinogenesis. Although changes in the protein levels and activity of epigenetic machinery players (e.g. DNMT and HDAC enzymes) can be accurately measured, establishing whether the change in the expression or activity represents a lasting adverse effect with phenotypic consequences or merely an adaptive response is more challenging. Similarly, changes in epigenetic patterns (levels and patterns of DNA methylation and histone modification marks) can be detected with increasing ease and accuracy. In both cases, it is critical to establish whether a given change in either of these two epigenetic layers is anchored to a phenotypic outcome. Nevertheless, assays that monitor the epigenetic patterns should be strongly preferred as they are more likely to detect lasting changes in the epigenome.

Another important consideration is that epigenetic changes that are identified in response to a specific carcinogen should be combined with assays that establish a causative or correlative link with adverse phenotype. In this regard, it has been suggested that classifying epigenetic-based phenotypes based on the gene pathway and ontology databases may be a valuable approach (243). For example, the Gene Ontology database that is being developed with the aim of describing genes and pathways in terms of their molecular functions and associated biological processes should be a useful tool in carcinogen evaluation. An important feature of this facility is that it should allow properties to be assigned to genes and pathways in a species-independent manner, although in terms of epigenetic testing an ideal database should be exclusively focused on epigenetic effects and sets of genes and pathways that are known to be influenced by epigenetic mechanisms.

It is well established that epigenetic patterns are species- and tissue specific; however, epigenetic changes are likely to also be exposure specific. Because the epigenome is considered as a cellular mechanism that responds to environmental exposures, many changes in epigenetic states are likely to be a consequence of an adaptive response to adverse exposures. The key question is how to distinguish adaptive from adverse responses in the epigenome. In order to detect adverse epigenetic changes, it is essential to have a comprehensive and detailed database for comparison. With the ongoing international efforts (such as the International Human Epigenome Consortium, http://ihec-epigenomes.net), it is hoped that the epigenomes (DNA methylomes) of a wide range of human and other cell types will be available, as well as the normal variability of the epigenome. The fact that the field of epigenetics is rapidly advancing means that in the near future, we are likely to be in a position to construct a 'normal' reference epigenome for comparison and identification of adverse effects of potential carcinogens. Nevertheless, experiments aimed at evaluating the epigenetic effect of a carcinogen should be carefully designed to include the appropriate control group that contains a normal epigenome for comparison.

### *Critical technological and biological issues in assessment of epigenetic carcinogens*

In recent years, we have witnessed an emergence of powerful technologies in epigenetics and epigenomics that allow the sensitive, high-throughput and genome-wide detection of epigenetic changes in

normal and cancer cells (244–246). These advances, notably those linked to the development and application of microarrays and massively parallel sequencing technologies, have accelerated epigenomic research and opened up new perspectives. A wide range of methods and approaches exist for the identification, quantification and mapping of changes in the epigenome. Although the earliest approaches were mostly qualitative, typically non-specific and at best useful for quantification of total epigenetic marks in cells, this field has seen considerable progress and development over the past decade.

Methods for DNA methylation analysis differ in their coverage and sensitivity, and the method of choice depends on the intended application and desired level of information. These methods include global methyl-cytosine content, degree of methylation at specific loci or genome-wide methylation maps. With the advent of more advanced and cost-effective technologies, notably DNA microarray platforms and massively parallel sequencing, it is possible to generate comprehensive maps of epigenomes with relative ease. Similarly, a wide range of robust and genome-wide approaches have been developed for analysis of histone modifications and non-coding RNAs. These technological advances will be instrumental in establishing the epigenome in normal and diseased tissues. Considering the intimate crosstalk between the different epigenetic mechanisms, there may be value in designing approaches that aim to interrogate all layers of the epigenome (DNA methylation, histone modifications and non-coding RNA-mediated gene silencing) in response to carcinogen exposure.

Despite the remarkable progress in epigenomics, challenges still remain with regard to the analysis and interpretation of the large data sets generated by the new sequencing platforms. An important challenge will be establishing the 'normal' state and the dynamic variation of the epigenome. A comprehensive understanding of the physiological variation of epigenetic states in different cell types will be critical to the capacity to discern between normal and abnormal epigenetic patterns. The development of new bioinformatics tools and epigenetic databases should facilitate these efforts (247).

Despite their genome-wide coverage, high resolution and cost effectiveness, most methods for epigenome analysis are not compatible with the analysis of cell subpopulations. It is particularly problematic when specific epigenetic effects, similar to genetic or other molecular effects, in mixed populations of cells need to be resolved (248). This difficulty is exemplified by the heterogeneous nature of tumour tissues, where the presence of normal ('contaminating') cells is common.

In applying epigenetic testing, exposure to carcinogens may induce changes in specific target subpopulations of cells (e.g. stem/progenitor cells) in a tissue. Due to an inability to resolve the epigenome of a single cell and the inevitable averaging of epigenetic data, carcinogen-induced effects may be masked by tissue heterogeneity. Therefore, in addition to the capacity to map the epigenome in great detail, further effort should focus on developing robust methods capable of isolating specific cell fractions and analyzing the epigenome of single cells. Although previously established protocols can be adapted, this area is in need of further development. In addition, significant attention should be paid to selecting appropriate cell populations or subpopulations for epigenetic testing. In this regard, the use of embryonic and tissue-specific stem/progenitor cells should be considered.

## Conclusions and perspectives

Unlike the genetic code, which is virtually the same in every single cell of an organism, the epigenetic code shows wide-ranging variability across different cell types and also in the same cells at different developmental stages and under the influence of various environmental stimuli. This plasticity of the epigenetic code poses a significant challenge in epigenetic testing. Current epigenetic studies, including major international sequencing projects, are expected to generate information for establishing the 'normal' epigenome of tissues and cell types as well as the physiological variability of the epigenome. This should facilitate studies focusing on individual carcinogens and adverse epigenetic effects associated with carcinogen exposure. It is noteworthy that more broadly than the categories of agents considered to date in the Monographs, other environmental exposures such as obesity, physical inactivity and stress are also likely to act through epigenetic mechanisms.

It is anticipated that chemical compounds may be classified as epigenetic carcinogens based on mechanistic evidence (in a development analogous to that applied to aristolochic acid, which was upgraded to a Group 1 carcinogen based on mechanistic data) (249). Therefore, the mechanistic epigenetic data may be used for non-genotoxic and non-receptor-mediated carcinogens (such as arsenic) or when the carcinogenicity data in humans are inconclusive. Identifying a priority set of potential epigenetic carcinogens [e.g. those classified by IARC as probably carcinogenic or possibly carcinogenic to humans (Groups 2A and 2B) that are known to be non-genotoxic] to be addressed in a systematic way may be a good starting point.

Several issues need to be resolved before epigenetic testing can be fully incorporated into carcinogen identification and evaluation and eventually used in policy decision making. In order to incorporate epigenetic data into carcinogen evaluation, it is necessary to consider which epigenetic marks are evaluated, which assays and model systems are used and how changes in the epigenome are interpreted in terms of their potential to contribute to carcinogenesis. The cellular heterogeneity of tumour and normal tissues represents an important challenge for the interpretation of epigenomic data. Because normal or tumour tissues are rarely composed of an identical cell type or clone, tissue purity needs to be carefully considered for accurately measuring epigenetic changes associated with specific exposures. For example, in analysing and interpreting epigenetic data associated with exposures, one may consider applying a recently developed set of analytical tools (247) for inferring changes in the distribution of different cell subpopulations using DNA methylation signatures, a method that circumvents the need for extensive flow cytometry sorting (250,251).

Next-generation sequencing will allow remarkable accuracy, sensitivity and deep read coverage of epigenetic changes; however, extending this approach to a defined cell subpopulation or to the single-cell level remains a challenge. In addition, almost all epigenetic profiling studies have been focused on identifying epigenetic changes associated with annotated genes. Further efforts aimed at improving our understanding of the functional impact of aberrant epigenetic changes occurring in non-genic regions of the genome will require a comprehensive analytical methodology capable of integrating epigenomic data and transcriptomic data as well as genetic data. The development and exploitation of the Gene Ontology database and the Encyclopedia of DNA Elements (ENCODE; nature.com/encode) should help in this task.

Understanding the epigenetic mechanisms by which epigenetic carcinogens promote cancer development will require bringing together dedicated teams, not only of molecular biologists, toxicologists, pathologists and oncologists but also of bioinformatics and computational experts, in order to establish epigenetic assays and translate epigenomic data into an efficient and systematic evaluation of carcinogens.

Despite these significant challenges, the remarkable advances in epigenetics have provided important insights into the epigenetic mechanisms underlying carcinogenesis. The inclusion of epigenetics in the agenda of the IARC workshops on 'Tumour Concordance and Mechanisms of Carcinogenesis', held in April and November 2012, is a testimony to a growing recognition of the importance of epigenetic mechanisms in carcinogenesis. Incorporating epigenetic mechanisms into carcinogen identification and evaluation and risk assessment will be an important legacy of the IARC Monographs Programme.

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