



Epigenetics and environmental exposures

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ABSTRACT

It is becoming increasingly apparent that genetic factors are inadequate to fully explain many processes that shape development and disease. For example, monozygotic twin pairs, despite sharing identical DNA sequences, are often discordant for many traits and diseases, indicating that the same genotype can give rise to distinct phenotypes. This points towards the involvement of additional factors that cannot be explained solely by the sequence of the genome. Epigenetic modifications, defined as heritable changes that do not alter the nucleotide sequence, emerge as key factors that regulate chromatin structure and gene expression and, together with genetic factors, provide the mechanistic basis to understand the biological effects of various classes of environmental exposures. Epigenetic mechanisms explain the ability of certain chemical compounds to initiate biological perturbations that can lead to malignancy, despite being weak mutagens or lacking mutagenic activity altogether—a view that challenges old beliefs and opens new avenues in public health. The field of epigenetics also explains the causal link between certain infectious diseases and cancer, a relationship that was first observed over a century ago and was initially discounted, then fell into oblivion and more recently re-emerged as an important concept in biology. A key feature that distinguishes epigenetic modifications from genetic changes is their reversible nature. This provides exciting prophylactic and therapeutic perspectives, some of which already materialised with the approval of the first drugs that modulate the epigenetic machinery, reinforcing the idea that our genes are not our destiny.

The human genome provided an invaluable and long-awaited glimpse into the relationship between genotype and phenotype. However, as links between mutations and disease predisposition were unveiled, it also became apparent that significant phenotypic discordance exists even among monozygotic twins, which are genetically identical.^{1 2} This pointed towards the involvement of additional factors that shape the phenotype and cannot be explained by DNA sequence variation alone.

Chromosomal changes that are heritable, but do not involve alterations in the DNA sequence, are known as epigenetic modifications. Unlike the genetic profile, which is identical among the cells within an organism, the epigenetic profile is highly dynamic and reversible, and varies among cells in the same organism and, in the same cell, between various states, such as health versus disease, or in response to environmental perturbations.³

In a comparison between monozygotic twin pairs, Fraga *et al*⁴ found that epigenetic modifications, such as DNA methylation and histone acetylation, became increasingly divergent with age.

This explained that gene expression profiles, although almost identical between 3-year-old twin pairs, became very different by age 50, illustrating that the phenotype, even in genetically identical individuals, is differentially sculptured by epigenetic marks during their lifetimes. In the first longitudinal study to examine epigenetic changes during childhood, Wong *et al*⁵ examined three promoter regions in monozygotic and dizygotic twin pairs at ages 5 and 10 and found highly discordant DNA methylation levels that changed in time even between genetically identical individuals. These findings underscore the need to better understand epigenetic modifications that, together with genetic factors, shape gene expression and biological processes.

EPIGENETIC MODIFICATIONS

Three classes of epigenetic modifications were described: DNA methylation, post-translational histone modifications and non-coding RNA-mediated signalling pathways. DNA methylation at CpG dinucleotides, the most frequent epigenetic change described to date in vertebrates, involves the covalent attachment of a methyl group to position five of cytosine to generate 5-methyl cytosine.⁶ The methyl group protrudes into the major groove of the double-stranded DNA and can recruit protein complexes that post-translationally modify histones, compacting the chromatin and silencing genes, or can displace transcription factors that bind DNA.⁷ CpG-rich regions exist in the promoters of ~60% of RNA polymerase II transcribed human genes,⁶ where they are unmethylated, and in repetitive genomic sequences, where they are heavily methylated. CpG hypermethylation at promoter regions leads to loss of function, while global hypomethylation at repetitive sequences causes genomic instability. Both types of modification were implicated in cancer development and progression.

The second class of epigenetic changes involves the post-translational modification of the 15–30 amino acid N-terminal unstructured histone tails. These modifications, which include methylation, acetylation, phosphorylation, ADP-ribosylation and ubiquitylation, alter chromatin condensation.⁷ Histone modifications, and the complex combinatorial patterns that they generate, extend the information that can be encoded in the genome, a concept that became known as ‘the histone code’.

Regulatory pathways mediated by small non-coding RNA molecules, collectively known as RNA interference, represent the third class of epigenetic modifications. RNA interference can be mediated by exogenous RNA molecules, which are known as small interfering RNAs or siRNA, or by endogenous RNA molecules, known as microRNAs or miRNA.³

miRNAs are 19–25 nucleotide long non-coding RNA molecules that bind to the 3' untranslated region of mRNA molecules, and by suppressing mRNA translation or enhancing their degradation, they negatively control gene expression at the post-translational level.^{3–8} Over 1000 miRNAs were identified in the human genome. Each of them is thought to have several targets, and each target is probably regulated by multiple miRNAs.⁸ Some miRNAs act as oncogenes, while others function as tumour suppressor genes, and many of them are altered in human malignant tumours.^{8,9}

ENVIRONMENTAL CHANGES AND EPIGENETIC MODIFICATIONS

For a long time, it was assumed that chemicals are able to cause cancer only by mutating the DNA. However, a growing body of scientific evidence reveals that this 'carcinogenesis equals mutagenesis' paradigm is not accurate. Twenty years ago, Ashby and Tennant¹⁰ examined 301 chemicals tested by the US National Toxicology Program and found that from 162 (53%) that were carcinogens, 64 (40%) were not genotoxic, illustrating the importance to focus on carcinogenic mechanisms other than genotoxicity. For many environmental agents, it is becoming increasingly clear that biological perturbations leading to cancer may occur even in the absence of mutagenesis, via epigenetic changes. Epigenetic changes are clinically relevant in ways that years ago seemed unimaginable. For example, Nobeyama *et al*¹¹ found that tissue factor pathway inhibitor-2, encoding a protein that suppresses the invasiveness of malignant melanoma, was methylated in 29% of metastatic lesions but in none of the primary tumours examined. This pointed towards gene expression and phenotypic differences between metastatic tumours and the primary tumour they originated from, a finding that presents significant therapeutic applications.

ENDOCRINE DISRUPTORS

Bisphenol-A (BPA) is an oestrogenic endocrine disruptor and one of the most widely used chemicals worldwide. As a synthetic polymer, BPA has found wide industrial applications, to manufacture polycarbonate plastics used in bottles and containers that store food and drinks, and epoxy resins that coat food cans. Monomers can leak into food or drinks during heating, under the action of acids or bases, or upon decay.¹² A study on 394 adults in the USA detected BPA in 95% of the urine samples at concentrations >0.1 µg/litre,¹³ and a study conducted in Mexico City found that 80% of the pregnant women examined had detectable urinary BPA at concentrations between <0.4 and 6.7 µg/litre, and women who delivered ≤37 weeks of gestation had higher urinary BPA levels than those who delivered after 37 weeks.¹⁴

An informative animal model, the *Agouti viable yellow* (A^{vy}) mouse, provides an excellent tool to examine epigenetic changes caused by environmental and nutritional exposures and was valuable in understanding the effects of BPA. In this model, a retrotransposable element, known as intracisternal A particle (IAP), is inserted upstream from the transcriptional start site of the *Agouti* allele, which encodes the yellow pigment responsible for the fur colour. Transcription of the *Agouti* gene normally initiates from a hair cycle-specific promoter and occurs for a very limited time during the follicle growth cycle, and the yellow pigment gives, on the black hair background, the brown or agouti coat colour. The A^{vy} metastable allele can undergo epigenetic modifications in response to environmental perturbations, and as a result, the gene becomes differentially expressed in genetically identical individuals.^{15,16} A cryptic

promoter within the retrotransposable element contains several CpG sites, and their methylation is inversely related to the expression level of the yellow pigment. When these sites are hypermethylated, pigment expression occurs only in hair follicles and at low levels, just like in wild-type mice, but when they are hypomethylated, expression becomes constitutive and occurs in all tissues, and is responsible for the yellow fur colour. In addition, ectopic expression of the pigment also increases susceptibility to obesity, diabetes and cancer.¹⁶

By using this model, Dolinoy *et al*¹⁵ showed that maternal BPA exposure can establish stable epigenetic alterations in the form of hypomethylation at nine distinct CpG sites upstream of the A^{vy} IAP cryptic promoter and change the phenotype in the offspring, with a shift of the fur colour towards yellow. DNA methylation at this locus was similar in tissues originating from the three germ layers, indicating that epigenetic patterning is sensitive to BPA exposure during early development.

Additional studies reported that BPA causes epigenetic changes in several cell types. Yaoi *et al*¹⁷ surveyed the methylation of approximately 2500 chromosomal loci to examine the impact of low-level (20 µg/kg of body weight) BPA exposure on the developing murine forebrain and found changes in signal intensity at 48 sites (1.9%). Certain spots were specifically induced at days 12.5 or 14.5 during development, while others showed changes at both stages, indicating that maternal exposure to BPA can establish epigenetic changes at various chromosomal loci and at specific times during development. Weng *et al*¹⁸ reported gene silencing mediated by CpG promoter methylation in breast epithelial cells exposed to low BPA levels (4 nm) and provided the first piece of evidence that interindividual differences in susceptibility to low-dose BPA may exist in humans, and Prins *et al* (2008) found that low doses of BPA (10 µg/kg of body weight/day), comparable to those measured in the blood of human fetuses at term, increased the susceptibility to preneoplastic prostate changes during ageing by epigenetic mechanisms.¹⁹

Methylation changes are not the only epigenetic mechanism reported for BPA. In two placental cell lines derived from the first trimester villous and extravillous cells, Avissar-Whiting *et al*²⁰ found 25 miRNAs differentially expressed in one cell line, 60 differentially expressed in the second one and 21 miRNAs in common between the two sets, in response to BPA exposure. One miRNA overexpressed in both cell lines, miR-146a, is known for its involvement in several malignant tumours. No miRNAs were significantly changed in a third cell line, derived from terminally differentiated third trimester extravillous cells, indicating that these epigenetic perturbations only occur during a specific time of embryonic development.

Additional endocrine disruptors cause epigenetic modifications. Newbold *et al*²¹ found, in a mouse model, significant uterine gene expression changes after neonatal diethylstilbestrol (DES) exposure, and Tang *et al*²² reported that neonatal DES exposure epigenetically reprograms nucleosomal binding protein 1 expression and its interaction with ovarian hormones in adulthood, providing a fascinating glimpse into the two-step early life reprogramming of adult diseases. Some endocrine disruptors have transgenerational effects. Vinclozolin, a fungicide with antiandrogenic activity, was shown to affect promoter methylation in the rat sperm epigenome for three generations after exposure,²³ and a significantly higher proportion of boys born to daughters with in utero DES exposure had hypospadias compared to boys born from unexposed mothers.²⁴

Epigenetic changes caused by endocrine disruptors are important, particularly as several studies document a decline in

reproductive health, in several locations worldwide, that started in the middle of the 20th century, coincided with an >20-fold increase in the use of natural and synthetic chemicals and cannot be solely explained by genetic factors.²⁵

METALS

With the exception of chromium, which forms DNA adducts, most carcinogenic metals are weak mutagens and act by epigenetic mechanisms.²⁶ Nickel compounds, linked to occupational and environmental exposures, have carcinogenic effects despite not being a known mutagen.^{27–29} In vitro and in vivo experiments reveal that nickel compounds silence gene expression by methylating DNA, an effect explained by the ability of Ni²⁺ to substitute Mg²⁺ in the DNA phosphate backbone and increase chromosome condensation.^{26, 29} This establishes heterochromatin regions where the access of cellular proteins to the respective genomic segments becomes more difficult. When the silenced regions contain genes relevant to cancer initiation or progression, such as tumour suppressor genes or senescence genes, their inactivation may lead to disease.²⁹

Nickel compounds also induce global post-translational histone modifications. Golebiowski and Kasprzak³⁰ reported decreased acetylation of histones H2A, H2B, H3 and H4 in a time-dependent and concentration-dependent manner in human and rat cell lines upon nickel exposure. Chen *et al*²⁸ found that at ≤1 mM concentrations, nickel decreased a specific histone demethylase and increased global H3K9 monomethylation and dimethylation in several cell lines. In response to soluble nickel compounds at levels showing minimal cytotoxicity, Ke *et al*³¹ described three histone modifications—H3K9 dimethylation, increased H2A and H2B ubiquitylation and reduced acetylation, which was also associated with a transgene silencing.

Cadmium, an occupational and environmental carcinogen linked to several types of human cancer, has very low levels of mutagenicity, and its carcinogenic mechanisms have been elusive for a long time.³² Takiguchi *et al*³³ reported that mammalian and bacterial DNA methyltransferases were inhibited in rat liver cells exposed to concentrations of up to 2.5 μM cadmium, resulting in concentration-dependent decrease in global DNA methylation after 1 week. More recently, Doi *et al*³⁴ reported that cadmium downregulates two DNA methyltransferases in chick embryos, causing global DNA hypomethylation, a potential explanation for the ventral body wall defects caused by this compound.

Until recently, chromium was thought to cause cancer only through its ability to damage DNA, but epigenetic mechanisms are becoming increasingly apparent.²⁷ Klein *et al*³⁵ reported for the first time that potassium chromate, a carcinogen, causes aberrant DNA methylation and silences a reporter gene in a mammalian cell line. Ali *et al*³⁶ found increased aberrant methylation in the promoters of three tumour suppressor genes in lung cancers of chromate workers compared to non-chromate lung cancer controls, and concordant methylation of multiple loci was more often observed among chromate workers. Sun *et al*³⁷ found that hexavalent chromium, at 5–10 μM concentrations, establishes global and local, gene-specific histone methylation changes in lung cancer and in non-cancerous bronchial epithelial cells.

In the first study to examine the link between maternal lead burden and genomic DNA methylation from the cord blood, Pilsner *et al*³⁸ found that lead levels in the patella and the tibia, which reflect cumulative exposure, were inversely related to the cord blood methylation levels of the long interspersed nuclear

element-1 (LINE-1) and AluI repetitive sequences global methylation markers, respectively. The authors did not find a correlation between DNA methylation and cord blood lead levels, which reflect recent exposure.

BENZENE, POLYCYCLIC AROMATIC HYDROCARBONS AND PERSISTENT ORGANIC POLLUTANTS

Benzene and aromatic hydrocarbons have increasingly emerged as environmental hazards in occupational or non-occupational settings. In the first study to link low levels of a common environmental carcinogen to epigenetic changes in human cancers, Bollati *et al*³⁹ examined traffic police officers and gas station attendants from Milan, Italy and reported that low-level airborne benzene exposures that are common in western countries cause a dose-dependent global hypomethylation in the LINE-1 and AluI repetitive sequences. In addition, the authors described hypermethylation and hypomethylation at specific promoters known to undergo epigenetic changes in human cancers.

As a group of chemicals, polycyclic aromatic hydrocarbons (PAHs) include thousands of compounds ubiquitously distributed in the environment. An interesting fact about PAHs is that for many years, the focus was on their ability to cause genotoxic damage while their potential to induce epigenetic modifications was largely ignored.⁴⁰ Benzopyrene, a prototype PAH, is an environmental and occupational carcinogen found in vehicle emissions and in cigarette smoke. In the first study showing that benzopyrene causes epigenetic changes, Sadikovic *et al*⁴¹ conducted a genome-wide analysis of benzopyrene-treated cells and found 775 genes that were hypoacetylated and 1456 that were hyperacetylated after exposure. Many of the affected genes had fundamental cellular roles in processes including DNA replication, repair and carcinogenesis.

Cigarette smoke is a complex mix of over 4000 chemicals. In one of the first studies linking in utero tobacco exposure to DNA methylation changes in the offspring, Breton *et al*⁴² found two genes, encoding a receptor tyrosine kinase important for cell survival and a receptor tyrosine phosphatase important for central and peripheral nervous system development that exhibited consistent methylation changes as a result of maternal smoking. Toledo-Rodriguez *et al*⁴³ found, in adolescents exposed to cigarette smoke in utero, an almost fourfold increased methylation in the promoter and 5'-untranslated region of brain-derived neurotrophic factor, a gene that shapes brain plasticity during development.

In the first study that examined the effects of smoking on miRNA expression, Schembri *et al*⁴⁴ found 28 miRNAs that were differentially expressed, and 82% of them downregulated, in smokers. One of these, miR-218, is also downregulated in several cancers, and the authors showed that modulating its levels changed the expression of its target genes. Xi *et al*⁴⁵ found that cigarette smoke condensate causes an early and significant increase in miR-31 that is apparent within 24 h after exposure and persists for 20 days after the exposure stops, and Maccani *et al*⁴⁶ found that three miRNAs important for growth and development are significantly downregulated in the placenta of smoke-exposed mothers compared to controls.

The first study to link persistent organic pollutant (POP) exposure to DNA methylation in humans found, in a Greenlandic Inuit population, which has one of the highest POP blood levels worldwide, an inverse relationship with the methylation of *LINE-1* and *Alu* repetitive elements. This finding opens important questions with regard to the epigenetic effects of these compounds.⁴⁷

DIET

Studies have long indicated that adversity during development shapes the risk of adult-onset disease decades later, a concept that became known as the developmental origins of health and disease or the Barker Hypothesis. The impact of nutritional influences during development on disease risk later in life was studied extensively as a result of a sad period in history, the Dutch Hunger Winter, a period of abrupt and intense famine that affected the western part of the Netherlands at the end of World War II. Heijmans *et al*⁴⁸ found that individuals who were nutritionally deprived in very early stages of development showed, six decades later, lower DNA methylation levels in *IGF2*, an imprinted gene with key roles in growth and development, than their same-sex, unexposed sibling. These differences were not found in individuals exposed to famine late during gestation, indicating that epigenetic changes might be particularly sensitive during early development.

Xiang *et al*⁴⁹ reported that selenium, an essential trace element that protects against several types of human cancer, but may also negatively affect DNA integrity after unregulated intake, can epigenetically restore the expression of several tumour suppressor genes that were silenced by hypermethylation.

Recent findings also promise to unveil the teratogenic mechanisms of alcohol, a major cause of non-genetic developmental delay. Zhou *et al*⁵⁰ conducted a genome-wide methylation analysis on neural stem cells exposed to alcohol and, for the first time, revealed significant changes in the promoter methylation pattern and in neural stem cell migration and differentiation, indicating that alcohol exposure alters epigenetic programming during neural stem cell development.

Ichi *et al*⁵¹ found that folic acid establishes epigenetic changes in *Hes1* and *Neurog2*, two genes involved in the development of the murine nervous system. Folic acid fortification, a public health accomplishment, reduced the incidence of neural tube defects, and many studies documented a 20–40% decrease in the colorectal cancer risk among individuals with the highest folate intake. At the same time, an emerging concept is that folic acid could also have unintended consequences by inducing epigenetically mediated changes during development,⁵² and several studies reveal that it accelerates the growth of already existing tumours, pointing towards a dual modulatory effect, depending on dose and timing.⁵³ This underscores the need to better understand the interface between epigenetics, genetics and nutrition.

Even though epigenetic changes caused by chemicals and food are studied separately, Lee *et al*⁵⁴ point out that, particularly in context of the large numbers of chemicals present in the food chain, and the potential for synergistic interactions, these two classes of environmental factors cannot be separated from each other, and their impact should be studied together.

INFECTIOUS DISEASES

Microorganisms were causally implicated in slightly over 20% of all human cancers. Viruses (hepatitis B, papilloma and the Epstein–Barr virus), bacteria (certain *Helicobacter pylori* serotypes) and parasites (*Schistosoma haematobium*) are among the most important pathogens linked to carcinogenesis. The mechanistic links between microorganisms and cancer have been elusive for many years, but recent findings reveal that many pathogens cause epigenetic reprogramming in host cells.

Maekita *et al*⁵⁵ found increased methylation at several CpG islands in the gastric mucosae of individuals infected with *H. pylori* compared to uninfected individuals. Mongolian gerbils

experimentally infected with *H. pylori* exhibited hypermethylation at several promoters from the gastric mucosa.⁵⁶ Methylation levels started to increase 5–10 weeks after the start of the infection, reached high levels by 50 weeks and decreased 10–20 weeks after the infection was eradicated but remained higher than in uninfected animals.⁵⁷ Cyclosporin A, which suppresses inflammation without affecting bacterial colonisation, abolished this aberrant methylation, indicating that it is not the pathogen itself, but the inflammatory process, that is responsible for the hypermethylation.^{56 58} Methylation appears to consist of a permanent component that occurs in gastric stem cells and a temporary component that results from methylation in progenitor and differentiated cells. It is thought that the temporary component is the one that disappears after eradicating the infection.⁵⁷

Nakajima *et al*⁵⁹ demonstrated, for the first time, the existence of gene specificity in the DNA hypermethylation induced by infection. Methylation of specific genes that occurs in a significant number of cells in the mucosa establishes an 'epigenetic field for cancerisation' or 'epigenetic field defect', marking a site with high risk for subsequent malignant transformation.⁵⁸ Epigenetic fields for cancerisation were also described for other tumours, including breast, colon and liver cancer. In some malignant tumours, such as stomach cancer, the inactivation of genes appears to occur more frequently by epigenetic mechanisms, such as aberrant methylation, than by genetic mechanisms.⁶⁰

PHYSICAL AGENTS: UV RADIATION

Nandakumar *et al*⁶¹ showed, for the first time, that chronic exposure to UV-B in mice increases the expression and the activity of DNA methyltransferases, leading to aberrant DNA hypermethylation, which could stimulate other epigenetic mechanisms, such as histone modifications, that culminate in carcinogenesis. This revealed that the silencing of tumour suppressor genes could be involved in the pathogenesis of skin cancers.

SOCIAL INFLUENCES

Social influences, acting through hormone signalling pathways, are one of the most recent additions to the group of environmental factors that induce epigenetic changes. Weaver *et al*⁶² compared rats engaged in high versus low licking and grooming (LG) and arched-back nursing (ABN), two types of maternal behaviour that are apparent during the first week after the pups are born, and showed that these maternal tactile influences establish epigenetic changes at the glucocorticoid receptor in the offspring hippocampus.

Exon 1₇ in the rat hippocampal glucocorticoid receptor promoter contains the binding site for nerve growth factor-inducible protein A (NGFI-A). Weaver *et al*⁶² found that the CpG dinucleotide from site 16 within this exon, which is the NGFI-A binding site, is rarely methylated in pups born to mothers with high LG–ABN but is always methylated in pups born from mothers with low LG–ABN. In pups born to high LG–ABN mothers, these methylation changes were associated with increased histone H3K9 acetylation and higher NGFI-A binding to the glucocorticoid receptor promoter. Cross-fostering generated a methylation pattern reflecting the rearing mother's behaviour. For the first time, this revealed that maternal care establishes epigenetic marks that shape chromatin organisation and gene expression in the offspring. Subsequent research found that differences in maternal care establish epigenetic changes over broad genomic areas that involve both coding and non-coding regions in the adult rat hippocampus.⁶³

In the first study to reveal protein synthesis changes in individuals who committed suicide, McGowan *et al*⁶⁴ found rRNA promoter hypermethylation and decreased rRNA expression in the hippocampus of individuals with a history of abuse or serious neglect, compared to individuals who died accidentally or committed suicide but did not have a history of abuse or neglect. Methylation levels in the hippocampus were not significantly correlated with those in the cerebellum, indicating that hypermethylation was specific to the hippocampus, and supporting the idea that early life adversity exerts specific epigenetic changes in the nervous system.

Roth *et al*⁶⁵ found that in rats, psychosocial stress comparable to human post-traumatic stress disorder (PTSD) increased *Bdnf* methylation in the dorsal hippocampus and downregulated its mRNA in the dorsal and ventral hippocampus, but not in other PTSD-relevant regions. This study, for the first time, linked traumatic stress during adulthood to region-specific epigenetic changes in *Bdnf* and revealed that DNA methylation remains an active process that can be shaped by environmental factors in the adult nervous system.

EPIGENETICS AND PUBLIC HEALTH

While recent years witnessed significant progress in understanding the biological impact of environmental exposures, an important take-home lesson is the insufficient attention that epigenetic factors have received, relative to genetic ones. Interdisciplinary efforts should increasingly focus on unveiling epigenetic mechanisms and pathways that shape development and disease. As epigenetics reveals, many environmental agents that appeared from epidemiological studies to be carcinogens, despite their inability to mutate DNA *in vitro*, are able to establish biological perturbations by changing chromosome compaction and gene expression. Epigenetic mechanisms emerge as a ‘unifying theme’ that, in combination with genetic factors, will assume fundamental roles in explaining the biological effects of chemical, physical, biological and social factors. This dynamically expanding field has already started to impact clinical medicine and should become an important instrument for public health and public policy decisions.

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