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Epigenetic memory in response to environmental stressors

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Abstract

Exposure to environmental stressors, toxicants and nutrient deficiencies can affect DNA in a number of ways. While some exposures cause damage and alter the structure of DNA, there is increasing evidence that the same or other environmental exposures, including those experienced during fetal development *in utero*, can cause epigenetic effects which modulate DNA function and gene expression. Some epigenetic changes to DNA that affect gene transcription are at least partially reversible (i.e. they can be enzymatically reversed after cessation of exposure to environmental agents), but some epigenetic modifications seem to persist even for decades. To explain the effects of early life experiences (such as famine and other exposures to stressors) on the long-term persistence of specific patterns of epigenetic modifications, such as DNA methylation, we propose an analogy with immune memory. We propose that an epigenetic memory can be established and maintained in self-renewing stem cell compartments. We suggest that the observations on early life effects on adult diseases and the persistence of methylation changes in smokers support our hypothesis, for which a mechanistic basis, however, needs to be further clarified. We outline a new model based on methylation changes. Though these changes seem to be mainly adaptive, they are also implicated in the pathogenesis and onset of diseases, depending on individual genotypic background and/or types of subsequent exposures. Elucidating the relationships between the adaptive and maladaptive consequences of the epigenetic modifications that result from complex environmental exposures is a major challenge for current and future research in epigenetics.

Introduction

Epigenetic events, including DNA methylation and histone modifications, are increasingly recognized as key mechanisms involved in cellular responses to environmental stimuli and in the etiology of disease (1, 2). Here, we consider the role of DNA methylation in these processes. The addition of a methyl group to cytosines at CpG sites within *cis*-regulatory elements such as promoter and enhancer sequences usually leads to suppression of transcription and therefore to a reduced expression of the corresponding gene. The main mechanisms driving this association with transcription involve proteins such as MeCP2 and MBD2, which bind to methylated cytosines and facilitate the assembly of repressed chromatin domains. The absence of methyl-CpG-binding proteins at unmethylated promoters enables chromatin to maintain a relatively open configuration that allows transcription factors access to their cognate target sequences in DNA [3]. Reduced methylation may lead to genome instability, particularly if it occurs in repeat sequences such as LINE1 transposable elements, which have a high CpG density (4). Methylation levels in white blood cells (WBC) and in other tissues in humans are potentially influenced by fluctuating exposures induced by smoking [5], diet [6-8] or air pollution [9]. Sometimes the events involved in inducing changes in methylation are very remote and persist over time, such as the impact of *in utero* exposure to the Dutch famine on adult phenotype. The latter has been attributed to changes in gene methylation, though with small absolute changes in methylation levels and mainly in imprinted genes [10]. In addition to cytosine methylation, other mechanisms, involving DNA and/or histone modifications and small RNAs, are also implicated in epigenetic changes, but they are not discussed further in this paper.

Studies in animals also point to the existence of remarkably stable DNA methylation changes that can be induced by exposure to nutritional, behavioural or other environmental stimuli, and which then persist long-term. Rodent offspring that are exposed to changes in maternal nutrition during fetal development or neonatally, exhibit metabolic disturbances with physiological consequences that are associated with stably altered patterns of DNA methylation and other epigenetic marks in somatic tissues (11-15). Similarly, studies of the epigenetic effects of stressful experiences in rodents have demonstrated changes in DNA methylation patterns associated with genes involved in regulating neuroendocrine signaling as a consequence of stressors experienced either during early life (16-18) or in adulthood (19). Direct exposure of rodents to the chemical environmental contaminant Bisphenol A has also been demonstrated to exert strong biological and long-lasting epigenomic effects through altering DNA methylation levels (20, 21).

Taken together, the observations on the effects of nutritional, behavioural or other environmental stressors [22] suggest that there are long-lasting mechanisms underlying these heritable changes that are transmitted across many cell generations. Here, we propose a hypothesis that DNA methylation is a crucial component of cellular responses to environmental signals, acting within self-renewing human stem cell populations that persist across the life course, which may become enriched under selective pressure.

Hypothesis: methylation memory

We hypothesize that a long-lasting epigenetic change (called here "epigenetic memory") can be likened to the immune memory of Burnet's clonal selection theory of *adaptive immunity*. Such epigenetic memory may be activated in response to changes in exposure to a wide range of exogenous or endogenous agents, including toxicants, nutrients, and behavioural stimuli, analagous to exposure to exogenous antigens from infectious pathogens. Since the resulting epigenetic changes are known to be observable many years after the initial stimulus or exposure, some of them must be embedded in stem cells since these changes persist for much longer than the lifetime of some of the mature terminally differentiated cells in which they are observed (e.g. WBC or epithelial cells). Accordingly, we hypothesize that, just as a clone of B-cells carrying a specific antibody is activated and amplified in response to reinfection of the body by a pathogen bearing the antigen that initially generated the B-cells, so self-renewing stem cells have a similar behavior: the DNA methylation status of specific genes would be altered by a particular exposure early in life and may persist in a state of primed responsiveness and alter transcription of these genes,- allowing rapid adequate cellular responses if the same or a related exposure occurs at a later time. Cells which have been epigenetically primed to respond effectively to renewed stimulus exposure would be expected to have a growth/survival advantage which would promote their clonal expansion. For example, such epigenetic changes predisposing genes involved in detoxification to rapid and high levels of transcription would be positive adaptive responses that enhance cellular and organismal survival if individual genotypic background is favorable.

Antibodies are continuously produced via somatic hypermutation and V(D)J recombination in B-cells. The renewed encounter with a specific antigen leads to clonal amplification of the B-cell that carries the corresponding antibody. In the case of detoxification of exogenous or endogenous agents, we hypothesize that repeated encounters of key cell regulatory molecules (e.g. receptor proteins such as AhR - see below) with such agents lead to persistent DNA methylation changes which place AhR-responsive detoxifying genes in a transcriptionally activated or activation-

competent state. We suggest that changes in the patterns of CpG methylation associated with such genes persist as adaptive epigenetic memories within stem cell compartments that can be activated and amplified by clonal expansion when the toxicant or other stimulus is subsequently re-encountered. Such beneficial epigenetic memories could then render cells competent to mount more rapid and effective transcriptional responses to environmental stimuli, including beneficial stimuli such as dietary. This hypothesis recognizes that whilst these epigenetic modifications may enable mature, differentiated non-proliferative tissues to respond to an exposure to a stimulus directly, it also postulates that persistence of an epigenetic memory of exposure in stem cells could enable the rapid production, mobilization and potentially widespread dissemination of differentiated cells with beneficial capabilities.

Whilst exposure to particular toxicants may introduce into stem cells epigenetic changes that modify the transcriptional capabilities of genes that are specifically toxicant-responsive, other environmental circumstances, such as nutrient deficiencies, may affect the *global* methylation status of cells, including stem cells and their differentiated, post-mitotic progeny. For example, reduced availability of nutrients that support one-carbon metabolism such as methionine, choline, betaine and folate can cause global hypomethylation and also effects on specific CpGs.

Global hypomethylation, frequently observed following toxic exposures and nutritional deficiencies, may in fact constitute a general cellular response that may render all or many inactive genes primed for activation, and favour increased transcription of already active loci. Such *transcriptional plasticity* could facilitate the global re-calibration of cellular phenotypes to accommodate altered circumstances, which might be necessary in times of toxic stress or nutrient restriction, for example. Since the broad phenotypic plasticity of pluripotent stem cells in the mammalian embryo is due, at least in part, to global hypomethylation (23), it is possible that the presence of hypomethylated loci in adult stem cell reservoirs in adult organs may also confer broader sensitivity to exposures arising in sub-optimal environments. Moreover, should nutritional circumstances improve, the induced phenotypic plasticity of stem cells could facilitate further adaptive responses more effectively than post-mitotic cells in which phenotypic plasticity might be limited. Stem cells carrying unmethylated CpGs could thus be selected to maintain an “alert” status following a situation of deficiency, in readiness to respond protectively in case of future re-encounters with similar environmental challenges or threats. Whilst such long-lived epigenetic changes would tend to serve a beneficial/protective purpose, it is possible that, over time, they may also interact with accumulated changes induced in cells by events later in life (e.g. other exposures or ageing), becoming maladaptative and end up contributing to disease pathogenesis. The latter

parallels the induction of B cell neoplasia and autoimmunity by chronic exposure to a structurally-related exogenous antigen (24). Another mechanism could be, for nutrition, the organism being prepared for one nutritional environment but meeting another – the "mismatch" idea.

Examples of long-lasting methylation changes

For the sake of simplicity we consider two broad types of observations on DNA methylation:

- global hypomethylation that is frequently observed after dietary deficiency, toxic exposures or in pre-cancerous states (usually measured in white blood cells, WBC)
- hypo- or hyper-methylation at specific genomic sites or pathways observed after exposure to toxic agents (such as tobacco smoke or bisphenol A) or in cancerous tissues.

The first category of change we consider is a somewhat global effect produced by fundamental environmental challenges that affect basic biochemical and molecular processes, such as folate deficiency. More targeted effects on particular sites associated with single genes or pathways are instead the consequences of signalling by specific types of stimuli. The two mechanisms can co-exist depending on the type of insult. We use case studies to test if such a hypothesis of a general and a specific methylation memory holds true for various types of challenges at various times in the lifecourse.

A state of global "hypomethylation" may be induced in proliferation-competent cells that are exposed to a stimulus and then undergo mitosis to give rise to the tissue of interest, or alternatively, hypomethylation may result when a post-mitotic tissue is exposed to an environmental challenge (for example when measured as an average across multiple or all probes in an epigenetic-wide association study, EWAS, or in LINE-1 and Alu, i.e. high copy long repeat sequences). In either situation, detection of hypomethylation for a single probe occurs when an excess of cells (which may or may not arise from proliferation of stem cells) exhibits low levels of methylation at the CpG site contained within the probe sequence. Hypermethylation for a single probe is detected when an excess of cells is methylated at the corresponding CpG (Figure 1). Caution is needed when the possibility of global methylation is being considered, because we currently have only a partial understanding of the extent to which a generalized hypomethylation process may be similar across tissues and indeed the extent to which such hypomethylation is fully genome-wide: most studies of methylation at specific sites do not consider genuinely global changes because many of the epigenome-wide techniques (e.g. the Illumina 450K array) have limited coverage, so that the possibility of specific effects occurring at some CpG sites are difficult to rule out. Therefore, whilst

there is mounting evidence that some exposures induce broadly global changes in methylation patterns, and that other exposures elicit more specific changes within the epigenome by targeting a particular biological signalling pathway, the dichotomy is an approximation for the sake of our hypothesis.

Examples

1. **Global hypomethylation and altered one-carbon micronutrient availability**

Global DNA hypomethylation is known to result from deficiencies either in methyl group availability or in enzymes responsible for DNA methylation. Nutrients involved in one-carbon metabolism, such as folate, methionine, vitamin B12, choline and betaine, play critical roles in determining methyl group availability and in maintaining DNA methylation. Folate, a water soluble B vitamin, aids in the transfer of one-carbon units and is involved in multiple cellular processes, including specific amino acid synthesis, DNA replication and DNA methylation. It cannot be synthesized *de novo* by mammalian cells and, therefore, its cellular level depends on dietary intake. Folate deficiency alters the balance of the pool of nucleotides needed for the synthesis of DNA, leading to dUMP accumulation/misincorporation in DNA and thus potentially to chromosomal changes and genetic instability. Of relevance for DNA methylation, folate also plays an important role in the synthesis of methionine as a cofactor for the enzyme methionine synthetase. Methionine is a precursor of S-adenosyl methionine (SAM), the universal methylation donor required for the methylation reaction catalyzed by DNA methyltransferases (DNMTs). Thus, factors that limit SAM supply could have an important impact on DNA methylation and histone methylation. Global hypomethylation resulting from reduced supplies of one-carbon nutrients may create a state of transcriptional permissiveness that renders tissues more readily responsive to external factors. This state may then enable epigenomic adaptations that could, for example, facilitate more efficient, or "thrifty", utilisation of scarce nutrients. Moreover, global DNA hypomethylation may constitute a background of hypomethylation, not significant at that level, but which favors overriding of the threshold for gene transcription, if an additional DNA hypomethylation or epigenetic change occurs at a specific gene in a given tissue.

Recent studies of the seasonal variation in the one carbon micronutrient intake of mothers at the time of conception further demonstrate a strong link between folate, betaine, choline and methionine availability and the establishment and long-term persistence of distinct patterns of DNA methylation in offspring (25, 26). In these studies, variable patterns of DNA methylation established in the early embryo were found to persist in infants at many different loci, termed "*metastable*

epialleles", in a manner that reflected one carbon micronutrient availability to mothers at the time of offspring conception. In a population based study of two European cohorts, a significant association was observed between maternal plasma folate levels during pregnancy and genome-wide patterns of DNA methylation in newborns (27). Consistent with these findings, it has been suggested that maternal nutrition contributes a "metabolic imprint" to the offspring epigenome during embryonic development, which then persists in progeny over the long-term as a pattern of metastable epialleles (28).

2. Exposure to Bisphenol A and patterns of DNA methylation and gene transcription

Bisphenol A (BPA) is a chemical which has multiple effects on gene methylation. It is widely used in the chemical synthesis of epoxy resins and polycarbonate plastics and is a ubiquitous environmental contaminant, implicated in endocrine disruption and increased risks for disorders of the nervous, immune and reproductive systems, including cancer and obesity. As a stable ligand for oestrogen and other steroid hormone receptors, BPA exerts appreciable effects on animal and human fertility, and in experiments to investigate its epigenetic effects, it has well-characterized impacts on DNA methylation that affect the expression of many genes. The adverse effects of maternal BPA exposure on offspring are strong when exposure occurs either during gestation or postnatally during lactation, and they persist in the tissues of progeny as metastable epialleles, long after the period of exposure has ended (20), suggesting that this compound may exert a substantial impact on the epigenetic programming of developing tissues and/or in stem/progenitor cells. Global methylation analysis suggested that BPA increases the overall levels of genomic methylation by ~15% (29), although more detailed studies of the epigenomic effects of BPA - focusing on transcription units and associated regulatory sequences - revealed that in the liver, whilst a large number of genes are hypermethylated in response to BPA, many others are hypomethylated (30). **A similarly complex picture has emerged from studies of the effects of BPA in the brain, where sex-specific, brain region-specific increases and decreases in DNA methylation levels have been described (31).**

3. Smoking-associated hypomethylation

In addition to global or generalized changes, we propose a model in which environmental exposures to toxicants can elicit distinct long-term and shorter-term impacts on patterns of DNA hypomethylation at specific loci. This proposal is based on observations related to smoking, an exposure with broad and well-characterised health effects (32), which we use here as a case study.

In a series of studies on tobacco smoking we found that hypomethylation of several genes in WBC and also in lung tissue of healthy subjects was associated with smoking (33-37). This finding has been replicated in many other studies of WBC, buccal cells and cord blood of smoking mothers (38-50). To investigate the dynamics of methylation in smoking we conducted epigenome-wide analyses in a population sample of 1,000 subjects [36]. When we examined the distribution of methylation changes by time since smoking cessation we observed the pattern reported in Figure 2. While for many CpGs, methylation reverted back to levels of never-smokers, for other CpGs hypomethylation was still present after 30-40 years since smoking cessation. The stability of some of these methylation changes is not compatible with the short half-life (death/replication rate) of WBC, and instead suggests that long-term changes to patterns of DNA methylation are induced in hematopoietic stem cells of the bone marrow by exposure to tobacco smoke, which then persist in the stem cell compartment for decades and are transferred to differentiated, circulating progeny of these stem cells even after smoking cessation.

The gene that was most affected by methylation changes in the majority of these studies was *AHRR*, encoding the repressor of the Ah Receptor (AhR), that in turn regulates transcription of specific target genes in response to cell-permeable toxicants from the external environment. In our study we found that the list of CpGs with persistent tobacco-induced methylation changes included a number of CpGs associated with the *AHRR* locus. We also compared methylation levels of 49 *AHRR* probes in WBC with methylation in lung macrophages from the literature, finding that the effect of smoking was broadly similar in the two tissues (Figure 3), with most CpGs being hypomethylated in current smokers and former smokers compared to never smokers, despite the large difference in baseline expression levels (very low in WBC, high in lung macrophages). *AHRR* expression was upregulated by smoking in both tissues. In a mouse model of smoking we observed an initial decrease in expression of *AHRR* at 3 days of exposure and a significant increased expression after longer-term exposure (28 days) [36]. These observations provide a clear example of a gene-specific transcriptional regulatory pathway activated by the exposure and a gene-specific epigenetic hypomethylation pattern that exhibits long-term persistence, most likely in hemopoietic stem cells, as a molecular memory of the exposure.

4. Multiple methylation and transcriptional changes in ex-smokers

It is likely that the prominent methylation changes in the *AHRR* gene are consequences of exposure to a particular class of toxicants/carcinogens in tobacco smoke. The obvious candidates are polycyclic aromatic hydrocarbons, including benzo(a)pyrene, which bind to AhR and are likely to be involved in lung carcinogenesis according to mutational signatures (Alexandrov et al, submitted). However, tobacco smoke is a very complex mixture and has a range of different impacts on health (including cardiovascular disease) and therefore it is likely that a more complex epigenetic reaction/adaptation is elicited than simply *AHRR* hypomethylation.

We have conducted a preliminary evaluation of the implications of long-lasting CpG-methylation changes by focusing on genes harboring CpG sites with significant methylation changes surviving for at least 10 years post-smoking cessation (518 CpGs; 339 unique genes) [36]. Bioinformatics analysis of these genes (unpublished results) yielded the list of significant gene ontology (GO) terms shown in Table 1, which can be interpreted as reflecting the cell's epigenetically-determined readiness for the upregulation of various categories of interacting cellular pathways and processes which influence cellular fate. It is noted that GO terms reflect not only the specific states or processes after which they are named but also underlying cellular activities of mechanistic relevance, and should be interpreted as such. For example, the top term in Table 1 ("growth plate cartilage development") is a developmental term implicating cell matrix and stroma interactions, fundamental components of the wound healing response. The GO terms identified are linked to a number of genes with global regulatory impact as judged by the number of functions to which they are associated (hub genes), some of which suffer substantial demethylation in smokers as well as ex-smokers for >10 years. These GO terms and associated hub genes relate to:

- development and morphogenesis, with a prominent role for genes like the retinoic acid receptors RARA and RARG, coupled by the differentiation-inducing SMAD interacting proteins to TGFBR2, the proto-oncogene SKI, the hedgehog/WNT signalling induced gene CSRNP1 and the histone demethylase ARID5B;
- activation of metabolism and protein synthesis, including broad metabolic (ASNS, NFE2L2), transcriptional (RARG, VIM, E2F1, TNF, ATF4) and translational (RPS18, RPL12, RPL23A, EEF1D) alertness, protective nonsense-mutated mRNA decay (SMG6), all of which reflect the cell's state of increased readiness to respond to future stress;

- response to oxidative stress/detoxification of free radicals (key genes involved: GPX, NFE2L2, responsive to AhR signaling) and anti-apoptosis – it is noted that suppression of oxidative stress-induced apoptosis, constitutes a fundamental mode of defence against toxicant-induced cell death;
- differentiation and wound healing (TGFBR2, INHBA, GDF11, SMAD3, NFE2, SKI), regulation of the cell cycle and proliferation (CDKN1A, MINK1, AKT1) and, particularly interesting, stemness (SKI, PIM3, RALA, RRAS, TNFAIP8, PURA), reflecting the induction of stem cell-like properties as being necessary for tissue repair; angiogenesis (ATOH8, NFE2L2, NFE2), inflammation (TNFAIP, TNF) and immune response modulation (CD44, BCL3, THBS1, MYO1G), which are fundamental components of the wound-healing process.

The potential upregulation of cellular metabolism-related processes (e.g. "positive regulation of gene expression", "translational elongation", "positive regulation of reactive oxygen species metabolic process") entails increased energy demands and would therefore be subject to strong thermodynamic restrictions depending on the energy available to cells, as reflected in the GO term "cellular response to glucose starvation". Finally, the potential for increased growth and/or survival is reflected in the GO terms «regulation of mitotic cell cycle», «positive regulation of programmed cell death» and « negative regulation of apoptotic process». This is consistent with the idea that persistent hypomethylation in stem cells, by facilitating cellular responses to subsequent exposures by readiness to transcribe relevant genes in daughter cells, promotes clonal expansion in mitotic cells via increased proliferation rate and/or reduced apoptosis frequency. Thus the overall picture of the long-lived smoking-induced epigenetic changes suggests the establishment of a multi-layered, spatially and temporally emergent, homeostatic response phenotype.

Discussion

The significance of global hypomethylation in different tissues (i.e. an altered ratio between cells carrying unmethylated vs.4 methylated CpGs) is still not well understood. We suggest that global hypomethylation is a cellular response to changes in nutritional circumstances such as famine, lack of nutrients such as folate, or other systemic environmental challenges. A causal relationship between hypomethylation and dietary shortage in, for example, methionine, folate, vitamin B12 and choline, is supported by the evidence reviewed above. Dietary factors may thus influence the supply of methyl groups available for the formation of S-adenosylmethionine (the co-enzyme involved in methyl group transfer) or modify the utilization of methyl groups. Moreover global systemic DNA hypomethylation may favor exceeding the threshold for gene expression if an additional epigenetic change occurs in specific genes of a given tissue.

Perhaps the most intriguing finding so far has come from the study of the long-term effects of tobacco smoke through an epigenome-wide methylation approach. The Ah Receptor (AhR) is well known for regulating responses to an array of environmental chemicals. A growing body of evidence suggests that the AhR also plays a key role in modulating critical aspects of cell function including cell growth, death, and migration [51]. *AHRR* is highly conserved in evolution [51]. While its most studied function is the mediation of the effects of exogenous chemicals such as dioxin and PAHs on the cell [52], *AHRR* is also involved in many other functions including the effects of tobacco smoking in pregnancy (53, 54). AhR is antagonized by the preventive agent and antioxidant resveratrol [55]. The studies we conducted in smokers were in white blood cells, implying that epigenetic "memory" elicited by smoking was likely established in hematopoietic stem cells. However, we also investigated the lung tissue of smokers and non-smokers. Methylation levels of the *AHRR* gene probes were significantly decreased ($P<0.001$) and expression increased ($P=0.0047$) in the lung tissue of current smokers compared with non-smokers (36). This was further validated in a mouse model of smoke exposure with similar results (36). There is no data yet on the lungs of ex-smokers.

It is likely that the prominent methylation changes in the *AHRR* gene are the expression of a particular class of toxicants/carcinogens, particularly benzo(a)pyrene, that bind to AhR and leave characteristic mutational signatures also found in lung cancer cells (Alexandrov et al, submitted). However, tobacco smoke is a very complex mixture with a range of different impacts on health and therefore it is likely that a more complex epigenetic reaction is elicited. This is in fact what we have observed by bioinformatic analysis of ex-smokers. Seen in the above context, the long-lived epigenetic changes induced by smoking can be thought of as serving the role of long lasting memory, providing an optimized pool of adaptive scenarios to tissue damage. On the other hand, because of their stochastic nature, these responses are also subject to disruption by detrimental structural events (mutations, insertions, deletions) which may arise from various exposures including smoking and which would determine whether they ultimately serve an adaptive or a maladaptive role. For example, the disruption of the balance between inherited genetic background, developmental, differentiating, immune regulatory and inflammatory stimuli during the wound healing process, by the induction of mutations in critical genes as a result of exposure to mutagens, may provide the basis for establishment of oncogenic niches. The latter may result in an aberrant deviation of the healing process, turning the induction of stemness from a potentially beneficial adaptation into a maladaptive (carcinogenic) response. Also, whereas suppression of apoptosis would protect the individual cell from toxicant-induced death, in the presence of mutations it could lead to cancer.

DNA methylation changes as “readiness” for plastic responses

There is still limited evidence that an exposure may cause a similar pattern of gene-specific methylation changes in different tissues, regardless of the gene's tissue-specific expression (see in particular the example of *AHRR* in WBC and lung). While the generality of such a phenomenon is currently unknown, a similarity across tissues may reflect the presence of common, locus-specific elements of chromatin architecture related to the operation of common gene regulatory networks across tissues that are more highly connected in functional terms than within-tissue transcriptional regulatory networks (56-58). Accordingly, we hypothesize that the pattern of DNA methylation changes following a particular exposure, and their persistence, extends to other tissues (especially in stem cells), including tissues that constitute targets for exposure-related disease.

The mechanisms linking DNA methylation and gene expression (e.g. demethylation in promoter CpGs being associated with increased transcription) involve many players in addition to the DNA methylation enzymes and methyl-CpG binding proteins, e.g. histone-modifying proteins, chromatin remodeling enzymes, other forms of methylation than just cytosine, and sequence-specific DNA binding-transcription factors. Thus, modulation of DNA methylation constitutes for the cell a means of producing heritable changes that can prime genes for transcription activation events that may not necessarily be initiated immediately, and which may be reversed if the need arises. An additional characteristic of DNA methylation is that its alteration at multiple CpG sites is usually necessary in order for change in expression to occur, thus providing cells with a means of modulating their levels of *readiness* for adapting gene expression. For example, one may speculate that the long persistence of the majority of the *AHRR*-associated CpGs modified by tobacco smoking (13 of a total of 20 tobacco-modified CpGs remained hypomethylated and 1 hypermethylated for up to 47 years after smoking cessation; ref. 36) may provide the cell with a mechanism of quickly re-activating epigenetic control of expression of this key regulatory gene if the need arises, by completing the methylation changes of the remaining CpGs. Therefore exposure-induced methylation changes can "*prepare the ground*" without automatically leading to significant changes in expression unless additional conditions are fulfilled, something that may occur in different tissues and at different times. When such conditions eventually arise in target tissues, in combination with the existing, altered methylation pattern, they then lead to significant changes in expression.

Taken together, the studies of the long-term epigenetic effects of exposure to environmental agents in early life, such as famine, micronutrient availability and chemical toxicants such as tobacco

smoke or Bisphenol A, suggest that self-renewing stem cell reservoirs in developing and adult tissues can acquire epigenetic memories that are adaptive, conferring additional elements of phenotypic plasticity to multipotent cells that enable beneficial responses to subsequent exposures. However, the broadening of phenotypic plasticity in stem cells by environmental exposures may also be maladaptive if subsequent exposures are encountered that increase cell vulnerability to disease-causing phenotypic changes or further destabilize stem cell identity in ways that extinguish self-renewal and/or multipotency. Conrad Waddington originally recognized that developmental processes are buffered against some environmentally-induced or stochastic phenotypic changes, through a genetically determined process he termed canalization (59). However, the phenotypic robustness produced by canalization can be compromised by mutations in developmental regulatory genes, some of which are now known to encode components of epigenetic mechanisms that regulate gene transcription. The chaperone protein Hsp90, for example, has been identified as a suppressor of phenotypic variation in many eukaryotes, through its regulatory interactions with a wide range of client proteins that are involved in the regulation of signaling pathway activities and epigenetic processes (60, 61). Nevertheless, even though canalization promotes robustness, many developmental and physiological processes still remain sensitive to environmental signals. A key challenge for future research is therefore to elucidate the processes that engender adaptive and maladaptive (disease-causing) epigenetic changes in response to environmental exposures. In particular, further studies are warranted of the roles of DNA methylation and other forms of chromatin modification in mediating the impacts of salient environmental stimuli, including chemical toxicants, on the maintenance, differentiation and oncogenic transformation of stem cells.

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Table 1: Significant ($p<0.05$) GO terms ("biological process") associated with 339 unique genes differentially methylated for >10 years post-smoking cessation (Chatziioannou and Kyrtopoulos, unpublished).

Rank	Term id	Term Definition	Enrichment	p-value	corr^d p
1	GO:0003417	growth plate cartilage development	4/8	3.6730e-5	0.0028
2	GO:0006928	movement of cell or subcellular component	10/87	0.0001	0.0041
3	GO:0043154	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	9/77	0.0003	0.0059
4	GO:0006414	translational elongation	10/101	0.0005	0.0086
5	GO:1902176	negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	4/15	0.0006	0.0110
6	GO:0042149	cellular response to glucose starvation	5/28	0.0009	0.0141
7	GO:0030168	platelet activation	15/212	0.0009	0.0147
8	GO:0043068	positive regulation of programmed cell death	3/8	0.0011	0.0156
9	GO:0045599	negative regulation of fat cell differentiation	6/43	0.0011	0.0202
10	GO:0007265	Ras protein signal transduction	8/76	0.0012	0.0205
11	GO:0060395	SMAD protein signal transduction	7/60	0.0013	0.0214
12	GO:2000379	positive regulation of reactive oxygen species metabolic process	5/32	0.0017	0.0224
13	GO:0060325	face morphogenesis	5/34	0.0023	0.0301
14	GO:0007346	regulation of mitotic cell cycle	7/71	0.0034	0.0304
15	GO:0060021	palate development	7/75	0.0046	0.0305
16	GO:0046685	response to arsenic-containing substance	3/13	0.0048	0.0334
17	GO:0010628	positive regulation of gene expression	16/280	0.0052	0.0350
18	GO:0007266	Rho protein signal transduction	6/59	0.0056	0.0360
19	GO:0043066	negative regulation of apoptotic process	25/527	0.0068	0.0404
20	GO:0006415	translational termination	7/88	0.0109	0.0415
21	GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	9/119	0.0058	0.0430
22	GO:0032147	activation of protein kinase activity	6/62	0.0072	0.0445
23	GO:0007369	Gastrulation	4/32	0.0112	0.0476
24	GO:0043408	regulation of MAPK cascade	5/50	0.0120	0.0492

Figure 2- Distribution of probe-specific methylation changes (number of perturbed probes, mainly hypomethylated, on vertical axis) according to time in years since smoking cessation (on horizontal axis). Stronger hypomethylation (vertical axis) is closer to smoking cessation. Increasing methylation levels approach those of never smokers several years after smoking cessation. After many years the number of signals levels-off, suggesting the existence of sites whose methylation status remains altered even more than 35 years after smoking cessation. From reference [36] with approval of the authors.

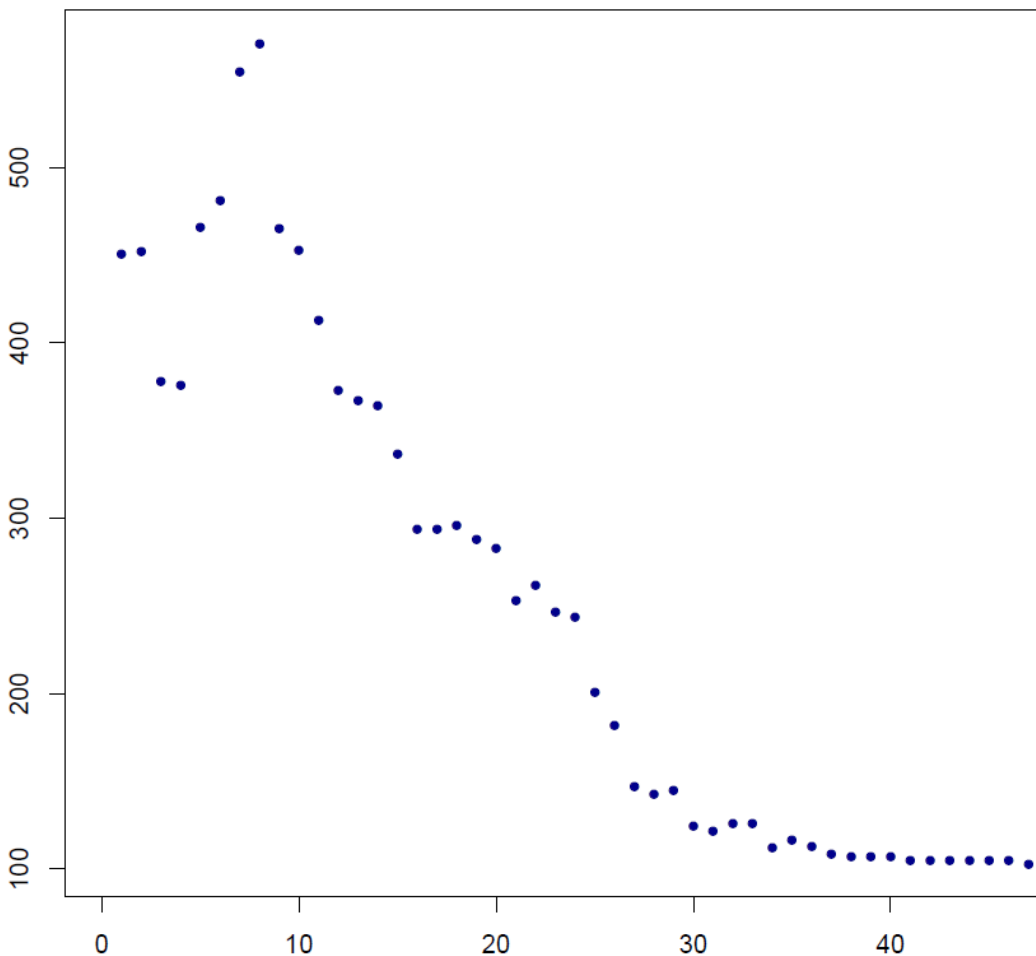

Figure 3 - Comparison of smoking effects on the methylation of 49 AHRR CpG sites in blood leukocytes and lung alveolar macrophages at which the effects of smoking are significant (FDR<0.05) in either tissue. From reference [46] with approval of the authors.