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Review Article

The Social Environment and the Epigenome

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The genome is programmed by the epigenome. Two of the fundamental components of the epigenome are chromatin structure and covalent modification of the DNA molecule itself by methylation. DNA methylation patterns are sculpted during development and it has been a long held belief that they remain stable after birth in somatic tissues. Recent data suggest that DNA methylation is dynamic later in life in postmitotic cells such as neurons and thus potentially responsive to different environmental stimuli throughout life. We hypothesize a mechanism linking the social environment early in life and long-term epigenetic programming of behavior and responsiveness to stress and health status later in life. We will also discuss the prospect that the epigenetic equilibrium remains responsive throughout life and that therefore environmental triggers could play a role in generating interindividual differences in human behavior later in life. We speculate that exposures to different environmental toxins alters long-established epigenetic programs in the brain as well as other tissues leading to late-onset disease. Environ. Mol. Mutagen. 49:46–60, 2008.

Key words: DNA methylation; demethylation; maternal care; gene environment interactions

INTRODUCTION

Biology in general has been genocentric for the last nine decades, nevertheless it is becoming clear that the fate of a gene is not defined by the DNA sequence per se but also by the manner by which the gene is marked and programmed by chromatin modification, DNA methylation, and noncoding RNA. Epigenetic programming of gene expression is stable and long-term but yet reversible and responsive. A change in gene programming by chromatin could have the same impact as a genetic polymorphism leading to either enhancing or silencing of expression of a gene. Thus, interindividual differences in epigenetic markings would result in interindividual phenotypic differences.

Most of the attention in the field of DNA methylation has focused on the normal processes sculpting the DNA methylation pattern during development [Razin and Shemer, 1995]. The common wisdom has been that once DNA methylation patterns were formed during development, they remained stable thereafter [Razin and Riggs, 1980]. This classic model predicted that any epigenetic variations would form exclusively during gestation but not later in life. An alternative hypothesis that is emerging recently suggests that it is plausible that DNA methylation might change later in life and thus provide a platform through which the environment could sculpt the genome and affect the phenotype throughout life. If such epigenetic changes in response to the environment occur in the germ cells they might be transmitted to future generations as well [Anway et al., 2005].

Recent data imply that environmental exposures might alter the epigenome after birth supporting the hypothesis that DNA methylation and chromatin modification machineries remain active and dynamic throughout life even in postmitotic cells [Meaney and Szyf, 2005]. Indeed, recent data suggest that the DNA methylation pattern in the hip-

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pocampus is responsive to environmental exposures even in the adult [Weaver et al., 2004, 2005] and that DNA methylation-demethylation events participate in learning processes in the hippocampus [Miller and Sweatt, 2007]. An intriguing prospect is that the response of the epigenome to environmental insults throughout life is not just an accidental aberration leading to pathology but a biological mechanism that serves as a medium for the adaptability of the genome to altered environments during life. If this is so, it implies that there are signaling pathways, which link extracellular environmental exposures and epigenetic machineries in mature somatic cells.

An important issue is defining the range of environmental exposures, which might affect the epigenome later in life. It is crucial to delineate the epigenetic changes associated with specific exposures and to determine whether they are limited to chemical exposures such as altered diets, drugs and toxins or whether the social environment affects the epigenome as well. The prospect that the social environment might sculpt our genome through modifying the epigenome is intriguing and might provide an explanation for the well-established relationship between socioeconomic status and physical health. Recent data suggest that social exposure early in life could alter epigenetic programming, which remains stable throughout life [Weaver et al., 2004]. A critical question is what are the signaling events linking the social and behavioral exposure and the state of chromatin and DNA modification? Recent data showing rapid methylation-demethylation events during the process of long term potentiation and fear conditioning in the mouse suggest a possible link between psychosocial conditions triggering in brain activity and the epigenome [Levenson et al., 2006; Miller and Sweatt, 2007]. Another intriguing prospect to consider is that altered epigenetic states are not just an effect of behavioral exposures but that they could also affect behavior. This suggests a loop through which social exposure affect epigenetic states. These epigenetic states in turn affect social-behavior as well as behavioral pathologies. Several chemical and environmental exposures might affect behavior and behavior pathology through epigenetic programming.

An important but unresolved question is whether there is a critical period for such exposures during embryogenesis and early after birth or whether exposures late in life might have an epigenetic impact as well.

In summary, epigenetics provides a mechanism to explain interindividual variations in human behavior and behavioral pathologies, and links social and chemical environmental exposures to behavioral and physiological outcomes.

CHROMATIN MODIFICATION

The epigenome consists of chromatin and its modifications as well as a covalent modification by methylation of cytosine rings found at the dinucleotide sequence CG [Razin, 1998]. The epigenome determines the accessibility of the transcription machinery, which transcribes the genes into messenger RNA. Inaccessible genes are therefore silent whereas accessible genes are transcribed. We therefore distinguish between open and closed configurations of chromatin [Groudine et al., 1983; Marks et al., 1985; Ramain et al., 1986; Grunstein, 1997; Varga-Weisz and Becker, 2006]. Recently another new level of epigenetic regulation by small noncoding RNAs termed microRNA has been discovered [Bergmann and Lane, 2003]. microRNAs regulate gene expression at different levels; silencing of chromatin, degradation of mRNA, and blocking translation . microRNAs were found to play an important role in cancer [Zhang et al., 2007] and could potentially play an important role in behavioral pathologies as well [Vo et al., 2005]. The basic building block of chromatin is the nucleosome, which is formed of an octamer of histone proteins. There are five basic forms of histone proteins termed H1, H2A, H2B, H3, and H4 [Finch et al., 1977] as well as other minor variants, which are involved in specific functions such as DNA repair or gene activation [Sarma and Reinberg, 2005]. An assortment of modifications of the histone N-terminal tails by methylation [Jenuwein, 2001], phosphorylation, acetylation [Wade et al., 1997], and ubiquitination [Shilatifard, 2006] are believed to form a histone code which defines the level of expression of genes [Jenuwein and Allis, 2001]. For example, one of the most consistent histone modifications which mark active promoters is acetylation of the N-terminal of H3-histones at the K9 residue [Perry and Chalkley, 1982; Lee et al., 1993]. In contrast, tri-methylation and di-methylation of the same K9 or the K27 residue signals promoter inactivity and inhibits acetylation. H3 K4 methylation on the other hand is characteristic of histones associated with active genes [Nakamura et al., 2002].

Histone modifications are catalyzed by histone modifying enzymes such as histone acetyltransferases (HAT), which acetylate histone tails and histone deacetylases (HDAC) that deacetylate histone tails [Kuo and Allis, 1998]. Another group of important enzymes are the histone methyltransferases (HMT) and the histone demethylases [Shi et al., 2004; Tsukada et al., 2006]. The balance of these activities determines the state of histone modification and thus the level of expression of the associated genes.

A critical point for understanding how extracellular signals triggered by the environment might affect the state of chromatin configuration of specific loci in the genome is the targeting of histone modifying enzymes to specific loci. Specific transcription factors and transcription repressors recruit histone-modifying enzymes to certain genes and thus define the gene-specific profile of histone modification [Jenuwein and Allis, 2001]. Signaling pathways, which are known to be triggered, by extracellular

signaling could activate these factors. A good example is the HAT CREB binding protein CBP [Ogryzko et al., 1996], which is activated in response to increased intracellular cAMP. We will discuss in this article one biological model illustrating how maternal care could trigger a signaling pathway, which results in chromatin reconfiguration [Meaney and Szyf, 2005].

DNA METHYLATION PATTERNS AND THEIR MAINTENANCE

DNA methylation is part of the covalent structure of the DNA [Razin and Riggs, 1980]. This differentiates it from chromatin, which is associated with DNA but is not part of the DNA molecule itself. DNA methylation is therefore an extremely stable epigenetic mark. This stability of the chemical bond between a methyl group and the cytosine ring has led to the supposition that this mark is irreversible in mature tissues. Thus, it was not considered plausible that the environment would influence gene methylation once the pattern was programmed during embryogenesis.

DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine onto the 5′ position of the cytosine ring residing in most cases at the dinucleotide sequence CG [Adams et al., 1975; Wu and Santi, 1985; Ho et al., 1991; Cheng et al., 1993]. What distinguishes DNA methylation in vertebrates is the fact that not all CGs are methylated, but there is a cell-specific pattern of distribution of methylation on CG dinucleotides [Razin and Szyf, 1984]. It was proposed that DNA methylation in critical regulatory regions marks genes for silencing. The DNA methylation pattern is sculpted during development by series of methylation events catalyzed by de novo DNMTs and demethylation events by demethylases [Razin and Kafri, 1994]. This pattern was believed to remain fixed thereafter since it was proposed that the enzymatic processes which are responsible for altering DNA methylation patterns de novo DNMTs and demethylases are inactive in mature somatic cells. Razin and his coworkers have proposed the model that the DNA methylation is copied faithfully in somatic cells since the maintenance DNMT1 which is present in somatic cells has a preference for a hemimethylated substrate [Razin and Riggs, 1980]. Since hemimethylated sites are generated during DNA replication when a nascent unmethylated C is synthesized across a methylated C in the template parental strand, the DNMT accurately copies the methylation pattern of the template strand [Gruenbaum et al., 1982]. This paradigm of a stable DNA methylation pattern formed in development and faithfully maintained thereafter by maintenance DNMT dominated our thinking for two decades. If this model is true then the only time where environmental interventions might change DNA methylation pattern is during the critical periods in embryogenesis and spermatogenesis [Anway et al., 2005] when these patterns are formed.

In support of this hypothesis three distinct phylogenic DNMTs have been identified in mammals. DNMT1 shows preference for hemimethylated DNA in vitro, which is consistent with its role as maintenance DNMT, whereas DNMT3a and DNMT3b methylate unmethylated and methylated DNA at an equal rate, which is consistent with a de novo DNMT role [Okano et al., 1998]. Several lines of evidence challenge this attractive and elegantly simple picture of inheritance of DNA methylation patterns. First, maintenance methylation of repetitive elements was shown to require the cooperation of the so called ''de novo'' methyltransferases [Liang et al., 2002]. Second, DNMT1 and DNMT3B were found in same complexes [Kim et al., 2002] in somatic cells which would be unexplained if the only methylation activity required in somatic cells is copying the DNA methylation pattern during replication. Why is there a need for a de novo DNMT in addition to maintenance DNMT? Moreover, the recruitment of DNMTs to specific gene targets suggests that our original picture of maintenance DNMT moving along with the replication fork [Gruenbaum et al., 1983; Leonhardt et al., 1992; Araujo et al., 1998] needs to be revisited. This challenge is further supported by recent data showing that a mutant DNMT1 defective in the replication fork targeting is still able to maintain most of the DNA methylation in the human genome [Spada et al., 2007]. Third, it is becoming clear that not only are DNMTs targeted to specific genes by sequence specific factors but they are also required to reside on these sequences to maintain their methylation state [Fuks et al., 2001; Di Croce et al., 2002; Brenner et al., 2005; Vire et al., 2006; Burgers et al., 2006]. The targeting of DNMTs suggests that maintenance methylation is not just automatic copying of a template pattern but it requires a positive identification of a specific sequence. If the only signal for methylation is the methylation state of the paternal strand, why do we need to target DNMTs to specific sequences? Why do we need to keep the DNMTs on the gene past the point of DNA replication?

The new data point to a model whereby DNA methylation patterns are actively maintained by DNMTs which are targeted to methylated sequences and that DNMT1 is required to reside there even after the replication fork has passed along. The factors, which target DNMT to specific genes, could serve as a conduit through which firing of extra and intracellular signaling pathways might be converted to specific methylation events in the genome. The fact that the presence of these factors is constitutively required suggests that DNA methylation is dynamic and that demethylation events would remove the methyl mark in the absence of DNMT. We will discuss below examples from recent studies of DNA methylation in the brain supporting the hypothesis that DNA methylation is main-

tained by loci-specific balance of methylation and demethylation in postmitotic tissues (Fig. 1).

SILENCING OF GENE EXPRESSION BY DNA METHYLATION RESULTS IN A PHENOTYPIC CHANGE IN ABSENCE OF A GENOTYPIC ALTERATION

DNA methylation in critical sites silences genes by two principal mechanisms as well as other variations of these mechanisms. First, methylation in critical sites inhibits the binding of transcription factors to their recognition elements [Comb and Goodman, 1990; Inamdar et al., 1991]. For example, the binding of MYC/MAX to their recognition element is inhibited by DNA methylation [Prendergast and Ziff, 1991]. Second, methylation of a regulatory region of DNA recruits methylated DNA binding proteins such as MeCP2 to the gene [Nan et al., 1997; Hendrich and Bird, 1998; Fujita et al., 1999; Ng et al., 1999]. These methylated-DNA binding proteins recruit chromatin modification enzymes such as HDACs and HMT SUV39, which in turn introduce silencing histone modifications resulting in silencing of chromatin. Silencing by DNA methylation is a stable change in gene expression programming. Thus, any random or programmed event of DNA methylation in critical sites in response to an environment insult or trigger might result in a change in phenotype similar to a mutation in the same sequence. For example, methylation of tumor suppressor genes silences them in a stable manner [Baylin et al., 2001], which is seemingly indistinguishable from a polymorphism or a deletion that totally ablates the gene.

There are two important differences between silencing by epigenetic and genetic mechanisms. First, a germ line mutation will result in the same genetic change in all tissues, whereas a somatic alteration in DNA methylation is usually cell specific. Second, silencing by methylation is reversible. Inhibitors of DNA methylation [Jones and Taylor, 1980; Cheng et al., 2003] and HDAC inhibitors could bring about demethylation and activation of genes silenced by DNA methylation. It is tempting to speculate that similar to the situation in cancer where somatic silencing of tumor suppressor genes is a result of an aberrant methylation event, silencing by methylation could affect behavior-regulatory genes in the brain. Methylation in the brain as well as other mature tissues might occur as a consequence of either pathological or adaptive physiological mechanisms.

We have now preliminary evidence that specific genes in the human hippocampus are more methylated in victims of suicide than in control subjects supporting the hypothesis that pathological somatic DNA methylation is not limited to cancer [MacGowan et al., unpublished data]. However, whereas there is general acceptance that de novo DNA methylation takes place in dividing cancer cells, there is yet general reluctance to accept that such mechanisms are at work in normal postmitotic somatic cells. We argue here based on recent data that somatic changes in DNA methylation are not limited to cancer and that they could potentially come about also in mature neurons.

One line of evidence supporting the concept that there is a lifelong drift in DNA methylation in normal somatic tissue comes from studies of hypermethylation events in aging tissues. It is tempting to speculate that lifelong environmental exposures lead to the hypermethylation observed in aging tissue [Ahuja et al., 1998; Issa, 2000]. Similarly, a recent study of monozygotic twins has revealed that a difference in DNA methylation emerges later in life suggesting an environmental rather than a genetic basis for the lifelong DNA methylation drift [Fraga et al., 2005].

In summary, we propose that DNA methylation is dynamic in postmitotic tissues and that the DNA methylation dynamic equilibrium is not just limited to cancer cells (Fig. 1). It is possible that the dynamic equilibrium is altered by either pathological or adaptive mechanisms in response to extra and intracellular signaling. This will lead to a change in either gene silencing or activation.

REVERSIBILITY OF DNA METHYLATION IN SOMATIC TISSUES

There is general agreement that during development both de novo methylation and demethylation events shape and sculpt the mature cell-specific DNA methylation pattern [Razin et al., 1984; Frank et al., 1990, 1991; Brandeis et al., 1993; Kafri et al., 1993; Razin and Shemer, 1995]. Active demethylation was reported for genomic DNA upon induction of Epstein Barr virus lytic cycle [Szyf et al., 1985] for the myosin gene in differentiating myoblast cells [Lucarelli et al., 2001], for Il2 gene upon T cell activation [Bruniquel and Schwartz, 2003] and the *INTERFERON GAMMA* gene upon antigen exposure of memory CD8 T cells [Kersh et al., 2006]. However, if DNA methylation is plastic in mature postmitotic tissue and responsive to the environment throughout the life of an organism, mechanisms must exist to enable both introduction of new DNA methylation sites by de novo methylation and mechanisms for removal of DNA methylation by demethylation. This has been a contentious and controversial issue that has not been resolved yet. We have previously proposed that DNA methylation is a reversible signal and isolated a demethylation activity from lung cancer cells [Ramchandani et al., 1999]. We later proposed that the METHYLATED DOMAIN DNA BINDING PROTEIN 2 (MBD2) bears a demethylation activity [Bhattacharya et al., 1999]. However, other groups disputed this finding [Ng et al., 1999]. Later data from our laboratory further supported the demethylation activity of MBD2 [Detich et al., 2002, 2003a,2003a]. A very recent

publication has used the assay described by Ramchanadni et al., to measure demethylase activity in white matter from multiple sclerosis patients [Mastronardi et al., 2007] and demethylase activity was also assayed recently in the nuclear extracts of chicken erythroid cells [Ramachandran et al., 2007]. Recent data support the view that DNA methylation is dynamic in postmitotic tissues in the brain [Weaver et al., 2004, 2005; Miller and Sweatt, 2007] and in neurons in vitro [Levenson et al., 2006].

There has been reluctance to accept the idea that an enzymatic activity removes methyl groups directly from the cytosine ring [Wolffe et al., 1999]. It is interesting to note that a similar reluctance has been directed at the notion that histone methylation is reversible. However, the discovery of histone demethylases in recent years has proven this concern to be ill founded [Shi et al., 2004]. A number of indirect mechanisms for demethylation have been therefore proposed which do not require direct removal of the methyl bond. These mechanisms involve a repair mechanism which removes either the 5mC base (glycosylase) [Jost, 1993; Zhu et al., 2000b] or the 5mCp nucleotide (nucleotide excision and patch repair) [Barreto et al., 2007] followed by passive demethylation through incorporation of a new unmethylated cytosine or a patch of nucleotides in the repair process in the absence of DNA methylation.

In summary, we propose here that demethylase activity is not limited to particular points during embryogenesis but is part and parcel of the DNA methylation equilibrium in many or all cell types throughout life (Fig. 1). We propose that DNA demethylase contributes to maintenance of the DNA methylation equilibrium in postmitotic cells. Although we have no evidence for a dynamic DNA methylation pattern in other cell types, there is evidence that DNA methylation pattern is dynamic in neurons [Weaver et al., 2004, 2005; Miller and Sweatt, 2007]. We propose that targeting plays an important role in demethylase action as it does in DNMT action as discussed in the previous section. One of the most critical challenges is to identify the demethylases involved in this equilibrium and unravel the signaling pathways that deliver them to specific genes.

THE RELATIONSHIP BETWEEN CHROMATIN AND DNA METHYLATION

The tight correlation between DNA and chromatin structure was reported almost three decades ago by Razin and Cedar [1977]. It was believed for a long time that this is a unidirectional relationship. That is, the state of DNA methylation defines chromatin structure; methylated DNA precipitates a closed chromatin configuration while unmethylated DNA maintains chromatin in an open configuration. As discussed above, this hypothesis was supported by the discovery of methylated DNA binding proteins that recruit chromatin modification enzymes to methylated genes such as MeCP2 [Meehan et al., 1992; Nan et al., 1997]. However, it is becoming clear that the relationship between chromatin configuration and DNA methylation is bidirectional. There is accumulating evidence showing that changes in chromatin structure would alter DNA methylation patterns. Moreover, the targeting of DNA methylation enzymes to genes is guided by chromatin modifying enzymes. Since it has been known for some time that chromatin configuration is dynamic and responsive to cellular signaling pathways, this relationship provides a link between the extracellular environment and the state of DNA methylation. That is signaling pathways, which activate chromatin-modifying enzymes could potentially result in altering DNA methylation patterns.

There is genetic and epigenetic data linking chromatin modeling and modifying enzymes to DNA methylation. In humans and mice mutations in the SWI-SNF proteins, which are involved in chromatin remodeling, result in defects in DNA methylation. A growing list of histone modifying enzymes interact with DNMT1, such as HDAC1 and HDAC2, the histone methyltransferases SUV3-9 and EZH2, a member of the multiprotein Polycomb complex PRC2, which methylates H3 histone at the K27 residue [Fuks et al., 2000, 2003b; Rountree et al., 2000; Vire et al., 2006]. DNMT3a was recently also shown to interact with EZH2 which targets the DNA methylation-histone modification multiprotein complexes to specific sequences in DNA [Vire et al., 2006].

An exciting recent development in understanding how DNA methylation is targeted to specific tumor suppressor genes in cancer is the discovery that sites that are regionally hypermethylated in cancer are also targets of the histone methyltransferase EZH2 [Vire et al., 2006; Schlesinger et al., 2007]. Interestingly, a very recent article links oncoproteins such as PML-RAR to EZH2 in targeting DNMTs to specific promoters [Marker, 2007]. Taken together this data support a mechanism whereby sequence specific factors such as certain oncogenes target chromatin-modifying enzymes to specific loci and they in turn recruit DNMTs to methylate these loci and stably silence the associated genes by regional DNA hypermethylation. We propose that if a sequence specific factor, which targets DNMT is inactivated, then DNMT is removed from the gene, the DNA methylation equilibrium is tilted toward DNA demethylation and the gene is demethylated by demethylases. Thus, the pattern of methylation is maintained by the constitutive presence of these sequence selective factors on the target genes. Some of these factors might be responsive to intracellular signaling pathways (Fig. 1).

Similar to DNA methylation, demethylation is targeted by transacting factors to specific genes. For example, the transcription factor NF-kappa B targets demethylation activity to the *Immunoglobulin kappa* chain enhancer [Kirillov et al., 1996]. It stands to reason that NF-kappa B targets chromatin-modifying enzymes which lead to an active chromatin configuration.

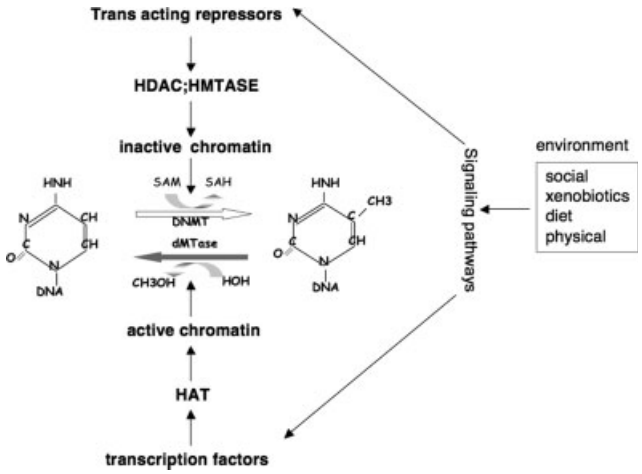

Fig. 1. The dynamic and responsive DNA methylation pattern; a model. A balance of methylation and demethylation reactions determines the DNA methylation state. Active chromatin facilitates DNA demethylation while inactive chromatin facilitates methylation. Different environmental signals trigger pathways in the cell that activate sequence specific factors which recruit chromatin modifying enzymes to specific loci resulting in either activation or inactivation of chromatin.

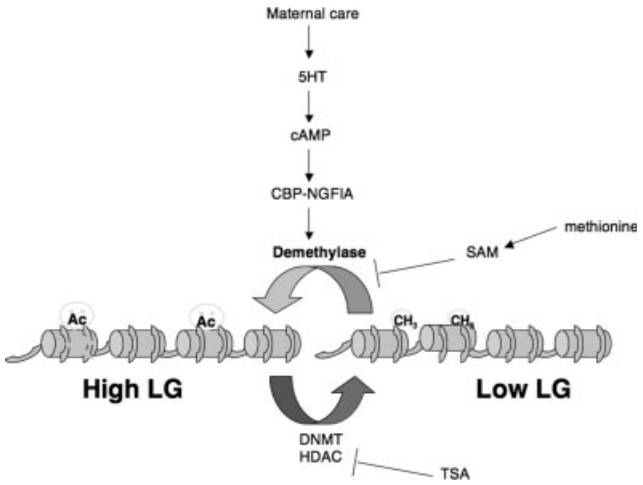

Fig. 2. Epigenetic reprogramming by maternal care; a model. Maternal licking and grooming in the rat triggers activation of 5HT receptor in the hippocampus leading to increase in intracellular cAMP, activation of the transcription factor NGFIA and recruitment of the HAT CBP to the GR exon 1_7 promoter. Acetylation of histone tails facilitates demethylation. In offspring of Low licking and grooming mothers this process is reduced in comparison with offspring of High licking and grooming mothers leading to differential epigenetic programming of the GR promoter. In the adult rat the epigenetic state is reversible. TSA a HDAC inhibitor increases histone acetylation and facilitates demethylation and epigenetic activation of the gene in the offspring of the Low licking and grooming mothers. Conversely, injection of methionine to adult offspring of the High licking and grooming mothers leads to increased SAM, inhibition of demethylation, increased DNA methylation, and reduced activity of the GR exon 1_7 promoter gene.

Indeed, in contrast to DNMTs, which are recruited by chromatin silencing enzymes such as SUV39 [Fuks et al., 2003a] and EZH2 [Vire et al., 2006; 2007], demethylation is facilitated by histone acetylation [Cervoni and Szyf, 2001; Cervoni et al., 2002]. Pharmacological acetylation using HDAC inhibitors such as TSA [Cervoni and Szyf, 2001] or valproic acid [Detich et al., 2003a] trigger replication-independent active demethylation of transiently transfected in vitro methylated plasmids and causes genomic demethylation [Milutinovic et al., 2007; Ou et al., 2007]. Interestingly TSA facilitates DNA demethylation in adult hippocampal neurons as well [Weaver et al., 2004], suggesting that the activity of TSA is not limited to cells in culture or to cycling cells. The pharmacological data with HDAC inhibitors might explain why certain transcription factors target DNA demethylation to specific genes. Several transcription factors recruit HATs to genes and their mode of action is similar to TSA. By increasing histone acetylation these factors facilitate the access of demethylation activities to their target genes.

We propose that demethylation activity targeted by sequence specific factors to genes is constitutively present in cells including postmitotic neurons. The demethylation activity is counterbalanced by the DNMTs recruited by repressing factors such as PML/RAR and EZH2. The maintenance of DNA methylation patterns is dependent on the preservation of the balance of these factors. Extra or intracellular signaling pathways could trigger activation of one of these factors result in loci specific histone acetylation and tilt the balance toward demethylation. An interesting example is the ubiquitous transcription factor CREB binding protein (CBP), which is activated by increased cAMP in the cell and is recruited to specific loci by different transcription factors such as CREB and AP-2 [Purucker et al., 1990; Uchida et al., 2002; Braganca et al., 2003]. CBP is also an acetyl transferase, which acetylates histones [Ogryzko et al., 1996]. We will discuss below the possible involvement of CBP in mediating demethylation in response to maternal care [Weaver et al., 2007]. Such a mechanism provides a conduit through which both the chemical and the social environment could affect our epigenome and thus gene expression and function (Fig. 1). It is tempting to speculate that this reversible DNA methylation pattern plays a physiological role in mediating adaptive responses to changing environments as well as mediating methylation changes with pathological consequences. Future studies are required to analyze the components of this machinery in somatic cells and to test the hypothesis that it remains active throughout life in some or all cell types.

HOW COULD BEHAVIOR MODULATE DNA METHYLATION? THE DYNAMIC PATTERN OF METHYLATION IN NEURONS

It stands to reason that certain chemicals would interfere with DNA methylation enzymes and thus result in an

alteration in DNA methylation. It is also widely accepted that chemicals as well as altered dietary intake would affect DNA methylation during gestation [Simmons, 2007] especially during gametogenesis [Anway et al., 2005] at a point when methylation machineries are highly active and cells are undergoing rapid cell division. It is difficult to accept however that environmental agents could affect DNA methylation patterns throughout life well after tissues and organs are formed and their methylation pattern is established. The model proposed here offers a possible mechanism for alterations in methylation in adult tissue by proposing that the DNA methylation machinery remains active throughout life, and thus sensitive to xenobiotics. An even more provocative idea is that the social environment could influence the physical state of modification of the genome. Future experiments are required to fully support the hypothesis presented here that the DNA methylation pattern is dynamic throughout life and responsive to both the chemical and even the social environment. Nevertheless, two relatively recent lines of data provide some support for this hypothesis.

One line of evidence comes from studies of epigenetic changes during long-term potentiation and fear conditioning by Sweat's group [Levenson et al., 2006; Miller and Sweatt, 2007] and the other line of evidence is our study of epigenetic programming by maternal care [Meaney and Szyf, 2005].

EPIGENETIC PROGRAMMING BY MATERNAL CARE

Epigenetic programming by maternal care is an example of how epigenetic programming of the offspring is triggered by maternal behavior, or how the epigenetic program of one subject is affected by the behavior of another subject. Epigenetic markings of DNA methylation, histone acetylation, and transcription factor occupancy bear the memory of maternal behavior. This programming by maternal behavior is stable and long lasting, but nevertheless is reversible by agents that interfere with either the methylation (methionine) or histone deacetylation machinery (TSA)[Weaver et al., 2004, 2005]. Thus, the maternal care model typifies the first principles of epigenetic programming which are stability and relative plasticity.

In the rat, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (i.e., High LG mothers) over the first week of life show increased hippocampal GR expression, enhanced glucocorticoid feedback sensitivity, decreased hypothalamic corticotrophin releasing factor (CRF) expression, and more modest HPA stress responses compared to animals reared by Low LG mothers [Liu et al., 1997; Francis et al., 1999]. Cross-fostering studies suggest direct effects of maternal care on both gene expression and stress responses [Liu et al., 1997; Francis et al., 1999]. These studies supported an epigenetic mechanism since the fostering mother and not the biological genetic mother defined the stress response of its adult offspring. We have demonstrated that the GR exon 1_7 promoter is programmed differently in the hippocampus of offspring of the High and Low LG maternal care and that differences which emerges between day 1 and 8 after birth remains stable thereafter. These differences include histone acetylation, DNA methylation, and the occupancy of the promoter with the transcription factor NGFI-A [Weaver et al., 2004]. A comprehensive analysis of the hippocampus transcriptome of the adult offspring of High and Low LG maternal care revealed differences in a few hundred genes. This suggests a wide change in epigenetic programming in the brain of the offspring, which was a consequence of maternal care. We are now applying whole epigenome methods to obtain a comprehensive map of these epigenetic changes.

EPIGENETIC PROGRAMMING IN THE HIPPOCAMPUS BY MATERNAL CARE EARLY IN LIFE IS REVERSIBLE LATER IN THE ADULT

These experiments raised a number of basic questions. The first question is whether this epigenetic programming by maternal care is reversible? The classic concept of epigenetic programming in early development viewed developmental epigenetic programming as fixed since it was not believed that the enzymatic machineries required to generate new methylation pattern would be present in adult tissue, particularly it was not believed that this machinery was required in non dividing cells as discussed above. We therefore addressed the question of whether the epigenetic programming early in life could be modulated during adulthood.

We injected the HDAC inhibitor TSA [Yoshida et al., 1990] into the brain. TSA is not a demethylating agent, nor is it an histone acetylating agent. TSA would induce an epigenetic change only if the machineries required for the modulation of chromatin and DNA methylation were found in neurons and associated with the GR exon 1_7 promoter and only if the epigenetic state was an equilibrium of modifying and demodifying enzymes. TSA induces replication-independent demethylation in cell culture [Cervoni and Szyf, 2001]. TSA induces histone acetylation by inhibiting HDACs and thus tilting the histone acetylation equilibrium toward acetylation. We proposed that this open chromatin structure induced by hyperacetylation facilitated the interaction of demethylases with methylated DNA and thus tilted the DNA methylation equilibrium toward demethylation [Cervoni and Szyf, 2001]. TSA injected into brains of adult offspring of Low LG maternal care increased acetylation, reduced methylation, activated GR exon 1_7 promoter to levels indistinguishable from adult offspring of High-LG maternal care and

reduced stress responsivity to the levels of offspring of High-LG [Weaver et al., 2004].

We similarly reasoned that if the DNA methylation and chromatin state is in a dynamic equilibrium even in adult neurons, it should be possible to revert the epigenetic programming in the other direction toward increased methylation. We therefore injected methionine, the precursor of SAM the methyl donor of DNA methylation reactions, into the brain of the adult offspring of different maternal care mothers. SAM was shown to inhibit active demethylation [Detich et al., 2003b] and to stimulate methylation [Pascale et al., 1991]. Methionine treatment was previously shown to increase SAM and DNA methylation levels in the brain [Tremolizzo et al., 2002; Grayson et al., 2005]. Methionine treatment of the offspring of High LG maternal care changed the DNA methylation state of GR exon 1_7 promoter and expression of GR in the hippocampus as well as increased their stress responsiveness and reduced the time that these animals spent in the open field.[Weaver et al., 2005, 2006]. Methionine does not methylate DNA, DNMTs do. The DNMTs need to be poised to methylate GR exon 1_7 promoter. Taken together, the TSA and methionine experiments support the basic hypothesis proposed in this article that epigenetic programs in the brain are maintained by a dynamic equilibrium of methylating and demethylating enzymes, a balance which could be shifted by agents which either inhibit demethylases or stimulate DNMTs. Thus, despite the remarkable stability of epigenetic programs they are nevertheless reversible (Fig. 2).

MECHANISM LINKING MATERNAL CARE AND EPIGENETIC REPROGRAMMING

Although the idea that a behavioral exposure might result in a physical change to chromatin or DNA of specific loci seems far-fetched at first glance, basic concepts of how epigenetic programming event are targeted to specific loci point toward a possible working hypothesis. The basic idea being that behavioral exposures fire signaling pathways in the brain which in turn activate sequence specific factors that target HATs to specific targets facilitating DNA demethylation. We have started to decipher the molecular events which link maternal licking and grooming and epigenetic changes at the GR gene -locus. In vivo and in vitro studies suggest that maternal LG or postnatal handling, which increases maternal LG, increases GR gene expression in the offspring through a thyroid hormone-dependent increase in serotonin (5-HT) activity at 5-HT_7 receptors, and the subsequent activation of cyclic adenosine 3′, 5′ monophosphate (cAMP) and cAMP-dependent protein kinase A (PKA) [Meaney et al., 1987, 2000; Laplante et al., 2002]. Both the in vitro effects of 5-HT and the in vivo effects of maternal behavior on GR mRNA expression are accompanied by increased hippocampal expression of NGFI-A transcription factor. The GR exon 1_7 promoter region contains a binding site for NGFI-A [McCormick et al., 2000]. Interestingly, NGFI-A was previously shown to regulate transcription of the transcriptional coactivator and histone acetyl transferase CREB binding protein CBP by both repression and activation under different cellular challenges [Yu et al., 2004]. Signaling pathways that result in increased cAMP also activate CBP [Chawla et al., 1998]. NGFI-A and CBP are recruited to the GR exon 1_7 promoter in response to maternal care which explains the increased acetylation and demethylation observed in offspring of high LG-ABN [Weaver et al., 2007]. Tissue culture experiments demonstrated that recruitment of NGFI-A to the GR exon 1_7 promoter resulted in replication-independent DNA demethylation. The recruitment of NGFI-A to the promoter facilitates the interaction of MBD2, a protein proposed to be involved in replication-independent DNA demethylation, with the promoter [Weaver et al., 2007]. Further experiments are required, including specific knock down of NGFI-A CBP and MBD2 in vivo to fully demonstrate the pathway linking exposure to maternal-care and demethylation of specific loci. Nevertheless, these experiments chart a feasible route leading from a behavioral exposure to a chemical change in chromatin (Fig. 2).

EPIGENETIC DYNAMISM AND ITS IMPLICATIONS ON HUMAN PHYSIOLOGY AND PATHOLOGY

The maternal care study raises a number of nodal questions. First, a critical question is whether the epigenetic-reversibility potential is part and parcel of the normal physiological homeostasis and response to the environment in the brain and other tissues throughout life, or whether this could only come about by harsh interventions such as pharmacological manipulation of epigenetic proteins. Are such responses adaptive enabling us to respond to our changing environment? A related question is whether such epigenetic readjustments later in life might result in late-onset pathologies? The maternal care model illustrates that significant epigenetic programming arises after embryogenesis is completed and that it could be triggered by social cues? Do social and behavioral experiences affect epigenetic programming throughout life or are they limited to critical points perinatally? Can behavioral exposures at different points in life trigger changes in physiological homeostasis that might contribute to late-onset pathologies? Can behavioral-mediated epigenetic reprogramming alter responses to xenobiotics and environmental toxins? Can behavioral-mediated epigenetic reprogramming affect methylation patterns in the germ line and be transmitted transgenerationally? Do environmentally driven epigenetic variations play a role in shaping societal change and in formation of social behavior or culture?

Although we cannot answer these questions at this stage, it is self evident that they have broad ranging implications on our understanding of social, physiological and pathological processes and their interrelationships. The cardinal question is whether the well documented plasticity of the epigenome during early development and gametogenesis extends beyond the period in life when epigenetic patterns are laid down.

EVIDENCE THAT THE DYNAMIC DNA METHYLATION PATTERN PARTICIPATES IN PHYSIOLOGICAL NEURAL FUNCTION IN THE BRAIN

Perhaps the best support for the idea that a dynamic DNA methylation and chromatin configuration plays role in normal physiology derives from recent experiment by Sweatt's group on long-term potentiation in hippocampal slices in vitro and fear conditioning in vivo. In the first set of experiments they have shown that treatment of nondividing neurons in hippocampal slices in culture with a protein kinase c activator phorbol-12,13-diacetate results in rapid increase in histone acetylation and demethylation of the *reelin* gene, a gene implicated in synaptic plasticity and learning [Levenson et al., 2006]. This is evidence that active demethylation activity is present in postmitotic neurons. These data are also consistent with the idea that increased acetylation triggers replication-independent demethylation [Cervoni and Szyf, 2001]. The PKC signaling cascade is involved in induction of synaptic plasticity and long-term memory formation.

The fact that the gene-locus specific demethylation was induced by activation of a signaling pathway PKC further supports the hypothesis presented here that signaling pathways link between the behavioral signal and the chemical modification of DNA. Protein kinase C is known to activate a number of transcription factors including the transcription factors which interact with AP-1 such as c- JUN [Boyle et al., 1991]. It will be interesting to examine whether some of these transcription factors lead to site-specific demethylation by the mechanisms proposed here for NGFI-A.

The second experiment was done in vivo using a contextual fear conditioning model revealed a concurrent bidirectional change in methylation, which accompanied contextual fear conditioning. Reelin was demethylated and its transcription increased, while the memory suppressor gene, protein phosphatase1 (PP1) was de novo methylated and its transcription was inhibited [Miller and Sweatt, 2007]. What is remarkable about this result is that it all happened within 1 hr after induction of fear conditioning suggesting that acquisition of memory in the adult animal involves a dynamic rapid change in DNA methylation. This experiment also illustrates that both methylation and demethylation activities are present in adult neurons on several genes and that physiological signals could tilt the balance in either direction. The fact that DNA methylation could change in such a rapid speed in a nondividing cell supports the idea that DNA methylation could serve as a physiological signal in addition to its role as a developmental signal as has been previously proposed [Ramchandani et al., 1999]. Dynamic DNA methylation changes might play a role in synaptogenesis by rapidly turning on and off specific genes involved in this process. Thus, DNA methylation might be involved in the dynamic sculpting of the genome in response to learning and in storing the information in the form of epigenetic memory.

It is interesting to note that both de novo methylation and demethylation of different genes took place simultaneously. These concurrent opposite effects could not be mediated by a global change in methylation enzymes although the overall levels of DNMT3A and DNMT3b transcripts increased during fear conditioning [Miller and Sweatt, 2007]. Any global change, such as an increase in the concentration of methylation enzymes, should have led to a concurrent effect in the same direction in both genes. The concurrent opposite effects observed in this experiment could be simply explained however by the concept of ''targeting'' of DNA methylation enzymes which was discussed in this article. If the signal transduction pathway launched by the memory acquisition process activated simultaneously two kinds of factors with different sequence specificities and different chromatin modification partners, then the epigenetic effects would be dissimilar in distinct genes.

This model offers great opportunity to dissect the molecular chain linking from exposure to contextual fear down to specific changes in DNA methylation. One approach will be to identify the trans-acting factors, which reside downstream to PKC and target *reelin* and *PPI* similar to the study described above for the maternal care model.

In summary, this study provides clear evidence that the DNA methylation pattern is dynamic in the brain in a normal physiological context. It remains to be seen whether this is true for other adult tissues as well. This dynamic plasticity of the DNA methylation in adult tissue provides a template for different environmental exposures to act upon and to affect the phenotype. Epigenetic alterations brought about by a response to the environment could potentially result in either adaptive or pathological consequences.

EPIGENETIC PLASTICITY AND LATE ONSET PATHOLOGY

The dynamic plasticity of the DNA methylation patterns revealed by these studies and its responsiveness to both chemical and behavioral environment raises the possibility that errors in DNA methylation might emerge during adulthood and lead to changes in gene expression and

emergence of late onset pathologies [Feinberg, 2007]. Perhaps the best example of a disease where aberrant methylation is involved in pathology is cancer where both gain of methyl groups and loss of methyl groups occur concurrently in the same tumor [Baylin et al., 2001]. Although the phenomenon of aberrant methylation in cancer is extremely common, we still do not know what triggers these changes. Whereas the relationship between activation of certain oncogenic pathways by genetic mutations and aberrant methylation is partly understood, we do not know whether certain environmental exposures could lead to the same results. For example, activation of RAS [MacLeod et al., 1995], down regulation of Rb [Slack et al., 1999], or upregulation of the Wnt signaling pathway [Campbell and Szyf, 2003] leads to increased expression of DNMT1. Certain oncogenes could target DNMT to tumor suppressor genes and thus bring about regional hypermethylation [Fuks et al., 2001; Di Croce et al., 2002; Brenner et al., 2005; Vire et al., 2006]. An interesting possibility is that aberrant DNA methylation of cancer related genes could also come about through activation of signaling pathway by different environmental exposures, which might also include social exposures. There are no data to support such a hypothesis but we cannot afford to ignore it. A link between normal variations in social and environmental exposures and epigenetic modifications of cancer related genes is an intriguing area that should be explored in the future. A model linking environmental stress and chromatin reprogramming through environmental modulation of HSP90 has been proposed [Ruden et al., 2005].

Genetic defects in genes encoding the DNA methylation and chromatin machinery exhibit profound effects on mental health. A classic example is RETT syndrome, a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in females which is caused by mutations in the methylated DNA binding protein MeCP2 [Amir et al., 1999]. Mutations in MeCP2 and reduced MeCp2 expression were also associated with autism [Nagarajan et al., 2006; Ben Zeev Ghidoni, 2007; Herman et al., 2007; Lasalle, 2007]. ATRX a severe, X-linked form of syndromal mental retardation associated with alpha thalassaemia (ATR-X syndrome) is caused by a mutation in a gene which encodes a member of the SNF2 subgroup of a superfamily of proteins with similar ATPase and helicase domains which are involved in chromatin remodeling [Picketts et al., 1996]. The ATRX mutation is associated with DNA methylation aberrations [Gibbons et al., 2000]. Although these genetic lesions in the methylation machinery were present through development and are thus fundamentally different from methylation changes after birth, these data nevertheless support the hypothesis that DNA methylation defects could lead to mental pathologies as well. Thus, it is possible that environmental exposures which would affect the activity of the methylation machinery would also lead to behavioral and mental pathologies.

There are some data indicating aberrant methylation in late onset mental pathologies although it is unclear whether these changes in DNA methylation originated during embryogenesis or later in life as a response to an environmental exposure. The gene encoding *REELIN* a protein involved in neuronal development and synaptogenesis, which is implicated in long-term memory was found to be methylated in brains of schizophrenia patients. The methylation of *REELIN* was correlated with its reduced expression and increased DNMT1 expression in GABAergic neurons in the prefrontal cortex [Chen et al., 2002; Costa et al., 2002, 2003; Grayson et al., 2005; Veldic et al., 2007].

Our preliminary data exhibit hypermethylation of the GR exon 1_f promoter and its reduced expression in hippocampi of subjects who committed suicide relative to age matched control subjects [McGowan et al., unpublished data]. The central position of GR in control of the HPA axis might suggest involvement of GR exon 1_7 promoter through the HPA axis in mediating the conditions leading to suicide. Our data suggest that this reduction in GR promoter function is epigenetically controlled.

The promoters of the genes encoding rRNA were found to be heavily methylated in hippocampi from subjects who committed suicides relative to controls [McGowan et al., unpublished data]. Methylation of rRNA defines the fraction of rRNA molecules which are active in a cell, the output of rRNA transcription defines to a large extent the protein synthesis capacity of a cell [Brown and Szyf, 2007]. Protein synthesis is critical for learning and memory. Thus, it is tempting to speculate that there is a reduced capacity for protein synthesis required for learning and memory in brains of suicide victims, which is epigenetically determined. This might be involved in the pathology leading to suicide. Thus, evidence is emerging that similar to cancer aberrant DNA methylation is involved in psychopathologies. However, it was unclear whether the epigenetic aberrations documented in brain pathologies were present in the germ line, whether they were introduced during embryogenesis or whether they were truly late onset changes.

The possible role of DNA methylation aberration in other late onset diseases such as asthma, stroke, type II diabetes, and osteoarthritis has been speculated, however there is very limited data to support the hypothesis as of yet. However some interesting observations were reported in literature. In systemic lupus erythematosus (SLE) there is Global DNA hypomethylation in CD4+ T cells which was proposed to be associated with its pathogenesis [Richardson, 2002]. It was recently shown that the methylated DNA binding protein MBD2 which we implicated in demethylation and in driving metastasis [Bhattacharya et al., 1999; Detich et al., 2002; Szyf, 2003; Campbell

et al., 2004] and MBD4 a glycosylase which was also implicated in demethylation [Zhu et al., 2000a] are elevated in T cells from lupus patients [Balada et al., 2007]. Thus, DNA hypomethylation, which is hallmark of cancer, is not unique to this disease and might characterize several other late onset diseases. It is tempting to speculate that global hypomethylation results in induction of expression of disease related genes [Cornacchia et al., 1988; Sawalha and Jeffries, 2007]. It is interesting to note that drugs and nongenotoxic agents, which induce global hypomethylation such as hydralazine and procainamide, induce lupus like symptoms [Cornacchia et al., 1988]. This observation provides an example of how xenobiotics, which are commonly prescribed could bring about global hypomethylation and precipitate a late-onset disease. In this respect it is also interesting to note that there is small concordance rate in monozygotic twins with SLE suggesting a strong effect of the environment [Huang et al., 1997]. Thus, it is tempting to speculate that different environmental exposures could trigger lupus through either stimulating demethylation or inhibiting methylation.

If indeed global hypomethylation plays a role in several late onset diseases, it will be extremely important to develop screens to identify different environmental pollutants, pharmaceuticals, and xenobiotics which induce global hypomethylation. One question that needs to be answered is how does the same process of global hypomethylation result in different disease states? Is there disease related specificity or tissue specificity to different global demethylating agents? Is the effect stochastic, and would we observe different gene selectivity in different tissues? Do agents, which act on different components of the chromatin modification and DNA methylation machineries, trigger different pathological consequences?

The cardinal role of hypomethylation in metastasis [Szyf et al., 2004a,2004b; Szyf, 2005; Shukeir et al., 2006] and autoimmune disease and the prospect that global hypomethylation might trigger other late onset disease are compelling reasons why we could not ignore the possibility that several or environmental exposures of both behavioral and chemical nature might drive global hypomethylation. It is therefore critical to understand the cellular pathways which respond to these exposures and trigger global hypomethylation.

SUMMARY AND PROSPECTIVE

The realization that the genome is programmed by the epigenome and that this programming might be as important as the sequence itself in executing genome functionality offers a new approach to the long-standing mystery of gene-environment interactions. This understanding is of utmost important to environmental toxicology. While genotoxicity has been the main focus of attention for those attempting to understand the impact of environmental hazards on the genome for the last few decades, it is becoming clear that epigenetic mechanisms should be now considered. Epigenetic aberrations might have similar consequences to genetic damage as far as gene expression and the resulting phenotype are considered. Epigenetic marks though potentially reversible are stable and could be long lasting. The emerging understanding that late onset diseases such as cancer might have an epigenetic origin points to the importance of developing screens to identify epigenetic environmental hazards. Perhaps one of the finest examples of how the epigenome mediates the effects of the environment on our genome comes from studies of endocrine disruptors [for a review see Jirtle and Skinner, 2007]. Endocrine disruptors cause epigenetic changes by DNA methylation, which are heritable in rodents and can promote disease across subsequent generations. [Anway et al., 2005]. These observations put forward the thought-provoking notion that environmental exposures in one generation could have an impact on phenotype and disease susceptibility on generations to come. Interestingly, exposure to endocrine disruptors affect female mate preference in rodents three generation removed from the exposure, raising the possibility that epigenetics is a yet unappreciated force in evolution [Crews et al., 2007]. If environmental exposures could alter the epigenetic information in a heritable fashion then it could serve as a mechanism for environmental directed evolution.

New data from behavioral studies is shedding new light on the relationship between the social environment and epigenetic programming. It has also illustrated the potential lifelong dynamic nature of the epigenome. The relationship between behavior and the epigenome is bilateral, behavior could result in epigenetic programming and epigenetic programming could affect behavior. Similarly behavior might affect susceptibility to environmental toxins while environmental toxins might have a long-term effect on behavior through affecting epigenetic reprogramming. Another important principle that is emerging from these studies is that behavioral parameters should be taken into consideration in our analysis of the environmental impact on the epigenome.

The dynamic equilibrium of DNA methylation provides a template for environmental hazards to act upon. The environment could act through cellular signaling pathways leading from cell surface receptors down to transacting factors, which deliver chromatin–modifying enzymes to specific sequences. The dynamic epigenome has obviously adaptive and physiological roles in the crosstalk between our environment and our inherited genome, but could at the same time serve as a target for environmental hazards (Figs. 1–2). Unraveling, the conduits between the environment and our genomes should have an important impact on our health and protection from environmental hazards.

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