Dynamic epigenetic responses to childhood exposure to violence

Jonathan Mill, March 2012

PART 1: The evidence base

The outcome: Altered DNA methylation

With the exception of a few rare somatic mutation events, the sequence of nucleotides comprising an individuals' genome is identical across all cells in the body and remains unchanged from the moment of conception onwards. But DNA is structurally much more complex than a simple string of As, Cs, Gs and Ts, and at a functional level the genome is anything but static. Although each of the >50 trillion cells (representing over 200 distinct cell-types) in our bodies contains the same DNA sequence, each has a unique phenotype characterised by a specific pattern of gene expression that is in a constant state of flux. It is not only the gene-encoding DNA sequence that is important in determining the phenotype of a cell, but the degree to which specific genes are functionally active at any particular time in development. Sequencing the genome was thus only the first step in our quest to understand how genes are expressed and regulated. Sitting above the DNA sequence is a second layer of information (the 'epigenome') that regulates several genomic functions, including when and where genes are actively expressed. Epigenetics refers to the reversible regulation of various genomic functions, occurring independently of DNA sequence, mediated principally through changes in DNA methylation and chromatin structure. Epigenetic processes are essential for normal cellular development and differentiation, and allow the long-term regulation of gene function through non-mutagenic mechanisms [1].

DNA methylation, occurring primarily at cytosine-guanine dinucleotides (CpG) and catalysed by a group of enzymes called DNA methyltransferases (DNMTs) [2], is the best understood epigenetic modification modulating the transcriptional plasticity of mammalian genomes. The methylation of CpG sites acts to disrupt the binding of transcription factors and attract methyl-binding proteins that initiate chromatin compaction and gene silencing [2]. Because methylated cytosines are liable to spontaneous mutation, CpG dinucleotides are less common in the genome than would be predicted by chance, and primarily occur in clusters called 'CpG islands' which are often found around gene promoters and are typically unmethyated [2]. The post-translational modification of histones, the basic proteins around which DNA is wrapped to form nucleosomes, comprises the other major type of epigenetic mechanism related to gene expression. A number of covalent histone modifications, occurring at specific residues, have been described (e.g. acetylation, methylation, phosphorylation, SUMOylation, and ubiquitylation), which together constitute a complex 'histone code' modulating gene expression via alterations in chromatin structure [3]. Condensed chromatin (heterochromatin), in which the DNA and histone proteins are tightly packed, acts to block the access of transcription factors and other instigators of gene expression to DNA, and is thus associated with repressed transcription. Conversely, an open chromatin conformation (euchromatin) allows the cells' transcriptional machinery to access DNA and drive transcription. While often investigated independently, epigenetic modifications to DNA and histones are not

mutually exclusive, and clearly interact in a number of ways; it is apparent that the classification of epigenetic mechanisms in terms of either gene activation or suppression is too simplistic [3].

Like the DNA sequence, the epigenetic profile of somatic cells is inherited down cell-lineages during mitosis. Unlike the DNA sequence, which is stable and strongly conserved, epigenetic processes are often tissue-specific, developmentally-regulated and highly dynamic. A growing body of evidence shows that the epigenome changes dramatically over the life-course, and is strongly correlated with age [4-10]. This is an important observation given that the prevalence of many chronic diseases increases with advancing age. There is also mounting evidence that epigenetic processes are influenced by a range of factors in the environment. DNA methylation, for example, has been shown to vary as a function of nutritional, chemical, physical, and psychosocial factors. Because epigenetic changes can be stably inherited through mitosis in somatic cell lineages, they provide a mechanism by which the environment early in life can lead to long-term alterations in cellular phenotype, potentially regulating health outcomes later in life. It is pertinent that the epigenome appears to be particularly labile during a number of key periods [11] including pre- and perinatal development when the rate of DNA synthesis is high and the epigenetic marks needed for normal tissue differentiation and development are being established [12].

Epigenetic mechanisms therefore represent a major link between genes and the environment, with environmentally-induced epigenetic changes suggested to account for much of the phenotypic discordance in monozygotic (MZ) twins. A study by Fraga and colleagues examined DNA methylation and histone acetylation in 80 pairs of MZ twins, ranging from 3 to 74 years of age, using a combination of global and locus-specific methods [9]. They found that one-third of MZ twins had a significantly dissimilar epigenetic profile, with older twins and those with a history of non-shared environments being the most disparate, suggesting that environmental factors may shape the epigenome over the life-course. A recent longitudinal twin study from our group subsequently highlighted marked changes in DNA methylation at several neuropsychiatric candidate genes occurring across early childhood [13]. Our data suggest that environmental influences are important factors accounting for inter-individual DNA methylation differences, and that these influences are locus-specific across the genome. Of note, the observation of dynamic changes in DNA methylation over time highlights the importance of longitudinal research designs for epigenetic research (see below).

Importance for physical health
The correct regulation of gene activity is critically important for normal functioning of the genome; even genes that carry no disease predisposing polymorphisms can be rendered harmful if they are not expressed at the appropriate level in the correct type of cell at the right time of the cell cycle. Because epigenetic processes mediate appropriate patterns of cellular development and function, aberrant DNA methylation signatures have been implicated in a number of human pathologies, most notably cancer and rare imprinting disorders [1]. Recently it has been proposed that epigenetic mechanisms play a pivotal role in human health and disease, with increasing evidence for their involvement across the broad spectrum of chronic complex illnesses, including physical, physiological, and mental disorders [1; 14].

Epigenetic dysfunction can explain many of the epidemiological, clinical, and molecular peculiarities associated with complex disease; for example the incomplete concordance between monozygotic (MZ) twins [13; 15], gender differences in prevalence [16], specific windows of environmental vulnerability [12], and parent-of-origin effects [17]. Environmental mediation of the epigenome provides a potential mechanism for the gene-environment interactions currently being uncovered across the spectrum chronic disorders. Environmental influences mediated through epigenetic effects are speculated to cause a diverse range of human diseases, including type 1 diabetes [18], chronic kidney disease [19], respiratory disease [20], chronic fatigue syndrome [21], alcohol dependence [22], major psychosis [23] and Parkinson's disease [24]. Of note, changes in DNA methylation following early life stress have been associated with long-term changes in gene expression and behavior [25] and may contribute to both psychiatric disorders [26] and physiological disturbances [27] later in life.

Evidence linking juvenile violence victimization to altered DNA methylation
Epigenetic processes provide a potential mechanism for how early-life experiences (e.g. violence victimization or maltreatment in infancy) can become manifest at a cellular / molecular level, influencing long-term changes in gene function and increased disease risk. Although there is mounting interest in epigenetic epidemiology, with a focus on exploring if and where the epigenome (i.e. the overall epigenetic state of a cell) is influenced by specific environmental exposures, the field is still early in its infancy and susceptible to new ideas and approaches. Specific research into the epigenetic consequences of childhood violence victimization in humans is extremely limited (currently review/hypothesis articles greatly outnumber empirical studies), although a stronger research base exists using rodent models aiming to establish links between early-life adversity and epigenomic plasticity. To date, virtually all research into the epigenetic consequences of early-life psychological adversity has been performed within the context of the neuropsychiatric / psychological phenotypes, focusing primarily on DNA methylation changes near *a priori* candidate genes involved in stress-response pathways, neural plasticity, and normal neurobiological functioning. It is therefore unclear how applicable the results from these studies are for understanding pathways to poor physical health; recent advances in genomic technology mean that unbiased genome-scale studies of DNA methylation across multiple samples are now feasible and that more widespread changes to the methylome in response to early-life stressors such as violence victimization are likely to be identified in the near future.

Recent research using rodent models provides direct evidence for the role of early life stress on the epigenome; a number of detailed reviews have been recently published on this subject [25; 28]. Perhaps the best known example is a study by Weaver and colleagues [29], who observed that variation in maternal care in rats alters DNA methylation and histone acetylation at a specific transcription-factor binding sequence motif upstream of the glucocorticoid receptor gene (*Nr3c1*) in the hippocampus of the offspring, directly affecting transcription and subsequent stress-responses in adulthood. A cross-fostering design was used to infer a causal relationship between maternal care and epigenetic differences, and it was discovered that the changes in DNA methylation could be reversed using epigenetic drug treatments. Despite stimulating research into the epigenetic consequences of

early-life adversity, these seminal findings await convincing replication; a recent study reported no changes in DNA methylation in the same region of *Nr3c1* in the hippocampus following a model of maternal separation in rat [30], highlighting the need to confirm the link between early life stress and epigenetic alterations at this locus. Early life stress in rodents has also been shown to bring about epigenetic changes at the arginine vasopressin gene (*Avp*), with a regulatory region in the gene being hypomethylated following maternal separation [31]. Similar changes following an environmental stressor have been observed in several other genes including *Bdnf* [32; 33], *Crh* [34], *Dlgap2* [35], *Mecp2, Cnr1* and *Crhr2* [36], suggesting that epigenetic changes may occur in multiple neurobiological pathways in response to stress. Finally, a recent study from our group reports altered DNA methylation levels in the hippocampus across promoter regions of *Nr3c1*, *Avp* and *Nr4a1* in a study of maternal separation in two strains of mice; interestingly the changes were strain-specific suggesting that epigenetic responses to an adverse environment may differ as a function of genetic background (Kember et al, in revision).

Research on epigenomic changes in humans occurring in response to early-life adversity is considerably more limited, and the results often difficult to interpret given the biological, technical and methodological issues inherent in how the studies have been implemented [37] (see below for more discussion). For this reason, I would suggest treating some of these findings with caution! Much of the published human literature focuses on a single candidate gene (*NR3C1*), encoding the glucocorticoid receptor. Building on their maternal separation work in rats, McGowan and colleagues report elevated *NR3C1* promoter DNA methylation in postmortem hippocampal tissue from depressed suicide patients who suffered from a history of early-life abuse and neglect compared to suicide patients not exposed to early-life adversity [38]. In contrast, however, no such epigenetic changes were identified in hippocampal samples from a cohort of depressed patients [39] suggesting that altered *NR3C1* DNA methylation may be specific to depressed individuals who have experienced early-life adversity. Perroud et al (2011) report increased DNA methylation in blood at a similar region of the *NR3C1* promoter in adults with a history of childhood maltreatment, and Oberlander and colleagues find that prenatal exposure to stress is also associated with DNA methylation changes at this locus in cord blood [40]. Finally, a recent study reports that healthy adults reporting a history of childhood adversity (parental loss and maltreatment) display an increase in *NR3C1* promoter DNA methylation in leucocytes [41]. There is a limited body of evidence for epigenetic changes in other candidate genes being associated with early childhood adversity. Beach and colleagues, for example, report that DNA methylation in a CpG island upstream of the serotonin transporter gene (*SLC6A4*) is associated with self-report childhood abuse using DNA from transformed lymphoblastoid cell-lines [42]. These human studies are all characterized by relatively small changes in DNA methylation, and the biological significance of the data is yet to be established. More systematic epigenetic analyses using genome-wide microarrays have been performed in adults exposed to stress and suffering from post-traumatic stress disorder (PTSD), although the observed changes are again small and often hard to interpret given limitations in the study design. Interestingly, however, several studies report differential DNA methylation in genes related to immune function and inflammation in PTSD patients [43; 44].

Establishing causality in epigenetic epidemiology (…and other musings about study design and interpretation)

Epigenetics is a relatively new, but rapidly expanding, area of investigation and optimal research methods are still being developed. In undertaking epigenetic research (or when interpreting previously published data) it is important to take into account a number of biological, technical and methodological issues [37]. It is unlikely that the simple "brute-force" approaches that have been used relatively successfully in genetic association studies are valid for epigenetic analyses. The results of GWAS are relatively easy to judge. Quality control steps are well-defined and reported, individually testing every genetic variant is straightforward, and levels of genome-wide statistical significance are clear. For epigenomic studies, the analytical methodology is very much under construction. In genetic studies, many of the epidemiological principles about designing studies with respect to selection biases, confounding, batch effects and appropriateness of controls could largely be replaced by the simple rule 'bigger-is-better'. This is not true for epigenetic epidemiology; as discussed above, the epigenome is not a static entity like the genome, necessitating the use of more conventional epidemiological approaches[45]. Further complicating matters is the fact that for the most powerful study designs in epigenetic epidemiology (including studies of discordant monozygotic twins[15] particularly when longitudinally sampled[13], and early exposure studies with long-term follow-up[46]) the number of eligible individuals for whom relevant biological materials have been archived in existing epidemiological cohorts is often limited.

As empirical research starts to identify epigenetic changes occurring in the context of early-life adversity and physical health, it will be important to establish cause and effect; disease-associated differentially methylated regions may arise prior to illness and contribute to the disease phenotype or could be a secondary effect of the disease process, or the medications used in treatment[47]. It may be difficult (perhaps impossible?) to obtain directly causal evidence linking childhood trauma or abuse to altered DNA methylation and subsequent disease given the ethical issues involved in implementing the optimal longitudinally-sampled study design. Animal models of early-life stress can overcome many of these issues, as exemplified by the clever cross-fostering design used in the Weaver et al study in rats [29], and are likely to be a powerful research tool in examining the direct effects of adversity on the epigenome.

Most human epigenetic studies focus specifically on DNA methylation [37], neglecting other layers of the epigenome like histone modifications that are also likely to be important in influencing disease phenotypes. The good news is that recent advances in genomic technology mean that genome-scale studies of DNA methylation across multiple samples are now feasible. In practice, however, there is a compromise between coverage and precision in epidemiological studies that likely incorporate a large number of samples. A large (and growing) number of methods exist for assessing DNA methylation both genome-wide and at specific sites[48], and one problem relates to our inability to compare results across studies that have used different platforms. Furthermore, our basic understanding of the methylome (i.e. the whole of DNA methylation marks on the genome) is in its infancy, and we are still learning about the specific localization of the features that, when differentially methylated, regulate gene expression and are thus relevant for epigenetic epidemiologists to study. For example, most current research focuses on promoter

CpG islands; although these features are often enriched for DNA methylation marks influencing the expression of genes, recent work suggests that other regions of the methylome outside of promoters, including intergenic CpG island shores[49] and intragenic CpG islands [50], may ultimately be more important for regulating phenotypic variation.

Whatever we do, it may never be enough to fully account for epigenetic differences between tissues and cells. In many respects large, comprehensively phenotyped and longitudinally-sampled epidemiological studies, like the E-Risk and the Dunedin cohorts, are an ideal resource for epigenetic epidemiology. In nearly all such studies, however, whole blood or buccal cells are the only biological material that have been archived. Blood is a heterogeneous tissue and any DNA methylation difference between groups could be confounded by differences in the cellular composition of whole blood samples, for example resulting from the immune response to sub-clinical infection. The good news is that fewer than perhaps expected DNA methylation differences exist between leukocyte types, and controlling for cellular heterogeneity may be possible in biobanks with a simple blood cell count[51]. Another key question for epigenetic epidemiology concerns the extent to which easily accessible peripheral tissues (such as blood) can be used to ask questions about inter-individual phenotypic variation manifest in inaccessible tissues such as the brain, visceral fat, and other internal organs and tissues. Cross-tissue comparisons of the methylome within the same individual are currently underway to establish the relationship between epigenetic patterns in blood with other tissues; data from our group using pre-mortem blood and post-mortem brain samples from the same individuals suggests that although tissue-specific methylation differences far exceed inter-individual variation, between-individual differences appear to be correlated across brain and blood (Davies et al, in review). Although these analyses are crucial, the results may not be generally applicable; higher inter-tissue concordance may be present for DNA methylation changes induced early in development (and potentially propagated soma-wide) than for changes occurring during ageing that are more likely to remain tissue-specific[47; 52]. Efforts to obtain biopsies (subcutaneous fat, muscle etc.) and post-mortem material in subsets of longitudinal biobanks will greatly increase their value for epigenetic studies, despite the problems associated with cellular heterogeneity that also holds for such samples.

Finally, because there is a considerable interest in epigenetic research in both the scientific and popular press, it is important that epigenetics should avoid some of the hype that surrounded the early days of genetic epidemiology. After the draft human genome sequence was announced in 2001, it was widely perceived that we would soon understand the causes of most common diseases and how to treat them. This expectation was not realistic, but not always renounced by geneticists. Currently, many scientists outside the field are disappointed by results of human genetics, and in particular GWAS, despite their overall considerable success. Very much like genetics, epigenetics will not be able to deliver the miracles it is sometimes claimed it will.

Effect moderation and mediation
It is often suggested that environmental mediation of the epigenome, such as in the examples described above in the context of early-life stress, provides a mechanism behind gene-environment (G X E) interactions. Strictly, this is not the case as G X E

interactions, by definition, also require the involvement of a specific genotype [53]. There are, however, several pathways through which epigenetic plasticity could mediate G X E interactions:

    i)   genotype exerts an effect on gene function via epigenetic effects, which in turn are susceptible to environmental mediation

    ii)   the environment mediates (or unmasks) the expression of pathogenic polymorphisms via epigenetic changes

    iii)   genotype alters the sensitivity of promoter regulatory regions to epigenetic changes in response to the environment

At the most basic level, it is intuitive that the pathogenic effect of a polymorphism associated with disrupted gene function is likely to be dependent upon the degree to which that particular variant is actually expressed. It is thus plausible that risk could be exaggerated or suppressed if expression is directly influenced by environmental factors via processes such as DNA methylation. Of particular interest are so-called 'metastable epialleles'; loci that can be epigenetically modified to produce a range of phenotypes from genetically identical cells [54]. Many of these loci have been shown to be environmentally-sensitive, and particularly affected by the prenatal environment of the developing foetus. A classic example of how such a mechanism could explain gene-environment interactions is provided by the agouti viable yellow allele (A^{vy}) inbred mouse strain, which demonstrates a range of coat-colour and metabolic phenotypes, depending upon the epigenetic state of a large transposable element inserted upstream of the agouti gene. The transposon contains a cryptic promoter, which expresses a phenotype characterized by yellow fur and various detrimental metabolic features such as diabetes and obesity. When the transposon is methylated, this phenotype is not expressed; the mice have brown fur and are metabolically healthy. Interestingly, DNA methylation across this region (and thus phenotype) can be manipulated in offspring by altering the diet of pregnant mothers [55; 56]. Enriching the maternal diet with methyl-donor supplements increases offspring DNA methylation, leading to gene expression changes associated with brown fur and metabolic health. Gene-environment interactions may also result when genetic polymorphisms alter the ability of a specific region of the genome to be epigenetically altered in *response* to an environmental pathogen. The interplay between the genome, the environment, and epigenetic processes is further complicated by the fact that some DNA alleles and haplotypes are themselves associated with a specific epigenetic profile [57]. As an example, allele-specific epigenetic modifications have been associated with 'risk' polymorphisms in psychiatric candidate genes including several mediating G X E involving early-life adversity such as the serotonin receptor gene (*5HTR2A*) [58] and that encoding brain-derived neurotrophic factor (*BDNF*) [59]. Given the known influence of environmental factors on epigenetic regulation, the cis-regulation of DNA methylation by genetic variation would suggest a common pathway behind both genetic and environmental effects and a potential mechanism for G X E interaction.

PART2: Implications for prevention and intervention
In addition to highlighting specific molecular changes and biological pathways involved in the response to severe stresses early in life, the discovery of epigenomic changes occurring as a result of violence exposure in childhood could have potential implications for prevention and intervention given the dynamic regulation of epigenetic phenomena; epigenetic disruption is potentially reversible, and thus a

realistic target for intervention (pharmacological and/or behavioural). Numerous agents have been discovered that can alter DNA methylation and histone modifications, and several of these are already being tested in ongoing clinical trials. So-called 'epigenetic drugs' are being developed for a range of disorders, most notably cancer [60], and many currently-used psychopharmacological agents have strong effects on the epigenome [61]. One potential obstacle to the widespread use of these agents is that drugs which target the epigenome globally can have unexpected (and potentially pathogenic) effects on the transcription of genes which are not the desired target.

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