SYMPOSIUM

The Dynamic Nature of DNA Methylation: A Role in Response to Social and Seasonal Variation

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Synopsis An organism's ability to adapt to its environment depends on its ability to regulate and maintain tissue specific, temporal patterns of gene transcription in response to specific environmental cues. Epigenetic mechanisms are responsible for many of the intricacies of a gene's regulation that alter expression patterns without affecting the genetic sequence. In particular, DNA methylation has been shown to have an important role in regulating early development and in some human diseases. Within these domains, DNA methylation has been extensively characterized over the past 60 years, but the discovery of its role in regulating behavioral outcomes has led to renewed interest in its potential roles in animal behavior and phenotypic plasticity. The conservation of DNA methylation across the animal kingdom suggests a possible role in the plasticity of genomic responses to environmental cues in natural environments. Here, we review the historical context for the study of DNA methylation, its function and mechanisms, and provide examples of gene/environment interactions in response to social and seasonal cues. Finally, we discuss useful tools to interrogate and dissect the function of DNA methylation in non-model organisms.

Introduction

Epigenetics and DNA methylation

Over the past century, epigenetic mechanisms have been identified with their roles in regulating gene function within cell lineages and/or in the germ line understood. Mechanisms including the covalent modification of DNA (Greenblatt et al. 1994; Elliott et al. 2010), histone modifications, microRNAs (Chuang and Jones 2007), long non-coding RNAs (Mercer et al. 2009), and long-range folding of chromatin (Woodcock and Ghosh 2010) are a few of the mechanisms. Among these, DNA methylation which is the covalent modification of the 5′-cytosine rings via methylation has been widely studied. DNA methylation can provide a dynamic response to environmental cues, and here we review examples of its dynamic responses to the environment.

DNA methylation: the first half century

DNA methylation, discovered >60 years ago, has been described in many taxa of animals (bivalves, echinoderms, amphibians, and mammals) (Bird and Taggart 1980; Tweedie et al. 1997). Historically, however, the study of genetics in tractable model systems overshadowed interest in investigating epigenetic mechanisms such as DNA methylation (Van Speybroeck 2002). This was largely a consequence of DNA methylation being reported as absent in three model systems central to molecular biological analysis, *Caenorhabditis elegans* (Simpson et al. 1986), *Drosophila melanogaster* (Rae and Steele 1979; Bird and Taggart 1980; Urieli-Shoval et al. 1982; Patel and Gopinathan 1987), and *Saccharomyces cerevisiae* (Proffitt et al. 1984). As a consequence, understanding of DNA methylation was technically and conceptually limited. However, the absence of

evidence is not evidence of absence since more recent reports found DNA methylation both in some strains of yeast (Tang et al. 2012) and in fruit flies (Lyko et al. 2000).

Interest in the role of DNA methylation in development arose in parallel with discoveries that it was integral to differentiated states of cells and to a regulator of gene function. This led to the suggestion that it was potentially an important mechanism in the fields of cancer and developmental biology (Riggs and Jones 1983). This idea arose from the discovery that a cytidine analog inhibitor of DNA methyltransferases (DNAMTs), the enzyme that catalyzes the DNA methylation reaction, could induce pluripotency and differentiation in 3T3 cell lines (Taylor and Jones 1979).

In cancer biology, aberrant DNA methylation is largely reported at gene-specific loci across the genome leading to the programmed hypomethylation of pro-oncogenic genes (Feinberg and Vogelstein 1983a, 1983b) and to hypermethylation of tumor suppressors genes (Greger et al. 1989; Herman et al. 1995). Aberrant DNA methylation was shown to be regulated by nodal oncogenic (MacLeod and Szyf 1995) and tumor-suppressing pathways (Slack et al. 1999). Inhibition of DNAMT enzymes in turn inhibited tumorigenesis (Ramchandani et al. 1997) and is now being tested in clinical trials in humans as an attractive anticancer target. Metastasis, on the other hand, was found to be driven by demethylation of DNA (Pakneshan et al. 2004) and inhibition of DNA demethylation inhibits cellular invasion and metastasis *in vivo* (Shukeir et al. 2006). Additionally, global loss of DNA methylation has an effect as well on genomic stability and could be involved in the chromosomal aberrations observed in cancer (Gama-Sosa et al. 1983; Eden et al. 2003). Further, it has been proposed that 5-methylcytosine increases mutability rate of C->T transitions and may serve as an endogenous substrate for oncogenic mutations (Greenblatt et al. 1994). Thus, DNA methylation has become an important factor within the context of a diseased state. In addition, during development, changes in genomic DNA methylation were reported in the primordial germ cells and in pre-implantation embryos in mice. In both instances, genomic erasure of DNA methylation situates cells as pluripotent substrates for genomic methylation and cell differentiation (Mayer et al. 2000; Hajkova et al. 2002; Inoue and Zhang 2011).

In 2004, Weaver et al. showed that maternal behavior of rats could differentially methylate the glucocorticoid receptor (GR) gene of their pups, thus reducing the receptor's ability to transcribe GR, and ultimately lowering stress responsiveness behavioral outcomes. This seminal study showed that DNA methylation could be induced by behavioral interactions and, in turn, causes subtle alterations in the nervous system and hence behavioral responses. Within a few years of this finding, many investigators showed a role for DNA methylation in a variety of behaviors, including memory (Miller et al. 2010), some pathological states of mental health (Sweatt 2013), and human diseases in addition to cancer (Robertson 2005). These reports revealed the dynamic nature of DNA methylation that matched neural plasticity over a range of time scales.

Functions of DNA methylation

The DNA methylation process has been hypothesized to be evolved to silence evolutionarily accumulated selfish genes (Yoder and Bestor 1998) and transposable elements (Zemach et al. 2010), and to regulate the transcription of genes (Razin and Riggs 1980). In addition, it has been posited to regulate gene splicing (Shukla et al. 2011), X-inactivation, and parental genomic imprinting (Li et al. 1993). Considering the variability of DNA methylation across different species, it is likely to assume various roles. For example, in invertebrate genomes, DNA methylation has been lost to varying degrees and is largely isolated to exonic gene-body elements (Glastad et al. 2011; Sarda et al. 2012).

In mammals, CpG dinucleotides serve as the substrate to which a methyl group is added. Since CpGs are relatively rare in the genome and uniquely arranged, they are thought to be important for transcriptional regulation (Bird 1986). In the genome, regions of higher CpG density comprise CpG islands (CGI) defined as 200 bp windows with a >50% GC content and an observed (within a given sequence) to expected (within the genome) CpG ratio >60% (Fatemi et al. 2005). Mammalian genomes contain CGI islands that can vary in size between 300 and 3000 bp and are found upstream of 40% of transcriptional start-sites. Interestingly, the inverse relationship between a gene's methylation and its expression has been linked to proximal areas of CGI (defined as CGI shores) (Irizarry et al. 2009) and not the CGI itself. DNA methylation also occurs within a non-CpG context in embryonic stem cells but is mostly absent in somatic tissues (Ramsahoye et al. 2000; Ziller et al. 2011). It should be noted that the mammalian genome is

largely methylated in most tissues (70–90%) (Ehrlich et al. 1982).

Mechanistically, DNA methylation has been suggested to cause steric hindrance to transcriptional activators within the major groove of DNA thus blocking expression of a particular gene. In addition, it has been shown to bind several transcriptional repressors such as members of the methyl-binding domain (MBD) family. These act to silence transcription through the recruitment of histone-modifying complexes to block transcription by condensing DNA into heterochromatic complexes (Razin and Riggs 1980; Choy et al. 2010).

DNA methylation toolkit

The methylation of DNA is catalyzed by a family of DNAMTs with highly conserved domains that add methyl moieties from the methyl donor, *S*-adenosyl-methionine. DNMT1, the first characterized methyltransferase (Bestor 1988), was characterized as a maintenance methyltransferase that targets hemi-methylated daughter strands of newly replicated DNA (Flynn et al. 1996; Bacolla et al. 1999; Fatemi et al. 2001). DNMT3A and DNMT3B catalyze the *de novo* methylation of DNA (Okano et al. 1999). Both were initially discovered to be upregulated during embryogenesis and have been implicated in generating patterns of *de novo* DNA methylation during cell differentiation.

The discovery of MBD proteins MBD1-4 and MeCP1 and 2 (Meehan et al. 1989; Nan et al. 1993) provided further understanding of DNA methylation. This family of proteins, characterized with a MBD domain, was shown to have various roles mitigating the function of methylcytosine. Subsequently, several groups have defined their function with roles in gene repression (Jones et al. 1998; Nan et al. 1998) and activation (Collins et al. 2004; Bienvenu and Chelly 2006). For example, MBD2 has been characterized as a transcriptional repressor capable of interacting with histone machinery to form heterochromatin (Ng et al. 1999; Zhang et al. 1999) and as a demethylase capable of inducing transcriptional activation (Bhattacharya et al. 1999; Alvarado et al. 2013).

How is DNA methylation removed? Demethylation through cytosine excision repair has been suggested to occur through growth arrest and DNA-damage-inducible protein 45 (Gadd45a/b) (Wu and Zhang 2014). Similarly, the recently-discovered function of the 10- to 11-translocation (TET) protein family has revealed that hydroxylation of methyl cytosine may be an oxidative intermediate of demethylation. Considering its mechanism, 5-hydroxymethylcytosine has been reported to possibly regulate transcriptional activation as seen in embryonic stem cells (Tahiliani et al. 2009) and the central nervous system (Kriaucionis and Heintz 2009; Lister et al. 2009). Other reports have suggested that, following TET-mediated hydroxylation, either activation induced deaminase (AID/APOBEC) and/or thymine DNA glycosylase (TDG) + MBD4 mediate a demethylation reaction on methylcytosine (Wu and Zhang 2014).

Seasonal regulation and DNA methylation

Many seasonal cues such as temperature and light (Epperson and Martin 2002; Ruby et al. 2002) can have profound effects on the regulation of transcriptional mechanisms in natural environments. Seasons provide predictable environmental changes to which an animal can evolve plastic transcriptional programs producing adaptations to metabolism and reproduction. While the exact role DNA methylation plays in these scenarios has yet to be fully explored, evidence shows that DNA methylation regulates many seasonally related candidate gene pathways (Figure 1).

Hibernation requires seasonally regulated metabolism and mammals conserve energy in the winter by undergoing prolonged bouts of torpor interspersed with brief arousals back to euthermia. During torpor, energy expenditures drop to as low as 1–5% of euthermic rates (Carey et al. 2003) and are accompanied by a suite of transcriptional changes (Srere et al. 1992). Since DNA methylation is largely conserved among vertebrate mammals, we believe that such a mechanism might be central for the differential gene expression that accompanies seasonal change. For example, the hibernation-specific protein-27 (HP-27), known to be upregulated in the blood of hibernating chipmunks (Kondo and Kondo 1992), is regulated by DNA methylation in a tissue-specific manner under the transcriptional control of upstream stimulatory factor-1 (USF1) (Fujii et al. 2006). Considering the mechanisms involved, DNMT3B, a *de novo* methyltransferase, is regulated by BmaL-regulated circadian rhythms in the liver (Maekawa et al. 2012) that underlie metabolic depression during hibernation in ground squirrels (Ruby et al. 2002). In examining circadian rhythms of the superchiasmatic nuclei during natural diurnal cycles, DNA methylation is shown to be remarkably plastic (Azzi et al. 2014). Considering the importance of these rhythms in response to seasonal
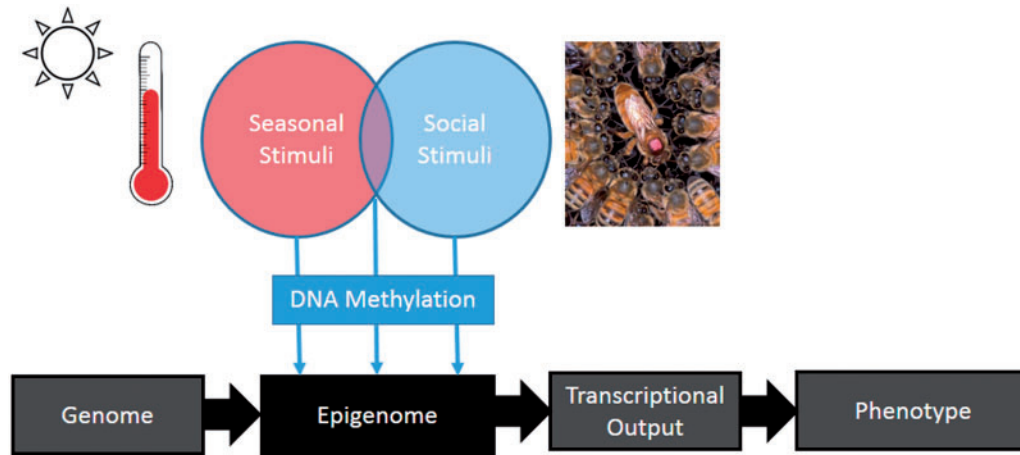
Fig. 1 Summary of how social and seasonal stimuli can affect phenotype through transcriptional outputs where gene transcription defines a specific trait.

change, we speculate their role may be integral for adapting to altered photoperiods.

Since most genomic repertoires of seasonal hibernators are strikingly similar to non-hibernators, we propose that the hibernating phenotype does not necessarily require novel genes but may result from unique epigenetic reconfiguration of its genome. Harris et al. (2000) has proposed a hypothesis of "hibernator as neonate" suggesting that the genetic predisposition for heterothermy is expressed to some extent in all neonates. Further, this hypothesis suggests that heterothermy in hibernation results from the continued expression in the requisite genes. Given the widespread conservation of heterothermy and epigenetic processes, we propose that a mechanism such as DNA methylation could complement an ancestral mammalian capacity for heterothermy (Geiser 1998).

In many vertebrates, seasonally regulated photoperiod changes regulate the development of discrete reproductive phenotypes (Dawson et al. 2001; Goldman 2001; Stevenson and Ball 2011). These distinct phenotypes may be a consequence of epigenetic changes though this is not known. However, in hamsters, changes in photoperiod induce thyroid-hormone-dependent induction of discrete reproductive phenotypes. DNA methylation has been shown to regulate this process through control of type III deiodinase gene in the hypothalamus (Stevenson and Prendergast 2013). Similarly, in humans, the season of conception among rural Gambians can result in aberrant DNA methylation of metastable epialleles (Waterland et al. 2010). This study showed that nutritionally challenged parents that conceived during the rainy season generated offspring with a unique signature of methylation when compared with others conceived out of the rainy season.

Seasonal variation is a broad term to describe a predictable change in environment that elicits an adaptive response that is not solely limited to metabolic regulation or reproduction. For example, the seasonal plasticity of coat color that produces crypsis to avoid predation in the case of deer mice and patterning that results from mutations in the agouti gene (Linnen et al. 2009, 2013). Interestingly, Waterland and Jirtle (2003) showed that the agouti gene promoter could be hypermethylated through dietary folate, a methyl donor, and capable of modulating coat coloring. While there is no direct link between DNA methylation of the agouti gene and seasonal plasticity of crypsis, we propose that DNA methylation may provide plasticity to such genes with important ecological and evolutionary consequences.

Role of social environment

Changes in behavior enable animals to face the challenges of feeding, evading predators, and breeding, but the specific cellular and molecular mechanisms that mediate such behaviors are largely unknown. While genetically set behavior patterns provide a functional basis for behavior, they do not account for the plasticity and dynamic nature of behavior (Figure 1). Since social interactions can regulate context-specific behaviors, they can shape the development and physiology of the brain through neuroanatomical and molecular changes in gene expression (Davis and Fernald 1990; Burmeister et al. 2005; Robinson et al. 2008; Maruska and Fernald 2011; Fernald 2012).

Early social interactions can have lasting effects on gene transcription through DNA methylation. As previously mentioned, maternal licking and grooming can alter DNA methylation and stress responsiveness through the GR (Weaver et al. 2004). These interactions have been described in models of maternal separation (Murgatroyd et al. 2009) and abuse (Roth et al. 2009) modifying the genes expressing arginine vasopressin and brain-derived neurotropic factor, respectively. While deprived social environments can induce diseased states of DNA methylation, social interactions can also protect changes in DNA methylation. For example, neuropathy-induced genomic CpG demethylation in the prefrontal cortex of mice can be protected by enrichment through social interactions with littermates (Tajerian et al. 2013).

Do epigenetic marks influence behavior, particularly social status? DNA methylation at gene-specific promoters in the brain has revealed an interesting signature unique to social status in animals. For example, in mammals, paradigms of social defeat and environmental enrichment both have stable effects on DNA methylation. Resilience in mice treated in a social-defeat model is accompanied by hypermethylation of the corticotrophin releasing factor gene and influences behavioral outcomes (Elliott et al. 2010). Furthermore, in humans, signatures of DNA methylation assayed from many different tissues can serve as biomarkers that categorize components of social behavior or status. For example, genomic signatures of DNA methylation assayed from blood are capable of categorizing socioeconomic status in children (Borghol et al. 2012) and aggression in adults (Guillemin et al. 2014). Similarly, peer rearing in rhesus macaque monkeys provides aggressive phenotypes with distinguishing signatures of methylation between mother-reared and isolated controls (Provencal et al. 2013).

There are also examples of DNA methylation responding to social and nutritional cues in insects. However, differences in DNA methylation in insects occur primarily in exonic areas of the genome (Lyko et al. 2010; Bonasio et al. 2012) suggesting a possible role in alternative splicing (Shukla et al. 2011; Foret et al. 2012). In bees, nutritional cues were shown to regulate patterns of DNA methylation between worker and queen castes (Kucharski et al. 2008). Furthermore, within worker castes, reports also show that complex social tasks result in unique task-specific signatures of gene methylation that may alternatively regulate genes through splicing (Lockett et al. 2012). Furthermore, in bees, DNA methylation can reversibly mark behavioral subcastes of workers that forage and nurse, respectively (Herb et al. 2012).

Molecular investigation of DNA methylation in non-model systems

To discover whether DNA methylation plays any role in social or seasonal phenotypes alone or in response to other exogenous factors, it must be localized and its effects tested. Specifically, in a given tissue, a genome will likely have many genes that are methylated during development and remain so throughout life. To identify these, a paradigm needs to be identified to compare animals in at least two distinct, semi-natural states. If a fully-annotated and sequenced genome does not exist, there are many possibilities for discovering whether DNA methylation might be implicated in different phenotypes. Here, we describe the use of restriction enzymes to assay genomic methylation and identify DNA methylation at specific loci and pharmacology targeted toward DNA methylation machinery. With the cost of sequencing dropping dramatically, sequencing of non-model organisms may become more common.

Methylation sensitive restriction digestion

To study DNA methylation, it is essential to be able to identify genomic loci that show differential methylation or, if possible to assay methylation across the genome. The use of restriction enzymes has been particularly useful in this endeavor as there are many isoschizomer pairs that can be methylation sensitive and insensitive thus capable of generating different patterns of digestion, dependent on DNA methylation. For example, the restriction enzymes *Msp*I and *Hpa*II have been particularly useful in assaying DNA methylation within a CpG context (Waalwijk and Flavell 1978). These enzymes identify methylated loci for sequencing, using methylation-sensitive, amplified fragment-length polymorphisms (MS-AFLP) (Xiong et al. 1999) and are widely applicable to ecological epigenetics in plants and animals (Schrey et al. 2013). Furthermore, the specific fragments identified from MS-AFLP screen can be sequenced at a low cost and situated within certain genomic loci given closely related and sequenced reference genomes. In the same vein, *Hpa*II and *Msp*I generate sticky ends that can be pyrosequenced to generate genomic indices of DNA methylation with the luminometric methylation assay (LUMA) (Karimi et al. 2006). These approaches have been particularly useful in identifying toxicological impacts of mercury

on DNA methylation in polar bears, mink, yellow perch, and chickens (Pilsner et al. 2010; Basu et al. 2013; Head et al. 2014). Taken together, a technique such as LUMA can identify genomic changes in methylation and MS-AFLP can identify specific loci worthy of follow-up studies.

Pharmacology of DNA methylation machinery

To find causal link to DNA methylation, cancer biologists have produced a suite of drugs capable of affecting DNA methylation. Specifically the cytidine analogs, 5-aza-cytidine and 5-aza-decytidine are well-characterized nucleoside analogs that incorporate into DNA during replication and halt DNMT1 maintenance methylation, thus demethylating the genome (Ghoshal et al. 2005). In non-dividing tissues, 5-aza-decytidine may have limited effects due to their replication-dependent mechanism; other drugs that target *de novo* methyltransferases would be better. For example, RG108 targets the conserved C-terminal domain of DNMTs, and can be employed to inhibit *de novo* methylation (Schirrmacher et al. 2006). In order to hypermethylate the genome, approaches are limited to providing methyl-donors, such as *S*-adenosyl-methionine and *S*-adenosyl-homocysteine, to the methylation reaction (Charles et al. 2012). Unfortunately, methylation in general is a ubiquitous process and its substrates may have confounding effects on other subcellular processes relevant to RNA, protein, and lipids. Thus, such pharmacological approaches should be used to complement one another to identify mechanisms central to DNA methylation.

Concluding remarks

Within the past decade, the study of DNA methylation has expanded into many fields. Given its dynamic nature, it may function widely to modulate genomic responses to a given environment. However, the intrinsic regulation of such a mechanism still begs many questions about gene regulation. For example, at what temporal scales (minutes to years) are DNA methylation signatures laid down and removed? Is the methylated state of a gene a permanent molecular memory or the maintenance equilibrium between DNA methylases and demethylases? Does DNA methylation initiate a transition to seasonally/socially-related transcriptional phenotypes, or is it a downstream signature of these environmental changes? The phenotypic variability seen throughout the animal kingdom suggests that adopting new models for DNA methylation studies may reveal how these epigenetic modifications can lend plasticity to a static genome by environmental interactions.

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