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Review

Cytosine methylation in insects: new routes for the comprehension of insect complexity

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Abstract: Cytosine methylation is one of the most studied epigenetic modifications and its occurrence has been deeply studied in mammals and plants. DNA methylation (together with other epigenetic modifications of DNA and histones) plays an important role in different processes. Indeed, several morphological and/or behavioural traits may origin as a consequence of the epigenetic modulation of genes so that identical genes can results in different "morphs". Despite considerable progress during recent years, many questions remain since it is largely unknown how the environment triggers alterations in the epigenome. In the present review we discuss the use of aphids and honey bees as epigenetic experimental model to understand how cytosine methylation is directly or indirectly linked to environmental factors. Indeed, the epigenetic changes of DNA could be at the basis of unexpected morphological differences explaining also complex traits.

Keywords: epigenetics; cytosine methylation; transcription control; aphid; honey bees

1. Introduction

DNA methylation occurring at cytosine residue is one of the most studied epigenetic modifications and it is based on the presence of 5-methylcytosine (5mC) [1,2].

According to literature data, 5mC is found mostly within CpG dinucleotides in the DNA of mammalian somatic cells [2,3], where cytosine methylation can be present at both promoter and gene

bodies, but with opposite effects on transcription [4]. In particular, according to almost all experimental data, cytosine methylation can stabilize or lock genes in a silent state if methylated at promoters [5], whereas it can favour transcription if methylated cytosines occur within gene bodies [5].

Cytosine methylation is also involved in several fundamental processes, other than transcription control, such as transposon silencing, genomic imprinting and X chromosome inactivation [2] and it is essential for regulating the development processes [6,7,8].

The mammalian DNA methyltransferases (DNMTs) catalyse the transfer of the methyl group from *S*-adenosyl-L-methionine to cytosine and can be distinguished in DNMT1 (that is thought to function as the major maintenance DNA methyltransferase), DNMT3A and DNMT3B (that act as *de novo* methyltransferases active on unmethylated DNA) and DNMT2, a well-conserved protein with homologs in plants, yeast, *Drosophila* and mammals, whose function (if present) is still not clear [9,10].

The mapping of 5mC in the genome has been examined in various models, such as the flowering plant *Arabidopsis thaliana* [11,12,13], and it has been suggested that some environment-dependent epigenetic marks can be heritable suggesting that, at least in plants [14,15,16], epigenetic modifications may be propagated across a variable number of generations orchestrating a flexible heredity of some advantageous phenotypic traits [14,15,16].

Differently from what reported in mammals and plants, insect researches in epigenetics are still in its infancy [17,18], but the availability of easy and high throughput approaches for the study of the epigenetic modifications of both DNA and histones [19] revealed some important similarities in the epigenetic machinery across taxa (in particular from insects to mammals). For instance, both insects and mammals show dynamic changes in cytosine methylation during development. In honeybees, methylation of specific CpGs correlates with age and behaviour [20,21]. Similarly, cell differentiation and ageing are associated with significant changes in the methylome in mammals. As a whole, there is a relevant set of data suggesting a general mechanistic theory of commitment and reprogramming across different levels of biological organisation and complexity [10,22].

Nevertheless, several differences can be present comparing insects and mammals [23]. Firstly, most of the 5mC in insects is found in the context of non-CpG dinucleotides, which rendered traditional CpG-specific assays ineffective [23]. Secondly, methylation in insects appears to be largely restricted to gene bodies and it is generally absent from promoters and transposons. In particular, the silencing of DNMT resulted in a sort of “burst” of transposon mobilization in both plants and mammals, whereas methylated mobile elements have never been observed in insects [17,18,23,25] so that the ancient and conserved role of DNA methylation seems to be related to transcription and potentially to alternative splicing [24], whereas transposon control and gene repression appear to be unique to mammals among metazoans [17,18,23,25].

Several aspects related to the role of DNA methylation in insects have been already revised by different Authors frequently focussing on *Drosophila* [17,18,25], so that in the present review, we discussed the role of cytosine methylation in view of the evolution of different morphological and/or behavioural traits as a consequence of the epigenetic modulation of genes. In particular, we reviewed the role of cytosine methylation on phenotypic variability so that identical genomes can results in different insect “morphs”, as reported in aphids and honey bees.

2. From a Single Genome to Different Morphologies and Reproductive Modes: an Aphid Epigenetic Tale

Aphids (Hemiptera: Aphididae) are sap-sucking insects that conquered most of the world's biomes [26]. They reproduce primarily by apomictic parthenogenesis, a form of reproduction whereby adult females give birth to female progeny in the absence of male fertilization and without any kind of meiotic recombination [27] so that it has been suggested that aphid offspring represent a genetically identical clone [28]. In view of this assumed genetic identity, Janzen [29] referred to aphid species as a single evolutionary individual able to exploit a much larger geographic region and its resources [30].

Actually, aphid clones within a same species can differ in a wide number of phenotypes such as: colour and size [31,32], intrinsic rate of increase [33], ovariole number [28], reproductive modes [31], ability to transfer pathogenic plant viruses [34], and susceptibility/resistance to predators, parasites, pathogens and pesticides [31,35,36]. Furthermore, the offspring of a same mother can differ for the presence/absence of wings [37], colour patterning (i.e. green versus orange) [32], reproductive modes (sexual reproduction or parthenogenesis) [38,39] and the occurrence of polymorphisms due to castes [40–43].

In particular, many aphid species show wing polyphenism in which winged morphs appear in response to changes in environmental factors in order to facilitate migration to new host plants or habitats [28,37,44]. Reproductive polyphenism, in which sexual reproduction and parthenogenesis are switched depending on seasonal conditions, is also exhibited by many aphid lineages [38,39]. In social aphids, caste polyphenism results in the production of soldier aphids which appear to defend their gall (nest) [40,45,46,47]. Lastly, aphid colour may depend on carotenoids, whose synthesis is strictly regulated by environmental factors [48,49]. Indeed, the synthesis of pigments in a given aphid clone is density- and frequency-dependent so that optimal conditions trigger a strong carotene synthesis (resulting in orange aphids), whereas a high population-density and cold temperatures produce a green pigmentation of aphids [28–50].

In contrast to the common thought that equates aphid clonality with molecular and genetic identity, aphids are therefore able to create a repertoire of variants with distinct behavioural and physiological traits and these "flexible" phenotypes contribute to their remarkable adaptations [28–50].

Even if the molecular machinery at the basis of most of these polyphenisms has been not fully understood, Dombrovsky et al. [50] clearly assessed that green aphids can also be obtained by treating parthenogenetic orange aphids with inhibitors of DNA methyltransferases. As a consequence of this treatment, many sites in the green variant genome were hypomethylated (whereas they were densely methylated in orange aphids) and the morph distribution was drastically modified. In view of the epigenetic basis, each of these variants (orange and green) can generate the other phenotype [50].

In aphids, DNA methylation was originally observed within the E4 esterase genes in insecticide-resistant strains of the peach potato aphids, *Myzus persicae* [51–52], where the amplified E4 esterase genes were highly expressed only when they were methylated [53]. At present, we know not only that 5mC is present in aphid genes (0.69% of all of the cytosines were methylated), but also that two copies of DNMT1, a DNMT2, a DNMT3 and a gene distantly related to the other DNMTs

(that has been called Dnmt3X since it lacks some key amino acids thought to be necessary for DNMT function) are encoded by the aphid genome [54]. Furthermore, several proteins involved in the recognition of DNA methylation (such as the methyl-CpG binding proteins MECP2 and NP95) are present in the aphid genome [54], overall suggesting that aphids have a functional DNA methylation system.

The analysis of the 5mC densities in the aphid genome showed that many methylated loci were associated to enhancers likely regulating gene expression from a long distance in the linear sequence and then acting closely to the promoters by chromosome folding [32].

As a whole this strongly suggests that covalent modification of DNA induced by the environment might have a broad effect on aphid genes by global modification of euchromatin/heterochromatin structure in chromosomes. Furthermore epigenetic stable marks might be transmitted through generations in clonality context and the sexual barrier in fall could preserve those that are advantageous for the wave of clonal individuals the next spring [32].

The trans-generational transmission of epigenetic marks is one of the most intriguing research fields not only in evolutionary biology, but also in medicine [55]. Aphids are therefore extremely intriguing models to explore this feature because their asexual phase generates an environment-oriented repertoire of variants, whose molecular bases can be properly studied. In particular, aphids can be used not only to study short-term maternal effects consisting of environmental information being passed from the mother to the first and second generations of progenies (three telescopic generations are co-existing in aphids: the mother, the embryos and the nascent embryos inside the mature ones), but also for long-term non-allelic heritability associated to extensive DNA methylation and orchestrated by the environmental pressures [32,56].

3. From a Single Genome to Castes: the Epigenetic Origin of Eusociality in Honey Bees

Division of labour is required to achieve biological complexity at all levels of organisation, from cells to organisms and to insect societies [57]. For example, in the evolution of multicellularity, cells aggregate to form higher-level individuality in multicellular organisms and perform specialised and mutually dependent roles [58]. Interestingly, at least in developmental terms, the transition from uni- to multi-cellular organization may be easy. For instance, in some bacteria [59], algae [60], and numerous myxobacteria, myxomycetes and cellular slime molds [61], the transition to multicellular organization is an inducible response to environmental stimuli mediated by epigenetic changes in some genes. Indeed, epigenetic modification of gene expression patterns, a hallmark of cellular differentiation in multicellular organisms, also characterizes many unicellular organisms [62]. Taking also into account that many of the developmental requirements for multicellular organization, including cell adhesion, cell-cell communication and coordination, and programmed cell death (PCD), existed in ancestral unicellular organisms [58], it could be “easy” to acquire multicellularity so that the evolution of multicellularity has been reconsidered as a “minor major” transition [58].

Likewise, in the evolution of eusociality, insects collaborate to form higher-level individuality in the form of the social colony, in which different insects perform specialised and mutually dependent roles as reproductive queens or non-reproductive workers [63]. Indeed, recent publications

assessed that DNA methylation, microRNAs and alternative splicing are involved in the regulation of queen and worker caste differentiation in social insects [18].

Recent experimental data evidenced that these evolutionary processes towards increased complexity have been governed by common epigenetic regulatory mechanisms suggesting that epigenetics has been recruited multiple times during evolution favouring the presence of very different levels of biological organization [63].

The developmental of castes in social insects can be described in terms of potency, in the same way as cell differentiation, such that totipotency is defined as an individual's ability to develop into any specialised phenotype (i.e. queen or worker) [63]. Even if this is almost true, actually highly eusocial species (like the honey bee *Apis mellifera*) differentiate early in larval development, whereas primitively eusocial species (such as *Polistes* paper wasps), retain the ability to switch castes/behaviours throughout adulthood [63].

As assessed by Patalano et al. [63] and Foret [64], honey bees and other social insects possess a complete set of DNMT [64–68], differently from what observed for instance in dipteran and coleopteran species where only genes coding for DNMT2 have been reported [23].

Functional assessments of the role played by the DNA methylation during the development of castes have been demonstrated in *A. mellifera*, where more than 500 genes showed a different cytosine methylation pattern in queens in respect to workers [69] and the silencing of genes coding for the DNMT3 resulted in the development of more queens than workers [70]. In particular, Shi et al. [71] demonstrated that DNMT3 knockdown caused demethylation of several genes, including the gene *dynactin p62* that is typically highly methylated in worker honeybees in respect to queens. Furthermore, after DNMT3 knockdown, emerging adults showed queen-like traits, both phenotypically (larger size, larger ovaries, and queen-like morphological traits) and in their methylation patterns strongly suggesting that DNA methylation played a direct causal role in honey bee caste determination. Furthermore, it has been suggested that the methylation pattern can also influence the splice site choice since caste-associated methylation patterns tend to be enriched in exon regions of genes involved in alternative splicing sites [69].

Interestingly, there is a close analogy between cell reprogramming to a totipotent state (such as the induced pluripotent stem cells in mammals) and caste reprogramming in social insects. Indeed, in primitively eusocial *Polistes* wasps, the loss (or the removal) of the queen results in the phenotypically reprogramming of an adult worker becoming a new queen [72,73] suggesting a functional similarity of the epigenetic mechanisms directing phenotypic plasticity and reprogramming in mammalian cells and social insects [63].

Hymenoptera are therefore intriguing experimental models not only for the study of the mechanisms (such as inclusive fitness) that favoured the success of eusociality from an evolutionary point of view, but also for the comprehension of the epigenetic mechanisms at the basis of castes, comparing eusocial, primitive eusocial and solitary species [64].

4. Conclusion

Variation of the phenotype is a central issue in biology because it is the basis for individuality, adaptation of populations to environmental fluctuations and the evolution of biodiversity [74,75].

Phenotypic variation can be produced by genetic differences, environmental influences and stochastic developmental events. The genetic component (including changes in cis-regulatory elements) and the stochastic developmental events are rather well investigated in insects. For instance, mutations in cis-regulatory sequences, such as enhancers and promoters, may affect development by altering gene expression contributing to phenotypic diversity within and between species [76]. Despite the limited number of mechanistic studies published to date [76], the analysis of how cis-regulatory elements can diverge addressed long-standing questions about the genetic mechanisms at the basis of the phenotypic evolution.

At present the role of epigenetics remained a largely untouched field of research for several years, but met the interest of the scientific community in the last two decades. In particular, the current emphasis on epigenetics is related to the possibility that epigenetics may drive the presence of new phenotypes over short evolutionary timescales, differently from the long time necessary for the selection of mutations. Even if mutations are the driving force of progressive evolution, epigenetics can drive the first appearance of new phenotypes also in the absence of mutation in the coding region and at the cis-regulatory elements.

Furthermore, epigenetics, as the term suggests, is a major turn away from molecular biology's Central Dogma, since it suggests that the flow of information moves from DNA to RNA and then on proteins. Differently, epigenetics makes possible inheritance systems through which non-sequence-dependent DNA variations can be transmitted in cell, tissue, and organismal lineages, using non coding RNA (such as microRNA) as effectors to transmit information to DNA. Thus, current epigenetics not only offers new insights into gene regulation and heredity, but it also profoundly challenges the way we think about evolution, genetics and development. Most interestingly, it suggests testable mechanisms whereby environmental factors can influence genetic expression in order to better understand the delicate interplay between genetic and environmental influences.

At present, different insect species have been studied at the epigenetic level (such as *Drosophila melanogaster*), but aphids and social insects seems to be ideal test grounds for studying the role of epigenetic mechanisms because they can derive multiple phenotypes (also including behavioural differences) from the same genome.

The ability to carry out controlled genetic crosses and other straightforward genetic manipulations (historically, the primary consideration for granting the "model organism" status) has been one of the greatest challenges for many eusocial insect species owing to difficulties in laboratory breeding and inherent limitations in generating large numbers of reproductive individuals. However, several specific eusocial species, such as the bee *A. mellifera*, the ant *Cerapachys biroi* and the wasp *Polistes metricus,* show a particularly unique potential either for the experimental generation of large numbers of reproductive individuals or for controlled crossing.

Differently from eusocial species, aphids do not have any limitation for breeding large populations in insectary due to their short generation time and their natural huge fitness, but may present some difficulties in laboratory crossing due to their parthenogenetic reproduction. However, a large repertoire of morphological variants can be observed in the parthenogenetic generations and crossing, even if difficult, is possible in laboratory for different species, such as *Acyrthosiphon pisum* and *M. persicae*.

Environmental factors can have long-lasting effects on gene expression and chromatin. Despite considerable progress during recent years, many questions remain since it is still largely unknown how the environment triggers alterations in the epigenome. Unravelling the underlying molecular mechanisms will be a daunting task, but aphids and bees could allow us to understand how the observed methylation and chromatin alterations are directly or indirectly linked to environmental factors that can be experimentally manipulated during their breeding and crossing.

For instance, aphid phenotype may be changed by environmental stimuli that regulate the presence/absence of wings and the colour patterning. The presence of clonal generations (due to the apomictic parthenogenetic reproduction of aphids) allows to study if aphids are capable to transmit across generations epigenetic marks as a signature of a transient and singular environmental episode. Furthermore, by a simple modification of the photoperiod in the insectary, aphids may generate a sexual generation so allowing to examine the transmission of a specific epigenetic mark in sexuales comparing the presence of epigenetic inheritance in clonality and sexuality. Lastly, DNMT inhibitors can be easily furnished by artificial diet so that it can be verified if observed chromatin alterations are directly linked to environmental stimulus and can be abolished inhibiting the DNA methylation machinery.

Similarly, the behaviourally and reproductively distinct queen and worker female castes derive from the same genome as a result of differential intake of royal jelly and are implemented in concert with DNA methylation. Adding specific miRNAs to royal jelly may help to better understand if microRNA mediates DNA methylation of target genes or if the regulation of the microRNA expression is mediated by DNA methylation better explaining the origin of castes. Lastly, the power of honey bees is likely to be in their rich source of general neurobiological principles, particularly in brain development and synaptic plasticity, the transferability of which can be examined in other nervous systems (including non-insects models).

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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