Published in final edited form as:

Trends Parasitol. 2013 June ; 29(6): 295–303. doi:10.1016/j.pt.2013.04.003.

Small RNAs: a new frontier in mosquito biology

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Abstract

The discovery of small non-coding RNAs has revolutionized our understanding of regulatory networks governing multiple functions in animals and plants. However, our knowledge of mosquito small RNAs are limited. We discuss here the state of current knowledge regarding the roles of small RNAs and their targets in mosquitoes, and describe the ongoing efforts to understand the role of the RNA interference (Randi) pathway in mosquito antiviral immunity and transposon silencing. Providing a clear picture into the role of small RNAs in mosquito vectors will pave the way to the utilization of these small molecules in developing novel control approaches that target mosquito immunity and/or reproductive events. Elucidation of the functions of small RNAs represents a new frontier in mosquito biology.

Keywords

microRNA; short interfering RNA; Piwi-interacting RNA; immunity; reproduction; blood digestion; virus

The small RNA world

The discovery of RNAi and small non-coding RNAs represents one of the most significant breakthroughs in molecular biology. RNAi is an evolutionarily conserved mechanism of sequence-specific gene silencing that is triggered by endogenous or invading double-stranded RNAs (dsRNAs) arising from hairpin structures, transposable elements (TEs), or virus infections. The finding that small dsRNA molecules control gene expression and viral infection has significantly altered our understanding of gene regulatory networks and antiviral immunity. In addition, RNAi has become a fundamental tool of gene knockdown for functional characterization, and raises hopes for the development of novel therapeutic

strategies for many human diseases and innovative insecticidal RNAi-based approaches for pest management.

The RNAi pathway in animals includes several branches that function in silencing endogenous genes, RNA viruses, and mobile genetic elements. The three classes of regulatory small RNAs in animals include: microRNAs (miRNA) (see Glossary), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) (Figure 1). Regulatory small RNAs are categorized based on their size and interaction with particular types of Argonaute (Ago) proteins. Typically in insects, 22 nt miRNAs are loaded into Ago-1, 21 nt siRNAs interact with Ago-2-containing effector complexes, and 24–31 nt piRNAs associate with the Piwi-subfamily of Ago proteins. Much of what is known about small RNA function and antiviral RNAi in insects is based on studies in the fruit fly, *Drosophila melanogaster*. However, as the genetic tools required for the study of small RNAs become established in non-drosophilid insects, more information regarding the function of small RNAs is becoming available in other organisms including mosquitoes (Table 1).

Mosquitoes are insect pests of high medical importance because they serve as vectors for many devastating human diseases, including malaria, Dengue (DENV) and yellow fever viruses, and West Nile virus (WNV). To control these disease vectors properly a deeper understanding of the molecular mechanisms underlying mosquito blood-meal processes, reproductive events, and anti-pathogen immunity is of paramount importance because these functions constitute an interconnected triangle that defines the ability of the female mosquito to transmit disease pathogens. Thus, unraveling small RNA function in mosquitoes is crucial to developing a complete picture of the regulatory processes of mosquito physiology and host—pathogen interactions, and may lead to the development of novel vector control strategies.

Mosquito miRNAs

MicroRNAs are approximately 22–23 nt non-coding endogenous RNAs that post-transcriptionally regulate transcript levels and the translational status of mRNA (Figure 1A). In insects, canonical miRNA loci are transcribed by RNA polymerase II to produce the primary miRNA (pri-miRNA) [1]. The pri-miRNA is processed in the nucleus by the microprocessor complex, consisting of the RNase III enzyme Drosha and a dsRNA-binding protein Pasha, liberating an approximately 70 bp stem—loop structure known as the precursor miRNA (pre-miRNA) [2,3]. The pre-miRNA is exported to the cytoplasm in a RanGTP/Exportin-5 dependent manner [4,5]. In the cytoplasm, another RNase III endonuclease, Dicer-1 (Dcr-1), and its dsRNA-binding partner, Loquacious (Loq), cleave the terminal loop structure of the pre-miRNA, releasing a ~22 nt miRNA duplex [6–8]. The duplex strands are sorted and loaded into an Ago-1 protein to complete its action on target sequences through the RNA-induced silencing complex (RISC). The RISC complex typically targets the 3′-untranslated region (UTR) of target transcripts, promoting translational repression and/or mRNA degradation [9]. More recently, several reports have revealed a novel mRNA—miRNA interaction that can lead to the stabilization of mRNA [10].

In the fruit fly, *D. melanogaster*, miRNAs have been identified as key regulators of many developmental processes, such as apoptosis, cell growth, metabolism, neural development, cell signaling pathways including WNT/Wg and Notch signaling, and many other important developmental processes [11]. Until recently, relatively little was known regarding the roles of miRNAs in other non-drosophilid insects. However, recent studies have indicated miRNA involvement in pea aphid (*Acyrtosiphon pisum*) and cockroach (*Blattella germanica*) reproduction [11], metamorphosis in the silkworm (*Bombyx mori*), brown planthopper (*Nilaparvata lugens*), and *Heliconius melpomene* [11], and silk production in the silkworm *B. mori* [11], and immune response in *Plutella xylostella* [12]. As with other non-drosophilid insects, knowledge of mosquito miRNAs is limited mostly to their identification using direct cloning and high-throughput sequencing approaches. The first experimental evidence of mosquito miRNAs was produced from direct cloning in *Anopheles gambiae* [13]. Additional experimental and high-throughput sequencing verified and expanded the list of *bona fide* conserved and species-specific miRNAs in various mosquito species [14–16]. Despite the ongoing efforts for mosquito miRNA discovery and expression profiling, the role of miRNAs in mosquito biology is largely unknown; however, recent studies into the function of mosquito miRNAs are revealing important roles of miRNAs in mosquito functions and anti-pathogen immunity (Tables 1 and 2).

Insights into miRNA regulation of mosquito blood digestion and egg development

Several miRNA library and functional analyses have provided clues to the regulation and function of miRNAs during mosquito blood digestion and ovarian development [13–15,17,18]. The effect of blood-feeding on miRNA expression in the mosquito midgut resulted in an induction of aga-miR-34, aga-miR-317, and the mosquito specific aga-miR-1174 and aga-miR-1175 miRNA cluster [13]. Another study reported that miR-989 was predominately expressed in the ovaries in *Anopheles stephensi* and the yellow fever mosquito *Aedes aegypti* [14]. Expression analysis of miRNAs in the *Ae. aegypti* blood-fed midgut also indicated that the majority of miRNAs are induced 24 h after blood-feeding [15]. Small-scale expression profiling experiments of conserved miRNAs in the *Ae. aegypti* fat body [17] and whole body mosquitoes [18] also showed upregulation of many miRNAs after blood-feeding. Differential expression of miRNAs post blood-meal indicates functions related to mosquito reproduction and blood-meal processes. Interestingly, DGCR8 (the mammalian Pasha homolog) was shown to work as a heme-binding protein [19]. When bound to heme, DGCR8 forms a highly stable complex and enhances pre-miRNA processing *in vitro*. The potential of mosquito Pasha to bind heme has not been characterized; however, the heme-binding ability of DGCR8 and contribution to miRNA turnover may shed some light into the induction of mosquito miRNAs post blood-meal (Box 1).

The first functional analysis of a mosquito miRNA was performed in *Ae. aegypti* on the conserved miRNA aae-miR-275 [17]. Expression profiling of aae-miR-275 in the female mosquito fat body suggested regulation by the steroid hormone 20-hydroxyecdysone (20E). Indeed, *in vitro* fat-body culture with 20E displayed an activated aae-miR-275 response. Inactivation of aae-miR-275 resulted in a dramatic defect in blood digestion in female mosquitoes. At 24 h post blood-meal, blood that had entered the midgut during feeding was regurgitated into the crop, an anterior portion of the digestive system normally used for

housing nectar (Figure 2). Blood in the crop and residual blood in the posterior midgut remained undigested, and as a result ovary maturation was severely inhibited. The exact role of aae-miR-275 in regulating digestive system processes and the aae-miR-275 targets contributing to this phenotype have not been identified. This study has raised a question concerning involvement of miRNAs in regulating the functions in female mosquitoes that are activated by blood-feeding (Box 1).

miRNAs play important roles in mosquito—pathogen interactions and innate immunity

MicroRNA expression patterns in female mosquitoes also change in response to pathogen infection [13,16]. In *An. gambiae* female midguts infected with the malaria model organism *Plasmodium berghei*, aga-miR-34, aga-miR-1174, and aga-miR-1175 displayed downregulation whereas aga-miR-989 exhibited significant upregulation [13]. Dcr-1 and Ago-1 knockdown in *P. berghei*-susceptible mosquitoes resulted in a substantial increase in *Plasmodium* oocyst numbers. In addition, polysome profiling of actively translated mRNAs in *An. gambiae* mosquitoes after *Plasmodium falciparum* infection revealed enhanced associations of Dcr-1, Dcr-2, and Drosha with polysomes [20]. These studies suggest that the miRNA machinery play a role in regulating mosquito response to infection by the malaria parasite.

In another study, differential expression analysis of miRNAs in *Aedes albopictus* C7/10 cells and *Culex quinquefasciatus* mosquitoes infected with WNV displayed downregulation of miR-989 and induction of miR-92 [16]. The role of these miRNAs and their putative targets in contributing to vector competence and anti-pathogen immunity has not been determined; however, differential expression of miR-989 during both *P. berghei* and WNV infection implies a function in mediating an immune response [13,16]. More recently, aae-miR-375 was detected in female mosquitoes post blood-meal [18]. Aae-miR-375 complementary target sites were identified and confirmed in the 5'-UTRs of the Toll immune pathway components *Cactus* and *REL1*. Female mosquitoes treated with mimic aae-miR-375 molecules resulted in downregulation of *Cactus* and upregulation of *REL1* transcripts. In addition, enhanced DENV infection was observed in *Ae. aegypti* Aag2 DENV-infected cells treated with an aae-miR-375 mimic, possibly due to an upregulation of Cactus, an inhibitor of NF- κ B transcription factor activation, thereby blocking the expression of innate immunity genes and antimicrobial peptides. MicroRNAs are known to target various signaling pathways at multiple levels; aae-miR-375 may also work to downregulate Rel1, further disrupting the Toll signaling pathway.

aae-miR-2940 and aae-miR-12 mediate Wolbachia infection

Successful introduction of the maternally inherited, endosymbiotic bacterium *Wolbachia pipientis* into *Ae. aegypti* mosquitoes results in decreased lifespan, reduced feeding success, and arthropod-borne virus (arbovirus) replication [21]. Although *Ae. aegypti* is not a natural host for *Wolbachia*, this is an excellent model system to characterize host—*Wolbachia* interactions. In *Ae. aegypti* mosquitoes infected with the *Wolbachia* strain wMelPop-CLA, 35 miRNAs displayed differential expression in response to this endosymbiont [22]. Two miRNAs, aae-miR-2940 and aae-miR-309a-2, were exclusively expressed in *Wolbachia*-infected mosquitoes. An aae-miR-2940 seed match was identified in the 3'-UTR of the

mosquito metalloprotease m41 ftsh transcript. Surprisingly, aae-miR-2940 induced the expression of m41 ftsh transcripts, perhaps through mRNA stabilization, and both aae-miR-2940 and metalloprotease were crucial for *Wolbachia* replication. In the same experiment, aae-miR-12 was upregulated in *Ae. aegypti* infected with wMelPop-CLA [23]. Two candidate target genes, DNA replication licensing factor MCM6 and monocarboxylate transporter MCT1, were bioinformatically and experimentally identified to contain aae-miR-12 complementary sites within their open reading frames (ORF). Inhibition of aae-miR-12 in *Wolbachia*-infected Aag2 mosquito cell lines resulted in a reduction of *Wolbachia* density.

Although these studies provide insight into miRNA and potential target function during *Wolbachia* infection, further work *in vivo* is required to confirm that these transcripts are in fact authentic miRNA targets contributing to phenotypes observed *in vitro*. The benefit of *Wolbachia* regulation of these miRNAs and the exact roles their targets play in host—*Wolbachia* interactions remain unknown and warrant further analysis. Interestingly, miR-12 was previously reported to arise from an intronic miRNA cluster containing miR-12 and miR-283 in *Ae. aegypti*, and miR-12, miR-304, and miR-283 in *An. gambiae* and *Drosophila* [14]. The role this miRNA cluster plays in regulating its host gene is unknown; however, these intronic miRNAs could work in parallel to regulate host gene expression and may play important roles in host-*Wolbachia* interaction.

Mosquito siRNAs

The siRNA pathway is subdivided into an endogenous and exogenous pathway, depending on the origin of the dsRNA trigger. Whereas endo-siRNAs are derived from the convergent transcription of overlapping genes [24–26] or structured loci [24,27], exo-siRNAs are processed from the dsRNA byproducts of virus replication or other foreign genetic material (Figure 1B). In flies, dsRNA is recognized and processed by the RNase III enzyme, Dicer 2 (Dcr-2) [28]. The resulting siRNA duplexes are then incorporated into the RISC. The siRNA duplexes contain a passenger strand, that will be removed from the activated RISC, and a guide strand that provides sequence specificity to the RISC. The guide strand directs Ago-2, an essential RISC component possessing endonuclease activity, to complementary RNAs in the cell, leading to their sequence-specific degradation or translational repression [29–31].

The role of antiviral RNAi in modulating arbovirus replication in mosquito vectors

It has been shown that the antiviral exo-siRNA pathway plays crucial roles in defense against RNA arboviruses in *Drosophila* and mosquitoes [32]. Virus-derived siRNAs have been detected in mosquitoes and/or mosquito cell lines infected with arboviruses possessing single-stranded (ss) positive-sense RNA [33–38], ss negative-sense RNA [39,40], and dsRNA genomes (Table 1) [39]. Although dsRNA virus replicative intermediates appear to be a common substrate for the production of these small RNAs [32,37,38], more recent work suggests secondary structures or, in the case of dsRNA viruses, the genomes themselves also may serve as a biogenic source of siRNAs [39,40]. Thus, the precise molecular pattern recognized and cleaved by Dcr-2 in the infected cell may be somewhat virus-specific (Box 1). To counteract host RNA silencing immune pathways, the genomes of many plant and

some insect viruses have evolved to encode proteins that suppress RNA silencing [41]. These virulence factors confer an evolutionary advantage to the pathogen. However, it remains unclear if mosquito-borne viruses encode similar proteins. Infections of the vertebrate host with these viruses are acute and are often associated with disease; however, the natural maintenance cycles of these pathogens generally require the establishment of persistent non-lethal infections in the mosquito host. The mechanism by which this occurs is not well understood, but an antiviral response directed by siRNAs may be important to the process (Box 1). RNAi-mediated knockdown of genes involved in the mosquito siRNA pathway indicate that Dcr-2, R2D2, and Ago-2 are crucial for restricting viral RNA accumulation, replication, and dissemination [42,43]. The expression of heterologous flock house virus (FHV) B2 proteins from recombinant alphaviruses also increases virus replication in mosquitoes and results in dramatic mortality [33,44]. The B2 protein of FHV is a well-characterized dsRNA-binding protein that interferes with RNA silencing by preventing protein components of the siRNA pathway from accessing the dsRNA byproducts of virus replication [45–47]. The pathogenesis associated with infection of mosquitoes with recombinant alphaviruses expressing B2 proteins is similar to an enhanced disease phenotype that has been described in *dcr-2* null mutant flies infected with various RNA viruses [33,48–50].

Similar to the *Drosophila* RNAi pathway, the mosquito RNAi machinery is undergoing rapid positive selection [51,52]. This suggests an evolutionary arms-race between mosquito-borne viral pathogens and their disease vectors (Box 1). The counteracting activities of antiviral immune responses and mechanisms of virus-mediated immune modulation may determine optimal equilibrium points conducive to pathogen transmission. Multiple mechanisms have been posited to explain how mosquito-borne viruses evade and/or suppress the RNAi pathway (Box 1). Virus-derived siRNAs have been found to map to ‘hot-’ and ‘coldspots’ in both alphavirus and flavivirus genomes [33,36,37]. Interestingly, high-frequency hotspot siRNAs derived from Semliki Forest virus (SFV) were found to have poor antiviral activity relative to the less frequent coldspot siRNAs, suggesting that arboviral genomes may have evolved so that siRNAs generated from highly targeted regions are less effective at inhibiting virus replication. However, whether or not this is a general feature of all arboviruses remains unclear, as does a molecular basis for the phenomenon. Recently, a non-coding subgenomic flavivirus RNA (sfRNA) produced by WNV and DENV was shown to interfere with RNAi activity in both insect and mammalian cells [53]. Although the results suggest that the flavivirus sfRNA may be a viral suppressor of RNA silencing (VSR), they were largely obtained by examining the effects of the isolated non-coding RNA on functional reporter constructs, an approach that may not reflect an authentic function for the sequence during viral infection. Studies involving isolated proteins or sequences and reporter constructs often exaggerate suppressor activity [54]. The role of the mammalian RNAi machinery in antiviral defense remains unresolved, leaving open the possibility that proteins or sequences found to possess suppressor activity in viruses naturally infecting mammalian cells actually evolved to counter interferon (IFN)-mediated antiviral responses, which are also induced by dsRNA. Thus, confirmation of the putative suppressor activity awaits experiments in which the replication of a sfRNA-deficient WNV or DENV is rescued in an RNAi null background.

A novel class of endogenous siRNAs

Although many questions remain regarding the role of the *exo*-siRNA pathway in the transmission of mosquito-borne viruses, even less is known regarding the role of *endo*-siRNAs in disease vectors (Box 1). The *Drosophila* *endo*-siRNA pathway has been reported to be involved in functions as diverse as TE silencing [25] and metabolism [55,56]. Recently, however, a novel class of transcript-derived *endo*-siRNAs was identified in *Ae. aegypti* mosquitoes infected with Sindbis virus (SINV) (Table 1) [57]. These siRNAs were derived from the entire length of the target transcripts and crossed exon-exon boundaries on the antisense strands, suggestive of biogenesis by an RNA-dependent RNA polymerase (RdRP) (Figure 3). Although there is little evidence for an RdRP in mosquitoes or other dipterans, an RdRP is encoded by the SINV genome. Because this class of *endo*-siRNAs was only observed in SINV-infected mosquitoes, and not in uninfected controls, it is possible the SINV-encoded RdRP is responsible for the conversion of the ss mRNAs into dsRNAs. However, further work is needed to determine if this is indeed the case, as well as any potential benefit to the infecting virus or mosquito host.

Mosquito Piwi-interacting RNAs

Piwi-interacting RNAs (piRNAs) are small RNAs 24–30 nt in length that are thought to arise from ssRNA precursors (Figure 1C) [58]. In *Drosophila*, the piRNA pathway functions in safeguarding the somatic and gamete genome of the ovary from TE activity, maintaining genome integrity and stability [58]. The pathway is Dcr-independent, but involves three members of the Piwi-subfamily of Ago proteins: Piwi, Ago3, and Aubergine (Aub) [59,60]. Biogenesis of piRNAs involves primary piRNA processing and a ‘ping-pong’ amplification pathway [59,60]. Primary piRNAs are derived from genomic loci termed piRNA clusters, in which ssRNA transcripts are believed to be cleaved by the ss-specific nuclease, Zucchini (Zuc), to generate an initial pool of piRNAs that are loaded into Piwi and target TEs [61,62]. The ‘ping-pong’ amplification is initiated through piRNA target sequences, in which Ago-3 and Aub amplify piRNAs in a slicer-dependent amplification loop. Ago-3 and Aub do not appear to be expressed in the *Drosophila* soma [59,60,63–65].

Piwi RNA function in TE silencing

As nearly half (47%) of the *Ae. aegypti* genome is comprised TEs [66], it seems plausible that piRNAs might be responsible for controlling their remobilization (Box 1). However, examination of *Ae. aegypti* small RNA libraries unexpectedly revealed a relatively low percentage (19%) of piRNAs mapping to TE sequences, by comparison to 50% of piRNAs in *Drosophila* [67]. Although a relatively poor annotation of the *Ae. aegypti* genome may account for some of the discrepancy, the results may also indicate that some aspects of TE regulation differ between *Ae. aegypti* and *D. melanogaster*. Mutations in Piwi-clade Ago proteins have been shown to increase expression of TEs in the fly ovaries [68,69]. Similar experiments will be necessary to clarify the role of piRNAs in regulating TEs in the mosquito germ line.

Novel piRNA-like small RNAs in antiviral immunity

No clear role for the *Drosophila* piRNA pathway has been established outside the germline. Although a simplified, alternative version (Piwi-dependent, but Aub- and Ago-3-independent) of the piRNA pathway has been demonstrated in the somatic cells that surround ovarian germ cells [69,70], piRNA pathways do not appear to operate more broadly in the fruit fly soma. Nevertheless, the identification of virus-derived piRNAs in a *Drosophila* ovary somatic sheet (OSS) cell line first suggested an antiviral role for piRNAs [71]. The existence of viral piRNAs in mosquito cells was first suggested by experiments profiling small RNAs during infection with various arboviruses, albeit only from the size range of the small RNA products (Table 1) [72,73]. Several studies subsequently reported production of virus-derived small RNAs exhibiting many similarities to ping-pong-dependent piRNAs in mosquitoes and cells infected with various arboviruses (Table 1) [74–76], including those with negative-strand RNA genomes [75,76]. Unlike the ping-pong-dependent piRNAs that had been described previously, from repetitive elements or piRNA clusters, production was demonstrated in the mosquito soma [74]. Further, these small RNAs were shown to be capable of modulating the pathogenesis of alphavirus infections in *dicer-2* null mutant mosquito cell lines defective in viral siRNA production [74], suggesting that the non-canonical piRNA pathway may provide robustness to the mosquito antiviral response (Figure 4 and Box 1). However, additional work is required to understand fully the role of piRNA pathway in mosquito–virus interactions.

Evolution of small RNAs in mosquitoes

Much of what is known about animal small RNAs stems from studies in *Drosophila*, *Caenorhabditis elegans*, and *Mus musculus*. Comparative analysis of small RNA loci, function, and RNAi effector proteins between these species has been vital in understanding the evolution of small RNAs in animals; however, aside from *Drosophila* studies, relatively little is known about the evolutionary diversity of small RNAs and their respective processing machineries in other insects, the most abundant animal group. Phylogenetic analyses of Argonaute family proteins demonstrated an expansion of *Ae. aegypti* and *Culex pipiens* Ago and Piwi subfamilies [77]. Ago-1 is represented as a single genomic locus in *An. gambiae*, *C. pipiens*, and *D. melanogaster*, whereas Ago-2 was also identified as a single locus in *Ae. aegypti*, *An. gambiae*, and *D. melanogaster*, but may be present in two copies in *C. pipiens* [77]. Two gene copies were previously reported for *Ae. aegypti* Ago-1, termed Ago-1a and Ago-1b [77]; however, alignment of the proposed Ago-1 isoforms revealed an extra sequence at the N-terminus in the putative B isoform but not in the A isoform, whereas the remainder of the Ago-1 amino acid sequences shared 100% similarity [17]. These results suggest different haplotypes instead of two individual genes for Ago-1. In addition, comparative analysis of piRNA pathway components suggests that the *Ae. aegypti* and *C. quinquefasciatus* genomes have undergone a significant expansion of piRNA pathway effector proteins (Ago-3, and 7 different Piwis) since diverging from *Drosophila* [77]. Interestingly, transcripts from the entire repertoire of Piwi-subfamily genes are expressed in the soma of virus-infected mosquitoes, although additional work will be necessary to determine if infection induces or upregulates transcription of any of these genes [74].

Genome-wide efforts in insect miRNA discovery and expression profiling have identified highly conserved lineage- and species-specific miRNAs in insect genomes [11]. These studies have demonstrated that a core set of miRNA families is conserved across highly divergent animal species including insects and mosquitoes in particular. These miRNAs exhibit a notable phylogenetic pattern of conservation across animal species, suggesting that miRNA families have accumulated throughout organismal evolution. Recently, miRNA libraries originating from *Episyrphus balteatus*, *Megaselia abdita*, *Tribolium castaneum*, *B. mori*, *Ae. aegypti*, *An. gambiae*, *C. quinquefasciatus*, and several *Drosophila* species have been used to provide a phylogenomic estimate of fly relationships [78]. In addition, many species- and lineage-specific miRNAs have been identified in the mosquito [13–15]. The mosquito-specific miRNAs, aga-miR-1174, aga-miR-1175, and aga-miR-989, are mid-gut-specific [13]. An adult female-specific miRNA, termed miRx2, is highly expressed in the mosquito ovaries post blood-meal, suggesting a possible role in mosquito reproductive event [14]. Although the exact roles of these miRNAs remain to be determined, these data suggest that lineage-specific miRNAs in mosquitoes have elusive roles in mosquito-specific events.

Antiviral RNAi proteins in *Drosophila* appear to be undergoing rapid adaptive evolution [51]. Components of the siRNAi pathway (Dcr-2, R2D2, Ago-2) are among the most rapidly evolving genes in *Drosophila*, whereas components of the miRNA pathway (Dcr-1, Loq, and Ago-1) do not exhibit significantly elevated rates of evolution [51]. This suggests an evolutionary arms-race with viral pathogens, in which adaptive mutations in the virus are countered with adaptations to the RNAi machinery. *Ae. aegypti* genes involved in the miRNA pathway also appear to be undergoing rapid, positive, diversifying selection [79]. In addition, similar to *Drosophila*, most of the positive selection is occurring in protein-coding regions of unknown function. Thus, it is possible that components of the mosquito miRNA pathway have evolved roles that differ from those of the *Drosophila* miRNA machinery.

Concluding remarks

With the advent of improved bioinformatic and genetic tools for the study of small RNAs in mosquito disease vectors, a greater understanding of the roles of small RNAs in these medically important insects is beginning to emerge. These studies highlight the importance of small RNAs during the adult mosquito life cycle and antiviral immunity. Although miRNAs have been heavily studied in model organisms with well-established classical genetic and transgenic tools, until recently little work had been done investigating their role in non-drosophilid insects and in insect development. However, limited genetic tools and a paucity of computational approaches to unravel miRNA function and identify targets remain a severe hindrance to understanding the role of miRNAs in mosquito biology. Steps must be taken to provide and enhance important tools needed for vector biologists to study complex gene regulatory pathways involving miRNAs and other regulatory small RNAs. The use of miRNA inhibitors [17,22] and miRNA mimics [18] has been successful in identifying functions of several mosquito miRNAs. However, the newly established transgenic GAL4—UAS system in *Ae. aegypti* [80] and *An. gambiae* mosquitoes [81] will allow the utilization of the miRNA sponge [82] or Tough decoy RNA [83,84] inhibition technologies, or the ectopic over-expression of miRNA hairpins, to evaluate spatiotemporal functions of

miRNAs in mosquitoes. These invaluable tools for the study of mosquito miRNAs will further enhance miRNA functional studies in the mosquito.

Despite the presence of efficient antiviral RNAi in mosquitoes, arboviruses are still adept at establishing persistent infections in vector species, permitting viral replication, dissemination, and transmission. Thus, understanding the small RNA pathways targeting viruses in the mosquito, and the mechanisms by which viruses modulate these pathways, promises substantial new insights into the transmission of mosquito-borne viruses.

Glossary

Arbovirus

a group of viruses that are transmitted by arthropod vectors

Argonaute (Ago)

the core component of RISC. Argonaute proteins are highly conserved between species, and many organisms encode multiple members of the family, which are subdivided in animals into the Argonaute (Ago) and Piwi clades. In insects the Ago subfamily consists of Ago1 and Ago2, both of which play functional roles in the miRNA and siRNA pathway. The Piwi clade of Argonaute proteins involves Piwi, Aubergine (Aub), and Argonaute3 (Ago3) and are associated with piRNA biogenesis and function

Dicer (Dcr)

an RNase III endonuclease that processes long dsRNAs and precursor miRNAs into siRNAs and miRNAs, respectively. In insects there are two genes in the Dicer family, Dicer-1 and Dicer-2, which play functionally distinct roles in the siRNA/miRNA pathway

MicroRNAs (miRNA)

small 22 nt endogenous non-coding RNAs that regulate gene expression at the post-transcriptional level by typically targeting the 3'-untranslated region of mRNAs, resulting in translational inhibition and/or mRNA degradation

MicroRNA Sponge

RNA molecules with tandemly arrayed miRNA antisense sequences that can sequester miRNAs from their endogenous targets. Stably expressed miRNA sponges are valuable to achieve spatiotemporal miRNA loss-of-function

Pasha/DGCR8

a dsRNA-binding protein in insects that forms the Microprocessor complex with the RNase III enzyme Drosha to cleave primary miRNAs, liberating a 70 nt stem-loop structure known as the precursor miRNA. The mammalian Pasha homolog, DGCR8, has been shown to work as a heme-binding protein

Piwi-interacting RNAs (piRNA)

small RNAs 24–30 nt in length that are thought to arise from ssRNA precursors derived from TEs, intergenic regions, and some protein-coding genes. piRNAs function in

preserving genome integrity and stability but have recently been identified to play a role in antiviral immunity

RNA-dependent RNA polymerase (RdRP)

an enzyme that catalyzes the copying of RNA from an RNA intermediate. RdRP genes appear to have been lost in many animal species, including *Drosophila* and mosquitoes, but are encoded by many viruses and are essential for RNA virus replication

RNA-induced silencing complex (RISC)

a multiprotein complex that incorporates one strand of siRNA or miRNA duplex, using the siRNA or miRNA as a template for recognizing complementary mRNA, resulting in mRNA degradation or translational inhibition

Small interfering RNAs (siRNAs)

small 21 nt non-coding RNAs derived from endogenous loci or foreign genetic material that work to silence invading viruses, TE activity, and some protein-coding genes

Tough decoy RNA

a hairpin-shaped decoy RNA containing two miRNA recognition sites that compete with a miRNA target, leading to long-term miRNA loss-of-function

Viral suppressors of RNAi (VSR)

a countermeasure adopted by viruses to suppress the antiviral RNAi response by producing proteins or RNA decoy mechanisms that block the generation or activity of viral siRNAs

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Box 1**Outstanding questions**

- What roles do miRNAs play in regulating functions activated by blood-feeding in female mosquitoes?
- Does mosquito Pasha share a similar function to the ability of mammalian DGCR8 to bind heme, and does this characteristic contribute to increased miRNA turnover in female mosquito after blood-feeding?
- What regulatory mechanisms link mature miRNA expression with blood-feeding in mosquitoes?
- Can mosquito-specific miRNA be utilized for developing novel approaches for mosquito control?
- What mechanisms are responsible for generating the biogenic ‘hotspots’ present in the profiles of small RNAs mapped back to infecting viral genomes, and what is their biological significance?
- What is the role of small RNA pathways in the establishment and maintenance of life-long, persistent, non-lethal infections of the vector host by mosquito-borne viruses?
- How do mosquito-borne viruses modulate the RNA-based immune response of the vector to maintain effectively their natural transmission cycles?
- What roles do mosquito-specific small RNA pathways play in controlling the activity of TEs and regulating gene expression?

Figure 1.

Three main classes of regulatory small RNAs. **(A)** miRNAs are transcribed by RNA polymerase II (RNA pol) to yield a primary miRNA (pri-miRNA) transcript, which is cleaved by Drosha to produce the precursor miRNA (pre-miRNA). These pre-miRNAs are exported from the nucleus to the cytoplasm, where they are further processed by Dcr-1 to generate an miRNA and miRNA* duplex. Once loaded into the RNA-induced silencing complex (RISC), predominately Ago-1-associated RISC, the miRNA strand guides translational repression and/or mRNA degradation of target mRNAs. **(B)** The siRNA pathway is subdivided into an endogenous and exogenous pathway, depending on the origin of the dsRNA trigger. Endo-siRNAs are derived from the convergent transcription of overlapping genes and structural loci; exo-siRNAs are processed from the dsRNA byproducts of virus replication or other foreign genetic material. dsRNAs derived from these sources are cleaved by the RNase III enzyme, Dcr-2, generating siRNA duplexes. These duplexes are then incorporated into a RISC. After removal of the passenger strand from the activated RISC, the remaining guide strand directs Ago-2 to complementary RNAs in the cell, resulting in their degradation. **(C)** piRNAs are transcribed from genomic loci termed piRNA clusters. Cleavage of these ss transcripts by Zucchini (Zuc) generates an initial pool of primary piRNAs, which are loaded into Piwi. Secondary piRNAs are generated in a

slicer-dependent amplification loop. Piwi- or Aub-bound piRNAs direct the cleavage of target strands, generating piRNAs that will be bound by Ago-3 and vice versa.

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Figure 2. miRNA-275-depleted *Aedes aegypti* female mosquitoes. Antagomir depletion of miR-275 drastically affects the ability of the female mosquito to digest blood. Female mosquitoes injected with a miR-275 specific antagomir (miR-275-ant) at day 1 post-eclosion displayed large portions of blood in the crop (Cr), an anterior portion of the digestive system normally used for housing nectar. Blood in the crop and residual blood in the posterior midgut (Mg) remained undigested (modified from [15], with permission).

Figure 3.

A novel class of mRNA-derived endo-siRNAs induced by virus infection. **(A)** Endogenous siRNAs 21 nt in length (shown in grey) derived from genes in uninfected *Aedes aegypti* mosquitoes do not cross exon-exon boundaries and are less abundant near these junctions (vertical broken line), ruling out the involvement of an RNA-dependent RNA polymerase (RdRP) in the biogenesis of these products. **(B)** In *Ae. aegypti* mosquitoes infected with Sindbis virus (SINV), novel 21 nt siRNAs mapping the length of the target transcript are detected. These siRNAs cross exon—exon boundaries (shown in black) on the antisense target strand, suggesting the involvement of an RdRP in their biogenesis.

Figure 4.

RNA silencing pathways implicated in mosquito antiviral immunity. Replication of arboviruses in mosquito cells results in the production of dsRNA from a potentially diverse array of sources. The RNase III enzyme, Dcr-2, is believed to recognize these pathogen-associated molecular patterns (PAMPs) to generate siRNA duplexes. The putative pattern-recognition receptor (PRR) and PAMP responsible for induction of the non-canonical piRNA pathway has not yet been identified. However, small RNAs generated by both pathways are believed to associate with members of the Argonaute family of proteins. Although guide strands derived from the siRNA duplexes are thought to direct the Ago-2-containing RNA-induced silencing complexes (RISCs) to complementary viral RNAs in the cell leading to their degradation, virus-derived piRNAs are believed to associate with Piwi-subfamily Ago proteins (Ago-3; Piwi 1-7), or a subset of these proteins, initiating a slicer-dependent amplification loop, which may also be restrictive to virus replication. How the two pathways are coordinated is not currently understood.

Table 1

Major discoveries of small RNA function in mosquito

Class	Putative function	Species	Refs
miRNA	Blood-feeding processes	<i>Aedes aegypti</i>	[13,15,17,18]
	Reproductive processes	<i>Anopheles stephensi</i> and <i>Aedes aegypti</i>	[14]
		<i>Aedes aegypti</i>	[17]
	<i>Plasmodium</i> infection	<i>Anopheles gambiae</i>	[13,14]
	Viral infection	<i>Aedes albopictus</i> and <i>Culex quinquefasciatus</i>	[16,18]
	<i>Wolbachia</i> infection	<i>Aedes aegypti</i>	[22,23]
Exogenous siRNA	O'yong nyong virus infection	<i>Anopheles gambiae</i>	[42]
	Sindbis virus infection	<i>Aedes aegypti</i>	[33,43,44]
	West Nile virus infection	<i>Culex quinquefasciatus</i>	[73]
	Dengue virus infection	<i>Aedes aegypti</i>	[34,35]
	Semliki Forest virus infection	<i>Aedes albopictus</i>	[38]
Endogenous siRNA	Sindbis virus infection	<i>Aedes aegypti</i>	[57]
piRNA	West Nile virus, Sindbis virus, and La Crosse virus infection	<i>Aedes albopictus</i>	[73,75]
	Dengue virus infection	<i>Aedes aegypti</i>	[72]
	Rift Valley fever virus infection	<i>Aedes albopictus</i>	[76]
	Chikungunya virus infection	<i>Aedes aegypti</i> and <i>Aedes albopictus</i>	[74]

Table 2 Known functions and targets of *Aedes aegypti* microRNAs

miRNA	Target	Function	Ref.
aae-miR-275	Unknown	Blood digestion and ovarian development	[17]
aae-miR-375	<i>Cactus</i> ; <i>REL1</i>	Viral immunity	[18]
aae-miR-2940	<i>m41 ftsH</i>	<i>Wolbachia</i> infection	[22]
aae-miR-12	<i>MCM6</i> ; <i>MCT1</i>	<i>Wolbachia</i> infection	[23]