Toward silencing the burden of malaria: progress and prospects for RNAi-based approaches

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The discovery of RNA interference (RNAi) is one of the most significant of recent years, with potential for application beyond the laboratory to the clinic. As a tool for functional genomics, RNAi has permitted the characterization of genes in organisms that had previously remained recalcitrant to targeted gene manipulation. Efforts to understand its mode of action have revealed a central role in gene regulation and host defense. Finally, as a therapeutic tool, it has shown enormous promise in the control of a large array of diseases. Here we examine how RNAi is revolutionizing malaria research in an organism, the Anopheles mosquito, that until recently was essentially resistant to genetic study, and show how its application in both the mosquito vector and the Plasmodium parasite might ultimately lead to new ways of controlling and perhaps even eradicating this devastating disease.

THE BURDEN OF MALARIA

Despite intense efforts, malaria remains a leading cause of morbidity and mortality worldwide. The World Health Organization estimates that nearly half a billion clinical cases of malaria occur each year, with over one million deaths (1,2). Almost 90% of these deaths occur in sub-Saharan Africa, where the risk of severe and potentially fatal Plasmodium falciparum infection is highest in young children. Recent estimates suggest that without immediate action, the number of malaria cases is likely to double over the next two decades (3). This resurgence is the result of a combination of factors, such as the breakdown of existing control programs and health infrastructure and the rapid spread of resistance to the most effective and affordable antimalarial drugs and insecticides used to control the parasite and mosquito vector populations, respectively. Therefore novel means of controlling malaria, based on integrated control strategies and innovative approaches, have been proposed. These will require an exquisite understanding of the biology of the malaria parasite and the interactions that occur between it, the mosquito vector, and the vertebrate host.

EXPLOITING WEAKNESSES IN THE PLASMODIUM LIFE CYCLE FOR MALARIA CONTROL

The life cycle of the malaria parasite (Figure 1) involves a complex relationship with a vertebrate host, in which it undergoes an asexual stage of development, and an Anopheles mosquito vector, where its sexual development occurs. This relationship has been honed over millennia of co-evolution to enable the parasite to partially evade both the human and insect immune systems and ensure its own survival (4). Infection starts with a bite of an infected mosquito, when the motile sporozoite is introduced into the skin together with mosquito saliva. The sporozoite enters the bloodstream and is rapidly carried to the liver where it invades a hepatocyte. Within the hepatocyte, the parasite undergoes a period of differentiation and multiplication to produce the pre-erythrocytic schizont, containing thousands of merozoites. The merozoite, when released from the hepatocyte, enters the bloodstream and invades an erythrocyte, differentiating through trophozoites to an erythrocytic schizont. This contains a small number of merozoites which, when released, go on to invade new erythrocytes. After a number of intra-erythrocytic cycles, a proportion of merozoites undergo sexual differentiation to produce male and female gametocytes, which are then picked up in the blood meal of an Anopheles mosquito. Once inside the midgut lumen, the gametocytes start to differentiate almost immediately. Fertilization between the microgamete and macrogamete produces a zygote that slowly elongates into a motile, invasive ookinete. The ookinete passes through the peritrophic matrix that encases the blood bolus and traverses the midgut epithelium. Upon reaching the basal lamina, the parasite transforms itself into an oocyst that bursts after approximately 7 to 16 days, depending on the parasite species and temperature, releasing thousands of sporozoites into the hemocoel. These sporozoites then specifically recognize and invade the mosquito’s salivary glands, and upon the next blood feeding are injected into the vertebrate host, initiating a new cycle of infection.

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The successful completion of this cycle is a fine balance between host factors, the agonists, which promote parasite development, and include molecules on the surface of both the mosquito and vertebrate host tissues that function as receptors for parasite surface ligands and the antagonists that negatively regulate it, such as components of the mosquito and vertebrate immune systems. While agonists and antagonists to Plasmodium development in the vertebrate host have generally been well characterized, due in part to the ability to culture P. falciparum in vitro (5–7) and the existence of the genetically tractable Plasmodium berghei murine model of malaria (8), relatively little was known of the molecular biology of the mosquito vector until recently. The urgent need, however, to address the resurgence of malaria has prompted the development of efficient molecular and genetic tools for Anopheles mosquitoes over the past few years, aimed at a better understanding of the genetic basis of mosquito-pathogen interactions. This effort has led to the achievement of a number of prominent technological milestones, including the development of an efficient germline transformation system for Anopheles stephensi (9), an important urban vector species in Asia, and Anopheles gambiae (10) mosquitoes, the principal African malaria vector, and the parallel completion of the A. gambiae genome sequence (11). The adaptation of RNAi techniques to Anopheles mosquitoes (12,13) has since provided the missing link to perform functional studies in the mosquito vectors of human malaria, that could ultimately provide an important contribution to vector control strategies. One of the driving forces behind these studies is the idea that mosquitoes can be genetically engineered to be refractory to pathogen development, and with an effective genetic drive mechanism, a pathogen-susceptible mosquito population may be replaced with a refractory one (14,15).

It has become increasingly apparent that intrinsic (genetic) factors govern the ability of a vector to support parasite development. Biological evidence for this is compelling, for among the hundreds of anthropophilic Anopheles species, only about 70 are malaria vectors, and only about 40 of these are regarded as important (16). Identifying which genetic factors shape the vector-parasite relationship may be crucial to identifying new genetic means of controlling mosquito-borne diseases (reviewed in Reference 17). Recent evidence strongly suggests that RNAi can play a key role in this.

### Table 1. Impact of RNAi-Mediated Knockdown of Mosquito Genes Upon Plasmodium berghei Development

<table>
<thead>
<tr>
<th>Gene Class</th>
<th>Target Gene</th>
<th>Effect Upon Oocyst Development</th>
<th>Effect Upon Parasite Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
<td>LANB2 (laminin y1)</td>
<td>58.8% decrease</td>
<td>Not stated</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>CTL4</td>
<td>96.5% decrease</td>
<td>94.1% decrease</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CTLMA2</td>
<td>56.5% decrease</td>
<td>50.2% decrease</td>
<td>20</td>
</tr>
<tr>
<td>Antagonist</td>
<td>TEP1</td>
<td>5-fold increase</td>
<td>Not stated</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>LRIM1</td>
<td>70.9% increase</td>
<td>70.8% increase</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>SRPN2</td>
<td>Not stated</td>
<td>97% decrease</td>
<td>22</td>
</tr>
</tbody>
</table>

RNAi, RNA interference.
regulators of the prophenoloxidase (PPO) activation pathway that invokes the melanization reaction have also recently been shown to be indirectly protective of the parasite within the midgut (22). The RNAi-mediated knockdown of the serine protease inhibitor (serpin) SRPN2, speculated to be an inhibitor of a PPO-activating enzyme, for example, reduced parasite prevalence by some 97% as a result of increased ookinete lysis and melanization (22).

Although no satisfactory model has yet been proposed to explain how these agonists and antagonists are mutually regulated and their effects coordinated in response to Plasmodium infection, recent evidence seems to indicate a role for NF-κB-like transcription factors in this process. REL2-F, the A. gambiae ortholog of Drosophila Relish (23,24), for example, appears to coordinate a number of the anti-Plasmodium immune responses in A. gambiae, including the expression of LRIM1 (25). It might be that a series of interconnected signaling pathways regulate a specific and coordinated repertoire of responses against Plasmodium.

These examples described above (summarized in Table 1) clearly show how RNAi technologies can be exploited to unravel the molecular processes underlying Plasmodium development within the mosquito. As success or failure of parasite transmission is seemingly dependent upon a fine balance between positive and negative mosquito factors (26), any alteration to this equilibrium should consequently affect parasite development and thus could be utilized for malaria control, for instance by rendering a previously competent mosquito species refractory to Plasmodium transmission.

It should be noted, however, that to date these RNAi studies have used the P. berghei mouse malaria model. Molecular factors that determine the development of the human malaria parasite, P. falciparum, might and probably do differ from those of the mouse parasite (27). It will be crucial over the coming years to extend studies of parasite-vector interactions to the human malaria parasite, P. falciparum.

But could RNAi itself be utilized as a transmission blocking approach? Certainly the results of the functional genomic studies completed thus far suggest that loss-of-function of mosquito factors that are essential for parasite development can negatively affect Plasmodium development, such that this approach could be used to prevent transmission.

RNAi FOR MALARIA CONTROL: THERAPY OR FALLACY?

RNAi can, and indeed has been made inheritable in Anopheles mosquitoes by stably transforming the mosquito with a transgene that contains two copies of the target gene arranged in an inverted repeat configuration (13). Hairpin RNA is expressed in vivo whenever the inverted repeat is transcribed from an upstream promoter (Figure 2). By placing dsRNA expression under the control of a tissue- and time-specific promoter, dsRNA expression can be tailored to coincide spatially and temporally with the journey of the parasite through the mosquito. The cell autonomous nature of transgenic RNAi in Anopheles (13), implies that the loss-of-function phenotype would only be observed in those cells where target gene and dsRNA are co-expressed, avoiding the pleiotropic effects of the loss of that gene in other non-target tissues. Conceptually, both parasite receptors and immune components protective of the parasite are putative targets for engineering parasite resistance through RNAi and, in principle, mosquito strains that have been rendered refractory to malaria transmission could be released in the field to replace wild-type, permissive popula-

Figure 1. The life cycle of Plasmodium falciparum. Sporozoites injected by an Anopheles mosquito into the vertebrate host invade liver cells. Here the sporozoite differentiates to produce a pre-erythrocytic schizont, containing thousands of merozoites. These are released into the bloodstream where they invade erythrocytes, differentiating through trophozoites to produce an erythrocytic schizont. These contain a few merozoites that, when released, invade new erythrocytes and repeat the erythrocytic cycle. After a number of intra-erythrocytic cycles, a proportion of merozoites terminally differentiate into gametocytes and are acquired by the mosquito upon blood-feeding. Within minutes, gametocytes develop into gametes that fuse to form a zygote. After approximately 24 h, the motile ookinete invades the midgut epithelium and, upon reaching the basal lamina, differentiates into an oocyst. After approximately 16 days, the oocyst ruptures, releasing thousands of sporozoites into the haemoceol (gray). The sporozoites then migrate to the salivary glands and enter the salivary duct lumen from where they are injected into the vertebrate host upon the next blood-feeding. The average number of Plasmodium parasites at each developmental stage (red) (56) and host tissues (upper case) are shown.
Figure 2. A versatile RNA interference (RNAi) toolbox for Anopheles mosquitoes. Double-stranded RNA (dsRNA) homologous to the target gene is delivered by either of two methods: (A) injection of in vitro-transcribed dsRNA into individual adult mosquitoes or (B) by the expression of dsRNA in situ from a stably integrated transgene that contains two copies of the target gene arranged in an inverted repeat (IR) configuration. Thus, hairpin RNA is expressed in vivo whenever the IR is transcribed from an upstream promoter. By using promoters that strictly control gene expression in a tissue- and time-specific manner, dsRNA expression can be tailored to coincide precisely with the development of Plasmodium within the mosquito. Transgenic RNAi can be further enhanced by making its expression conditional. Here, for example, the IR is coupled to a promoter that is activated only upon Plasmodium infection. In uninfected mosquitoes (C) its expression remains absolutely quiescent, and target gene expression is unaffected. (D) Infection with Plasmodium triggers dsRNA expression and concomitant silencing of the target gene. No such Plasmodium-activated promoters, however, have yet been characterized. (i) dsRNA delivered by either means is cleaved into small interfering RNAs (siRNAs) by Dicer (red). The siRNA/Dicer complex is then recruited by the ribonucleotide protein complex (RNP; blue) and transferred to the RNA-induced silencing complex (RISC; purple). (ii) RISC becomes activated by unwinding of the siRNA duplex by an RNA helicase. (iii) The sense strand dissociates from RISC and is replaced by the target messenger RNA (mRNA) complementary to the antisense siRNA strand. A nuclease activity (not shown) then cleaves the mRNA, leading to posttranscriptional silencing of the target locus. P, promoter; nt, nucleotides.

It is however wise to be cautious. First, as protein expression by RNAi is not generally suppressed entirely, a complete blockade of transmission is unlikely to be achieved, since a single ookinete succeeding in evading immune clearance and developing into an oocyst is competent to produce hundreds of potentially infective sporozoites, each of which is capable of producing clinical malaria. Second, no single gene appears sufficient on its own to eliminate transmission. Considering the highly inbred nature of the mosquito strains commonly used in laboratory experiments when compared to the highly polymorphic field populations, where susceptibility or refractoriness to Plasmodium transmission is possibly determined by a more complex combination of factors, a combinatorial approach targeting multiple genes would probably be needed. Furthermore, in areas where multiple parasite species are present, this approach would have to target all Plasmodium species that infect humans, and their respective mosquito vectors, to have any significant impact upon malaria transmission.

A further concern is whether the effects of silencing from stably integrated transgenes wane over time. There is increasing speculation that this occurs with some integrated transgenes, meaning the efficacy of transmission blocking would be compromised. Furthermore, the RNAi transgene might itself be subject to selection pressure if its expression negatively affected the fitness of transgenic mosquitoes (28). The RNAi gene would consequently be lost within the space of a few generations and would not be effectively disseminated throughout a field population. Indeed there is already some evidence of fitness costs associated with the knockdown of immunomodulators like SRPN2, whose loss-of-function phenotype results in the spontaneous deposition of melanin and decreased mosquito survival (22). Negative selection pressures like these could, however, theoretically be limited by conditionally expressing the RNAi gene: an advantage over relying on gene-replacement technologies that would permanently knockout target gene expression. One possible means of doing this would be to couple the RNAi gene to a promoter activated only during Plasmodium infection, such that at all other times it is quiescent and the mosquito is effectively wild-type (Figure 2). Such a Plasmodium-induced promoter, however, remains to be identified. In the mean time, mosquito promoters that are time- and tissue-specific are instead utilized to drive transgene expression in organs relevant to parasite development. A battery of midgut-specific promoters, like that of the regulatory region of the carboxypeptidase gene (29), whose
expression is induced in the gut of female mosquitoes following a blood meal, have been identified and tested in transgenic mosquitoes (30,31). The characterization of salivary gland-specific promoter regions (32–35) will also be useful to prevent parasite development in these tissues and hence block transmission to the vertebrate host (Figure 2).

An alternative to replacement strategies with mosquitoes impaired in their capacity to transmit malaria is population eradication achieved through the release of genetically sterile males. Already proven as an effective means of eliminating the New World screwworm, Cochliomyia hominivorax, from the southern states of the USA, Mexico, and all of Central America (36), this approach (the Sterile Insect Technique; SIT) relies exclusively upon the release of large numbers of sexually competent but genetically sterile males over large areas. Mating of released sterile males with wild females is nonproductive, such that if large numbers of males are released over a sufficient period of time, local eradication or suppression of the mosquito population will ensue. The number of sterile males released depends on many factors, including the presence or absence of a genetic system to drive sterility into field populations, but in general, releases in the range of a 10:1 excess of released material compared to field populations are performed. Crucially, the release of females must be avoided as it would not only fail to contribute to population suppression, but also contribute to disease transmission. In previous release programs involving anopheles mosquitoes, separation of the two sexes has been achieved by complicated or inefficient procedures based upon the size of the pupae and on pseudo-linkage of sex chromosomes to insecticide resistance alleles. It has been proposed that RNAi could be utilized to facilitate the genetic sexing procedure by, for instance, targeting sex-determining genes to produce a male-only population. Male sterility could then be achieved by irradiation. The feasibility of RNAi to generate such genetic sexing strains has already been demonstrated in the Mediterranean fruitfly, Ceratitis capitata, where targeting of the key transformer gene of its sex determination pathway resulted in a strong sex ratio bias in favor of male individuals (37). Genetic regulation of sex determination in Anopheles remains largely uncharacterized but, given its conservation between Diptera (38–40), a combination of comparative genomics and functional RNAi studies should lead to the identification of potential genetic targets for the creation of male-only populations. Recently, the A. gambiae homolog of doublesex, the final double-switch gene in the somatic sex determination cascade, has been isolated and characterized (41). Manipulation of this gene could thus be a possible means of generating genetic sexing strains in Anopheles. Importantly, the promoter region of the β2-tubulin gene from A. gambiae has been identified and shown to drive the sperm-specific expression of an enhanced green fluorescent protein (EGFP) reporter in A. stephensi mosquitoes from early stages of development (42). Thus, this promoter may be a candidate to drive the expression of dsRNAs specifically in the mosquito’s testes in order to target key spermatogenesis genes and induce male sterility.

**dsRNA: A DESIGNER ANTIMALarial?**

In the future, a more practical use of RNAi with therapeutic value in the fight against malaria may be emerging. The widespread resistance to common anti-Plasmodium drugs is urging the development of new antimalarials and effective vaccines (43). Current antimalarials act on a limited number of biological targets, and the next challenge is to identify new classes of drugs that attack novel molecular targets and whose therapeutic lifespan is not compromised by the rapid development of resistance. However, little information on the function of different Plasmodium proteins is available, slowing the identification of appropriate therapeutic targets and hence the development of effective drugs for malaria control (44). Gene targeting by homologous recombination is routinely achieved in Plasmodium species (45) but is necessarily time-consuming, preventing large-scale multi-target screenings. RNAi, in combination with the genome sequence of P. falciparum (46) however, may allow the possibility of reinvigorating antimalarial drug design. Electroporation of dsRNA into erythrocytic cultures of P. falciparum in vitro would deliver a fast and efficient means to study gene function in the malaria parasite and determine which factors are required for parasite growth, providing crucial information for selecting new chemotherapeutic targets. Whether true RNAi phenomena exist in Plasmodium however, remains a highly contentious issue (47,48). While the few publications to date have all described characteristic RNAi-like silencing phenomena [loss-of-function-like phenotypes, reduction in cognate messenger RNA (mRNA) levels, and the presence of approximately 25 nucleotide RNA species] (49–54), there is no genetic evidence to support its existence in Plasmodium. Unlike Trypanosoma brucei and some other protozoa, P. falciparum possesses no significant homologs of the RNAi pathway genes Dicer, Piwi, PAZ, or RdRP within its published genome sequence (48,55). Thus, it is impossible to discount that the gene silencing effects observed in the RNAi studies undertaken to date are the result of antisense effects. Nevertheless, the urgent need to develop new therapeutics for malaria control warrants a thorough assessment of the possible application of novel technologies like this.

In recent years, the use of small interfering RNAs (siRNAs) has been shown to hold great promise in gene therapy, attracting major interest from biopharmaceutical companies. A new class of therapeutic RNAi drugs are thus being developed that can target genes previously considered inaccessible to conventional therapeutics. While this approach holds great promise for the treatment of challenging human diseases, such as cancer, neurodegenerative diseases, and chronic infections, its use for malaria therapy is limited by the acute nature of this disease, which requires the use of fast-acting drugs. In the sole in vivo study published to date, injection of P. berghei-infected mice with siRNAs targeting the P. berghei...
cysteine protease berghepain resulted in its specific down-regulation (54). Injections resulted in only approximately 0.01% of the siRNA being internalized into the parasite, and the observed 40%–50% reduction in berghepain mRNA levels did not alter the parasitemia of the siRNA-treated mice. More studies will be needed to elucidate the mechanisms of gene silencing observed in *Plasmodium* and to assess the therapeutic potential of RNAi in this important parasite.

**PERSPECTIVE**

With genome-wide siRNA studies being instigated in hepatocytes in culture to dissect the molecular requirements of sporozoite invasion (www.cnix-bioscience.com/index.php?id=prklid=14&plg=en), all members of the malaria triad are now subject to intensive RNAi-based research efforts that should help reveal novel molecular targets for the development of new drugs, vaccines, and vector control tools.

These studies are bound to proliferate over the coming years and will hopefully help reduce the burden of malaria for the millions currently living under the threat of this devastating disease.

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**COMPETING INTERESTS STATEMENT**

The authors declare no competing interests.

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