Wolbachia uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*

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Edited by Anthony A. James, University of California, Irvine, CA, and approved April 21, 2011 (received for review April 6, 2011)

The obligate endosymbiont *Wolbachia pipientis* is found in a wide range of invertebrates where they are best known for manipulating host reproduction. Recent studies have shown that *Wolbachia* also can modulate the lifespan of host insects and interfere with the development of human pathogens in mosquito vectors. Despite considerable study, very little is known about the molecular interactions between *Wolbachia* and its hosts that might mediate these effects. Using microarrays, we show that the microRNA (miRNA) profile of the mosquito, *Aedes aegypti*, is significantly altered by the *w*MelPop-CLA strain of *W. pipientis*. We found that a host miRNA (aae-miR-2940) is induced after *Wolbachia* infection in both mosquitoes and cell lines. One target of aae-miR-2940 is the *Ae. aegypti* metalloprotease gene. Interestingly, expression of the target gene was induced after *Wolbachia* infection, ectopic expression of the miRNA independent of *Wolbachia*, or transfection of an artificial mimic of the miRNA into mosquito cells. We also confirmed the interaction of aae-miR-2940 with the target sequences using GFP as a reporter gene. Silencing of the metalloprotease gene in both *Wolbachia*-infected cells and adult mosquitoes led to a significant reduction in *Wolbachia* density, as did inhibition of the miRNA in cells. These results indicate that manipulation of the mosquito metalloprotease gene via aae-miR-2940 is crucial for efficient maintenance of the endosymbiont. This report shows how *Wolbachia* alters the host miRNA profile and provides insight into the mechanisms of host manipulation used by this widespread endosymbiont.

Estimated to infect more than 65% of all insect species, *Wolbachia pipientis* are maternally inherited, Gram-negative endosymbiotic bacteria (1, 2). They manipulate host reproductive systems through a variety of strategies, including cytoplasmic incompatibility, male killing, feminization, and parthenogenesis (3), and also provide direct mutualistic benefits to hosts in certain contexts (4–6). Recently, a life-shortening *W. pipientis* strain (*w*MelPop-CLA) was successfully introduced into *Aedes aegypti* (7), the main mosquito vector of dengue viruses. Mosquitoes carrying this *Wolbachia* strain exhibit an ~50% reduction in adult female lifespan (7), as well as altered feeding success (8, 9) and activity levels (10). Interestingly, *Ae. aegypti* infected with *Wolbachia* show significantly reduced replication of arboviruses, such as dengue (8, 11) and Chikungunya (8), as well as filarial nematodes (12) and *Plasmodium* (8). Despite considerable advances over the last 20 years in understanding the descriptive phenomenology of *Wolbachia* infections and their almost ubiquitous distribution in insects, relatively little is known about the underlying mechanisms that the bacteria use to mediate their diverse effects on hosts.

MicroRNAs (miRNAs) are nonprotein coding 18- to 25-nucleotide RNAs that play significant roles in regulating a range of cellular processes, including development, differentiation, apoptosis, and immunity (13, 14). Within the past decade, more than 10,000 miRNA sequences from plants, animals, insects, protozoans, and viruses have been deposited in miRNA databases (15). Dramatic changes in the expression levels of cellular miRNAs have been documented recently in response to bacterial and viral infections in animals and plants. For example, infection with the bacterium *Helicobacter pylori* induces expression of miR-155, which regulates the cAMP pathway in T cells both in vivo and in vitro (16). *H. pylori* infection also down-regulates miR-218, resulting in an increase in gastric carcinogenesis (17). Bacterial lipopolysaccharides (*Salmonella* and *Escherichia coli*) lead to the induction of miR-155, miR-132, and miR-146a expression in immune cells (18). miR-218 directly targets NF-κB, and overexpression of miR-218 has been shown to inhibit cell proliferation and apoptosis. In human cells, the Epstein–Barr virus (EBV) has been shown to trigger miR-21, miR-155, and miR-146a expression, which might be involved in the development of EBV-associated Burkitt's lymphoma (19). A liver-specific miRNA, miR-122, targets the 5′ UTR of hepatitis C virus and enhances virus replication (20). Similarly, HIV uses host miRNAs that target its genes to its own advantage (21).

Recently, 86 distinct miRNAs were reported from *Ae. aegypti* after large-scale deep sequencing of small RNAs (22). The role of these miRNAs in the mosquito's biology is largely unknown, however. Here we investigated differential expression of cellular miRNAs in *Ae. aegypti* infected with the *Wolbachia* strain *w*MelPop-CLA. Microarray and Northern blot analyses indicated differential expression of a number of cellular miRNAs. We analyzed one of the induced miRNAs, aae-miR-2940, at the functional level and found that it regulates expression of a mosquito host gene, metalloprotease m41 ftsh. Silencing of the target gene and inhibition of aae-miR-2940 using a synthetic inhibitor led to reduced *Wolbachia* replication and density in cells and mosquitoes, suggesting that the endosymbiont manipulates the host gene via induction of this cellular miRNA to replicate efficiently in the host.

Results

***Wolbachia* Infection Leads to Differential Expression of Mosquito miRNAs.** We tested the hypothesis that *Wolbachia*-infected *Ae. aegypti* would display differential expression of cellular miRNAs using miRNA microarrays covering 386 insect miRNAs (miRBase v.14) and 105 *Ae. aegypti*-specific custom miRNAs (22). miRNA expression levels were compared between *w*MelPop-CLA (+Wol) infected and tetracycline-treated (−Wol) female mosquitoes of the same genetic background at days 4 and 12 post-pupal emergence. In total, 35 miRNAs (13 from *Ae. aegypti*)

Author contributions: S.A. designed research; M.H., F.D.F., and L.A.M. performed research; S.L.O. and S.A. contributed new reagents/analytic tools; M.H., F.D.F., L.A.M., and S.A. analyzed data; and M.H., F.D.F., S.L.O., and S.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105469108/-/DCSupplemental.

showed a significant change in expression level ($P < 0.01$; t test) in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 1), some being homologs represented from different species (e.g., miR-989). Northern blot analyses were used to validate the microarray results specifically for *Ae. aegypti* custom miRNAs (Fig. 2). The 13 *Ae. aegypti* miRNAs that showed significant differential expression in the microarray analysis were analyzed by Northern hybridization to confirm their expression status. Two of the *Ae. aegypti* miRNAs that did not show significant differential expression ($P > 0.05$; aae-miR-2943-1 and –275, not included in Fig. 1) were also analyzed by Northern hybridization to confirm their expression status. Let-7 was also included in the Northern blot analysis because it is a conserved miRNA. In addition, three *Ae. aegypti* miRNAs (aae-miR-2940, -309a-2, and -970) appeared on miRBase after the microarray analysis was carried out and were included in the hybridization analyses. Densitometry graphs comparing the intensity of Northern blot signals in +Wol and −Wol samples are shown in Fig. S1. Northern blot analyses were replicated only once for all miRNAs except aae-miR-2940, which was replicated at least five times as it was chosen for further analysis; therefore, the statistics presented in Fig. S1 are based on differences in the densitometry readings, not on differences between biological replicates. Two miRNAs, aae-miR-2940 and aae-miR-309a-2, showed exclusive induction in +Wol mosquitoes, whereas aae-miR-2943-1 and aae-miR-970 at 4 d and 12 d after emergence and aae-miR-308* and aae-miR-2941–2 at 4 d after emergence were up-regulated in +Wol mosquitoes relative to −Wol mosquitoes (Fig. 2). Aae-miR-989, aae-miR-210, and aae-miR-988 expression was down-regulated in +Wol mosquitoes compared with −Wol mosquitoes at 12 d postemergence. Expression levels of the remaining miRNAs remained the same. Aae-miR-970 expression was down-regulated in both +Wol and −Wol mosquitoes at 12 d postemergence,

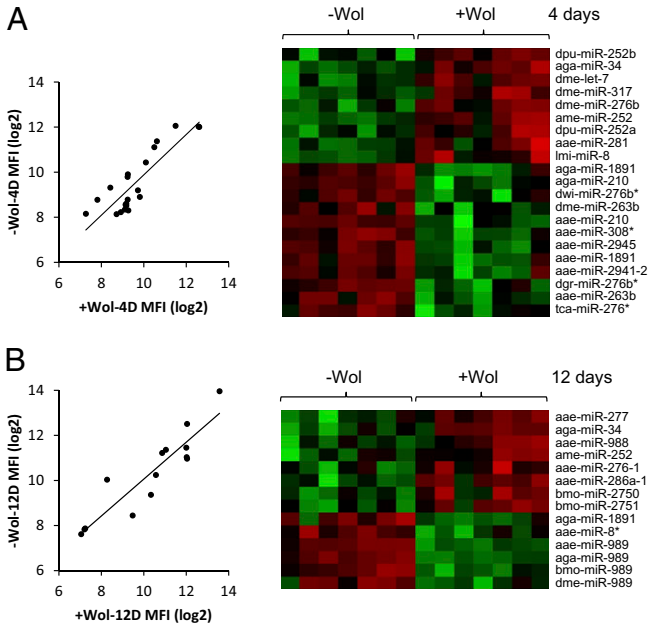

Fig. 1. *Wolbachia* infection alters adult mosquito cellular miRNA expression profile, as determined using microarrays. Expression profiles of miRNAs that were significantly up or down-regulated in 4-d-old (*A*) and 12-d-old *(B) Wolbachia*-infected female *Ae. aegypti* (+Wol) compared with uninfected (−Wol) female mosquitoes are shown. MFI, mean fluorescent intensity. The corresponding miRNA microarray color map shows expression levels of insect miRNAs in −Wol and +Wol samples. Each miRNA was replicated seven times on each chip. Red and green indicate up-regulation and down-regulation of expression, respectively.

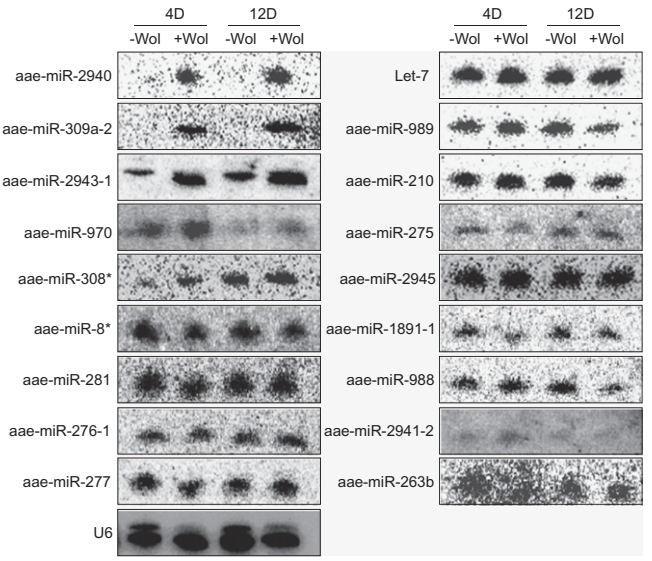

Fig. 2. Validation of expression profile of miRNAs differentially expressed in mosquitoes. Aae-miRs that were significantly up-regulated or down-regulated in *Wolbachia*-infected (+Wol) and uninfected (−Wol) 4-d-old and 12 d-old females were analyzed by Northern hybridization with probes specific to each miRNA. The same blot was used multiple times after removal of probes. U6 was used as a control to demonstrate equal loading of samples.

suggesting that its expression might be age-related (Fig. 2). Aae-miR-286a-1 did not produce a signal on Northern hybridization even after several attempts, perhaps due to a low expression level not detectable by this technique. Putative targets of miRNAs analyzed by Northern hybridization were identified by bioinformatic analysis (Table S1).

Aae-miR-2940 Regulates Expression of a Mosquito Metalloprotease Gene. Induction of aae-miR-2940 only in *Wolbachia*-infected mosquitoes led us to investigate its possible function in the host-bacterium interaction. Using BLAST searches of the *Ae. aegypti* genome, we identified the candidate target gene, metalloprotease m41 ftsh (GenBank ID: XM_001660643), and subsequently confirmed it with the RNAHybrid and RNA22 software. Target sequences with complete complementarity to the aae-miR-2940 seed region were predicted in the 3′ UTR of the metalloprotease gene at nucleotides 2123–2145 (Fig. 3*A*). Using Northern blots with specific probes binding to the target gene, we found that transcript levels of the metalloprotease were induced in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 3*B*). The difference was consistent with the up-regulation of aae-miR-2940 in +Wol mosquitoes compared with −Wol mosquitoes (Fig. 2), suggesting that by binding to the 3′ UTR of the target gene, the miRNA could enhance mRNA transcript levels and/or the stability of the metalloprotease mRNA.

We performed two additional independent experiments to confirm the specific interaction of aae-miR-2940 with the metalloprotease m41 ftsh target sequences and resulting transcript up-regulation. In the first experiment, we initially confirmed the expression of aae-miR-2940 in an *Ae. albopictus* cell line previously infected with the *w*MelPop-CLA strain (C6/36.*w*MelPop-CLA; ref. 23) and confirmed its reduction in *Wolbachia*-uninfected C6/36 cells (Fig. 4*A*). Compared with whole −Wol mosquitoes, in which aae-miR-2940 was not present in detectable levels on Northern hybridization (Fig. 2), a low level of the miRNA was detected in C6/36 cells in the absence of *Wolbachia* (Fig. 4*A*). This could be related to the fact that cell lines are usually clones of a single type of cell from an organism; C6/36 cells are derived from the salivary glands. Differential expression of miRNAs in

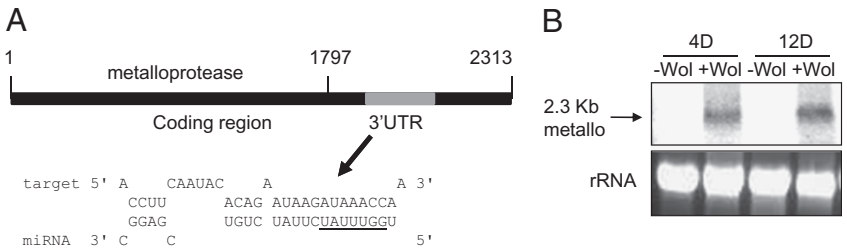

Fig. 3. Aae-miR-2940 target prediction and expression levels of the corresponding target. (*A*) The *Ae. aegypti* metalloprotease gene was predicted to be the best target with complete seed region (underlined) complementarities. The target sequence was identified in the 3′UTR of the metalloprotease gene. (*B*) Northern blot analysis of total RNA extracted from *Wolbachia*-infected (+Wol) and uninfected (–Wol) female *Ae. aegypti* at 4 d and 12 d after emergence. The blot was hybridized with a probe specific to the metalloprotease (metallo) gene. rRNA is shown to indicate equal loading of samples.

various tissues within an insect is well established (24). As a result, when whole mosquitoes are analyzed, a mixture of tissues is analyzed that might not contain detectable quantities of transcripts. However, up-regulation of the target gene was distinctly greater in C6/36.*w*MelPop-CLA cells than in control C6/36 cells (Fig. 4*A*). We cloned both target and mutated target sequences (in nucleotides complementary to the aae-miR-2940 seed region) from the 3′ UTR of the metalloprotease gene, along with their flanking nucleotides, downstream of the GFP gene in the pIZ expression vector (Fig. 4*B*), resulting in the constructs pIZ/GFP-target and pIZ/GFP-Δ1target, respectively. In another control construct, pIZ/GFP-Δ2target, the complementary sequences to the aae-miR-2940 seed region were deleted from the target sequences (Fig. 4*B*). The constructs were transfected into C6/36.*w*MelPop-CLA cells. After 48 h, significantly higher GFP transcript levels were detected in cells transfected with pIZ/GFP-target compared with those transfected with pIZ/GFP-Δ1target or pIZ/GFP-Δ2target ($P < 0.0001$, ANOVA) (Fig. 4*B*).

In a second, independent experiment, we transfected *Ae. aegypti* Aag2 cells with a specific synthetic inhibitor of aae-miR-2940; the sequence is provided in *Materials and Methods*. Control cells were transfected with an inhibitor of random miRNA sequence. After 48 h, we observed lower transcript levels of the mosquito metalloprotease gene in cells transfected with the miRNA inhibitor compared with cells transfected with the control inhibitor (Fig. 4*C*). These results confirm that aae-miR-2940 specifically targets the metalloprotease gene and induces its expression.

RNAi-Mediated Silencing of Metalloprotease Reduces *Wolbachia* Density. We tested whether the metalloprotease gene is critical to *Wolbachia*'s persistence in insect cells using in vitro synthesized dsRNA specific to the gene's coding region in an RNAi experiment. Equal numbers of *Wolbachia*-infected Aag2 (Aag2.*w*MelPop-CLA) cells were transfected with dsRNA of metalloprotease and with dsRNA of GFP and mock infection as controls. RT-qPCR confirmed silencing of metalloprotease (Fig. 5*A*). Using live/dead cells staining with Trypan blue, we confirmed that the metalloprotease knockdown had no substantial effect on cell viability, with ∼1% of cells dead across all treatments at the time of sampling. qPCR using the *Wolbachia*-specific *wsp* gene showed a significant reduction in density of the bacteria in the metalloprotease-silenced cells compared with control cells at 72 h posttransfection ($P = 0.0415$, *t* test) (Fig. 5*B*). To examine whether the same effect could be reproduced in mosquitoes, +Wol mosquitoes were mock-injected (with sterile water), injected with dsRNA specific to GFP (control), or

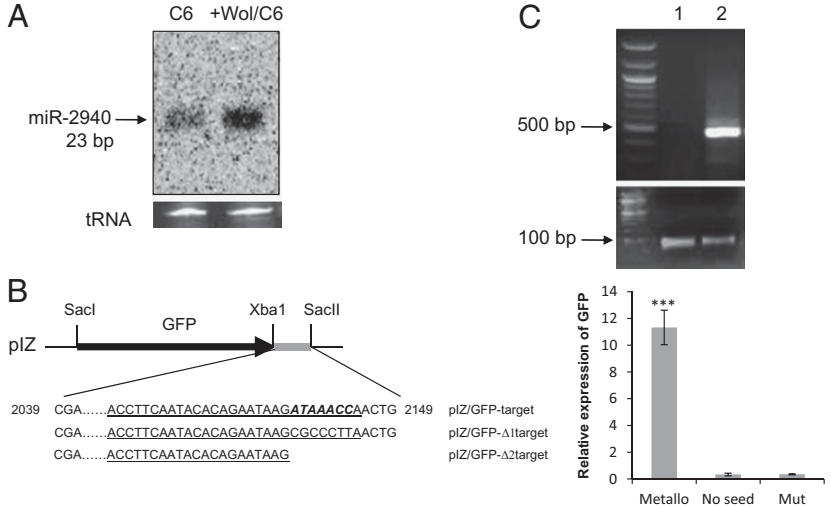

Fig. 4. Target validation of aae-miR-2940. (*A*) Northern blot analysis of expression of aae-miR-2940 in *Wolbachia*-infected C6/36.wMelPop-CLA (+Wol/C6) and uninfected C6/36 (C6) cells. (*B*) Cloning strategy of the metalloprotease (metallo) target, mutated target, and target with deleted sequences complementary to the miRNA seed region from the metalloprotease 3′UTR under the GFP reporter gene ORF in the pIZ vector, denoted as pIZ/GFP target, pIZ/GFP-Δ1 target, and pIZ-GFP-Δ2 target constructs, respectively. Constructs were transfected into C6/36.*w*MelPop-CLA cells that overexpress aae-miR-2940. RT-qPCR analysis indicated that GFP expression was significantly higher ($P < 0.0001$) in pIZ/GFP-target (metallo) transfected cells than in pIZ/GFP-Δ1target (mut) and pIZ/GFP-Δ2target (no seed) transfected cells. Target sequences are underlined, and seed region complementary sequences are shown in bold italic type. (*C*) RT-PCR analysis of metalloprotease gene expression in Aag2.*w*MelPop-CLA cells at 48 h posttransfection with a synthetic inhibitor of aae-miR-2940 (1) and a control inhibitor with random sequences (2). Actin gene expression was analyzed in both experiments as a loading control.

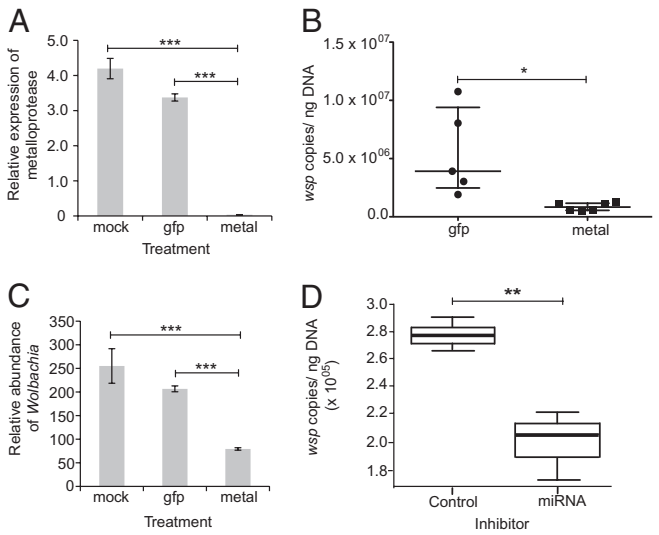

Fig. 5. RNAi-mediated silencing of the metalloprotease gene. (*A*) RT-qPCR analysis of the metalloprotease gene relative to actin in Aag2.*w*MelPop-CLA cells 48 h after transfection with mock, GFP, and metalloprotease (metal) dsRNAs. Three biological replicates were analyzed for each transfection. Asterisks indicate a significant difference between transfection with metalloprotease dsRNA and the other treatments ($P < 0.0001$; *t* test). (*B*) Differences in the density of *Wolbachia* in Aag2.*w*MelPop-CLA cells transfected with the metalloprotease (MP) and GFP dsRNAs examined by qPCR ($P = 0.0415$; *t* test). (*C*) Differences in the density of *Wolbachia* in +Wol adult mosquitoes injected with sterile water, GFP and the metalloprotease (metal) dsRNAs, analyzed using qPCR. Five biological replicates were analyzed per injection type. Asterisks indicate significant difference between mosquitoes injected with metalloprotease dsRNA and the other treatments ($P < 0.0001$; *t* test). (*D*) A significant difference in the density of *Wolbachia* in Aag2.*w*MelPop-CLA cells transfected with aae-miR-2940 inhibitor versus control inhibitor (random sequence) ($P < 0.0001$; *t* test), analyzed by qPCR. Three biological replicates were analyzed for each transfection.

injected with dsRNA specific to the metalloprotease gene. Gene knockdown and the density of *Wolbachia* were analyzed using qPCR. Consistent with cell culture results, *Wolbachia* density was significantly lower in mosquitoes injected with metalloprotease dsRNA compared with those injected with control dsRNA (GFP) or water ($P < 0.0001$, *t* test) (Fig. 5*C*). These results suggest that the metalloprotease gene is critical to *Wolbachia*'s maintenance in mosquitoes.

Aae-miR-2940 Is Required for *Wolbachia* Replication. We tested whether aae-miR-2940 plays a role in *Wolbachia* replication by transfecting Aag2.*w*MelPop-CLA cells with a synthetic inhibitor of the miRNA. At 72 h posttransfection, qPCR results using the *wsp* gene indicated significantly lower *Wolbachia* density was in cells transfected with the aae-miR-2940–specific inhibitor compared with cells transfected with a control inhibitor of random sequence (Fig. 5*D*; $t = 4.898$; df = 4; $P = 0.008$). These results suggest that the miRNA aae-miR-2940 is critical to communication between *Wolbachia* and host that allows the bacteria to persist in mosquito cells.

Discussion

Despite considerable research into the biology of *Wolbachia*, the mechanisms by which the bacteria manipulate host environments to ensure their own survival have proven elusive. Recently, several cellular miRNAs have been implicated in host–pathogen interactions in animals and plants. Here we have reported differential expression of cellular miRNAs in response to *Wolbachia* infection in the mosquito *Ae. aegypti*. Functional analysis of one of the induced miRNAs revealed a role in the regulation of cellular proteins that appears to be fundamental to the ability of *Wolbachia* to colonize and persist in mosquito host cells.

Based on microarray and Northern blot analyses, the expression profiles of several cellular miRNAs were either up-regulated or down-regulated in mosquitoes infected with *Wolbachia*. This is consistent with previous reports on the differential regulation of host miRNAs in response to bacterial infection in human cells (17, 18). We observed induction of aae-miR-2940, aae-miR-309a-2, aae-miR-2943-1, and aae-miR-970 at 4 d and 12 d after emergence and of aae-miR-308* and aae-miR-2941-2 at 4 d after emergence in *Wolbachia*-infected mosquitoes. Based on our target predictions in the *Ae. aegypti* genome for these miRNAs, we focused on aae-miR-2940 for functional analyses. We found one potential target in the 3′ UTR of an *Ae. aegypti* gene, metalloprotease 41 ftsh. Transcript levels of the target gene analyzed by Northern hybridization showed significant up-regulation in *Wolbachia*-infected mosquitoes. We confirmed the interaction of aae-miR-2940 with the 3′ UTR target sequence through the use of a synthetic inhibitor of aae-miR-2940 in the *Ae. aegypti* Aag2 cell line, as well as a GFP-based reporter construct. We found down-regulation in transcript levels of the target gene in Aag2.*w*MelPop-CLA cells transfected with aae-miR-2940 inhibitor, confirming a functional role of aae-miR-2940 in transcriptional regulation of the metalloprotease gene. We detected a similar induction of GFP transcripts when a GFP-metalloprotease target sequence construct was expressed in C6/36.*w*MelPop-CLA cells. Interestingly, RNAi-mediated silencing of the metalloprotease gene in both Aag2.*w*MelPop-CLA cells and +Wol mosquitoes led to a significant decline in *Wolbachia* density, suggesting a critical role for this protein in the maintenance of the *Wolbachia* infection in mosquito cells.

In most scenarios, interaction of miRNA with its target leads to the suppression of the target gene by either degradation of the target mRNA or inhibition of translation (25). However, in addition to the aae-miR-2940 reported in this study, there are a few examples in which up-regulation of the target gene has been documented. An miRNA discovered in mouse embryonic stem cells, miR-4661, up-regulates both mRNA and protein expression of its target gene IL-10 in Toll-like receptor–triggered macrophages (26). miR-4661 competitively binds to the 3′ UTR of IL-10 and protects it from degradation by tristetraprolin, an RNA-binding protein that normally mediates rapid degradation of IL-10 mRNA. In another example, involving a pathogen, hepatitis C virus uses a liver-specific miRNA, miR-122, to enhance viral replication (27, 28). miR-122 protects viral RNA from degradation or from inducing innate immune responses to the RNA terminus (29).

Metalloproteases are known to enable temporal control of many cellular processes by regulating the protein stability of specific and critical regulators (30, 31). Although the precise function of the metalloprotease gene in the *Wolbachia*–insect interaction is unclear, it appears to play a critical role. In the beetle *Callosobruchus chinensis*, a metalloprotease ftsh gene, presumably transferred from *Wolbachia* to the insect genome, was expressed at significantly higher levels compared with several other genes investigated in association with *Wolbachia* infection (32). Thus, metalloproteases may be critical for *Wolbachia* symbiosis in general. It will be interesting to examine whether the manipulation of these molecules might facilitate horizontal transfer of *Wolbachia* into naïve hosts, a key objective of applied studies that seek to introduce *Wolbachia* into anopheline mosquitoes for malaria control.

In conclusion, we have shown that the miRNA profile of the dengue virus vector *Ae. aegypi* is altered by the endosymbiont *W. pipientis*. A host miRNA, aae-miR-2940, was found to be up-regulated in *Wolbachia*-infected mosquitoes and mosquito-derived cell lines. A target of the miRNA was determined to be the host's metalloprotease 41 ftsh gene, which was up-regulated

in *Wolbachia* infection. Inhibition of the miRNA led to reduced expression levels of the target gene. In addition, silencing of the metalloprotease gene led to significant reductions in *Wolbachia* density both in vitro and in vivo. This report describes a fundamental role for miRNAs in the manipulation of the host intracellular environment to favor the *Wolbachia* endosymbiont. Manipulation of host miRNAs also might be responsible for other aspects of the *Wolbachia*–host interaction, such as various reproductive phenotypes, and thus merits further study.

Materials and Methods

Mosquitoes and Insect Cells. *Ae. aegypti* infected with the *w*MelPop-CLA strain of *Wolbachia* (+Wol) and a *Wolbachia*-free, tetracycline-cured line (–Wol) generated previously (7) were used for experiments. Insects were reared at 25 °C with 80% relative humidity and a 12-h light regime. Larvae were maintained with fish food pellets (TetraMin; Tetra) and adults were offered 10% sucrose solution ad libitum. *Ae. aegypti* Aag2 cells were infected with *Wolbachia* (denoted by Aag2.*w*MelPop-CLA) as described for the C6/36.*w*MelPop-CLA cell line (23). *Wolbachia* levels were checked after several cell passages postinfection and throughout the experiment using PCR and FISH, as described previously (23). The Aag2.*w*MelPop-CLA cell line remained stably infected (>80% of cells infected) throughout the course of the experiment. Aag2 and Aag2.*w*MelPop-CLA cells were maintained in growth media in a 1:1 mixture of Mitsuhashi–Maramorosch and Schneider's insect media (Invitrogen), supplemented with 10% FBS. *Ae. albopictus* C6/36 and C6/36.*w*MelPop-CLA cells were grown as described previously (23).

miRNA Microarray Analysis. Total RNA was extracted from female mosquitoes (+Wol and –Wol) at 4 d and 12 d postemergence using TRI Reagent (Molecular Research Centre) and sent to LC Sciences for miRNA microarray analysis. Available insect miRNAs (a total of 386 insect miRNAs; miRBase, release 14.0), in addition to custom miRNA probes from *Ae. aegypti* (a total of 105 miRNAs) (22) were printed on chips in seven replicates and hybridized with Cy3-labeled small RNAs isolated from mosquitoes. Four samples included small RNAs from 4-d-old and 12-d-old female mosquitoes from the same generation (+Wol and –Wol). In addition, probes were included on each chip to control for quality of chip production, sample labeling, and assay conditions. Data were analyzed using ANOVA and *t* tests. Normalization of expression was performed using a cyclic LOWESS method (33).

Northern Blot Hybridizations. Small RNA was isolated from total RNA extracted from mosquitoes as above using the PureLink miRNA isolation kit (Invitrogen). Small (20 μg) RNA samples were run on 15% urea denaturing polyacrylamide gels, electroblotted to nylon membranes by a semidried Western blotting apparatus (Bio-Rad), and UV cross-linked. DNA oligonucleotides (21 mer) with reverse complementarity to specific miRNA sequences were labeled with [α-^{32}P]dCTP using terminal nucleotide transferase. All probe hybridizations and washes were done at 50 °C. Blots were exposed to a phosphorimager screen overnight, and radioactive signals were detected using a phosphorimager scanner. In cases when a blot was used multiple times, the probe was removed by washing blots with boiling 0.1% SDS twice for 30 min each time. Stripping of the probe was confirmed by scanning the blots as described above. Transcripts of target and nontarget genes were detected by Northern blot analyses of total RNA (10 μg) run on 1.2% agarose formaldehyde gels.

miRNA Target Studies. NCBI BLAST, RNAHybrid, and RNA22 software (IBM) were used to find potential targets of aae-miR-2940 in the *Ae. aegypti* genome. Expression profiles of target genes were confirmed by Northern blot analyses with gene-specific probes. A 111-bp fragment from the metalloprotease 3′UTR (2039–2149; XM_001660643) containing the target sequences and a mutated construct with the complementary sequences to the aae-miR-2940 seed region mutated were cloned into pIZ/V5-His vector (Invitrogen) downstream of GFP using XbaI and SacII restriction sites, resulting in pIZ/GFP-target and pIZ/GFP-Δ1target constructs (Fig. 4*B*). In another control construct, the complementary sequences to the aae-miR-2940 seed region were removed (pIZ/GFP-Δ2target). Aae-miR-2940 and control inhibitors were synthesized by Genepharma and used in transfection studies at a concentration of 100 μM/mL. Gene expression was analyzed at 72 h after transfection by RT-PCR and Northern blot analysis. Expression levels of the GFP gene were analyzed by RT-qPCR using three biological replicates, each with three technical replicates.

RNAi-Mediated Gene Silencing and qPCR of *Wolbachia* Density. For RNAi-based experiments, dsRNA was synthesized in vitro using the Megascript transcription kit (Ambion). The T7 promoter sequence (5′-TAATACGACTCACTA-TAGGG-3′) was incorporated in both forward and reverse primers designed to amplify 500 bp of *Ae. aegypti* metalloprotease (forward: 5′-CCCGGAC-CAAGTCCTAGTA-3′; reverse: 5′-CAACTCTTTCGGCACGTAA-3′) and the jellyfish GFP genes. For dsRNA synthesis, 1 μg of PCR product was incubated for 4 h at 37 °C, DNase-treated, and precipitated by the lithium chloride method following the manufacturer's instructions (Ambion). A total of 2 μg of dsRNA was used for transfection of Aag2.*w*MelPop-CLA cells. The cells were transfected again with the same reagents at 48 h after the first transfection. At 24 h after the second transfection, cells were collected for genomic DNA isolation. Gene silencing was confirmed by RT-qPCR using specific primers to the metalloprotease gene. Three biological replicates with three technical replicates were analyzed. Live/dead cell staining with Trypan blue was performed to confirm that silencing of the metalloprotease gene had no effect on cell viability. After staining, cells were counted with a hemocytometer. Cells were randomly sampled from three replicates of each transfection, and two independent hemocytometer counts were performed for each replicate. GFP and metalloprotease RNAi experiments in cells (Fig. 5*B*) were represented by five and six biological replicates, respectively. For qPCR (see below), each biological sample had two technical replicates. Control inhibitor and miRNA inhibitor experiments (Fig. 5*D*) had three biological replicates each, with two technical replicates per biological sample.

Wolbachia density was determined in total cell genomic DNA by qPCR with primers targeting the *wsp* gene (34). The reaction consisted of 2 μL of diluted DNA (10 ng), 5 μL of Platinum Sybr Green mix (Invitrogen), and 1 μM of each primer (forward: 5′-ATCTTTTATAGCTGGTGGTGGT-3′; reverse: 5′-GGAGTGATAGGCATATCTTCAAT-3′), in 10 μL total volume. Reactions were performed in duplicate in a Rotor-Gene thermal cycler (QIAGEN) under the following conditions: 50 °C for 2 min; 95 °C for 2 min; and 45 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 10 s, followed by the melting curve (68 °C to 95 °C). Melting curves for each sample were analyzed after each run to check the specificity of amplification. A standard curve was generated for the *Wolbachia wsp* as described previously (8). The standard was diluted to known concentrations and run in parallel with the samples to determine the absolute number of *wsp* gene copies in each DNA sample, as described previously (8). Gene copy numbers were calculated using the Rotor-Gene software, and *t* tests were used to test for statistically significant differences in means between groups.

To knock down the metalloprotease gene in +Wol mosquitoes, 500 ng dsRNA (metalloprotease) in 69 μL of sterile water was injected in the thorax of CO_2-anesthetized mosquitoes at 4 d after emergence. Control mosquitoes were injected with either sterile water or 500 ng of dsRNA GFP, also in 69 μL of liquid. All mosquitoes were collected at 3 d after injection. The relative ratio of *Wolbachia* copies to mosquito genomic DNA was determined by qPCR using the *wsp* gene and the mosquito gene *RpS17* with primers as described previously (8). Five biological replicates per treatment were analyzed, and qPCR reactions were performed in triplicate with cycling conditions detailed above. The *t* test was used to compare differences in means between different treatments.

Inhibition of aae-miR-2940 in *Wolbachia*-Infected Cells. An inhibitor for aae-miR-2940 (5′-GCCUCGACAGAUAAGAUAAACCA-3′) and a control inhibitor (a random sequence; 5′-UCUACUCUUUCUAGGAGGUUGUGA-3′) were synthesized by Genepharma. Here 100 ng of the aae-miR-2940 inhibitor or the control inhibitor was transfected into 10^6 Aag2.*w*MelPop-CLA cells using Cellfectin transfection reagent (Invitrogen). Cells were collected at 72 h after transfections, and total DNA was extracted. qPCR analyses were performed as above.

ACKNOWLEDGMENTS. This project was funded by a grant from the Australian Research Council (DP110102112, to S.A.) and a grant from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health Initiative of the Bill and Melinda Gates Foundation and the National Health and Medical Research Council (to S.L.O.).

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