Video Article

Methods to Investigate the Regulatory Role of Small RNAs and Ribosomal Occupancy of *Plasmodium falciparum*

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Abstract

The genetic variation responsible for the sickle cell allele (HbS) enables erythrocytes to resist infection by the malaria parasite, *P. falciparum*. The molecular basis of this resistance, which is known to be multifactorial, remains incompletely understood. Recent studies found that the differential expression of erythrocyte microRNAs, once translocated into malaria parasites, affect both gene regulation and parasite growth. These miRNAs were later shown to inhibit mRNA translation by forming a chimeric RNA transcript via 5' RNA fusion with discreet subsets of parasite mRNAs. Here, the techniques that were used to study the functional role and putative mechanism underlying erythrocyte microRNAs on the gene regulation and translational potential of *P. falciparum*, including the transfection of modified synthetic microRNAs into host erythrocytes, will be detailed. Finally, a polysome gradient method is used to determine the extent of translation of these transcripts. Together, these techniques allowed us to demonstrate that the dysregulated levels of erythrocyte microRNAs contribute to cell-intrinsic malaria resistance of sickle erythrocytes.

Video Link

The video component of this article can be found at https://www.jove.com/video/53214/

Introduction

Malaria, caused by apicomplexan parasites of the genus *Plasmodium*, is the most common human parasitic disease, globally infecting approximately 200 million people each year and causing around 600,000 deaths[1]. Of the five *Plasmodium* species that infect humans, the most relevant to human disease are *P. falciparum* and *P. vivax*, due to their widespread distributions and potential for severe malaria complications. The life cycle of the malaria parasite requires infection of both mosquitoes and humans. When an infected mosquito bites a human, the parasites travel through the bloodstream to the liver, where an initial round of replication occurs. After merozoites rupture from the host hepatocyte, they infect nearby red blood cells, initiating either asexual or sexual replication. The asexual stage of replication, which lasts 48 hr in *P. falciparum*, is the focus of this study since it is both the source of most malaria symptoms and is easily recapitulated *in vitro*.

While a number of public health initiatives, including improved anti-malarial therapies, have somewhat reduced the burden of malaria globally, the continuing emergence of drug resistant parasites presents a problem for malaria control efforts. One area which may suggest new therapeutic approaches is the study on how various genetic variants confer resistance to malaria. In malaria-endemic regions, a variety of erythrocytic polymorphisms are quite common[2,3]. These mutations, with sickle cell being perhaps the most prominent, are often associated with substantial resistance to the onset of symptomatic malaria infection[4]. The underlying mechanisms by which they cause erythrocytes to resist malaria infection are incompletely understood. Parasitized erythrocytes with hemoglobin mutations are subject to enhanced phagocytosis through enhanced cellular rigidity and dehydration, which is associated with decreased invasion by *P. falciparum*[5]. The HbC allele also affects protein expression at the erythrocyte surface and with the remodeling of the cytoskeleton, further inhibiting parasite development[6,7]. Finally, *P. falciparum* grows poorly within homozygous sickle (HbSS) erythrocytes[8,9] *in vitro*, suggesting intrinsic erythrocytic factors of malaria resistance. However, while all of these mechanisms appear to play a role, they do not fully explain the mechanisms behind sickle cell resistance to malaria.

One potential set of erythrocytic factors which remain poorly understood is the large pool of miRNA present within mature erythrocytes. MicroRNAs are small non-coding RNAs, 19-25 nt in size, which mediate translation and/or stability of target mRNAs by base pairing within the 3' UTR. They have been implicated in the control of mammalian immune responses, including the suppression of virus replication[10], and were shown to confer resistance to viruses in plants They have also been shown to regulate several erythrocytic processes, including erythropoiesis[11,12] and iron metabolism[13]. Previous studies identified an abundant and diverse population of erythrocytic miRNAs, whose expression was dramatically altered in HbSS erythrocytes[14,15]. Since mature erythrocytes lack active transcription and translation, the functional role of these erythrocyte miRNAs remains unclear. As significant material exchange occurs between the host cell and *P. falciparum* during the

intraerythrocytic developmental cycle (IDC)[16], it was speculated that the altered miRNA profile within HbS erythrocytes may directly contribute to cell-intrinsic malaria resistance.

These studies ultimately led to the development of a pipeline to isolate, identify and functionally study the role of human miRNA within the malaria parasite, *P. falciparum*, which indicated that those host/human miRNAs ultimately covalently fuse and then translationally repress parasite mRNA transcripts[17]. This provided an example of the first cross-species chimeric transcripts formed by trans-splicing and implicates that this miRNA-mRNA fusion could be occurring in other species, including other parasites. All trypanosome mRNAs are trans-spliced with a splice-leader (SL) to regulate the separation of polycistronic transcripts[18]. Since *P. falciparum* lacks orthologs for Dicer/Ago[19,20], it is possible that erythrocyte miRNAs hijack similar SL machinery in *P. falciparum* to integrate into target genes. Recent studies in *P. falciparum* have in fact indicated the presence of 5' splice leader sequences[21]. This study details the methods that led to the discovery of human-parasite miRNA-mRNA fusion transcripts, including both transcriptomic and translational regulation techniques. The overall goals of these methods are to investigate the effects of small RNAs in the gene regulation, phenotypes and translation potential of *P. falciparum* transcripts.

The initial identification of human-parasite chimeric transcripts relied upon usage of RNA analysis techniques, such as real-time PCR, transcriptome sequencing and EST library capture, which included both total and small RNAs, rather than using techniques which only isolated small RNAs. Isolating all RNA together in one large pool, rather than separately, allowed the identification of both translocated human small RNAs in the parasite as well as the presence of these small RNA sequences as part of a larger sequence. This then required an analysis of the translation state of these fusion mRNAs to determine the functional consequences of these fusions.

While extensive efforts on characterization of the parasite's genome and transcriptome have added to the understanding of the parasite's biology[22-25], far less is known about the translational regulation of the mRNA transcriptome across the life cycle of *P. falciparum*[26]. This limited understanding of the parasite's proteome has hindered both understanding of the parasite's biology and the ability to identify new targets for the next generation of anti-malarial therapeutics. This gap in the understanding of the parasite's cellular biology has persisted largely due to the lack of adequate techniques to investigate translational regulation in *P. falciparum*. One recent paper described the use of ribosomal footprinting of *P. falciparum* to determine the global translation status[21]. One well established measurement of translational potential of transcripts is the number of associated ribosomes determined by polysome profiling. However, when this technique is applied to *P. falciparum*, it is unable to recover most polysomes and captures predominantly monosomes. Recently, several groups[27,28] have optimized *P. falciparum* polysome techniques by lysing the erythrocyte and parasite simultaneously to preserve the polysomes and characterize the ribosomal occupancy and translational potential of these malaria parasites during their asexual development in host red cells[28].

Collectively, these methods demonstrate that the observed fusion of human miRNA and parasite mRNAs modulates parasite protein translation of those fusion mRNAs, which was demonstrated using previously reported methods[27], and is a major determinant of malaria resistance in HbAS and HbSS erythrocytes[17]. These methods would be useful in any system looking to identify and functionally explore RNA splicing events, whether those fusion RNAs are within *P. falciparum* or other eukaryotic systems.

Protocol

1: Isolation of Small-sized RNAs from *P. falciparum* During the IDC

1. Obtain malaria parasites in asexual culture[29].
   NOTE: The required culture size will vary based upon the desired application; however, a 10 ml culture at 3-5% parasitemia and 5% hematocrit provides ample RNA for real-time PCR (RT-PCR). The technique was initially optimized for asynchronous cultures, but when specific time points during the infection cycle are desired, ring-stage parasites can be synchronized by sorbitol synchronization no later than 10-12 hr post-invasion. Synchronization should be repeated after one cycle (approximately 48 hr)[29] .
2. Pool infected cells together (~5 x 10^9 RBCs at 3-5% parasitemia) and fill cold PBS to the top of the tube and centrifuge at 800 x g for 5 min without brake to pellet the red cells.
3. After centrifugation, remove the supernatant carefully using an aspirating pipette, attached to a vacuum, since the erythrocytic pellet is easy to dislodge. Wash the pellet once more with cold 1x PBS.
4. Resuspend cell pellet in cold 0.15% saponin (in 1x PBS) and place on ice for 30 min.
5. Centrifuge cells at 1,500 x g for 12 min at 4 °C, remove supernatant (which will be dark red in color) and resuspend pellet in cold PBS. Repeat this step once more.
   NOTE: In order to ensure that the microRNAs present were reflective of microRNAs within parasites, and not erythrocytic contamination, a number of parasite isolation conditions were tested: 1) saponin lysis, 2) saponin lysis combined with RNaseA treatment of host cell miRNAs and 3) Methyl-beta-cyclodextrin treatment to remove the parasite from the host erythrocyte. All treatments gave similar results.
6. Lyse the isolated parasite pellet using the recommended 600 µl of lysis buffer. This volume of lysis buffer is sufficient for cultures up to ~30 ml at 2% hematocrit and 5% parasitemia.
   NOTE: For larger parasite cultures, this volume can be increased. It is important to ensure complete lysis of the parasite pellet by thorough vortexing for at least 1 min. Also, for ease of extraction, the lysed samples can be transferred to a 1.7 ml microcentrifuge tube.
7. Add 60 µl, or 1/10 volume of the lysis buffer used in step 6, of homogenate additive (2 M sodium acetate, pH 4).
8. Leave samples on ice for 10 min.
9. Add 600 µl, or a volume equal to the amount of lysis buffer added in step 6, of acid phenol choloroform to each sample and vortex for 1 min.
10. Separate the aqueous and organic phases by centrifuging the samples at 10,000 x g for 5 min. This spin is longer than that suggested by the kit to ensure that all hemoglobin/hemozoin is in the organic phase.
11. Collect the upper (aqueous) layer and transfer it to a fresh tube. Repeat steps 9 and 10 if the aqueous phase is white or (more likely) brown in color, which suggests protein contamination.
12. Determine the volume of the resulting supernatant by pipette and then add 1.25 volumes of 100% ethanol (~800 µl) and mix by inverting the 1.7 ml microcentrifuge tube 5 times.
13. Pass each sample through a provided RNA-binding filter cartridge (several different of which are available commercially) by either centrifugation at 14,000 x g for 1 min or by using a vacuum manifold.

14. Wash the filter cartridge with 700 µl of the miRNA wash buffer 1 using a microcentrifuge, spinning at 14,000 x g for 1 min.
15. Wash the filter cartridge with 500 µl wash buffer 2/3 twice by centrifuging at 14,000 x g for 1 min each.
16. Elute RNA with 100 µl DEPC-water, which has been preheated to 95 ºC, in two washes of 50 µl, centrifuging at 14,000 x g for 1 min each. Measure the concentration of RNA via UV absorbance at 260nm.
    NOTE: An RNA gel (either TBE-urea acrylamide or denaturing formaldehyde agarose gel) can be used to assess the size distribution of the isolated RNAs.
    NOTE: This RNA is suitable for analysis by microarray, RNA-sequencing, northern blot and ribonuclease protection assay.

2: Quantitation of Small-sized RNAs from *P. falciparum* During the IDC

1. Determine the concentration of individual samples isolated in step 1 using UV spectrophotometry at 260 nm.
2. Generate real-time PCR primers for the desired RNA species. For miRNA alone, we used premade miRNA qPCR assays, while for mRNAs or fusion miRNA-mRNA we designed primers for SYBR green. For the fusion RNAs, the forward primer was the miRNA sequence itself (miR-451: AAACCGTTACCATTACTGAGTT), while the reverse was a gene-specific primer ~100 bp downstream of the miRNA in the mRNA sequence (PKA-R: ATCGAACATGCTGTGAACAT).
3. Make cDNA from the RNA samples using either a hairpin primer for miRNAs (use 20-40 ng of RNA) alone or random hexamers for mRNA and/or miRNA-mRNA fusion species (using 1 µg of RNA). Mix RNA and primers for 5 min at 65 °C, then cool to room temperature. Afterwards, add reverse transcriptase mix (0.5 µl reverse transcriptase, 0.5 µl RNase inhibitor, 1 µl dNTPs (2.5 mM each), 4 µl 5x buffer, 2 µl 0.1M DTT, 1 µl H_2O).
    1. Run samples on a thermocycler capable of detecting either SYBR green or TaqMan probes. SYBR green quantitative PCR[30] for specific genes was run as a 3-step PCR, 95 °C for 15 sec, lowest primer annealing temperature minus 5 °C for 30 sec, and 68 °C for 30 sec, for 40 cycles, using a commercial SYBR green master mix. miRNA real-time PCR was run as a two-step PCR, 95 °C for 15 sec and 60 °C for 1 min, for 40 cycles.
    2. Quantify assays using the ΔΔCt method and normalize them against either Rab GTPase (PF08_0110) or 18S rRNA [31].

3: Introduction of Small RNAs into Malaria Parasites

1. Pellet 300 µl of red blood cells per transfection (250 µl of RBCs, approximately 1.5 x 10^9 cells, will ultimately be used) in complete malaria media by centrifugation at 800 x g for 5 min at brake 1.
2. Wash the erythrocytes twice with RPMI and resuspend in complete cytomix (120 mM KCl; 0.15 mM $CaCl_2$; 10 mM K_2HPO_4/KH_2PO_4, pH 7.6; 25 mM HEPES, pH 7.6; 2 mM EGTA, pH 7.6; 5 mM $MgCl_2$; pH adjusted with KOH) at 50% hematocrit after centrifuging at 800 x g for 5 min at brake 1.
3. Transfer the cells to a 0.2 cm electroporation cuvette.
4. Add 10 µg, or appropriate amount, of custom synthetic oligonucleotides to the cuvettes and resuspend the cultures.
5. Adjust the electroporator to 310 V/950 µF and deliver a pulse to each cuvette.
6. Resuspend the 300 µl of RBCs within the cuvette in pre-warmed complete malaria media and plate them in 24 well plates and incubate at 37 °C in an airtight container with malaria blood gas mix (3% O_2, 5% CO_2).
7. After 4 hr, infect transfected RBCs with synchronized late trophozoites to an approximate final parasitemia of 1%.
8. Add freshly transfected RBCs every 4-6 days to infected cultures and determine the % parasitemia by FACS using YoYo-1 staining, as indicated in section 4.

4: Determination of Erythrocyte microRNAs Effect Upon Parasite Infection Rate

1. Take 100 µl of resuspended culture by micropipettor and place in a 1.7 ml microcentrifuge tube. Wash twice with 1 ml of 1x PBS and centrifuge in a tabletop microcentrifuge by centrifugation at 800 x g for 5 min.
2. Resuspend pellet in 1 ml of 0.025% glutaraldehyde. Incubate samples for 20 min at RT.
    NOTE: samples can be stored here for several weeks at 4 °C. Afterwards, pellet the infected RBCs by centrifugation at 800 x g for 5 min. Wash twice with 1 ml of 1x PBS.
3. Resuspend pellet in 1 ml of 0.015% saponin. Incubate at 4 °C for 15 min. Pellet infected RBCs by centrifugation at 800 x g for 5 min. Wash twice with 1 ml of 1x PBS. Repeat.4.3. Resuspend cells in 1 ml of 1x PBS containing 1 µl (1,000X) DNA stain.
4. Determine the degrees of parasitemia on a flow cytometer, at 488 nM excitation and ~530 nM (FL-1) emission.
5. First, gate the flow cytometer based upon FSC and SSC to focus on RBCs. Then measure the degrees of parasitemia (infected RBCs) in the gated RBC by fluorescence in the FL-1 channel.
    NOTE: later stage parasites are 10X more fluorescent than earlier stage parasites, and ~100X above uninfected RBCs. Also, collecting at low speed tends to reduce noise. Finally, this approach has been compared to ^{3}H-hypoxanthine incorporation and Giemsa staining, both as reported previously[13], and all techniques gave similar results.

5: Biotin-tagging and Elution of miRNA-mRNA Fusion Products

1. Directly order custom synthetic RNA oligonucleotides with desthiobiotin (Db-) covalently linked to the 5' end.
2. Transfect uninfected RBCs with Desthiobiotin-conjugated (Db-) miRNA and unmodified miRNA (negative control), as indicated in step 3.
3. Infect transfected RBCs with *P. falciparum* parasites to a starting parasitemia of 0.5% (step 1.1).
4. Extract parasite RNA 4 days (96 hr) later as described above in step 1.
5. Incubate 10 µg of parasite RNA with 50 µl packed streptavidin beads, added via micropipette, and rotate on a microcentrifuge tube rotator for 1 hour at 4 °C.
6. Pellet the beads at 800 x g for 30 sec and wash with 20 mM KCl and 1/1,000 RNase Inhibitor.

7. Elute RNAs from the beads by competition with 2 mM biotin and with 200 µl RNA capture elution buffer (20 mM KCl, 1/1,000 RNase Inhibitor and 2 mM biotin) at 4 °C overnight with rotation.
8. Determine the degree of enrichment of indicated *P. falciparum* transcripts using SYBR green qRT-PCR, and microRNA enrichment (positive control) by TaqMan qPCR as indicated in step 2.

6: Polysome Separation to Determine the Ribosomal Occupancy of *P. falciparum*

1. Place 5 ml of 15% sucrose solution (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM cycloheximide, 1 mM DTT, 1 mM PMSF, 40 U/ml RNase Inhibitor, and 15% sucrose) into a SW41 ultracentrifuge tube (14 x 89 mm).
2. Using a 5 ml syringe, add 5 ml of 50% sucrose solution (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM cycloheximide, 1 mM DTT, 1 mM PMSF, 40 U/ml RNase Inhibitor, and 50% sucrose) underneath the 15% sucrose using a long steel needle, then remove the needle.
3. Seal the top of the gradient tube with Parafilm, tilt the tube slowly onto its side, in a stable position to prevent rolling, so it is lying horizontally on the tabletop. Keep the tube on its side for at least 2 hr. While waiting, start with step 6.5.
4. After 2 hr, slowly tilt the tube back upright and transfer the tube to ice, allowing it to cool for at least 15 min prior to sample loading (step 14).
5. Collect enough *Plasmodium*-infected blood stage culture to generate 100-500 µg of total RNA, or roughly a 100 ml asynchronous culture at 3-5% parasitemia. Add 1/10th that volume of culture medium containing 10x (2 mM) cycloheximide (CHX) and incubate at 37 °C for 10 min.
    1. Pellet cultures by centrifugation at 500 x g for 7 min with the centrifuge (brake 1), then wash twice with room temperature 1x PBS containing 200 µM CHX. Resuspend the parasite cultures in 1x PBS containing 200 µM CHX and store on ice.
6. Estimate the volume of the RBC pellet. Add lysis buffer (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM CHX, 1 mM DTT, 1 mM PMSF, 40 U/ml RNase Inhibitor, 1% (v/v) IGEPAL CA-630, and 0.5% (w/v) DOC) to the RBC pellet to a final volume of 4.25 ml. Incubate the lysate at 4 °C for 10 min while rotating.
7. Transfer the lysate to microcentrifuge tubes, and then centrifuge the samples in a microcentrifuge at 16,000 x g and 4 °C for 10 min.
8. Prepare sucrose cushions by pipetting 1.25 ml of cold 0.5 M sucrose cushion solution (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM cycloheximide, 1 mM DTT, 1 mM PMSF, 40 U/ml RNase Inhibitor, and 0.5 M sucrose) into a SW55 ultracentrifuge tube (13 x 51 mm).
9. Carefully layer 3.75 ml of lysate supernatant (from step 7) on top of the sucrose cushion using a syringe and small (~27) gauge needle. Store the remaining lysate (~500 µl) at -80 °C to extract total RNA levels, as indicated in section 1.
10. Load the samples into an ultracentrifuge rotor, pre-chilled to 4 °C, which can hold 13.2 ml tubes. Centrifuge the samples at 366,000 x g and 4 °C for 146 min.
11. Using a pipette, transfer the supernatant into a 15mL conical tube. Store the supernatant at -80 °C for RNA isolation of free (unbound by ribosomes) RNAs.
12. Resuspend the ribosome pellet in 500 µl of ribosome resuspension buffer (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM cycloheximide, 1 mM DTT, 1 mM PMSF, and 40 U/ml RNase Inhibitor) by pipetting for 5 min.
13. Centrifuge the samples in a microcentrifuge at 16,000 x g and 4 °C for 10 min.
14. While keeping the gradient from step #4 on ice, remove the Parafilm, then carefully layer the ribosome suspension on top of the gradient using a syringe and small (~27) gauge needle.
15. Load the tube into a pre-chilled ultracentrifuge rotor and centrifuge the loaded gradients at 200,000 x g and 4 °C for 180 min. When the spin is completed, store centrifuged gradients at 4 °C until ready to load onto the fractionator.
16. Load an empty ultracentrifuge tube into the gradient fractionator (this can be a tube used in a previous run), then wash with RNAse-free water for 5 min at 100 x 10 speed.
17. During the wash in step 6.16, set the sensitivity of the UV absorbance detector. A good starting sensitivity is 0.2, but this may have to be adjusted in later runs depending on the A_{254} signal (which varies based on parasite number, *etc.*).
18. Once the detector sensitivity is set, set the baseline signal to zero while water is flowing through the detector.
19. After washing the fraction collector, reverse the fluid flow until the lines are empty and then remove the empty ultracentrifuge tube.
20. Run 60% sucrose solution through the fractionator until it exits the needle apparatus.
21. Insert the tube containing the first gradient at the top of the loading chamber and tighten the seal, taking care not to over-tighten. Then, pierce the bottom of the tube with the needle.
22. Reset the fluid flow speed to 12.5 x 10 (controlled by the flow speed control knob on the front of the pump), then set the automated fraction collector to collect 18 sec fractions (~330 µl/fraction) in microcentrifuge tubes.
23. Start forward flow of the 60% sucrose solution.
24. When the first drop of the gradient solution drops into the microcentrifuge tube, immediately begin both fraction collection and live recording of the A_{254} signal.
25. Once a sharp drop is observed in the A_{254} signal, corresponding to the interface between the 50% and 60% sucrose solutions, stop both the fluid flow and A_{254} recording.
26. Store the gradient fractions at -80 °C for later use.
27. Reverse the fluid flow at 100 x 10 speed until the 60% sucrose solution empties out of the ultracentrifuge tube.
28. If there are additional gradients to fractionate, remove the empty ultracentrifuge tube from the loading chamber and restart from step 6.21. If all samples are completed, wash the apparatus as was performed in steps 6.16 and 6.19.

Representative Results

Global profiling of human microRNA in *P. falciparum*

The techniques presented here were used to extract microRNAs from parasites in a variety of conditions. One thing to note is that RNA extractions for uninfected RBCs were performed as indicated in[32], which often served as a reference point for the microRNA data presented in both this article and the original study[29]. Those previous studies also demonstrated that HbSS erythrocytes possessed far greater levels of certain miRNAs, miR-451 in particular. The techniques indicated above were sufficient to demonstrate the changes in miRNA abundance in HbS

(HbAS or HbSS) infecting parasites. RNA samples were extracted as indicated above and normalized against 18S rRNA. Parasite samples from HbSS RBCs, for example, do show a significant increase in levels of several microRNAs, including miR-451 (**Figure 1**). The uptake of miRNA from the host erythrocyte is also consistent with previous studies[34,35].

Transfection of HbAA erythrocytes with miR-451 increased HbAA miR-451 to levels similar to HbSS erythrocytes (**Figure 1**). Flow cytometry was then used to measure parasite infection, in order to assess the effect of miRNA translocation upon parasite growth. Based upon that, HbAA erythrocytes transfected with miR-451, miR-223 and miR-181a, along with an ssDNA oligo, were examined for the effects of microRNAs upon parasite growth. Transfection of ssDNA or miR-181a had no effect upon parasitemia (**Figure 2A**-**D**) while transfection with miR-451 markedly reduced parasitemia, which could be mitigated only by co-transfecting an antisense miR-451 oligo.

Blocking of microRNAs by transfection of 2'-O-Me antisense microRNAs demonstrated a reciprocal effect. HbSS erythrocytes, with naturally higher miR-451 levels, were transfected with antisense miR-451 or 2'-O-methyl oligonucleotides (**Figure 3**). Inhibition of miR-451 increased parasite growth in both HbSS erythrocytes (**Figure 3C**-**F**). Parasite percentages were calculated from at least 3 independent transfections, then averaged and presented as fold change relative to HbSS growth (**Figure 3F**).

Capture of miRNA-mRNA fusion RNAs by biotin capture assay

After transfection of 5'-desthiobiotin-miR-451 (5'Db-miR-451) and 5'Db-miR-181, as indicated in methods section 5, it was determined that the observed miRNA sequence originated with human microRNA. qRT-PCR analysis indicated that transfection of 5'Db-miR-451 resulted in enrichment of *PKA-R* fusion transcripts (**Figure 4**).

Polysome separation to determine the ribosomal occupancy of *P. falciparum*

A typical A_{254} trace is shown in **Figure 5A** (with northern confirmation of rRNA content in **Figure 5B**), with the fraction density increasing from left to right on the graph (*i.e.* lighter fractions to the left). The initial A_{254} is quite high due to absorbance by hemoglobin in the early fractions, but quickly drops to the baseline. The first small peak corresponds to the small ribosomal subunit (40S), followed quickly by the second peak, which is slightly larger and corresponds to the large ribosomal subunit (60S). The third peak, which is generally the largest for *P. falciparum*, corresponds to the monosome peak (80S). Due to its height, the 80S should be used as a guide to adjust the sensitivity of the UV detector for subsequent runs (Step 6.17). Also, due to the size of the 80S peak, it is common for the 60S signal to blur like a "shoulder" into the start of the monosome peak, if both the 60S and 80S signals are large.

Following the monosome peak are the peaks corresponding to polysomes. Each successive peak represents a polysome fraction representing progressively larger numbers of ribosomes (2, 3, 4, *etc.*). Each polysome peak is also smaller than the previous peak, decreasing in height by 2-3 fold with each successive polysome number. Typical runs will allow the resolution of polysomes composed of 5-7 ribosomes, though resolution of up to a 9-mer polysome has been observed in some runs with a large amount of starting material. Higher order polysomes compose the remainder of the trace, and cannot be resolved under these gradient conditions.

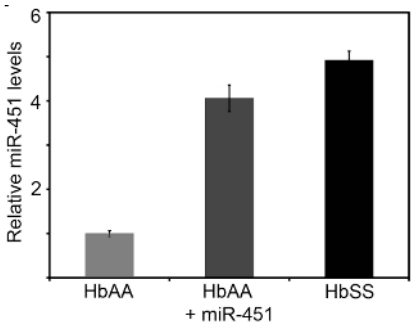

Figure 1: MiR-451 are elevated in HbSS erythrocytes. Intraparasitic miR-451 levels, as determined by real-time PCR and normalized against 18S rRNA, from parasites isolated from the indicated erythrocyte types. Values are mean ± SE.

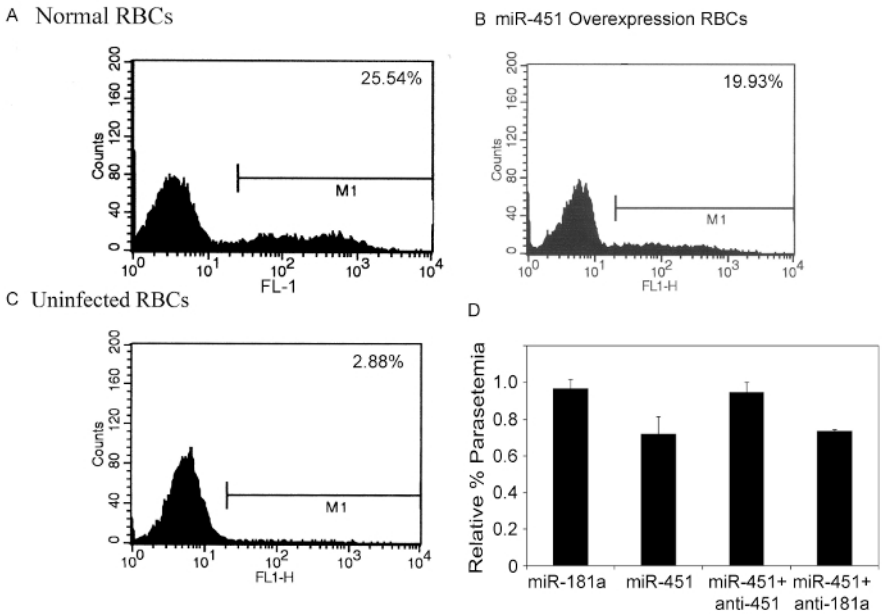

Figure 2: Overexpression of miR-451 in normal erythrocytes inhibits parasite growth. (**A**-**C**) Representative FACS plots indicating infection rates, as a percentage of total RBC, are indicated by m1, for the shown erythrocyte types. (**D**) Composite infection rates derived from FACS and normalized against untransfected HbAA RBCs. Values in panel A-C are representative FACS plots while D is mean ± SE.

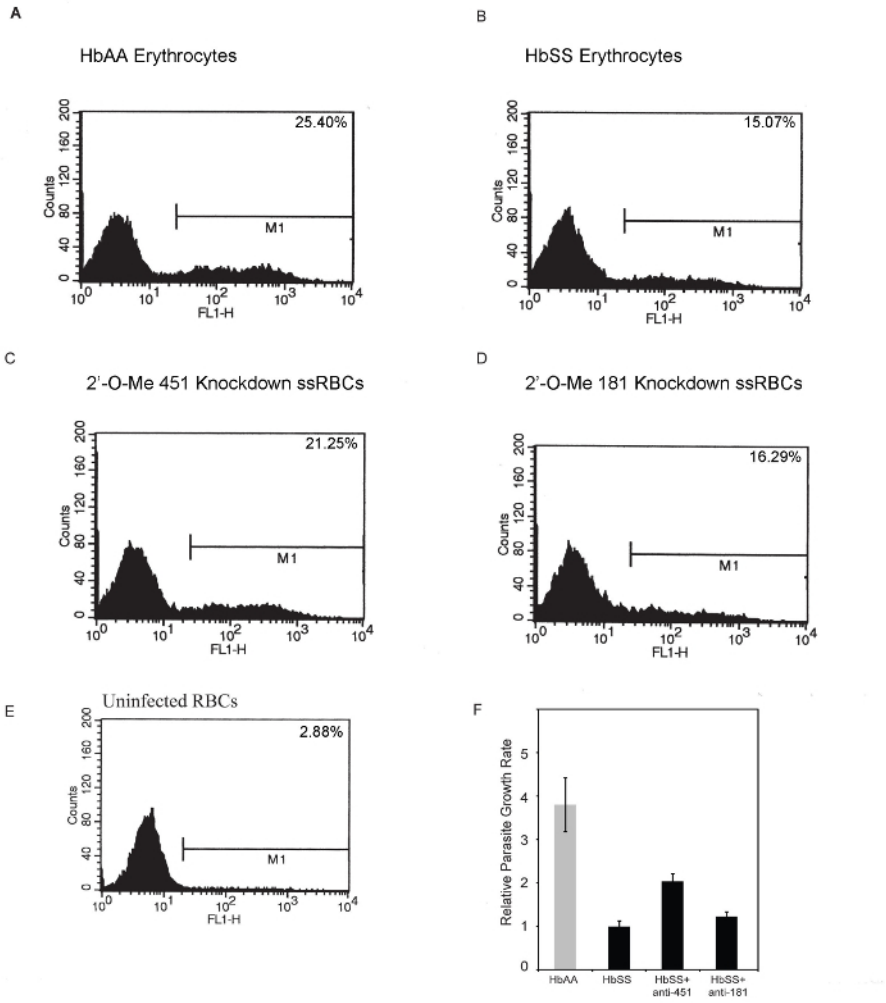

Figure 3: Inhibition of miR-451 in sickle cell erythrocytes elevates parasite growth. (**A**-**E**) Representative FACS plots indicating infection rates, as a percentage of total RBC, are indicated by m1, for the shown erythrocyte types. (**F**) Composite infection rates derived from FACS and plotted as a normalized value against untransfected HbAA RBCs. Values in panel A-E are representative FACS plots, F is mean ± SE.

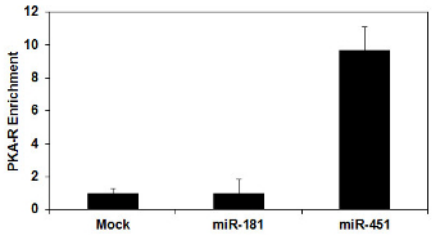

Figure 4: Transfection of miR-451 enriches for *PKA-R* fusion transcripts and shows that the fused miRNA is erythrocytic in origin. Enrichment of the indicated desthiobiotin-conjugated miRNA-mRNA fusion transcripts that have been captured by streptavidin. Values were measured using real-time PCR and plotted as a relative value versus non-transfected parasites. Values are mean ± SE.

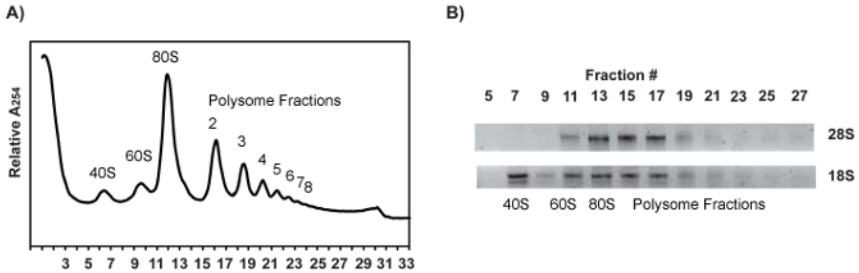

Figure 5: Parasite ribosomes can be isolated via sucrose gradient. (**A**-**B**) Example traces of a polysome profile extracted from *Plasmodium falciparum*. (**A**) Trace of a polysome profile extracted from *Plasmodium falciparum*, with the 40S, 60S, 80S and Polysome peaks (with #s indicating the number of ribosomes associated) displayed. (**B**) Northern blot of 18S and 28S rRNA across the ribosomal fractions. Values are mean ± SE.

Discussion

HbS is one of the most common hemoglobin variants in malaria endemic areas, largely because it provides protection against severe malaria caused by *P. falciparum*. The techniques needed to characterize the role of human microRNA in the gene regulation of *P. falciparum* are detailed throughout this manuscript. By extracting total RNA in such a way as to include all small RNAs, and by performing a relatively straightforward parasite lysis procedure, these fusion RNAs were able to be identified through a variety of independent techniques.

These steps are relatively straightforward but are sensitive to problems and contamination if not followed properly. During the parasite extraction, it is critical to perform saponin lysis to remove contaminating host erythrocytes. This is particularly true during the saponin lysis step of the RNA isolation. The miRNA component of the host red blood cells is several orders of magnitude greater than that of the parasite, in part due to most red blood cells being uninfected, so it is vital to remove as much erythrocytic contamination as possible. It is also important to maintain the malaria culture in a healthy and proper stage (if a specific point in the infection cycle is required) to obtain an accurate miRNA profile. Similarly, during the phenol-chloroform extraction, it is vitally important to make sure the aqueous phases are clear and, if not, repeat the phenol-chloroform extraction to avoid any remaining contamination.

Also, in the desthiobiotin capture experiment, it is critical to elute with an excess of biotin and to use the minimum of streptavidin beads, so as to ensure proper specific elution. The elution of bound RNAs through competition with biotin, rather than complete denaturation of the beads themselves, served to dramatically reduce the background RNA enrichment. Finally, the desthiobiotin capture experiment was highlighted as one important way to demonstrate how these miRNAs were captured, but several other techniques were also used in the previous studies to demonstrate these fusion RNAs, such as northern blots and ribonuclease protection assays[29]. Such methods of using the transfected microRNAs to retrieve the modified transcripts may have broader application, such as the purification of associated protein complexes that may mediate the transport and processing of the transfected miRNAs.

While various genome-wide approaches have been used to study the malaria proteome and transcriptome[22-25], there are few methods to globally examine translational regulation in *P. falciparum*[21,26]. This methodological limitation precludes the global analysis of the translational regulation in *P. falciparum*. One of the most common experimental approaches to study translational regulation is polysome profiling, which assesses overall translational activity based on the ribosomes loaded onto specific mRNAs of interest. Detailed in this manuscript is an optimized polysome profiling method for use in malaria parasites that lyses both the infected erythrocyte and the parasite within. Previous methods did not recover the majority of polysomes and made it seem as though malaria parasites did not have substantial populations of polysomes. The use of a lysis buffer with a high concentration of potassium acetate and magnesium made it possible to lyse both red blood cells and parasites while solubilizing membrane-bound ribosomes to allow the capture of intact polysomes.

This cost-effective method of ribosomal purification and profiling will help to bridge the gap in existing knowledge between the malaria transcriptome and proteome and enable the genome-wide analysis of the translational state of *P. falciparum*. This approach has been used to compare the translation state of the early and late stage blood stage parasites[28], which have tightly coordinated gene expression. Data on the ribosomal loading of transcripts can be readily obtained and analyzed to determine the density of ribosomes on specific mRNAs. Furthermore,

when combined with sorbitol synchronization, changes in ribosomal density can be used to establish how translation is regulated throughout the parasite life cycle. Isolation of ribosomes using this approach requires a large amount of culture, which unfortunately limits the stages of the malaria life cycle in which it can be performed. The large amount of input material will also limit its use both to *in vitro* lab strains of *Plasmodium falciparum*, and may restrict its applicability to other infectious organisms. Anyone using this procedure will also be able to screen for genes that show unusual translation profiles, in particular transcripts which are not associated with ribosomes, reflecting alternative modes of gene regulation. Finally, this approach will enable us to evaluate how novel antimalarial agents affect protein translation and to identify translation-based mechanisms of drug resistance. This pipeline has great utility in identifying and determining the post-transcriptional regulation in many experimental systems.

Disclosures

The authors have no competing financial interests to declare.

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