Published in final edited form as:

Int J Parasitol. 2015 August ; 45(0): 579–583. doi:10.1016/j.ijpara.2015.04.004.

Functional analysis of microRNA activity in *Brugia malayi*

Canhui Liu^a, Denis Voronin^b, Catherine B. Poole^c, Saheed Bachu^b, Matthew B. Rogers^d, Jingmin Jin^c, Elodie Ghedin^e, Sara Lustigman^b, Larry A. McReynolds^c, and Thomas R. Unnasch^{a,*}

^aGlobal Health Infectious Disease Research Program, Department of Global Health, College of Public Health, University of South Florida, 3720 Spectrum Blvd. Suite 304, Tampa, FL 33612 USA

^bNew York Blood Center, Lindsley F. Kimball Research Institute, 310 East 67th Street, New York, NY 10065 USA

^cNew England Biolabs, 240 County Road, Ipswich, MA 01938-2723 USA

^dDepartment of Surgery, University of Pittsburgh, Children's Hospital, Pittsburgh, USA

^eDepartment of Biology, Center for Genomics & Systems Biology, New York University, New York, NY 10003 USA

Abstract

The complement of the *Brugia malayi* miR-71 microRNA was inserted into the 3' untranslated region (UTR) of a reporter plasmid, resulting in a decrease in reporter activity. Mutation of the seed sequence restored activity. Insertion of the 3' UTRs from two algorithm-predicted putative target genes into the reporter resulted in a similar decrease in activity; mutation of the predicted target sequences restored activity. These experiments demonstrate that *B. malayi* microRNA targets may be predicted using current algorithms and describe a functional assay to confirm predicted targets.

Keywords

Filariasis; Transfection; Genetic regulation; miRNA

MicroRNAs (miRNAs) have received increasing attention as a mechanism of control of gene expression in a wide variety of organisms (Ghildiyal and Zamore, 2009). miRNAs regulate gene expression at the post-transcriptional level, either by cleavage (Yekta et al., 2004) or translational repression (Petersen et al., 2006) of their target mRNAs. Recent deep sequencing projects have revealed the presence of many miRNA homologues in nematode parasites (reviewed in (Britton et al., 2014)). These include *Ascaris suum* (Wang et al.,

*Corresponding author. Thomas R. Unnasch, Department of Global Health, 3720 Spectrum Blvd, Suite 304, Tampa, FL 33612, USA. Tel.: 813-974-0507; fax: 813-974-0992. tunnasch@health.usf.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2011), *Brugia pahangi* (Winter et al., 2012), *Dirofilaria immitis* (Fu et al., 2013) and the human filarial parasite *Brugia malayi* (Poole et al., 2010, 2014). While some putative miRNAs in parasitic nematodes are highly conserved, a large proportion appear to be novel (Winter et al., 2012). Furthermore, many of the putative parasitic nematode miRNAs are restricted to certain lifecycle stages, suggesting that they function in developmental control of gene expression (Winter et al., 2012). Other studies have suggested that the parasite miRNAs may play a role in adaptation to life in the vertebrate host (Britton et al., 2014) and possibly in the development of drug resistance (Devaney et al., 2010).

miRNAs are generally 20 – 25 nucleotides (nt) long. Mature miRNAs interact with a number of different proteins, including members of the Argonaute family, to form the RNA induced silencing complex (RISC), which in turn binds to the target mRNA(s) of the miRNA. miRNAs contain a 5' sequence necessary for selective RISC-mediated mRNA binding. This “seed sequence” is located at nt positions 2 – 8 of the miRNA (Brennecke et al., 2005). The seed sequence is responsible for initial recognition of target sites within mRNAs. Recognition is mediated through sequences complementary to the miRNA within the non-coding portion of the mRNA (generally the 3' untranslated region (UTR)). Recognition of a target mRNA results in binding of the miRNA-containing RISC to the target. This double stranded structure results in blockage of translation of the cognate mRNA (Petersen et al., 2006), and may subsequently reduce its stability (Fabian and Sonenberg, 2012). mRNA degradation is catalyzed by the RISC complex, which cleaves the miRNA-mRNA duplex. This can occur through two mechanisms. The first is direct cleavage, which generally occurs in the central region of the duplex (the slicer domain) (Kawamata et al., 2009). Alternatively, the RISC complex can affect cognate mRNA stability in the mRNA-miRNA duplex by shortening the poly (A) tail of the mRNA at the 3' end and by facilitating de-capping at the 5' end of mRNAs (Fabian and Sonenberg, 2012).

We utilized a transient transfection system to study the mechanism for miRNA gene regulation in the filarial parasite *B. malayi*. The plasmid pBmHSP70/GLuc (Fig. 1A) was employed as a backbone for all these studies. pBmHSP70/GLuc was originally designed as a marker for transfection of developmentally competent *B. malayi* infective stage larvae (Xu et al., 2011). It consists of the *B. malayi* HSP70 promoter driving the expression of a secreted *Gaussia princeps* luciferase (GLuc) reporter gene. The first intron of the *B. malayi* HSP70 gene was inserted into the open reading frame (ORF) of the GLuc gene. The intron sequence contains the trans-splicing motif that is necessary and sufficient to direct trans-splicing of transgenic mRNAs in transfected *B. malayi*, ensuring that the GLuc transcripts are correctly cis- and trans-spliced (Liu et al., 2007). Finally, BmHSP70/GLuc contains the 3' UTR derived from the *B. malayi* HSP70 gene cloned downstream of the stop codon of the GLuc ORF.

The existing sequence of the 3' UTR was initially mutated to introduce a complement of microRNA-71 (miR-71), using the Gene Tailor in vitro mutagenesis kit (Invitrogen, USA), as previously described (Liu et al., 2009) (Fig. 1B, C). miR-71 was chosen for this study as it is highly expressed in microfilariae (Poole et al., 2014) and adult female parasites (Winter et al., 2012). Deep sequencing analysis of the miRNA population demonstrated that miR-71 was one of the five most abundant miRNAs present in the isolated embryo preparations used

in the transient transfection studies (T. R. Unnasch and L. McReynolds, unpublished data). The mutated plasmid was then transiently transfected into isolated *B. malayi* embryos and the amount of GLuc activity assayed using the dual luciferase assay as described in the legend to Fig. 1. Insertion of the miR-71 complementary sequence into the 3' UTR of pBmHSP70/GLuc (Fig. 1C) resulted in a 75% reduction in reporter activity when compared with the activity observed in embryos transfected in parallel with the parental plasmid (Fig. 1E). These data suggested that the miR-71 present in the embryos was interacting with the synthetic miR-71 recognition site incorporated into the mutant plasmid, reducing expression of the GLuc reporter.

To explore the sequence requirements necessary for miR-71-mediated reduction of reporter activity an additional series of mutants was prepared, modifying the sequence recognized by the miR-71 seed sequence, the putative slicer domain, and the complement of the 3' end of the miR-71 miRNA sequence (Fig. 1D). These mutants were then assayed for reporter activity in transfected *B. malayi* embryos using the dual luciferase assay. Mutation of the sequence complementary to the miR-71 seed sequence restored 80% of the activity seen with the parental plasmid, suggesting that recognition of the seed sequence was necessary for miR-71-mediated reduction in reporter activity (Fig. 1E). In contrast, mutation of the putative slicer domain (a CC to GG change located in the middle of the miRNA target sequence; Fig. 1D) did not restore activity, while mutation of the 5' end of the recognition sequence (complementary to the 3' end of miR-71) resulted in reporter activity that was approximately 50% of that seen with the parental construct lacking the miR-71 recognition sequence (Fig. 1E).

Quantitative northern blot analysis was then conducted on RNA samples extracted from the transfected embryo preparations, as described in the legend to Fig. 1. The amount of GLuc reporter gene-derived mRNA present in each transfected embryo preparation corresponded almost exactly to the amount of normalized GLuc reporter activity detected in each sample (Fig. 1F). This suggested that the miR-71 miRNA was acting, at least in part, on the level of mRNA stability to down-regulate the expression of the GLuc reporter.

To identify putative targets of miR-71 in *B. malayi*, all putative 3' UTRs were analyzed in a genome-wide scan. Because the *B. malayi* miR-71 sequence is identical to *Caenorhabditis elegans* miR-71, the *C. elegans* miRNA miR-71-3p and miR-71-5p sequences were used to search for target sequences using ComiR (Coronnello and Benos, 2013), which integrates the predictions obtained from three target prediction tools: miRanda (John et al., 2004), PITA (Kertesz et al., 2007), and TargetScan (Lewis et al., 2005). By design, ComiR predicts the target genes of miRNAs by considering multiple miRNA binding events on the same mRNA and generates output scores for each target identified. The analysis was run on predicted 3' UTRs for each *B. malayi* coding sequence (WormBase: https://www.wormbase.org/species/b_malayi/sequence). Due to limited annotation of the 3' UTRs, these were represented by 500 nt of sequence downstream of each ORF. We identified 135 *B. malayi* genes for which ComiR scores were above 80% for both the miR-71-3p and miR-71-5p targets; these were then searched against *C. elegans* genes to find orthologs. ComiR scores were then compared across orthologs in both species, and only genes for which scores were similar were kept for further analysis. This analysis resulted in the

identification of six genes whose 3' UTRs were predicted to encode miR-71 recognition sites. The amount of mRNA encoded by each of these genes was quantified by real time qPCR in microfilariae and adult female parasites. These stages were chosen for analysis as previous studies have indicated that miR-71 levels are 3 – 7 fold higher in microfilariae than in adult females (Poole et al., 2014). Total *B. malayi* RNA was extracted from 1×10^6 microfilariae and from five female adults provided by the Filariasis Research Reagent Resource Center (FR3, Division of Microbiology and infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA), using a Trizol-based method and RNA mini kit (Ambion, USA). Purified RNA was treated with 1 U of DNase I (PureLink, Ambion) at 37°C for 30 min. Treated RNA (approximately 1 µg) was used as a template for cDNA synthesis with SuperScript III (Invitrogen) priming with random hexamer primers. This cDNA was used as a template in quantitative reverse transcription (RT)-PCR assays with SYBR green and gene-specific primers (Supplementary Table S1) to quantify expression of the six genes identified bioinformatically as putative targets of miR-71. All amplifications and fluorescence quantifications were performed using an Applied Biosystems ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA). The

Ct-based method was used to analyze gene expression levels, normalized with the *B. malayi* NADH dehydrogenase subunit 1 gene, as previously described (Li et al., 2004). All data were collected from at least three biological replicates and each biological replicate was also tested in three technical replicates. The expression of all six genes with putative miR-71 target sequences was higher in adult females than in microfilariae (Table 1). As the amount of miR-71 has previously been reported to be 5 – 7 times higher in microfilariae than in adult parasites (Poole et al., 2014), the finding that expression of all six genes was higher in adult parasites was consistent with the hypothesis that expression of these genes might be in part regulated by miR-71, suggesting that these mRNAs did indeed contain active miR-71 recognition sites in their 3' UTRs.

To assess the accuracy of the prediction of the miR-71 target sequences, two 3' UTRs containing putative miR-71 targets examined in the study described above (Bm13889 and Bm7472; Fig. 2A) were chosen for experimental confirmation. Specific target sites within these two 3' UTRs were predicted by identifying locations that overlapped in the TargetScan and PITA outputs (the miRanda outputs were not used in the specific site selection). The 3' UTRs for these two genes were then substituted for the HSP70 3' UTR in pBmHSP70/GLuc as described in the legend to Fig. 2. The plasmids containing the Bm13889 and Bm7472 3' UTRs were then transiently transfected into *B. malayi* embryos and the reporter activity compared with embryos transfected with the parental pBmHSP70/GLuc plasmid using the dual luciferase assay. Both constructs containing the 3' UTRs with the putative target sites for the miR-71 miRNA produced levels of GLuc that were approximately 25% of those produced by the parental construct, which lacks a miR-71 target site (Fig. 2B). Mutation of the putative target sites (i.e. the complements of the miR-71 seed sequence present in each UTR) in both constructs restored reporter activity to levels that were not significantly different from those seen in embryos transfected with the parental construct (Fig. 2B). These data suggest that the ComiR algorithm was accurately predicting miR-71 recognition sites in the Bm13889 and Bm7472 3' UTRs.

Taken together, these experiments demonstrate that transfection of reporter constructs into *B. malayi* embryos may be used to probe the details of post-transcriptional regulation of gene expression by miRNA in this parasite. Given the development of methods to produce developmentally competent transgenic *B. malayi* (Xu et al., 2011), and to introduce miRNAs into this parasite through soaking (Britton et al., 2014), the approach described here may prove useful in exploring the roles that developmentally regulated miRNAs play in controlling developmental processes of human filarial parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Parasite material used in this study was obtained through the Filariasis Research Reagent Resource Center (FR3), Division of Microbiology and infectious Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, USA. This work was supported by a grant from the United States NIAID (Project # 1R56AI101372-01A1) to SL, EG and TRU.

References

- Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. *PLoS Biol.* 2005; 3:e85. [PubMed: 15723116]
- Britton C, Winter AD, Gillan V, Devaney E. microRNAs of parasitic helminths - Identification, characterization and potential as drug targets. *Int. J. Parasitol. Drugs Drug Resist.* 2014; 4:85–94. [PubMed: 25057458]
- Coronnello C, Benos PV. ComiR: Combinatorial microRNA target prediction tool. *Nucleic Acids Res.* 2013; 41:W159–W164. [PubMed: 23703208]
- Devaney E, Winter AD, Britton C. microRNAs: a role in drug resistance in parasitic nematodes? *Trends Parasitol.* 2010; 26:428–433. [PubMed: 20541972]
- Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. *Nat. Struct. Mol. Biol.* 2012; 19:586–593. [PubMed: 22664986]
- Fu Y, Lan J, Wu X, Yang D, Zhang Z, Nie H, Hou R, Zhang R, Zheng W, Xie Y, Yan N, Yang Z, Wang C, Luo L, Liu L, Gu X, Wang S, Peng X, Yang G. Identification of *Dirofilaria immitis* miRNA using illumina deep sequencing. *Vet. Res.* 2013; 44:3. [PubMed: 23331513]
- Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* 2009; 10:94–108. [PubMed: 19148191]
- Higazi TB, DeOliveira A, Katholi CR, Shu L, Barchue J, Lisanby M, Unnasch TR. Identification of elements essential for transcription in *Brugia malayi* promoters. *J. Mol. Biol.* 2005; 353:1–13. [PubMed: 16154590]
- Higazi TB, Unnasch TR. Biolistic transformation of *Brugia malayi*. *Methods Mol. Biol.* 2013; 940:103–115. [PubMed: 23104337]
- John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol.* 2004; 2:e363. [PubMed: 15502875]
- Kawamata T, Seitz H, Tomari Y. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. *Nat. Struct. Mol. Biol.* 2009; 16:953–960. [PubMed: 19684602]
- Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nat. Genet.* 2007; 39:1278–1284. [PubMed: 17893677]
- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 2005; 120:15–20. [PubMed: 15652477]

- Li BW, Rush AC, Tan J, Weil GJ. Quantitative analysis of gender-regulated transcripts in the filarial nematode *Brugia malayi* by real-time RTPCR. *Mol. Biochem. Parasitol.* 2004; 137:329–337. [PubMed: 15383303]
- Liu C, Chauhan C, Katholi CR, Unnasch TR. The splice leader addition domain represents an essential conserved motif for heterologous gene expression in *B. malayi*. *Mol. Biochem. Parasitol.* 2009; 166:15–21. [PubMed: 19428668]
- Liu C, de Oliveira A, Higazi TB, Ghedin E, DePasse J, Unnasch TR. Sequences necessary for trans-splicing in transiently transfected *Brugia malayi*. *Mol. Biochem. Parasitol.* 2007; 156:62–73. [PubMed: 17727976]
- Petersen CP, Bordeleau ME, Pelletier J, Sharp PA. Short RNAs repress translation after initiation in mammalian cells. *Mol. Cell.* 2006; 21:533–542. [PubMed: 16483934]
- Poole CB, Davis PJ, Jin J, McReynolds LA. Cloning and bioinformatic identification of small RNAs in the filarial nematode, *Brugia malayi*. *Mol. Biochem. Parasitol.* 2010; 169:87–94. [PubMed: 19874857]
- Poole CB, Gu W, Kumar S, Jin J, Davis PJ, Bauche D, McReynolds LA. Diversity and expression of microRNAs in the filarial parasite, *Brugia malayi*. *PLOS One.* 2014; 9:e96498. [PubMed: 24824352]
- Wang J, Czech B, Crunk A, Wallace A, Mitreva M, Hannon GJ, Davis RE. Deep small RNA sequencing from the nematode *Ascaris* reveals conservation, functional diversification, and novel developmental profiles. *Genome Res.* 2011; 21:1462–1477. [PubMed: 21685128]
- Wang JL, Liu YH, Lee MC, Nguyen TM, Lee C, Kim A, Nguyen M. Identification of tumor angiogenesis-related genes by subtractive hybridization. *Microvasc. Res.* 2000; 59:394–397. [PubMed: 10792972]
- Winter AD, Weir W, Hunt M, Berriman M, Gilleard JS, Devaney E, Britton C. Diversity in parasitic nematode genomes: the microRNAs of *Brugia pahangi* and *Haemonchus contortus* are largely novel. *BMC Genomics.* 2012; 13:4. [PubMed: 22216965]
- Xu S, Liu C, Tzertzinis G, Ghedin E, Evans CC, Kaplan R, Unnasch TR. *In vivo* transfection of developmentally competent *Brugia malayi* infective larvae. *Int. J. Parasitol.* 2011; 41:355–362. [PubMed: 21118694]
- Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science.* 2004; 304:594–596. [PubMed: 15105502]

Highlights

- Insertion of a recognition site for a microRNA (miRNA) reduces reporter gene expression in *Brugia malayi*.
- Mutation of the seed and 5' end sequences of the miRNA recognition site partially restore expression.
- miRNA target prediction algorithms developed for other organisms can predict miRNA targets in *B. malayi*.
- Transient transfection of *B. malayi* embryos can be used to functionally assay miRNA recognition sites

Fig. 1.

Effect of the introduction of the microRNA-71 (miR-71) sequence on reporter activity in transiently transfected *Brugia malayi* embryos. (A) Schematic representation of the parental plasmid pBmHSP70/GLuc showing the location of restriction sites and ampicillin resistance gene (Amp^r). (B) Sequence of the *B. malayi* BmHSP70 3' UTR in pBmHSP70/GLuc. The region of the 3' untranslated region (UTR) mutated to create a miR-71 complementary sequence is double underlined. (C) Mutations introduced in the 3' UTR to generate a miR-71 recognition sequence. (D) Nucleotide (nt) changes made to the miR-71 target sequence to generate the seed, slicer and 3' end mutants. In C and D, colons (:) indicate sequence identity and dashes (–) indicate an insertion or deletion. (E) *Gaussia princeps* luciferase (GLuc) reporter activity in embryos transfected with parental, miR-71 native and mutated miR-71 containing pBmHSP70/GLuc plasmids. Plasmids were biolistically transfected into *B. malayi* embryos together with BmHSP70 (nts –394 to –1)/luc (Higazi et al., 2005), a plasmid which contains the minimal BmHSP70 promoter driving the expression of a firefly luciferase reporter gene. BmHSP70 (–394 to –1) lacks the sequences necessary for trans-splicing of the transgenic mRNA encoded in the first intron of the BmHSP70 gene and does not contain the BmHSP70 3' UTR. Despite lacking these features, reporter gene

activity produced from embryos transfected with this construct is comparable with that seen in embryos transfected with constructs that contain the sequences necessary for proper mRNA maturation in *B. malayi* (Liu et al., 2007). BmHSP70 (–394 to –1)/luc was included in the transfections as an internal standard to control for transfection efficiency. Transient transfections were performed as previously described (Higazi and Unnasch, 2013). In brief, embryos collected from five gravid adult female parasites were biolistically transfected with 1.35 mg of DNA-coated 0.6 μ m gold beads at a pressure of 1100 PSI using a BioRad PDS-1000/He™ System (BioRad Hercules, CA, USA). Beads were prepared by precipitating 15 μ g each of experimental GLuc plasmid and the BmHSP70 (–394 to –1) internal standard plasmid onto 5 mg of 0.6 μ m gold beads (BioRad), following the manufacturer's instructions. Transfected embryos were cultured for 48 h. The amount of GLuc activity was then determined and normalized to the amount of firefly luciferase using the Promega dual luciferase assay (Promega, Madison, WI, USA). Columns represent the means and error bars the S.D.s of six independent transfections. The activity of the embryos transfected with all constructs was significantly different from that seen in embryos transfected with the parental construct ($P < 0.05$; Student's *t* test). WT, wild type. (F) Transgenic mRNA levels and GLuc reporter activity in embryos transfected with parental and miR-71 target containing pBmHSP70/GLuc plasmids. Total RNA was extracted from transfected embryos using Trizol (GIBCO/BRL, USA). Twenty micrograms of total RNA per lane were resolved on a 1.2% agarose gel prepared with Rapid Formaldehyde-Free RNA gel buffer (Amresco, Solon, OH, USA) and the RNA transferred to a nitrocellulose membrane, as previously described (Wang et al., 2000). The northern blot was probed with a labeled PCR product derived from the *Gaussia* luciferase open reading frame (ORF), stripped and re-probed with a labeled PCR product derived from the firefly luciferase ORF that served as the internal standard for transfection efficiency. Probes were labeled by random priming with the Digoxigenin High Prime DNA labeling and detection kit (Roche Applied Science, USA). Blots were hybridized in DIG Easy-Hyb buffer (Roche Applied Science) containing the probes overnight at 42°C. The blots were washed twice for 5 min per wash in 2 $\times$ SSC, 0.1% SDS at room temperature and then twice for 15 min per wash in 0.5 $\times$ SSC, 0.1% SDS at 65°C. RNA present in each lane was quantified using a Molecular Dynamics Laser Densitometer (GE Healthcare, USA) and the Image Quant software program provided with the instrument (GE Healthcare). The amount of GLuc mRNA in each sample was then normalized to the amount of the internal standard firefly luciferase reporter mRNA present in each sample to control for differences in transfection efficiency and the amount of RNA loaded in each lane.

A**Bm13889**

GAGGGAC

TGCCTACATACTACTTCGTCTGTATGTGGCTATCAGTTCTTTTCAGTTCTGGTTTGAAGCAGTAGCCATCCTT
 CCTCGTTATTCTAATTTATTTTTTAATATTGCATATTGCTTCACTCTCATGATTCATTTATTTGTGAATTTG
 CTGATTTGGTACTCACCAGTTAGAAACGGAATTTTAACTGATTTGAAATTTTTTCGCGGGAAGGTTCTAGTAT
 TTTTTTTAAAAGTTCTTGCATAATTCTAGTGATTTTCATTTGTGCGACATTTTTCATAATGATTCCAGTGAT
 AATTTAAGATGATTGCTCAAGTATTCAACGAGGTGTTGCAAATGTTACCAACAATTAACTGTTTGAAACTG
 AAAAAATATTGAAAATAACTGAAAATCTTTTCAGCTGAAG

Bm7472

TGTCTTCATCACTCAGCAAAAGTTAAATATGACAGGCTGTAATGGTACAATGTATTTTCACTATTAAAGATA
 GA
 AGGAAATAAGCAAATATGCTGAAGACTGCCATTTTAGTCTTTAAGAAATGTAGATATTCAGATTAGGAATTC
 GAGGGAC
 TCTTTCACTACTCATCATTTGTTATCTGTGGTATTTATGGTCAGTTTTCATTCAGTCAATTCAAGTGATAC
 TGACAAATTGCTTTGTTAGAATTGTCTATTGCGCTCATATATTTTCACAATAATCAGTTATCAGTTATACAC
 AAAATGATTTTTCAGTTGTATGAAATATCAATAAAATAGGTTTGTGTTTGAATATCGGTTCCACATTTTCAG
 TTTATCAGATAGAGATTAAGGAAGATGTACTTTAAAATAA

B **Fig. 2.**

Analysis of putative *Brugia malayi* microRNA-71 (miR-71) target 3' untranslated regions (UTRs) in the transient transfection system. (A) Sequences of 3' UTRs chosen for analysis in the transient transfection system. The putative miR-71 target sequences (complementary to the putative miR-71 seed sequence) are underlined. The mutated sequences are indicated in the text over each of the predicted targets. The putative 3' UTRs were amplified from *B. malayi* genomic DNA using Maxima Hot Start PCR Master Mix (Thermo Scientific, USA). The primers used in the amplification of the 3' UTR derived from Bm13889 were

BM13889F (5' GGTCTAGATGCCTACATACTACTTCGTCTGT 3') and Bm13889R: 5' GGTCTAGACTTCAGCTGAAAAGATTTTCAGTT 3'. For the Bm7472 3' UTR, the primers were Bm7472F (5' GGTCTAGATGTCTTCATCACTCAGCAA 3') and Bm7472R (5' GGTCTAGATTATTTTAAAGTACATCTTCCTTAATCTCT 3'). All primers contained a GG clamp at their 5' ends, followed by a synthetic *Xba*I site (underlined) to facilitate subsequent cloning. Amplification conditions for the 3' UTRs consisted of 4 min at 95°C followed by 40 cycles consisting of 95°C for 30 s, 50°C for 30 s and 1 min at 72°C. The reaction was completed with a final extension at 72°C for 7 min. Following amplification, the amplicons were cloned into the pCR2.1 TA cloning vector (Invitrogen, USA), and their sequences confirmed. Clones containing the correct sequence were then digested with *Xba*I to liberate the insert, and the fragment was cloned into *Xba*I digested pBmHSP70/GLuc, replacing the HSP70 3' UTR. The orientations of inserts were determined by PCR, and the sequences of those in the correct orientation were confirmed by DNA sequencing. Mutations were prepared using the Gene Tailor mutagenesis kit (Invitrogen). (B) Activity of constructs containing native and mutated 3' UTRs containing putative miR-71 target sequences. *Gaussia princeps* luciferase (GLuc) activity was normalized to the amount of firefly luciferase activity as described in Fig. 1. Columns represent the means and error bars the S.D.s of six independent transfections. Asterisks (*) indicate constructs that produced activities that were significantly different from what was detected in embryos transfected with the parental construct pBmHSP70/GLuc ($P < 0.05$; Student's *t* test).

Table 1

Expression of genes predicted to contain microRNA-71 (miR-71) target sites in adult females and microfilariae of *Brugia malayi*.

Coding Sequence (CDS, Gene ID)	Predicted function	Relative expression in adult females versus microfilariae (mean fold increase $\pm$ S.D.)
Bm3992	Membrane-associated protein	3.1 $\pm$ 0.4
Bm6639	Acetyl-choline esterase	2.0 $\pm$ 0.04
Bm7472	Ammonium transport protein	3.6 $\pm$ 0.04
Bm9342	Mitogen-activated protein kinase	4.7 $\pm$ 0.5
Bm9199	Serine-threonine kinase, BMA-SEL-5 protein (suppressor/enhancer of Lin-12)	1.4 $\pm$ 0.07
Bm13889	BMA-RER-1, Retention in endoplasmic reticulum protein (protein transportation, intracellular trafficking)	3.3 $\pm$ 0.2