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In vivo and in vitro knockdown of *FREP2* gene expression in the snail *Biomphalaria glabrata* using RNA interference

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Abstract

RNA interference (RNAi) is reported here for the first time for *Biomphalaria glabrata*, the snail intermediate host for the human parasite *Schistosoma mansoni*. The fibrinogen-related protein 2 (FREP2) gene, normally expressed at increased levels following exposure to digenetic trematode parasites, such as *S. mansoni* or *Echinostoma paraensei*, was targeted for knockdown. Double-stranded RNA (dsRNA) corresponding to specific regions of the *FREP2* gene was introduced into snails by direct injection into the hemolymph, 2 days prior to exposure to trematodes, or added to co-cultures of *B. glabrata* embryonic (Bge) cells and *E. paraensei* sporocysts. After introduction of *FREP2* dsRNA, expression levels of *FREP2* were significantly reduced, to 20–30% of control values. In addition, we were able to disrupt expression of the house-keeping *myoglobin* gene, further confirming the feasibility of RNAi for *B. glabrata*. Cross-reactivity in RNAi has not been observed either among four *FREP* gene subfamilies or between *FREP2* and *myoglobin*. Establishment of RNAi techniques in *B. glabrata* provides an important tool for clarifying the function of genes believed to play a role in host-parasite interactions, specifically between *B. glabrata* and its trematode parasites, including *S. mansoni*.

Keywords

RNA interference (RNAi); Fibrinogen-related protein (FREP); *Biomphalaria glabrata*; *Echinostoma paraensei*; *Schistosoma mansoni*; Host–parasite interactions; Innate immunity; Comparative immunology

1. Introduction

RNA interference (RNAi) is a process by which the introduction of exogenous double-stranded RNA (dsRNA) corresponding to a specific messenger RNA (mRNA) sequence results in a significant reduction in level of the targeted mRNA [1–3]. This technique is a powerful post-transcriptional gene silencing technique that is providing insight into gene function in plants and animals [4–8]. RNAi has been used to assess gene function in invertebrates and vertebrates under in vivo and in vitro conditions. Most in vivo RNAi studies have been performed with invertebrates, mainly arthropods, with the aim of directly assessing phenotype changes. These invertebrates include *Caenorhabditis elegans* [9,10], digenetic trematodes [11,12], silkworms [13], moth *Spodoptera liturs* [14], *Drosophila* [15–17], mosquitoes [18,19], honeybee [20], and ticks [21]. In vitro RNAi assays have also been

widely applied to cell lines from humans and model organisms such as *C. elegans* [10], *Drosophila* [22,23], mosquitoes [24], and mice [25,26].

Successful application of RNAi in a broad range of eukaryotic organisms suggests that it may be possible to employ this promising technique in mollusks. Indeed, RNAi has been reported to disrupt neuronal nitric oxide synthase (nNOS) gene function in the pond snail *Lymnaea stagnalis* [27], and to inhibit the CCAAT/enhancer-binding protein gene (ApC/EBP) in the marine mollusk *Aplysia* [28]. However, RNAi has not yet been reported in the snail *Biomphalaria glabrata*, a widely studied intermediate host of the trematode *Schistosoma mansoni*. This helminth infects 83 million people worldwide [29]. *B. glabrata* is used as a model to explore the nature of the defense responses of snails to digenetic trematodes like *S. mansoni* and *Echinostoma paraensei*. A large, diverse family of hemolymph proteins termed fibrinogen-related proteins, or FREPs is present in *B. glabrata* [30,31]. Previous work suggests that FREPs are involved in molluscan internal defense because they are capable of precipitating soluble trematode antigens and binding to trematode sporocysts. Although considerable data on the structure, diversity, and expression of *FREPs* have accumulated recently [32–36], the precise roles of *FREPs* in defense and perhaps other aspects of snail physiology require further clarification. Classical genetic knockout techniques are not presently available in mollusks, nor are they likely to soon be developed. RNAi could serve as a powerful alternative tool to assess snail gene function in both in vivo and in vitro studies. Towards this end, we here report our studies to develop RNAi to knockdown expression of *FREP* genes in *B. glabrata*, as a necessary prelude to exploring their role in anti-trematode defense.

FREP2, a member of *FREP* gene family, was chosen as a target gene because its transcripts appear in greater abundance in individuals of the M line strain of *B. glabrata* after exposure to either *E. paraensei* or *S. mansoni*, and in snails of the BS-90 (also called Salvador) strain after exposure to *S. mansoni* [36]. In addition, *FREP2* has a relatively simple gene structure with one immunoglobulin superfamily (IgSF) domain [32]. Moreover, like all *FREPs* currently known, *FREP2* is comprised of both a specific IgSF region and a more conserved fibrinogen (FBG) region. This provides a useful opportunity to assess the specificity of dsRNAs targeted to these regions in the RNAi experiments described below.

2. Materials and methods

2.1. Parasites, Bge cells and snails

The life cycles of *S. mansoni* and *E. paraensei* were maintained in the laboratory as described by Stibbs et al. [37] and by Loker and Hertel [38], respectively. The *B. glabrata* embryonic (Bge) cells originally established by Hansen [39] were obtained from the American Type Culture Collection (ATCC CRL 1494). Bge cells were maintained at 26 °C in complete Bge cell medium [39], supplemented by 50 µg/µl gentamicin sulfate (Sigma) and 5% fetal bovine serum (FCS) (Sigma) as described by Coustau et al. [40]. The BS-90 and Mline strains of *B. glabrata* were maintained in the laboratory [39].

2.2. Co-culture of *E. paraensei* miracidia/sporocysts and Bge cells

Bge cells were seeded at 2×10^5 cells per well (500 µl culture medium) in a 24-well plate for 1 day prior to addition of dsRNA (or 5 µl nuclease-free water for the control). Miracidia were added 2 days after the addition of dsRNA or nuclease-free water. To maintain physical separation between the Bge cells and parasites, *E. paraensei* miracidia were added in sterile plastic inserts (0.4 µm membrane pore size; Corning Incorporated, NY, USA), and co-cultured with Bge cells in the same well. After co-culture for a specified number of days, the parasites were removed and Bge cells were collected for RNA extraction.

2.3. dsRNA synthesis

FREP2 dsRNA was synthesized following the method of Clemens et al. [22]. Sequence-specific primers were designed from *FREP2* cDNA sequences (GenBank accession No. AY012700). The primers used for generating *FREP2* dsRNA (537 bp) were: forward 5'-TAATACGACTCACTATAGGGTCGCTACCACTTCGACTTGTT-3', reverse 5'-TAATACGACTCACTATAGGGCGTGGGACTGGCTCTTGATAT-3'. The sequence underlined is T7 promoter sequence (same for *myoglobin* primers below). The primers span the region that encodes the signal peptide (SP), IgSF, and interceding region (ICR) of *FREP2*, a region known to be specific to this particular *FREP* gene [35]. A full-length *FREP2* cDNA was cloned from M line snails and the sequence was confirmed by sequencing. Using these primers, this specific sequence was amplified from the *FREP2* cDNA template and purified using the High pure PCR purification kit (Roche Molecular Biochemicals). dsRNA was synthesized from these purified templates using the MEGAscript RNAi kit (Ambion). Synthesized dsRNA was dissolved in nuclease-free water and stored at -20 °C and its integrity was routinely verified by non-denaturing agarose gel electrophoresis.

Myoglobin, a house-keeping gene [41] whose expression would not be expected to change as a consequence of exposure to trematode infection, was chosen as a control. *Myoglobin* dsRNA (541 bp) was synthesized in the same manner as described above. The primers designed from Gen-Bank accession number U89283 were: forward primer 5'-TAATACGACTCACTATAGGGACAACCAGCCCACTGAAA-3', and reverse primer 5'-TAATACGACTCACTATAGGGCGTGGGCGAATAATGACTTT-3'. The forward and reverse primers are in the 5'UTR (untranslated region) and 3'UTR, respectively [41]. The cDNA template for synthesis of the *myoglobin* dsRNA was cloned from BS-90 snails.

2.4. Introduction of dsRNA into snails

Regardless of the amount of dsRNA applied, the volume for all injections was 5 µl throughout this study. For sham injections, the same amount of nuclease-free water was injected. Snails of 6–9 mm shell diameter were used. Injections were made into the post-renal sinus just anterior to the digestive gland, using a BD Ultra-Fine™ II syringe, needle G 31 (BD Consumer Healthcare, New Jersey, USA). Two days after introduction of dsRNA, snails were exposed individually to ~30 miracidia of *S. mansoni* or *E. paraensei*, depending on the strain of snails used (i.e., BS-90 for *S. mansoni*; M line for *E. paraensei*). For the combination of M line snails and *E. paraensei*, the status of infection was checked under a dissecting microscope. Only snails with sporocysts visible in the heart were considered as “infected” and used for RNA extraction. Total RNA samples were extracted from the whole body of snails. Five snails were used for each treatment within each separate experiment. We first investigated different doses of *FREP2* dsRNA on RNAi knockdown of *FREP2* transcript abundance. BS-90 snails were injected with 0.1, 1.0 and 5.0 µg per snail of *FREP2* dsRNA.

In vitro RNAi was attempted by introduction of dsRNA into the Bge cultures, followed by exposure to *E. paraensei*. Five µg dsRNA (dissolved in 5 µl nuclease-free water), or 5 µl nuclease-free water as a control, were added directly to the culture medium in each well. About 2000 miracidia of *E. paraensei* were loaded per well at two days after addition of dsRNA. Total RNA was extracted from the Bge cells at 1, 2, 4, and 6 days after addition of miracidia (i.e., 3, 4, 6, and 8 days post-introduction of dsRNA).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from Bge cells or the whole body of individual snails using the NucleoSpin RNA II kit (BD Biosciences). cDNA was generated using random hexamers from the total RNA samples (ThermoScript RT-PCR kit; Invitrogen).

2.6. Real-time quantitative PCR

Quantitative PCR (qPCR) was used to monitor the quantitative expression of *FREPs* and *myoglobin* genes. qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems). All primers used for qPCR assay are listed in Table 1. In order to avoid amplification and detection of injected dsRNA, the qPCR primers were designed to amplify regions that did not overlap with the regions targeted by dsRNA. Since FBG of *FREP2* has no introns, additional primers which span intron 3 (at the ICR region) were used to monitor genomic contamination (Table 1). *18S* rDNA was used as an internal reference for qPCR, as described by Hertel et al. [36]. To test the specificity of RNAi, qPCR was also performed for *FREPs*, 3, 4, 6, 7, and 13.

qPCR was carried out on a Sequence Detection System 7000 (Applied Biosystems) using a SYBR Green PCR Core Reagents kit (Applied Biosystems), as used previously to assess differential expression of *FREPs* [36]. To quantify expression level of the target genes (*FREP2* and *myoglobin*), the synthesized cDNA used for qPCR was diluted 10-fold with nuclease-free water. For the *18S* rDNA (internal reference gene), 1000-fold dilution was used for qPCR because of the high number of *18S* rDNA copies. For each primer/cDNA sample combination, qPCR reactions were performed in duplicate. Twenty-five μ l of qPCR reaction mixture contained the following reagents: cDNA derived from 20 ng total RNA for *FREP2* or *myoglobin* amplification, or cDNA derived from 0.2 ng total RNA for *18S* rDNA amplification; $1 \times$ SYBR Green PCR buffer, 250 μ M each of dATP, dCTP, dGTP, and 500 μ M dUTP, 3mM MgCl₂, 0.1 μ M of primers, and 0.625U AmpliTaq Gold DNA polymerase. Cycling parameters were: 1 cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. Dissociation curves were analyzed at the end of each run to determine the purity of product and specificity of amplification.

2.7. Northern blots

For each of the three groups to be compared, 45 μ g total RNA was obtained by pooling from extractions from the whole bodies of ten individual BS-90 snails. RNA was separated on a 1% formaldehyde agarose gel. Electrophoresis and RNA transfer, hybridization, and blot-washing were performed according to the supplier's instruction (NorthernMax™ System, Ambion) with modifications suggested by Zhang and Loker [35]. The *FREP2* probe used in this study is identical to the *FREP2* probe previously used [35]. The probe sequence overlaps the region from which dsRNA of *FREP2* was derived. In addition to loading the same amount of RNA per lane, the staining intensity of ribosomal RNA (rRNA) was used as a standard to assure that equal amount of RNA was loaded in each lane.

2.8. Statistical analyses

Relative gene expression data were obtained using the $\Delta\Delta$ Ct method [42], using *18S* rDNA as the internal standard. The formula is: Fold change = $2^{-[\Delta\Delta\text{Ct}]}$, where $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{target}} - \text{Ct}_{18\text{S}})_{\text{treatment}} - (\text{Ct}_{\text{target}} - \text{Ct}_{18\text{S}})_{\text{control}}$. Each threshold value Ct is the mean of duplicate measurements. Before using the $\Delta\Delta$ Ct method for quantitation, validation experiments were performed according to the sequence detection system user bulletin #2 (Applied Biosystems) to demonstrate that efficiencies of target amplification and reference *18S* rDNA amplification were approximately equal.

All data were analyzed using statistical software SPSS10.3. Independent sample *t*-tests were used to determine *FREP2* expressional differences between dsRNA-treated groups and their corresponding non-dsRNA treated groups in both in vivo and in vitro experiments at various times of post-dsRNA treatment. One-way ANOVA was used to analyze induction of the *FREP2* expression in Bge cells, BS-90 and M line snails at various times post-exposure to parasites. Effect of different doses of *FREP2* dsRNA on knockdown of *FREP2* expression in BS-90 snail, induction of *FREP2* expression in Bge cells after exposure to different numbers of sporocysts and expression change of *myoglobin* gene in BS-90 snails at various times after introduction of *myoglobin* dsRNA were analyzed by one-way ANOVA. Post hoc multiple comparisons were made using Fischer's least significant difference test. Differences were considered as significant at $P < 0.05$.

3. Results

3.1. Validation of qPCR experiments by $\Delta\Delta C_t$ method

For the $\Delta\Delta C_t$ method to be valid, the amplification efficiencies of the target and reference genes should be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to examine the correlation between ΔC_t ($C_{t_{\text{target}}} - C_{t_{18S}}$) and several dilutions of template cDNA [42]. A plot of the log cDNA dilution versus ΔC_t was generated for each target gene tested (plots not shown). The slopes (b) of regression lines for *FREPs* 2, 3, 4, 6, 7, 13, and *myoglobin* were -0.049 , -0.068 , 0.075 , 0.054 , 0.042 , 0.094 , and -0.068 , respectively. The absolute values of all slopes obtained were smaller than 0.1 ($|b| < 0.1$), suggestive of equivalent efficiencies of amplification [42]. Therefore, $\Delta\Delta C_t$ calculation was used for the relative quantification of target genes selected in this study.

3.2. Knockdown of *FREP2* expression in BS-90 snails

For this study, two snail-parasite combinations were chosen to determine if RNAi could be documented for the *FREP2* gene. The initial experiments were done with BS-90 snails exposed to *S. mansoni*. Fig. 1 shows that, compared to uninfected controls, significant increases occurred in *FREP2* expression in BS-90 snails at 2 days post-exposure (DPE) to infection (9.7 ± 1.6 fold increase, $P < 0.01$) and at 4 DPE (13.1 ± 2.9 fold increase, $P < 0.01$). This trematode-provoked increase in host *FREP2* transcript abundance thus provided a good target for which to test protocols to achieve knockdown.

At 2 days post-injection (DPI), the snails were exposed to *S. mansoni*. *FREP2* transcript abundance at 4 DPE was significantly reduced compared to controls when individual snails were injected with dsRNA at a dose of 1 μg ($43.7 \pm 17.4\%$ of control, $P < 0.05$) or 5 μg ($16.5 \pm 5.8\%$ of control, $P < 0.01$) (Fig. 2). We, therefore, chose the dose of 5 μg per snail for our further RNAi experiments.

To determine the temporal dynamics of RNAi knockdown, dsRNA was injected into snails and at 2 DPI the snails were exposed to *S. mansoni*. Expression levels were found to be significantly decreased compared to snails that were exposed to the parasites but not injected with dsRNA. On 2 and 4 DPE, the expression levels were $34.3 \pm 7.3\%$, and $22.9 \pm 5.9\%$ relative to controls (i.e., no dsRNA injected; $P < 0.05$) (Fig. 1).

To further confirm that RNAi had been achieved, we performed Northern blot analysis to monitor changes in *FREP2* expression. We examined RNA samples from control snails and snails exposed for 2 days to *S. mansoni* that had been treated with dsRNA or not. Compared to controls, *FREP2* expression in snails exposed to *S. mansoni* was much higher than that of normal snails. In RNAi-treated snails, the expression level was obviously diminished compared to the snails that were exposed to parasites but received no dsRNA (Fig. 3). The pattern noted was thus consistent with data generated by qPCR. Unexpectedly, in addition to

FREP2 message, a fuzzy band of a size equivalent to, and smaller than, that of injected dsRNA was noted on the blot.

3.3. Knockdown of *FREP2* expression in M line snails

Using the same approach as described in Section 3.2, we examined the effect of RNAi on *FREP2* abundance in M line snails. First, *FREP2* expression was shown to be elevated in M line snails at 1, 2, 4, and 8 DPE to *E. paraensei* (4.9 ± 0.7 , 3.1 ± 0.4 , 7.5 ± 0.6 , and 3.6 ± 0.6 fold increases relative to non-exposed controls, respectively, all $P < 0.01$) (Fig. 4). This confirmed that *E. paraensei* exposure activated *FREP2* expression.

For experimental snails, 5 μ g *FREP2* dsRNA was injected followed 2 days later by exposure to *E. paraensei*. Controls were sham-injected and then exposed to *E. paraensei* at the same time. *FREP2* expression in experimental snails was observed to be significantly reduced at 2 DPE ($42.2 \pm 9.9\%$ of controls, $P < 0.01$) and 4 DPE ($20.0 \pm 6.9\%$ of controls, $P < 0.01$) (Fig. 4). The largest RNAi effect was seen at 4 DPE. These results generally agree with the findings observed in the BS-90 snails.

3.4. Knockdown of *FREP2* expression in Bge cells

In vitro RNAi experiments were conducted in Bge cells, the only available *B. glabrata* cell line. Like the in vivo RNAi studies described above, we first determined whether exposure to *E. paraensei* miracidia/sporocysts was able to heighten *FREP2* expression in the Bge cells, and how many parasites are required for the activation. As shown in Fig. 5, there is a positive correlation between the number of miracidia added to cultures and the level of *FREP2* expression observed in Bge cells. The expression levels observed from the cells that were co-cultured with 2000 miracidia was significantly higher than that of the non-exposed control cells ($P < 0.05$).

This was followed by experiments to determine the timing of *FREP2* expression after exposure to the parasite. *FREP2* expression was significantly increased at 4 and 6 days post-addition of 2000 miracidia (5.2 ± 1.0 and 6.0 ± 0.5 fold of the non-exposure control, respectively; both $P < 0.01$) (Fig. 6).

After introduction of dsRNA to the Bge cultures, followed by exposure to *E. paraensei*, significant decreases of *FREP2* expression were observed at 4 DPE ($38.2 \pm 6.8\%$ of non-RNAi treated control, $P < 0.05$) and 6 DPE ($46.9 \pm 14.8\%$ of non-RNAi treated control, $P < 0.05$).

3.5. Knockdown of myoglobin expression in BS-90 snails

Injection of *myoglobin* dsRNA resulted in significant decreases of *myoglobin* expression in BS-90 snails at 2, 3 and 4 DPI (i.e., $40.0 \pm 9.3\%$, $31.9 \pm 4.3\%$ and $34.1 \pm 8.5\%$ of control levels, respectively, all $P < 0.05$) compared to sham-injected controls (Fig. 7).

3.6. Specificity of RNAi in snails

To test the specificity of RNAi, BS-90 snails were injected with *FREP2* dsRNA, *myoglobin* dsRNA or nuclease-free water 2 days before exposure to *S. mansoni* miracidia. We detected significant reduction in expression of the targeted gene level at 4 DPE, but no RNAi effect was observed for the non-target gene (Fig. 8A).

Further RNAi off-target experiments were performed using members of the *FREP* gene family. The expression of six *FREP* genes, i.e., *FREPs* 2, 3, 4, 6, 7, and 13, was examined in BS-90 snails that were injected with *FREP2* dsRNA and exposed to *S. mansoni*. Significant reduction of gene expression was observed for *FREP2* and *FREP6* ($P < 0.01$), but no

reduction was observed in transcript abundance for the other four *FREP* genes (Fig. 8B). Knockdown of *FREP6* is likely due to the high nucleotide identity (96%) between *FREP2* and *FREP6*, which will be further discussed below. In conclusion, extensive cross-reactivity in RNAi was not observed.

4. Discussion

We chose three systems to investigate in this study: BS-90 snails—*S. mansoni*, M line snails—*E. paraensei*, and Bge cells—*E. paraensei*. BS-90 snails are resistant to *S. mansoni* whereas M line snails are susceptible. A previous study showed the expression of *FREP2* in the BS-90 snails is upregulated after exposure to *S. mansoni* whereas M line snails have no response to schistosome infection, suggestive of a role for *FREP2* in resistance [36]. Our study confirmed that *FREP2* expression could be provoked in BS-90 snails after exposure to *S. mansoni*. Development of RNAi in the BS-90—*S. mansoni* combination provides a plausible way to evaluate the function of FREPs in defense against *S. mansoni* in resistant snails. Search for anti-schistosome molecules in the snail intermediate host is a long-term goal relevant to schistosomiasis control efforts. The M line—*E. paraensei* system has been studied in our lab as a model of host–parasite interactions, and FREPs were originally identified in M line snails infected with *E. paraensei* [30]. In addition to attempts to induce RNAi in vivo, we were interested in determining if, in principle, RNAi could be demonstrated in vitro, so we chose cells of the Bge cell line as potential targets for *FREP2* knockdown. Here, we note that Bge cells were originally derived from embryonic snails so the exact cell lineage they represent is not well characterized. Although Bge cells have some properties consistent with those of hemocytes, it should not be assumed they are representative of hemocytes derived from juvenile or adult snails. Before proceeding with RNAi experiments, we first demonstrated an increase in *FREP2* expression following exposure to *E. paraensei* in the latter two model systems. This is the first report that *FREP2* expression can be upregulated in Bge cells following exposure to trematode larvae. Development of RNAi in a cell line will provide a potentially valuable alternative means to evaluate snail gene functions.

We then observed that injection of *FREP2* dsRNA resulted in a 77–80% reduction of *FREP2* transcript abundance in BS-90 and M line snails. Similar results were found for the *myoglobin* gene (68% reduction). Generally, knockdown of transcript abundance of 70% or more is considered to be an effective application of RNAi (Ambion). The efficiency of RNAi varies, depending on the species, tissue, or genes targeted. For example, knockdown of *Toll*, *Dif* and *Drs* genes in adult *Drosophila* were 26, 47 and 62%, respectively [15], and the knockdown of aminopeptidase (slapn) in *Spodoptera litura* larvae was up to 95% based on the intensity of RT-PCR bands [14].

The RNAi knockdown we achieved was transient. For both BS-90 and M line snails, significant reduction in *FREP2* expression was not noted at 3 DPI, was achieved at both 4 and 6 DPI, and was gone by 10 DPI. Similar dynamics were noted for *myoglobin*. Goto et al. [15] reported that knockdown in fruitflies following direct injection of dsRNA lasted for at least 1 week [15]. In the planarian *Girardia tigrina* the RNAi effect on targeted transcript was apparent up to 3 weeks post-injection as determined by in situ hybridization [43]. In general, the mechanisms explaining longevity of the RNAi effect remain unknown. As the snails were exposed to infection 2 days following dsRNA injection, and sporocysts of *S. mansoni* are typically killed within 3 DPE in resistant snails [44,45], a significant level of knockdown would be achieved for much of the critical interval determining parasite success or failure. Thus even though transient, RNAi could still provide a unique opportunity to test the role of FREPs in defense against trematodes.

In the in vitro studies, the direct introduction of dsRNA into culture medium successfully knocked down targeted gene expression in snail cells. Clemens et al. [22] reported that dsRNA was capable of specifically blocking the production of targeted proteins in *Drosophila* cell lines when added directly to the culture media, without using a transfection reagent [22]. These results indicate that some cell lines effectively take up dsRNA. The transmembrane protein SID-1 has been found to transport dsRNA into *Drosophila* cells [46].

In addition to confirming our RNAi results, the Northern blot showed that a considerable amount of the injected dsRNA was still present in snails at 4 DPI. Our explanation is that this band represents dsRNA that was still present in the injected snails, either in intact or partially degraded form. It is possible that the dsRNA detected in the Northern blot was present in extracellular compartments, such as hemolymph because RNA was extracted from the whole body of snails.

It has been reported that off-target effects can be a potential problem for RNAi [47–49]. We first demonstrated that limited or no cross reactivity occurs between *FREP2* and *myoglobin*, and then showed that with *FREP* subfamilies non-specific knockdown of expression was not observed except for *FREP6*. This may be explained by the fact that the available *FREP6* sequence (ICR and FBG regions, a total of 625 bp) has 96% nucleotide identity with *FREP2*. In the same region, the identity between *FREP2* and *FREP4*, the only other *FREP* with a single IgSF domain for which the complete gene sequences are known, is only 60% [32]. Although these results indicate that cross-reactivity is not extensive among *FREP* subfamilies, it does suggest the cross-reactivity may occur between two sequences with high nucleotide identity. This provides useful information for the design of future RNAi experiments to further minimize off-target effects for the purpose of knockdown of specific genes.

Many aspects of the RNAi effect in snails await further clarification. Our future studies will emphasize how to maintain the RNAi effect for longer periods of time. We also intend to examine how RNAi specifically affects gene expression in hemocytes, and how to develop an effective approach to examine the corresponding changes in protein abundance.

In summary, our data for the first time demonstrated that RNAi based on *FREP2* and *myoglobin* genes can be achieved in two strains (BS-90 and M line) of the snail *B. glabrata* and its cell line (Bge cells). RNAi thus may be applied to assess snail gene functions, an approach that will become more useful once the *B. glabrata* genome, now being actively sequenced, has been obtained. RNAi will also be a useful tool to reveal the genes critical in mounting effective defense responses against medically important parasites such as *S. mansoni* that depend on their molluscan hosts for their larval development.

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Fig. 1.

Effect of timing on knockdown of *FREP2* expression in BS-90 snails after exposure to *S. mansoni*. Open bars show *FREP2* expression levels at different days post-exposure (DPE). The symbol “#” indicates a significant elevation ($P < 0.01$) of expression of *FREP2* in the sham-injected snails at the given time point post-exposure to the parasite, relative to non-exposed, sham-injected snails. Filled bars show the expression of *FREP2* after introduction of dsRNA (5 μ l at 1 μ g/ μ l). In this experiment snails were exposed to *S. mansoni* at 2 days post-injection (DPI). An “*” indicates a significant reduction ($P < 0.05$) in *FREP2* expression in snails injected with dsRNA compared to sham-injected snails at the same time post-exposure to the trematode.

Fig. 2.

Effects of different doses of *FREP2* dsRNA on knock-down of *FREP2* expression in BS-90 snails after exposure to *S. mansoni*. Snails were injected with different doses of dsRNA, followed by exposure to 30 *S. mansoni* miracidia at 2 DPI of dsRNA. RNA was extracted from the snails at 4 DPE. Five snails ($n = 5$) were used in each experiment. The symbols “*” and “**” indicate $P < 0.05$ and $P < 0.01$, respectively.

Fig. 3.

Northern blot analysis showing RNAi effect in BS-90 snails. Lane 1 shows RNA from control snails at 4 days post sham-injection. Lanes 2 and 3 represent RNA from snails that were either sham-injection (lane 2) or injected with dsRNA (lane 3), followed by exposure to *S. mansoni* for 2 days (i.e., 4 DPI). For each lane, total RNA was pooled from the whole bodies of 10 snails. The upper image shows the intensity of *FREP2* probe binding to mRNA. The lower photos show the staining intensity of rRNA from lanes, as loading controls. Approximate molecular sizes (kilobase) are indicated on the right.

Fig. 4. Effect of timing on knockdown of *FREP2* expression level in M line snails after exposure to *E. paraensei*. Open bars show *FREP2* expression levels in M line snails. The symbol “#” indicates a significant elevation ($P < 0.01$) of expression of *FREP2* in the sham-injected snails at the given time point post-exposure to *E. paraensei*, relative to non-exposed, sham-injected snails. Filled bars show the expression of *FREP2* after introduction of *FREP2* dsRNA (5 μ l at 1 μ g/ μ l). Snails were exposed to *E. paraensei* at 2 DPI. An “*” denotes a significant reduction ($P < 0.05$) in *FREP2* expression in snails injected with dsRNA, compared to sham-injected snails at the same time post-exposure to the trematode.

Fig. 5.

Induction of *FREP2* expression in Bge cells after exposure to different numbers of *E. paraensei* miracidia. Bge cells were seeded at 2×10^5 cells per well in 500 μ l medium, one day prior to addition of parasites. Parasites were in inserts to maintain separation between them and the Bge cells. RNAs was extracted from the cells 4 day after addition of parasites. An “*” indicates significance when compared to control ($P < 0.05$). Three wells were used for each treatment.

Fig. 6.

Knockdown of *FREP2* expression level in Bge cells after exposure to *E. paraensei*. Bge cells were seeded as described in Fig. 5. After one day of cell culture, 5 μ g *FREP2* dsRNA was applied to the culture medium. Two thousand miracidia were added 2 days after the dsRNA. Bge cells were harvested at different DPE, i.e., 1, 2, 4 and 6 days for RNA extraction. Open bars show *FREP2* expression levels in the Bge cells at different DPE. The symbol “#” indicates significant elevation ($P < 0.01$) of expression of *FREP2* in the sham-injected snails at the given time point post-exposure to the parasite, relative to the non-exposed cells. Filled bars show the expression of *FREP2* after introduction of *FREP2* dsRNA (5 μ l per well). An “*” denotes a significant reduction ($P < 0.05$) in *FREP2* expression in the cells in which the

FREP2 dsRNA was added compared to cells at the same time post-exposure to the trematode, but with no involvement of RNAi. The analysis was based on three independent wells for each experiment.

Fig. 7.

Expression of *myoglobin* in BS-90 snails was reduced after introduction of *myoglobin* dsRNA (5 μ l at 1 μ g/ μ l). The sham-injection (control) was conducted in each experiment, and the expression value is presented by a solid horizontal line indicated by 1.0 in the figure. An “*” indicates a significant reduction ($P < 0.05$) in *myoglobin* expression relative to non-RNAi treated snails at 2, 3 and 4 DPI.

Fig. 8.

Specificity of RNAi effects in *B. glabrata*. (A) RNAi cross-reactivity did not occur between *myoglobin* and *FREP2* in BS-90 snails. The injection of *FREP2* dsRNA resulted in a significant reduction in *FREP2* expression ($*P < 0.01$) compared to sham-injected controls, whereas *myoglobin* expression was not significantly altered. Conversely, the injection of *myoglobin* dsRNA resulted in a significant reduction in *myoglobin* expression ($*P < 0.01$) compared to sham-injected controls, whereas *FREP2* expression was not significantly reduced. As before, all snails were exposed to *S. mansoni* at 2 DPI of dsRNA or of nuclear-free water (sham-injection). qPCR data were collected at 4 DPE (i.e., 6 DPI). (B) Expression level of *FREP* genes (*FREPs* 2, 3, 4, 6, 7 and 13) in BS-90 snails after injection of dsRNA of *FREP2* (5 µg per snail). There were two groups of snails, one injected with *FREP2* dsRNA (5 µg) and the other sham-injected as a control. Five snails were analyzed in each group by qPCR. We divided the expression values for each *FREP* gene by their corresponding control expression value. Accordingly, the control expression for each gene is 1 (presented by a solid horizontal line indicated by 1.0 in the figure). Values < 1 represent downregulation and values > 1 represent upregulation. $*P < 0.01$.

Table 1

Primers used for qPCR

Gene	Primer	Sequence (5'-3')	GenBank No.
<i>FREP2</i>	Forward	TGGAATTACTAAGAGTGTACGTTTACTGA	AY012700
	Reverse	GGGTTTTCCCACTTAATCTAG TTCTATTT	
<i>FREP2*</i>	Forward	GCTGTAGCTCTGAGTTATATACAAGATCGT	AY012700
	Reverse	GGCTCT TGATATTTGAATGCTATCC	
<i>FREP3</i>	Forward	CAATCTTTC AACTGCCTCGATAAA	AY028461
	Reverse	GGGTGGATAGCTCATTTTGCTT	
<i>FREP4</i>	Forward	AAGAAAATCATTTAGCAGCCTTGAG	AY012701
	Reverse	GCAAGACAGTTGCAGGTTTTGT	
<i>FREP6</i>	Forward	ATGGATAAACCCAGGCTTACGAA	AY012702
	Reverse	CACGTTTCTCTTAATGTTACAATTCATGAT	
<i>FREP7</i>	Forward	CGT CTGGTTTTCTTCCAATCTTTATT	AY028462
	Reverse	TCTGGTTGGACATCAATGACAAG	
<i>FREP13</i>	Forward	CAACTGCCTTAATGGAAGTAAAAAATC	AF515468
	Reverse	AGATGT TATTTGATCGTCTCCCTTTC	
<i>Myoglobin</i>	Forward	GGAATTCATGCAATCAATGCA	U89283
	Reverse	TCT CTTTAATGTTTTCGAGATCTTC	
<i>18S rDNA</i>	Forward	CGCCCGTCGCTACTATCG	U65224
	Reverse	ACGCCAGACCGAGACCAA	

Note: *FREP2** primers located at the interceding region (ICR) of *FREP2* were used to check for absence of contamination with genomic DNA.