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Schistosoma mansoni: Off-target analyses using nonspecific double-stranded RNAs as control for RNAi experiments in schistosomula

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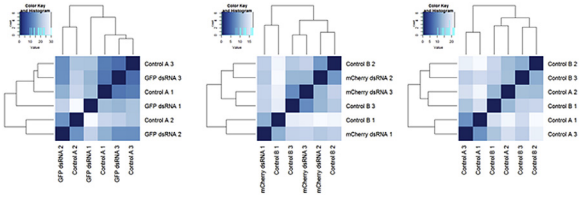
HIGHLIGHTS

- Extrinsic factors generate greater experimental variability than most used RNAi controls.
- Nonspecific genes GFP/mCherry are suitable controls for RNAi assays in schistosomula.
- Untreated parasites could also be set as controls for RNAi assays in schistosomula.
- This work sets parameters that will enhance RNAi work in *S. mansoni*.

GRAPHICAL ABSTRACT



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ABSTRACT

RNA interference is a well established and widely used reverse genetic tool available for gene functional studies in trematodes. This technique requires the use of nonrelevant double-stranded RNA as control. However, several authors have reported inconsistencies associated with RNAi. We used RNASeq to analyze genes affected by nonspecific dsRNA exposure. We found only few genes presenting altered expression in schistosomula exposed to GFP or mCherry nonspecific-dsRNAs, most of them encoding uncharacterized proteins. Correlation analysis revealed that there are more differences among biological replicates, than due to treatment with nonspecific controls. These observations are of key relevance to other RNAi gene function assessment in other organisms.

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1. Introduction

Gene silencing using specific dsRNA is an informative and powerful reverse genetic tool to examine gene function by loss or reduction of specific transcripts. It is widely used in many organisms including parasites such as *Schistosoma mansoni* (Andrade et al., 2014; Boyle et al., 2003; Kotze and Bagnall, 2006; Mourão et al., 2009; Skelly et al., 2003). Most of the RNA interference (RNAi) assays in trematodes have been performed in *Schistosoma* sp., mostly in *S. mansoni*. Dicer and RISC-associated proteins (piwi/argonaute orthologues) are coded in the genome and transcribed in schistosomes, indicating that post-transcriptional gene silencing could occur (Verjovski-Almeida et al., 2003). Therefore, the use of RNAi to suppress schistosome gene function was possible and had been pursued by different groups as the primary reverse

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genetics tool available in helminthes. The first two reports of RNAi targeted manipulation of endogenous gene expression in a parasitic flatworm were in *S. mansoni*. Skelly and collaborators (Skelly et al., 2003) suppressed the expression of a cathepsin B gene in *S. mansoni* by soaking schistosomula in dsRNA for 6 days. Lower levels of cathepsin B were identified by immuno-staining and by enzyme activity measurements, and the suppression was checked at the RNA level using RT-qPCR. In another report, Boyle and collaborators (Boyle et al., 2003) targeted two *S. mansoni* genes: SGTP1, a facilitated diffusion glucose transporter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *S. mansoni* larvae treated *in vitro* for 6 days with specific dsRNA showed 70–80% reduction at the transcript level, while non-targeted transcripts were not affected. The phenotype of parasites exposed to SGTP1 dsRNA was 40% reduction in glucose absorption (Boyle et al., 2003). Since then, a number of experiments based on RNAi have been performed in *Schistosoma* targeting diverse life stages in culture: miracidia, sporocysts, schistosomula and adult worms, demonstrating the feasibility of manipulation of gene expression and shedding light on the functions of several schistosome genes.

Despite the clear success of RNAi use in schistosomes, there are critical reports on the reproducibility of experiments among other pitfalls, especially off-target effects (Geldhof et al., 2007). To date there are 49 papers that used RNAi to knockdown different genes in various life stages of *S. mansoni*, using long double-stranded RNA (dsRNA) or small interfering RNA (siRNA). For those using siRNAs, the commonly used controls are commercial irrelevant scrambled siRNAs or mutated versions of the siRNAs. Regarding the use of dsRNA, 85% of the papers used a nonspecific dsRNA as control. The most frequently used controls are dsRNA of an irrelevant non-schistosome RNA, GFP (green-fluorescent protein from pCRII plasmid vector) and mCherry (*Discosoma* sp. mCherry fluorescent protein, GenBank AY678264) accounting for 18% and 11% of the reports, respectively. In most studies, the authors used quantitative real-time PCR (qPCR) to measure the levels of knockdown. The endogenous genes commonly used in qPCR normalization are α-tubulin, GAPDH, 18S rRNA or cytochrome C oxidase I. Additionally, dsRNAs specific for paramyosin, actin, alkaline phosphatase and proteasome subunit beta type-4 were used as controls.

Since its discovery, among the main concerns of any study involving functional analysis by RNAi are around the occurrence and extent of off-target effects (disturbance in the expression of genes non-related or similar to the specific gene target). Off-targeting has been a concern for experiments using schistosomes and other helminthes, in addition to issues regarding the reproducibility of different methods used to deliver dsRNAs in RNAi experiments (Geldhof et al., 2007; Mourão et al., 2009; Stefanić et al., 2010). It has also been described that the dsRNA itself can have an effect on the parasites which emphasizes the need for adequate controls (Geldhof et al., 2007). Furthermore, we have observed that for some genes, the transcript expression levels of controls treated with different nonspecific dsRNAs diverge from untreated controls. Based on the above mentioned issues and taking into account discrepancies observed in our experience working for years with functional analysis using RNAi of several *S. mansoni* genes, we decided to globally investigate the effects of two of the most frequently used nonspecific dsRNA controls, GFP and mCherry. We used an RNASeq approach in order to globally investigate off-target effects.

2. Material and methods

2.1. Parasites

Here, we used the LE strain of *S. mansoni* that was maintained at Centro de Pesquisas René Rachou – FIOCRUZ by passages using *Biomphalaria glabrata* as the intermediate host. Schistosomula were obtained by mechanical transformation of cercariae (Basch, 1981) and cultured in Glasgow Minimum Essential Medium (GMEM) (Sigma-Aldrich, Germany) supplemented with 0.2 μM triiodothyronine (Sigma-Aldrich, Germany); 0.1% glucose; 0.1% lactalbumin (Sigma-Aldrich, Germany); 20 mM HEPES; 0.5% MEM vitamin solution (Gibco, Thermo Fisher Scientific, USA); 5% Schneider's Insect Medium (Sigma-Aldrich, Germany); 0.5 μM Hypoxanthine (Sigma-Aldrich, Germany), 1 μM hydrocortisone (Sigma-Aldrich, Germany), 1% Penicillin/Streptomycin (Gibco, Thermo Fisher Scientific, USA) and 2% heat-inactivated Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, USA).

2.2. dsRNA synthesis

The sequences of primers for dsRNA synthesis were taken from the literature (Mourão et al., 2009; Stefanić et al., 2010) and synthesized by IDT (IDT, USA). A T7 promoter tag was added to the 5′ end of all PCR primers. A fragment of 360 bp of the open reading frame for GFP was amplified from the plasmid vector pCRII-GFP (Thermo Fisher Scientific, USA) (GFP_dsRNA_Forward 5'-taatacgactcactatagggTCTTCAAGTCCGCCATG-3' and GFP_dsRNA_Reverse 5'-taatacgactcactatagggTGCTCAGGTAGTGGTTGTC-3') and a 711 bp fragment for mCherry (mCherry_dsRNA_Forward 5'-taatacgactcactatagggTGGTGAGCAAGGGCGA-3′ and mCherry_dsRNA_Reverse 5'-taatacgactcactatagggTTACTTGTACAGCTCGTCC-3′) were used as nonspecific RNAi controls. In lowercase are the T7 promoter sequence and in capital letters are the specific sequences for each gene. Following amplification, PCR products were separated on 1% agarose gels and purified using QIAquick Gel Extraction Kit (Qiagen, Germany), sequence identity was confirmed by Sanger sequencing. The dsRNAs were generated from PCR products using the T7 RiboMAX Express RNAi Kit (Promega, USA) according to the manufacturer instructions (Mourão et al., 2009; Stefanić et al., 2010). dsRNAs were analyzed by 1% agarose electrophoresis to check the integrity and annealing of the dsRNA transcript. In order to have a positive dsRNA knockdown control, a SmERK (Smp142050) gene fragment of approximately 500 bp was also amplified by PCR. Primers for this gene were previously designed and used by Andrade et al. (2014). cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Transcript levels were verified by RT-qPCR on an ABI 7500 RT-PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) using Power SYBR® Green Master mix (Applied Biosystems, Thermo Fisher Scientific, USA) and using the Cytochrome C oxidase I gene (GenBank AF216698) (Oliveira et al., 1998) as endogenous control to normalize expression levels. Post-RNAi transcript levels were analyzed using the comparative Ct method (Livak and Schmittgen, 2001) and expressed as percentage of difference compared to the untreated control.

2.3. Parasite exposure and RNA extraction

In order to clarify the effects in gene expression triggered by largely used nonspecific controls in schistosomula transcriptomes, we performed RNASeq-based deep sequencing in schistosomula untreated and treated with dsRNA of GFP or mCherry and the positive control SmERK. Schistosomula (~500,000 worms) were cultivated in flasks containing 10 mL GMEM supplemented as above mentioned. For each treatment, 100 nM of dsRNA were added after transformation. Untreated controls (A and B) contained only supplemented medium and parasites. Incubations were carried for 2 days at 37 °C under 5% CO_2 and 95% relative humidity. Three biological replicates of each one of the four conditions

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