Journal of Bacteriology and Parasitology

Mourão and Gava, J Bacteriol Parasitol 2017, 8:5
DOI: 10.4172/2155-9597.1000325

Commentary Open Access

RNA Interference and Nonspecific Controls in Parasitic Helminths

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Received date: October 11, 2017; **Accepted date:** October 30, 2017; **Published date:** November 03, 2017

Abstract

Despite of the remarkable success of RNA interference (RNAi) application in the free-living nematode *Caenorhabditis elegans*, the exploitation of this powerful technique to helminths parasites with complex life-cycle has been a challenge for parasitologists. It has proved to be effective only for certain parasite species and specific target genes. To date, RNAi is the only methodology available for reverse genetics in trematodes and combined to rescue studies (such heterologous complementation) have been the only alternative to genetic manipulation in nematodes and helminths parasites, thus this subject is of great interest to the scientific community involved in the field.

The RNAi technique is widely used to assess gene function in helminths parasites, in order to elucidate their role in parasite development, mechanisms of drug resistance, and validate therapeutic targets for disease control.

After fifteen years of the first report of RNAi in parasitic helminths, many advances have been achieved, but pitfalls remain as challenges in gene expression manipulation in these organisms. In addition to the methodological particularities of the RNAi technique for each group of helminths, there are still other reasons behind the slow progress of RNAi in those parasites, such; the lack of homology between genes related to parasitism and genes of model organisms and the complex life cycle of these organisms, which results in difficulties for *in vitro* cultivation.

At this point, a wide assortment of approaches for doubled stranded RNA "delivery" has been proposed. Thus, deeper studies on fundamental aspects of the RNAi methodology in parasitic helminths, such as off-target and the use of controls, can be useful in determining the reason of variations between and within species, facilitating the experimental design and the use of RNAi in the study and eradication of helminths parasites.

Keywords: Helminths; RNA interference; Gene function

Introduction

According to the World Health Organization (WHO), approximately 24% of the world's population is infected with helminths and more than 200 million people worldwide are infected with schistosomes [1]. It is well known that helminths parasitic diseases directly impact human and livestock around the world, associated with considerable economic losses. The scarce parasitology-related research aggravates the situation by the lack or limited alternatives to disease control, such as vaccination and/or effective drugs. The WHO has been convening the scientific society to translate the achieved knowledge on the molecular basis of complex biology parasites to unravel broader knowledge that could lead to effective interventions [2].

Given the medical and veterinary importance of helminths infections, there is urgent need for expanding research findings towards drugs and vaccines, thus is necessary to have a comprehensive knowledge of the parasite's biology. In order to build up information to elucidate helminths biology and host-parasite interaction towards discovery of new drug targets, comes along the feasibility of systems and tools to manipulate gene expression to make sense of so much data generated by genome and transcriptome sequencing. Among very few techniques available for gene characterization in parasitic helminths, RNA interference (RNAi) is the most accessible and employed tool [3-5].

The discovery of RNAi-based gene silencing in *Caenorhabditis elegans* [6] and the succeeding knowledge that RNAi was a pervasive phenomenon occurring in plants and animals, provided the first real perspective for the application of reverse genetics approach to achieve gene function assessment in parasitic helminths [7]. RNAi is fairly conserved and intrinsic mechanism of gene regulation that drives silencing at the transcriptional or posttranscriptional level, protection against viral infection, control of epigenetic modifications/heterochromatin formation, regulation of genome stability, and restriction of transposon movement. The consequence of RNAi is loss-of-function phenotype that, ideally, would be similar to a null mutant [8,9]. Within the context of parasite-based research, gene function studies generate target-validation data in that RNAi-induced phenotypic changes can support drug target validation [10-13].

One obstacle of using this technique in helminths parasites has been the lack of consistency of knockdown, not only in different species, but also within genes in the same species [3-5,14]. Even though there have been several suggestions to explain those inconsistencies [15-17], there remain a number of studies concerning questions in which RNAi has not been successful and therefore, unpublished.

Gene knockdown in *C. elegans* has been highly successful, however, the success of the translation of RNAi approaches from *C. elegans* model to parasitic nematodes has been rather variable. This could be

attributed to the absence of defined RNAi effectors in specific nematodes [16]. Nonetheless, this explanation has been ruled out in recent comparative genomic analysis that shows a similar coverage of RNAi functional protein complexes in both, parasitic nematodes in which silencing has been successful and the ones it has failed [18]. This supports the broad applicability of RNAi in nematodes and suggests that variable results of RNAi approaches could be credited to other factors, such, adverse culture conditions, choice of dsRNA/siRNA sequence and/or differing cuticle permeability, which could interfere with the RNAi methodology and its outcomes [2].

Besides the RNAi-related methodological particularities for each group of helminths, two main reasons are behind the slow and disappointing development of RNAi in this group: (i) the apparent lack of homology between some of *C. elegans* genes and parasites' genes, especially those involved in the parasitic lifestyle and host-parasite relationship [14] and (ii) the complexity of the parasitic life cycles, which includes the difficulties for *in vitro* culture of developmental stages and the lack of immortal cell lines.

Many reports of success and pitfalls have raised great interest and doubts for the application of RNAi in helminths parasites. This technique requires the use of non-relevant double-stranded RNA as control. As great users of this technique to fulfill the biology gaps after the release of *Schistosoma mansoni* genome, we sought to know whether the nonspecific controls our group employ and are used in 29% of work with RNAi in *S. mansoni*, could generate off-target in the larval stage of the parasite. To address that, we used RNASeq to globally analyze genes affected by nonspecific dsRNA exposure in our article entitled "*Schistosoma mansoni*: Off-target analyses using nonspecific double-stranded RNAs as control for RNAi experiments in schistosomula" [19].

The off-target is one of the factors commonly associated with discrepancies and unexpected results when using the RNAi technique. It consists of the altered expression of unrelated or homologous genes triggered by the presence of dsRNAs/siRNAs. In addition, it has been described that the dsRNA itself may have an effect in parasites, which evidences the need for adequate controls [14]. Based on the above mentioned and taking into account the deviations observed in our own experience working with functional characterization of various *S. mansoni* genes using RNAi, we decided to investigate the effects of the two most commonly used nonspecific dsRNA controls (GFP and mCherry) on the schistosomula transcriptome, searching for possible genes affected by exposure to these nonspecific dsRNAs.

With this strategy, we found that groups of schistosomula that were not exposed to dsRNAs (untreated controls) and schistosomula treated with dsRNAs (GFP or mCherry) are transcriptionally equivalents. It is worth noting, that untreated parasites presented slightly more Differential Expressed Genes (DEGs) among biological replicates than when compared to groups treated with the nonspecific controls (GFP or mCherry-dsRNA). We report very few genes presenting altered expression in the larval stage exposed to GFP (6 DEGs, P-value<0.01) and mCherry (3 DEGs, P-value<0.01) nonspecific-dsRNAs, most of them encoding uncharacterized proteins and none of them is routinely used as endogenous control in the normalization of qPCR experiments. In addition, correlation analysis also showed that, overall, there are more differences between biological replicates than due to treatment with these nonspecific controls. Therefore, extrinsic factors generate greater experimental variability in *Schistosoma mansoni* than the exposure to GFP and mCherry-nonspecific dsRNA controls commonly used in RNAi experiments.

Despite the highly discussed experimental variations and inconsistencies in RNAi assays, our results show that GFP and mCherry-dsRNAs are suitable controls for RNAi assays in schistosomula, since we provide experimental evidence that schistosomula treated with these nonspecific dsRNAs are transcriptionally similar to the untreated parasites. Moreover, those results could lead to discussion on the requirement of unspecific controls use for RNAi experiments in *Schistosoma* spp., since carrying out two controls for each experiment increase the expense, amount of samples to be processed, and might not be enlightening.

Acknowledgements

The authors thank the Program for Technological Development in Tools for Health-PDTIS FIOCRUZ for use of its facilities. This work was supported by the European Commission's Seventh Framework Programme for research, under Grant agreement no. 602080 (A-ParaDDisE), CAPES Programa PCDD-Programa CAPES/Nottingham University (3661/2014), FAPEMIG (CBB-APQ-0520-13).

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