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RNA interference in parasitic nematodes of animals: a reality check?

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RNA interference (RNAi) is widely used in *Caenorhabditis elegans* to identify gene function and has been adapted as a high-throughput screening method to identify genes involved in essential processes. The technique has been applied to parasitic nematodes with variable success and we believe that inconsistent outcomes preclude its use as a robust screen with which to identify potential control targets. In this article, key issues that require clarification are discussed, including the mode of delivery of double-stranded RNA to the parasite, the developmental stage targeted and, perhaps of most importance, whether the RNAi pathway (as defined by studies in *C. elegans*) is fully functional in some parasitic nematodes.

RNA interference

Specific gene silencing by RNA interference (RNAi) was first described in *Caenorhabditis elegans* and has been developed in this organism for high-throughout functional genomics [1–3]. The ease with which RNAi can be used in *C. elegans*, together with the fact that the RNAi mechanism itself is conserved in a large number of organisms [4–6], has almost automatically led to the assumption that RNAi could be applied to related parasitic nematodes. However, recent evidence indicates that the application of RNAi to parasitic nematodes is not as straightforward as was expected [7,8] and efficacy has been extremely variable [9–11]. This variability is particularly intriguing. Some genes in *C. elegans*, particularly some neuronal genes, are less susceptible than other genes to RNAi [12]. However, most genes targeted in parasites are not neuronal and some are, in fact, potential homologues of genes that are susceptible to RNAi in *C. elegans* [10]. So, why do parasitic nematodes seem to be less susceptible to RNAi than does *C. elegans*? Is it because of suboptimal culture conditions and/or methods of double-stranded (ds)RNA delivery – as recently suggested by Zawadzki *et al.* [13] – and, if so, is it possible to overcome these hurdles? Alternatively, is the classical RNAi pathway functional in parasitic nematodes and are the effects that have been observed to date caused by as-yet-undefined interactions? (Box 1)

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Culture conditions, method of delivery and parasite stage

Most studies have evaluated RNAi in parasitic nematodes using the soaking technique applied to *C. elegans* [14], with worms maintained in a simple medium containing the dsRNA at concentrations usually in the mg/ml range. Often, culture fluids are supplemented with liposome preparations (such as lipofectin) to increase the efficiency of RNA uptake into cells. However, parasitic nematodes will not survive for more than a few days under these conditions, with the result that apparently lethal RNAi phenotypes can arise because the worm is already severely compromised. It is recognized [13] that there is a need to develop appropriate culture conditions that enable the nematode parasite to survive for prolonged periods (weeks or months) or, better still, to continue development through to sexually mature adult worms. For strongyloid parasites such as *Haemonchus contortus*, the complexity of the medium required to achieve this precludes routine use [15]. Moreover, there is the possibility that the medium could interfere with the efficiency of dsRNA, or short interfering (si)RNA, uptake by nonspecifically binding to the RNA species.

Zawadzki *et al.* [13] attributed the success of RNAi in *C. elegans* to the ability to maintain the nematode throughout its life cycle. However, other related free-living nematodes (such as *Oscheius tipulae* and *Pristionchus pacificus*) that are cultured in a similar way to *C. elegans* do not seem to be susceptible to gene silencing by RNAi [16]. Although improved culture methods are likely to enhance the ability to monitor RNAi effects, it cannot be assumed that effective RNAi will be possible in all parasitic nematodes, even with the development of suitable culture systems.

The route of dsRNA delivery is also likely to be important. Because of the ease with which it can be carried out, soaking is most commonly used for RNAi in parasitic nematodes. However, microinjection remains the 'gold standard' for an effective RNAi in *C. elegans* [17] and *Caenorhabditis briggsae*. Indeed, studies have indicated [18] (M.K. Montgomery, personal communication) that *Caenorhabditis* strains (e.g. *C. briggsae*) can vary in their susceptibility to RNAi by feeding or soaking but silencing can be induced by microinjection. This indicates differences between nematodes in the uptake and, possibly, the spread of dsRNA, which could also apply to parasitic species. Following the soaking of *H. contortus*

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Box 1. Factors requiring further research to optimize RNAi in parasitic nematodes

- The presence of fully functional RNAi pathways
- Culture conditions to maintain the target parasite stage
- Uptake of dsRNA by the parasite stage under investigation
- Delivery of dsRNA to maximize uptake
- Transmission of dsRNA to subsequent developmental stages in the life cycle
- The site of expression of the target gene
- The level of expression of the target gene
- The capacity for spreading throughout the worm tissues
- The capacity of RNAi-treated worms to retain infectivity to examine *in vivo* effects

larvae in fluorescently labelled dsRNA, uptake was found to be relatively weak in third-stage larvae (L3) compared with L2 and L4 stages [10], which might reflect the lack of functioning mouthparts in L3s [19]. Electroporation has been used to introduce dsRNA into several trichostrongylid nematodes of sheep and cattle [9–11]. This seems to be the most effective means of delivery [9] but it remains unclear at which anatomical site the dsRNA penetrates the worm and to what extent it spreads in the tissues. In addition, this approach requires careful optimization because pulses of current of the wrong length or intensity cause cell damage or rupture [20]. Electroporation of dsRNA has been used effectively in schistosomes but it is interesting that the time taken to detect a decrease in specific transcript level varies depending on the developmental stage targeted. This indicates that early schistosomula might be incapable of and/or not susceptible to RNAi [21].

To date, the life stages tested for parasitic nematodes range from newly hatched L1 of *Trichostrongylus colubriformis* [9] to adult *Nippostrongylus brasiliensis* [22]. Clearly, it would be much easier if all experiments could be carried out using the free-living larval stages. However, from the viewpoint of parasite control, the most relevant genes are likely to be transcribed in the parasitic life stages. For RNAi to be a truly effective tool in parasitic nematodes, it is desirable that the silencing RNA molecules are passed from one stage to the next and, therefore, mediate RNAi when gene transcription is 'switched on'. This is the case in *C. elegans*, in which an interference effect can persist for several days and could, in some cases, be inherited by subsequent generations [14]. However, it is not yet clear whether this mechanism is present in parasitic nematodes. It would be an important disadvantage if it were not because this would mean that the parasite stage would have to be selected based on the transcription pattern of each target gene.

The RNAi pathway – absence of key genes?

Although improved culture methods and dsRNA delivery routes should enable more-effective RNAi, it is also important to consider whether the classical RNAi pathway, as detailed in Ref. [8] and summarized in Ref. [13], is present in parasitic nematodes. In an attempt to understand the limited reliability and efficiency of RNAi in parasitic nematodes, we searched currently available parasite genome and expressed sequence tag (EST) datasets for genes that are known to be involved in the RNAi pathway in *C. elegans* [23]. Similar to the results of Zawadzki *et al.* [13], we were unable to identify *rde-4*, *rde-2*, *sid-2* and *rsd-2* homologues in the *H. contortus* and *Brugia malayi* genomes (currently 95% and 98% coverage, respectively) and the available nematode EST datasets.

These observations could be crucial. *C. elegans* with mutations in the gene *rde-4* are deficient in RNAi induced by dsRNA but not by siRNA, which shows the essential role of RDE-4 in siRNA generation, at least in *C. elegans*. As emphasized in Refs [8,13], it is possible that an *rde-4* homologue has yet to be sequenced from parasitic nematodes or that *rde-4* in parasitic nematodes is considerably divergent from *C. elegans rde-4* and the *Drosophila melanogaster* homologue *r2d2* [24]. However, if its absence is confirmed, this raises at least two possibilities: either that the function of RDE-4 is undertaken by a different protein in parasitic nematodes or that *rde-4* has been lost during the evolution of parasitism.

The first possibility could be addressed by using *in vitro* gel shift and northern–western blot assays such as those reported by Tabara *et al.* [25]. The identity of RNA-binding proteins could then be determined by mass spectrometric analysis and subsequent database searches. The second possibility is pure speculation but perhaps the parasite within its host has been protected from direct exposure to foreign (viral) DNA or RNA and has lost a key stimulant for the process. However, if this were the case, how would one explain the increasing number of reports that describe apparently successful RNAi in several parasitic nematodes? A possibility is that standard dsRNA preparations, which are used at relatively high concentrations, contain contaminating partially degraded dsRNA, antisense RNA or siRNAs at a concentration that is sufficient to induce RNAi downstream of the interaction between RDE-4 and DICER, two key components of the RNAI pathway [13,23]. It is noteworthy that siRNA is more efficiently delivered in *T. colubriformis* than is the longer dsRNA [9], although the converse is true in *C. elegans* [26].

In recent studies, we have noted considerable variation in the outcome of RNAi experiments (from total transcript suppression to no effect) between different batches of dsRNA directed at the same target gene [11]. These outcomes could reflect differences in the amount of 'contaminating' siRNA in each batch. A recent report described nonspecific concentration-dependent repression and stimulation of mammalian gene expression by siRNAs [27]. The authors were attempting to identify targets of transcription factors by examining expression profiling after siRNA treatment of mammalian tissue-culture cells. Several genes were affected, including those involved in cell signalling, cytoskeletal organization, metabolism and cell adhesion. Such nonspecific effects could cloud the interpretation of RNAi experiments to identify drug targets and highlight the importance of appropriate controls [8]. In addition, a requirement to demonstrate a reduction in specific transcript levels, by inclusion of reverse-transcription (RT)–PCR data regarding target and control genes, would enable a more meaningful and consistent interpretation of the data.

The possible absence of the *sid-2* and *rsd-2* genes could infer the lack of an effective uptake and spreading pathway for RNAi. In this case, the outcome of RNAi might be affected by the transcription pattern of the target gene, the cell type it is expressed in and whether the gene is expressed close to a surface that is accessible to the dsRNA solution, such as in the intestinal and reproductive tracts. The demonstration that siRNA can move between mammalian cells through gap junctions provides a possible explanation for variability because silencing might occur only for target genes that are expressed in accessible cell types [28].

In *C. elegans*, dsRNA can induce both post-transcriptional and transcriptional gene silencing [23]. Post-transcriptional silencing is the classical RNAi, in which the target transcript is destroyed. By contrast, transcriptional silencing blocks the actual transcription of a gene. Is it possible that only the latter mechanism is functional in parasitic nematodes? The actual mechanism and the proteins involved in transcriptional silencing are not yet fully characterized but it is thought that the RDE-4 protein is not essential in this process. This might also explain why, in some cases, it can take days before an effect on transcript level is detectable. The treatment of parasites with dsRNA would interfere with the transcription of the target gene but target transcripts already present at the time of the treatment would not be destroyed. This proposal means that the period needed to observe the effect using RT–PCR would depend on transcript stability and turnover rate. The effect would also vary depending on the gene and species of nematode.

Concluding remarks

This article highlights a requirement for specific analyses of RNAi machinery in parasitic nematodes, analyses that are likely to provide the key to developing RNAi as a useful tool for determining gene function. In particular, improved culture methods are required to investigate the longer-term effects of RNAi [13]. Although RNAi seems to be possible in some nematodes, caution is required when interpreting the outcome of these experiments. It is important to demonstrate that observed phenotypes are due to specific transcript reduction and not nonspecific, toxic effects of dsRNA or unsuitable parasite maintenance. Given that the value of RNAi would be in selecting control targets from the wealth of gene information that is becoming available, it is important to examine further the variable efficacy of RNAi in different nematodes, different developmental stages and different target genes [9–11].

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