The development of RNA interference (RNAi) in gastrointestinal nematodes

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SUMMARY

Despite the utility of RNAi for defining gene function in *Caenorhabditis elegans* and early successes reported in parasitic nematodes, RNAi has proven to be stubbornly inconsistent or ineffective in the animal parasitic nematodes examined to date. Here, we summarise some of our experiences with RNAi in parasitic nematodes affecting animals and discuss the available data in the context of our own unpublished work, taking account of mode of delivery, larval activation, site of gene transcription and the presence/absence of essential RNAi pathway genes as defined by comparisons to *C. elegans*. We discuss future directions briefly including the evaluation of nanoparticles as a means to enhance delivery of interfering RNA to the target worm tissue.

Key words: Animal parasitic nematode, RNAi, delivery, pathways.

INTRODUCTION

Gene silencing by RNA interference (RNAi) was originally characterized in the free-living nematode *Caenorhabditis elegans* (Fire *et al*. 1998). The technique has since then been applied to a wide variety of organisms to study gene function including protozoa (Ullu *et al*. 2004), amphibians (Nakano *et al*. 2000), insects (Kennerdell and Carthrew, 1998) and a number of parasitic helminths including *Fasciola hepatica* (McGonigle *et al*. 2008), *Schistosoma mansoni* (Correnti *et al*. 2005) and nematodes infecting plants (Rosso *et al*. 2005; Urwin *et al*. 2002), animals (Hussein *et al*. 2002) and humans (Aboobaker and Blaxter, 2003). While transcript levels have been reduced for some target genes in parasitic nematodes, using current methods, RNAi seems to be inconsistent with variable results between species and between genes. Several laboratories have aimed to address this by testing different delivery methods and targeting different genes and different parasite stages. In this review, we focus on studies attempting to develop RNAi methodology in *Nippostrongylus brasiliensis*, the parasitic nematode in which RNAi effects were first reported (Hussein *et al*. 2002). We consider approaches to improve double-stranded RNA (dsRNA) or small inhibitory RNA (siRNA) uptake, discuss recent findings from *Haemonchus contortus* (Samarasinghe *et al*. 2011) and consider why RNAi may be less effective in parasitic nematodes compared to *C. elegans*.

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DELIVERY OF RNA

Soaking of adult worms

In *C. elegans*, RNAi can be effectively induced by injection of dsRNA into any site of the worm (Fire *et al*. 1998) by feeding on bacteria expressing dsRNA (Timmons *et al*. 2001) and by soaking in dsRNA solution (Tabara *et al*. 1998). Knockdown of target mRNA is depends on several proteins that enable uptake, spreading and processing of exogenous dsRNA. Spreading can be largely ascribed to the transmembrane proteins SID-1 (*S*ystemic RNA *I*nterference *D*efective) and SID-2. SID-1 forms channels in the membrane to enable dsRNA diffusion (Feinberg and Hunter, 2003) whilst SID-2 is mainly located in the intestine and is involved in the uptake of exogenous dsRNA into the cells (Winston *et al*. 2007). Together, these proteins facilitate systemic spread of the dsRNA in *C. elegans*, as demonstrated by effective silencing achieved simply by soaking worms in media containing dsRNA. Indeed, this mode of delivery has been used in a large-scale survey of *C. elegans* gene function (e.g. Maeda *et al*. 2001).

Given this background, initial experiments were performed in *Nippostrongylus brasiliensis* by soaking adult parasites with dsRNA for AChE B, a secreted acetylcholinesterase, and this treatment resulted in knockdown of both this and two other closely related

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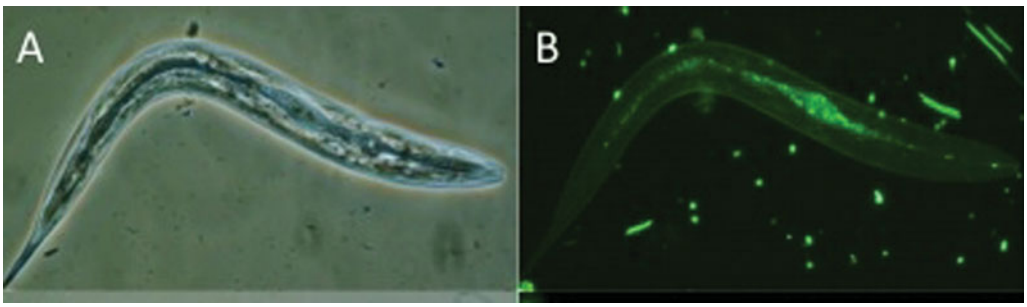


Fig. 1. *Nippostrongylus brasiliensis* larvae ingest bacteria. Both L1 and L2 (shown) feed on *E. coli*. Panel A, bright field; Panel B, fluorescence. Parasites do not develop beyond the L1 stage on NGM plates in the absence of bacteria.

variants of the enzyme (Hussein *et al*. 2002). Parasite motility was unaffected, suggesting that neuromuscular AChEs were not significantly affected, and this was also suggested by visualisation of enzyme activity in a polyacrylamide gel-based assay, although this is not quantitative (Hussein *et al*. 2002). Soaking adult worms with dsRNA for another variant, AChE A, also resulted in suppression of secreted enzyme expression, but we failed to observe knockdown by RT-PCR of six other targets by this method, homologues of ubiquitin, paramyosin (*unc-15* in *C. elegans*), calponin (*unc-87*), protein disulphide isomerase 3 (PDI-3), phospholipase A2 (PLA2) and β-tubulin (unpublished data). The latter was somewhat surprising in that this gene appears to be particularly susceptible to knockdown in other parasitic nematodes following dsRNA soaking (Aboobaker and Blaxter, 2003; Geldhof *et al*. 2006; Kotze and Bagnall, 2006; Samarasinghe *et al*. 2011).

As expression of secreted cholinesterases could be suppressed in adult *N. brasiliensis* worms maintained *in vitro*, the effect of this treatment on establishment of parasites in the host intestinal tract was examined. Parasites which had been cultured for 24 h in dsRNA for AChE B were used to reinfect rats by oral gavage, and the animals sacrificed 6 days later. A significant reduction in recovery of parasites with suppressed AChE was observed in two independent experiments, but this was matched by two which showed no difference and by others in which no parasites were recovered from control groups (unpublished data). Interpretation of these data was hampered by the very low recovery of adult worms which presumably resulted from extended maintenance *in vitro*. In addition, in the absence of sequence information for neuromuscular AChEs, we could not rule out effects on these enzymes which, by analogy with *C. elegans*, would be required for normal movement and presumably the ability to maintain position in the jejunum (Culotti *et al*. 1981; Selkirk *et al*. 2005).

As a major objective of RNAi is to examine the role of gene products on parasitism (i.e. development in the definitive host), application to infective larval stages is generally more useful. This approach was recently taken to investigate *in vivo* effects of gene silencing in *H. contortus* (Samarasinghe *et al*. 2011) and *Ascaris suum* (Chen *et al*. 2011). Pre-soaking of infective L3 larvae of *H. contortus* in dsRNA for an H11 aminopeptidase gene resulted in significant reductions in worm burden, egg output and aminopeptidase activity in recovered worms (Samarasinghe *et al*. 2011). Similarly, pre-soaking of infective larvae of *A. suum* for 72 h in dsRNA for an enolase gene resulted in shorter worms compared to those derived from control larvae following infection in mice (Chen *et al*. 2011). These studies indicate that essential gene function can be identified by *in vivo* RNAi, but by using current methods only those genes susceptible to reliable knockdown can be monitored in this way.

Ingestion of bacterially expressed dsRNA

Feeding *C. elegans* on bacteria engineered to express dsRNA has been used very effectively for RNAi (Timmons *et al*. 2001) and is the method of choice for genome-wide functional screens (e.g. Kamath *et al*. 2003). Lawns of *Escherichia coli* HT115, deficient in RNaseIII, support development of *N. brasiliensis* from newly hatched first stage larvae (L1) through to infective third stage larvae (L3), a process which takes 7 days at 25 °C (i.e. similar to incubation in faecal cultures), and these L3 s are fully infectious, completing their life cycle in rats. Both L1 and L2 feed on *E. coli*, demonstrable by the use of bacteria expressing Green Fluorescent Protein (Fig. 1). Suppression of a number of target mRNAs has been tested in this system by feeding worms with *E. coli* HT115 expressing dsRNA for *N. brasiliensis* paramyosin, calponin, protein disulphide isomerase-3 and phospholipase A2. The first two targets were selected as suppression of expression might be predicted to result in alterations in motility (Fraser *et al*. 2000; www.wormbase.org) but this was not observed with any consistency, and further investigation indicated there was no reduction in transcript levels for any of these targets. This concurs with the failure of feeding bacteria expressing dsRNA to *H. contortus* (Geldhof *et al*. 2006) and *Heligmosomoides polygyrus* (Lendner *et al*. 2008) to elicit RNAi. The only species of animal-parasitic nematode in which this method has been documented to have a phenotypic effect is

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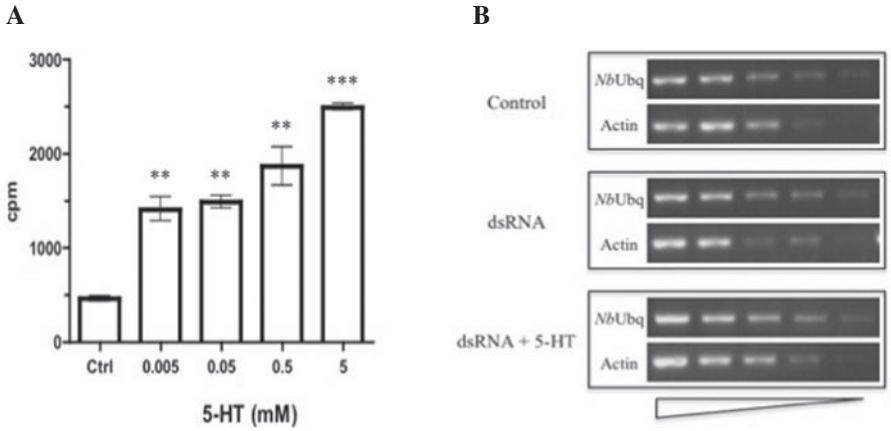


Fig. 2. Serotonin enhances the rate of feeding in activated L3, but does not enhance RNAi. A. Larvae were activated for 47 h at 37 °C, then incubated in the absence (Ctrl) or presence of the indicated concentrations of 5-HT for 1 h before quantitative assessment of feeding by uptake of ^{125}I-BSA (Huang *et al*. 2010). Data are presented as the mean ± S.E. of assays in triplicate. Values for uptake which differ significantly from controls (no 5-HT) are indicated (**$P<0{\cdot}005$; ***$P<0{\cdot}0001$). B. Activated larvae were exposed to 1 mg ml^{-1} *N. brasiliensis* ubiquitin dsRNA with or without 0·5 mM serotonin (5-HT) for 1 hr before electroporation. Parasite RNA was isolated 48 h after treatment and levels of mRNA for ubiquitin and actin determined on 10x serially diluted cDNA.

Trichostrongylus colubriformis (Issa *et al*. 2005). In the case of *H. polygyrus*, it was noted that L1 ingest many fewer bacteria than the corresponding stage of *C. elegans* (Lendner *et al*. 2008). In an attempt to increase the liberation of dsRNA from bacteria in the parasite intestine, we transfected bacteria with pLysE, a plasmid expressing bacteriophage T7 lysozyme, but this proved to be toxic and inhibited larval development (unpublished data).

Activation of feeding in infective larvae

Infective larvae are developmentally arrested in strongylid nematodes and do not feed, presenting a problem in terms of delivery of dsRNA or siRNA. Chemical exsheathment of *N. brasiliensis* L3 did not stimulate feeding, but maintenance at elevated temperature (37 °C) proved to be sufficient (Huang *et al*. 2010). Infective larvae begin feeding after a lag period of 3 to 4 h at 37 °C, reaching a maximum of 90% of the population feeding by 48 h (Huang *et al*. 2010). A variety of regimens of soaking temperature-activated larvae with dsRNA or siRNA for up to 72 h have been tested, followed by semi-quantitative RT-PCR for up to 48 h post-treatment. Whilst we could inconsistently detect knockdown of AChE B transcripts, ubiquitin and β-tubulin proved refractory to suppression. Lipofectin, lipofectamine and polyethyleneimine (PEI), the latter a cationic transfection reagent which condenses nucleic acids into positively charged particles, have all been tested without success (Huang, 2010).

Resorcinol (Rosso *et al*. 2005) and octopamine (Urwin *et al*. 2002) stimulate uptake of dsRNA by plant-parasitic nematodes. Resorcinol is toxic to *N. brasiliensis* larvae at similar concentrations used by Rosso *et al*. (2005) and blocks uptake of fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA). However, serotonin, known to stimulate pharyngeal pumping in *C. elegans* (Avery and Horvitz, 1990) and *Ascaris suum* (Brownlee *et al*. 1995), elicited a dose-dependent increase in uptake of BSA by activated infective larvae up to a maximal concentration of 5 mM (Huang *et al*. 2010). Nevertheless, inclusion of serotonin in further experiments in which worms were soaked in dsRNA still failed to elicit suppression of ubiquitin transcripts (Fig. 2).

Electroporation

Electroporation has been used in a variety of species of parasitic nematodes in RNAi procedures with variable success (Geldhof *et al*. 2006; Issa *et al*. 2005; Visser *et al*. 2006 ; Lendner *et al*. 2008; Chen *et al*. 2011). We have attempted to use electroporation to introduce dsRNAs and siRNAs into larval and adult *N. brasiliensis* but, as with soaking experiments, this only effected partial and inconsistent knockdown of AChE expression, with no effect on three other target mRNAs. Luciferase activity has been used effectively as a marker to optimise conditions for electroporation of *S. mansoni* schistosomula with mRNA for firefly luciferase (Correnti and Pearce, 2004). These conditions were used successfully to suppress expression of cathepsin B (Correnti *et al*. 2005), and this methodology was further confirmed by others to be more effective than soaking with transfection reagents in this species (Krautz-Peterson *et al*. 2007). Attempts to introduce mRNA for firefly luciferase into activated L3 or adult *N. brasiliensis* via a square wave electroporator were unsuccessful however, despite using a range of conditions and efficient

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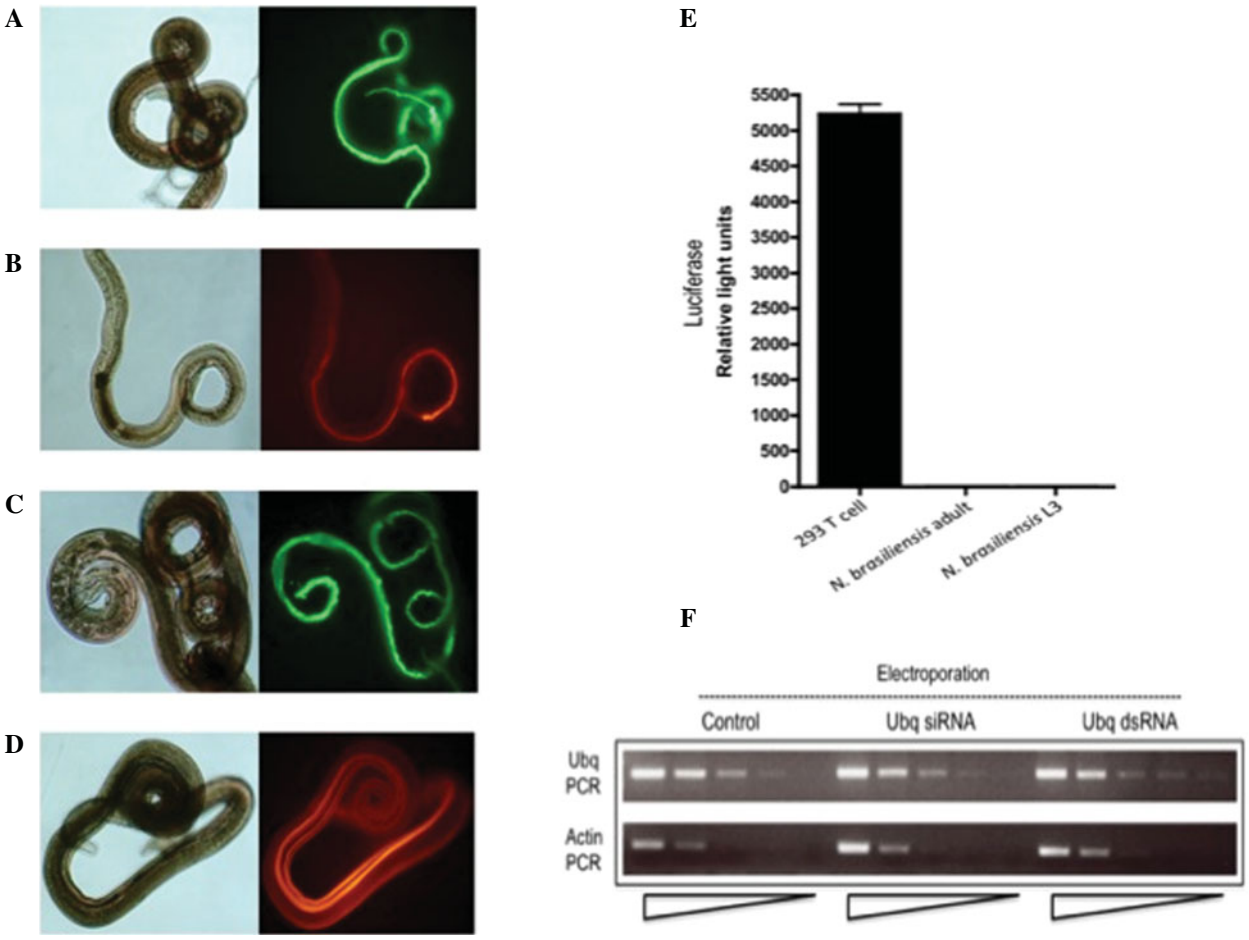


Fig. 3. Electroporation does not enhance uptake of dsRNA. A: Adult *N. brasiliensis* were incubated for 2 h with FITC-labelled bovine serum albumin (A and C) or TRITC-labelled *β*-tubulin dsRNA (B and D). Parasites in panels C and D were subsequently electroporated. Both dsRNA and BSA accumulated in the pharynx and intestine in a manner indistinguishable from soaking without electroporation. E: Electroporation of *N. brasiliensis* adult and larval parasites with luciferase mRNA does not result in detectable expression. A range of conditions were used (Huang, 2010) and parasites were assayed at 3 h (shown here) and 24 h post-electroporation. T cells (293 cell line) were used as positive controls and assayed 3 h post-electroporation. Results are presented as the mean + S.E. of assays performed in triplicate. F: Electroporation of adult parasites with siRNA or dsRNA for ubiquitin did not result in RNAi. Levels of mRNA for parasite ubiquitin and actin were determined as for Fig. 2 on 10x serially diluted cDNA.

recovery of luciferase activity from electroporated mammalian cells (Fig. 3 and Huang, 2010). This is perhaps not surprising, as the cuticle would present an impermeable barrier to electroporation, although cuticular pores appear to exist in some nematode species (Martin *et al*. 1987; Tilney *et al*. 2005). In the absence of cuticular pores, electroporation would only be possible via an inside-out process into cells which are connected to the exterior, such as those present in intestinal epithelia, secretory or excretory glands or sensory organelles such as amphids, and moreover this process would require electrical conductivity to the outside medium. Electroporation of *H. polygyrus* using a wide range of conditions also failed to effect RNAi, although the authors did observe focal points of fluorescence between the cuticle and hypodermis when using Cy3-labelled siRNA as a probe (Lendner *et al*. 2008). Electroporation of *N. brasiliensis* with labelled dsRNA, siRNA or bovine serum albumin (BSA) did not result in internalisation of fluorescence unless preceded by an extended period of incubation, in which case macromolecules accumulated in the pharynx and intestine in a manner indistinguishable from soaking without electroporation (Fig. 3). We thus concluded that, in *N. brasiliensis* at least, electroporation does not provide a viable means of delivery of RNA.

Alternative methods of delivery

Particle bombardment is an alternative approach for delivering nucleic acids into worms (Davis *et al*. 1999; Wilm *et al*. 1999; Higazi *et al*. 2002; Jackstadt *et al*. 1999), but this approach is generally harmful to *N. brasiliensis* (Ball, unpublished data). Microinjection with constructs designed to produce an endogenous hairpin dsRNA has been routinely used for RNAi in *C. elegans* (Tavernarakis *et al*. 2000; Johnson *et al*. 2005). However,

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microinjection-mediated introduction of RNA was shown to damage *H. contortus* larvae (Geldhof *et al.* 2006), probably due to high internal pressure. This approach is generally not suitable for most parasitic nematodes, as the inability to complete the life cycle *in vitro* means that it would be very difficult to select transformants, and survival of microinjection procedures tends to be very low. Microinjection of dsRNA has, however, been used in free-living adult female *Strongyloides stercoralis*, resulting in specific knockdown of mRNA in F1 progeny (Lok, 2007). The same effect was not achieved following dsRNA soaking of adult worms, suggesting that delivery of dsRNA directly into nematodes may overcome apparent gene insensitivity to RNAi.

Nanoparticles are an emerging technology with potential application for interfering RNA delivery (Britton *et al.* 2011). They are very small in size but provide a large surface area for attachment of RNA and current data, in other organisms, suggest their use can enhance dsRNA uptake. Of particular note, *C. elegans* showed effective uptake of fluorescent silica or polystyrene based nanoparticles, this being evident in the intestine and pharynx inititially. This first phase was followed by translocation to the gonad and even early embryos (Plustoka *et al.* 2009). Nanoparticles have been used with success for the delivery of dsRNA to mosquito larvae intestinal cells following feeding (Zhang *et al.* 2010).

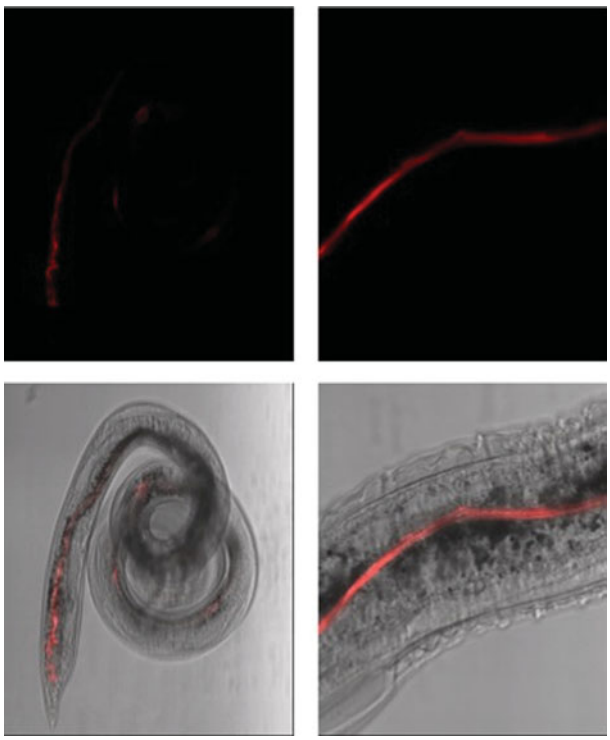


Fig. 4. Ingested dsRNA appears to accumulate in the intestinal tract without further uptake and dissemination. Adult *N. brasiliensis* were incubated with 10 μg ml^{-1} Cy3-labelled dsRNA for 16 h and examined by confocal microscopy. RNA accumulated in the intestinal lumen and did not appear to be transported at significant levels into other tissues.

UPTAKE OF DSRNA/SIRNA – THE LIMITING FACTOR?

Collectively, the data obtained from multiple animal-parasitic nematode species suggest that uptake of dsRNA or siRNA is a limiting factor in RNAi. Confocal microscopy has indicated that, although larval or adult *N. brasiliensis* ingested Cy3-labelled dsRNA, this accumulated in the intestinal lumen and did not appear to be transported at significant levels into other tissues (Fig. 4). Findings from a recent analysis of factors potentially impacting the susceptibility of *H. contortus* to RNAi are consistent with both initial uptake and transport of dsRNA being factors limiting RNAi efficacy (Samarasinghe *et al.* 2011). This study assessed whether the level of target transcript or site of gene expression influenced susceptibility to RNAi by soaking. Target genes represented by a high number of expressed sequence tags (ESTs) in the *H. contortus* L3 stage were not reproducibly silenced. In contrast, four out of six genes putatively expressed in the intestine, excretory cell or amphids were consistently silenced by RNAi. This suggested that genes expressed at a site accessible to the environment were more likely to be susceptible to RNAi by soaking, which may reflect uptake of dsRNA at a sufficient level only into these cell types.

RNAi pathway genes

The efficacy of environmental (soaking or bacterial feeding) RNAi in *C. elegans* seems to be an anomaly even among free-living nematodes. Differences in the SID-2 transporter are responsible for the lack of environmental RNAi in *C. briggsae*, a deficiency that can be overcome by transgenic expression of the *C. elegans sid-2* gene (Winston *et al.* 2007). Poor conservation of *sid-2* and other RNAi pathway genes is therefore likely to influence RNAi success in parasitic species.

The molecular processes and components necessary for a functional RNAi pathway have been extensively investigated in *C. elegans* and summarized by Grishok (2005). These are outlined briefly as follows (Geldhof *et al.* 2006): the dsRNA is recognized by a protein complex containing a double-stranded RNA-binding protein, Rde-4, as well as the dsRNA-specific ribonuclease, Dicer, which cleaves dsRNA into small inhibitory RNAs (siRNAs). The DexH box helicase Drh-1 and Rde-1 argonaute protein are thought to transport the siRNAs to the RNA induced silencing complex (RISC). This interaction results in the activation of the silencing complex, which targets homologous mRNA transcripts by a base-pairing mechanism. Once bound, the target transcript is degraded by the RISC, resulting in gene knock-down.

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In *C. elegans* the silencing effect is systemic, spreading to most cells through the activity of the SID and RSD proteins, and siRNAs are subsequently recognized by the RISC protein complex. Thus, RNAi is induced by dsRNA, the RNAi effect can be systemic and, at least in *C. elegans*, is heritable (Grishok, 2005).

In a preliminary analysis to examine conservation of RNAi pathway genes in parasitic nematodes, we screened the available *H. contortus* genome database (at the time with approximately 6-fold coverage). Sequences of 17 proteins with defined functions in the RNAi pathway of *C. elegans* were used in BLAST searches against the *H. contortus* genome database (Geldhof *et al.* 2007). Genes encoding potential homologues of *dcr-1, drh-1, rde-1, rde-3, sid-1, eri-1, rrf-1, tsn-1, vig-1, rrf-1, ego-1, mut-7* and *rsd-6* were readily identified, but other genes thought to be essential for a functional and systemic RNAi pathway were not found; these included *rde-2, rde-4, sid-2* and *rsd-2*.

The absence of *rde-4* was particularly noteworthy, given that the protein product binds double-stranded RNA, and is considered critical for complex formation with Dicer and generation of short interfering RNAs (e.g. Parrish and Fire, 2001). Standard RNAi is inoperative in *C. elegans* mutants lacking *rde-4* (Tabara *et al.* 1998). At the time, we tested the efficacy of RNAi against several *C. elegans* homologues of genes of interest to us in *H. contortus* (Clark, Geldhof and Knox, unpublished observations). All were susceptible to RNAi in wild-type *C. elegans*, but not in C. elegans mutants lacking *rde-4*. *Rde-4* was also not obviously apparent in the *Brugia malayi* genome, nor in available *S. mansoni* gene datasets. The authors (Geldhof *et al.* 2007) suggested that perhaps other mechanisms for RNAi activation are operative in helminths. Interestingly, a study in *C. elegans* using transgenic arrays to express dsRNA showed that when high concentrations of trigger dsRNA were produced within the nematode, the RNAi pathway was activated even in *rde-4* mutants (Habig *et al.* 2008). This suggested that it is possible to overcome the absence of *rde-4* by increasing the levels of dsRNA. It was proposed that this may reflect titration of dsRNA destabilising factors (e.g. adenosine deaminases; Habig *et al.* 2008) and/or more effective binding of Dicer to abundant dsRNA, without the need for RDE-4.

Recently, Dalzell *et al.* (2011) published the outcome of a study which focused on RNAi effector diversity in nematodes. Again, *rde-2, rde-4, rsd-2* and *sid-2* were not detected in the genome of *Haemonchus* nor of several other parasitic nematodes using genome datasets which had been considerably expanded since the initial analysis by Geldhof *et al.* (2007). The authors (Dalzell *et al.* 2011) also noted that the *Meloidogyne* species of plant-parasitic nematodes lacked orthologues of SID-1, SID-2 and RSD-2, yet showed systemic RNAi effects after soaking in dsRNA, indicating that there may be alternative uptake proteins in use or even alternative mechanisms of transport. How RNAi works in parasites in which the currently defined RNAi machinery is clearly deficient merits attention, and may be key to achieving consistent RNAi effects for target genes. The absence of *rde-2, sid-2* and *rsd-2* homologues in the *H. contortus* genome would suggest that the RNAi effect would not spread through the worm and to the germline. The absence of *rde-4,* based on findings from *C. elegans,* suggests that the RNAi pathway would not be active unless high concentrations of dsRNA were present. In these scenarios, it may be anticipated that gene silencing would only be observed in regions of the worm directly accessible to delivered dsRNA and where the concentration of dsRNA was sufficiently high to activate the pathway. Recent findings from *H. contortus* RNAi (Samarasinghe *et al.* 2011) are consistent with this hypothesis.

FUTURE PERSPECTIVES

Although the study of Samarasinghe *et al.* (2011) has improved the success rate of RNAi in *H. contortus* by focusing on genes expressed in the intestine, amphids or excretory cell, not all of the genes targeted showed a decrease in mRNA level. In addition, it has not yet been tested whether the same approach improves RNAi success when applied to *N. brasiliensis* and other parasitic species. Alternative means therefore should to be exploited for RNA delivery. Receptor-mediated endocytosis has been demonstrated to be utilised for uptake of RNA in both insect and mammalian cells (Saleh *et al.* 2006; Wolfrum *et al.* 2007), and it might therefore be productive to investigate suitable ligands for conjugation which could promote uptake in parasitic nematodes. Given that nematodes are deficient in sterol biosynthesis and require exogenous cholesterol for development (Hieb and Rothstein, 1968; Kurzchalia and Ward, 2003), this would appear to be one suitable molecule for conjugation, and indeed chemically stabilised siRNAs conjugated to cholesterol have been used for systemic delivery to mammalian cells *in vivo* (Soutschek *et al.* 2004). Further understanding of the requirements for effective RNAi in parasitic nematodes will also help with potential improvement strategies. Successful transgenesis of *Strongyloides* spp. (Junio *et al.* 2008) may allow knock-out or knock-in of specific pathway genes to identify the limiting steps. Recently, Xu *et al.* (2011) reported chemical transfection of infective L3 larvae of *B. malayi in situ*, with subsequent stable expression of a luciferase reporter gene. Generation of transgenic parasites therefore has the potential to enhance RNAi silencing mechanisms and also provide a means of expressing dsRNA from hairpin constructs which may overcome the

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limitations of environmental RNAi. Further approaches to improving dsRNA delivery in parasitic nematodes are needed to reliably study gene function on a large scale.

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