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Short interfering RNA-mediated knockdown of drosha and pasha in undifferentiated *Meloidogyne incognita* eggs leads to irregular growth and embryonic lethality

Johnathan J. Dalzell [a], Neil D. Warnock [a], Michael A. Stevenson [a], Angela Mousley [a], Colin C. Fleming [b], Aaron G. Maule [a,*]

[a] *Molecular Biosciences – Parasitology, School of Biological Sciences, Queen's University Belfast, Belfast, UK*
[b] *Agri-Food Biosciences Institute, Belfast, UK*

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ABSTRACT

Micro-(mi)RNAs play a pivotal role in the developmental regulation of plants and animals. We reasoned that disruption of normal heterochronic activity in differentiating *Meloidogyne incognita* eggs may lead to irregular development, lethality and by extension, represent a novel target for parasite control. On silencing the nuclear RNase III enzyme drosha, a critical effector of miRNA maturation in animals, we found a significant inhibition of normal development and hatching in short interfering (si)RNA-soaked *M. incognita* eggs. Developing juveniles presented with highly irregular tissue patterning within the egg, and we found that unlike our previous gene silencing efforts focused on FMRFamide (Phe-Met-Arg-Phe-NH_2)-like peptides (FLPs), there was no observable phenotypic recovery following removal of the environmental siRNA. Aberrant phenotypes were exacerbated over time, and drosha knockdown proved embryonically lethal. Subsequently, we identified and silenced the drosha cofactor pasha, revealing a comparable inhibition of normal embryonic development within the eggs to that of drosha-silenced eggs, eventually leading to embryonic lethality. To further probe the link between normal embryonic development and the *M. incognita* RNA interference (RNAi) pathway, we attempted to examine the impact of silencing the cytosolic RNase III enzyme dicer. Unexpectedly, we found a substantial up-regulation of dicer transcript abundance, which did not impact on egg differentiation or hatching rates. Silencing of the individual transcripts in hatched J2s was significantly less successful and resulted in temporary phenotypic aberration of the J2s, which recovered within 24 h to normal movement and posture on washing out the siRNA. Soaking the J2s in dicer siRNA resulted in a modest decrease in dicer transcript abundance which had no observable impact on phenotype or behaviour within 48 h of initial exposure to siRNA. We propose that drosha, pasha and their ancillary factors may represent excellent targets for novel nematicides and/or in planta controls aimed at *M. incognita*, and potentially other parasitic nematodes, through disruption of miRNA-directed developmental pathways. In addition, we have identified a putative *Mi-eri-1* transcript which encodes an RNAi-inhibiting siRNA exonuclease. We observe a marked up-regulation of *Mi-eri-1* transcript abundance in response to exogenously introduced siRNA, and reason that this may impact on the interpretation of RNAi-based reverse genetic screens in plant parasitic nematodes.

1. Introduction

A number of *Caenorhabditis elegans* mutants which display aberrant revisions of normal developmental patterns have been identified; the resultant defects may present in any tissue and may impact on cell division patterns, cell cycle phase lengths or terminal differentiation. In this way, heterochronic mutants suffer from temporal disruption of cellular fate, which may impact on other maturation events and tissue patterning (Moss, 2007).

These heterochronic genes were first discovered in screens of *C. elegans* stem cell-like seam cells (Nimmo and Slack, 2009). *Lin-4*, the first such gene was found to encode an RNA hairpin-loop structure which was processed to form a mature anti-sense RNA with complementarity to the 3′ untranslated region (UTR) of *lin-14* (Lee et al., 1993), a transcription factor which positively regulates the development of certain neuronal cells, in part through regulation of the cytoplasmic zinc-finger protein *lin-28*. *Lin-4* mutants embryonate and pass L1 as wild type, however the inception of L2 developmental circuits results in the reiteration of L1-specific cell lineage differentiation patterns. It transpired that *lin-4* was

* Corresponding author. Address: Molecular Biosciences – Parasitology, School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL Northern Ireland, UK. Tel.: +44 (0)28 9097 2059; fax: +44 (0)28 9097 5877.
E-mail address: a.maule@qub.ac.uk (A.G. Maule).

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the first of a large family of micro-(mi)RNAs to be characterised, most of which function in the translational repression of gene products, and act through a double-negative feedback loop (Nimmo and Slack, 2009). There are five other miRNA genes in addition to *lin-4* which have been implicated in heterochrony; namely, *let-7*, *mir-48*, *mir-84*, and *mir-241* which form one family, and *mir-237* which shares significant similarity with *lin-4* (Nolde and Slack, 2009).

The biosynthesis of miRNAs, short interfering (si)RNAs and other non-coding (nc)RNAs centres around the endonucleolytic processing of double-stranded (ds)RNA precursors. Primary (pri)-miRNAs (RNA polymerase II transcripts) are processed into imperfect stem-loop dsRNAs, or precursor (pre)-miRNAs, by the nuclear RNase III enzyme drosha (RNasen, in humans) in conjunction with the cofactor pasha (Partner of droSHA, or DGCR8 (DiGeorge syndrome Critical Region 8) in humans), whereupon they are translocated to the cytosol by an exportin and further processed into 21–25 nucleotide (nt), 3′ two nt overhang miRNAs by another RNase III enzyme, dicer. The mature miRNA molecule is the single guide strand incorporated into the functional RNA-induced silencing complex (RISC).

The guide strand (denoted miRNA) of a miRNA–miRNA* duplex (formal denotation of the miRNA helix) is loaded into RISC on the basis of relative thermodynamic stability of the strand's 5′. It is thought that miRNAs are preferentially directed to and loaded into a specific subset of non-slicing argonaute proteins (AGOs) which mediate translational repression within the RISC. The vast majority of characterised animal miRNAs bind imperfectly to sites located primarily in the 3′ UTR of transcripts, through Watson–Crick base pairing of the miRNA seed region (∼bp 2–8 of the miRNA) (see Tay et al., 2008, for some exceptions). It is thought that the UTRs are commonly successful miRNA binding sites because ribosomal activity in these regions is less likely to dislodge the miRNA strand (Gu et al., 2009). However, it seems likely that many of the exceptional miRNAs which do function within the open reading frame (ORF) of transcripts may do so because of local secondary structure in the transcript which slows the ribosome and allows a greater proportion of successful inhibitory events. It may even be possible that certain miRNAs function purely through steric hindrance, as is the case for a number of modified anti-sense oligonucleotides (Summerton, 2007). The mechanism of translational inhibition elicited by miRNA binding is not fully understood, as it impacts on both translational initiation and elongation (reviewed in Kloosterman and Plasterk, 2006). However, binding of RISC to the transcript 5′, deadenylation of the poly-A tail and sequestration in cytoplasmic foci (known as P-bodies or GW-bodies) for exonucleolytic degradation, are all proposed mechanisms of translational repression (Jinek and Doudna, 2009).

As the miRNA pathway is heavily implicated in the developmental regulation of plants and animals we reasoned that drosha, dicer and pasha, the two main biosynthetic RNase III enzymes of the RNA interference (RNAi) pathway and a key cofactor in miRNA maturation processes, respectively, would prove critical to the functionality of miRNA pathways in the root knot nematode *Meloidogyne incognita*. Consequently, this would impact on normal heterochronic activity, leading to irregular development and decreased viability. Plant parasitic nematodes (PPNs) impose a significant economic burden on global plant cultivation enterprise, through loss of yield which results from the parasitic interaction. Root knot nematodes are responsible for a large proportion of these losses and *M. incognita* is considered one of the most damaging, capable of maintaining a viable parasitism in over 2000 flowering plant species (Bird et al., 2009).

The efficacy of RNAi in root knot nematode egg stages has already been demonstrated (Fanelli et al., 2005), and lethality associated with gene knockdown is seemingly more practicable in *C. elegans* during embryogenesis, demonstrable through the injection of dsRNA constructs into hermaphrodite gonads (e.g. Pispa et al., 2008). The apparent increase in RNAi sensitivity of the embryo relative to larval or adult stages may be due to one or any combination of the following explanations: (i) the increased expression of otherwise rate-limiting RNAi pathway components during embryogenesis; (ii) lower expression or altered distribution of RNAi inhibitors; (iii) a more favourable RNAi penetrance and/or propagation dynamic; or possibly (iv) more limited plasticity of embryonic biology, rendering the embryo incapable of full dynamic recovery. Taking into account the published support for such a strategy, we considered that silencing miRNA pathway components in embryonic egg-stage *M. incognita* could provide a strong target for control, in that if successful, this would not only compromise embryonic development, but could also impact on further larval transitions and maintenance; potentially important considerations for the implementation of successful in planta control. The fact that *M. incognita* undergoes its first moult within the egg could provide increased egg-stage sensitivity to miRNA pathway disruption.

The siRNA exonuclease ERI-1 acts as an RNAi inhibitor in *C. elegans*, together with the RNA-dependent RNA polymerase RRF-3, adenosine deaminases (ADAR-1/-2), other exonucleases (ERI-3/-5/-6/7), uncharacterised protein products (LIN-15b), and possibly other as yet unidentified inhibitors. In addition, inhibitors of miRNA-specific elements of the RNAi pathway are known, and include the miRNA exonucleases XRN-1, XRN-2, and the little known SOMI-1 (suppressor of over-expressed miRNA). We investigate the flux of a putative *Mi-eri-1*, and find that transcript abundance is up-regulated in response to even low amounts of exogenously introduced siRNA. Interestingly, we found that inhibitor up-regulation was life-stage-dependent. This phenomenon has been linked to the speed and severity of target transcript rebound following a successful knockdown event in mice (Hong et al., 2005), and it seems likely that this function may be conserved in other organisms. This could impact on the application of RNAi to reverse genetics applications in PPNs, and may prove a confounding factor in the interpretation of results.

2. Materials and methods

2.1. Target identification by reciprocal BLAST analysis

A range of drosha/RNasen, dicer and pasha protein sequences were used in a BLAST search of the predicted proteins, expressed sequence tags (ESTs), 10× scaffold reads and unplaced reads generated by the *M. incognita* genome project (Abad et al., 2008). Likely candidates were further ratified by reciprocal BLAST analysis in addition to predicted motif and domain architecture using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/), and the NCBI Conserved Domain Database (CDD) software (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). We aimed to avoid targets present in the *M. incognita* genome as multiple pseudo-allelic copies, to avoid complications related to potential co-expression and redundancy (Abad et al., 2008). All three targets were represented by strong single-copy candidates (see Supplementary Fig. S1). Subsequently, primers were designed to sections of the predicted ORF of the main candidates (see Supplementary Table S1 for sequences), using EST-supported segments where possible, PCR amplified from cDNA, cloned, sequence-verified, aligned as contigs and found to match the predictions (http://meloidogyne.toulouse.inra.fr/cgi-bin/consortium/meloidogyne.cgi). In addition, a putative *Mi-eri-1* transcript was identified in the genome-predicted proteins. A 260 bp internal fragment of the predicted *Mi-eri-1* was amplified and sequence validated as before (see Supplementary Tables S1 and S2).