A growing molecular toolbox for the functional analysis of microRNAs in Caenorhabditis elegans

Jeanyoung Jo and Aurora Esquela-Kerscher

Abstract

With the growing number of microRNAs (miRNAs) being identified each year, more innovative molecular tools are required to efficiently characterize these small RNAs in living animal systems. Caenorhabditis elegans is a powerful model to study how miRNAs regulate gene expression and control diverse biological processes during development and in the adult. Genetic strategies such as large-scale miRNA deletion studies in nematodes have been used with limited success since the majority of miRNA genes do not exhibit phenotypes when individually mutated. Recent work has indicated that miRNAs function in complex regulatory networks with other small RNAs and protein-coding genes, and therefore the challenge will be to uncover these functional redundancies. The use of miRNA inhibitors such as synthetic antisense 2′-O-methyl oligoribonucleotides is emerging as a promising in vivo approach to dissect out the intricacies of miRNA regulation.

Keywords: microRNA; Caenorhabditis elegans; miRNA inhibitors; antisense 2′-O-methyl oligoribonucleotides

MicroRNAs (miRNAs) belong to a large class of small non-coding RNAs that have captured the attention of scientists and clinicians alike due to their importance during animal development and their close correlation to human disorders such as cancer [1, 2]. MiRNAs are extensively processed in the nucleus and the cytoplasm by the RNase III enzymes Drosha and Dicer, respectively, before generating the mature single-stranded ~22-nt RNA species [3]. MiRNAs are subsequently loaded into a large multi-protein miRNA ribonucleoprotein complex (miRNP) and function to negatively regulate gene expression by associating in a sequence-specific manner typically within the 3′-untranslated region (3′-UTR) of their target messenger RNA (mRNA) transcripts resulting in translational inhibition and/or mRNA degradation [4]. Animal miRNAs bind to their targets with incomplete complementarity and this interaction allows for bulges, loops and G-U base pairing to occur outside of the miRNA ‘seed’ region (nt 2–8 of the miRNA that generally bind perfectly with its target). The founding members of the miRNA family, lin-4 and let-7, were first identified in Caenorhabditis elegans via forward genetic screens and found to act as ‘developmental switches’ that control the timing of the larval and adult transitions [5–7]. Since this discovery, thousands of miRNA genes have been identified in various organisms ranging from insects, fish, birds, mammals, plants and viruses by primarily using small RNA cloning.

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deep sequencing and bioinformatic approaches [8]. While the number of miRNAs identified continues to climb (approximately 1424 miRNA genes have been validated in the human genome thus far), our ability to tease out their biological roles in the intact organism has lagged behind. Indeed, only a fraction of animal miRNAs have been well-characterized and are found to direct essential events related to cellular differentiation, proliferation, immune surveillance, metabolism and lifespan [1].

A major challenge in the field is to devise high-throughput methods to determine the functional significance of miRNA genes as well as identify the targets that these small RNAs control. Target identification is difficult due to the imperfect association of the miRNA to the mRNA target and current tools such as computational prediction algorithms and microarray analysis result in high false positive and false negative rates. This issue is compounded by the fact that a single miRNA can regulate the expression of multiple mRNA targets and therefore can modulate distinct genetic pathways simultaneously. Innovative experimental tools are required to understand the biological roles of miRNAs within and across closely related RNA families in the intact animal and to probe how certain miRNAs function in complex biological networks with other non-coding RNAs and protein coding genes during development and in the adult.

Use of the simple and genetically amenable C. elegans animal model has been extremely successful in studying miRNA regulation and function (Table 1). Genome-wide RNAi suppressor screens performed in miRNA loss-of-function mutant backgrounds [9] and pull-down assays to identify miRNA targets loaded into miRNP complexes [Argonaut (ALG-1) HITS-CLIP screens] [10, 11] have provided insight into the multitude of mRNA targets controlled by small RNAs. The generation of animals carrying transgenic arrays consisting of miRNA promoter elements driving green fluorescent protein (GFP) expression has been a valuable method to determine the in vivo spatial and temporal expression patterns of miRNAs throughout development and discern biological activity [12–16]. C. elegans overexpression studies that employ extrachromosomal arrays expressing transgenes for individual miRNAs such as lin-4, let-7, mir-48, mir-61 and mir-84 result in obvious phenotypes that have aided in the characterization of their functional roles [7, 17–21]. However, ectopic expression of miRNAs in general can result in a bias to downregulate artificial targets or drive miRNA expression at the wrong time or place compared to wild-type expression patterns making the phenotypes observed difficult to interpret. These studies (as well as miRNA promoter::GFP expression studies described above) are also hindered by the fact that high-copy transgenic arrays are not expressed in the germ line. Therefore, the use of specialized techniques to achieve single/low copy expression such as microparticle bombardment or Mos1 mediated Single Copy transgene Insertion (MosSCI) would be required for the analysis of miRNAs in this tissue [22, 23].

Large scale deletion studies in C. elegans have revealed that with the exception of lin-4 [6], let-7 [7], lsy-6 [12] and mir-1 [24] individual elimination of 91 additional miRNAs fail to produce overt phenotypes [25]. These results indicate that the majority of miRNA genes are individually not crucial for viability or developmental processes and that many miRNAs likely possess redundant functions. In support of this notion, recent work has shown that in the case of the mir-35, mir-51, mir-58 and let-7 families in C. elegans, multiple closely related miRNA members sharing identical ‘seed’ sequences must be deleted in combination in order to uncover gross morphological abnormalities and embryonic lethal

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<td><strong>Approach</strong></td>
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<td>RNAi suppressor screens in miRNA deletion backgrounds</td>
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phenotypes [26–28]. Interestingly, Alarez-Saavedra and Horvitz [26] found that for 12 additional miRNA families tested in the worm, the deletion of multiple miRNA members within these individual families failed to produce any measurable defects and therefore the authors concluded that most miRNAs are not essential and do not regulate important developmental functions. In contrast, Brenner and colleagues showed that loss of 25 out of 31 individual miRNAs assayed in sensitized genetic backgrounds, such as \textit{alg-1} loss-of-function mutants, resulted in obvious mutant phenotypes and implies that functional redundancies must exist between certain miRNAs, across individual miRNA families or with other non-miRNA genes [29]. Taken together, these miRNA deletion studies have been hampered by a lack of phenotypes observed when miRNAs are individually eliminated due in part to functional redundancies as well as the difficulties in interpreting embryonic lethal and sterile phenotypes. This approach also has limitations when attempting to study miRNA genes that exist in clusters, which often share common promoter elements, or miRNAs located within introns of protein-coding genes, since deletion mutations in these sites could alter the function of multiple genes.

Given the shortcomings of currently available technologies, additional methods are needed to interrogate miRNA activity \textit{in vivo} within biological networks, at specific stages of development, under certain environmental conditions as well as in specific cell populations of the worm in order to determine their biological roles. Fortunately, Zheng and colleagues have recently added another reagent to the \textit{C. elegans} toolbox that can potentially address many of these issues—the development of dextran-conjugated antisense 2′-O-methyl oligoribonucleotides that block miRNA activity within the living animal by competitively binding to endogenous miRNAs and preventing association with their miRNA targets [30].

Zheng's group recognized that \textit{C. elegans} researchers lacked an effective method to experimentally inactivate miRNAs in nematodes throughout various stages of development. The ideal reagent would need to be resistant to degradation, potent at low concentrations, nontoxic to the developing animal and possess high specificity to block the activity of individual miRNAs without cross-reacting with closely related miRNA family members. Furthermore, the miRNA inhibitors would be most useful in functional assays if they could be mixed into 'cocktails' in which select miRNAs could be inactivated in groups to uncover functional redundancies. Hutvagner and colleagues [31] first described the use of antisense oligoribonucleotides to inactivate miRNAs in \textit{C. elegans} 6 years ago. In this early study, oligonucleotides carrying 2′-O-methyl modifications (to prevent degradation by nucleases) and antisense to \textit{let-7} were injected directly into the body cavity of early larval stage (L2/L3) animals resulting in hypodermal defects and bursting vulval phenotypes that resembled abnormalities observed in \textit{let-7} deletion mutants [7] (Figure 1, left panel). Although promising, these first-generation \textit{C. elegans} miRNA inhibitors could not produce phenotypes in the progeny of adult animals injected within the germ line. This approach requires the injection of antisense oligoribonucleotides into individual larval stage nematodes, which is extremely laborious, technologically challenging and impossible to use to study miRNA function in the embryo and at early larval stages of development. Zheng and colleagues [30] theorized that the miRNA inhibitors used by Hutvagner’s group were not transmitted to the progeny of the injected animals due to poor uptake and cellular retention. To circumvent these problems, the authors conjugated one, four or eight copies of 2′-O-methyl oligoribonucleotides antisense to specific miRNAs with dextran, a nontoxic polysaccharide that is highly soluble and well retained in cells (Figure 1, right panel). These dextran-conjugated miRNA inhibitors were also labeled with a fluorescent rhodamine marker to allow the researchers to track phenotypes only in animals successfully delivered the reagent.

As a proof-of-principle experiment, Zheng and his group injected the syncytial germ line of adult hermaphrodites with dextran-conjugated oligoribonucleotides antisense to \textit{lin-4} [30]. Loss-of-function phenotypes for \textit{lin-4} have been well characterized and include complete absence of egg-laying structures (the vulvaless phenotype), reiteration of early larval division patterns in the hypodermal seam cells and absence of adult alae formation in the nematode [5, 6]. The authors found that progeny carrying dextran reagents conjugated with one or four copies of the antisense \textit{lin-4} 2′-O-methyl oligoribonucleotide exhibited potent inhibition of \textit{lin-4} activity (100% showed egg-laying defects at 20\,\mu M). Importantly, injection of \textit{C. elegans} with
dextran-conjugated oligoribonucleotides antisense to the highly homologous *lin-4* family member *mir-237* did not result in egg-laying defects or any other obvious abnormalities, mirroring the lack of phenotypes reported in animals genetically deleted for miR-237 [25] and demonstrating the high specificity of the antisense reagent.

This technique holds great promise as a powerful *in vivo* experimental tool that can be applied to a large range of miRNA genes. Indeed, Zheng et al. [30] found that the use of dextran-conjugated antisense oligoribonucleotides with dextran, to promote cellular uptake and retention, as well as rhodamine, a fluorescent marker to assist in tracking successful retention of the complex. This reagent is injected into the syncytial germ line of adult animals and miRNA inhibition can be observed in the progeny for up to ~10–15 h post-L4 molt. This technique can successfully inhibit the activity of two miRNAs in combination, making it a suitable approach to study miRNA functional redundancies during embryonic and larval development.

**Figure I:** Antisense 2'-O-methyl oligonucleotides are effective agents to block miRNA activity in *C. elegans*. (A) Hutvanger and colleagues [31] devised first generation antisense 2'-O-methyl (Me) oligonucleotides (oligos) against *let-7* that can be injected into early stage *C. elegans* larvae and block miRNA activity into adulthood. This technique cannot inhibit miRNA activity in the progeny of injected adult hermaphrodites and is therefore unsuitable for large-scale screens. (B) Zheng et al. [30] modified this technique by conjugating miRNA antisense 2'-O-methyl oligoribonucleotides with dextran, to promote cellular uptake and retention, as well as rhodamine, a fluorescent marker to assist in tracking successful retention of the complex. This reagent is injected into the syncytial germ line of adult animals and miRNA inhibition can be observed in the progeny for up to ~10–15 h post-L4 molt. This technique can successfully inhibit the activity of two miRNAs in combination, making it a suitable approach to study miRNA functional redundancies during embryonic and larval development.
limited and unsuitable for studying miRNA function in treated adult progeny for processes such as fertility and aging. Furthermore, these dextran-conjugated antisense oligoribonucleotides do not appear to completely eliminate miRNA function in the hypodermis of treated progeny, even at the earliest larval stages. When characterizing another classic lin-4 loss-of-function phenotype, the reiteration of L1 larval fates in hypodermal seam cells, Zheng and colleagues noted that progeny treated with antisense lin-4 oligoribonucleotides exhibited a repeat of L2 larval division patterns (rather than L1 fates) in seam cells, suggesting that this miRNA was reduced but not absent in this tissue during early larval development. In terms of overall toxicity, high doses (>50 μM) of dextran-conjugated miRNA inhibitors injected into nematodes showed detrimental effects in the treated progeny, specifically increased embryonic lethality and egg-hatching defects.

Although dextran-conjugated antisense 2'-O-methyl oligoribonucleotides should be effective in characterizing the role of the 207 miRNA genes currently identified in the C. elegans genome, more work needs to be done to optimize these miRNA inhibitory-based tools. Synthetic antisense oligoribonucleotides that carry alternative RNA modifications to improve their stability such as phosphorothioate linkages or locked nucleic acids (LNAs, 2'-O, 4'-C-methylene bridge), which are proven to be less toxic and more potent in mammalian systems, may be good alternatives for in vivo studies in C. elegans. Unfortunately, a significant disadvantage of using antisense technologies devised by the Hutvagner and Zheng groups for large-scale functional screens is the reliance of single worm microinjection to deliver these reagents to the animals in contrast to other methods, i.e. delivery by soaking. Existing miRNA inhibitor strategies in C. elegans are also not amenable to block miRNA activity in a cell type-specific manner, at certain times during the worm lifecycle or to assay effects in multiple generations.

Looking forward, this technology will likely move toward the generation of C. elegans carrying transgenes expressing miRNA sponges—a method devised by Ebert and colleagues for use in mammalian cells [32]. MiRNA sponges function as decoy targets to inhibit miRNA activity and consist of a tandem array of miRNA-binding sites engineered within the 3'-UTR of a reporter gene driven by a strong promoter. The miRNA complementary sites in the sponge constructs are mismatched for nt 8–11 of the mature miRNA sequence and allow stable binding and sequestering of the miRNA away from its endogenous targets. These miRNA sponges could be engineered to interrogate specific biological processes within the worm in both a temporal and spatial manner by regulating transgenic expression of the miRNA inhibitors using tissue specific or heat shock promoters or via conditional elements based on Cre, FLP or MEC-8-dependent splicing technologies [33–36]. Potentially, these inhibitors could even be designed to inhibit the activity of multiple miRNA genes. The future is bright for miRNA research and with a growing molecular toolbox available in C. elegans, this model organism will undoubtedly remain at the forefront to interrogate miRNA networks in living systems.

Key Points

- MicroRNAs (miRNAs) are ~22 nt non-coding RNAs that negatively regulate gene expression post-transcriptionally by binding to the 3'-UTRs of messenger RNA (mRNA) targets. Thousands of miRNAs have been identified in plant, animal and viral systems but only a small portion have been biologically characterized.
- The C. elegans animal model has successfully been used for studying miRNA function and regulation. Molecular tools such as RNAi suppressor screens, miRNA promoter::GFP fusion constructs as well as overexpression and deletion analysis have been employed in vivo to determine the role of miRNAs during development in the nematode. These approaches possess technical limitations that make it difficult to elucidate the physiological significance of miRNAs or how they interact within intricate networks with other small RNAs and protein-coding genes.
- Antisense 2'-O-methyl oligonucleotides are promising reagents that inhibit small RNA activity in the living nematode by physically binding to endogenous miRNAs and blocking their interaction with miRNA targets. Dextran-conjugated 2'-O-methyl oligoribonucleotides can inactivate multiple miRNAs in a single experiment and therefore could potentially be used to study functional redundancies. However, these miRNA inhibitors are laborious to deliver via single animal microinjection and have limited potency in treated progeny during the C. elegans lifecycle.
- More innovative and high-throughput approaches are required to aid in the characterization of the >200 miRNAs that exist within the C. elegans genome. In the future, miRNA sponges could be adapted to inhibit C. elegans miRNA activity in a temporal and spatial manner.

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