Chapter 13
MicroRNAs of Filarial Nematodes: A New Frontier in Host-Pathogen Interactions

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Abstract Metazoan parasites, especially nematodes, are a highly diverse group of large organisms that typically sustain an infection for long periods of time with relatively modest pathology. This state is achieved by the release of parasite-derived immunomodulatory molecules which coerce the host into providing a relatively safe niche in which the parasite is able to carry out the host-housed aspects of its life cycle. It has recently been recognized that parasitic nematodes release microRNAs (miRNAs) in culture and in mammalian hosts, primarily in exosome-like vesicles, and that these parasite-derived miRNAs may target host genes involved in the immune response. This review focused primarily on data from filarial nematodes, which occupy tissue niches in humans and other animals, and provides a perspective on possible biological roles of these molecules and their therapeutic and evolutionary implications.

13.1 Nematodes and Parasitism

Nematodes are one of the three phyla that constitute the group of animals commonly called parasitic helminths or worms. About 25,000 species of nematodes have been described (Zhang 2013), although over a million may exist (Kumar et al. 2012). Nematodes present a remarkable diversity in habitat and life histories. Some are found in water, others are terrestrial; living freely, like the model organism *Caenorhabditis elegans*, while other species have adopted parasitic lifestyles in plants or animals. Parasitism can be defined as a relationship between one (parasitic)

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species diverting resources from another (host) species, the interaction benefiting the former species but being detrimental to the latter (Sorci and Garnier 2008). To reproduce, a parasite must spend all or part of its lifecycle in or on another organism. Adoption of parasitic lifestyles by nematodes is an ancient and widespread evolutionary phenomenon and presents a great diversity of life strategies (Poulin 2011; Sorci and Garnier 2008).

The origin of the phylum Nematoda is likely to be marine, but parasitism is not uncommon in these organisms and may have emerged at least 15 times independently (Blaxter and Koutsovoulos 2015). Five separate events are likely to have occurred to give rise to parasitism of mammals (Blaxter and Koutsovoulos 2015), in which they cause a range of diseases. In many cases, the involvement of an intermediate host (e.g., in an indirect life cycle) is required to transmit or facilitate the dissemination of the infective stage to the definitive host (Poulin 2011). Intermediate hosts are often arthropods (e.g., mosquitoes and flies), or gastropods (e.g., snails and slugs) (Anderson 2000).

Parasitic nematodes face multiple challenges in their lives, most notably physical barriers and immune defenses associated with host species. An evolutionary arms race underlies all host-parasite interactions in which hosts have constrained the detrimental impact of parasites, while the latter have learned how to overcome host responses to permit transmission of the next generation of parasites. This negotiation has resulted in highly specialized interspecific relationships, characterized by complex molecular dialogues between hosts and parasites and in a narrow window for an infection to be successful but not rapidly fatal. The often chronic and non-fatal nature of nematode infections, which persist for months to many years in their hosts, is due to their highly evolved abilities to hide from the host immune system and to modulate or suppress it to their own advantage (Allen and Maizels 2011).

The most well-known immunomodulatory molecules are excretory/secretory (E/S) proteins, released by helminths into their environment. These proteins facilitate the establishment of parasites in their hosts, creating a suitable environment for them to thrive. Classically, helminths evoke a skewed type 2 and regulatory immune response in the host that prevents excessive parasite-induced toxicity (inflammatory self-damage) while permitting reproductive success by the parasite. Th2 immune responses classically involve interleukin (IL)-3, IL-4, IL-5, IL-9, IL10, and IL-13, and the immunoglobulins IgG1, IgG4, and IgE. CD4 + T helper 2 cells, eosinophils, basophils, mast cells and alteRNAtively activated macrophages are cell populations that characterize Th2 responses (Allen and Maizels 2011). In parallel, helminths induce the proliferation of a regulatory T cell subset (Treg), promoting the secretion of IL-10 and TGF-β, dampening the inflammatory component of Th2 reactions (Diaz and Allen 2007; Siles-Lucas et al. 2015). For example, secretions from intestinal nematodes inhibit T cell proliferation and macrophage production of nitric oxide, harmful to the worm (Rzepecka et al. 2006). Similarly, filarial nematodes release cytokine homologs (i.e., MIF-1 of *Brugia malayi*), protease inhibitors (serpin-2, cysteine proteases, etc., of *B. malayi*), or other sorts of molecules with potent anti-inflammatory effects (i.e., ES-62 of *Acanthocheilonema viteae*), to cite only a

few examples (Hewitson et al. 2009; Pastrana et al. 1998; Pineda et al. 2014; Stepek et al. 2004; Zang et al. 1999).

In addition to proteins, there is growing evidence for a role for non-coding RNAs (ncRNA) in host-pathogen interactions and immune regulation, especially microRNAs (miRNAs), on which this chapter is focused. miRNAs are short (≈20 nucleotides) non-protein coding RNA molecules ubiquitously present in eukaryotes with important and widespread regulatory functions in gene expression. The translation of genes targeted by miRNAs is usually repressed; typically, the stability of the targeted messenger RNA (mRNA) is compromised (Bartel 2004; Griffiths-Jones et al. 2008; Rana 2007). Most miRNAs are of intergenic origin. A transcribed long primary miRNA is processed in the parent cell nucleus into a precursor miRNA. Precursor miRNAs adopt a characteristic hairpin conformation and are exported to the cytoplasm, where they are subjected to further enzymatic trimming. Mature miRNAs occur as single-stranded entities incorporated in the RNA-induced silencing complex (RISC) (Kim et al. 2009). miRNAs bind to mRNA targets, usually in the 3′ untranslated region, with partial complementarity, and can interact with multiple genes (Kim et al. 2009). The miRNA mechanism of action presents some parallels to RNA interference, relying on the same protein machinery (Rana 2007). miRNAs are cell or tissue-specific, and in humans, may reflect a pathophysiological status. miRNAs fulfill a multitude of functions by targeting many genes; it is nonetheless worth mentioning that miRNAs play key roles in the development of innate and adaptive immunity events, regulating inflammation following pathogen recognition and the development of B and T cells (Gracias and Katsikis 2011; Mobergslien and Sioud 2014).

13.2 miRNAs of Nematodes

miRNAs are not restricted to the inside of cells, as they have also been found circulating in cell-free biofluids. Extracellular vesicles (EVs), comprising exosomes (30–100 nm Ø) and microvesicles, have been identified as major vehicles for miRNA transport, together with proteins and other molecules, protecting them from degradation. miRNAs incorporated into EVs are selectively chosen for export, and do not reflect the proportions observed in the parent cell (Villarroya-Beltri et al. 2014). EVs mediate cell-to-cell communication, sometimes reaching distant recipient cells (Raposo and Stoorvogel 2013). Upon uptake and integration by a recipient cell, the miRNA cargo is functional (Chen et al. 2012; Ramachandran and Palanisamy 2012). Understandably, these discoveries raised questions about a potential role for miRNAs in mediating informational exchange between a host and parasite species. The fate of EVs has been studied in a number of host-parasite associations, summarized elsewhere by Coakley and colleagues (Coakley et al. 2015). Helminths, too, release EVs, and miRNAs found in helminth-derived EVs have been characterized (BeRNAl et al. 2014; Buck et al. 2014).

The first miRNA discovered was *lin*-4 in the early 1990s, followed a few years later by *let*-7, both initially reported in the free-living nematode *C. elegans* (Lee et al. 1993). *C. elegans* is an exceptionally valuable experimental model because its developmental life history has been extensively well-characterized, it has a known, fixed number of somatic cells with known origin, a fully sequenced and highly annotated genome with an abundant toolkit for functional genomics, and a sophisticated capacity to adapt to its environment, by for instance, arresting larval development and reducing metabolic activity (dauer state) if conditions are unfavorable (Brenner 1974; Felix and Braendle 2010). *lin*-4 and *let*-7, and many others discovered subsequently, regulate temporal development of the worm. Together with *lin*-14 and *lin*-28, *lin*-4 controls the cell lineage organization in the lateral hypodermis in the transitions from first- through third-stage larvae (L3). Similarly, *let*-7, in conjunction with *lin*-41 and *lin*-29, ensures normal developmental timing from the late L3 through the adult stage (Rougvie 2001).

Research in *C. elegans* has illuminated the general importance of miRNAs in development and physiology, and has provided mechanistic insights into the genesis and function of these key regulatory molecules (Hoogstrate et al. 2014; Lima and Pasquinelli 2014). These advances stimulated research on parasitic nematodes which analyzed miRNA populations in extracts of whole organisms, including the filarial parasites *B. malayi* (Poole et al. 2010, 2014), *B. pahangi* (Winter et al. 2012) and *Dirofilaria immitis* (Fu et al. 2013), the tissue parasite *Angiostrongylus cantonensis* (Chang et al. 2013; Chen et al. 2011; Li et al. 2014), several gastrointestinal parasites (Ahmed et al. 2013; Liu et al. 2011; Shao et al. 2014; Xu et al. 2013; Zhao et al. 2013) and plant parasitic nematodes (Ding et al. 2015; Wang et al. 2015). Assembly of the menu of parasitic nematode miRNAs is an important first step that enables characterization of their biological functions. Initial efforts in this direction have identified a *B. malayi* miRNA that is highly expressed in L3 larvae and may play a role in regulating transmission of this parasite (Winter et al. 2015). Comparative analyses of miRNAs in parasitic species also provide insight into adaptations that may be associated with parasitism; in this regard, Wang et al. (2015) identified 4 miRNAs that are found in a very diverse set of parasitic species, but are absent from *C. elegans*. The functional roles of these miRNAs should be a high priority for research.

13.3 miRNAs Released by Filarial Nematodes

Although it is important to analyze the developmental and physiological roles of miRNAs in parasitic nematodes, little progress has yet been reported in this area. Most interest to date has been devoted to the potential roles of miRNAs released by parasites in regulating the host-parasite interaction. Evidence that parasitic nematodes release miRNA-containing EVs in culture and in the host is beginning to accumulate (Buck et al. 2014; Hansen et al. 2015; Zamanian et al. 2015). Based on their life style, which includes larval and adult stage residence in inteRNAl tissues

of the host, we propose that filariae provide particularly fertile models in which to investigate the roles of miRNAs secreted by parasitic nematodes.

Filarial nematodes are important pathogens of humans and animals. They belong to the Clade III group of nematodes, in the Order Spirurida. The causative agents of lymphatic filariasis (*Wuchereria bancrofti*, *B. malayi* and *B. timori*), river blindness (*Onchocerca volvulus*) and the eye worm (*Loa loa*) are prominent examples of filarial parasitic nematodes of humans. Species of veterinary importance include the dog heartworm (*Dirofilaria immitis*), and the cow tissue parasite *O. ochengi*, among many others.

The availability of genome data is an essential prerequisite for the study of small RNAs. Over 100 nematode genomes are available, although in various stages of completion and annotation, either having been reported or as subjects of an ongoing sequencing project (www.nematodes.org). Among filarial species, the genomes of *B. malayi*, *D. immitis*, and *L. loa* are published (Desjardins et al. 2013; Ghedin et al. 2007; Godel et al. 2012), while genomes of another 11 filariae are currently in progress.

miRNAs have been sequenced in a rapidly increasing number of filarial organisms. To date, the sequence repository miRBase (release 21; (Griffiths-Jones et al. 2008)) contains 115 precursor and 107 mature high confidence miRNA sequences of *B. malayi*. miRNAs found in other filarial species are summarized in Table 13.1.

The numbers of miRNA candidates identified in the worm's environment (host blood, nodule fluid or culture media) have differed substantially among studies. For example, we identified over 200 putative parasite miRNAs in plasma obtained from dogs infected with *D. immitis*, but only 21 in plasma obtained from *O. volvulus*-infected humans (Tritten et al. 2014b). A separate study identified 6 *O. volvulus* miRNAs in human blood (Quintana et al. 2015). Circulating miRNAs in *O. ochengi*-infected cows were described twice independently, from different host biofluids, reporting 62 and 10 sequences (Quintana et al. 2015; Tritten et al. 2014a),

Table 13.1 Reported miRNAs of filarial nematodes

Species	Worm extract	Secretions	Source
B. malayi	**145** (Poole et al. 2014)	**Yes** (Zamanian et al. 2015)	Culture media
B. pahangi	**104** (Winter et al. 2012)	N/A	–
L. loa	N/A	**Yes** (Tritten et al. 2014a)	Host blood
O. volvulus	N/A	**Yes** (Quintana et al. 2015; Tritten et al. 2014b)	Host blood
O. ochengi	N/A	**Yes** (Quintana et al. 2015; Tritten et al. 2014a)	Host blood/nodule fluid
L. sigmodontis	N/A	**Yes** (Buck et al. 2014)	Host blood
D. immitis	**1063** candidates (Fu et al. 2013)	**Yes** (Tritten et al. 2014b)	Host blood

Sequences were obtained from culture media and/or host biofluids
N/A: data not available

while 22 predicted nematode miRNAs were found in plasma from *L. loa*-infected baboons (Tritten et al. 2014b) and 16 from plasma of mice infected with *Litomosoides sigmodontis* (Buck et al. 2014). Due to variations in methodological aspects, sample storage conditions and infection burdens (often incompletely known), direct comparison of these studies is difficult. However, parasite localization may contribute significantly to the variation in miRNA sequence diversity and abundance: all *D. immitis* dog stages reside in blood (adults and microfilariae), as do microfilariae of *L. sigmodontis*, while others occupy host tissues (*O. volvulus* and *O. ochengi*), with only indirect access to blood.

13.3.1 Stage-Specific miRNA Release

miRNAs are well-known to be differentially expressed across tissues and developmental stages. Several studies have reported variation of miRNA expression (up to >twentyfold) between embryonic, larval and adult stages of *C. elegans* (Karp et al. 2011; Kato et al. 2009). Larval stages in particular show an enrichment of many miRNAs. Studies in *C. elegans* have revealed variation in the expression of miRNAs related to ageing, stress-resistance, dauer state, etc. (Abbott 2011; de Lencastre et al. 2010; Karp et al. 2011; Pincus et al. 2011). With some exceptions, miRNAs in the same family (e.g., sharing a common seed sequence) tend to display similar expression patterns (Guo et al. 2014). Based on experiments with *B. pahangi* tissue extracts from L3 larvae and adults, Winter and colleagues identified 69 miRNA sequences in common to both stages, while 6 were unique to L3 and 10 to adults (Winter et al. 2012).

EVs containing small RNAs by *B. malayi* are released much more abundantly by larvae than adult males or females (Zamanian et al. 2015), despite the greatly differing amounts of worm tissue incubated in culture media to collect EVs (adults being 15–50 times larger than L3s). That study also confirmed in nematodes that miRNAs exported through EVs are present in proportions quite distinct from those in whole worm extracts. For example, Bma-*let-7* was significantly enriched in EVs released by L3s compared to its overall abundance at this stage. In support of this, *let-7* is known to regulate the L3-L4 larval transition in *C. elegans* (Rougvie 2001), while in *B. pahangi*, 3 members of the *let-7* family were shown to be up-regulated in L3 larvae as a result of invasion of the definitive host, from insect vector to mammal (Winter et al. 2015). Hence, it is hypothesized that EVs and their contents are biologically relevant and specific to infective stages (Zamanian et al. 2015).

In *D. immitis* E/S miRNA profiles established from culture media, we observed that miRNAs are abundantly produced by adult worms (especially females) in comparison to microfilariae (L1 larvae), and overlap substantially with the profile obtained from infected dog blood (manuscript in preparation). None of the most abundant miRNAs was exclusively released by one stage (not shown). The miR-100 family was among the most abundant sequences. In *C. elegans*, the conserved *mir-51/mir-100* family is an essential player in embryonic development,

specifically in the early morphogenesis of the pharynx, but also in growth and male mating (Shaw et al. 2010). Understanding the role of the miR-100 miRNAs released by parasites may not be resolvable through studies in a free-living species, but may have to await the development of more robust techniques to alter gene expression in parasites.

13.3.2 Species-Specific and Shared Filarial miRNA Sequences

miRNAs are generally thought to be highly conserved across animal species. However, a large proportion of miRNAs released from filariae appear to be species-specific (Fig. 13.1). Interestingly, only miR-71 and miR-100a were found in the secretions of all 6 examined species, miR-100c and miR-34 have been reported from all but *O. volvulus*, and miR-100d and *lin-4* from all but *L. sigmodontis*. Due to the large number of candidate miRNAs from other filariids that do not have a known homolog in the *B. malayi* secretome, its profile may be substantially different from the other 5 species. Confirming this possibility would require a detailed genomic search to determine if the other filariid miRNAs are truly absent in this species. In this context, it must be recognized that other explanations, including differences in

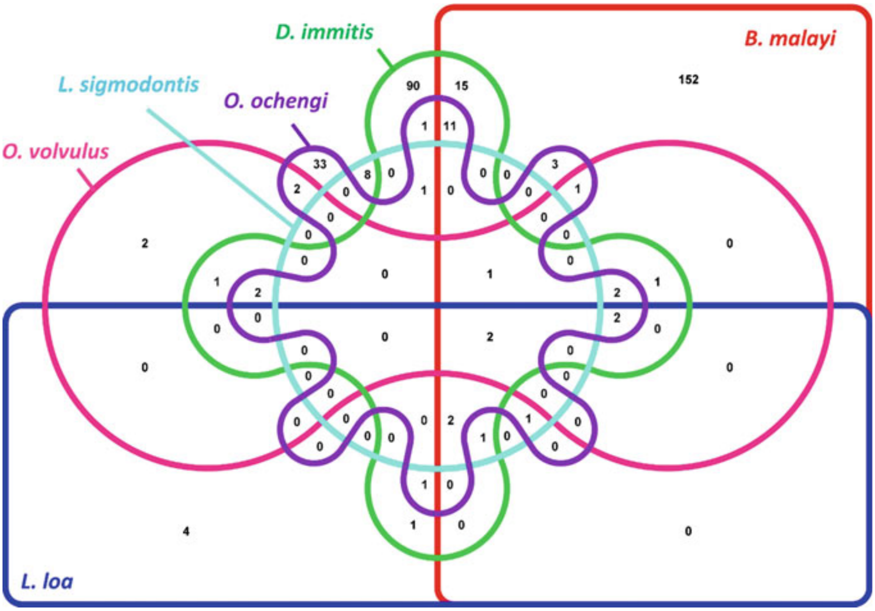

Fig. 13.1 Venn diagram of E/S miRNAs from filarial nematodes. Data are derived from the studies cited in Table 13.1. Only sequences of 18–24 nucleotides were included

the experimental milieu (i.e., host blood versus culture media), the generally unknown worm burden in vivo, the localization of the parasite in the host, and methods used for analysis and annotation may underlie this result.

Although abundant evidence supports the hypothesis that the E/S products of parasitic nematodes exert potent and profound effects on host immune response pathways, it is not yet known to what extent these molecules act at a local vs. systemic level. In this regard, parasite-derived miRNAs exported into the environment near the worm may fulfill functions that reflect the host-parasite interface at the site of infection, and diffusion of miRNAs from that site to the general circulation may be unrepresentative of the composition of the local milieu. In addition, due to the artificial nature of parasite culture systems, the miRNAs released in vitro may fail to represent accurately the miRNA profile secreted in vivo. We have begun to investigate this possibility through experiments with *D. immitis*; the population of miRNAs detected in culture media from adult males, adult females and microfilariae overlapped but were not identical to the population of *D. immitis* miRNAs detected in plasma from infected dogs (manuscript in preparation). Importantly, the most abundant miRNAs found in infected dog plasma were generally also detected in culture supeRNAtants. However, as all stages of this parasite examined so far in culture exist in the canine host bloodstream, this dataset does not directly address the local-systemic question proposed above; resolving this possible source of variation will require additional experiments in animal models of non-bloodstream filarial species. Based on currently available data, it would be premature to conclude that the suite of miRNAs so far reported from filarial parasites represents significant phylogenetic or functional differences; this is clearly an area that warrants further systematic research.

13.3.3 Predicted Host Targets of Filarial miRNAs and Cross-Species Transfer

In *C. elegans*, miRNAs possess many known functions as endogenous gene regulators, most notably fine-tuning critical transition steps in its complex life history. As noted, similar studies in parasitic species are only now beginning (Winter et al. 2015). Research in this area will be guided by the availability of bioinformatics tools that enable the identification of candidate miRNA targets, searching for perfect complementarity between the miRNA seed sequence and a mRNA, and considering aspects such as partial complementarity of the rest of the miRNA sequence, the free energy of the duplex formed by the short and the long RNA, site accessibility, position, etc. (Zheng et al. 2013). It is also common practice to further narrow down target searches using comparative analyses of sequences in orthologous species, reinforcing the statistical significance (Friedman et al. 2009; Rehmsmeier 2006; Zheng et al. 2013). A given miRNA can regulate multiple targets (Friedman et al. 2009), and clustered miRNAs are often co-expressed. There is also some evidence for a functional relationship between targeted transcripts,

i.e., in a biological pathway (Hausser and Zavolan 2014). Nevertheless, it must be kept in mind that miRNAs act in concert with transcription factors to regulate genes, and function in multi-level regulatory networks (Le Bechec et al. 2011).

It is reasonable to hypothesize that miRNAs released by worms into the host environment have been evolutionarily selected for export and function. Given the well-known immunomodulatory properties of E/S proteins, the research community rapidly envisioned that miRNAs of parasitic origin must have biological significance at the host-parasite interface. EVs from parasitic flatworms and nematodes filled with E/S products have been reported to be inteRNAlized by mammalian cells in culture (Buck et al. 2014; Marcilla et al. 2012; Zamanian et al. 2015). Of interest in this regard is that a number of nematode secreted miRNAs show identical sequences, or at least identical seed sequences, to host miRNAs. For instance, bma-*let-7*, along with bma-miR-1, bma-miR-9, bma-miR-92 and bma-miR-100b, found in EVs from *B. malayi* L3, are sequences with perfect sequence identity with human miRNAs (Zamanian et al. 2015), and hence, at least theoretically, could regulate the expression of host genes. Bma-*let*-7 was strongly enriched in EVs (Zamanian et al. 2015). Among other, diverse targets in humans, endogenous *let*-7 acts as a repressor of RAS and other oncogenes, and negatively influences cell proliferation (Johnson et al. 2007; Zamanian et al. 2015). Importantly, *let*-7 regulates several aspects of the immune system including macrophage polarization (Almanza et al. 2010; Banerjee et al. 2013; Chen et al. 2007). As algorithms that can predict miRNA–mRNA matches improve, it will be increasingly possible to identify high-quality host candidate targets for parasite-derived miRNAs (Fromm et al. 2015). Proof that parasite-derived miRNAs affect host gene regulation in situ is the next challenge facing this rapidly burgeoning field.

Recently, it has been reported that horizontal transfer of functional miRNAs can occur between parasitic nematodes and host cells in culture, with the postulate that parasites use miRNA release to mediate host immune suppression. Indeed, EVs from nematode parasites were observed to be taken up by cell lines in vitro. *B. malayi*-derived EVs were inteRNAlized by murine macrophages, via the phagocytic route, which elicited the classical activation of these immune cells (Zamanian et al. 2015), a response that was previously observed following exposure to dead or moribund worms, but which is somewhat opposed to typical immune responses to live worms or purified E/S proteins (Allen and MacDonald 1998; Osborne et al. 1996; Taylor et al. 2000). Similarly, miRNAs (miR-200, *let*-7, and miR-425; all 3 are homologous to mouse miRNAs) found in culture supeRNAtants of the murine gastrointestinal nematode *Heligmosomoides polygyrus* were predicted to interact with the 3′ UTR of the mouse gene *Dusp1* (Buck et al. 2014). DUSP1 is thought to attenuate acute inflammatory responses by inducing production of arginase by macrophages, a process shown to be protective in *H. polygyrus* infections (Anthony et al. 2006; Buck et al. 2014; Nelin et al. 2007). The study also showed the uptake of parasite EVs containing RNA and proteins, including an Argonaute homolog, by intestinal cells in vitro and provides the first evidence of immune suppression in vivo. Mice treated with *H. polygyrus* exosomes failed to mount the expected type 2 innate immune response following exposure to an allergenic fungus (Buck et al. 2014).

The immune suppression was observed to be local, as the parasite does not have direct contact with blood. In serum from mice infected with the filarial nematode *L. sigmodontis*, the adults of which reside in the pleural cavity, several putative miRNA sequences were detected, matching the parasite's genome (Buck et al. 2014). Hence, the localization of the parasite likely dictates the potential for a local versus systemic delivery of miRNAs to host cells.

13.4 Applications and Therapeutic Perspectives

Because miRNAs are present in biofluids (blood, urine, cerebrospinal fluid, sputum, etc.), stably packaged and protected in vesicles, and expressed in a tissue- or even cell-specific manner, they represent potentially valuable biomarkers for diagnostic or prognostic applications. In humans, miRNA profiles are dysregulated in all types of cancer and are tumor-specific, and thus have been widely tested for diagnostic purposes (Cortez et al. 2011). Some circulating pathogen-derived miRNAs are sufficiently distinct from host sequences to be candidate biomarkers of parasitic infections (Hoy et al. 2014; Manzano-Roman and Siles-Lucas 2012; Siles-Lucas et al. 2015). Previously, we investigated the diagnostic potential of two abundant *D. immitis* miRNAs found in dog blood, miR-71 and miR-34 (Tritten et al. 2014b). Using a stem-loop reverse transcription, quantitative PCR approach (Chen et al. 2005; Kramer 2011), we amplified *D. immitis* miR-71 and miR-34 from infected dog blood. These assays differentiated between infected and uninfected animals, but did not correlate well with the measured microfilariae counts (Tritten et al. 2014b); adult worm burdens in these dogs were unknown, and the relative proportion of these miRNAs released by microfilariae vs. adults is unknown. Nevertheless, these results suggest that an appropriately stage-specific (or stage-selective) miRNA may be of use for estimating worm burdens in infections for which such measures are difficult or impossible,

A wide range of miRNA-based therapies is currently being evaluated in human clinical trials. For example, miR-34 has been recognized as a tumor suppressor, and an injectable miRNA mimic in an ionizable liposome is undergoing clinical trials (Bader 2012). Another example is "Miravirsen", an antisense oligonucleotide that is used to sequester miR-122, a host miRNA required by the hepatitis C virus for its stability and propagation (Janssen et al. 2013).

Will miRNAs contribute one day to an improved control of nematode infections? The question has been raised by several authors (Britton et al. 2014; 2015; Schwab et al. 2015; Siles-Lucas et al. 2015). With the growing availability of parasitic nematode genomes, and affordable sequencing technologies, miRNA discovery has evolved at an increasingly rapid pace. In addition, technologies to engineer stable, chemically modified miRNA mimics, antagonists, sponges, etc., and administer them in suitable carriers are becoming increasingly available (Baumann and Winkler 2014). However, the fact that genes may be regulated by many different miRNAs raises issues of complexity in target validation, and for

efficacy in the implementation of miRNA-based treatments, as targeting a single miRNA may prove to be therapeutically insufficient (Baumann and Winkler 2014). Further substantial challenges reside in the precise targeting of administered miRNA-based treatment to the target tissue or cell population, and in obtaining a physiologically observable effect (Siles-Lucas et al. 2015).

For helminthologists, extra challenges and open questions apply. Given the parallel mode of action between RNAi and miRNA-induced repression, and the so far limited success of RNAi experiments in parasitic nematodes, miRNA-mediated strategies to interfere with parasite development or physiological function may be very challenging to develop (Britton et al. 2015; Maule et al. 2011). Besides this, proving that hosts recognize and respond to parasite EVs in situ, determining whether all parasite EVs are equally recognized, identifying how parasite miRNA populations are sorted and selected for export, and deriving a more quantitative understanding of miRNA-exosome stoichiometry, all are challenges that remain to be resolved (Chevillet et al. 2014; Coakley et al. 2015). However, for therapeutic utility, perhaps the most important challenge is to identify parasite-derived miRNAs that are essential and sufficient to achieve host immunomodulation. If multiple miRNAs contribute to the successful establishment of infection, it will be difficult to convert a normally permissible host into a non-permissible host by interfering with the functions of parasite miRNAs in host fluid compartments. If, however, a few parasite miRNAs can accomplish the necessary immunomodulation, targeting them through current approaches may enable the host to kill or eliminate the parasite immunologically.

Host miRNAs, too, have appeared to change in response to parasitic infections (El-Assaad et al. 2011; He et al. 2013). It will be crucial to define them, not only to better understand their possible role in parasite-induced pathology, but also to identify their possible roles in host defense mechanisms, and further elements in their targeted genes that may be required for the outcome of an immune response (Britton et al. 2015).

13.5 Evolutionary Considerations

It has long been known that host-parasite interactions are often quite specific or selective; this is especially true for filarial parasites. For example, the host ranges of parasites that cause onchocerciasis or lymphatic filariasis in humans is highly restricted, while the closely related parasite *D. immitis* successfully infects only canids (and felids to a limited extent). The biological bases underlying the success or failure of a parasitic nematode in a particular host remain almost entirely unknown, but it is reasonable to propose that parasite-induced immunomodulation is a key variable in determining this outcome. The molecular negotiations carried out by the parasite include the exchange of several types of molecules. While protein components of parasite E/S products have been the focus of much of this work (e.g., Hewitson et al. 2009; McSorley et al. 2013), the effects of parasite

secreted proteins have rarely been shown to be host species-specific. Since single nucleotide changes can profoundly alter miRNA-mRNA interactions, it may be more likely that selection for host-specific miRNA interactions is a key driver of the evolution of host-parasite specificity, a hypothesis that remains to be rigorously evaluated.

13.6 Considerations of Other ncRNA Species

miRNAs are arguably the most well-characterized (and the most abundant, with endogenous small-interfering (si)RNAs; (Winter et al. 2012)) type of ncRNA in filarial nematodes, but are not the only class of these molecules exported in EVs. It is worth noting that miRNAs may not be the only ncRNA species involved in host-parasite cross-talk. In *H. polygyrus*-derived exosomes, a set of Y RNAs was reported along with miRNAs (Buck et al. 2014). Also, endogenous siRNAs and piwi-interacting RNAs have been discovered in *B. pahangi* whole-worm extracts (Sarkies et al. 2015; Winter et al. 2012). There is yet no biological evidence for a role of other parasite ncRNAs in host modulation, but it would be unwise to ignore the possibility.

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