Characterizing small RNA cargoes of exosomes from *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae)

by

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Abstract

Exosomes are extracellular communication vesicles utilized in both mammalian and insect systems. Most exosomal research to date has been conducted in mammalian systems, specifically in the field of biomedical research due to their ability to carry protein and RNA cargoes in both short- and long-range signaling pathways within an organism. In mammalian research, exosomes are important as both diagnostic mechanisms for detecting cancer and potentially other diseases, but also as vehicles for carrying therapeutic molecules throughout the body, including across the blood brain barrier.

In insects, much less is known about exosomes. Previous studies have shown that exosomes play important roles in diverse aspects of insect biology and physiology, including in maintaining signaling gradients or carrying cargoes in developing embryo, in altering female mating behavior by enhancing egg laying and reducing mating receptivity, and in enhancing pathogen transmission to and from their insect vectors. More recently, evidence also suggests that exosomes may play a role in systemically spreading RNA interference (RNAi) responses within insects. However, there is a lack of information on the specific composition and cargoes that are carried by exosomes in insect systems. Such basic information is vital to understanding the roles that exosomes play in insect biology and physiology, as well as understanding how exosome-based signaling changes following stimulation.

In this study, small RNAseq was conducted on exosomal RNAs isolated from cultured *Diabrotica undecimpunctata* cells (southern corn rootworm, SCR). The cells were either untreated or treated with double-stranded RNA (dsRNA) specific to a non-target gene (*enhanced green fluorescence protein, EGFP*) to determine how exosome cargoes change according to

changing physiological conditions. Sequencing yielded over 10 million and 12 million reads, respectively, and small RNA reads from both sets of data showed a multimodal size distribution with main peaks at 28, 21-22, and 32 nt. Subsequent analysis identified miRNAs from the read data for each sample. Many miRNAs detected from SCR exosomes were homologous to miRNAs from the well characterized beetle, *Tribolium castaneum*, but unique miRNA sequences (912 unique miRNAs overall,616 of these were found in the control samples and 557 from the dsEGFP treated samples) were also predicted from the sequencing data. The miRNAs were compiled together and named in order of abundance, and isomiRNAs were detected and classified for naming with 812 unique sequences and 98 various isomiRNAs. No significant differences in the miRNA profiles were observed between treated and untreated samples; however, analysis of the small RNA reads from exosome RNA from dsEGFP-treated cells were also mapped to the EGFP gene sequence.

This study shows that exosomes from SCR cells carried RNA cargoes in both untreated and dsRNA-treated systems, and that exosomes may contribute to spreading RNAi signals by carrying small RNA sequences corresponding to the dsRNA that was used for treating the parent cells. These results provide a firm foundation to continue characterizing insect exosomes and the roles that they play in insect biology and physiology. Future experiments will enhance these findings by also analyzing mRNA and protein cargoes carried by SCR exosomes and correlating our *in vitro* results with *in vivo* experiments. Subsequent experiments may also explore the biosynthetic pathways that produce exosomes in insects and the roles of specific miRNAs that are carried by exosomes.

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Chapter 1 - Literature Review

1. Introduction

Exosomes are small extracellular messenger vesicles, about 30-100 nanometers in size, that are released from most cell types (Thery et al. 2009). Originally, they were not considered to be functionally significant and at best were thought of as waste-disposal vessels, but recent research has suggested that they are involved in many biological activities including within development, the immune system, and anti-tumor activity (Wolf 1967, van den Boorn et al. 2013).

In insects, exosomes were also largely ignored until researchers observed that messages were moving via lipid-based vesicles during development. Originally identified as argosomes in experiments on development in *Drosophila* (Greco 2001), researchers noted that green fluorescent protein that was anchored to membranes was able to spread in imaginal discs. Since then, exosomes have been identified in several species with roles in development, disease transmission, and immune responses in insects (Beckett et al. 2013, Corrigan et al. 2014, Vora et al. 2018).

While not much is known about exosomes in insects, extensive research in mammals has found exosomes in all bodily fluids (Beckett et al. 2013) and revealed much of the mechanisms that result in exosome synthesis and export, as well as their functional roles in mammalian physiology. Given the lack of information about insect exosomes, these studies in mammals are invaluable for understanding how exosome formation may occur and what roles exosomes may fulfill in insects. Here we will review the current literature on how exosomes form, are loaded with protein or nucleic acid cargo, are exported, and what biological functions they may be

fulfilling in insects, using the current knowledge base in mammalian and nematode systems to inform our analysis.

2. Exosome Synthesis and Export

Exosomes are formed within cellular endosomes through invaginations of the endosomal membrane (Gruenberg and van der Goot 2006, Trajkovic et al. 2008, Simons and Raposo 2009, Lakhal and Wood 2011, Lawson et al. 2016). Once endosomes begin forming internal vesicles they are referred to as multivesicular bodies (MVB). During MVB formation, tissue- or cell-specific cargoes, including proteins, lipids, and RNAs, are loaded into exosomes as they are formed within the MVB (Thery et al. 2001). Upon maturation, the MVB fuses with the plasma membrane of the cell of origin, releasing the vesicular cargoes, including exosomes, into the extracellular space. Exosomes are then free to deliver their cargoes to target cells where they are taken up via endocytosis. Here, we will discuss the specifics of exosome synthesis, loading, and export.

2.1. Biosynthesis

Very little data are available on the specific mechanisms of synthesis of exosomes in insect cells, and so we must extrapolate from extensive studies in mammalian systems in order to help understand the process in arthropods. As noted above, MVBs form through invagination of endosomal membranes to create intraluminal vesicles (ILVs) inside the MVBs (Gruenberg and van der Goot 2006, Trajkovic et al. 2008, Simons and Raposo 2009, Lakhal and Wood 2011, Colombo et al. 2014, Lawson et al. 2016) (Figure 1). MVBs can contain a variety of different ILVs, including exosomes and microvesicles. Exosomes specifically are synthesized through

both endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways (Simons and Raposo 2009, Kanemoto et al. 2016).

ESCRT is a membrane sculpting complex that creates vesicles through a variety of protein complexes (Beer and Wehman 2017). ESCRT-0 is necessary for gathering exosome cargoes and then recruiting ESCRT-1 and ESCRT-II to the site of vesicle formation. These complexes work together and are involved in the budding of the EV within the MVB membrane (Kowal et al. 2014, Beer and Wehman 2017) (Figure 1). Once this is done, ESCRT-III is recruited and pulls the sides of the EV together and causes scission of the new vesicle. The ESCRT-III associated protein, Alix, is important for promoting budding of new vesicles within the MVB and is frequently associated with exosomes and other ESCRT proteins (Baietti et al. 2012, Beer and Wehman 2017) (Figure 1). Once the new EV is created, the ATPase, Vps4, allows for ESCRT dissociation from the endosomal membrane. There are contradictory reports over the role of ESCRT-III-associated Vps4 in potential scission of exosomal membranes and dissociation of ESCRT, though. One study shows that inhibition of Vps4B in HeLa-CHTA cells increased exosome secretion (Colombo et al. 2013), while a different study in MCF-7 cancer cells determined that silencing both Vps4A and Vps4B led to a decrease in secretion but silencing either isoform alone showed no significant differences in secretion levels (Baietti et al. 2012). This may be due to slightly different mechanisms of exosome creation between healthy mammalian cells and cancer cells.

2.2. Cargo loading

Exosomal cargoes, including proteins, lipids, mRNAs, and miRNAs, are loaded into the vesicle during the process of exosome formation (Valadi et al. 2007, Belting and Wittrup 2008, Moldovan et al. 2013, Sun et al. 2013). As the early endosome or the later MVB continues EV

formation, the cargoes must be recognized and brought to the MVB for loading into the nascent vesicles (Simons and Raposo 2009). This process, like exosome synthesis, involves both ESCRT-dependent and ESCRT-independent mechanisms. For the ESCRT-dependent mechanism, ESCRT-0 is responsible for recognizing EV cargoes and bringing those together before recruiting ESCRT-I, II and -III for vesicle formation as mentioned in the above section (Figure 1). In this process, it is not fully understood how specific cargoes are loaded into exosomes, but that ESCRT-0 binds to ubiquitylated proteins in the cell and on the surface of MVBs (Robbins and Morelli 2014, Wei et al. 2021). After binding to the MVB membrane, ESCRT-I and -II cause budding of the future exosome and during this process, cargoes can enter the forming exosome before ESCRT-III cleaves the budding vesicle into the completed exosome.

Other studies suggest ESCRT-independent processes, mainly involving tetraspanin family proteins, are responsible for loading exosome cargoes (van Niel et al. 2006, Simons and Raposo 2009, Trajkovic et al. 2008, van Niel et al. 2011, Vlassov et al. 2012, Kanemoto et al. 2016). Tetraspanins are a family of proteins that contain four transmembrane domains that form complexes with themselves and other proteins and are fused into the exosomal membrane to facilitate cargo loading (Rana et al. 2012) (Figure 1). CD36, one of these proteins, worked in sorting specific proteins into exosomes in melanoma cells (van Niel et al. 2011). In rats, the tetraspanin, TSPAN8, was able to alter mRNA content and protein composition of secreted exosomes (Nazarenko et al. 2010). A variety of tetraspanins have been identified in exosomes and play key roles in cargo loading, mainly protein loading, within the forming vesicles.

More recently, Wei et al. (2021) determined that Rab31 controls exosome formation independently of ESCRT proteins. Researchers were investigating Rab GTPases with currently unknown functions in exosomal biosynthesis using epidermal growth factor receptor (EGFR).

Overexpression of Rab31 led to MVB targeting by EGFR which suggested that it was potentially involved in exosome formation. Further investigation determined that Rab31 increased exosome production and packaging of EGFR. Together, this suggests that Rab31 may be important in ESCRT-independent exosome biosynthesis.

Zhang et al. (2015) summarized four potential modes of sorting miRNAs into exosomes, though the underlying mechanisms for these modes remain unclear. The first of these is the neutral sphingomyelinase 2 (nSMase2)-dependent pathway (Kosaka et al. 2013). Expression levels of nSMase2 in breast cancer cells was related to the amount of exosomal miRNAs found; overexpression of nSMase2 led to an increase in miRNAs whereas suppression reduced the number of miRNAs. It is currently not known how this is important, but it may have to do with exosomal ceramides as nSMase2 is important for ceramide production. The second potential mode is miRNA motif and sumoylated heterogenous nuclear ribonucleoproteins (hnRNPs)-dependent pathway (Villarroya-Beltri et al. 2013). This pathway allows for specific miRNA loading into exosomes through sumoylated hnRNPs recognizing specific motifs on 3' portions of miRNAs. Researchers discovered three hnRNPs that could potentially be involved in this process as they can all bind specific miRNAs: hnRPNA2B1, hnRPNA1, and hnRPNC, though only the binding motif for hnRNPA2B1, GGAG is currently known. The third mode was a 3'-end of the miRNA sequence-dependent pathway (Koppers-Lalic et al. 2014). This group used exosomes from B cells as their model and determined that the 3' end of miRNA sequences contains a signal for sorting into exosomes. This was found by discovering that in endogenous miRNAs, if the 3' end is uridylated they are mainly found in exosomes while 3' ends of miRNAs that are adenylated are kept in the B cell for presentation. The final mode mentioned is through the miRNA-induced silencing complex (miRISC)-related pathway (Frank et al. 2010). One of the

major components of the miRISC complex is Ago2 which has been identified in exosomal protein analyses. Knockout of Ago2 in HEK293T cell-derived exosomes was shown to decrease the types or abundance of important mammalian miRNAs (Guduric-Fuchs et al. 2012). Colocalization of other integral miRISC components and MVBs has also been reported (Gibbings et al. 2009) as well as an inverse relationship between MVB abundance and the amount of miRISCs in a cell (Squadrito et al. 2014).

2.3. Export

Exosomes must be released from the cell of origin in order to deliver their cargoes to cells throughout an organism. The MVB-containing exosomes and other vesicles will fuse with the plasma membrane to release their contents from the cell and into the extracellular space (Trajkovic et al. 2008, Simons and Raposo 2009, Lakhal and Wood 2011, Zhang et al. 2015, Lawson et al. 2016). This process is controlled by both ESCRT-dependent and ESCRT-independent mechanisms.

ESCRT is important for many steps in exosome biogenesis and secretion (Figure 1). Inhibition of Hrs and Stam1, proteins associated with ESCRT-0, led to a decrease in secretion in multiple mammalian cell types (Colombo et al. 2013, Kowal et al. 2014). In *Drosophila*, ESCRT-0 subunit Hrs and the accessory factor Alix were found to be required for exosome secretion from male accessory gland cells (Corrigan et al. 2014). In *C. elegans*, ESCRT-I and ESCRT-III are important for the release of various microvesicles, including exosomes, after recruitment to the plasma membrane (Wehman et al. 2011, Beer and Wehman 2017) (Figure 1). To show that ESCRT is important for budding of the plasma membrane, ESCRT-0 and ESCRT-II proteins were suppressed (Wehman et al 2011). This suppression significantly suppressed

vesicle release. Knockdown of ESCRT genes in *Drosophila* wing imaginal discs inhibited the release of exosomes carrying developmentally important Hh (Gradilla et al. 2014).

Exosome secretion is also controlled through ESCRT-independent methods. This was determined through suppression of ESCRT proteins as this did not completely suppress exosome release in *Drosophila* gland cells or in *C. elegans* embryos (Wehman et al. 2011, Corrigan et al. 2014, Beer and Wehman 2017). This was further supported in *Drosophila* S2 cells as knockdown of ESCRT-0 and ESCRT-1 genes did not disrupt EV release (Beckett et al. 2013). Various members of the Rab family of GTPases have been tested in different mammalian cell types and are believed to be involved in MVB docking to the plasma membrane before fusion and vesicle release (Savina et al. 2005, Hsu et al. 2010, Ostrowski et al. 2010, Baietti et al. 2012, Kowal et al. 2014, Beer and Wehman 2017) (Figure 1). Rab27 knockdown results in a decrease in MVB fusion to the plasma membrane and therefore exosome release (Ostrowski et al. 2010, Zhang et al. 2015). Additionally, in *Drosophila* and *C. elegans*, Rab7 seems to play a role in exosome secretion as well, but it is not known what specific role is fulfilled by this protein (Wehman et al. 2011, Corrigan et al. 2014). Rab11 is required for exosome release form wing imaginal discs in *Drosophila* S2 cells and is believed to be important in docking and fusion to the plasma membrane (Corrigan et al. 2014, Beer and Wehman 2017). First demonstrated in mammalian cells, Rab35 is believed to be important in fusion to the plasma membrane (Hsu et al. 2010). This was further supported in *Drosophila* as knockdown of Rab35 led to a decrease in exosome release from male accessory gland cells (Corrigan et al. 2014).

ESCRT-independent mechanisms are beginning to be determined, mainly in the field of biomedical research. In neural cells, inhibition of sphingomyelinase led to a decrease in exosome secretion through decreased ceramide production (Trajkovic et al. 2008, Kowal et al. 2014). In

addition to Rab GTPases, induced cholesterol accumulation in MVBs of neural cells increased the secretion of exosomes and other vesicles (Strauss et al. 2010). There is also evidence that soluble NSF-attachment protein receptor (SNARE) proteins are involved in MVB fusion for release of exosomes (Gross et al. 2012, Kowal et al. 2014, Beer and Wehman et al. 2017) (Figure 1). Vamp7 and Syntaxin-5 are SNARE proteins found on MVBs and the plasma membrane, respectively, that are necessary for plasma membrane fusion (Fader et al. 2009, Hyenne et al. 2015). In *Drosophila*, Ykt6 and Syx1a are both important SNAREs involved in microvesicle and exosome secretion (Gross et al. 2012, Gradilla et al 2014, Koles et al. 2012). While some SNARE proteins have been identified and partially characterized, it is still not known whether there are interactions between the proteins that are important for their function or if there are interactions with other currently uncharacterized proteins that may be important for exosome secretion.

Once secreted from cells, exosomes can mediate both short- and long-range specific signaling and can target specific tissue or cell types (Sun et al. 2013, van den Boorn et al. 2013, Corrigan et al. 2014). This means that exosomes do not fuse with only nearby cells; they can also specifically target a certain tissue type no matter the distance from the originating cell. Short-range signaling was observed in the wing disc of developing Drosophila as exosomes helped maintain the Hh gradient necessary for normal wing development (Gradilla et al. 2014). In contrast, long-range exosome signaling was demonstrated by Alvarez-Erviti, et al. (2011). In this study, exosomes harvested from dendritic cells transfected with a plasmid for expressing an exosomal protein, Lamp2b, that was fused with a brain-specific rabies protein, rabies viral glycoprotein (RVG), were transported to target sites in the brain following injection into the tail.

Once the exosome reaches the target cell, they must be taken into the cell cytoplasm to deliver cargoes. The exact mechanisms for this internalization and processing are currently unknown, though recent studies have given some insight into potential mechanisms. Using dendritic immune cells, it was determined that exosomal transmembrane proteins directly interact with receptors on the target cell (Munich et al. 2012, Zhang et al. 2015). Specifically, dendritic cell derived exosomes express TNF, FasL, and TRAIL that are able to induce apoptosis in target cells, as well as stimulate NK cells. Exosomal lipid rafts are also important in cellular recognition of exosomes. When lipid-rafts were experimentally disrupted in breast cancer cells, exosomes were unable to be internalized into the target cell (Koumangoye et al. 2011).

Once recognized, the exosome has multiple potential ways of entering the target cell to release their cargoes (Mulcahy et al. 2014). Some exosomes may fuse with the plasma membrane directly to deliver cargoes to the cytoplasm, while others are internalized through clathrin-mediated endocytosis or phagocytosis, as was determined with leukemia cell exosomes only being taken up by macrophages (Feng et al. 2010, Mulcahy et al. 2014). Direct fusion of exosomes to the plasma membrane has been shown as well using fluorescent lipid dequenching of exosomes taken up by melanoma cells under acidic conditions (Parolini et al. 2009, Mulcahy et al. 2014). Clathrin-mediated endocytosis has been implicated many times as a mechanism of uptake as blocking any stage of the process leads to a reduction in the number of exosomes internalized (Mulcahy et al. 2014). Exosomes that are internalized will be taken into intracellular endosomes where they will merge with this intracellular membrane (Mulcahy et al. 2014, Zhang et al. 2015). From there, the exosome components will either be recycled into other EVs or will mature with the endosome into a lysosome and undergo degradation.

After exosome uptake via endocytosis, the cargoes of exosomes are capable of inducing changes in the physiology of the target cells (Belting and Wittrup 2008, Valadi et al. 2007). Translation of mRNA cargoes can occur and cause differences in the level of transcripts that are isolated from the donor cells and the targets, which continues to suggest that there are intricate selection mechanisms in place for exosome cargoes as well as their targets.

3. Exosome Cargoes

The contents of exosomes appear to be dependent on the cell type of origin, leading to the possibility of many different potential roles that exosomes may be fulfilling for an organism (Forterre et al. 2014). These cargoes include proteins, mRNAs, and miRNAs that may carry out different functions within the target cells.

3.1. Proteins

Because exosomes can be secreted by many cell types, it is likely that some proteins found both on and in exosomes are unique to the cell type of origin. Additionally, exosomal proteins will most likely differ between different organisms as well (Vlassov et al. 2012). Using mass spectrometry, over forty thousand proteins associated with exosomes have been found and uploaded into the online database ExoCarta (Mathivanan and Simpson 2009, Keerthikumar et al. 2016). These include proteins from normal mammalian systems as well as those from cancer systems in order to potentially compare if there are any differences in the two states. This information may lead to understanding what normal protein cargoes are found within exosomes, as well as how these potentially change due to changing conditions, differences between organisms, and different origin tissues.

Because mammalian exosomes are formed from endosomes, the protein components of these two membrane compartments are very similar. Exosomal proteins include membrane and

transport proteins, tetraspannins, heat shock proteins, proteins involved with MVB biogenesis, and lipid-related proteins and phospholipases (Subra et al. 2010, Vlassov et al. 2012). Specifically, exosomal proteins generally include Alix, Hsp70, CD9 and CD36 that come from the process of exosomal biosynthesis (Thery et al. 2009, Simons and Raposo 2009, Vlassov et al. 2012, Lawson et al. 2016). Many of these are used as markers in both mammalian and insect exosome research.

Jorgensen et al. (2015) created an improved method of surface protein detection using antibodies for three known human exosomal proteins, CD9, CD63, and CD81, combined with many other antibodies to try to detect previously unknown proteins. By using antibodies of these three known proteins, it made for easier detection of exosomes using antibody profiling. This method has been utilized to detect new surface proteins and how these proteins change under different biological conditions (Just et al. 2020). In this study, exosomes and other extracellular vesicles were isolated from human plasma before and after blood flow-restricted exercise. The researchers determined that this stress altered the surface proteins on the vesicles by increasing Alix, ITGA2B, NCAM, and IL2RA expression and decreasing Flot1 expression. This is important as it gives more information on how the roles of exosomes may change in an organism during different physiological states due to changes in protein contents of exosomes.

3.2.1. mRNA

In addition to carrying protein cargoes from site to site, exosomes also carry nucleic acids, including mRNAs and miRNAs that can induce physiological responses in the target tissues or cells. Exosomes secreted from mast cells were found to be carrying 1,300 different mRNAs as well as miRNA cargoes that functioned mainly in basic cellular processes like development, protein synthesis, and post-transcriptional RNA modifications (Valadi et al. 2007,

Vlassov et al. 2012). The mRNA transcript levels differed between the exosome and the donor cells in that the most abundant mRNAs in exosomes were different than those in the donor cells. Some of the transcripts in the exosomes, including those involved in cell proliferation, immune responses, and angiogenesis, were entirely unique as they were not detectable in the donor cells at the time of detection. In addition, the researchers were able to determine that at least some of these mRNAs were full length as they were translated in the recipient cell. This is interesting because it shows that exosomes carrying mRNAs may be useful in creating a response in the recipient cell that may alter the state of this cell based on what the organism needs. This idea is supported by Skog et al. (2008) as exosomes from glioblastoma cells carried mRNA, miRNA, and proteins to human brain microvascular endothelial cells. The mRNAs, specifically, were translated and stimulated tubule formation by the target cells.

3.2.2. miRNAs

miRNAs are single-stranded noncoding RNAs that are typically 21-24 nucleotides in length and are derived from endogenous primary miRNAs (Carthew and Sontheimer 2009, Zhu and Palli 2020). miRNAs are utilized for post-transcriptional gene silencing of specific target mRNAs (Bartel 2004, Zhang et al. 2015). From these works, it was determined that miRNA cargoes of exosomes are very important in cell communication. Because exosomes can carry multiple miRNAs, there can be rapid changes in gene expression in the target cells (Vlassov et al. 2012)

miRNAs are significant cargoes of exosomes (Valadi et al. 2007, Vlassov et al. 2012, Just et al. 2020). There are differing opinions on how these miRNAs relate to the origin cells, however. Some papers report that the levels of miRNAs are significantly different from those in total cell lysates taken from the origin cells (Mittelbrunn et al. 2011, Zomer et al. 2010, Zhang et

al. 2015). Others, report that the miRNA content is similar to the origin cell (Rabinowits et al. 2009, Taylor and Gercel-Taylor 2008). The latter was determined using cancer cells, so there are potentially differences in the cargoes and the amounts of cargoes in exosomes from cancer cells when compared to healthy cells.

While there are differing reports on how the miRNA cargoes relate to the cell of origin, it is important to know that the miRNA cargoes are not necessarily subsets of those found in the cell that are then randomly packaged into exosomes. Instead, analysis of miRNA expression levels between cell lines and their exosomes has found that some miRNAs preferentially enter exosomes when compared to other cellular miRNAs (Guduric-Fuchs et al. 2012). The miRNAs that were found to preferentially be in exosomes are believed to be important to intercellular communication. miR-451, a human miRNA with a high expression ratio between the cell of origin and exosome, is interestingly processed by Ago2 within the target cell and not Dicer (Gudrich-Fuchs et al. 2012, Herrera-Carillo and Berkhout 2017). In the case of miR-451, the base-paired stem is 17 bp which is too short for Dicer processing so is instead loaded directly into Ago2. Once in Ago2, the RNase-H-like domain cleaves in the complementary portion of the RNA and the miRNA is then further processed by the Poly(A)-specific ribonuclease (PARN) to the mature form (Herrera-Carrillo and Berkhout 2017). The researchers suggested that Ago2 may be involved in directing miRNA towards exosomal secretion, but this is currently not well understood (Gudrich-Fuchs et al. 2017). Other studies suggest that exosomal miRNA expression levels can change depending on the physiological condition of the originating cell (Skog et al. 2008). Different expression levels of miRNAs are also noted between different cell types of the same organism. Using human cultured cells, it has been determined in many studies that there are differences in the miRNAs expressed in the exosomes from different cell types (Skog et al.

2008, Taylor and Gercel-Taylor 2008, Rabinowits et al. 2009, Guduric-Fuchs et al. 2012, Squadrito et al. 2014, Zhang et al. 2015).

4. Biological Roles

Exosomes seem to be most important in intercellular communication. In mammals, this has been most studied in the immune system (Beckett et al. 2013, Bhatnagar and Schorey 2007, Bhatnagar et al. 2007) and in signaling associated with diseased states (Jansen et al. 2013, Cheng et al. 2013, Kruger et al. 2014, Lawson et al. 2016). In insects, we see similar roles in communication in mating and reproduction (Corrigan et al. 2014, Gradilla et al. 2014, Beckett et al. 2013). Interestingly in insects, there may also be roles in disease transmission from the insect to the host (Atayde et al. 2015, Vora et al. 2018). Exosomes seem to play a part in many of the important functional roles within both mammals and insects and will be discussed in further detail below.

4.1. Mammals

The field of immunology is particularly interested in exosome research as recent work has shown that they can play an important role in antigen presentation and in the spread of infectious agents (Beckett et al. 2013). In the human immune system, antigen presenting cells (APCs) capture and present peptides to T cells leading to activation of cytotoxic T cells (Chaput and Thery 2011). Exosomes secreted by APCs can also lead to T cell stimulation both *in vivo* and *in vitro*, but exosomes from cells other than immune cells can also present and stimulate T cells, completely bypassing the need for APCs in this case (Thery et al. 2002, Admyre et al. 2006). This could be helpful in creating a fast immune response to cells infected with pathogens as cells can bypass the APC steps and instead directly activate an immune response.

In addition to stimulating immune responses, exosomes are involved in promoting immune responses as well (Greening et al. 2015). Exosomes from bacteria or macrophages infected with intracellular pathogens contain pro-inflammatory responses (Bhatnagar and Schorey 2007, Bhatnagar et al. 2007). This same type of response can be seen with tumor cells but exosomes can also work in the opposite way and hide infections and cancers from the immune system. In suppression of the immune response against cancerous cells, exosomes work to directly suppress the anti-tumor responses of both cytotoxic T cells and Natural Killer (NK) cells which leads to the body being unable to detect the tumor (Greening et al. 2015). In addition, tumor-derived exosomes can induce T cell apoptosis to further avoid detection. Tumor cells also create and secrete more exosomes when compared to normal cells, which may increase the likelihood of tumor cells remaining undetected within the body (van den Boorn et al. 2013). There is increasing evidence that exosomes are important for both suppressing tumor activity and protecting cancerous cells from the immune system, as well as protecting the entire organism from cancerous cells depending on the type of cell releasing exosomes and the signals that are being sent.

Exosome cargoes, as well as proteins associated with exosomes, have been targeted as biomarkers for profiling, diagnosing, and treating human disease (Jansen et al. 2013, Cheng et al. 2013, Kruger et al. 2014, Lawson et al. 2016). Exosomes are easily obtained in body fluids and their protein and RNA signatures are reflective of the cell of origin and the state of the organism at the time of isolation (El Andaloussi et al. 2013). With the research suggesting that exosomes are important for a variety of biological functions, biomedical researchers have begun looking at the potential of targeting exosomes to treat various disorders. Researchers working on cardiovascular disease were able to take exosomes from stem cells and show that these exosomal

cargoes were able to protect the heart from injury and improve heart function for 28 days after treatment (Lai et al. 2010, Arslan et al. 2013, Lawson et al. 2016). In a different study, exosomes isolated from cardiac progenitor cells from patients undergoing heart valve surgery were injected into the hearts of rats that had a permanent coronary artery ligation (Barile et al. 2014). This exosome treatment led to reduced cardiomyocyte apoptosis and scar size, as well as an increased amount of viable tissue in the affected area, altogether improving function of the entire system. In regenerative medicine, there are also a growing number of reports suggesting that exosomes from stem cells possess cytoprotective properties that inhibit apoptosis and stimulate proliferation of cells and neovascularization (Ratajczak et al. 2012, Lai et al. 2010, El Andaloussi et al. 2013).

4.2. Insects

In insects, exosomes are associated with several aspects of insect growth and survival, including in reproduction and development, host-pathogen interactions, and RNA interference. Here, we will briefly review the role of exosomes in each of these processes.

4.2.1 Reproduction and development

Reproduction and development are some of the most important physiological functions within all organisms. In *Drosophila*, males secrete peptides from the accessory glands that induce changes in females after mating, including enhancing egg laying and reducing the receptivity of the female to mating again (Chen et al. 1988, Chapman et al. 2003). The latter of these changes is in the best interest of the male as it increases their own reproductive fitness while decreasing that of mated females. Corrigan, *et al.* (2014) demonstrated the necessity of exosomes from the male accessory glands in inducing these changes in the females through interactions with the epithelial cells of the female reproductive tract.

After mating, development of the offspring begins within the egg. In embryonic tissues, cells show patterned and controlled release of exosomes which suggests a significant role in developmental intercellular communication and tissue polarity (Liegeois 2006, van den Boorn et al. 2013). Hedgehog (Hh) is a morphogen required for growth regulation and specification of cell fates from a distance within the embryonic segments (Gradilla et al. 2014). Hh is secreted by cells and distributed down a concentration gradient which leads to differential activation of target genes down this gradient (Briscoe and Therond 2013). Within *Drosophila* wing imaginal discs, it has been shown that Hh is produced and secreted from the posterior portion of this disc and moves towards the anterior portion. Cytonemes were originally thought to maintain this gradient and movement, but a more recent study suggested that exosomes are also involved in movement of Hh molecules (Bischoff et al. 2013). Gradilla et al. (2014) showed through live imaging that Hh could be transported in exosomes. In addition, staining Hh revealed significant clusters of exosomes within spaces of the wing discs which seems to give more evidence that the two can be associated together. To determine if exosomes are responsible for maintaining the Hh gradient seen in development, exosome formation was blocked in *Drosophila* using RNA interference (RNAi). This led to a reduction, but not a complete knockout, of the Hh gradient, suggesting that exosomes may be an important part of maintaining the gradient in conjunction with cytonemes.

Wingless (Wg) is expressed along the dorsal-ventral boundary of wing imaginal discs and then spreads throughout the prospective wing pouch where it controls patterning and growth (Strigini and Cohen 2000). Greco et al. (2001) proposed that Wg could be packaged into vesicles to facilitate the spread throughout the wing pouch because GFP expressed in Wg secreting cells was found to colocalize with Wg in receiving cells. Subsequently, Beckett et al. (2013) showed that Wg was located in exosomes in the extracellular space of *Drosophila* embryos using

immunoelectron microscopy. To further confirm this, Wg was fractionated and found to cofraction with Flo2, an exosomal protein. Loss of Flo2 led to a decrease in the range of the Wg gradient, suggesting that they are involved in the gradient, but were not the only component responsible for maintaining this gradient. The researchers also determined that exosomal proteins were not required for Wg secretion, like with the Hh gradient, which gives more evidence to support the idea that exosomes are only responsible for transport of Wg through the gradient, but not solely responsible for establishing the gradient.

4.2.2 Host-pathogen interactions

Exosomes have been shown previously to be important in disease, including cancers, but little is known about the role of exosomes in disease transmission in insects. In *Leishmania* infections, it has been shown using the J774 mouse macrophage cell line that infected cells release exosomes and other vesicles that contain large amounts of pathogen molecules that can induce changes in non-infected cells to make them more susceptible to infection (Hassani and Olivier 2013). Taking this, Atayde et al. (2015) investigated the role of exosomes in creating an infection within the sandfly host and potentially spreading the infection. This group found that the *Leishmania* parasites were able to secrete exosomes in the sand fly midgut. They also determined that these exosomes are passed with the parasite during an infected sand fly bite and can help the parasite infect the host.

Similarly, Vora et al. (2018) looked at exosomes in the salivary glands of mosquitoes to determine if they could potentially play a role in dengue virus (DENV) transmission. Isolated salivary exosomes contained DENV particles which suggest that they may play a role in virus transmission to the host. Knowing this, it is possible that other pathogens may also be spread via

exosomes or in relation with exosomes as the salivary glands are important to pathogen transmission.

4.2.3. RNAi

RNAi is a post-transcriptional gene silencing mechanism that was first discovered in the nematode *Caenorhabditis elegans* (Fire et al. 1998). This is a highly conserved pathway found across eukaryotic organisms that is believed to have initially evolved as an innate antiviral immune response (Swevers et al. 2013, Leggewie and Schnettler 2018, Zhu and Palli 2020). Researchers have adopted this process to suppress transcript levels of specific genes to determine gene functions, regulation, and interactions (Clemens et al. 2000, Saleh et al. 2006, Belting and Wittrup 2008, Nandety et al. 2015, Suzuki et al. 2017). In addition, RNAi is now in development as a tool for therapeutic and agricultural purposes, including for controlling insect pests (Baum et al. 2007, Price and Gatehouse 2008, Zhu and Palli 2020).

RNAi responses are induced by the presence of double-stranded RNAs (dsRNAs). These RNAs are then recognized and cleaved by Dicer into small interfering RNAs (siRNAs) (Zhu and Palli 2020). These are then bound to argonaute in the RNA Induced Silencing Complex (RISC). Once loaded into the RISC, the bound guide strand is used to bind and degrade complementary mRNAs resulting in the degradation of the complementary strand (Agrawal et al. 2003, Meister 2013).

To induce an RNAi response, dsRNA is typically either fed to or injected into insects, though for the purposes of agricultural pest control. Once introduced, cellular uptake and intracellular transport of dsRNAs are vital to initiate an RNAi response and to spread the RNAi response systemically throughout the organism. In insects, it is believed that dsRNA is taken into the cell through clathrin-mediated endocytosis, though there is evidence of different uptake

mechanisms in some coleopterans (Xiao et al. 2015, Cappelle et al. 2016, Pinheiro et al. 2019, Cooper et al. 2019). Once inside the cell, the core RNAi machinery recognizes the dsRNA and uses it as a guide to find and degrade complementary RNAs. This local response is sufficient to suppress the transcript levels of the target gene in the cell that took up the dsRNA, but export of the RNAi response is necessary to achieve the systemic responses observed in some insect species (Zhu and Palli 2020).

Until recently, the mechanisms permitting systemic RNAi responses in insects were unknown, and it was not clear if it was an active process or what form of RNA spread the systemic signal. Exosomes have been shown to carry systemic responses within Colorado potato beetle (CPB) and in red flour beetle (RFB) (Mingels et al. 2020, Yoon et al. 2020). In the TcA cell line from RFB, exosomes carry siRNAs (20-23 nt) that matched the dsRNA introduced into the cells (Mingels et al. 2020). Interestingly, researchers were also able to show that these exosomes were able to enter other cells, as well as spread the RNAi response to these non-treated cells. In the CPB cell line LD-S1, Yoon et al. (2020) showed that exosomes isolated from dsRNA treated cells contained various lengths of dsRNA. The researchers were also able to determine that dsRNA treated cells were able to trigger an RNAi response. These studies suggest that exosomes are important players in the spreading of systemic RNAi responses.

5. Applications

With the understanding of the roles of exosomes within organisms increasing, there are a lot of potential uses of these vesicles in both mammalian and insect applications. The main ones that will be discussed further here are the potential of exosomes as carriers for therapeutic purposes and disease markers, and for increasing RNAi responses in refractory insects.

5.1. Biomedical Research

Exosomes have recently been targeted as therapeutic delivery vehicles for important diseases. One of the first studies showing that exogenous RNA can be transported and delivered by exosomes used mice models for potential human applications. Because the brain is difficult to send delivery vectors and treatments to, exosomes seemed like a very promising carrier to be able to cross the blood brain barrier (BBB) to potentially treat disorders. As discussed briefly earlier in the paper, a peptide from the rabies genome was shown to be transferred in exosomes (Alvarez-Erviti et al. 2011, El Andaloussi et al. 2013). The exosomes containing the peptide of interest were isolated and injected into the tails of mice to test if the response could go from the posterior end of the animal all the way to the brain. Specific knockdown in regions of the mouse brain corresponding to the peptide was observed, suggesting that exosomes are capable of crossing the BBB and being taken up by cells. Importantly, little or no toxicity or immunogenicity was noted even after multiple treatments, which suggests that this could be a useful new delivery system for crossing the BBB.

Other studies also confirmed the ability of exosomes to transport exogenous biological materials. Zhang et al. (2010) determined that monocytes transfected with miR-150 could deliver this miRNA to the recipient cell and cause a biological response. Using hepatic cells transduced with vectors expressing siRNA and miRNAs, research showed that the cells were able to secrete exosomes and mediate an RNAi response in the recipient cells (Pan et al. 2012). All of these studies show great potential in creating exosomes that carry exogenous cargoes and using them in a variety of roles in order to treat complex diseases or create a specific response that can then potentially be used to study currently known physiological functions.

Additionally, there are now cancer diagnostics on the market that utilize exosomes circulating in blood (Sheridan 2016). In the ExoDx *Lung(ALK)*, exosomes are taken from a blood sample and RNA is harvested from them. This exosomal RNA is used to detect five different mutations that are associated with non-small cell lung cancer. There is also evidence that this same type of technology can be developed to detect pancreatic cancer (Melo et al. 2015). In this study of 250 patients, glypican-1 was found in circulating exosomes in all patients suffering from both early- and late-stage pancreatic cancer. There is further evidence that similar technologies may be useful in detection of breast cancer (Rupp et al. 2011, Moon et al. 2016, Liu et al. 2018), prostate cancer (Khan et al. 2012), ovarian cancer (Zhao et al. 2016), urinary cancer (Smalley et al. 2008), and potentially many others (Soung et al. 2017).

5.2. Insect Applications - RNAi

As noted above, RNAi is under development for use in insect control strategies (Baum 2007, Head 2017, Silver et al 2021). Unfortunately, insects vary greatly in the efficiency of their RNAi responses following exposure to dsRNA. For example, coleopterans typically show a robust response regardless of delivery method utilized (Bodermann et al. 2012, Powell et al. 2017, Prentice et al. 2017), while lepidopterans are generally refractory to RNAi no matter the delivery method (Terenius et al. 2011, Shukla et al. 2016, Cooper et al. 2019). In addition, some insects vary in their responses depending on the delivery method. In locusts there is this variation as microinjections will produce an efficient response, but feeding dsRNA does not induce an effective RNAi response (Luo et al. 2013, Song et al. 2019, Cooper et al. 2019).

There are many proposed ideas as to why there are such differences in RNAi efficiency including stability of dsRNA, cellular uptake, RNAi core machinery, gene target selection, and impaired systemic spread (Cooper et al. 2019, Zhu and Palli 2020). DsRNA is susceptible to

degradation by a variety of nucleases that have been found within the gut, hemolymph, and salivary glands (Arimatsu et al. 2007, Christiaens et al. 2014, Wynant et al. 2014, Wang et al. 2016, Song et al. 2017, Singh et al. 2017). These nucleases degrade the DNA and prevent it from entering cells and affecting transcript levels. The highest nuclease activities have been found in lepidopterans and hemipterans, which may be a major factor in why these groups are or can be refractory to oral transmission (Wang et al. 2016, Singh et al. 2017).

In addition to dsRNA stability, dsRNA uptake, differences in expression or activity of core RNAi machinery, selection of target genes, and impaired systemic spread of the RNAi signal can also limit the efficiency of RNAi responses in insects. There are many differences in dsRNA uptake noted in insects that have been reviewed previously and include differing responses to sizes of dsRNA or siRNA, as well as different responses to dsRNA introduction methods (Cooper et al. 2019). The core RNAi machinery gene copy numbers differ between insects which could also explain efficiency differences. Some have multiple copies of key enzymes while other insects are missing some components completely (Dowling et al. 2016). Additionally, there are differences in the expression of these key components between insects and life stages (Cooper et al. 2019). Target gene selection can be complicated in insects as there are wide variations in silencing ability of genes (Terenius et al. 2011, Silver et al. 2021). The systemic spread of an RNAi response is crucial for creating effective insect pest management. Despite this, there have been no amplification mechanisms discovered in insects and the signal spreading mechanics are not well understood (Cooper et al. 2019).

With so many things limiting RNAi efficiency in insects, it is important to find ways to try to overcome this. Because differences in response to a target gene can vary widely across insects and tissues, selecting an effective target gene is crucial to RNAi. Through screening of

multiple potential targets, the most efficient target can be determined (Silver et al. 2021). Additionally, encapsulating dsRNA or using nanoparticles bound to dsRNA can increase the dsRNA stability to increase efficiency (Zhang et al. 2010, Das et al. 2015, Cooper et al. 2020, Zhu and Palli 2020, Silver et al. 2021). Lipid encapsulation has also been shown to increase cellular uptake of dsRNA (Gurusamy et al. 2020). These work by either encapsulating dsRNA or binding it to a polymer based on opposing charges and then feeding or injecting into the insect. Genetic engineering of plants and microbes is potentially the most field applicable method to increase RNAi efficiency. Currently, there is a transgenic corn cultivar that produces dsRNA targeting western corn rootworm (Head et al. 2017). In addition to transgenic plants, there is work using viruses, fungi, and bacteria to produce dsRNAs and can be fed to insects for control (Silver et al. 2021). Despite all the promise that these methods show, no control method is effective against all insect species.

As noted above, exosomes play roles in systemic RNAi responses, and data suggests that their physiological attributes may also be exploited to enhance RNAi efficiency. Exosomes provide protection from enzymatic degradation to systemic RNAi signals, one of the main mechanisms that limits RNAi efficiency. Exosomes from *T. castaneum* cells that were treated with the nuclease RNase A were still able to carry a silencing response in TcA cells (Mingels et al. 2020). Similarly, nuclease protection was shown by Yoon et al. (2020) where exosomes containing dsGFP from Colorado potato beetle cells were treated with RNaseIII. In this experiment, dsRNA in exosomes was protected from degradation while naked dsRNA was not. In addition, exosomes may also provide methods for overcoming limited dsRNA uptake in insects. Previous studies suggest that dsRNA uptake relies on clathrin-mediated endocytosis to enter cells (Cappelle et al. 2016, Cooper et al. 2019), but exosomes may utilize other methods to

enter insect cells, including by direct fusion to the plasma membrane or phagocytosis as was discussed in an earlier section (Mulcahy et al. 2014, Zhang et al. 2015).

6. Conclusion

Exosomes play important roles in many biological functions across organisms, though more is known about exosomes in mammals than in insects. In mammals, exosomes are formed alongside other vesicles within MVBs (Gruenberg and van der Goot 2006, Trajkovic et al. 2008, Simons and Raposo 2009, Lakhal and Wood 2011, Lawson et al. 2016). ESCRT proteins, as well as their associated proteins, are important for biosynthesis, cargo loading, and export of exosomes (Baietti et al. 2012, Kowal et al. 2014, Beer and Wehman 2017). Rab GTPases (Savina et al. 2005, Hsu et al. 2010, Ostrowski et al. 2010, Baietti et al. 2012, Kowal et al. 2014, Beer and Wehman 2017) and SNARE (Gross et al. 2012, Kowal et al. 2014, Beer and Wehman et al. 2017) proteins are involved in docking of the MVB to the plasma membrane and export of exosomes from the cell. The limited data on biosynthesis and export in insects suggests that there are similar mechanisms in mammalian systems, but further studies are needed to determine whether the mechanisms of biosynthesis, cargo loading, and export of exosomes in insects are similar to the currently understood mammalian systems.

The importance of exosomes lies in their biological functions within organisms. In mammals, they are involved in intracellular communication. In mammals, the best understood examples are in the immune system and can work in both antigen presentation, as well as in promoting an immune response (Thery et al. 2002, Admyre et al. 2006, Bhatnagar and Schorey 2007, Bhatnagar et al. 2007, Beckett et al. 2013). In insects, the currently understood roles are in mating and development (Beckett et al. 2013, Corrigan et al. 2014, Gradilla et al. 2014) and in host-pathogen interactions or viral transmission to a new host (Atayde et al. 2015, Vora et al.

2018). More information on the roles of exosomes in insects are necessary, however, which will provide us with a greater understanding not only of the functions of exosomes in insects, but also the diversity of cargoes that exosomes carry in these organisms. At the same time, this information will lead to a better understanding of physiological processes within insects and may identify strategies to exploit these processes for insect pest control.

Importantly, there are many potential applications of exosomes in both biomedical research and in entomology. In mammalian systems, exosomes are being investigated and utilized as potential biomarkers for diseases such as cancer (Smalley et al. 2008, Rupp et al. 2011, Khan et al. 2012, Melo et al. 2015, Sheridan 2016, Moon et al. 2016, Zhao et al. 2016, Liu et al. 2018). Additionally, exosomes are being targeted for therapeutic purposes in disease as they are able to carry cargoes to specifically targeted cells and are able to cross the BBB (Zhang et al. 2010, Pan et al. 2012, Alvarez-Erviti et al. 2011, El Andaloussi et al. 2013). Knowing how important exosomes are to not only mammalian physiological processes, but also that they can be targeted and used for beneficial purposes like targeted therapeutics shows a potential for insect exosomes to be manipulated and designed for different purposes.

With more understanding of exosomal roles in insects, exosomes may be targeted for pest control purposes by carrying lethal cargoes to important organs within the insect. Additionally, further understanding how exosomes can carry systemic RNAi signals throughout an insect may lead to improved RNAi pest control products, but this could also be utilized in the laboratory for gene function studies. Targeted exosomal RNAi in earlier insect life stages may lead to the knockdown of transcript levels in the adults necessary to characterize gene functions that so far have not been able to be determined. Insect exosomes could be a large area of currently untapped potential that we are only now really understanding.

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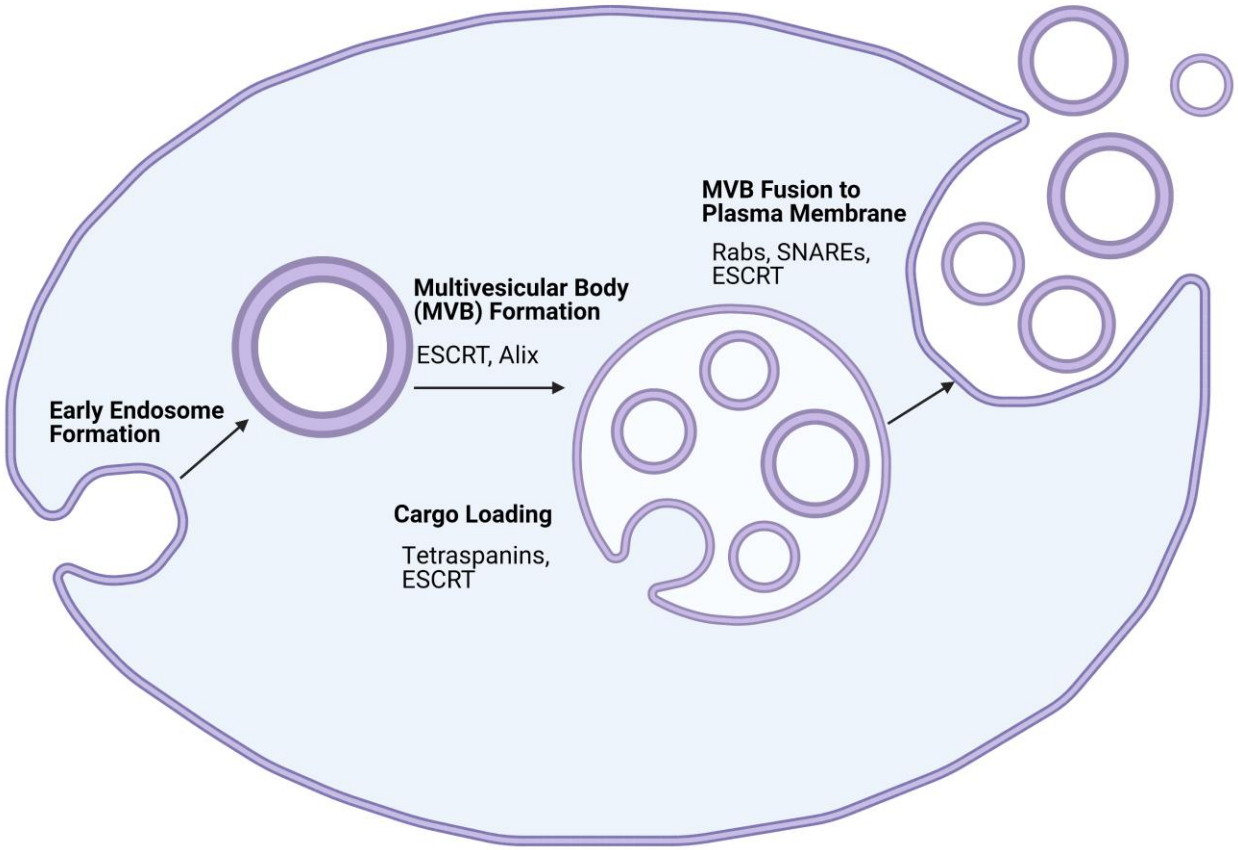

Figure 1.1. Exosome biosynthesis pathway and key proteins involved. Once the endosome is formed in the cytoplasm, various ESCRT proteins and Alix are responsible for the budding of new vesicles into the created MVB. During this period of budding, cargoes are loaded into exosomes and other vesicles. This process of cargo loading is controlled by tetraspanins and ESCRT proteins. One the MVB matures, there is fusion to the plasma membrane and release the vesicular contents. Fusion to the plasma membrane involves ESCRT, SNARE, and Rab proteins. Created with BioRender.com.

Chapter 2 - Characterizing Small RNA Cargoes of *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae) Exosomes

Introduction

Exosomes are extracellular messenger vesicles around 30-100 nanometers in size that are involved in many biological processes including anti-tumor activities, immunity, and development (Thery et al. 2009, van den Boorn et al. 2013). Because of this, they have become a growing area of biomedical research as potential therapeutic delivery vehicles to treat diseases (Alvarez-Erviti et al. 2011, El Andaloussi et al. 2013, Sheridan 2016) and as diagnostic tools for early detection of cancers (Smalley et al. 2008, Khan et al. 2012, Rupp et al. 2011, Melo et al. 2015, Moon et al. 2016, Zhao et al. 2016, Liu et al. 2018).

In mammals, exosome cargoes are loaded during the process of biosynthesis through recognition and recruitment by various means. ESCRT proteins and tetraspanins are responsible for loading protein and mRNA cargoes (van Niel et al. 2006, Simons and Raposo 2009, Trajkovic et al. 2008, van Niel et al. 2011, Vlassov et al. 2012, Kanemoto et al. 2016). miRNAs are also important cargoes in exosomes, but there are multiple potential mechanisms for loading these cargoes into exosomes (Zhang et al. 2015). These mechanisms include the neutral sphingomyelinase 2 (nSMase2)-dependent pathway which seems to regulate how many miRNAs are loaded into exosomes (Kosaka et al. 2013), the miRNA motif and sumoylated heterogenous nuclear ribonucleoproteins (hnRNPs)-dependent pathway where hnRNPs recognize specific motifs on the 3’ end of miRNAs (Villarroya-Beltri et al. 2013), the 3’-end of the miRNA sequence-dependent pathway in which the 3’ end of miRNAs contains a signal for sorting into exosomes (Koppers-Lalic et al. 2014), and finally the miRNA-induced silencing complex

(miRISC)-related pathway in which miRISC components such as Argonaute 2 assist in miRNA loading (Frank et al. 2010).

miRNAs are significant cargoes within exosomes and are important in cellular communication (Valadi et al. 2007, Vlassov et al. 2012, Just et al. 2020). They are utilized for post-transcriptional gene silencing of target mRNAs in cells (Bartel 2004, Zhang et al. 2015). Because exosomes can carry multiple miRNAs as cargoes and the roles of miRNAs in the target cells, there can be rapid changes in gene expression of the specific target cells (Vlassov et al. 2012). Interestingly, the miRNA cargoes of exosomes can either have similar compositions of RNAs and proteins to those of the originating cell (Taylor and Gercel-Taylor 2008, Rabinowits et al. 2009) or they can differ significantly from the originating cell (Zomer et al. 2010, Mittelbrunn et al. 2011, Zhang et al. 2015). Also, the composition and proportions of miRNA cargoes within exosomes can change based on the physiological condition of the originating cell (Skog et al. 2008, Gudrich-Fuchs et al. 2012). This suggests that exosome-based signaling is adaptable to different conditions and may be important for inducing specific responses in target cells.

In insects, much less is known about exosomes and what roles they may play in biological activities. In *Drosophila*, exosomes have been implicated in intercellular communication and establishing tissue polarity within developing embryos (Liegeois 2006, van den Boorn et al. 2013), in maintaining the Hedgehog gradient within the wing imaginal disc (Gradilla et al. 2014), and in spreading Wingless throughout the developing wing pouch (Greco et al. 2001, Beckett et al. 2013). Additionally, exosomes may play a role in host-pathogen interactions within insects. *Leishmania* parasites are secreted in exosomes in the infected sand fly midgut and exosomes are passed with the parasites during an infected sand fly bite (Atayde et al.

2015). Additionally, dengue viral particles were found in exosomes from the salivary glands of mosquitoes, suggesting a potential role in viral transmission to human hosts (Vora et al. 2018).

In addition to these roles, recent evidence suggests that exosomes may be important in systemic RNA interference (RNAi) responses within insects. Exosomes from cultured Colorado potato beetle (*Leptinotarsa decemlineata*) or red flour beetle (*Tribolium castaneum*) cells treated with dsRNA were able to induce RNAi responses in naïve cells (Mingels et al. 2020, Yoon et al. 2020). In addition, treatment with RNases had no effect on the ability of the exosomes to carry the RNAi signal, indicating that the exosomes provided protection from degradation by RNases.

Currently, there is little known about exosome cargoes and other roles that they may play within insects. Accordingly, this project characterized miRNAs from exosomes harvested from Du182a cells, a *Diabrotica undecimpunctata* (SCR) embryonic cell line (Lynn and Stoppleworth 1984), before or after treatment with double-stranded RNA (dsRNA). Small RNAseq was conducted on RNAs isolated from exosomes and miRNA sequences were identified and compared to those previously identified in *T. castaneum* (Herndon et al. 2020). Our analysis identified miRNA sequences that were consistent with those previously identified, but also revealed numerous new miRNAs associated with the Du182a cell line. Our analysis enhances our understanding of the cargoes of insect exosomes under normal conditions and demonstrates that they change and after treatment with dsRNA.

Materials and Methods

Exosomal RNA Harvest

Du182a cells (Lynn and Stoppleworth 1984) were acquired from the USDA-ARS Biological Control of Insects Research Laboratory (BCIRL) and grown in Ex-Cell 420 media

(Sigma-Aldrich, St. Louis, MO) with 5% fetal bovine serum. For exosome harvest, SCR cells were seeded onto 10 cm tissue culture treated plates and grown to 75% confluency. Complete growth media was removed from the plates and cells were washed with serum-free media and either treated with 1μg/mL of dsEGFP (~240 bp) in serum free media or with serum free media alone (control). This was done for three replicates per treatment for a total of 6 samples (G1-3, Un1-3). dsEGFP was designed for the 200-500 nt region of the 733 nt *EGFP* sequence. The dsEGFP was synthesized using the forward primer taatacgactcactatagggTGACCACCCTGACCTAC and the reverse primer TTGATGCCGTTCTTCTGC. The lower-case letters indicate the T7 polymerase initiation sequence on the 5' end. After 48 h, media was removed from the plates and exosomes were isolated with Total Exosome Isolation Reagent (Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Briefly, conditioned media was centrifuged at 2,000 g for 30 min at 4°C, the supernatant was collected, and one-half the volume of Total Exosome Isolation Reagent was added to the conditioned media. After mixing, the media plus reagent was stored at 4°C overnight, then centrifuged at 10,000 g for 1 h at 4°C. Total RNA was isolated from exosomes using Trizol Reagent (Fisher Scientific) according to the manufacturer's instructions and eluted in nuclease-free water.

Small RNA Library Generation

Small RNA library preparation was performed at the University of Kansas Medical Center – Genomics Core (Kansas City, KS). Fifty to 70 ng of total RNA was used in the TruSeq Small RNA library preparation protocol (Illumina #RS200-0012, San Diego, CA). Total RNA was ligated with 3' and 5' RNA adapters followed by a reverse transcription reaction and a 15

cycle PCR amplification which incorporated the Small RNA TruSeq index adaptor. Size selection of cDNA library constructs was conducted using 3% marker F gel cassettes – dye free with internal standards on the Pippin Prep size fractionation system (Sage Science, Beverley, MA) using a 125 – 160 bp size capture. Size captured library constructs were purified using the Qiagen MinElute PCR Purification kit (Qiagen cat#28004, Germantown, MD). An Agilent TapeStation 4200 was used with the High Sensitivity DNA1000 ScreenTape assay (Agilent #5067-5584, Santa Clara, CA) to validate the purified libraries. Following Agilent TapeStation QC of the library preparation and library quantification using the Roche Lightcycler96 with FastStart Essential DNA Green Master Mix (Roche – cat#06402712001, Indianapolis, IN), the RNA-Seq libraries were adjusted to a 2 nM concentration and pooled for multiplexed sequencing. Libraries were denatured and diluted to a concentration of 6 pM (based on qPCR results) followed by clonal clustering on an Illumina MiSeq using a MiSeq v3 reagent kit (Illumina MS-102-3001) with a 1x50 bp cycle sequencing profile with a single index read. Following collection, sequence data was converted from .bcl file format to fastq files and de-multiplexed into individual sample sequence data sets for further downstream analysis.

miRNA analysis

Untrimmed sequencing data was uploaded to the online data analysis platform Galaxy (http://usegalaxy.org/). FastQC (Babraham Bioinfromatics) was run on all samples to get the baseline quality control information. To prepare the sequencing data for further analysis, the sequences were run through the Galaxy tool FASTQ Groomer to correct the formatting. Sequences were then trimmed using Trim Galore (Babraham Bioinformatics) to remove Illumina adapters and low-quality sequences. Low quality sequences were defined as any reads below the

minimum quality phred score of 30. The sequencing data from the exosomal RNAs of dsEGFP treated cells were also mapped against the *EGFP* sequence using Bowtie2 in DNA Galaxy. Mapped sequences were viewed on IGV.

The trimmed RNAseq data were then uploaded to the online miRNA platform Chimira (EMBL-EBI, https://www.ebi.ac.uk/research/enright/software/chimira) which maps miRNAs against known miRNAs from miRbase (Vitsios and Enright 2015). We used a model beetle system, *Tribolium castaneum*, for mapping because it is well characterized. The data was also uploaded to mirnovo to predict potential novel SCR miRNA sequences (Vitsios et al. 2017). Mirnovo analysis was done using the universal animal prediction model with no input species. The results generated from mirnovo were tested against *T. castaneum* miRNAs in miRbase (Kozomara et al. 2019). The miRNAs generated were also examined for potential miRNA isoforms (isomiRNAs) that varied in length or had SNPs within the sequences. Additionally, the 5' and 3' ends of the miRNA sequences were analyzed to determine if any specific sequences were targeted for cutting in our miRNA sequences.

Naming SCR miRNA

miRNAs from both untreated and dsEGFP samples were compiled together and listed in order of sequencing depth, starting with the most abundant miRNA found throughout the samples. Samples that were homologous to *T. castaneum* sequences were named based on the existing miRNA sequence name in that species. Unique sequences were named in descending order, starting with Dun-miR-3 for *Diabrotica undecimpunctata*-miRNA-3, as Dun-miR-1 and Dun-miR-2 were homologous to *T. castaneum*. IsomiRNAs were characterized as having a single nucleotide polymorphism (SNP) within the sequence and/or having up to a 9 nt addition or

deletion compared to the most abundant miRNA of that type. All isomiRNAs found were named using the number from the most abundant isomiRNA of that type, followed by a letter (Dun-miR-3a, Dun-miR-3b, etc.).

Results

Small RNA Sequencing and Analysis

From the Small RNAseq run, we were able to collect 12,361,293 reads from the dsEGFP treated samples and 10,916,383 reads from the untreated samples. Of these reads, 11,415,354 reads from dsEGFP and 10,166,399 reads from the untreated samples were above the cutoff quality phred score of 30 and were used for further analysis. These reads all ranged from 17-50 nt in length. Figure 2.1 displays the sequence length distribution of unique exosomal RNAs from exosomes harvested from untreated cells (A) and dsEGFP treated cells (B), respectively. These RNAs have multimodal distributions with the main peak in both groups at 28 nt, and two minor peaks at 21-22 nt and 32 nt.

miRNA Analysis

To analyze miRNAs contained in Du182a exosomes, sequences from total RNA samples were uploaded to Chimira to determine if miRNAs matched miRNAs from the model beetle *T. castaneum*. This analysis gave 270 RNA sequences that aligned to known *T. castaneum* miRNAs. Figure 2.2 shows the percent frequency of the 13 most abundant miRNA sequences, all with over 10,000 hits from this data. The most abundant miRNA, Tca-miR-279e-3p, had 669,313 RNAs, or 55%, from alignment of the exosmal RNA sequences.

Unique miRNA sequences were then predicted from our data using mirnovo. There was a total of 616 miRNAs predicted from the untreated samples and 557 from the dsEGFP treated samples. From these samples, there were 912 unique miRNA samples overall. Figure 2.3 shows the sequence length distribution for the mirnovo results from RNAs isolated from exosomes from untreated (A) and dsEGFP treated (B) cells, respectively. Both graphs have bimodal distributions of miRNAs with the first peak at 21-22 nt and the second peak at 28 nt.

miRNAs were compiled together and listed in order of abundance for naming. During this, potential isomiRNAs were discovered among the sequences. IsomiRNAs were classified as sequences with up to 2 SNPs and/or up to 9 nt deletions or additions. With these identified, the 912 unique miRNA sequences between all the sequencing reactions were named in descending order of abundance and isomiRNAs were named based on the most abundant sequence. Tables 2.1 and A.1 and show the ten most abundant isomiRNA sequences and names, as well as the sequence lengths and the depth of the sequencing. In total, there were 812 unique miRNAs and 98 various isomiRNAs throughout the generated miRNAs.

Both ends of the miRNAs were analyzed for three nucleotide cut sequence motifs (Table 2.2, Table A.2). Table 2.2 shows the ten most abundant end motifs ordered by miRNA length and grouped into three nucleotide clusters. The most frequent 5’ end sequences started with T and contained at least one A in the sequence, though the most abundant sequence across all lengths was TTT. The most frequent 3’ end sequences throughout all miRNA lengths contained a C and/or a T nucleotide in the final two positions, with the most frequent motif being ACT across all lengths. Figure 2.4 visualizes the percentage of each nucleotide at each position at both the 5’ and 3’ cleavage sites.

Effects of dsRNA treatment on exosomal RNA composition

Small RNAs from the exosomes from untreated (A) and dsEGFP treated (B) cells were mapped against the *EGFP* sequence (accession number MN443913) using Bowtie2 to identify sequences that matched. Out of over 11 million reads from the dsEGFP derived analyzed reads, 3,005,070 reads aligned to the *EGFP* sequence at least one time. These reads were mainly from the area from which the dsEGFP was derived, but some did map to areas outside of this region. Most of these reads that aligned from the G3 replicate, with 2,498,973 reads (83.16% of the total reads) mapping to the EGFP sequence at least once. The small RNAs isolated from exosomes from the untreated control cells were also aligned to the *EGFP* sequence as a control. These reads consistently had less than 720 reads out of each sample, or 0.02% of the total reads, that aligned to the *EGFP* gene in both the target and non-target region.

With reads mapping to *EGFP* across the small RNA sequencing results, the further processed miRNA reads were then mapped with Bowtie 2 to the sense strand of *EGFP* (Figure 2.5). The antisense strand was also used with no differences noted in mapping. There were no sequences from the control mirnovo results that mapped to the *EGFP* sequence. Figure 2.6 shows the sequence length distribution of the RNAs that aligned to the gene. This data showed 61 unique predicted miRNAs had a single peak at 22 nt which corresponds to the typical miRNA size of 21-24 nt (Zhu and Palli 2020). The sequences that aligned ranged in size from 17-27 nt.

Discussion

Our goal with this work was to expand our understanding of the RNA cargoes carried by exosomes in insects. Previous research in mammalian systems suggest that exosomes carry RNA cargoes to other cells in the forms of mRNAs and small RNAs (Valadi et al. 2007, Skog et al.

2008, Vlassov et al. 2012, Just et al. 2020). Sequencing of the RNAs from SCR exosomes revealed a wide variety of small RNAs that conformed to a trimodal size distribution. A previous study in *T. castaneum* showed a similar size distribution of small RNAs with peaks at 21-22 and 28 nt (Wu et al. 2017). This may be a characteristic of insect small RNA populations but analysis of a wider array of small RNAs in insects is necessary to be sure.

Comparison of our sequencing results to the well-characterized model beetle *T. castaneum* revealed 270 matches to known *T. castaneum* miRNAs (Figure 2.2). The most abundant miRNA, Tca-miR-279e-3p, is known to be involved in a wide variety of processes in development and in adult insects (Sun et al. 2015, Ylla et al. 2017). This miRNA is critical for organismal viability, the normal cell specification of olfactory neurons, and in rhythmic behavior by acting on circadian pacemaker cells. Because it plays so many important roles in insects, it is not surprising that it is the most abundant miRNA found within these samples and highlights the important roles that insect exosomes may play in intercellular communication.

The functions of most of the other most abundant miRNAs that matched to the *T. castaneum* database are largely uncharacterized (Singh and Nagaraju 2008). Those that are characterized have mainly been determined based on gene ontology and not through functional studies. These miRNAs include Tca-miR-184-3p which is involved in cyclin-dependent protein kinase regulator activity, Tca-miR-276-3p which is involved in zinc ion binding and protein kinase activity, Tca-miR-275 which is involved in structure of the cytoskeleton, Tca-miR-71-3p which is involved in RNA polymerase II transcription mediator activity, Tca-let-7-5p which has translation elongation factor activity, Tca-bantam-3p which is involved in cell proliferation as well as regulates apoptosis, and Tca-miR-12-5p which has protein membrane transporter activity (Brennecke et al. 2003, Singh and Nagaraju 2008). Functional studies are still necessary to determine if these

miRNAs function as their ontology suggests or if they have other functions that have yet to be determined.

An array of potential isomiRNAs having up to 2 SNPs and/or up to 9 nt deletions or additions were identified from our sequencing results. Many of the isomiRNAs matched portions of known *T. castaneum* miRNA sequences, suggesting that they are involved in similar activities as the main miRNA. Three of the unique isomiRNAs matched to the multifunctional Tca-miR-279-3p and are likely important for a variety of processes in SCR cells. Additionally, isomiRNAs matching Tca-bantam-3p, Tca-miR-71-3p, and Tca-miR-276-5p were found in the SCR analysis. These are likely to be involved in the same processes that the main miRNAs are which include cell proliferation, RNA polymerase II activity, zinc ion binding, and protein kinase activity but further investigation is required to determine this. Other SCR isomiRNAs matched other *T. castaneum* sequences, but the activities of these are currently uncharacterized. Those that did not match known *T. castaneum* sequences, as well as those that did match sequences but are currently uncharacterized, require future functional studies to determine what roles they play in both *T. castaneum* and SCR systems.

Previous studies have looked at preferences of 5’ and 3’ miRNA cleavage sites in insects. Guan et al. (2018) noted that in Lepidopterans the sequence GGU is the conserved digestion site on both the 5’ and 3’ ends. In contrast, *T. castaneum* in the same study had more varied sequences on both ends, but that the second position contained mainly A an U. In this study, the same trend was there, but not as significantly at the 5’ end (Figure 2.4, Table 2.2). Here, the most common nucleotides in each position were A and T ranging from 16-61% at each site. At the 3’ end, there was a difference as the first position had mainly A and G (33% and 28% respectively), the second position was mainly C (43%), and the final position was mainly T (41%) and C

(32%). This is interesting as it seems that there is potential similarity in enzymes that process these small RNAs between *T. castaneum* and SCR due to the similarities in cut sites at the 5' end, but that these enzymes are not completely complementary due to differences at the 3' end. It is possible that the variations are due to different enzymes cutting the small RNAs that we sequenced, but the current work is not enough to draw any definitive conclusions on this and will require further investigation.

Previous studies have also indicated that the composition or proportion of small RNAs in exosomes may differ significantly from their originating cells (Zomer et al. 2010, Mittlebrunn et al. 2011, Zhang et al. 2015), whereas others found that the RNA content in exosomes were similar to the originating cells (Taylor and Gercel-Taylor 2008, Rabinowits et al. 2009). These apparently contrasting results may be the result of the use of different cell types in these experiments; however, the results also support the idea that exosomes and the different RNA cargoes that they carry are utilized in intercellular communication (Valadi et al. 2007, Skog et al. 2008, Vlassov et al. 2012, Zhang et al. 2015). Our experiments were not designed to investigate this aspect of exosome cargoes, however, comparison of exosome small RNA cargoes to the small RNA population of the SCR cells could provide insight into how RNA cargoes are loaded into exosomes in insects and whether this is a selective process as has been suggested by some in mammals.

The sequencing results also showed that exosome RNA cargoes changed in response to dsRNA treatment. After treatment with dsEGFP, small RNAs that mapped to the *EGFP* sequence were observed that were not seen in the control exosomes. Additionally, these RNA sequences mapped to the region of the *EGFP* sequence that corresponds to the dsRNA that was used for treatment. These results are important for two reasons. First, they indicate that exosome

RNA cargoes change according to the treatment of the cells from which they originate. Second, the presence of *EGFP* small RNA sequences in exosomes from cells treated with dsEGFP indicate that exosomes participate in systemic spread of RNA interference signals. These results are consistent with previous experiments where exosomes from dsRNA-treated *T. castaneum* and *Leptinotarsa decemlineata* induced an RNAi response in naïve cultured cells from each species (Mingels et al. 2020, Yoon et al. 2020). Future experiments are necessary to determine if exosomes also promote the spread of systemic RNAi signals *in vivo*.

Interestingly, most of the *EGFP* mapped reads in this study came from one of the sequencing replicates, G3. This could be due to a variety of reasons including variability in the response of the cells to the dsEGFP treatment between the replicates or problems with library preparation. Despite this, there were sequences that mapped in all three replicates which shows that all had some response to dsRNA treatment. Additionally, a very small proportion of reads from the untreated control group unexpectedly mapped to the *EGFP* sequence. After continuing down the analysis pipeline and focusing solely on the miRNA reads generated, these mapping sequences were lost. The initial mapping of the full small RNA reads may be due to areas of sequence similarities between the *EGFP* sequence and areas of the SCR genome captured in the sequencing results or could be the result of contamination of the control samples at some point during their preparation.

These preliminary results from cultured beetle cells are an important first step in enhancing our understanding of the RNA cargoes carried by exosomes. However, these results are generated from cultured cells from embryonic SCR, leaving some questions as to how relevant these results are to RNA cargoes in exosomes from whole SCR beetles or other insects. To validate the results from our *in vitro* model, our experiments should be extended to include

exosomes from different beetle tissues and life stages as well as other insect species. These additional future experiments will help identify conserved exosomal RNAs across insect species. Additionally, further experimentation with exosomes may reveal whether non-beetle insects use exosomes in RNAi responses and if exosomes could be a viable strategy for enhancing RNAi in refractory insects (Silver et al. 2021).

In addition, these additional experiments will help fill the gaps in our knowledge of the roles of exosomes in intercellular communication in insects. Previous studies show that exosomes are important in carrying cargoes in embryos in developing wing imaginal discs and helping determine tissue polarity (Greco et al. 2001, Liegeois 2006, Beckett et al. 2013, van den Boorn et al. 2013, Gradilla et al. 2014), as well as in delivering peptides in *Drosophila* during mating from the male to the female which causes her not to mate again and signaling for increases in egg laying (Chen et al. 1988, Chapman et al. 2003, Corrigan et al. 2014). Beyond this, there is little known about how exosomes function within insects so more studies will be required to determine their natural roles.

This study showed that exosomes isolated from the SCR cell line contained a variety of miRNAs. Many of these miRNAs mapped to the model beetle *T. castaneum* miRNAs and are involved in a variety of cellular processes, but there were many sequences that were unique to SCR. Additionally, small RNAs in exosomes from dsEGFP treated cells changed after treatment and were mapped to the *EGFP* sequence. These changes suggest that exosomes may play a role in the systemic RNAi responses and that exosomal RNA cargoes are responsive to external stimuli. More work is necessary in *in vivo* systems to confirm these results; however, these findings suggest that insect exosomes could have a significant impact in causing physiological changes in recipient cells as has been shown with mammalian exosomes.

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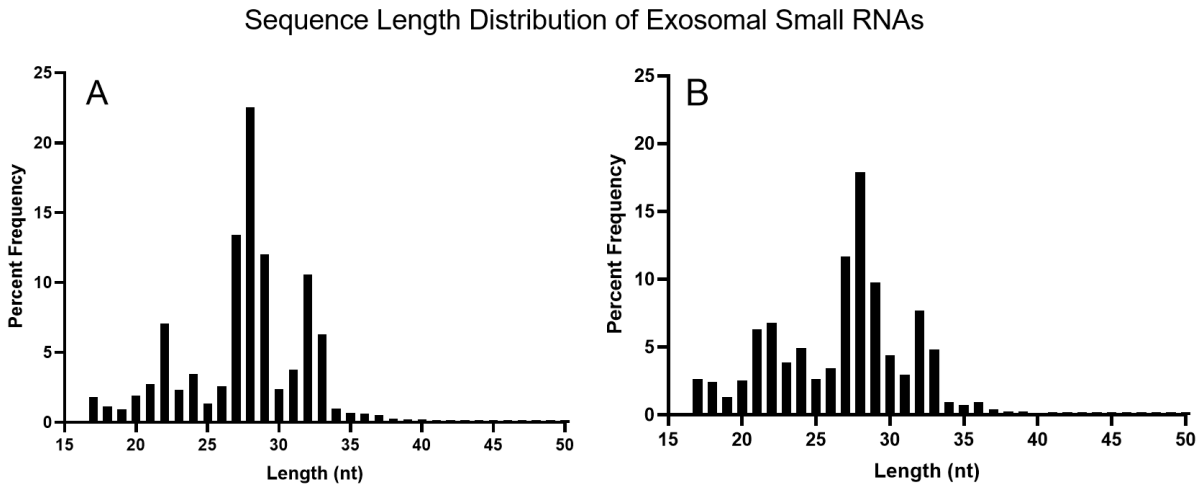

Figure 2.1. Sequence length distributions of exosomal small RNAs from untreated (A) and dsEGFP treated (B) cells. Both groups show a large peak at 28 nt *as well as* two smaller peaks at 21-22 nt and at 32-33 nt.

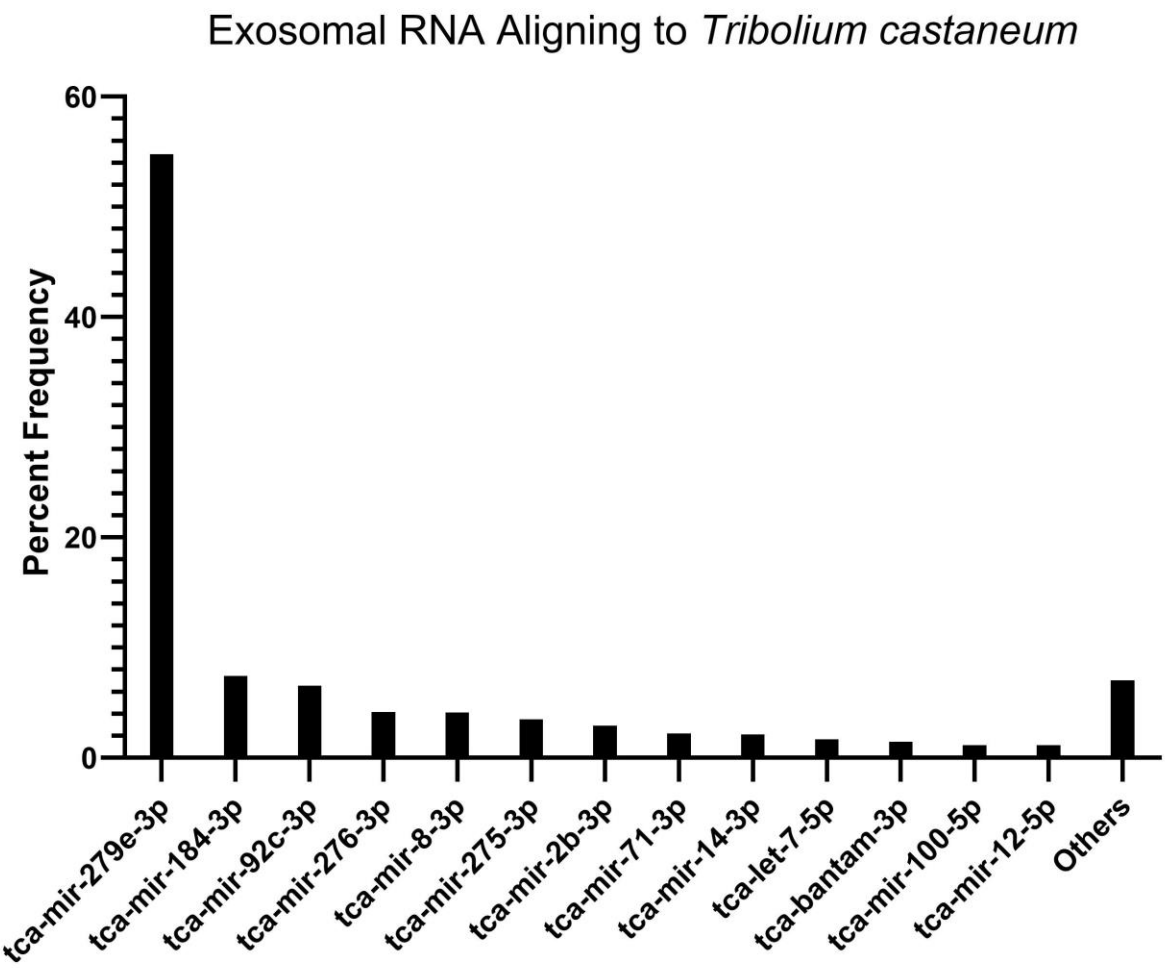

Figure 2.2. The abundance of small RNA reads from SCR exosomes that aligned to known *T. castaneum* miRNA sequences.

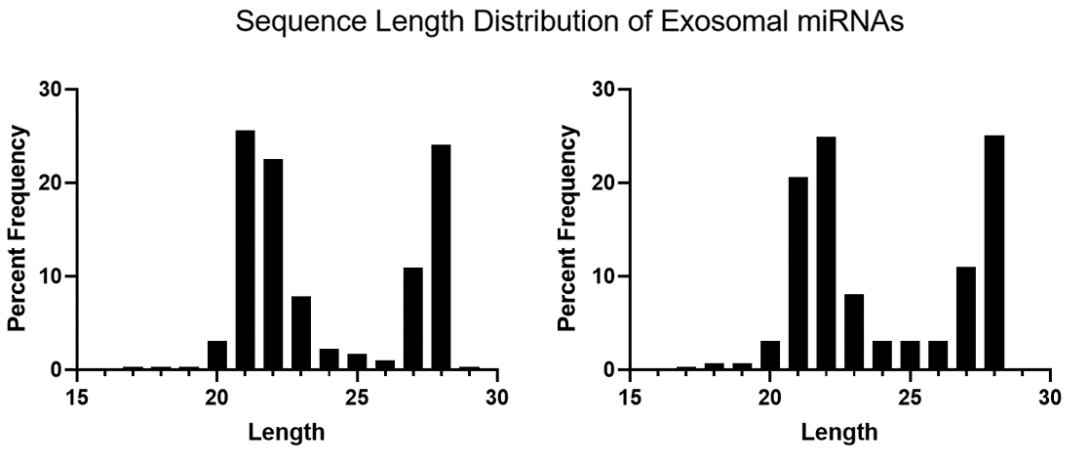

Figure 2.3. Sequence length distribution of unique miRNAs from exosomes from untreated (A) and dsEGFP treated (B) cells. There is a bimodal distribution in both data sets, with peaks at about 21-22 nt and at 28 nt.

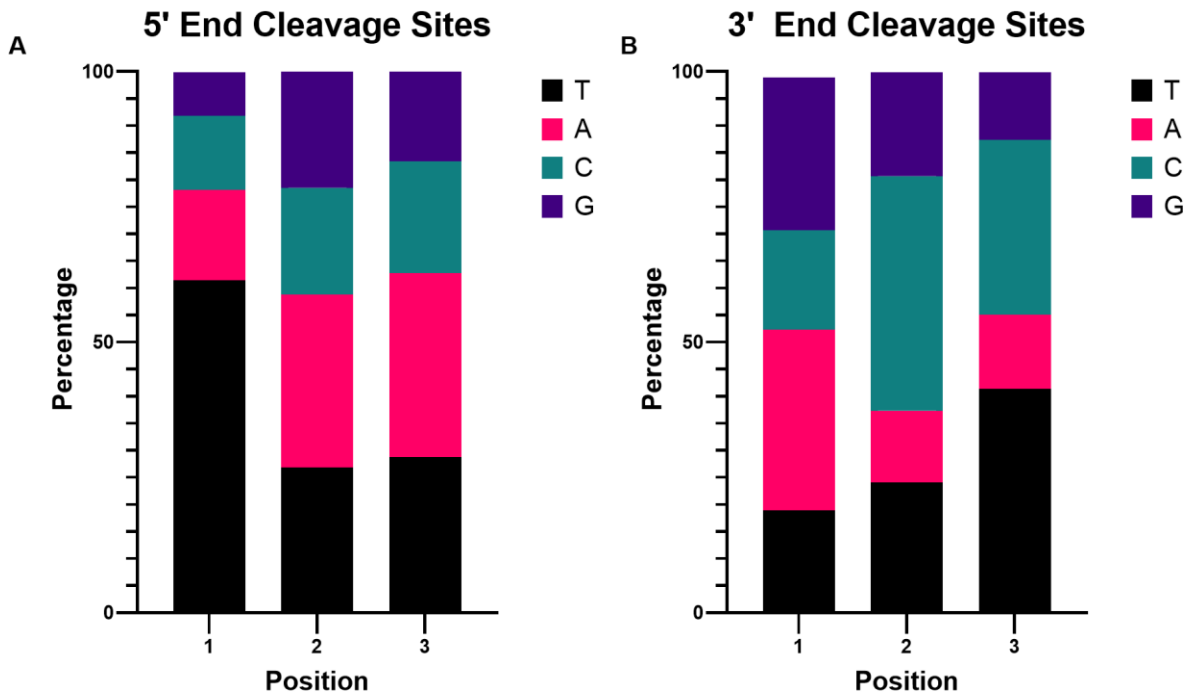

Figure 2.4. Percentage of each nucleotide at the (A) 5’ and (B) 3’ cleavage sites of the miRNA sequences.

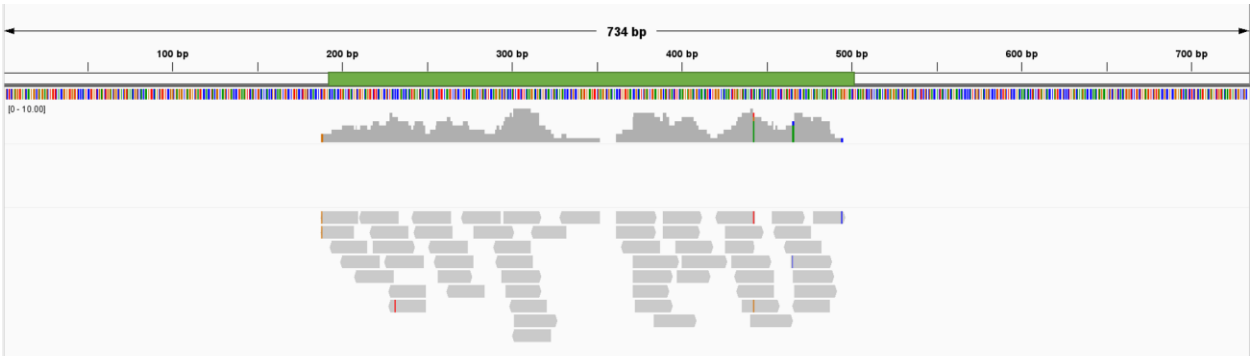

Figure 2.5. miRNA sequences from exosomes from dsEGFP treated cells aligned to the *EGFP* sequence. The green bar at the top is the area where the dsEGFP was designed.

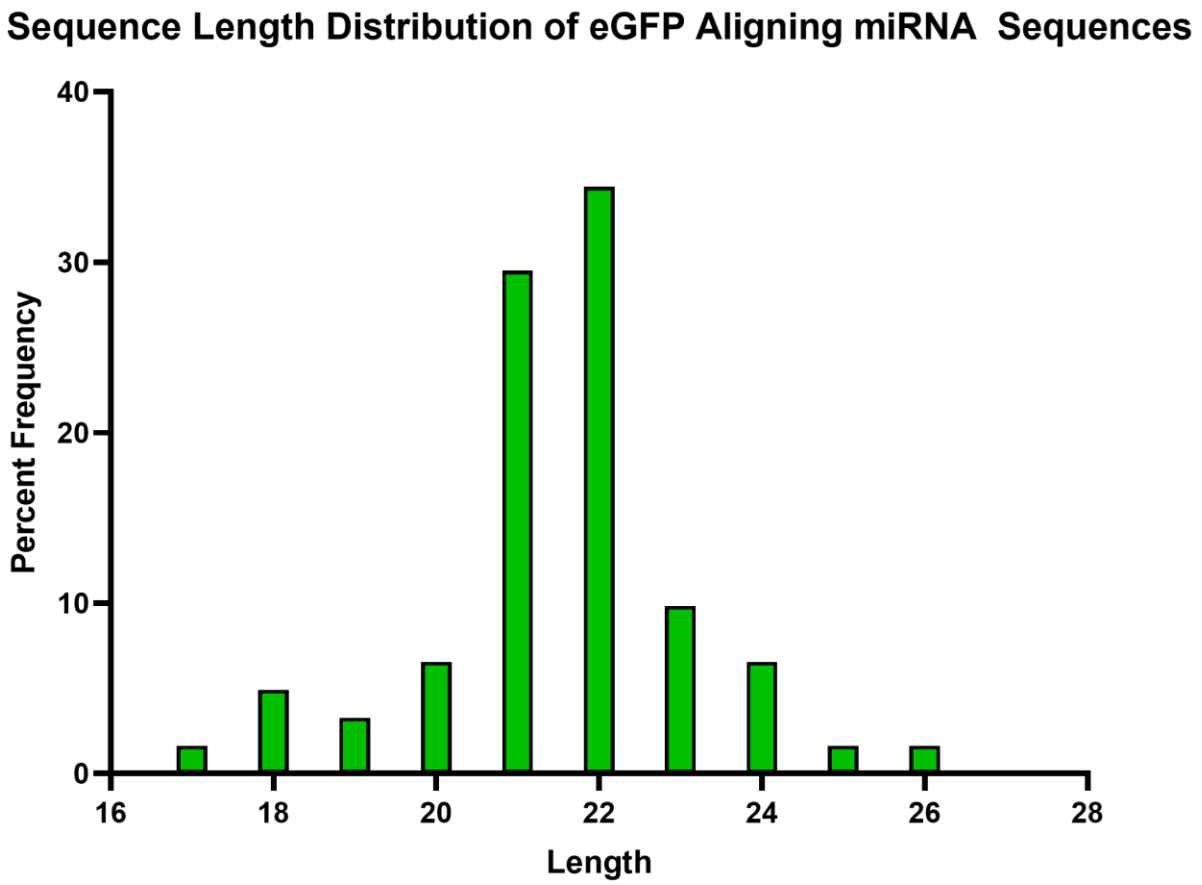

Figure 2.6. Sequence length distribution of unique RNAs isolated from exosomes from cells treated with dsEGFP that map to the *EGFP* sequence. This data shows a single peak at 21-22 nt which corresponds to the typical *si*RNA size.

Table 2.1. The ten most abundant miRNAs from SCR exosomes and their isomiRNAs.

Name	Iso-miR	Sequence	Length	T. castaneum Homology
Dun-miR-279	miR-279a	TGACTAGATGGAACACTCGCCT	22	Tca-miR-279e-3p
	miR-279b	TGACTAGATGGAACACTCTCCT	22	Tca-miR-279e-3p
	miR-279c	TGACTAGTTGGAACACTCGCCT	22	Tca-miR-279e-3p
	miR-279d	TGACTAGATGGAACACTCACCT	22	Tca-miR-279e-3p
	miR-279e	TGACTAGATCCACACTCATTA	21	Tca-miR-279a-3p
	miR-279f	TGACTAGATCCACACTCATTAA	22	Tca-miR-279a-3p
	miR-279g	TGACTAGATCCATACTCGTCT	21	Tca-miR-279a-3p
	miR-279h	CGTGACTAGATCCATACTCGT	21	Tca-miR-279a-3p
	miR-279i	TGACTAGATCCACACTCATTAT	22	Tca-miR-279a-3p
	miR-279j	AATGAGTGGAGATCCAGTAACACG	24	Tca-miR-279a-3p
	miR-279k	AATGAGTGGAGATCCAGTAACACT	24	Tca-miR-279a-3p
	miR-279l	TGACTAGATGGAACACACGCCT	22	Tca-miR-279e-5p
Dun-miR-3	miR-3a	CAGCCCTCAGCTTGGGGATTCGTC	24	
	miR-3b	CCTCAGCTTGGGGATTCGTC	20	
Dun-miR-4	miR-4a	CCGCATCGAGCTGAAGGGCATCGACT	26	
	miR-4b	CCGCATCGAGCTGAAGGGCA	20	
	miR-4c	CCGCATCGAGCTGAAGGGCATC	22	
	miR-4d	CGCATCGAGCTGAAGGGCATC	21	
Dun-miR-5		TCTTCTTCAAGGACGACGGCAACT	24	
Dun-miR-6	miR-6a	CCCTGGTGAACCGCATCGAGCTG	23	
	miR-6b	CCCTGGTGAACCGCATCGAGCT	22	
Dun-miR-15	miR-15a	CAGAAGAACGGCATCAACCCT	21	
	miR-15b	CAGAAGAACGGCATCAAC	18	
Dun-miR-16	miR-16a	GACCAATACAGCTAGACCTGGATCCTT	27	
	miR-16b	CGACCAATACAGCTAGACCTGGATCCTT	28	
Dun-miR-17		AGGAGGACGGCAACATCCTGGGGCA	25	
Dun-miR-18	miR-18a	TCAAGGAGGACGGCAACA	18	
	miR-18b	TTCAAGGAGGACGGCAACATC	21	
Dun-miR-19		CCCGGGCGGAACCACCT	17	

Table 2.2. Ten most abundant end motifs of SCR miRNAs.

Number of nt	5' End	Count	3' End	Count
17-20 nt	TCA	5	ACT	4
	CAC	2	GCC	3
	TAC	2	GTC	3
	TGA	2	ACC	2
	TGG	2	ACG	2
	TGT	2	CCA	2
	TTT	2	CCT	2
	AAC	1	GAC	2
	ACC	1	GAG	2
	AGT	1	GCA	2
21-23 nt	TTT	32	CCT	36
	TGA	31	ACT	33
	TAA	24	GCT	28
	AAA	20	GCC	24
	TCA	18	CTC	22
	TAG	17	ACC	21
	TAT	16	TCT	19
	TCT	16	AGT	15
	TTC	15	ATG	15
	TTG	13	TTC	14
24-26 nt	TAT	4	ACT	7
	AAT	3	AGT	3
	TAA	3	GCA	3
	TCT	3	GCT	3
	ACT	2	TTT	3
	ATT	2	ACA	2
	GAG	2	ACG	2
	GGG	2	CTT	2
	GTT	2	GAC	2
	TAC	2	GAT	2
27-29 nt	TAA	34	CCT	31
	TTT	28	ACT	27
	TGA	25	GCC	17
	TAT	23	ATT	16
	TGT	23	GCT	15
	TCA	19	GGC	13
	TTA	19	ATC	12
	TCC	15	ACC	11
	TGC	14	TCT	10

TTC	14	AGT	9

Appendix A - SCR miRNAs

Table A.3. Complete small RNA sequencing miRNA and isomiRNA data from SCR.

Name	Iso-miR	Sequence	Length	T. castaneum Homology
Dun-miR-279	miR-279a	TGACTAGATGGAACACTCGCCT	22	Tca-miR-279e-3p
	miR-279b	TGACTAGATGGAACACTCTCCT	22	Tca-miR-279e-3p
	miR-279c	TGACTAGTTGGAACACTCGCCT	22	Tca-miR-279e-3p
	miR-279d	TGACTAGATGGAACACTCACCT	22	Tca-miR-279e-3p
	miR-279e	TGACTAGATCCACACTCATTA	21	Tca-miR-279a-3p
	miR-279f	TGACTAGATCCACACTCATTAA	22	Tca-miR-279a-3p
	miR-279g	TGACTAGATCCATACTCGTCT	21	Tca-miR-279a-3p
	miR-279h	CGTGACTAGATCCATACTCGT	21	Tca-miR-279a-3p
	miR-279i	TGACTAGATCCACACTCATTAT	22	Tca-miR-279a-3p
	miR-279j	AATGAGTGGAGATCCAGTAACACG	24	Tca-miR-279a-3p
	miR-279k	AATGAGTGGAGATCCAGTAACACT	24	Tca-miR-279a-3p
	miR-279l	TGACTAGATGGAACACACGCCT	22	Tca-miR-279e-5p
Dun-miR-3	miR-3a	CAGCCCTCAGCTTGGGGATTCGTC	24	
	miR-3b	CCTCAGCTTGGGGATTCGTC	20	
Dun-miR-4	miR-4a	CCGCATCGAGCTGAAGGGCATCGACT	26	
	miR-4b	CCGCATCGAGCTGAAGGGCA	20	
	miR-4c	CCGCATCGAGCTGAAGGGCATC	22	
	miR-4d	CGCATCGAGCTGAAGGGCATC	21	
Dun-miR-5		TCTTCTTCAAGGACGACGGCAACT	24	
Dun-miR-6	miR-6a	CCCTGGTGAACCGCATCGAGCTG	23	
	miR-6b	CCCTGGTGAACCGCATCGAGCT	22	
Dun-miR-15	miR-15a	CAGAAGAACGGCATCAACCCT	21	
	miR-15b	CAGAAGAACGGCATCAAC	18	
Dun-miR-16	miR-16a	GACCAATACAGCTAGACCTGGATCCTT	27	
	miR-16b	CGACCAATACAGCTAGACCTGGATCCTT	28	
Dun-miR-17		AGGAGGACGGCAACATCCTGGGGCA	25	
Dun-miR-18	miR-18a	TCAAGGAGGACGGCAACA	18	
	miR-18b	TTCAAGGAGGACGGCAACATC	21	
Dun-miR-19		CCCGGGCGGAACCACCT	17	
Dun-miR-20		AGGACGACGGCAACTACAAGACC	23	
Dun-miR-21	miR-21a	CACCATCTTCTTCAAGGACG	20	
	miR-21b	GCACCATCTTCTTCAAGGACG	21	
	miR-21c	CGCACCATCTTCTTCAAGGACG	22	
Dun-miR-184	miR-184a	TGGACGGAGAACTGATAAGGGC	22	Tca-miR-184-3p
	miR-184b	TGGACGGAGAACTGATAAGGG	21	Tca-miR-184-3p
	miR-184c	TGGACGGAGAACTGATAAGG	20	Tca-miR-184-3p
	miR-184d	TGGACGGTGAACTGATAAGGGC	22	Tca-miR-184-3p
	miR-184e	TGGACGGAGAACTGATGGAAG	21	Tca-miR-184-3p
	miR-184f	CCTTGTCATTCTCACGCCCGGT	22	Tca-miR-184-5p

Dun-miR-22		GTGTCGGGCTTGGCGAGGAAGCGGGTC	27	
Dun-miR-23	miR-23a	TACGGACGAGAGAAAACTAAGGCTGAG	27	
	miR-23b	TACGGACGAGAGAAAACT	18	
Dun-miR-92	miR-92a	TATTGCACCAGTCCCGGCCTGA	22	Tca-miR-92c-3p
	miR-92b	AATTGCACTTGTCCCGGCCTGC	22	Tca-miR-92b-3p
Dun-miR-24		AGGTGAAGTTCGAGGGCGACA	21	
Dun-miR-25	miR-25a	CATCGACTTCAAGGAGGACGG	21	
	miR-25b	CATCGACTTCAAGGAGGACG	20	
Dun-miR-276	miR-276a	TAGGAACTTCATACCGTGCTCT	22	Tca-miR-276-3p
	miR-276b	AGCGAGGTATAGAGTTCCTACG	22	Tca-miR-276-5p
	miR-276c	AGCGAGGTATAGAGTTCCTAC	21	Tca-miR-276-5p
Dun-miR-2	miR-2a	TCACAGCCAGCTTTGATGAG	20	Tca-miR-2-3p
	miR-2b	TATCACAGCCAGCTTTGATGAGC	23	Tca-miR-2-3p
	miR-2c	TCACAGCCAGCTTTTATGAG	20	Tca-miR-2-3p
	miR-2d	CTCATCAAGTGGTTGTGAAATG	22	Tca-miR-2-1-5p
	miR-2e	CTCATCAAGTGGTTGTGAAACG	22	Tca-miR-2-1-5p
Dun-miR-26		TTTGCAGTCAGACCAAACATTCTCTATT	28	
Dun-miR-275	miR-275a	TCAGGTACCTGAAGTAGCGCGCG	23	Tca-miR-275-3p
	miR-275b	AGCGCTACCTCGGTATCTAGGCT	23	Tca-miR-275-5p
Dun-miR-27		ACTACAAGACCCGCGCCGAGGTGA	24	
Dun-miR-28	miR-28a	CGTAATTTTTGGTAGTCGGGACT	23	
	miR-28b	CGTAATTTTTGGTAGTCGGGAC	22	
	miR-28c	CGTAATTTTTGGTAGTCGGG	20	
Dun-miR-29		GCGGAACCGTCTTGCTGCGAGGCT	24	
Dun-miR-8	miR-8a	TAATACTGTCAGGTAAAGATGTCT	24	Tca-miR-8-3p
	miR-8b	TAATACTGTCAGGTAAAGATGTC	23	Tca-miR-8-3p
	miR-8c	CATCTTACCGGGCAGCATTAGA	22	Tca-miR-8-5p
Dun-miR-30		CGACCACATGAAGCAGCACGACT	23	
Dun-miR-71	miR-71a	TCTCACTACCTTGTCTTTCATG	22	Tca-miR-71-3p
	miR-71b	TCTCACTGCCTTGTCTTTCATG	22	Tca-miR-71-3p
	miR-71c	TCTCACTACCTTTTCTTTCATG	22	Tca-miR-71-3p
Dun-miR-32		TAGCGGCTGAAGCACTGCACGC	22	
Dun-miR-33		TTGTCGGCCATGATATAGACG	21	
Dun-miR-35		TTTGGTTGACGCACTTACTCGA	22	
Dun-miR-36		ATCAACCCTATAGTGAGTCGT	21	
Dun-miR-37		CCCGCGCCGAGGTGAAGTTCGAGGGC	26	
Dun-miR-38		CTACGTCCAGGAGCGCACCA	20	
Dun-miR-39		GGGTAGCGGCTGAAGCACTGCA	22	
Dun-miR-40		TGCACGCCGTAGGTCAGGGTG	21	
Dun-miR-41		GGTCTTGTAGTTGCCGTCTCC	21	
Dun-miR-277	miR-277a	GGGTGTTGTACCTGGTAGAGCAGTTTCC	28	Tca-miR-277-5p
	miR-277b	TAAATGCACTATCTGGTACGAC	22	Tca-miR-277-3p
	miR-277c	TAAATGCACTATCTGGTACGACA	23	Tca-miR-277-3p
Dun-miR-42		GCATGGCGGACTTGAAGAAGTC	22	

Dun-miR-bantam	miR-bantam-a	TGAGATCATTGTGAAAGCTGTTT	23	Tca-bantam-3p
	miR-bantam-b	TGAGATCATTGTGAAAGCTGTTTT	24	Tca-bantam-3p
Dun-miR-43	miR-43a	CAAGCTGGAGTACAACTACAAC	22	
	miR-43b	CAAGCTGGAGTACAACT	17	
Dun-miR-44		CGGGCATGGCGGACTTGAAGAA	22	
Dun-miR-45		TCTTGAACTTGTTGGTCCAATCTGTTT	27	
Dun-miR-46		GGGGAGGTACTGGGTTCTCGGGATA	25	
Dun-miR-47		TGGCCGACAAGCAGAAGAACGGCA	24	
Dun-miR-100	miR-100a	AACCCGTAGATCCGAACTTGTG	22	Tca-miR-100-5p
	miR-100b	AACCCGTAGATCCGAACTTGTGA	23	Tca-miR-100-5p
Dun-miR-48		ACGGTATTCTTGAGCCAAGGGCA	23	
Dun-miR-49		TTGCCGTCGTCCTTGAAGAAGA	22	
Dun-miR-let-7	miR-let-7a	TGAGGTAGTAGGTTGTATAGT	21	Tca-let-7-5p
	miR-let-7b	TGAGGTAGTAGGTTGTATAGTA	22	Tca-let-7-5p
	miR-let-7c	CTGTACAGCCTGCTAACTTTCC	22	Tca-let-7-3p
Dun-miR-12	miR-12a	TGAGTATTACATCAGGTACT	20	Tca-miR-12-5p
	miR-12b	TGAGTATTACATCAGGTACTGGT	23	Tca-miR-12-5p
Dun-miR-50	miR-50a	ATGGTGCGCTCCTGGACGTAGCC	23	
	miR-50b	TGGTGCGCTCCTGGACGTAGCC	22	
Dun-miR-51		CGTAATTTTTGTTCCCGTGGGGCT	24	
Dun-miR-52		GAAGGGCATCGACTTCAAGGAGGA	24	
Dun-miR-53		GTCTTGTAGTTGCCGTCGTC	20	
Dun-miR-54		TTATACGAAAACAGCTGCTCTAGAACT	27	
Dun-miR-55		AACGTCTATATCATGGCC	18	
Dun-miR-56	miR-56a	TGTGGCTGTTGTAGTTGTACT	21	
	miR-56b	TGTGGCTGTTGTAGTTGTACTC	22	
Dun-miR-57		TATTTACAAGAACTTCCTCGTCTAACGG	28	
Dun-miR-58		CTACAACAGCCACAACGTCTATAT	24	
Dun-miR-59		ATAAACCCAAGAGAATTTGTAGACTAT	27	
Dun-miR-60		TGTAAGGAGAGTTGAGCTGCAATATGTT	28	
Dun-miR-61		GAAGCACTGCACGCCGTAGGTC	22	
Dun-miR-62		TCTTCAAGTCCGCCATGCC	19	
Dun-miR-63		TTCTAAAACGGCTGAAGATCATGAGAA	27	
Dun-miR-64		TCACAGTGATAAGGAGGTAGTCTCAAC	27	
Dun-miR-65		TATCGGTTTCCTCTCTGCAATCGAAGA	27	
Dun-miR-66		TCCTGGACGTAGCCTTCGGGCA	22	
Dun-miR-67		TATTGGAAACGGCTGTCCAACTGATAGT	28	
Dun-miR-68		TAACTGAATCGACCACGAACTGAATTTG	28	
Dun-miR-69		CGTTCTTCTGCTTGTCGGCCA	21	
Dun-miR-70		ACTTGAAGAAGTCGTGCTGCTT	22	
Dun-miR-2944	miR-2944a	TATCACAGCCAGTAGTTACCT	21	Tca-miR-2944c-3p
	miR-2944b	TATCACAGCCAGTAGTTACCTACG	24	Tca-miR-2944c-3p
	miR-2944c	AAGGAACTCCTGGTGTGATATG	22	Tca-miR-2944c-5p

	miR-2944d	TTAAGACATCAAACCAATCGTTCACCT	27	Tca-miR-2944a-5p
Dun-miR-72		TACCGTCGACCAAAGTTTTATGAATAGG	28	
Dun-miR-73	miR-73a	CCGTAGGTCAGGGTGGTCACC	21	
	miR-73b	GTAGGTCAGGGTGGTCACCCT	21	
	miR-73c	GTAGGTCAGGGTGGTCACC	19	
Dun-miR-283		AAATATCAGCTGGTAATTCTGGG	23	Tca-miR-283-5p
Dun-miR-74		GATTCCCGGTATCGGAACCA	20	
Dun-miR-75		GTTGCCGTCGTCCTTAGAAGA	21	
Dun-miR-76		TATATTGGGACTTGAGATTAGGAAACTC	28	
Dun-miR-77		TCGGAGATATCCTCGAAAGGTGTAGAG	27	
Dun-miR-78		TTGAAGAAGATGGTGCGCTCC	21	
Dun-miR-11		CATCACAGGCAGAGTTCTAGCT	22	Tca-miR-11-3p
Dun-miR-79		TACGAGGACTTTATCAAAGCAACTGATT	28	
Dun-miR-80		CGAACTTCACCTCGGCGCGGGTC	23	
Dun-miR-81		GAAGTTGACCAGGAATTCTGTAATTCT	27	
Dun-miR-82		GGTGGCCCTCGAACTTCACC	20	
Dun-miR-87	miR-87a	GTGAGCAAAGATTCAGGTGTAT	22	Tca-miR-87b-3p
	miR-87b	GTGAGCAAAGTTTCAGGTGTGT	22	Tca-miR-87b-3p
	miR-87c	ACGCTTGAACTTTGTTTTTCCT	22	Tca-miR-87b-5p
	miR-87d	TAAATTCTGGTGTGTTGCGAC	21	Tca-miR-87b-3p
	miR-87e	TAAATTCTGGTGTGTTGCGACC	22	Tca-miR-87b-3p
Dun-miR-83		AGTGAGTCGTATTAGGATCCCC	22	
Dun-miR-84		TTACAATCATCTCCTAACTCGTGAGCCT	28	
Dun-miR-305	miR-305a	ATTGTACTTCATCAGGTGCTC	21	Tca-miR-305-5p
	miR-305b	ATTGTACTTCATCAGGTGCTCTGGT	25	Tca-miR-305-5p
	miR-305c	ATTGTACTTCATCAGGTGCTCTGTC	25	Tca-miR-305-5p
	miR-305d	TTGTACTTCATCAGGTGCTCTGG	23	Tca-miR-305-5p
	miR-305e	CGACACCTGTTGGAGTGCACT	21	Tca-miR-305-3p
Dun-miR-85		TAACTCTGATCCAACATGTAAAACTAT	27	
Dun-miR-86		CTTCAGCTCGATGCGGTTCACC	22	
Dun-miR-88		TTCTCCACTGAAGACCAACAAAGTTCTG	28	
Dun-miR-89		TATTGATTGGCCTGGTGTGGATGAACA	27	
Dun-miR-90		TTTCTTCTTGGGCAGTGAAATTGGGAGC	28	
Dun-miR-91		TACCGTCGGCCATAGTTTTATGAATAGG	28	
Dun-miR-93		TCTTGATACTCAAATACCTTTCGGCCCC	28	
Dun-miR-94		TGGTCGGGGTAGCGGCTGAAGC	22	
Dun-miR-95		TTTGAATCCGACGTTTGAACAGGGCTT	27	
Dun-miR-96		ATCCCACTTCTGACACCG	18	
Dun-miR-97		CTTCAGCCGCTACCCCGACCACA	23	
Dun-miR-98		TACTTGAGAATTTTCTGTCCATCCTGCC	28	
Dun-miR-99		TAGCCTTCGGGCATGGCGGACT	22	
Dun-miR-101		TTATAAATCTCGTCTGTCCCTGCACCC	27	
Dun-miR-317	miR-317a	TGAACACAGCTGGTGGTATCTCAGT	25	Tca-miR-317-3p
	miR-317b	TGAACACAGCTGGTGGTATCTC	22	Tca-miR-317-3p

	miR-317c	TGAACACAGCTGGTGGTATCT	21	Tca-miR-317-3p
	miR-317d	TGAACACAGCTGGTGGTATCTCT	23	Tca-miR-317-3p
Dun-miR-102		AACTCCATACCAATATTATCTTGCGAC	27	
Dun-miR-103		CTTGAAGAAGATGGTGCGCTC	21	
Dun-miR-104		TAGTGAGTCGTATTAGGATCCC	22	
Dun-miR-105		CTTGAAGAAGTCGTGCTGCTTC	22	
Dun-miR-106		TAGGTACTAGAGAAGATTTGATAGCCT	27	
Dun-miR-107		TAACCGATTGCAAAGCTCAGAACAGCGC	28	
Dun-miR-108		TAAGAAATGACACTTGATTTATTGGACC	28	
Dun-miR-3802		TTTCTCGGCATTCAATCACATTTTTATA	28	Tca-miR-3802-5p
Dun-miR-109		TGACAGTTGTGAGACAATGAATAAGAAT	28	
Dun-miR-110		GCACTTTACACTCTGACTTGATTCTGTT	28	
Dun-miR-111		GAAACGCGTGCGTCGTTCGCTC	22	
Dun-miR-112		AGCACGTTCTGTGTTTGACGGC	22	
Dun-miR-113		TTTGCATTCCACGACATGTCTATTCATC	28	
Dun-miR-114		TCGATAAACTGAATCTCTCGTTGGCCC	27	
Dun-miR-115		TTTATTACAAGACGTCAGACAAGGGCT	27	
Dun-miR-116		TACGTTGAACTAATTCTGCTTCTTGGGC	28	
Dun-miR-117		TGAACGAAAACGATTTGTAATTGGATTT	28	
Dun-miR-118		TACGAGGACTTTATGAAAGCAACTGATT	28	
Dun-miR-119		TCCGTAGAATAAATAACGCACTCACTAT	28	
Dun-miR-970		TCATAAGACACACGCGGCTGT	21	Tca-miR-970-3p
Dun-miR-120		TCACCGTGAACTCGAAGGCTGATCCATC	28	
Dun-miR-121		CTGGAGTACAACTACAACAGCC	22	
Dun-miR-122	miR-122a	TCGTTTCCCGGCCGATGCACCA	22	
	miR-122b	TTTTCCAATGGGTCTAGACTT	21	
	miR-122c	TTTTCCAATGGGTCTAGACTTT	22	
Dun-miR-3477		TAATCTCATTTGGTAACTGTGA	22	Tca-miR-3477-5p
Dun-miR-123		TACATCGGTAATAATTGACTGAAAGCCT	28	
Dun-miR-124		GTTTCATGGATCCGAGTTCGGTC	23	
Dun-miR-126		ATTGGCTCTGAGGATCGGGGC	21	
Dun-miR-6015		CTGCTTCATGTGGTCGGGGTA	21	Tca-miR-6015-5p
Dun-miR-127		TGCTACCACATGAAGCAGCA	20	
Dun-miR-128		TGACACAATACGAGAAGGTAAAAAGCC	27	
Dun-miR-129		TTCTACTGTCTTAACTCAAACTTAACT	27	
Dun-miR-130		TGCTGGAATTCTTTAAATCCTAAGGGAC	28	
Dun-miR-3851	miR-3851a	GAGGTGTAGCATAAGTGGGAGATGGC	26	Tca-miR-3851p-5p
	miR-3851b	GAGGTGTAGCATAAGTGGGAGATGG	25	Tca-miR-3851p-5p
	miR-3851c	TATTGACTGGGTTGGTACTGTCA	23	Tca-miR-3851d-5p
	miR-3851d	TATTGACTGGGTTGGTACTGT	21	Tca-miR-3851d-5p
Dun-miR-3827		TCATAAATCGATCTTCATTTGTTTCATT	28	Tca-miR-3827-3p
Dun-miR-131		TACAATTAGCCTGGGTAGATCCTGTAA	27	
Dun-miR-132		TAAAAGGATTCAGCAAGGCGAGGGTACT	28	
Dun-miR-133		TATTTGCACTCATCCGGCTCGAAGGACC	28	

Dun-miR-134		TTAAAGACTGTCGTGATGACTTAGGCT	27	
Dun-miR-135		ATAAACCCATCACCTGAACAATGGCGCC	28	
Dun-miR-136		TAGGTACTAGAGAAGATTTGATACTCT	27	
Dun-miR-1	miR-1a	TGGAATGTAAAGAAGTATGGA	21	Tca-miR-1-3p
	miR-1b	CCGTACTTCCTTACTATCCCATA	23	Tca-miR-1-5p
Dun-miR-137		TGCTTCATGTGGTCGGGGTAGC	22	
Dun-miR-138		TGCAAGGCAAAACAGACGTTGGTAAAGA	28	
Dun-miR-139		AGTTCACTGACCATCCGGATG	21	
Dun-miR-13	miR-13a	TATCACAGCCATTTTGACGAGTT	23	Tca-miR-13b-3p
	miR-13b	TATCACAGCCATTTTGACGAGTTT	24	Tca-miR-13b-3p
	miR-13c	TCATCAAGTTGGGTGTGACTTT	22	Tca-miR-13a-3p
	miR-13d	TCATCAAGTTGGGTGTGACT	20	Tca-miR-13a-3p
	miR-13e	TCATCAATTTGGTTGTGAATT	21	Tca-miR-13a-5p
	miR-13f	TCATCAATTTGGTTGTGAATTCT	23	Tca-miR-13a-5p
Dun-miR-3856		CGGAGTGACGTTTCGTTGCGTTG	23	Tca-miR-3856-3p
Dun-miR-140		GGCGGATGATGATACCGGGA	20	
Dun-miR-141		TTTTCACGCTACGGACTTACAGCCTAG	27	
Dun-miR-142		AAGTTGTACTCCAGCTTGTGCC	22	
Dun-miR-34		TGGCAGTGTGGTTAGCTGGTTG	22	Tca-miR-34-5p
Dun-miR-143		TGAAATTAGCTATATTTGCCGAAGGAC	27	
Dun-miR-144		CCTGGCCGACAAGCAGAAGAACG	23	
Dun-miR-145		TAGAGAACTCAATTCAAACATG	22	
Dun-miR-146		TGATAGGACTTTTAGATGATTTGCACC	27	
Dun-miR-147		TGAGCAGTAACTGAAATATCCCTTCGAC	28	
Dun-miR-148		TGAGTAGAATTATGCACTTGTGATTGGG	28	
Dun-miR-149		TGATGTCAAATTCGGAGACTCTTGCCT	27	
Dun-miR-150		TGAGTGCTTAATGTTGGAGAATTCTGGC	28	
Dun-miR-151		TACCTATCCTGACAGTAATTTCTGAAGC	28	
Dun-miR-152		ATGGCCGACAAGCAGAAGAACGG	23	
Dun-miR-153		TAAAAAGCCGGTACCTTAAAATTCTTCC	28	
Dun-miR-154	miR-154a	AGTTCTACAGTCCGACGATC	20	
	miR-154b	GAGTTCTACAGTCCGACGATC	21	
Dun-miR-155		TGATGTTGTTGACCTCAGGCTGGCAGT	27	
Dun-miR-156		ACAGAATTGATTATGTGGACT	21	
Dun-miR-157		AGAAGACTCTGTTTTTTTCGTCCGTGCT	28	
Dun-miR-158		TAGTAATGGATTCCTCAGTTTGGTCGAC	28	
Dun-miR-159	miR-159a	AACCCGAAAGGTCGAAAGAGG	21	
	miR-159b	AAACCCGAAAGGTCGAAAGAGG	22	
Dun-miR-160		TGCATTCCTTTTATCACGTCCGACAAGA	28	
Dun-miR-161		CGAGTTCGCTTTTACAGGTACT	22	
Dun-miR-162		AAGTTGCTCCTCAATTTTGGTTAAGGGC	28	
Dun-miR-163		CTACAAGACCCGGGTCTTGTA	21	
Dun-miR-164		TTTATGCGAACTTTGTGCACTCTCGGG	27	
Dun-miR-10	miR-10a	TCCGTGTGTCCGAAGTCTCTCTAGAACC	28	Tca-miR-10-3p

	miR-10b	TACCCTGTAGATCCGAATTTGT	22	Tca-miR-10-5p
Dun-miR-165		TGGGCGAGGTGAAGTGAGCC	20	
Dun-miR-166		TAAGAATTACGTTCGGGTGTAACAATTC	28	
Dun-miR-167		ATCAACCCTATAGTGAGTCGCC	22	
Dun-miR-168		TGTGACGTTTGTTCTTGATGTACTGCCT	28	
Dun-miR-169		TTAGACTTCCTGCACTGAGCA	21	
Dun-miR-170		TCTCCAGTGTGTGTTCTCATA	21	
Dun-miR-171		CCCAGCGTTAAACTGTATGAAGGAGGCC	28	
Dun-miR-172		TACCTGACACCTACCTGACGCATACCTG	28	
Dun-miR-173		ATAAATGATTGGAAGTACTCTGCATCT	27	
Dun-miR-174		GCTTGTGTTCCGTCTATGTTGC	22	
Dun-miR-175		TAACCGTGAACTCGAAGGCTGATCCATC	28	
Dun-miR-176		AGGGGAAAGATCTAGGCTTACT	22	
Dun-miR-177		TTTAGTAAGCGATTAGGATATGCCAGCC	28	
Dun-miR-178		TACGAGGACTTTATGGAAACAACTGAT	27	
Dun-miR-179		TAACAATGGAAAATGGCTGATAATCTGT	28	
Dun-miR-180		ACCCTATAGTGAGTCGTACTAT	22	
Dun-miR-181		TTGAAGTTCTTGGATCTGTAGGTAGGTC	28	
Dun-miR-182		TTGTGACGAAGTGCTCGGTGCTAAATCC	28	
Dun-miR-183		TCGGTCTGTAGTTTTGTGGAAAAGTGCC	28	
Dun-miR-185		TGTGTGGATTAGTATCTCCTTA	22	
Dun-miR-186		TGCTGGTAATGGAGAACAATTGGACGAG	28	
Dun-miR-187		TCCTTTCGGCATCCTGGACTTCTTTGG	27	
Dun-miR-188		TCGATGATCCGGTAACATGATGTTTCT	27	
Dun-miR-189		TCCAGAAGGTTCGAATTGTTGTTTTTAT	28	
Dun-miR-190		TATGTCTGCTATTTAATTCATTGAACCT	28	
Dun-miR-191		ACATTTTACAACAACAGGACT	21	
Dun-miR-192		TATTTGGTTGCATTGTGAGTCACGACT	27	
Dun-miR-3869		TTAAAGCTGAGAGACTGTTTAGAAAAGC	28	Tca-miR-3869-5p
Dun-miR-193		ATTTGTCACAATTAACTGGTGTAGGACT	28	
Dun-miR-194		TCATCGTCCCAGCTCTCATCCACAGAC	27	
Dun-miR-195		GACAGAAAATCGAAAGGATTC	21	
Dun-miR-196		TTGGTCTGTAGTTTTGTGGAAAAGTGCC	28	
Dun-miR-197		TGAAGACTCTGTTTTTTTCGCCCGTGCT	28	
Dun-miR-198		TTAGTGATTTGTTCTGGATACTTGGCC	27	
Dun-miR-199		TAGCAGGATGTACTTATTTTTGGTGCC	27	
Dun-miR-200		TACACACTTTTTGTCTGATAGTAACGCT	28	
Dun-miR-201		CCGTCGTCCTTGAAGAAGATG	21	
Dun-miR-202		GAATATGATGATTGTTCTATC	21	
Dun-miR-203		TAAAGCTTCTTTGGAAAATCCGGGTGC	27	
Dun-miR-932	miR-932a	TCAATTCCGTAGTGCATTGCAGT	23	Tca-miR-932-5p
	miR-932b	TGCAAGCAGTGCGGAAGTGAGG	22	Tca-miR-932-3p
Dun-miR-204		TTATAGCTTCAGGCCTGGGAATCTGCAC	28	
Dun-miR-205		TGCTAGATTTTTTTAGGCTGTAGTGACT	28	

Dun-miR-206	miR-206a	TAGGATCAATGATTTAAACTT	21	
	miR-206b	TAGGATCAATGATTTAAACTTA	22	
Dun-miR-207		TAACATGGCGACATATGACTCTACATT	27	
Dun-miR-208		TTCTGATTATTGCACCATGTTGACGCCT	28	
Dun-miR-209		TTTGTATGACGGATAGCGTTT	21	
Dun-miR-210		ACACTTTACACTCTGACTTGATTCTGTT	28	
Dun-miR-211		TAAAAGGATTCAGTAAGGCGAGGGTACT	28	
Dun-miR-212		TACGTCAGGGATATGAGGATTTTCAGA	27	
Dun-miR-213		TATTAACTAGTATGAGATCCGGTCTATT	28	
Dun-miR-214		TTTCTCGGCATTCAATAACATTTTTAT	27	
Dun-miR-215		TCAAAGCACTCTCGAGATCCGCGTACAC	28	
Dun-miR-216		TGTTTTCGAGATAGGCACAGAGTCGTC	27	
Dun-miR-217		TTCAAACGGACTTCAGCTGGTAACTTGC	28	
Dun-miR-218		TAGACATACCGTCTCAGGACT	21	
Dun-miR-219		TTTGGAATTCTTTTCTGGGCT	21	
Dun-miR-220		ACTGCCAAGAATTCGGACACTCTGC	25	
Dun-miR-221		TTGAAGTTCTTGGATCTGTTGGGAGGTC	28	
Dun-miR-222	miR-222a	TTGAACCTTTGGACTGTGGAA	21	
	miR-222b	TTTGAACCTTTGGACTGTGGAA	22	
Dun-miR-223		TGTGCGAGGATTAGGAAGATAAAAAAGT	28	
Dun-miR-224		TCGGATGGTGACCAAGAACATAGGTAGC	28	
Dun-miR-225		TTGACTGGAATAATTTTGATTGGCGAT	27	
Dun-miR-226		TTTGAGGAGTCCAATCTGTTGTGGGGA	27	
Dun-miR-227		CGCTGACGCAATTACATAATTTGACCTG	28	
Dun-miR-9	miR-9a	TCTTTGGTGATCTAGTTGTATGA	23	Tca-miR-9e-5p
	miR-9b	TCTTTGGTGATCTAGTTGTATGAT	24	Tca-miR-9e-5p
	miR-9c	TCTTTGGTGATCTAGTTGTATG	22	Tca-miR-9e-5p
	miR-9d	ATAAAGCTGGTTCAGCAAAGCA	22	Tca-miR-9e-3p
Dun-miR-228		TAGCTGACGCATACCTGACGCATACCTG	28	
Dun-miR-229		ACCTATGATGAAACGATGACGAGAC	25	
Dun-miR-230		TGATATAATTATCTGTATTTAAGGGCT	27	
Dun-miR-231	miR-231a	ACATGCTGACAATTCTGACCT	21	
	miR-231b	CACATGCTGACAATTCTGACCT	22	
Dun-miR-232		GGACGATTAGGTTATGTGTAGAATCGCC	28	
Dun-miR-233		TTGTAGTTGTGAATAAGCTTTGGCATGG	28	
Dun-miR-234		TGACTTTACTAGGCAATCGT	20	
Dun-miR-235		TCTCGATCCAACGTAGCATCCTTCTGGT	28	
Dun-miR-236		TACCTGACGCATACCTGACGCATACCTG	28	
Dun-miR-237		TTAACACGATAGCTGACGACCTGAGCGC	28	
Dun-miR-238		TTCTGCTTGTCGGCCATGATA	21	
Dun-miR-239		TAAAAGGATTCAGCAAGGTGAGGGTACT	28	
Dun-miR-240		TATTGGATATGCGACAAACTCTACTCT	27	
Dun-miR-241		TAACAAACACTTCCCAACTCTTCGCCT	27	
Dun-miR-242		CTGGCAGTTGAAGCACTGTGTGGGCCC	27	

Dun-miR-243		TACATTTGAGATTCAGAGGACC	22	
Dun-miR-244		TGACCGCATTGTAGTAGGAACTCGGGA	27	
Dun-miR-245		TTTAGTCTCTTGTAAGGCCATGACATC	27	
Dun-miR-246		TACACGAAGACATTCCACCCTATCCAAA	28	
Dun-miR-247	miR-247a	GTTGACACGATCGGTGTCGGAGCT	24	
	miR-247b	GTTGACACGATCGGTGTCGGAGCTT	25	
Dun-miR-248	miR-248a	TACAAATGTTGTCACTGGAGGCCTCTT	27	
	miR-248b	TACAAATGTTGTCACTGGAGGCCTC	25	
Dun-miR-249		AAGGCTACGTCCAGGAGCGCACC	23	
Dun-miR-250		TCACTGTCTTCTAATGTTGTTGTCTCCT	28	
Dun-miR-251		TTGGCATCTGGTACACTGGACA	22	
Dun-miR-253		TGAAAGCCTGTTGAATGGAAATAATTCC	28	
Dun-miR-254	miR-254a	AAATTCTTTAGACTCTGGCCT	21	
	miR-254b	AAAATTCTTTAGACTCTGGCCT	22	
Dun-miR-255		TTAGAACATAGAGACTAGTTGGTAGAT	27	
Dun-miR-256		TCCTAGCTGGTTGAACTGTTTTTGGCCT	28	
Dun-miR-257		TCAAAGAAACTCTCAGCTGTCATCCAGC	28	
Dun-miR-11617		TTTGTATGGATGGTACACATGGCACT	26	Tca-miR-11617e-5p
Dun-miR-258		TGCTTGTGAACTGAGATGAAATAACCT	27	
Dun-miR-259		TCAGAGACTGTAATTTCATCTAGAACT	27	
Dun-miR-260		TCTCGATCCAACATAGCATCCTTCTGGT	28	
Dun-miR-261		AAGTTGCTCCTCAATTTCGGTTAAGGGC	28	
Dun-miR-31		AGGCAAGATGTCGGCATAGCTGA	23	Tca-miR-31-5p
Dun-miR-262		TGAAGAACAATTTCTGGGCGAAT	23	
Dun-miR-263	miR-263a	TTTTGATCGATTACTTCAGGTAAAGCCT	28	
	miR-263b	TTTTGATCGATTACTTCAGGTAAAGCC	27	
Dun-miR-264		TTTACTTCTTTGAGTGGGGCT	21	
Dun-miR-265		TTTAAGGAGTTTATTAGGATATCGGCC	27	
Dun-miR-266		TGCGAGGATTAGGAAGATAAAAAAGT	26	
Dun-miR-267		TTTTGACTCGAAAGCCCATAAGGCATC	27	
Dun-miR-268		CACTTGACACTCAAAGAAGGATTCGGCC	28	
Dun-miR-269		TCCAAGCATTTCTCGTCAAGTTTCTATT	28	
Dun-miR-270		TGAGAGAACACACTGCAGAAA	21	
Dun-miR-271		TAAACTCTTGTAGAATGGATGATACACT	28	
Dun-miR-272		TATTTTCCTCTACAAAGTTCGCCGATG	27	
Dun-miR-273		TGTTCAGATCTGAATCCAAACTGAGATT	28	
Dun-miR-274		CACATTTGTAAGGTTTTTCTC	21	
Dun-miR-278		TACTCTAGAATACTCTGACTCACATGA	27	
Dun-miR-280		TGTTACGAGAAAGAAAAGGGATAGGACC	28	
Dun-miR-282		AAATGCTGATAGTACTCTCCC	21	
Dun-miR-284		CTGTTCTAGTTCTCTTTGTAGCCGCTGC	28	
Dun-miR-285		TATAGTGAGTCGTATTAGGATC	22	
Dun-miR-286		ATAAATGATTGGAAGTACTCCGCATCT	27	

Dun-miR-287		TGCGTAGATCTCCCGATTGGTGTGCTA	27	
Dun-miR-288	miR-288a	GCCTCGGTAGCGCTTAAGTC	20	
	miR-288b	GCCTCGGTAGCGCTTAAGTCT	21	
Dun-miR-289		CTACAGTTTCTCTTGTAAGGGGT	23	
Dun-miR-3857		TACTCGGTGGCTTGATACTGTCA	23	Tca-miR-3857-5p
Dun-miR-125	miR-125a	TCCCTGAGACCCTTACTTGTGA	22	Tca-miR-125-5p
	miR-125b	ACAAGTTTGTGACTCGGGTAT	21	Tca-miR-125-3p
Dun-miR-290		TTAAGTGATGAACGAAGCCACC	22	
Dun-miR-291		ACACTTTACACTCTGACTTCATTCTGTT	28	
Dun-miR-292	miR-292a	AGAGAGGGAAGTGTTCTGGCCT	22	
	miR-292b	AGAGAGGGAAGTGTTCTGGCC	21	
Dun-miR-293		TTACAGGAAATGGGAAATACAACTTAT	27	
Dun-miR-294	miR-294a	TTCTTTTCTGGGCTCTGGATC	21	
	miR-294b	ATTCTTTTCTGGGCTCTGGATC	22	
Dun-miR-295		CACCTTGATAATTTTTTGCGTTTAGTCT	28	
Dun-miR-296		ATCTATGATGAAACGATGACGAGAC	25	
Dun-miR-297		AGAAAGCCTGTGGAATGGAAATAATTCC	28	
Dun-miR-298		CCACCGTGATAATTTGTGCTC	21	
Dun-miR-299		ATTTGACACACTTAGCTCCACAATGGC	27	
Dun-miR-300		CCATATCGAAACCGGTTTTTAAAGCTC	27	
Dun-miR-301		TACAATTAGCCTGGGTAGATTCTGTAA	27	
Dun-miR-302		TATTTCAACTTCGTCTTCTTCAGAACT	27	
Dun-miR-303		TGTGTTACTAGAAATTGGCGGAACATA	27	
Dun-miR-304		TTACTGTGAATGTGGCCTAATAGAGAAC	28	
Dun-miR-306		TATATACCAGTGCCGTTATCTCGAAAAG	28	
Dun-miR-307		TTTAGTCTCTTGAAGAGCCATGACATC	27	
Dun-miR-309		TATTGAGTTTCGTGCACTGGTAGGCAC	27	
Dun-miR-310		TAAAACCTTCTTGAGTAGACTGGACGGC	28	
Dun-miR-308		GGCAGAATTTCCTCGTGAATGG	22	Tca-miR-308-5p
Dun-miR-311		ACGTCCAGGAGCGCACCATCT	21	
Dun-miR-312		TGTGCACAACTCTGTTTCGTAACCCACT	28	
Dun-miR-313	miR-313a	AATAATTATCTGTATATAGAATTGCCT	27	
	miR-313b	TAATAATTATCTGTATATAGAATTGCCT	28	
Dun-miR-314		TTCTGGCTGAGTGTCCAAACTCCTGGC	27	
Dun-miR-316		TCCAGAAGCAACTTGTAGGTCGGACAGT	28	
Dun-miR-318	miR-318a	TATTAAGAAAATCACGTAGAGCAGAACT	28	
	miR-318b	TATTAAGAAAATCACGTAGAGCAGAACTT	29	
Dun-miR-319		TATAGAACCTTTTCCTCTTTCCAGTATT	28	
Dun-miR-320		TCCTGTAGAAAGCTGATGATGTGTCTGC	28	
Dun-miR-321		TACTCAGTCAGTTTTAAGCATGTCGAAC	28	
Dun-miR-322		TCGTTTACTGGAGGACAATTTTGGCAAT	28	
Dun-miR-323		TGTAACTTCTTCCACTGCTCTGGACAT	27	
Dun-miR-324		TTACAGCGTCTTCAGTAGATTTTCCACT	28	
Dun-miR-325		TTCAAGCACAAATCTTCAGGGCT	23	

Dun-miR-326		TAATCTCTTCTAGGACCTGAAACAACCT	28	
Dun-miR-327		TTACTCGAATATTCTGGACTCTTGACT	27	
Dun-miR-328		TACTGGAGTAAGTAGATAGTGTCTG	25	
Dun-miR-329		CGAGATCGTGCGGCGCGTCCA	21	
Dun-miR-330		ATGCATGGACGATCAGTTAACAGT	24	
Dun-miR-331		TGTAATTTTTGGTAGTCGGG	20	
Dun-miR-332		ACTAGTTGGATTTGATGCTGTCA	23	
Dun-miR-333		TAAGAAATTTGAGAGAGTGGTTCGGTT	27	
Dun-miR-334		TACCCGAGTCTTGAACACTGAATGTAA	27	
Dun-miR-965		GGTGAAAAGTTGTACAGCTCATG	23	Tca-miR-965-5p
Dun-miR-2796		TTCTTTCGGCATCCTGGACTTCTTTGGA	28	Tca-miR-2796-5p
Dun-miR-335		TAAAGGCAAACTGGATTTCCCTCGCATC	28	
Dun-miR-336		GGAGATTGATGTCTGTGGATG	21	
Dun-miR-11641	miR-11641a	TTTGATCAAATATTAAGCTGTCATGACT	28	Tca-miR-11641-5p
	miR-11641b	TTTGATCAAATATTAAGCTGCCATGACT	28	Tca-miR-11641-5p
Dun-miR-337		TAAAAAAGGACAGAGAAGACGTAGCATC	28	
Dun-miR-338		TATTACAGAAAATCAGGAGCACGCACT	27	
Dun-miR-339		TATTGAAGGGCCAGTCTATCCAACCT	26	
Dun-miR-340		TGATGAACTCTGCTGCTGCTTCTTGCCT	28	
Dun-miR-341		TGCACGGAACCTGGATATCCCACA	24	
Dun-miR-342		TAAGAAATGCACTGGCCTATCTGTTC	26	
Dun-miR-343		TCATAAAAAACTGGTACAACTCTTATA	27	
Dun-miR-344		TCCCAAAATGCTGATAGTACTC	22	
Dun-miR-345		TTGGTTCTTTCTGACAAAACTCG	23	
Dun-miR-346		TATGAAATCTCGGAGCCAACTTGCGGT	27	
Dun-miR-347	miR-347a	AAATTCTGTCTGGATCAGGACT	22	
	miR-347b	AAATTCTGTCTGGATCAGGAC	21	
Dun-miR-348		TAGGACCATGAACAAATTCCCTTCGTT	27	
Dun-miR-349		ATTTGTGATGATATTAGACGAC	22	
Dun-miR-350		TCATAGCTATTCGGACTTTTGAGACGGC	28	
Dun-miR-351		TTTCCAATGGGTCTAGACTTA	21	
Dun-miR-352		TTTGTAACCCACTTAGACTGAAGGCCT	27	
Dun-miR-353		TCTCAAGGGTGAGTTCGTCTTGTCTT	26	
Dun-miR-354		TCTGCGTCAGGCTGACAGGCAG	22	
Dun-miR-355		CAACGTGAGTAGAAGTGGCCT	21	
Dun-miR-356		TCCTTTATCCACAATAATTGCAGAGCCT	28	
Dun-miR-357		TTACCCTGAACGGTGAATCACT	22	
Dun-miR-358	miR-358a	TACGACGTACCAGTTGAACTC	21	
	miR-358b	TACGACGTACCAGTTGAACTCG	22	
Dun-miR-359		AAAACCCCACCAGTTGAAGTCG	22	
Dun-miR-360		AATAATGATGACAGATCACTGCGCA	25	
Dun-miR-361		TAGCTGTGTATGTTAGTCCAACCTGATT	28	
Dun-miR-362		TTTTAGAGAACTGCTTTGAGCA	22	

Dun-miR-363		CATTTTCTTTAGAATCGGGCC	21	
Dun-miR-364		TGTATCGGCTTTGCACCACTTCCTGAAG	28	
Dun-miR-3841		TAAGAAAGGAAAAATCGGACGGGTCATC	28	Tca-miR-3841-5p
Dun-miR-365		TTTTCAGAACCTGCATGAAGGACAATGT	28	
Dun-miR-366	miR-366a	GGATCATACTCGAGTAGGACC	21	
	miR-366b	GGATCATACTCGAGTAGGAC	20	
	miR-366c	CGGATCATACTCGAGTAGGACC	22	
Dun-miR-367		GCCCCAGCGGGTGTTGACACAATG	24	
Dun-miR-368		TATTTGCTCGTTTAATGCTTGAAGCACT	28	
Dun-miR-369		TGTTTTTTGACATACAGGGCC	21	
Dun-miR-370		TGCCTTCATTTGCCAATCGTCTTACTGT	28	
Dun-miR-371		ATAAGGATTGGCTCTGAAGAC	21	
Dun-miR-372		TGTTAGCTTTCTGATTCCAATATAGATT	28	
Dun-miR-373		CGCTGACGCAATTGCATAATTTGACCTG	28	
Dun-miR-374		GCCCGTAGCGACCGTTGCCGATT	23	
Dun-miR-375		TCATTGTTTCGATTGCCGTTCA	22	
Dun-miR-376		AACGATTACCCTGAACGGTGAAT	23	
Dun-miR-377		TAGTTTGTCTGATGGTGCAAGT	22	
Dun-miR-378		GTTCCAATAGTGTAGCGGC	19	
Dun-miR-379		TGAAATTGTTGTATGTGGCTC	21	
Dun-miR-380		TTCGATCAAATATCAAGCTGTCATGACT	28	
Dun-miR-381		TGCTATTGCAGTGATAAGTAGGGCCATT	28	
Dun-miR-382	miR-382a	GAGAACACACTGCAGAAAAATC	22	
	miR-382b	GAGAACACACTGCAGAAAAAT	21	
Dun-miR-383		TACTATCCAATATGTAGCGAAGCCCTGT	28	
Dun-miR-384		TTTGGAATTATTTTCTGGGCT	21	
Dun-miR-385		CATAGATATGTGATCTGCACC	21	
Dun-miR-386		AAAAAATACTGCTACTGGGCC	21	
Dun-miR-387	miR-387a	TAAGCAGAACGGACAACTTTC	21	
	miR-387b	TAAGCAGAACGGACAACTTTCC	22	
Dun-miR-388		CAGCTTCACCTCGTAGGGGACC	22	
Dun-miR-389		TACATCAAACGACGAATATGCTCTGACT	28	
Dun-miR-390		CTAGGTACTCAATTAAAACTCG	22	
Dun-miR-391		TTAGAGATCCAGAACCCAGAA	21	
Dun-miR-392		CAAGATGATTTTTCTGTAGTGAGCCCT	27	
Dun-miR-393		TTTTCAGAACCTGCATTAAGAATAATGT	28	
Dun-miR-394		ACAACATAGAAGATGAGGTCT	21	
Dun-miR-395		TTTTCCAATGGGTCTGGACTT	21	
Dun-miR-396		TTAAAGCTTCAAGTGGTCCATATAAACT	28	
Dun-miR-397		ACTTTCGGGTGCTTGGATGTCCT	23	
Dun-miR-398		TGCTACAGCTTATTAAATTACCGTCGGC	28	
Dun-miR-399		TTTAATTTGTGTGTCGCGCAGA	22	
Dun-miR-400		TTTCTGCTTTAATCTGGAGCCT	22	
Dun-miR-401		TCGAGATCTCAATTCAAACATG	22	

Dun-miR-402		TCCACAAGACTGATTTTGCGTTTGCGTC	28
Dun-miR-403		TCTGAACAATTTACTTCACTAACTAAAT	28
Dun-miR-404		TGTTTGGATCCGGTTTATTGCAGAAAAG	28
Dun-miR-405		TAAATGTAAAATATCCAGGCC	21
Dun-miR-406		TAGTGAAAATCGCCCCAGGCC	21
Dun-miR-407	miR-407a	TTCGAGGATAAACCAGGGGTG	21
	miR-407b	TTCGAGGATAAACCAGGGGTGG	22
Dun-miR-408		TCCTTTATCCACCATAATTGCAGAGCCT	28
Dun-miR-409		GTATAGCATTCTGTTGGTGACGACT	25
Dun-miR-410		TAAAGCTAATTGTTGATGATAAAAAGGC	28
Dun-miR-411	miR-411a	TTTGTCTATTGTGTCCATCTAT	22
	miR-411b	TTTGTCTATTGTGTCCATCT	20
	miR-411c	TTTGTCTATTGTGTCCATCTC	21
Dun-miR-412		TGTTCAGATCTGAATCCGAACTGAGATT	28
Dun-miR-413	miR-413a	CTAAACAGTCGAGCTGGGTCCC	22
	miR-413b	TAAACAGTCGAGCTGGGTCCC	21
Dun-miR-414		GGAGTATCAGAGACTGGGTTCT	22
Dun-miR-415		AAAATTCTTTAGACTCTGGCC	21
Dun-miR-416		ACTTATGATGAATTTCGGCGGCT	23
Dun-miR-417		AAACTTGGTCTTGTAGTGAAC	21
Dun-miR-418		TTTTAGCTTCTGACTTCCAATCTAGGTT	28
Dun-miR-419		TTGGCTTCGGTTCTCTGGGGA	21
Dun-miR-420		TAAATTCTTCAGGACGCGCCGACTGAGC	28
Dun-miR-421		TTTTAGAAGACTGCTTTGAACA	22
Dun-miR-422		TGACTATTCGTTAAACTGATCTTAAGCT	28
Dun-miR-423		CAGTGAACTGGCCTGGGGCGA	21
Dun-miR-424		TCTGAGCAATATAACCTGGTTGTCGCCC	28
Dun-miR-425		TTCCAATTGTAGATAATCTACTTCCACT	28
Dun-miR-426		ATAGATATGTGATCTGCACCT	21
Dun-miR-427		TCCACAAGACTGATTTCGCGTTTGCGTC	28
Dun-miR-428		TAGATGAGTCAGTTAAAACATG	22
Dun-miR-429	miR-429a	TAATACTGTACTTGGGAATCC	21
	miR-429b	CTAATACTGTACTTGGGAATCC	22
Dun-miR-430		TGTGCACAAATCTGTTTCGTAACCCACT	28
Dun-miR-431		ATGAATCTTATACTTGACTGCA	22
Dun-miR-432		TTCTAGTTGTCGCACCAAGACTTGAAGG	28
Dun-miR-433		GTACGAGATCAGTCCAGGACC	21
Dun-miR-434		CAACATCTCTTTTACTCTGGA	21
Dun-miR-435		TTTTAGAAAACTGCTTTGAGCA	22
Dun-miR-436		CTCCAGTGACATTTGTAGAGC	21
Dun-miR-437	miR-437a	CAGAGAACCGAAGCCAAACCC	21
	miR-437b	CAGAGAACCGAAGCCAAACCCT	22
Dun-miR-438		TCAAACTTGCAAACATCAATAATGACGG	28
Dun-miR-439		GCGGTATAGCGATGAGGGACC	21

Dun-miR-440		ACTTGGTGGTTTTGATACTGTC	22	
Dun-miR-441		ATTCGTAGTGACTTAAGTTCC	21	
Dun-miR-442		ATGTCTGCTATAGGGAATTCCGAAACT	27	
Dun-miR-443		AGATATCAATTCGACTCAGTAGA	23	
Dun-miR-444		TGTTTCTAAGTGCTCTTGAATTCCAAAT	28	
Dun-miR-445		TAAAAAGAACAGAGAAGACGTAGGTAGC	28	-
Dun-miR-446		CCCAGAATGCTGATAGTACTC	21	
Dun-miR-447		TTCTGGAGGTTGGAGCCGGCGGACT	25	
Dun-miR-448		TCTGACAGTTGGGACCGGAAA	21	
Dun-miR-449		TGAGATCGATGATTTAAACTCA	22	
Dun-miR-450	miR-450a	GTGAACTGGCCTGGGGCGACTT	22	
	miR-450b	GTGAACTGGCCTGGGGCGACT	21	
Dun-miR-451		TAATGGGATTATTTCATTACGCTGAGCC	28	
Dun-miR-452		TTTGATAATGCTGGTCCAACTAGACT	26	
Dun-miR-453		ACATTTGAGATGCAGAGGACC	21	
Dun-miR-454		TCTGATCAGGCATTGCCGCGAA	22	
Dun-miR-455		TAATACTGGACGTAGCTCATC	21	
Dun-miR-456	miR-456a	TATATGCGCTTGCACACTGAA	21	
	miR-456b	TATATGCGCTTGCACACTGAAC	22	
Dun-miR-457	miR-457a	TACTATTTGTGTTGGAGTCC	20	
	miR-457b	ATACTATTTGTGTTGGAGTCCT	22	
Dun-miR-458		TTACCATCCGGATGTGAATTCAG	23	
Dun-miR-459		TTTTAGAGAACTGCTTTGAACA	22	
Dun-miR-460		TAATTCACCCTGAGGAAGGTCTTACGAC	28	
Dun-miR-461		TATCTGAACTGCAAGAAGTACTAGGA	26	
Dun-miR-462		TATACCGACTTAACTTTTTCTATGAGAC	28	
Dun-miR-463		TGCTGAATCCATGCGGGCTGCA	22	
Dun-miR-464		TTCTATCCAATATGTAGCGAAGCCCTGT	28	
Dun-miR-465		TTTTATCATCATTGTCCAGACCTCTACC	28	
Dun-miR-466		CGAAAATTACGTTTGAGGACT	21	
Dun-miR-467		TCCGATATTTAGTCCGACTTAT	22	
Dun-miR-468		CATCTTTATCATCGTTTGGCC	21	
Dun-miR-469		TGAACCGATTGTACTGAATAATAGCCT	27	
Dun-miR-470		CATGATCCATGATTTATACTCA	22	
Dun-miR-3813		TCAAAGGTGTCTACACGTCGAAGGAATC	28	Tca-miR-3813-3p
Dun-miR-471		CAATCCAAAGACTCAGACCCT	21	
Dun-miR-472		TCAAAACTGTTCGGAATTAAATAGCGTT	28	
Dun-miR-473	miR-473a	TTCGATTGAGATGACACGTAC	21	
	miR-473b	TTCGATTGAGATGACACGTACT	22	
Dun-miR-474		TTCCAGAATTTTGAGCCGTCTCTATAGT	28	
Dun-miR-475		TAGCTCATCTGTGAACGATCT	21	
Dun-miR-476		TCTGTTGGCCTGACCACTTTCCT	23	
Dun-miR-477		GTGCAATTTTTGGAAGACCCCC	22	

Dun-miR-11618		AAACATTGTCTGATCAAAGAGG	22	Tca-miR-11618a-3-3p
Dun-miR-478		TCGGCCAATACTTTGAAAAGT	21	
Dun-miR-479		TGCTATTCCAGTGATAAATAGGACCATT	28	
Dun-miR-480		AATTCACATCCGCATGGTCAG	21	
Dun-miR-481		TAACTTTTCAGAGGATGAACT	21	
Dun-miR-11621		TCTTGCTTAAAAGCATCTGAATAGCGCC	28	Tca-miR-11621-3p
Dun-miR-482		AAACTAAAACACCTCTGTCCC	21	
Dun-miR-483		GTTCATTGTCGTTTCTGCACA	21	
Dun-miR-484		TTTTCGCTTTTATGTAGAACAATTGCCT	28	
Dun-miR-485		CTTTAGGCTCAGCAGGGTACC	21	
Dun-miR-486		TGTAAAGTGCGAGACGTCTGAATAAGGC	28	
Dun-miR-487		TTTAGAGATAGTATTAGGGCT	21	
Dun-miR-488		TAGATTTGGCTGATAATTTGGATGGAGT	28	
Dun-miR-489		TGATGAAACAGTGCTGTATACT	22	
Dun-miR-490		ACTGTGTCAAATGTGGAGGCA	21	
Dun-miR-491		CAAGAATTTTGTAAGCGGTTC	21	
Dun-miR-492		TGATATATACTACGCTGGGCA	21	
Dun-miR-493		TCTCTTTCCAGCCTACCTTTGACTAGAC	28	
Dun-miR-494		CACACTCTCCAACATTTTCGGCACCTGA	28	
Dun-miR-495		GAGAAGCGTCAAGTAGAGTTTT	22	
Dun-miR-496		TAAAAACGAATGTCCTGACACT	22	
Dun-miR-497		TGATCGTAGCCAATTTTGAATGTCCGCT	28	
Dun-miR-498		TCTATGGGATTTAATTCGCAATGATATG	28	
Dun-miR-499		TGTCAGGATTCTGTGAGAAGTC	22	
Dun-miR-500		TCTCTTTCCAGCCTACCTTTGACTAAAC	28	
Dun-miR-501	miR-501a	ACTCATCCCCCTTTTGGTGAAGT	23	
	miR-501b	ACTCATCCCCCTTTTGGTGAAG	22	
Dun-miR-502		TTCCATAGAAAGAACTCTTCC	21	
Dun-miR-503		TGTCATTGGTCGCTGGGGGT	20	
Dun-miR-11632		AACTCACACAAGTAATGACTGTAATAAG	28	Tca-miR-11632-5p
Dun-miR-504		TTTCCGATATGAGTAGAACATC	22	
Dun-miR-505		TGAACCCACACTGTCGATTTTCTCTGCA	28	
Dun-miR-506		CACCAAAGCACACGTCTGTACG	22	
Dun-miR-507		CTCACTTCCCCCTCTGTTGGCC	22	
Dun-miR-508		TTTCGGAATCGTCTGCAGACGA	22	
Dun-miR-509		TAGAAAGGAAGAGATCTGACT	21	
Dun-miR-510		TCCAGAGGTTGGGTGTTGGCAGAG	24	
Dun-miR-511		TCAATCAACCATGGGAGCTTATCGTCT	27	
Dun-miR-512		AGGAAAACTCTACTTGACGCT	21	
Dun-miR-513		CTATGCCGTCTGTAACTCAGT	21	
Dun-miR-514		ATTCTGTAGAAGCCCAGGGCC	21	
Dun-miR-515		TCTTGCCTGTTCAATTCTACATCGAATT	28	

Dun-miR-516		TGTCGACAGGGAGATAAATCACT	23	
Dun-miR-517		TTCATCAAGCTCTTCGAGGCAC	22	
Dun-miR-518		AATGGAGGAAACAATTGGCCT	21	
Dun-miR-519		TCATGATCGGAAGTTACTACCGTTTT	26	
Dun-miR-520		TCTGTCCGGGAAGTATCTTAGCTTTAGT	28	
Dun-miR-521		TCCTTCGAACGTAACAGAATATTTGCCT	28	
Dun-miR-522		TACAGAGATAATTTTCTTCTCAGCGGCT	28	
Dun-miR-523		TCCTAAAACCGAAAATCCTGCAGACA	26	
Dun-miR-524		TGTCCAGGTAGTAGATGGCTCTTAAGAT	28	
Dun-miR-525		TGTCTGGATCTAAAGCCGAACTGTGCTT	28	
Dun-miR-526		TTACGTGATGATATCAAGATTCTGACC	27	
Dun-miR-527		CTAACTTTTCAGAGGATGAACT	22	
Dun-miR-528		TGTGCTAACATTGCTTACCGAAGGAGCT	28	
Dun-miR-529		TAAGTGAAACGTCTTCTGGGA	21	
Dun-miR-530		TTCATGAACACGAGCTGGCCC	21	
Dun-miR-14	miR-14a	TCAGTCTTTTTCTCTCCCCTAT	22	Tca-miR-14-3p
	miR-14b	TCAGTCTTTTTCTCTCCCCTGT	22	Tca-miR-14-3p
	miR-14c	TCAGTCTTTTTCCCTCTCCTAT	22	Tca-miR-14-3p
Dun-miR-531		TAGGAGAAGACGAATGATACT	21	
Dun-miR-532		AGTTGTTATAAGCAGTTCTTC	21	
Dun-miR-533		TCAAACTTGCAAGCATCAATAATGACGG	28	
Dun-miR-534		TGCACGCCAGCTGAACCAATCCTGATCC	28	
Dun-miR-281	miR-281a	AAGAGAGCTATCCGTCGACAGTA	23	Tca-miR-281-5p
	miR-281b	AAGAGAGCTATCCGTCGACAGT	22	Tca-miR-281-5p
Dun-miR-535		TGAGATGCAGAGGACCTTGTC	21	
Dun-miR-536		GTGTCATTTTTGCTGGAGGCC	21	
Dun-miR-537		TCATTGATTGGGCAGGGCGAC	21	
Dun-miR-538		TACTGGAGCCTTTCTCTGATGG	22	
Dun-miR-315		TTTTGATTGTTGCTCAGAAAGCC	23	Tca-miR-315
Dun-miR-539		TTTCAGATTTTCAAATTCAGTGGGATTC	28	
Dun-miR-540		TGATGAACTATTTGATGAAGACTGAATA	28	
Dun-miR-541		TGACAAGAAAACTTAAGGCCT	21	
Dun-miR-542		ATGTCAAATACAACTGTGGCT	21	
Dun-miR-543		ACCCTCGACATCTGCTGTCCT	21	
Dun-miR-544		GTTGAAATCGTGGGGAGGGCT	21	
Dun-miR-545		CAAGCATACAGACTGGGGATC	21	
Dun-miR-546		CAATTTTAAACAACGTTGGCT	21	
Dun-miR-547		TGTTAATAACAATTGCCGGCC	21	
Dun-miR-548	miR-548a	TCCTGTACTGAGCTGCCCCGAGT	23	
	miR-548b	TCCTGTACTGAGCTGCCCCGAG	22	
Dun-miR-549		TAACTTCCACAGTGCCTACTCGGTTTCT	28	
Dun-miR-550		TACCAAGATTAGCTTGTAGAACAGCTTT	28	
Dun-miR-551		AAAGTGAAGTATCTCCGGACT	21	
Dun-miR-552		TTCATCAAGATCTTCGAGGCAC	22	

Dun-miR-553		CACTCTGATAGCCAAATGCCTCGT	24	
Dun-miR-554		TTAGTACGGCTCAACACTTAAGT	23	
Dun-miR-555	miR-555a	TCTTGCTCATTTTGGTGAAAGT	22	
	miR-555b	TCTTGCTCATTTTGGTGAAAG	21	
Dun-miR-556		GTAGAACATCATTAGAGGGTC	21	
Dun-miR-557		GTTAAAATGATGCCGGTACTC	21	
Dun-miR-558		TTCACTCGACTTTGTAGCAAAAGCTTTC	28	
Dun-miR-559		TTAACCAAACTGTCCTGTATCGC	23	
Dun-miR-560		TGCTTATGACAGATAGGACCT	21	
Dun-miR-561		TGTTAGATTAGCCATTTCCATCGCCAGT	28	
Dun-miR-252		CTAAGTACTAGTGCCGCAGGAG	22	Tca-miR-252a-5p
Dun-miR-562		TAAAATCGCCGGTCCATTTCTAGG	24	
Dun-miR-563		ACCTGGAGATCGAAGAACCT	20	
Dun-miR-564		TTAACCAGTTGGCTGTCCCGATGAAGCT	28	
Dun-miR-565		CTTAGAGATAGTAATAGGGCT	21	
Dun-miR-566		ATGTAATATCTGATTGGGTGT	21	
Dun-miR-567		ATAAGTCACAATTAACTGGTTTAGGGCT	28	
Dun-miR-568		TGAGAACTCTAAAGAAAGTGCGTTGATT	28	
Dun-miR-569		TTTTGGGATCATGAGAGTCTC	21	
Dun-miR-570		CACTGAGAGGGCGTCTGACGA	21	
Dun-miR-571		TCCTAAAAATTCGCATTATTCTGAGCCT	28	
Dun-miR-572		ATCTCACACTCAAACATGTCT	21	
Dun-miR-573		ATGTCATTTTTGCTGGAGGCC	21	
Dun-miR-574		CTCCAATGTCACTGTTTGTAT	21	
Dun-miR-575		CAAAGAGCTTGATGAATAACT	21	
Dun-miR-576		TTCTCATTGTCAGCCTGAACAT	22	
Dun-miR-577		TTAGAGAATGGTAAGAGACTTAT	23	
Dun-miR-578		TTTTGAACCTTTGGACTGTGGA	22	
Dun-miR-980		TAGCTGCCTTTTGAAGGGC	19	Tca-miR-980-3p
Dun-miR-579		TGCAATAGTCGTTTCAGCATCTGAGTCT	28	
Dun-miR-580		TCAGAAAATCGGCTCCTAGTATGGGTG	27	
Dun-miR-581		ACAGATAGTGCTTGGTAGACC	21	
Dun-miR-582		GGGAACTCTATCTGTGACATGTG	23	
Dun-miR-583		TGTACTCGTTTTCGGTCCAACAGAAAGA	28	
Dun-miR-584		CAATTCTGCAACATCGGCACA	21	
Dun-miR-585		TACAAATTTTTCCAATGGGTC	21	
Dun-miR-586		AAACAAATGAAAACTCTCACT	21	
Dun-miR-587		TCACTCAGTCTCGGTAGAAACT	22	
Dun-miR-588		TAAAATCTTCACCCCCTTATAGGTGCCT	28	
Dun-miR-589		CTGCTTCATGTGGTCGGAGTA	21	
Dun-miR-590		TACTACGGGTAGGACAAACTCG	22	
Dun-miR-591		CTGAGAAGCGTCAAGTAGAGT	21	
Dun-miR-592		TTTCAATGACGATGTAGGCT	20	
Dun-miR-593		CAAGCTTTCCAGATGGTCACGTATTTGT	28	

Dun-miR-594		TCCGTTTTTAATTAGTTCGTT	21	
Dun-miR-595		CACGATTCATCTTCAGGGAC	20	
Dun-miR-596		TTCCAGCTGTCAGATTTGAATCGCGAAC	28	
Dun-miR-597		CCAGATGCCAATTGTATGGAC	21	
Dun-miR-598		TTTAATTTGTGTGTCGCGCAGG	22	
Dun-miR-599		TCTGTTACCCTCTGTTACCCT	21	
Dun-miR-3880		ACCACTTCCGTCTCTGTTGGCT	22	Tca-miR-3880-3p
Dun-miR-600		CACTATCACCTTTTCTGGCTGA	22	
Dun-miR-601		TCAACCTACCTTCGCCTGCCT	21	
Dun-miR-602		AAGATTTAATTAAACTGTGCTC	22	
Dun-miR-603		TGAACTTGAGGACTCCAACACT	22	
Dun-miR-604		TCTATCAGCAATGCAGGGCAC	21	
Dun-miR-605		TTTATGGAAGACTTCGAGACCC	22	
Dun-miR-606		CGAAATTCGAAGGTCTGCACA	21	
Dun-miR-607		CCAATGGGTCTAGACTTATTC	21	
Dun-miR-608		GTAGAATGTCCAAGTCGGTAATT	23	
Dun-miR-609		CTGAAGACGCAGCGTTAATTTTTGCTAG	28	
Dun-miR-610		TCTTCTTAGTTCTCCTACACTA	22	
Dun-miR-611		TAAGAACCTGACTACTCTCAAGCCGAGT	28	
Dun-miR-612	miR-612a	TAATACGGGTAGGCCAAACTC	21	
	miR-612b	TAATACGGGTAGGCCAAACTCG	22	
Dun-miR-613		TAATATCGGCCAGGTAGGATTCCCATC	27	
Dun-miR-614		GGGAAGGTACTGTGCTCTCGGGAT	24	
Dun-miR-615		ATCTTGTCTGGAGGTAAGGCT	21	
Dun-miR-616		ATCAAAAAATATCTCAGGGCT	21	
Dun-miR-617		TGCAAGTAGATCCGCTGAGCT	21	
Dun-miR-618		CGTGAATCCCTGTCCTGGCATT	22	
Dun-miR-619		AATCTTTAGAATGTGCGATTT	21	
Dun-miR-620		TATTAAATGGTTTGATACTGTCA	23	
Dun-miR-621		AAAAACTTAATCAATGGGGCC	21	
Dun-miR-622		TCTGTAGAATCTGCTGACGAGG	22	
Dun-miR-623		GTGATTGACCCGATGTTGGCC	21	
Dun-miR-624		AATGAAAGTCAACAAGGTATT	21	
Dun-miR-625		TAGATTGATGATCACGTTCTGTC	23	
Dun-miR-626		GTCAGTGAACTGGCCTGGGGA	21	
Dun-miR-627		CAGATCAATCACTGTAGGGTT	21	
Dun-miR-628		TCGTAGAGATCTGTCTGAAGCT	22	
Dun-miR-629		ACGTTCCGACGTCACGAGGAC	21	
Dun-miR-630		GTCGAATATCAAAGTAGGGAATG	23	
Dun-miR-631		TCCTTTGATAATGTGGGCCAGT	22	
Dun-miR-632		TTTGGGATTGCTCACTGTGCC	21	
Dun-miR-633		TCAAAATGGAGCTTACGATTC	21	
Dun-miR-634		ACAATTTAATTAACGTTGGCT	21	
Dun-miR-635		GGGAGTTGTGAAAGAAGGATTC	22	

Dun-miR-636	TTGCTCTTTTTAGAGGACTGCT	22	
Dun-miR-637	TGATTTAGTATCTGACCGTCT	21	
Dun-miR-638	TAGACAAGAGATGTAGGTAACCAGCGCT	28	
Dun-miR-639	TCACTCTTAACAAAATGTCTG	21	
Dun-miR-640	TGATACATCGTCTGCTCTTTCTTTGCCT	28	
Dun-miR-641	TATGACTGTACGATGTGCACTC	22	
Dun-miR-642	TTACACGTATCAATAGAACAGCTGCTAT	28	
Dun-miR-643	ACATTAGGATAGAAGAGGGTT	21	
Dun-miR-644	TGAGAAACTCTTGCATTAGCT	21	
Dun-miR-645	GGGACAGTTAGTTTTTGAAGATCGCGCC	28	
Dun-miR-646	TAGTTCACTGTCTCTTAACCCATTGACG	28	
Dun-miR-647	TAGCTGTTTTCTTGGGCAGGGCA	23	
Dun-miR-648	TACAACTGCAACAGCCACAAC	21	
Dun-miR-649	AATAGAGAGATTATCAGGGGT	21	
Dun-miR-650	GTTTTTCGCGAATATCTGGCT	21	
Dun-miR-651	TGAGCTGGAGACTCGGGATAAAGACAGG	28	
Dun-miR-652	CAGATGGTAGATTTCATATTTATGGAA	27	
Dun-miR-7	CAAGGAATCACTAATCATCCCAT	23	Tca-miR-7-3p
Dun-miR-653	ATAGAGAGATTATCAGGGGTA	21	
Dun-miR-654	TCCATGTTGTTGGTAGTCTTTGGGCATC	28	
Dun-miR-655	TAATTCTTTTATCTCAGGGTT	21	
Dun-miR-656	TATCTCCCCCTTTTTGGTGAAG	22	
Dun-miR-657	GAAGTCTAAACAGTCGAGCTGT	22	
Dun-miR-658	GGGGATAGCACAGTAGGATTG	21	
Dun-miR-659	TCAAAAACAATTTCTCTATCCCACCAGC	28	
Dun-miR-660	TCATAAAGGATTGTGCAAAGTCTTCACC	28	
Dun-miR-661	GGAGAGTACTGTCAGGATTCT	21	
Dun-miR-662	TTAAAGCCACAATACGGATTCTGTCACC	28	
Dun-miR-663	AAATATCAATTCGACCTAGTAG	22	
Dun-miR-664	ATTTTCGGCTTGTTATGTTAC	21	
Dun-miR-665	ATGAAAACTAATGTAGTGCCTC	22	
Dun-miR-666	TTTGTCACATCTTATAAAACTCG	23	
Dun-miR-667	CCGGAGAATGTTGAGAATTCCA	22	
Dun-miR-668	TCAGGGAGTGGTAAGAAACTC	21	
Dun-miR-669	AATATTTCTGCGATGGCTGCC	21	
Dun-miR-670	TACTAATAGGACTAGTTCTGAAGGCACC	28	
Dun-miR-671	TTCCATTGCTAGTCGCGTAGAG	22	
Dun-miR-672	TAATATAACGGACTTGGGATCT	22	
Dun-miR-673	AATTATAATATTAGGCAGGACT	22	
Dun-miR-674	TAAGTCTTTCCTAGATTATGAGATCGGC	28	
Dun-miR-675	TTTTAGAGGACTGCTTTGAACA	22	
Dun-miR-676	CACATAGACCAACCAGGGCTC	21	
Dun-miR-677	TACCAGTATGCGTTGGCTTTC	21	
Dun-miR-678	TCGGATTCAATCGCTAGTATAGATTGGC	28	

Dun-miR-679		TAAACGACCACCAACTCTTAATAAACCT	28	
Dun-miR-680		ATTCTCGACTTCACCCTGACCT	22	
Dun-miR-681		TCACAGACTATCTACAGATTGT	22	
Dun-miR-682		TTGTTAGAGCGAGAACCCGTAA	22	
Dun-miR-683		ACTCAGGACGTGAATATGGCC	21	
Dun-miR-684		TAAAAGCCACAATACGTATTTTGTCACC	28	
Dun-miR-685		TTTTAAGTTGATCCAGTGCCT	21	
Dun-miR-686		AATTCGTTGGGATTTCTGCCT	21	
Dun-miR-687		TACCCGGTATCAATGTATCGCTTTGACT	28	
Dun-miR-688		CGAGAGTGCTTTGAAACGTAC	21	
Dun-miR-689		TTCAGGATTGCTACCAAACTGTT	23	
Dun-miR-690		TAAAGTGCACGTCTACCTGCT	21	
Dun-miR-691		TCAAAACAGCCCAACACTCAAGT	23	
Dun-miR-692		TTTGTACTCTCTGATCAAACTCT	23	
Dun-miR-693		TGTCAAGCCCCTTTTGGTGAAG	22	
Dun-miR-694		TTTATACTGAAGACGGCTTTTC	22	
Dun-miR-695		AAAACTGTACCAGTTGAACTTG	22	
Dun-miR-696		CGGACTTGGAGAAGTCGTGCAA	22	
Dun-miR-697		AGATGGACTAGCAATGGGTTC	21	
Dun-miR-698		TGAAACCAGTTCTCAGAGGATT	22	
Dun-miR-699		TTTTCTACAACGGAGTGCAAGCTGTCTC	28	
Dun-miR-700		CCCAAGGAAGCTGATAGGTTC	21	
Dun-miR-701		GAGAAGACGTCGTTAGTTATGT	22	
Dun-miR-702		TTGTGGAAAACGAAGCTGGAGA	22	
Dun-miR-703		TTTTGAATATTTGCACTTTAGTCATCGG	28	
Dun-miR-704		TTATCTGGATTGGGTTTTCTCT	22	
Dun-miR-705		CATTATTATTGCGACTTGGGA	21	
Dun-miR-706	miR-706a	TTGCAGATTTTGTGACCAAATG	22	
	miR-706b	TTGCAGATTTTGTGACCAAATGA	23	
Dun-miR-707		ATTCATCAAGCTCTTCGAGACT	22	
Dun-miR-708		CTCCAATAAGGGTCTCTGAATT	22	
Dun-miR-709		TATCCGGGTGCTTAACAGTTGCTAACTC	28	
Dun-miR-710		TACCAGTAGTCTCTAACCACATT	23	
Dun-miR-711		ATGAGAAAGGAAGCTGGGCACT	22	
Dun-miR-712		TGTGCGATTTGATCAAGGCAGT	22	
Dun-miR-713		ATGTTTGGACCAACCACTGCCT	22	
Dun-miR-927		CAAAGCGTCTGGATTCTGAAT	21	Tca-miR-927a-3p
Dun-miR-714		ACAATGTGCCTCAAAGAGCTT	21	
Dun-miR-715		TAGTAGTGTACTGTATCATGCT	22	
Dun-miR-716		TGTGCCAGACTCTGTCAAATGCTTTACT	28	
Dun-miR-717		GAAGAGAAAATAAGGAGGACCT	22	
Dun-miR-718		ATCCCAAAGACTGTCTGGTTCT	22	
Dun-miR-719		CCACTTAGTCTGTTAACACCC	21	
Dun-miR-720		TGTGAAGAGTCAGAATCGGACATGCCT	27	

Dun-miR-721	TGAATGGGCATCTTTCGGTTTACGACTT	28
Dun-miR-722	TATGAGGAAGTTGAAGTAGAAATATGCT	28
Dun-miR-723	TGCTATCGCTTTGTTGAATTAAATGGCC	28
Dun-miR-724	TCGATAAATCAATTGTGGGACT	22
Dun-miR-725	ATCGAAATGCATTGTGGGATA	21
Dun-miR-726	GAAATTGAAGGCGGAAGGGGC	21
Dun-miR-727	TGATATCATTCTAAGTTGGTC	21
Dun-miR-728	TATACACATGTTCCTAGGGGC	21
Dun-miR-729	AAAGATGCATTCGATCAGGTCT	22
Dun-miR-730	ACTTTGGCTATGGAAATTGACC	22
Dun-miR-731	TCAAAACAAAATTTCCAATTGTCATGTC	28
Dun-miR-732	ACTCATCCCCTTTTTGGTGAAGT	23
Dun-miR-733	TAAGGCAGTTAGGACAAACTC	21
Dun-miR-734	TAATTCGGAAGTTGATTACACC	22
Dun-miR-735	TATTATATCCTCACTTAGGCCT	22
Dun-miR-736	TAAAACCTCGATATAACGGACT	22
Dun-miR-737	TGAGATATTTAAAACGCTGACC	22
Dun-miR-738	AAAGTGTGACAGTTCTTCTTC	21
Dun-miR-739	CATTGTAAAGTTTCCAGGTTTT	22
Dun-miR-740	AGAAGATGACGATACTTTGGCT	22
Dun-miR-741	ATTTTTCGAAGAAATAGGACC	21
Dun-miR-742	CATACGGATCCCAAAAGTGTTTTACCCT	28
Dun-miR-743	CCACTCTGATAGCCAAATGAAAA	23
Dun-miR-744	TATTTCACAACGGTCAAAAACC	22
Dun-miR-745	TTATACAGGTACGACAAACTCG	22
Dun-miR-746	TCAGTGCCGTTTGACAAGA	19
Dun-miR-747	TGAAAAAACCTGACGCACCTTATAGCCT	28
Dun-miR-748	TACGACTCGAATGGACCATGGATTTGCT	28
Dun-miR-749	AATGAATCGGAGCTTCTGCACT	22
Dun-miR-750	GGAGAATTTTTGATGGAGAGCT	22
Dun-miR-751	AACACCAGGATAAATGAACATT	22
Dun-miR-752	AGATTGTAGCTACATTTGTCCT	22
Dun-miR-753	TTAAAGCAACCCTTTTCGGCT	21
Dun-miR-754	ATTTGGCCAAGACTCTACAAC	21
Dun-miR-755	CAAACTTAGTACTTCTGTAGC	21
Dun-miR-756	CGACAAGGTATGTTTAACTCT	21
Dun-miR-757	TAAAAGCTCCAAGGTAGAACCTC	23
Dun-miR-758	TGTAACGAAATTTGCACGTCCT	22
Dun-miR-759	TATCAGTTCTCAATCATGGTC	21
Dun-miR-760	AACGGCTATGAATAACTATTC	21
Dun-miR-761	TGAGCAAGAATTTATTCGGGC	21

Appendix B - miRNA End Sequences

Table A.4. Complete list of end motifs by miRNA length.

Number of nt	5'	Count	3'	Count
17 nt	CAA	1	ACT	1
	CCC	1	CCT	1
18 nt	AAC	1	AAC	1
	ATC	1	ACA	1
	CAG	1	ACT	1
	TAC	1	CCG	1
	TCA	1	GCC	1
19 nt	GTA	1	ACC	1
	GTT	1	AGA	1
	TAG	1	GCC	1
	TCA	1	GGC	2
	TCT	1		
20 nt	TCA	3	GTC	3
	CAC	2	ACG	2
	TGA	2	ACT	2
	TGG	2	CCA	2
	TGT	2	GAC	2
	TTT	2	GAG	2
	ACC	1	GCA	2
	AGT	1	GGG	2
	CAT	1	ACC	1
	CCG	1	AGG	1
	CCT	1	ATC	1
	CGT	1	CCT	1
	CTA	1	CGT	1
	GAT	1	GCC	1
	GCC	1	GCT	1
	GGA	1	GGA	1
	GGC	1	GGT	1
	GGT	1	TCC	1
	GTC	1	TCT	1
	TAC	1		
	TGC	1		
21 nt	TGA	13	GCC	17
	TAA	12	GCT	17

TTT	12	CCT	16
AAA	11	CTC	14
ACA	10	ACT	13
CAA	10	TTC	11
AAT	7	TCT	10
ATT	7	ACC	9
TCA	7	ATC	7
TTC	7	CCC	6
CGA	6	GAC	6
TAG	6	GGA	6
ATC	5	GTC	5
CAT	5	TCC	5
GTT	5	ACA	4
TAT	5	AGT	4
TCT	5	CTT	4
CAG	4	GGC	4
CCA	4	GTA	4
TAC	4	GTT	4
TTG	4	TAC	4
ATA	3	AAC	3
ATG	3	ATA	3
CAC	3	ATG	3
CTG	3	GAA	3
CTT	3	GCA	3
GGA	3	TGT	3
GTA	3	AAA	2
GTG	3	AAG	2
TGC	3	AAT	2
TGG	3	ACG	2
TGT	3	AGC	2
TTA	3	ATT	2
AAC	2	CCA	2
ACG	2	CGA	2
ACT	2	CGT	2
AGA	2	GTG	2
AGG	2	TAT	2
AGT	2	TTA	2
CCC	2	TTT	2
CCG	2	AGA	1
CGT	2	AGG	1
CTA	2	CAC	1
CTC	2	CAG	1
GAA	2	CGG	1
GAG	2	CTG	1

	ACC	1	GGG	1
	AGC	1	GGT	1
	CGC	1	TTG	1
	GAC	1		
	GCA	1		
	GCC	1		
	GCG	1		
	GGG	1		
	GGT	1		
	GTC	1		
	TCC	1		
	TCG	1		
22 nt	TTT	17	CCT	18
	TGA	14	ACT	17
	TAA	9	ACC	10
	TAG	9	ATG	10
	TCT	9	GCT	8
	AAA	8	CTC	7
	TAT	7	GCA	7
	TCA	7	TAT	7
	TCC	6	TCG	6
	TGT	6	TCT	6
	TTC	6	TGT	6
	TTG	6	AGG	5
	CTA	5	AGT	5
	TAC	5	ATC	5
	TGG	5	CCC	5
	AAG	4	GCC	5
	ACT	4	ACA	4
	ATG	4	ACG	4
	ATT	4	AGA	4
	CAT	4	ATT	4
	CTC	4	GTC	4
	GAA	4	TGA	4
	GTG	4	TTT	4
	TCG	4	AAG	3
	TTA	4	GAA	3
	AAT	3	GAC	3
	AGA	3	GAG	3
	CAC	3	GGC	3
	CGG	3	TCA	3
	GAG	3	TCC	3
	TGC	3	TGG	3

	AAC	2	TTC	3
	ACC	2	AAC	2
	AGC	2	AGC	2
	ATA	2	CAC	2
	ATC	2	CCA	2
	CAG	2	CGA	2
	CCG	2	CTT	2
	CGT	2	TAA	2
	CTG	2	TGC	2
	CTT	2	TTA	2
	GGA	2	TTG	2
	GGG	2	CAA	1
	ACG	1	CAG	1
	AGG	1	CAT	1
	AGT	1	CGC	1
	CAA	1	CTA	1
	CCC	1	GGA	1
	CCT	1	GGT	1
	CGA	1	GTA	1
	CGC	1	GTG	1
	GCA	1	TAG	1
	GCT	1		
	GGC	1		
23 nt	ACT	5	AGT	6
	TAT	4	GTC	4
	TCA	4	TCA	4
	TGA	4	TGA	4
	TTA	4	ACT	3
	TAA	3	ATT	3
	TTG	3	GCT	3
	TTT	3	TCT	3
	AAC	2	AAT	2
	AAG	2	ACA	2
	AGG	2	ACC	2
	ATG	2	ATG	2
	CGA	2	CCT	2
	TAC	2	GCA	2
	TAG	2	GCC	2
	TCT	2	GGT	2
	TTC	2	GTT	2
	AAA	1	TCG	2
	ACG	1	AAA	1
	AGA	1	ACG	1

	AGC	1	AGA	1
	CAA	1	AGC	1
	CCA	1	ATA	1
	CCC	1	CAG	1
	CCG	1	CAT	1
	CCT	1	CGC	1
	CGG	1	CGG	1
	CGT	1	CTC	1
	CTA	1	CTG	1
	CTT	1	GCG	1
	GCC	1	GGG	1
	GGG	1	GTA	1
	GGT	1	GTG	1
	GTA	1	TAT	1
	GTC	1	TGG	1
	GTT	1	TTG	1
	TCC	1	TTT	1
	TGT	1		
24 nt	AAT	2	GCT	3
	TAA	2	ACG	2
	TAT	2	ACT	2
	TCT	2	GAT	2
	ACT	1	TTT	2
	ATG	1	ACA	1
	CAC	1	AGG	1
	CAG	1	AGT	1
	CGT	1	ATG	1
	CTA	1	CGT	1
	GAA	1	GAG	1
	GCC	1	GCA	1
	GCG	1	GGA	1
	GGG	1	GTC	1
	GTT	1	TAT	1
	TCC	1	TCT	1
	TGA	1	TGA	1
	TGC	1		
	TGG	1		
25 nt	ATT	2	ACT	2
	TAC	2	GAC	2
	AAT	1	GCA	2
	ACC	1	AGT	1
	ACT	1	ATA	1

	AGG	1	CTC	1
	ATC	1	CTG	1
	GAG	1	CTT	1
	GGG	1	GGT	1
	GTA	1	GTC	1
	GTT	1	TGC	1
	TGA	1	TGG	1
	TTC	1		
26 nt	TAT	2	ACT	3
	TTT	2	GGC	2
	CCC	1	ACA	1
	CCG	1	AGT	1
	GAG	1	CCT	1
	TAA	1	CTT	1
	TCA	1	GGA	1
	TCC	1	TTC	1
	TCT	1	TTT	1
	TGC	1		
27 nt	TTT	12	CCT	10
	TAT	9	ACT	8
	TTA	9	TCT	7
	TAC	8	GCC	5
	TGA	8	ATC	4
	TAA	6	TAT	4
	TCA	6	CCC	3
	TAG	4	CTT	3
	TGT	4	GAC	3
	ATA	3	GAT	3
	TCG	3	GCT	3
	TTC	3	TAA	3
	TGC	2	ACC	2
	AAC	1	AGA	2
	AAT	1	ATA	2
	ATG	1	GAA	2
	ATT	1	GAG	2
	CAA	1	GGA	2
	CAG	1	GGC	2
	CCA	1	GTC	2
	CTG	1	GTT	2
	GAA	1	AAC	1
	GAC	1	ACA	1
	GTG	1	AGT	1

	TCC	1	ATG	1
	TCT	1	ATT	1
	TTG	1	CAC	1
			CAT	1
			CTA	1
			CTC	1
			GGG	1
			GGT	1
			GTG	1
			TAG	1
			TGA	1
			TGC	1
			TGG	1
			TTT	1
28 nt	TAA	28	CCT	21
	TAC	20	ACT	19
	TGT	19	ATT	15
	TGA	17	GCC	12
	TTT	16	GCT	12
	TCC	14	GGC	11
	TAT	13	ACC	9
	TCA	13	AGC	8
	TGC	12	AGT	8
	TCT	11	ATC	8
	TTC	11	TGT	7
	TTA	10	CTG	6
	TAG	6	GAC	6
	TTG	5	GTT	6
	TCG	4	TCC	6
	CAC	3	GTC	5
	AAG	2	AAC	4
	ACA	2	AAG	4
	AGA	2	AAT	4
	ATA	2	AGG	4
	CGC	2	CGG	4
	CTG	2	AGA	3
	GGG	2	CTC	3
	AAC	1	CTT	3
	ATT	1	TAT	3
	CAA	1	TCT	3
	CAT	1	TGC	3
	CCC	1	TTC	3
	CGA	1	ATA	2

	GCA	1	CAC	2
	GGA	1	CCC	2
			CGC	2
			GGT	2
			TTT	2
			AAA	1
			ACG	1
			ATG	1
			GAG	1
			GAT	1
			GCA	1
			GGA	1
			GGG	1
			TAG	1
			TGA	1
			TGG	1
			TTG	1
29 nt	TAT	1	CTT	1