THESIS

CROSS-KINGDOM MICRORNA DETECTION AND INFLUENCE OF DIET ON ENDOGENOUS EQUINE MICRORNAS

Submitted by

Lisa C. Nulton

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2014

Master's Committee:

Advisor: Jason E. Bruemmer

Gerrit Bouma
Tanja Hess

ABSTRACT

CROSS-KINGDOM MICRORNA DETECTION AND INFLUENCE OF DIET ON ENDOGENOUS EQUINE MICRORNAS

Every year the equine industry spends millions of dollars on research to enhance equine performance, whether it be reproductive or athletic. Equine nutrition is a leading contributor to equine performance and health, with poor nutrition and diet being associated with an ever-increasing incidence of metabolic related disorders. However, there is a lack of clinical markers to diagnose disease processes associated with poor nutrition or nutrient absorption. It has been well established that changes in nutrient intake can have a direct impact on health, but new evidence has emerged indicating that diet can influence endogenous miRNA and consequently gene expression. Also, recent research has identified the presence of exogenous plant miRNA in mammalian serum as a result of food consumption, but the role these miRNAs have on physiology is unclear. miRNAs are small, non-coding RNA molecules that post-transcriptionally regulate mRNA to silence gene expression via translational repression or degradation. We first hypothesized that diet-derived plant miRNAs can be detected in serum and tissue. For this study, twelve horses were randomly assigned to one of three groups (n=4/group) and fed alfalfa hay, extruded corn and alfalfa hay, or rice bran and alfalfa hay. Additionally, multiple tissues were harvested from four necropsied horses for detection of plant miRNAs in tissue. Our results reveal the presence of plant miRNAs in the serum and tissue of horses. Using an mRNA target prediction database, we were able to identify several potential mRNAs that could be regulated by exogenous plant miRNAs. These results suggest that diet-derived plant miRNAs enter into the

circulation of the horse and are capable of being taken up by tissues. This data is important for understanding the role diet-derived plant miRNAs may have on equine biological processes. Furthermore, these results suggest a novel way in which plant miRNAs function as not only a nutrient in equine diet but also as a potential regulator of endogenous mRNA.

Due to the large influence diet can have on the health of horses, we were then interested in investigating the role of diet on endogenous miRNAs in the horse. We hypothesized that either a rice bran (high-fat) or corn (high-non-structural carbohydrate) diet will alter the relative endogenous miRNA profile in horse serum exosomes. For this study we utilized the same serum samples collected from the feed trial horses in the previous study. Our results discovered 37 differentially expressed ($P \leq 0.05$) miRNAs in horses fed the corn diet high in NSC (starch and simple sugars) 23 days after feed treatment began. Pathway analysis of significantly different ($P \leq 0.05$) miRNAs indicated gastrointestinal disease, hepatic system disease, connective tissue disorders and inflammatory diseases as the primary predicted networks in which the differentially expressed miRNAs are involved. The horses fed rice bran (high-fat diet) exhibited 11 miRNAs that were differentially expressed on day 23. Cellular development, growth and proliferation were predicted to be the primary networks associated with these differentially expressed miRNAs. Finally, 11 miRNAs were different at day 23 in horses fed alfalfa. Ingenuity pathway analysis (IPA) revealed that these miRNAs are associated with connective tissue disorders and inflammatory response. This data indicates that dietary changes can result in altering endogenous miRNA. This information is important to understanding the influence nutrition has on gene expression and thus on health and disease.

ACKNOWLEDGMENTS

Many people in my life deserve thanks for their continuous support throughout my pursuit of a Master's degree. First, I would like to thank my primary adviser, Dr. Jason Bruemmer, for your guidance, support, and for all the knowledge you have shared. I would also like to thank my committee members Dr. Gerrit Bouma and Dr. Tanja Hess for your encouragement, patience, and contributions to my research. I have enjoyed working with all of you and am thankful for the all of the opportunities I was provided throughout my career as a graduate student. I owe much of my success and professional development to all of you.

Also, I would like to sincerely thank all my fellow graduate students that have worked with me over the last two years and have become close friends. To Ellie Cleys, Kristin Klohonatz, and Vanessa Enriquez, I would have never learned anything in the lab if it wasn't for the three of you. I could never thank you enough for the time and energy you put forth in teaching me and the patience all of you had for my mistakes and questions. To Aeriel Belk, your support in the lab was greatly appreciated and your cheerful attitude was always a breath of fresh air. To Kathleen Eddy, your friendship and encouragement was always valued. I would have never been able to make it without all of you. Working at the Animal Reproduction and Biotechnology Laboratory with all of you was an incredible experience.

However, I would have never gotten to where I am today without the endless support, mentorship, and inspiration from Dr. Fred Hofsaess "Doc". Your continuous guidance and

encouragement gave me the confidence to pursue my goals. Thank you so much for teaching me the importance of loving what you do and for always being there when I needed advice.

Most importantly, I would like to thank my family. Mom and Dad, your unwavering faith in me is to thank for where I am today. I don't think that either one of you knew what you were getting into when you allowed me to start riding lessons as a child or even when I bought Toby. But, as my passion for a career with horses grew your support stayed strong. You have given me access to all the opportunities I could ever ask for and have taught me the value of determination and a strong work ethic.

Finally, to Eric, you will never know all the ways in which your love, friendship, and belief in me helped me over the last few years. I do not think I would have achieved all that I have without you. At times, I think you believed I would never end my career as a student, but I will be forever grateful for your support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

It has been well-established that a horse's optimal health and performance is influenced greatly by food quality, exercise, metabolism, stress, and genetics. Horses' gastrointestinal tracts are built to manage continuous forage intake due to their evolutionary development as grazing animals. The majority of the domestic horse population is subject to diets consisting of grain concentrates and short feeding times. These "unnatural" feeding practices along with other environmental stressors, such as high exercise intensity, have impacted the health and performance of horses. However, evaluating performance in an animal such as the horse is difficult. In food production animals, performance can be measured via weight gain, weaning weight, or milk production. In the horse this is more subjective, making nutrition research results hard to implement in industry. There are also several pathologies associated with diet in the horse including Equine Metabolic Syndrome and laminitis. These disease states are not yet completely understood. Research investigating the affect of diet on gene expression and reliable, clinically relevant diagnostic markers associated with diseases related to poor nutrition is lacking. Recently, dietary influence of small RNA molecules (miRNAs) and the presence of food-derived exogenous miRNAs in mammalian serum have been identified (Zhang et al., 2012; García-Segura et al., 2013).

miRNAs are small, single stranded, non-coding RNAs that are 19-24 nucleotides in length (Krol, Loedige, et al., 2010). miRNAs have emerged as a critical post-transcriptional regulator of mRNA through binding of complementary sites in the 3' untranslated regions of the target gene. These miRNAs are known to play a key role in many developmental and cellular processes

including: developmental timing, tissue differentiation, maintenance of tissue identity, and sensing nutrient stress (Ambros, 2004; García-Segura et al., 2013). Furthermore, cancer and other diseases have been linked to the misregulation of miRNAs. These endogenous miRNAs can be packaged into exosomes and secreted from most cells into circulation (Valadi et al., 2007).

This study is the first to investigate the presence of exogenous diet-derived plant miRNAs as well as the influence of diet on endogenous miRNA expression profiles in the horse; providing insight into the novel idea of food-derived miRNAs entering circulation and their transport to tissues. Additionally, the direct effect on endogenous miRNA expression following either a diet higher in fat (rice bran), a diet higher in nonstructural carbohydrates (corn), or a control diet (alfalfa only) will be examined.

CHAPTER I: LITERATURE REVIEW

CROSS-KINGDOM microRNA REGULATION

Several studies have demonstrated health benefits, such as reducing risk of disease and managing type II diabetes, associated with plant-based diets (Kozuka et al., 2012a; Jenkins et al., 2013; Patrick et al., 2013). Also, it has been established that plant diets contribute to mammalian cell homeostasis particularly within the digestive system (Kozuka et al., 2012b). However, there is little understanding of the mechanisms related to the health effects that these diets seem to provide. Recent experimental studies have suggested a novel idea that diet-derived exogenous plant miRNAs are transferred to blood and can be transported to tissues of vertebrates (Zhang et al., 2012; Mu et al., 2014). With new evidence that reveals the potential for nutrients to have an influence on the regulation of genes, there has been great interest in attempting to repeat these results and determine the impact of exogenous miRNAs (xenomiRNAs) on the organism that ingests them.

It has been established that *C. elegans,* either soaked in a solution with double-stranded RNA (dsRNA) or feeding bacteria that express dsRNA, absorbs the molecules in the intestine resulting in RNA interference (RNAi) (Tabara et al., 1998a; Timmons and Fire, 1998). The transport of ingested environmental dsRNA, into an organism, is possible due to the action of SID-2, a transmembrane protein expressed in the intestine, binding the dsRNA for internalization. SID-1, a transmembrane protein present in cells sensitive to systemic RNAi, works as a channel for passive uptake of dsRNA into cellular cytoplasm of *C. elegans* (Winston et al., 2002; Feinberg and Hunter, 2003). This SID-2/SID-1 mechanism allows for RNAi in distant cells throughout the

organism. However, when either SID-1 or SID-2 are not expressed (in a nematode) the animal is resistant to RNAi mediated by ingested dsRNA (Feinberg and Hunter, 2003; Winston et al., 2007a). Although, SID-2 is not highly conserved, other organisms have been reported to internalize ingested dsRNAs via endocytosis (Price and Gatehouse, 2008). In addition to *C. elegans*, insects, shrimp, and two species of sponge have now been reported to have the ability to take in environmental RNA (Witwer and Hirschi, 2014).

SIDT1, a human ortholog of SID-1, has been reported to be localized at the cell membrane, increase siRNA passive internalization, and facilitate RNAi when overexpressed in pancreatic ductal adenocarcinoma cell lines (Duxbury et al., 2005). Moreover, others have demonstrated that siRNAs conjugated to cholesterols (Wolfrum et al., 2007) or encapsulated siRNAs (Kriegel et al., 2013) administered orally can be taken up by the intestine. Investigators also describe a contact-dependent uptake and intercellular transfer of human miR-21 in cultured human adenocarcinoma cells (Elhassan et al., 2012). This data demonstrates a potential mechanism in which orally administered small non-coding RNAs can be internalized by intestinal cells and play a role in RNAi in nematodes and insects. After years of research to determine a potential mechanism for oral delivery of RNA interference molecules in vertebrates, there is still very little data verifying the uptake of diet-derived RNAs in mammals. A recent publication described the detection of plant miRNAs in the serum and tissue of several mammals. This study revealed that diet-derived plant miRNAs entered general circulation of the mammalian organism that ingested it with no intervention to stabilize the ingested miRNA such as conjugation to cholesterols. XenomiRNAs were also detected in several tissue types of mice at levels comparable to endogenous miRNAs (Zhang et al., 2012). Additional data within this study

demonstrated mice fed a rice diet had a significant increase in plant miR-168a concentrations in serum and liver six hours after feeding as compared to animals on a chow diet, low in plant material. Researchers conducting this study also reported a down-regulation of the low-density lipoprotein receptor adapter protein 1 (LDLRAP1) in mice hours after dietary plant miRNA was consumed (Zhang et al., 2012). Although this is the first study that characterizes the ability of xenomiRNAs to be absorbed from food in mammals, there has been evidence that RNAs in a mother's breast milk may be available for absorption in an infant's intestinal tract and assist in gene regulation (Munch et al., 2013). There is currently no evidence of direct transfer of miRNAs from mother to infant, but pathway analysis has revealed potential interaction of exosomal miRNAs, in breast milk, with regulatory pathways in the infant.

Several investigators have now sought out to confirm the uptake of diet-derived miRNAs in vertebrates with little success. Researchers have reported an insignificant level of plant miRNAs in human, non-human primates, mice, and bees fed diets high in xenomiRNAs (Jiang et al., 2012; Witwer et al., 2013). There has also been little evidence to show an increase in plant miRNAs after administration through the diet (Snow et al., 2013; Witwer et al., 2013). There are several factors that may impact the analysis of xenomiRNAs in vertebrates including; consistent or appropriate endogenous miRNA normalizers, understanding of the uptake mechanisms, and digestion/metabolism differences in animal models or cell culture.

Zhang et al. (2012), showed that plant miR-168a, when transfected into human intestinal epithelial Caco-2 cells in culture, are packaged into microvesicles and released into culture media. They then treated HepG2 (hepatocyte carcinoma cell line) cells with the microvesicles

containing the plant miR-168a and detected a significantly elevated level of the plant miRNA in the HepG2 cells (Zhang et al., 2012). This suggests that xenomiRNAs that enter the small intestine can be transported into the intestinal epithelium and packaged into microvesicles to be released and transported to other tissues.

Recent data has identified plant derived exosome-like nanoparticles (EPDENs) in several fruits and vegetables including grapes, ginger, carrots, and grapefruit (Mu et al., 2014). These EPDENs are similar to mammalian exosomes in size, charge, and composition carrying proteins, lipids, and miRNAs. Using confocal microscopy, EPDENs were shown to co-localize with macrophages and stem cells within the intestinal tissue of mice fed EPDENs. This discovery provides additional evidence that mammalian intestinal cells are capable of taking up plant miRNAs.

MicroRNAS

MicroRNAs (miRNAs) are small, single stranded, non-coding RNAs that are 18-24 nucleotides in length. Endogenous miRNAs mediate post-transcriptional mRNA expression through the binding of complementary sites in the 3' untranslated regions of the target gene. miRNAs were first described as small RNAs capable of silencing mRNAs translation in *C. elegans* (Fabian et al., 2010). The miRNAs identified by investigators were lin-4 and let-7 and regulated the timing of larval development (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000). Shortly after the discovery of these two small RNAs investigators reported findings of both lin-4 and let-7 in worms, flies, and mammals (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miRNAs continued to be identified in many mammals (Chen et al., 2004) and plant

species (Aukerman and Sakai, 2003; Palatnik et al., 2003; Chen, 2005). It has since been reported that some plant miRNA families are conserved across plants (e.g. miR166); as others (e.g. miR156) are only well characterized in monocots (corn, rice, grasses) (Zhang et al., 2009). miRNAs have been demonstrated to play a key role in many developmental and cellular processes including: developmental timing, tissue differentiation, maintenance of tissue identity. Currently it is thought that miRNA play a regulatory role in approximately 50% of human protein coding genes (Krol, Loedige, et al., 2010). Furthermore, cancer and other diseases have been linked to the dysregulation of miRNAs (Krol, Loedige, et al., 2010).

Biogenesis

Primary miRNAs (pri-miRNAs) are transcribed from independent miRNA genes or part of introns of protein-coding genes by RNA polymerase II (pol II) in the cell nucleus (Lagos-Quintana et al., 2001; Lau et al., 2001; Meister and Tuschl, 2004). These transcripts are formed into pri-miRNAs, which are hairpin loop structures that are greater than 100 nucleotides long and have a 5' cap and a 3' poly (A) tail. Plant pri-miRNAs are highly variable in length, ranging from 250 to 2,500bp (Zhang et al., 2009). The folded end of the hairpin consists of an unpaired bulge (Lee et al., 2002). While still in the nucleus, RNase III enzyme Drosha (Dcl1/HYL1 in plants) and dsRNA-binding protein DGCR8 cleave the 5' and 3' arms of the pri-miRNA to produce precursor miRNA (pre-miRNA) (Grishok et al., 2001; Reinhart et al., 2002; Kim, 2005; Bologna et al., 2009). The approximately 70 nucleotide long hairpin pre-miRNA has a ~2 nucleotide 3' over hang. Pre-miRNAs in animals are transported to the cell cytoplasm by Exportin 5 (Lund et al., 2004). The pre-miRNA couples with RNase III enzyme Dicer and binding protein TRBP, resulting in cleavage of the hairpin loop and formation of a miRNA duplex (Cullen, 2004). In

plants, Dcl1 carries out this cleavage step while the pre-miRNA is still in the nucleus (Kim, 2005). The resulting miRNA duplex 3' overhang is methylated by Hua Enhancer 1 (HEN1) (Yu et al., 2005) and then transported out of the nucleus by the Hasty protein (HST) (Park et al., 2005). These processes are illustrated in Figure 1.

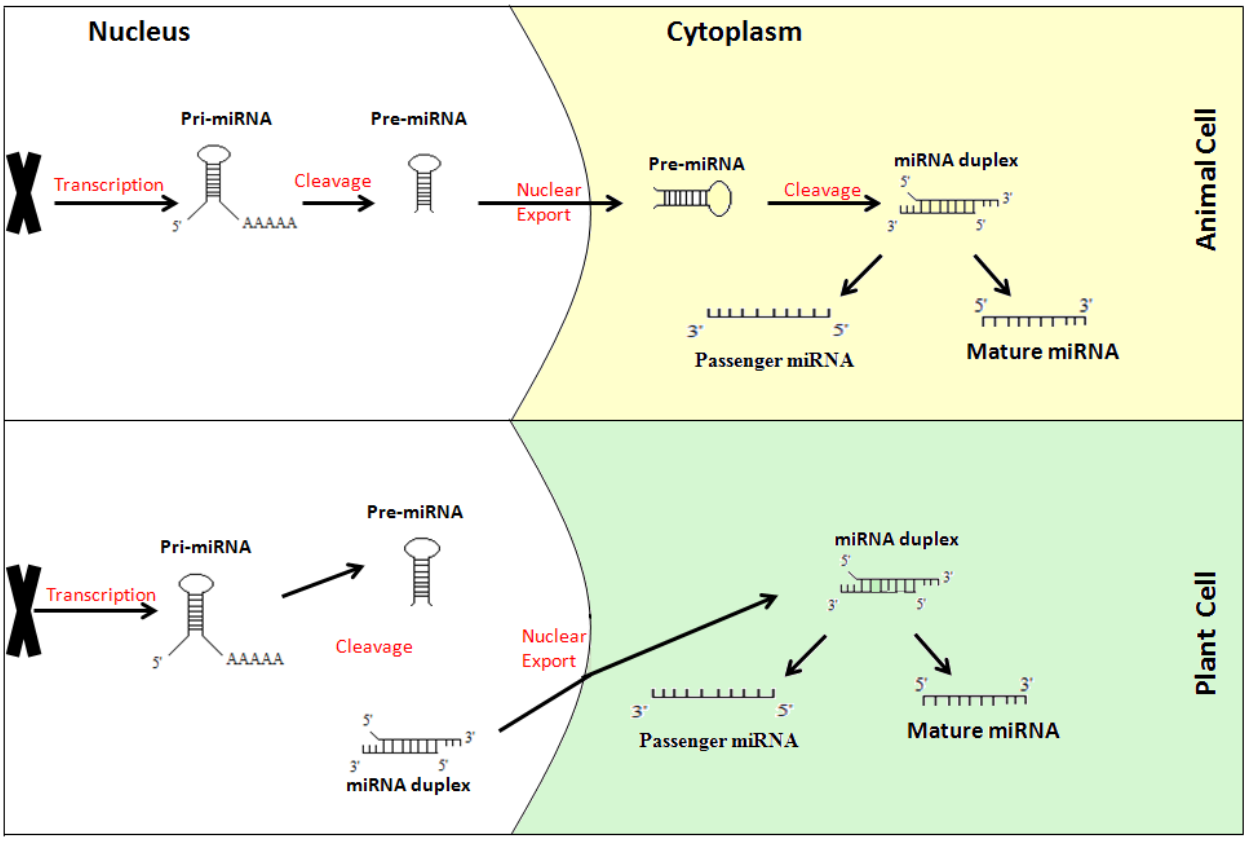


Figure 1: Depiction of miRNA biogenesis in animal verses plant

Argonaute proteins assist in the assembly of the miRNA-induce silencing complex (miRISC) with the mature strand of the duplex (Hutvágner and Zamore, 2002; Mourelatos et al., 2002). The miRISC complex consists of a mature miRNA, Argonaute 2 protein, and glycine troptophan protein (GW182) (Liu et al., 2005). In plants, Dcl1 associated miRNA duplex recruits Argonaute 1 protein and other undefined accessory proteins (Morel et al., 2002). The miRNA duplex is

loaded into Argonaute (AGO) proteins as dsRNA. The guide strand is identified as the stand with lower thermodynamic stability at the 5' end (Khvorova et al., 2003). The two strands are then unwound and the passenger strand is degraded, while the mature guide strand is retained in the Ago protein (Tomari et al., 2007; Kawamata et al., 2009). Although, the mechanism of the separation of the double stranded miRNA duplex is not fully understood, in both flies and humans it has been reported that the unwinding of the miRNA duplex is promoted by mismatches in the seed region or the mid-3' region (guide positions 12-15) (Tomari et al., 2007; Kawamata et al., 2009; Yoda et al., 2010). This process results in a single strand mature miRNA incorporated with the miRISC that is relatively stable in comparison to its precursors pri-miRNA and pre-miRNA (Winter et al., 2009).

Regulation of miRNA Biogenesis

The regulation of miRNA biogenesis is multifaceted. Due to the great deal of proteins and enzymes involved in the production of a single miRNA there are many levels in which they can be regulated. To begin, the potential of a miRNA binding to the gene it is transcribed from makes a perfect model for potential feedback mechanisms by repressing the translation of the mRNA that encodes for that miRNA (Kim et al., 2007; Tsang et al., 2007). Another pathway utilized to regulate the accumulation of miRNAs is through the control of processing. The regulation of Drosha, Dicer, and their associated RNA binding proteins in appropriate ratios results in control of miRNA biogenesis. For example, Drosha induces DGCR8 degradation by cleaving hairpins on the DGCR8 mRNA (Triboulet et al., 2009). This ratio is important because it has been reported that an excess of DGCR8 inhibits Drosha processing (Gregory et al., 2004). Also, a decrease in transactivation-responsive (TAR) binding protein, which generally assists

Dicer with pre-miRNA cleavage, will destabilize Dicer resulting in pre-miRNA defects (Chendrimada et al., 2005). Moreover, recent studies have identified a large number of accessory proteins that can regulate miRNA processing through Drosha or Dicer interaction or by binding to miRNA precursors. Most of these accessory proteins affect the processing of a broad range of miRNA precursors while some bind to specific miRNA families (Winter et al., 2009).

Function

Like mammalian miRNAs, plants have miRNAs that are preferentially expressed in specific tissues and at different developmental stages (Carrington and Ambros, 2003). Both rice and corn plants have exhibited specific miRNA expression patterns in seeds as well as miRNAs (miR-156a and miR-168) that are highly conserved across tissues and developmental state (Xue et al., 2009; Zhang et al., 2009). This indicates that plant and animal miRNAs play specific roles in development and function.

miRNAs play regulatory roles in gene expression and development. They cause translational repression, mRNA destabilization, or direct cleavage, but the mechanisms behind these functions are still not clear (Hutvágner and Zamore, 2002; Mourelatos et al., 2002; Song et al., 2004; Lim et al., 2005). Endogenous miRNAs act as a guide for the miRISC complex allowing for specific recognition and pairing with complementary sites of target mRNAs. Animal miRNAs bind target mRNAs in the 3' untranslated region of the target gene. Multiple binding sites are usually present, either for the same or different miRNAs (Doench and Sharp, 2004). Generally multiple sites are required for complete repression. The key in recognition of a target mRNA is that it must have a perfect base-pairing with the miRNAs seed sequence. This seed sequence is made

up of nucleotides in position 2-8 from the 5' end of the miRNA. Through the examination of the action of miRNAs in cell-free systems that mimic the action of miRNAs in cells, it was reported that the determination between miRNA action of inhibiting mRNA translation or causing deadenylation is dependent on the base-pairing of the seed sequence of the miRNA to the target mRNA (Mathonnet et al., 2007; Wakiyama et al., 2007). While this seed region needs to pair perfectly, the rest of the miRNA may have mismatched pairing (Doench and Sharp, 2004). However, if there is mismatched pairing in the seed region the complementarity of the 3' end of the miRNA (positions 13-16) becomes important. Complementarity of this region could rescue the translational repression that is impaired by the mismatching of the seed region. Although uncommon, it has been recently reported that animal miRNAs have to ability to associate with 5' UTR target sites. Interaction at this region of the target mRNA appears to have a stimulatory role, activating mRNA translation rather than causing repression of translation (Henke et al., 2008; Orom et al., 2008). For example, it has been reported that the addition of miR-122 to liver cell lines stimulates Hepatitis C virus translation.

Unlike animal miRNAs, plant miRNAs bind with near-perfect complementarity in coding sequences of their target endogenous mRNA, resulting in lesser target mRNAs for each miRNA to act upon (Naqvi et al., 2012). Although mammalian miRNAs tend to impair mRNA expression through translational repression, the perfect complementarity that plant miRNAs bind with allows for direct cleavage of the target mRNA through endonucleolytic cleavage (Palatnik et al., 2003). Plant miRNAs can also bind to target mRNAs with a mismatch at the expected cleavage site inhibiting its capacity to elicit degradation. This mechanism, known as 'target mimicry,' blocks the action of the miRNA-mediated regulation by the miRNA it sequesters

(Franco-Zorrilla et al., 2007). Finally, a small population of plant miRNAs are known to regulate mRNA expression through translational repression, similar to mammalian miRNAs (Brodersen et al., 2008). There is currently no evidence to indicate that plant miRNAs have the capacity to induce translation of target genes (Naqvi et al., 2012).

miRNAs are only capable of implementing their effects through the association with miRISC proteins (Bartel et al., 2004). While the details of the miRISC assembly are not fully understood in either mammalian or plant species, the argonaute (AGO) family of proteins are the key component of the miRISC functionality (Fabian et al., 2010). Mammals utilize four AGO proteins in miRNA repression; AGO1, 2, 3, and 4. AGO proteins consist of three highly conserved domains; PAZ, MID, PIWI (Peters and Meister, 2007). These domains interact with the 3' and 5' ends of the miRNA. Only AGO2 has an enzymatic action due to an RNaseH-like PIWI domain. This enzymatic action allows for endonucleolytic cleavage if the target mRNA is perfectly base-paired to the miRNA, as it is in plants (Liu et al., 2004). Glucine-tryptophan repeat protein (GW182) interaction with AGO is also greatly important for miRISC function and miRNA guided repression. Importantly, AGO2 and GW182 are augmented in MVBs and endosomes. This points to a role in loading and packaging miRISCs into exosomes for cell-cell communication (Gibbings et al., 2009). Mammals have three GW182 proteins: TNRC6A, -B, -C. GW182 proteins have a N-terminal region with glycine-tryptophan (GW) repeats which binds to AGOs. A glutamine (Q)-rich region, a domain with an unknown function, and a RNA recognition motif domain are also present on these proteins. It has been demonstrated that the interaction of QW182 proteins with AGOs is required for proper miRNA gene silencing (Eulalio et al., 2009). Reports have demonstrated the interaction of miRISC with several other proteins.

These proteins may play a role in the regulation of miRNA function. However, there is still very little understanding of how these proteins function in miRNA regulation (reviewed by Peters and Meister, 2007).

As stated above, miRNAs mediate translational repression of mRNAs via inhibition of translation or by facilitating their deadenylation which leads to degradation (Hutvágner and Zamore, 2002; Mourelatos et al., 2002; Song et al., 2004; Lim et al., 2005). It has also been reported that miRNAs are capable of up-regulating mRNA expression (Henke et al., 2008; Orom et al., 2008). Exploration into the mechanisms behind miRNA control of mRNA translation has resulted in multiple findings dependent on the concentration of miRNAs, miRNA binding sites in target mRNA, or growth stage of the cells. Also, the outcome of miRNA regulation is dependent on the target mRNA, meaning that the same miRNA can carry out a translational repression effect on one mRNA target, but triggering degradation of another (Alemán et al., 2007; Grimson et al., 2007). Currently, there are four proposed mechanisms in which miRNAs have been shown to repress translation. The first is the repression of protein synthesis by miRNAs after translation has been initiated. Original investigations of lin-4 reported inhibition of lin-14 translation. However, with this effect there was no reduction of mRNA levels or a shift in polysome fraction (Olsen and Ambros, 1999). Several studies using mammalian cell culture also reported the same observations (Seggerson et al., 2002; Petersen et al., 2006). Further investigation demonstrated that a synthetic mRNA reporter normally associated with polysomes even though translation was repressed by the siRNA (miRNA mimic). When initiation of translation was blocked, ribosomes dissociated more rapidly from the mRNA bound by the siRNA than control mRNAs (Petersen et al., 2006). One additional report describes repression of cap-independent translation. This data

suggests that miRNA binding is interfering with the elongation step of translation, potentially triggering dissociation of ribosomes (Petersen et al., 2006). In contrast, others reported mRNAs that possess internal ribosome entry sites (IRESs), a factor that mediates cap-independent translation (which does not require eIF4E), exhibited tolerance to miRNA translation repression (Pillai et al., 2005; Mathonnet et al., 2007; Wakiyama et al., 2007). The addition of internal ribosome entry site (IRESs) to target mRNAs inhibited the miRNA-mediated regulation (Humphreys et al., 2005; Pillai et al., 2005). The second mechanism in which miRNA may mediate mRNA translation is through co-translational protein degradation. Active target mRNA translation was observed, but undetectable protein levels led to the idea of co-translational protein degradation. Active translation was confirmed by mRNA targets exhibiting sensitivity to treatment with translation inhibitors (Nottrott et al., 2006). Kiriakidou et al. (2007), revealed yet another potential mechanism of miRNA gene regulation. Argonaute proteins exhibited sequence similarities with eIF4E, a protein essential for cap-dependent translation initiation. AGO2 was reported to bind to a cap structure analog of mRNAs, indicating competition with eIF4E (Kiriakidou et al., 2007). Finally, miRNAs appear to be able to repress translation by AGO2 recruiting eIF6, a ribosome inhibitory protein known to prevent productive assembly of the 80S ribosome. Depletion of eIF6 relieved the translational suppression of lin-4 miRNA in *C. elegans*. miRNA mediated translational repression was also reversed in human cells when eIF6 was removed (Chendrimada et al., 2007). There have also been several studies that have demonstrated animal miRNAs inducing degradation of target mRNAs. Examples show that when a miRNA expression is induced the target mRNAs will be down-regulated (Lim et al., 2005). In plants, the perfect complementarity with the target mRNA results in triggering endonucleolytic cleavage catalyzed by AGO protein within the miRISC (Palatnik et al., 2003).

Degradation

Even with the great deal of attention miRNAs have received in the past few years, turnover of miRNA is not completely understood. It has been demonstrated that miRNAs are highly stable molecules. After depletion of pol II, a miRNA processing enzyme, mature miRNAs have been recorded to have a half-life of hours or even days in cell lines (van Rooij et al., 2007; Krol, Busskamp, et al., 2010). This characteristic is not a universal feature of miRNAs as indicated by several examples of accelerated or regulated turnover. For example, miR29b has accelerated decay in cycling mammalian cells verses cells arrested in mitosis (Hwang et al., 2007). miRNAs can be tagged with the addition of certain groups (ubiquitin, methly group, adenine-tail) to signal its fate. The addition of ubiquitin (not yet identified in plants) is a signal for degradation, while the addition of a methyl group to the 3'-OH residue of a miRNA will stabilize its survival (Yu et al., 2005). It has also been reported in both plant and animal miRNAs that the addition of adenine residues to the 3'-hydroxyl group will contribute to miRNA stability (Katoh et al., 2009; Lu et al., 2009). Recently, a family of 3' to 5' exoribonucleases, small RNA degrading nuclease 1 (SDN1, SDN2, and SDN3), was identified as enzymes that stabilize several miRNAs when inactivated (Ramachandran and Chen, 2008). SDN1 has been described to only have the capacity to degrade single stranded small RNAs that range from 17-27bp long. Also, the 5' to 3' exonuclease XRN-2 increases the rate of mature miRNA degredation once the miRNA is released from the miRISC complex (Ramachandran and Chen, 2008; Chatterjee and Grosshans, 2009). Finally, argonaute proteins may also pay a role in miRNA degradation by simply protecting them from exonucleases while within the miRISC complex and then releasing the miRNA making them available to enzyme activity (Chatterjee and Grosshans, 2009). miRNAs

are not only regulated and degraded in the cell they are synthesized, but they can also be packaged into microvesicles for transfer to distant tissues.

EXOSOMES

Cells release a diverse population of vesicles. The diversity of these vesicles stems from the type and physiologic state of the cell. Exosomes, a subset of cell secreted vesicles, are microvesicles of endocytotic origin that range in size from 50 to 100nm (Pan et al., 1985). The function of exosomes are still not fully understood, but several reports have demonstrated the importance of exosomes in cell signaling events such as immune response. Early in the discovery of exosomes it was well established that they play a role in protein transport (Johnstone et al., 1987); their role in RNA transport was not shown until more recently (Valadi et al., 2007). Exosomes originate from budding off the inside of multivesicular bodies (MVBs), creating intraluminal vesicles. During invagination of the membrane, transmembrane proteins are incorporated and the cytosolic components are engulfed by the intraluminal vesicle (Van Niel et al., 2006). Instead of interacting with lysosomes, resulting in degradation, these intracellular MBVs move to the plasma membrane where they fuse and release their intraluminal vesicles or exosomes into the extracellular environment (Simpson et al., 2009). The mechanism that differentiates between MVBs destine for degradation or exocytosis is not clearly understood.

Evidence supporting the suggested role of exosomes in intercellular communication continues to grow. Initial analysis described the removal of plasma membrane proteins during the cell maturation process by exosomes (Johnstone et al., 1987). Since their discovery, exosomes have been described in a variety of cells including: reticulocytes (Pan and Johnstone, 1983), dendritic

cells (Théry et al., 1999), B cells (Raposo et al., 1996), T cells (Blanchard et al., 2002), mast cells (Raposo et al., 1997), intestinal epithelial cells (van Niel et al., 2001), and tumor cells (Mears et al., 2004). Exosomes have also been reported in a variety of biological fluids, such as urine (Pisitkun et al., 2004), saliva (Ogawa et al., 2008), breast milk (Admyre et al., 2007), amniotic fluid (Keller et al., 2007), plasma (Caby et al., 2005), serum (Keller et al., 2006), and follicular fluid (da Silveira et al., 2012). The presences of exosomes in a multitude of biological fluids suggest an action of exosomes on distant tissues. Also, this characteristic may indicate the potential role of exosomes as a biomarker for disease and allows for their use as a less invasive research tool.

All exosomes share some common characteristics including a lipid bilayer, generalized size, and a common protein makeup. Exosomal proteins can be present both on the outer surface and in the lumen. The proteins commonly present include: cytoplasmic proteins (tubulin, actin-binding), signal transduction molecules (protein kinases, heterotrimeric G-proteins), and heat-shock proteins (Hsp70, Hsp90). The tetraspanin proteins (CD9, CD63) have been described most commonly in exosomes and are used as exosomal markers (Keller et al., 2006). There have also been reports stating that exosomes may have the capacity to deliver prostaglandins to target cells (Vlassov et al., 2012). Valadi et al. (2007) were the first to report the significance exosomes play in the transport of miRNAs, other non-coding RNAs, and mRNAs. There have been conflicting reports on the makeup of the RNAs carried in exosomes. Some report that the RNAs present in the exosome are significantly different than the cell they originate from (Mittelbrunn et al., 2011); while others in cancer research, show that the miRNA content of the secreted exosomes is similar to the cells they originated (Taylor and Gercel-Taylor, 2008). No matter its content,

exosomes possess several mechanisms in which they interact with the target cell. First, exosomes can bind to the target cell through receptor-ligand interactions (Raposo et al., 1996). Exosomes can also fuse with the target cell membrane. This fusion allows for the transfer of surface proteins and potentially cytoplasm (Clayton et al., 2004). Lastly, internalization of the exosome by the target cell may occur through endocytosis (Morelli et al., 2004). Several studies support the idea that miRNAs can be specifically packaged into exosomes and, through ligand binding to target cells, delivered to specific target cells (Valadi et al., 2007). These findings would suggest that exosomes are involved in cell-cell regulatory mechanisms.

DIETARY REGULATION OF microRNA

There has been emerging evidence showing an impact of dietary changes on miRNA expression. Essential nutrients acquired through diet are important for normal growth, reproduction, and maintenance. Several studies have reported that changes in the intake of amino acids, carbohydrates, fatty acids, and vitamins have resulted in changes in miRNA expression. Davis C. and Ross S. (2008) reported that folate, retinoids, and curcumin can have cancer-protective effects by acting to modulate miRNA expression. Polyphenols, which are the most abundant antioxidants in the human diet, have been demonstrated to modulate the expression of miRNAs in the liver independent of structural differences between polyphenols (Milenkovic et al., 2012). Amino acid deficient diets have also been reported to lead to changes in miRNA expression (García-Segura et al., 2013). A methionine-choline-deficient diet was fed to mice and resulted in the up-regulation of miR-182, -183, -199a, -705, and -1224. Mice fed this diet also showed signs of diet induced nonalcoholic fatty liver disease, such as liver injury, and liver steatosis (Dolganiuc et al., 2009). The dysregulation of some of the up-regulated miRNAs have previously

been reported to be involved with nonalcoholic fatty liver disease (Gori et al., 2014). Some reports have demonstrated that proteins that are up-regulated in amino acid deficient diets compete with miRNAs for mRNA binding by binding the 3'UTR of a specific mRNA (García-Segura et al., 2013). This interaction may explain how miRNAs are regulated in amino acid deficient diets. For example, CAT-1 mRNA, which encodes the high affinity cationic amino acid transporter, is translationally repressed by miR-122. However, if cells are deprived of amino acids or subject to oxidative stress this suppression is relieved. It is suggested that this relief in miRNA repression is due to binding of the AU-rich element-binding protein HuR to the CAT-1 mRNA 3'UTR, inhibiting miR-122 binding even though miR-122 levels are unchanged (Bhattacharyya et al., 2006). The addition of essential amino acids to the diet has also shown an induction of miRNA expression. Within hours after administration of a mix of essential amino acids, one study observed an increase in the levels of miR-1, -23a, -208b, -499, and pri-miR-206 in human muscle biopsies (Drummond et al., 2009).

It has also been recognized that an increase or decrease in glucose intake can result in modulation of miRNA expression. Druz et al. (2012), demonstrated that murine epithelial like cells (B/CMBA.Ov), in a glucose deficient environment, accumulate reactive oxidative species which reduces histone deacetylase activity and consequently up-regulates miR-499h-5p. In contrast, induced hyperglycemia in type 1 diabetic mice triggers an increase in miR-21 expression in the kidney (Dey et al., 2011).

Fatty acids have also been shown to alter miRNA expression. Fish oil, which is rich in n-3 polyunsaturated fatty acids, has been demonstrated to prevent certain digestive cancers

(Davidson et al., 2009). Rats fed a carcinogenic compound resulted in a down-regulation of five tumor suppressor miRNAs (let-7d. miR-15b, -107, -191, -324-5p). However, when fish oil is added to the rats' diet this down-regulation is reversed. These results suggest that the chemo-preventative characteristic that fish oil demonstrates may act through changes in miRNA expression (Davidson et al., 2009). Additional studies demonstrated that rats fed a high fat low carbohydrate diet induced nonalcoholic fatty liver disease and down-regulated miR-27, -122, and -451 expression, while miR-200a, -200b, and -429 expression was up-regulated in the liver (Alisi et al., 2011). Finally, another study demonstrated that mice fed a high-fat diet resulted in an increase of miR-143 and -145 expressions. Mice with an increase in miR-143 showed an increase in fasting plasma insulin levels, impaired glucose, and insulin tolerance. These changes were reversed in mice deficient in miR-143. The results of this study show that miR-143 could be a candidate for type 2 diabetes and obesity therapies (Jordan et al., 2011).

Reports have shown that miRNAs may have therapeutic applications in dietary-derived diseases. For example, mice with an over-expression of let-7 demonstrate impaired glucose tolerance and insulin resistance. This is explained by the role let-7 plays in repressing components of the insulin-PI3K-mTOR pathway. Utilizing this knowledge, let-7 expression was silenced using an antimiR, which impedes let-7 binding to their target mRNAs, in genetically obese fed a high fat diet. An improvement of glucose tolerance and insulin sensitivity was observed (Frost and Olson, 2011; Zhu et al., 2011). miR-122 has also shown potential as a therapeutic target. This miRNA is highly expressed in the liver and plays an important role in the regulation of cholesterol and fatty acid metabolism (Esau et al., 2006). It has been reported that the inhibition

of miR-122 results in the increase of fatty acid oxidation, reduces plasma cholesterol levels, and decreases fatty acid cholesterol synthesis rate (Esau et al., 2006).

DIGESTION

The horse is a non-ruminant herbivore with a digestive tract that is roughly similar to that of the human. Horses have evolved as grazing animals with a diet that historically consists of fibrous feeds (roughage) high in structural carbohydrates that require microbial digestion in the specialized hindgut (Reed et al., 2009). Bacterial fermentation of forage in the cecum results in the production of volatile fatty acids, which are absorbed and used as the primary energy source (Evans et al., 1990). However, the modern day domestic horses' diet includes a large amount of starch-rich cereal grains, leading to an increased intake of nonstructural carbohydrates. The consumption of more starch and simple sugars leads to generalized increased glycemic response. This shift in roughage to grain ratio has been associated with an increase in gastrointestinal problems in the domestic horse (Ralston, 2007), chronic muscle diseases (McKenzie et al., 2003), and insulin resistance related to Equine Metabolic Syndrome (Geor et al., 2008).

Like all other mammals the first step in equine digestion occurs in the mouth, where teeth begin mechanically breaking down feed. In the horse, saliva only acts as a lubricant and pH buffer. Horses' saliva has very little enzymatic action due to its low concentration in amylase (Evans et al., 1990). Once feed is swallowed, the feed passes through the esophagus, and enters the stomach where it is subject to further digestion. The horses' stomach consists of a non-glandular and glandular section. The non-glandular section provides for a small amount of microbial fermentation. The glandular section of the stomach consist of three regions; cardiac, fundic, and

pyloric. The cardiac and pyloric regions secrete mucus to regulate pH within the stomach (Kararli, 1995). The fundic region contains parietal cells that secrete hydrochloric acid which denatures proteins to begin protein digestion. There is very little nutrient absorption that occurs in the stomach (Boron and Boulpaep, 2009).

The majority of nutrient absorption occurs in the small intestine. The small intestine contains several levels of folding to increase the surface area for nutrient absorption and enzymatic action. Folding of the intestinal wall creates projections and valleys that are termed villi and crypts. Every villus is lined with epithelial cells that have additional folding to create even smaller projections or microvilli. These epithelial cells can be categorized based on their function; enterocytes absorb nutrients, goblet cells secrete mucus, and enteroendocrine cells release gastrointestinal hormones. The crypts are also lined with differentiated epithelial cells; paneth cells, which secrete antibacterial peptides and endocrine cells (Cheng and Leblond, 1974). A lymph duct, vein, and artery reside within each villus.

Any material that is not digested in the small intestine passes into the large intestine of the horse. This material generally consists of non-starch polysaccharides and any undigested soluble carbohydrates. The large intestine of the horse consists of three sections: an enlarged cecum, colon, and rectum. There are no villi present in the large intestine, but microvilli are still present on the luminal side of the intestinal epithelium. The cecum and large colon house a large population of microorganisms such as bacteria and protozoa (Smith, 1965). Microbial digestion breaks down structural carbohydrates such as cellulose and hemicellulose. A small but significant amount of protein is also digested through microbial action. Here epithelial cells serve

to secrete mucus and bicarbonates to support an optimal pH of 7-8 necessary for microbial survival and function (de Fombelle et al., 2001).

Carbohydrate Metabolism

Most feed consumed by horses consists of either a structural carbohydrate (SC) or a nonstructural carbohydrate (NSC). The SCs include the highly fibrous portions of the plant such as the plant cell wall which includes hemicellulous, lignin, and cellulose. NSCs are the starches and sugars from the plant cell contents. No matter the ratio of roughage to concentrate in a horses' diet both SC and NSC are present (Ralston, 2007). The process of digestion and absorption of these two carbohydrates sources varies, however. This difference in digestibility is due to the type of linkages between the monosaccharide residues. Hemicellulose and cellulose both contain β1-4 linkages. The horse does not secrete enzymes in the small intestine to break the β1-4 linkage allowing SCs to pass through the small intestine intact and enter the cecum where they are broken down by microbial action. NSCs, such as starch and maltose, contain α1-4 and α1-6 linkages (de Fombelle et al., 2004). These NSCs are subject to hydrolysis and therefore amyloytic digestion. Digestion of simple sugars and starches begins in the stomach. Here, a small amount of microbial action results in the production of lactic acid. The majority of starches (amylose, amylopectine) are broken down into disaccharides by α-amylase produced by the pancreas in the small intestine. Disaccharides are then broken down into glucose, galactose, or fructose by digestive enzymes (maltase, sucrase, and lactase) embedded in the surface of the epithelial microvilli. All of these membrane protein enzymes cleave terminal α1-4 linkages (Binder and Reuben, 2009). Once the carbohydrate is broken down to monosaccharides they are transported by Na+ dependent glucose co-transporter type 1 (SGLT1)(glucose and galacotse) or

a GLUT-5 transporter (fructose) from the intestinal lumen across the apical membrane and into the epithelial cell. All three monosaccharides are transported across the basolateral membrane by a facilitated sugar transporter (GLUT2) (Dyer et al., 2002). The absorption of monosaccharides results in a much more pronounced glycemic response and more energy than SCs digested in the cecum and colon (Ralston, 2007). The hindgut (cecum and colon) supports a population of microorganisms responsible for microbial hydrolysis of SCs from plant fiber. The breakdown of the plant fiber results in the production of soluble sugars which are then subsequently fermented into volatile fatty acids (VFAs). VFAs such as acetate, propionate, and butyrate, are absorbed and utilized as an energy source. Any change in the forage to concentrate ratio will result in a change in the microbial population, luminal pH, content of VFAs, and lactate within the hindgut (Dyer et al., 2002). These changes may also include an increase in endotoxins, exotoxins, and vasoactive monoamines potentially leading to an increased risk of laminitis in the horse (Frank, 2011).

Protein Metabolism

While a small amount of intact proteins make it to the small intestine and are absorbed through endocytosis by the enterocyte, the majority of proteins begin digestion in the stomach. Proteolytic digestion begins in the stomach with the release of pepsinogens from chief cells and mucous cells, which are activated to pepsins. Pepsins initiate hydrolysis of proteins cleaving the N-terminal of the protein. This results in the formation of polypeptides which enter to small intestine. Polypeptides are then exposed to protein-splitting enzymes (trypsin, chymotrypsin, and carboxypeptidases) that are secreted from the pancreas and enter the small intestine via the pancreatic duct. Amiopeptidase, an enzyme found on the microvilli of the small intestine, works

to cleave peptides into amino acids, dipeptides, and tripeptides that are absorbed by the enterocytes. Oligopeptides are taken up by an H+/oligopeptide cotranspoter (PepT1). Amino acid absorption occurs via various transport systems with overlapping affinities that require sequential movement of amino acids across the apical and basolateral membranes of the intestinal epithelial cell. Any peptides will be hydrolyzed to single amino acids by intracellular peptidase before being transported out of the intestinal epithelial cell. Amino acids then enter the hepatic portal system and are carried to the liver where any amino acids needed by the liver are removed. The remaining amino acids then enter the general circulation and are taken up by cells that can utilize them (Binder and Reuben, 2009).

Fat Metabolism

Lipolytic digestion begins in the small intestine with the secretion of bile directly from the liver, horses do not store bile because they do not have a gallbladder. Bile acts as an emulsifier of lipids, breaking up large fat droplets and increasing the surface area for enzymatic action. Emulsified droplets contain triglycerides, diacylglycerols, and cholesterol esters. The surface of an emulsion droplet consists of fatty acids, manoacylglycerols, lysolecthins, and cholesterol. Lipase, secreted from the pancreas, then acts on the surface of the lipid droplet breaking triglycerides into monoglycerides and free fatty acids. The triglycerides at the core replace the outer layer causing the droplet to shrink. As the fatty acids, monoacylglycerols, and bile salts build up on the surface of the droplet they begin to bud off and forming micelles. The micelles are transported to the epithelial cells. Lipids are then absorbed by the enterocytes by either nonionic diffusion, incorporation into the enterocyte membrane, or carrier mediated transport. Once absorbed, the lipid is processed through the endoplasmic reticulum and re-synthesized into

triglycerides for storage in the body. Triglycerides are coated by apolipoproteins to form chylomicrons or VLDLs that are incorporated into vesicles in the Golgi apparatus. The vesicles then carry the chylomicrons or VLDLs to the basolateral membrane of the cell where they are released. Chyomicrons and VLDLs are then transported to lymphatic system, then enter circulation, and are deposited into tissues. Glycerol and short chain fatty acids pass through the enterocyte and enter the blood capillary (Binder and Reuben, 2009).

CONCLUSION

microRNAs play a significant role in both plant and animal gene regulation, contributing to cell homeostasis. As diet has been found to impact health and disease as well as endogenous gene expression, exploration into the mechanisms that modulate this could be useful in identifying diagnostic biomarkers and management of disease through diet. Currently, evidence that diet-derived plant miRNAs are present in the serum and tissue of animals and can affect gene expression, has demonstrated a novel role of miRNAs as a nutrient. This discovery, if confirmed, could allow for the development of oral therapies that alter gene expression to treat disease. Moreover, traditionally recognized nutrients, such as carbohydrates and amino acids, have emerged as potential regulators of endogenous miRNAs (García-Segura et al., 2013). If an understanding of the mechanisms behind dietary regulation of miRNAs is clearly established, dietary intervention could be utilized to effectively manage health by targeting gene regulation.

Horse health and performance is greatly impacted by nutrition. There are several diseases that have been linked to diet in the horse, including: Equine Metabolic Syndrome related insulin resistance, laminitis and chronic muscle diseases. The progression of these disease states and

their association with diet are not clearly understood. There is currently little research establishing clinical markers to diagnose equine diseases linked to nutrition. The goal of this study is to increase the understanding of diet-derived plant miRNA transfer to the horse and the impact diet has on endogenous miRNA serum profile. Using the horse as an animal model, we hypothesized that diet-derived plant miRNAs are present in serum and tissue of the horse. Secondly, we hypothesized that the relative miRNA expression in serum exosomes of the horse can be altered by diet.

CHAPTER 2: IDENTIFICATION OF PLANT MICRORNAS IN EQUINE SERUM AND TISSUE

INTRODUCTION

The feed animals consume contributes greatly to their health and performance. Yet, our understanding of the mechanisms that link diet to health is minimal. Data have shown that *C. elegans* (Tabara et al., 1998a; Timmons and Fire, 1998) and insects (Price and Gatehouse, 2008) are capable of taking up environmental double stranded RNA (dsRNA) through dietary sources. These exogenous dsRNAs have been shown to have an effect on mRNA silencing (Timmons and Fire, 1998; Tabara et al., 1998b; Price and Gatehouse, 2008). This phenomena had not been described in vertebrates until recently in the presence of diet-derived plant miRNAs in mammals (Zhang et al., 2012; Mu et al., 2014). miRNAs are small, non-coding RNAs that post-transcriptionally regulate mRNA expression and thereby have an important role in many biological processes (Fabian et al., 2010).

The reports of dietary transfer of plant miRNAs suggests that food is not only a source of energy and essential nutrients, but can also provide genetic information and regulation. Zhang et al. (2012) reported that plant miRNAs are capable of regulating endogenous gene expression in mice and therefore have potential to impact maintenance and performance. Also, exosome-like nanoparticles (EPDENs) have recently been reported in fruits and vegetables. The absorption of EPDENs by intestinal macrophages and stem cells has been described in mice, providing additional evidence of cross-kingdom miRNA interaction (Mu et al., 2014). This discovery, if

confirmed in other species, could open the door to development of new therapies, as well as a greater understanding of health benefits and diseases linked to diet.

Interestingly, the presences of diet-derived plant miRNAs has not been extensively investigated in herbivore mammals, such as horses. We hypothesized that diet-derived plant miRNAs can be detected in serum and tissue of horses.

MATERIALS AND METHODS

Animal Care and Feeding Protocols

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. Twelve horses were leased from Colorado State University and kept in groups in dry lots with free choice water and alfalfa hay 30 days prior to the initiation of the treatment period. These horses were then randomly separated into three groups (n=4) and confined to individual stalls throughout the 23 day feed trial. Three diet groups consisted of a control diet fed 100% alfalfa hay; a corn supplemented diet was fed 90% alfalfa hay and 10% ground corn mix; a rice bran supplement was fed 90% alfalfa hay and 10% pelleted rice bran (Appendix Table III). All diet groups were fed twice daily at 6:00am and 6:00pm. 10 mL of whole blood was collected from all horses everyday at noon via jugular venipuncture.

Throughout the trial the control diet consisted of 10 lbs of alfalfa twice daily. Beginning on day 1 of the trial the corn and rice supplements were fed at 0.25 lbs of the respective feed supplement with 10 lbs alfalfa twice daily for three days. The feed supplement was then increased to 0.5 lbs for each feeding for two days, after which the feed supplements were increased to 0.75 lbs per

feeding for two days. Finally, the feed supplements were increased to 1 lb per feeding, for a total of 2 lbs per day, for the remainder of the trial.

Horses utilized for tissue harvest were randomly chosen from horses euthanized as part of unrelated terminal study. The horses were stalled individually during feeding which consisted of 6.5 lbs of alfalfa pellets and 1 flake of grass hay twice daily (Appendix Table III). After 90 days the horses were transported to a small pasture and allowed free choice grass hay for 6 days prior to humane euthanasia. Tissue was also harvested from one horse that was only fed grass hay and pasture.

Serum and Tissue Collection

Jugular vein blood samples were collected daily. Samples were allowed to clot for 1 hour at room temperature and were centrifuged at 2,000 x g for 10 minutes to remove cellular debris. Serum was isolated and sequential centrifugation was performed at 300 x g for 10 minutes, 2,000 x g for 10 minutes, and 10,000 x g for 30 minutes (Théry et al., 2006). After each centrifugation step serum supernatant was transferred to clean tubes and the cellular debris was discarded. Serum supernatant was transferred into a new tube and stored at -80°C until RNA isolation.

Tissue samples were harvested from two year old mares (n=4) randomly chosen from a group that were euthanized as part of unrelated terminal study being performed at Colorado State University. Tissue was also collected from a mare (n=1) euthanized for an unrelated health problem. The primary research the horses were used for had no impact on the viability of the tissue samples collected for this study. Cross-section samples of fat, liver, heart, lung, kidney,

skeletal muscle, esophagus, jejunum, and cecum were collected and rinsed with sterile PBS. All tissue samples were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Serum Exosome Collection

Exosomes were isolated by incubating 400 μL of serum samples with 100 μL of Exoquick™ (System Biosciences, Inc. (SBI), Mountain View, CA). Samples were then stored for a minimum of 12 hours at 4 °C. Centrifugation at 1,500 x g for 30 minutes pelleted serum exosomes. Supernatant was removed and serum exosome pellets were re-suspended in 250 μL phosphate buffered saline (PBS)(pH 7.4). Samples were immediately processed for RNA isolation.

RNA Isolation

Both total serum RNA and serum exosomal RNA were isolated according to the manufacturer's protocols using TRI Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). After samples were lysed at room temperature and RNA, DNA, and protein were separated using chloroform the RNA phase was transferred to a clean tube. RNA was then precipitated using isopropanol and pellet was washed with 75% ethanol. Ethanol was removed, tubes were inverted, and RNA pellets were air dried for 5 minutes at room temperature. The RNA pellet was re-suspended in 20 μL of molecular grade water (Thermo Scientific, Wilmington, DE). All RNA samples were treated with DNA-free DNase Treatment (Invitrogen/Life Sciences, Grand Island, NY) to remove any genomic DNA. RNA purity and concentration was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). All RNA aliquots were stored at -80° C until further qRT-PCR analysis.

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Tissue was homogenized and lysed and subject to phase separation using chloroform. RNA was precipitated using isopropanol and the pellet was washed twice with 75% ethanol. All samples were air-dried for 5 minutes and re-suspended in 20 μL of nuclease free water. RNA was treated with DNA-free DNase Treatment (Invitrogen/Life Sciences, Grand Island, NY). RNA purity and concentration was measured as described above. RNA was diluted if the concentration was greater than 3,000 ng/μL. All samples were stored at -80° C until further qRT-PCR analysis.

Plant RNA Isolation

Total RNA was isolated from feed samples according to the manufacture's protocol using TRI Reagent (Molecular Research Center, Cincinnati, OH) with the following modifications. All plant samples were first subject to homogenization using a mortar and pestle that were previously cleaned using RNase (Denville Scientific, Metuchen, NJ), rinsed with 70% ethanol, followed by distilled water and let stand to dry. Ground plant materials were lysed by transferring into 5 mL tube and 1 mL of TRI Reagent was added. Samples were homogenized. A primary centrifugation step of lysate was performed at 12,000g for 10 min to remove any insoluble material such as extracellular material or polysaccharides. Supernatant was transferred into a clean tube and the pellet was discarded. Phase separation was carried out by the addition of 200 μL of chloroform. RNA phase was transferred into a clean tube. A modified RNA precipitation included the addition of 250 μL Isopropanol and 25 μL Salt Precipitation Solution (0.8M sodium citrate and 12.M NaCl). RNA pellet was washed with 75% ethanol and pellet was air-dried for 5 minutes and re-suspended in 20 μL of nuclease-free water. All RNA samples were

treated with DNA-free DNase Treatment (Invitrogen/Life Sciences, Grand Island, NY) to remove any genomic DNA. RNA purity and concentration was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). All RNA aliquots were stored at -80° C until further use.

Reverse Transcription

cDNA was generated according to manufacturer's protocol using TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). 1.5 μL 10x Reverse Transcription Buffer, 1 μL MultiScribe™ Reverse Transcriptase, 0.15 μL 100mM dNTPs, 0.19 μL RNase Inhibitor, 3 μL 5x Reverse Transcription Primer, and 5 μL RNA sample were combine into a 15 μL reaction. Reaction was incubated at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. cDNA was immediately used for PCR analysis.

Real Time PCR Analysis

Three mature plant miRNAs were selected for analysis due to previous reports of their expression in serum, or expression in the specific plant material feed (Xue et al., 2009; Zhang et al., 2009; Jiao et al., 2011; Zhang et al., 2012). The relative level of 3 mature plant miRNAs (Appendix Table II) was assessed in total equine serum and serum exosomes. Each real time PCR reaction contained 1.33 μL of cDNA, 1.0 μL 20x TaqMan® Small RNA Assay, 10.0 μL 2x TaqMan® Universal PCR Master Mix II (no UNG)(Life Technologies, Carlsbad, CA), and 7.67 μL nuclease-free water for a final reaction volume of 20 μL per reaction.. Real time PCR cycle conditions consisted of an enzyme activation at 95°C for 10 minutes, followed by 90 cycles at 95°C for 15 seconds to denature cDNA and 60°C for 60 seconds for annealing and extension.

miR-16, a miRNA commonly used as a normalizer in serum miRNA studies (Zhang et al., 2012), was used as an endogenous control. Also, a no template control was included.

Statistical Analysis

miR16 is a miRNA that is known to be conserved across mammalian tissues (Zhang et al., 2012) that had a standard deviation of less than 1.5 across all samples. Therefore, miR16 was used to normalize raw Ct values of plant miRNAs in serum. To determine the difference in plant miRNA levels between day 0 and day 23 within diets and across diets on day 23 of treatment samples were subject to a two-tailed, paired student's t-test with statistical significance at $P \leq 0.05$. Only horses that had plant miRNAs detectable at both time points were used. Target analysis of detectable plant miRNAs was performed to determine predicted mammalian gene targets using Plant Small RNA Target Analysis Server (Dai and Zhao, 2011).

RESULTS

To determine the presence of plant miRNA in equine serum and tissue, serum was collected from horses of the course of a 23 day feed trial and tissue was harvested from multiple horses. TaqMan® qRT-PCR analysis of alfalfa, rice, and corn samples, fed to horses during the feed trial, indicated the presence of ath-miR-156a in all samples. To determine if there is a specific feed effect on plant miRNA accumulation in serum, osa-miR-1866 and zma-miR-827 were identified to be specific to rice or corn, respectively (Table 1). The grass hay and pellet feed was fed to the horses and tissues were harvested. Feed was also analyzed for presence of these three plant miRNAs. Ath-miR156a was detected in both the grass hay as well as the pellet feed. Osa-miR-1866 was not at detectable levels in either feed but, zma-miR-827 was at detectable levels in the pellet feed (Table 2).

Plant miRNAs (miR-156a, miR-1866, and miR-827) were not evident in total serum RNA samples at day 0 or day 23 in any diet groups. However, when serum exosomes were examined, ath-miR156a was measurable in all diet groups at both day 0 and day 23 (Figure 3). There was no difference between days. Finally, neither osa-miR-1866 (rice specific miRNA) nor zma-miR-827 (corn specific miRNA) were identified in the rice fed or corn fed horses' serum exosomes, respectively.

The presence of plant miRNA in equine tissues (fat, liver, heart, kidney, lung, skeletal muscle, jejunum, cecum) was investigated. The tissue from the mare fed grass hay or pasture had detectable levels of ath-miR-156a in all tissues tested at a relatively invariable level. Osa-miR-1866 and zma-miR-827 were not present in any tissue samples from this mare (Figure 4A). Ath-miR156a was also detected in all tissues sampled from horses (n=4) fed both hay and pellet feed. Although, these horses were fed a pellet which contained zma-miR-827, this miRNA was not identified in any tissue sample (Figure 4B). Target analysis revealed four mammalian genes; ALG2, SUCLG2, HIF3A, and F11R as predicted targets of plant miRNA ath-miR-156a (Appendix Figure I).

Table 1: Plant miRNA Expression Across Feed Trial Feeds. Levels of plant miRNAs were detected using qRT-PCR in the alfalfa, rice, and corn feed to horses throughout the feed trial.

miRNA	RNA sample	Ct
Zma-miR827	Corn Feed	30.7
Zma-miR827	Rice Feed	undetectable
Zma-miR827	Alfalfa	undetectable
Osa-miR1866	Corn Feed	undetectable
Osa-miR1866	Rice Feed	33
Osa-miR1866	Alfalfa	undetectable
Ath-miR156a	Corn Feed	24
Ath-miR156a	Rice Feed	25.3
Ath-miR156a	Alfalfa	20.8

Table 2: Plant miRNA Expression Across Harvest Trial Feed. Levels of plant miRNAs were detected using qRT-PCR in the grass hay and pellet feed that was fed to horses prior to tissue harvest.

miRNA	RNA Sample	Ct
Ath-miR156a	Grass Hay	27.1
Ath-miR156a	Pellet	26.6
Osa-miR1866	Grass Hay	undetectable
Osa-miR1866	Pellet	undetectable
Zma-miR827	Grass Hay	undetectable
Zma-miR827	Pellet	31.6

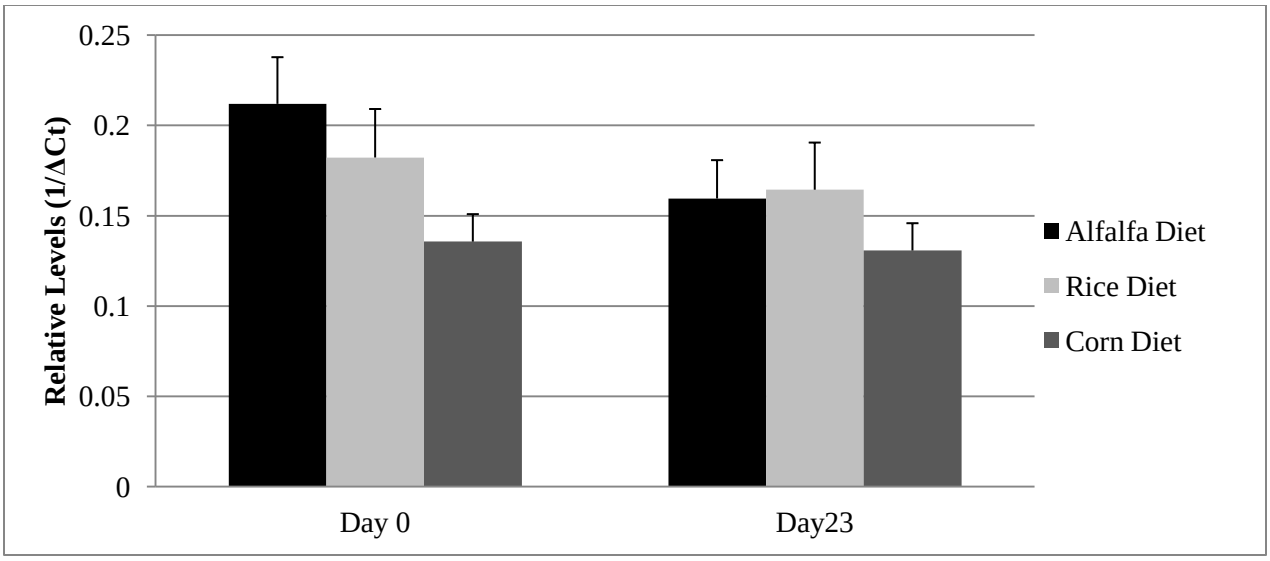


Figure 2: Relative Level of ath-miR156a in Serum Exosomes. qRT-PCR was performed to determine levels of ath-miR156a in serum exosomes across all diets at day 0 and day 23. Data was normalized to hsa-miR16.

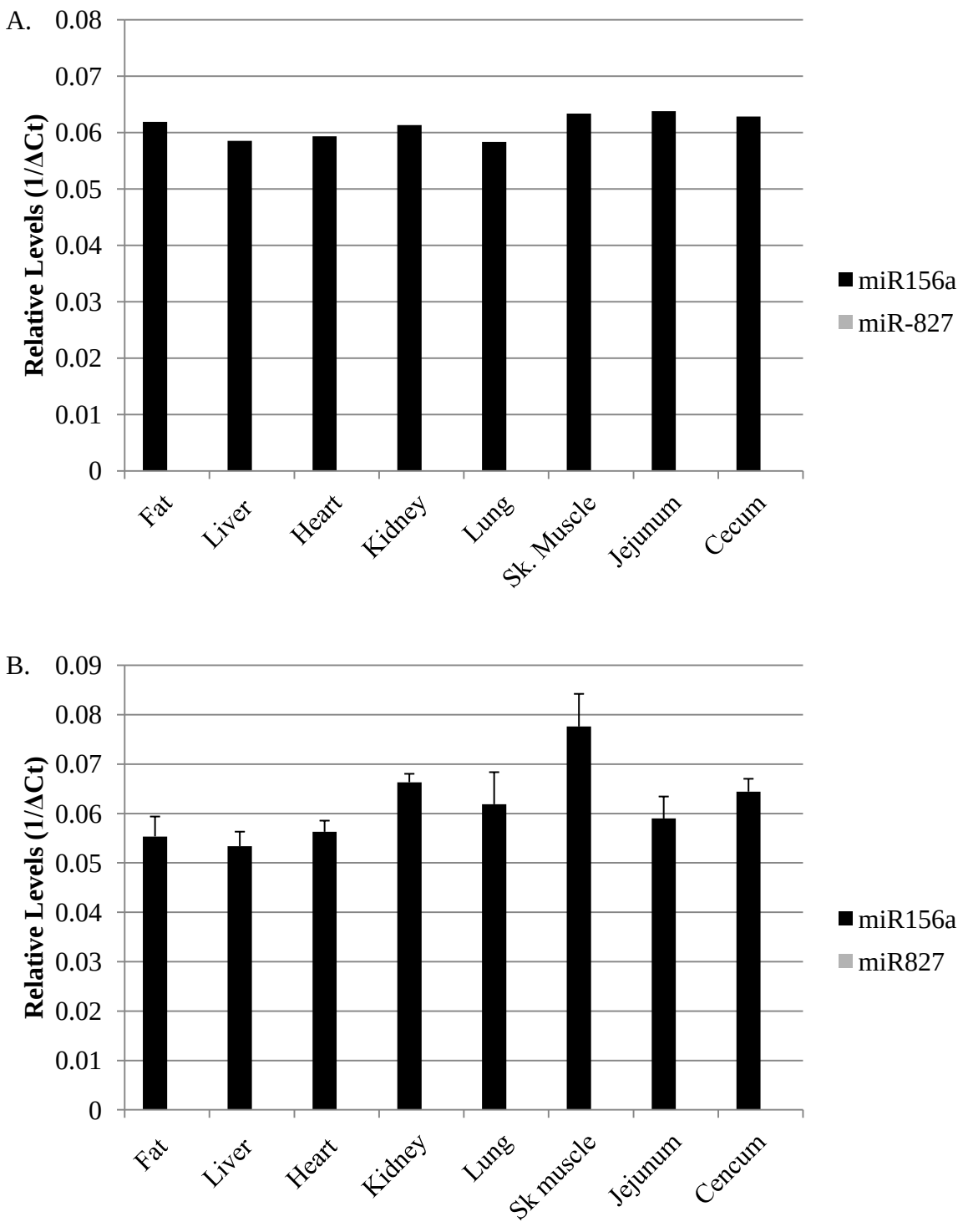


Figure 3: Relative Levels of Plant miRNA in Equine Tissue. Levels of ath-miR156a and zma-miR827 in equine tissue harvested from (A.) a horse that was only fed grass hay and pasture (B.) horses (n=4) that were fed both grass hay and pellet feed. Data was normalized to hsa-miR16

DISCUSSION

This experiment investigated the presence of diet-derived plant miRNAs in equine serum and tissue. qRT-PCR was performed on serum samples collected from horses fed corn, rice, or alfalfa only diets, on day 0 and day 23 of the feed trial. The results revealed that plant ath-miRNA-156a was present in exosomes within the serum of all horses with no difference between days or diet. Detection of xenomiRNAs within exosomes agrees with the previous data indicating that more than half of the diet-derived miRNAs are detected in microvesicles (Zhang et al., 2012). In contrast to Zhang et al. (2012), plant miRNAs were not detectable in total equine serum isolates at any time point in any diet analyzed. Plant specific miRNAs (osa-miR-1866 and zma-miR-827) were also not detected in the serum of horses. This could be due to a low concentration of these specific plant miRNAs in the feed as well as the extensive dilution within the horses' circulation. The plant miRNAs could have also been rapidly cleared from the blood and taken up by the tissues or degraded prior to blood sampling, which occurred 6 hours after feeding.

From equine tissue samples that were harvested, ath-miR156a was present at detectable levels. Although we did not investigate the effect ath-miR-156a had on equine tissue gene expression, Plant Small RNA Target Analysis (Dai and Zhoa, 2011) revealed four predicted mammalian gene targets included *ALG2, SUCLG2, HIF3A,* and *F11R* with similar target accessibility to that of plant target genes of ath-miR-156a. ALG2, a glycosyltransferase, and HIF3A, a protein associated with the adaptive response to hypoxia, are predicted to be translationally repressed by ath-miR-156a. SUCLG2, succinate-CoA ligase, GDP-forming beta subunit, and F11R, an adhesion protein, are predicted to be cleaved by ath-miR-156a. Further investigation must be done to determine the effect of ath-miR-156a on these genes.

While further investigation must be completed to understand how plant miRNAs are absorbed and enter circulation, previous findings suggest that plant miRNAs are absorbed by the luminal cells of the small intestine of mammals (Mu et al., 2014). The findings of this study support the idea that diet-derived plant miRNAs are packaged into mammalian exosomes, released into circulation, and taken up by equine tissues. Understanding the mechanisms other animals use for environmental small RNA absorption may be helpful to gain additional insight into equine xenomiRNA intestinal absorption. Nematodes take up ingested small RNAs through two transmembrane proteins, SID-1 and SID-2; mammals do not seem to express a gene similar to SID-2 (Winston et al., 2007b). However, SIDT1, a human homolog to *C. elegans* SID-1, has been reported to act as a channel for small RNA uptake and may play a role in the absorption of xenomiRNAs in mammals (Elhassan et al., 2012). Insects, on the other hand, have been reported to take in dsRNA through receptor mediated endocytosis (Price and Gatehouse, 2008). Interestingly, the most recently published findings to describe the absorption of diet-derived plant miRNAs show they are packaged in plant exosome-like nanoparticles (EPDENs) that are capable of withstanding the acidic environment of the stomach and intestine. Investigators reported that EPDENs, present in fruits and vegetables, contain RNAs, including miRNAs. Through confocal microscopy, it was shown that EPDENs are co-localized with both macrophages and intestinal stem cells (Mu et al., 2014). This raises a question of whether EPDENs are transferred to the blood intact or if the miRNAs are released in the intestinal cells and repackaged into mammalian exosomes. Mu et al (2012) also described miRNAs present in grape EPDENs that have seed sequences which match human miR-445 and -4662a, suggesting that miRNAs absorbed from grape derivatives can target mammalian mRNAs for translational repression.

Additional research would provide a clearer understanding of diet-derived miRNA transfer into mammalian circulation and tissue and could potentially lead to the utilization of xenomiRNAs as biomarkers of disease once the function of the plant miRNA in animal tissue is fully understood.

CHAPTER 3: THE EFFECT OF DIET ON MICRORNA PROFILE IN EQUINE SERUM

INTRODUCTION

Horses have evolved as grazing animals that typically eat highly fibrous roughage for up to 16 hours a day, requiring digestion of feed high in structural carbohydrates. The modern horse however, often is provided an additional energy source due to their use in high-level performance and competition. This additional energy source generally comes from cereal grains, such as corn, which are high in nonstructural carbohydrates (NSC), such as starch and simple sugars. Additionally, performance horses have an ever-increasing number of metabolic or nutrition related diseases that greatly impact athletic and reproductive performance, including: Equine Metabolic Syndrome and related insulin resistance, laminitis, and developmental orthopedic diseases (Ralston, 2007). Our understanding of the mechanisms that relate diet to disease in the horse is very limited due to the lack of research in this area.

Recent research has demonstrated a role of diet on the modulation of endogenous miRNAs (García-Segura et al., 2013) which are small non-coding RNAs that regulate mRNA translation (Fabian et al., 2010). This study will address the impact of rice bran (a high-fat diet), corn (high-NSC diet), and a forage only diet on endogenous miRNA expression. The hypothesis of this study is that diet will alter the relative endogenous miRNA profile in serum exosomes of the horse. The data from this study may help develop a deeper understanding of the role diet plays on endogenous gene expression and as a disease states.

MATERIALS AND METHODS

Animal Care and Feeding Protocol

All experiments were approved by the Colorado State University Institutional Animal Care and Use Committee. As stated in Chapter II above, twelve horses were leased from Colorado State University and kept in groups in dry lots with free choice water and alfalfa hay 30 days prior to the initiation of the treatment period. These horses were then randomly separated into three groups (n=4) and confined to individual stalls throughout the 23 day trial. Three diet groups consisted of a control diet fed 100% alfalfa hay; a corn supplemented diet was fed 90% alfalfa hay and 10% ground corn mix; a rice bran supplement was fed 90% alfalfa hay and 10% pelleted rice bran (Appendix Table III). All diet groups were fed twice daily at 6:00am and 6:00pm. 10 mL of whole blood was collected from all horses everyday at noon via jugular venipuncture.

Throughout the trial the control diet consisted of 10 lbs of alfalfa twice daily. Beginning on day 1 of the trial the corn and rice bran supplements were fed at 0.25 lbs of the respective feed supplement with 10 lbs alfalfa twice daily for three days. The feed supplement was then increased to 0.5 lbs for each feeding for two days, after which the feed supplements were increased to 0.75 lbs per feeding for two days. Finally on day 7, the feed supplements were increased to 1 lb per feeding, for a total of 2 lbs per day, for the remainder of the trial.

Serum Exosome Collection

Exosomes were isolated, as stated in Chapter II, by incubating 400 µL of serum samples with 100 µL of Exoquick™ (System Biosciences, Inc. (SBI), Mountain View, CA). Samples were then stored a minimum of 12 hours at 4 °C. Centrifugation at 1,500 x g for 30 minutes pelleted

serum exosomes. Supernatant was removed and serum exosome pellets were re-suspended in 250 μL phosphate buffered saline (PBS)(pH 7.4). Samples were immediately subjected to RNA isolation.

RNA Isolation

As stated in Chapter II, serum exosomal RNA were isolated according to the manufacturer's protocols using TRI Reagent for Blood Derivatives (Molecular Research Center, Cincinnati, OH). After samples were lysed at room temperature and RNA, DNA, and protein were separated using chloroform the RNA phase was transferred to a clean tube. RNA was then precipitated using isopropanol and pellet was washed with 75% ethanol. Ethanol was removed, tubes were inverted, and RNA pellets were air dried for 5 minutes at room temperature. The RNA pellet was re-suspended in 20 μL of molecular grade water (Thermo Scientific, Wilmington, DE). All RNA samples were treated with DNA-free DNase Treatment (Invitrogen/Life Sciences, Grand Island, NY) to remove any genomic DNA. RNA purity and concentration was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA aliquots were stored at -80° C until further use.

Reverse Transcription

Quantifiable cDNA were generated using the miScript II Reverse Transcriptase Kit (Qiagen, Valencia, CA) with 800 ng RNA, 4 μL 5x miScript HiSpec solution, 2 μL 10x Nucleic solution, 2 μL of miScript Reverse Transcriptase, and RNase-free water to reach a final volume of 20 μL. The reaction is incubated at 37°C for 60 minutes and then 95°C for 5 minutes. cDNA was then immediately used for PCR analysis.

Real Time PCR Analysis

The sequences for 340 equine mature miRNAs primers (Appendix Table I) used in this study were obtained in a *silico* detection model (Zhou et al., 2009) and have been previously used by this laboratory to profile miRNAs in equine serum exosomes (Cameron et al. 2012). Based on preliminary data, 190 of the 340 equine miRNAs were selected with a standard deviation of less than 2.0 and a Cp value of less than 37 for further analysis. qRT-PCR analysis was performed in 384 well plates. Each 6.5 μL reaction contained 3.0 μL 2x QuantiTect SYBR Green PCR Master Mix, 0.6 μL 10x miScript Universal Reverse Primer (Qiagen, Valencia, CA), 1.5 μL miRNA specific forward primer, and 0.1 μL of cDNA. Analysis was performed using the LightCycler 480 PCR system (Roche, Basel). Cycle conditions began with reaction initiation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds to denature, 55°C for 30 seconds to anneal, and 70°C for 30 seconds for extension. Melt curve analysis was performed at the end of 45 cycles to ensure single cDNA amplification. Amplification products were confirmed by analysis of amplification curves and singular melt peaks. Three endogenous controls were identified (eca-mir130a, eca-mir323-3p, and eca-mir767-5p) as miRNAs that had a standard deviation of less than 0.5 across all horses on day 0 and day 23. A no template control with a random miRNA specific forward primer was also included.

Statistical Analysis

To determine the change in equine exosomal miRNA levels between day 0 and day 23 of each dietary treatment, raw Cp values were normalized to the geometric mean of the 3 endogenous controls listed above. Only miRNAs detected in all samples were used. miRNA expression was compared between days of the same diet using a paired, two-tailed student's t-test with statistical

significance assessed at P≤0.05. Predicted target pathways of the significantly different miRNAs were identified using Ingenuity Pathway Analysis (Qiagen).

RESULTS

To establish the influence of diet on the serum exosome miRNA profile in the horse, qRT-PCR analysis of 340 equine miRNAs was performed. Initial profile analysis revealed 190 miRNAs were present in either day 0 serum exosomes or day 23 or both based on amplification curves and single melt peaks. These 190 miRNAs were then used for investigation of miRNA profiles in all horses at day 0 and day 23 of each diet group. Of the 190 miRNAs used, 3 (eca-mir130a, eca-mir323-3p, and eca-mir767-5p) were detected in all samples at levels that were not statistically different across diets and time points with a standard deviation of less than 0.5. The geometric mean of these three miRNAs was used to normalize all other Cp values.

Each diet resulted in exosomal miRNA profiles that were different (p≤0.05) 23 days after feed supplementation began, as compared to day 0. Horses fed the alfalfa diet exhibited 10 mature miRNAs that were higher in serum exosomes on day 23 compared to day 0; eca-mir-15b, eca-mir-93, eca-let-7g, eca-mir-191, eca-mir-361-5p, eca-mir-423-5p, eca-mir-125a-5p, eca-mir-132, eca-mir-142-3p, and eca-mir-335 (Figure 4A). eca-mir-181a was the only miRNA that was lower in serum exosomes isolated from horses on day 23 of alfalfa diet compared to day 0 (Figure 4B). 11 mature miRNAs were differentially expressed in day 23 serum exosomes isolated from horses fed the rice bran supplement compared to day 0. Of these, 7 miRNAs were higher in rice fed horses on day 23; eca-mir-15b, eca-mir-20b, eca-mir-195, eca-mir-301b3p, eca-mir-4863p, eca-mir-16, and eca-mir-129a3p (Figure 5A). The remaining 4 miRNAs were found to be lower on

day 23: eca-mir-33b, eca-mir-382, eca-mir-541, and eca-mir-598 (Figure 5B). Results of exosomal miRNA profiling of horses fed the corn diet indicated a total of 37 mature miRNAs that were differentially present at day 23 of diet supplementation compared to day 0. The 17 following miRNAs were shown to be higher in day 23 serum exosomes: eca-mir-15b, eca-mir-20b, eca-mir-93, eca-mir-195, eca-mir-4863p, eca-mir-17, eca-mir-20a, eca-mir-25, eca-mir-106a, eca-mir-106b, eca-mir-148a, eca-mir-191, eca-mir-197, eca-mir-221, eca-mir-361-5p, eca-mir-423-5p, and eca-mir-451 (Figure 6A). The remaining 16 miRNAs were lower in exosomal serum of horses fed the corn diet on day 23: eca-mir-301b-3p, eca-let-7a, eca-let-7c, eca-let-7d, eca-let-7g, eca-mir-23a, eca-mir-23b, eca-mir-26a, eca-mir-27b, eca-mir-29a, eca-mir-29c, eca-mir-30b, eca-mir-30d, eca-mir-30e, eca-mir-100, eca-mir-143, eca-mir-199a-5p, eca-mir-378, eca-mir-652, and eca-mir-664 (Figure 6B). Fold changes and p-values for all miRNAs are in Tables 3-5.

Ingenuity pathway analysis (IPA) was performed on miRNAs that were differentially expressed on day 23 of each diet group when compared to day 0. Pathway analysis of the 11 miRNAs that were differentially expressed in serum exosomes on day 23 of the alfalfa only diet group revealed targeted network functions related to connective tissue disorders and inflammatory response (Appendix Figure II.I). Pathways predicted to be regulated by the 11 miRNAs differentially expressed at day 23 compared to day 0 of the rice fed horses that pathway related to cellular development, growth and proliferations networks as well as skeletal and muscular system development and function (Appendix Figure II.II). The miRNAs that were statistically different on day 23 in exosomal serum from horses fed the corn diet were also subject to IPA analysis. This revealed predicted target networks associated with gastrointestinal disease, hepatic

system disease (Appendix Figure II.III), connective tissue disorder, inflammatory disease, and inflammatory response (Appendix Figure II.IV). No further analysis of pathway predictions was completed regarding this data.

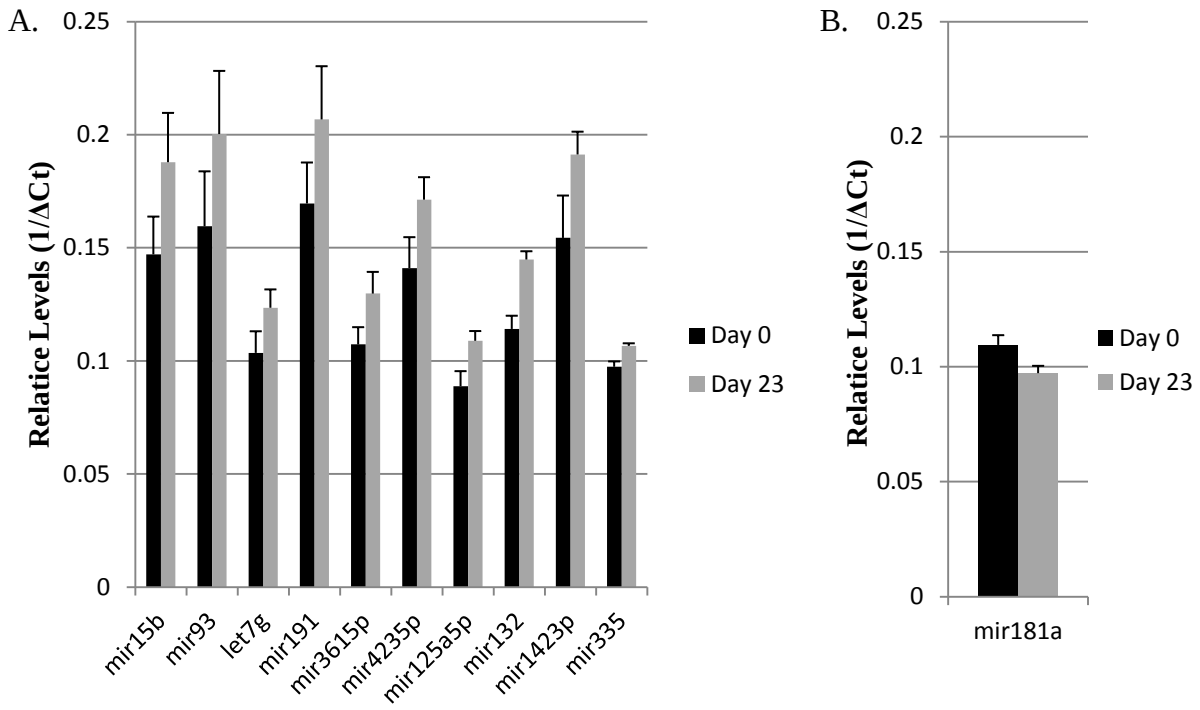


Figure 4: Serum Exosomal miRNAs in Day 23 vs Day 0 Samples from Alfalfa Diet. Levels of relative miRNAs that were shown to be (A.) significantly higher on day 23 or (B.) significantly lower on day 23 in serum exosomes when compared to day 0.
$P \leq 0.05$

Table 3: T-Test p-Values and Fold Changes for Significantly Higher Exosomal miRNAs in Alfalfa Diet. P-values and fold changes of miRNAs that were (A.) significantly higher or (B.) significantly lower in serum exosomes of Alfalfa diet horses at day 23 compared to day 0 of feed trial. $P \leq 0.05$

A.

mature miRNA	p-Value	Fold Change
eca-mir-15b	0.02227	1.27697
eca-mir-93	0.04687	1.25557
eca-let-7g	0.01919	1.19262
eca-mir-191	0.03622	1.2191
eca-mir-361-5p	0.04629	1.20966
eca-mir-423-5p	0.04473	1.2145
eca-mir-125a-5p	0.04447	1.22619
eca-mir-132	0.04156	1.26885
eca-mir-142-3p	0.04683	1.23888
eca-mir-335	0.01401	1.09466

B.

mature miRNA	p-Value	Fold Change
eca-mir-181a	0.00842	1.1263

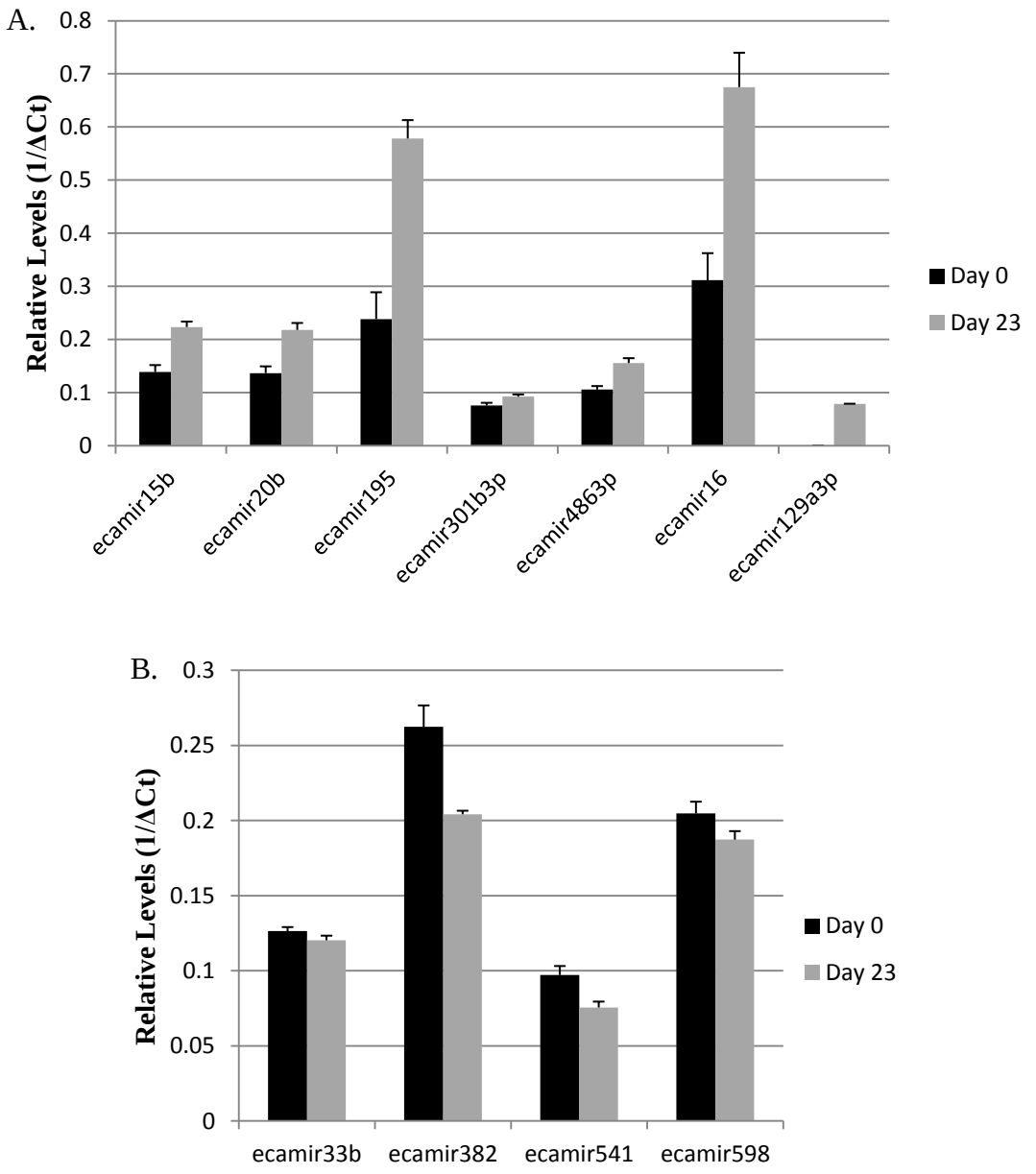


Figure 5: Serum Exosomal miRNAs in Day 23 vs Day 0 Samples from Rice Diet. Levels of relative miRNAs that were shown to be (A.) significantly higher or (B.) significantly lower on day 23 in serum exosomes when compared to day 0.
$P \leq 0.05$

Table 4: T-Test p-Values and Fold Changes for Significantly Higher Exosomal miRNAs in Rice Diet. P-values and fold changes of miRNAs that were (A.) significantly higher or (B.) significantly lower in serum exosomes of Rice diet horses at day 23 compared to day 0 of feed trial. $P \leq 0.05$

A.

Mature miRNA	p-Value	Fold Change
eca-mir-15b	0.02908	1.61157777
eca-mir-20b	0.04854	1.59494097
eca-mir-195	0.02781	2.42526015
eca-mir-301b-3p	0.04761	1.22091801
eca-mir-486-3p	0.0328	1.4709373
eca-mir-16	0.048	2.16726263
eca-mir-129a-3p	5.3E-07	

B.

Mature miRNA	p-Value	Fold Change
eca-mir-33b	0.00229	1.05046207
eca-mir-382	0.03445	1.28556375
eca-mir-541	0.02782	1.28583993
eca-mir-598	0.03543	1.0932931

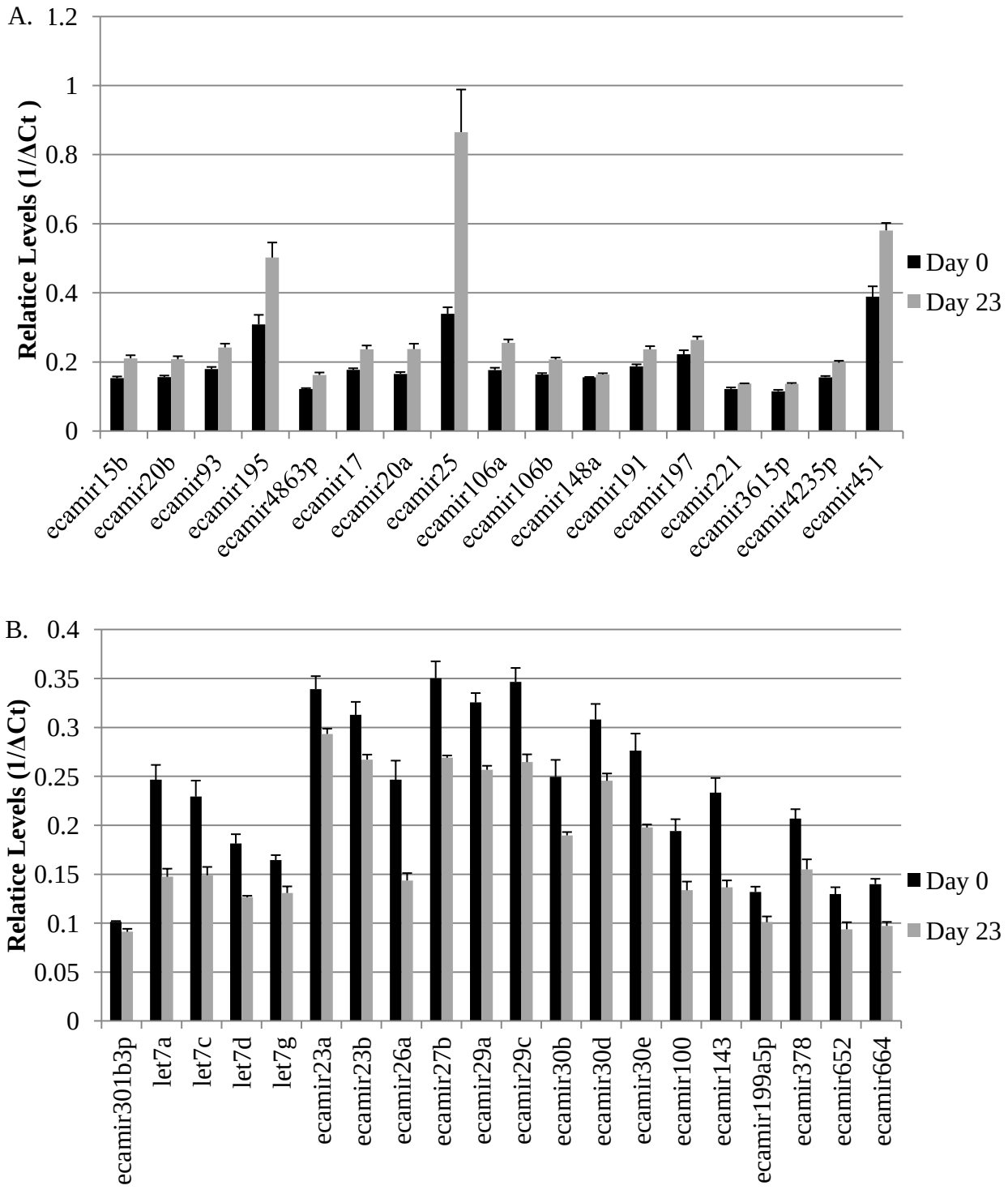


Figure 6: Serum Exosomal miRNAs in Day 23 vs Day 0 Samples from Corn Diet. Levels of relative miRNAs that were shown to be (A.) significantly higher or (B.) significantly lower on day 23 in serum exosomes when compared to day 0.
$P \leq 0.05$

Table 5: T-Test p-Values and Fold Changes for Significantly Higher Exosomal miRNAs in Corn Diet. P-values and fold changes of miRNAs that were (A.) significantly higher or (B.) significantly lower in serum exosomes of Corn diet horses at day 23 compared to day 0. P≤0.05

A.

Mature miRNA	p-Value	Fold Change
ecaeca-mir-15b	0.0098724	1.375819848
ecaeca-mir-20b	0.0199405	1.330717885
eca-mir-93	0.0209977	1.351025127
eca-mir-195	0.0267596	1.626775104
eca-mir-4863p	0.0050531	1.332777717
eca-mir-17	0.0135106	1.338402171
eca-mir-20a	0.0345492	1.432544193
eca-mir-25	0.0261424	2.548062709
eca-mir-106a	0.0100888	1.447976731
eca-mir-106b	0.0070653	1.26282608
eca-mir-148a	0.0447912	1.055195591
eca-mir-191	0.0271992	1.263481827
eca-mir-197	0.0320578	1.189009109
eca-mir-221	0.0316777	1.127459786
eca-mir-361-5p	0.0329414	1.188046373
eca-mir-423-5p	0.009825	1.268017217
eca-mir-451	0.0103466	1.49362117

B.

Mature miRNA	p-Value	Fold Change
eca-mir-301b-3p	0.0398068	1.111754616
eca-let-7a	0.0036666	1.672531171
eca-let-7c	0.029273	1.540284511
eca-let-7d	0.0162381	1.43405983
eca-let-7g	0.010586	1.553775395
eca-mir-23a	0.0104352	1.156274897
eca-mir-23b	0.0165694	1.171479921
eca-mir-26a	0.0192821	1.716347318
eca-mir-27b	0.0194411	1.30207878
eca-mir-29a	0.0030579	1.267924704
eca-mir-29c	0.0025366	1.310150643
eca-mir-30b	0.0287076	1.315025168
eca-mir-30d	0.0472505	1.255385189
eca-mir-30e	0.0126037	1.397427285
eca-mir-100	0.0386513	1.453573244
eca-mir-143	0.0083126	1.707881909
eca-mir-199a-5p	0.0383867	1.305421805
eca-mir-378	0.0320579	1.334225148
eca-mir-652	0.0149046	1.383169186
eca-mir-664	0.0190582	1.439157589

DISCUSSION

The idea of diet directly impacting health is well established, especially in the horse. It has become common for the domestic horse to be fed two meals a day consisting of roughage and a grain concentrate, especially high-level performance horses that require an increase in energy and nutrient demand. Several studies have demonstrated feeding a high volume grain concentrate meals to horses is linked to digestive, metabolic (Ralston, 2007; Frank, 2011) and orthopedic diseases (Williams et al., 2001). Glucose tolerance and insulin sensitivity have been researched in depth has they relate to diets high in starch and sugar in the horses. Studies have determined that high starch and sugar diets result in increased plasma glucose levels, insulin levels, and lower insulin sensitivity than horses fed forage only diets or grain concentrates high in fat and fiber (Williams et al., 2001; Hoffman et al., 2003). Also, recent research indicates a role of diet in altering miRNA expression (Davidson et al., 2009; García-Segura et al., 2013).

While studies have indicated the impact of diet on overall equine health (Williams et al., 2001; Hoffman et al., 2003; Ralston, 2007; Frank, 2011), this study provides insight into the influence of diet on miRNA expression that regulate gene expression. Here we investigated the change in miRNA profiles using qRT-PCR. Horses were fed either a forage only diet (alfalfa), a diet higher in fat and fiber (rice bran), or a higher starch and sugar diet (corn). The rice bran feed was approximately 5 times higher in fat and 3 times higher in fiber than the other feeds. The corn feed was approximately 2.5 times higher in NSC than the rice bran feed and approximately 5.5 times higher than alfalfa.

Analysis of serum exosomes revealed horses fed a high starch and sugar diet had 37 differentially expressed miRNAs by day 23 of the feed trial. Of the 37 miRNAs, several members of the let-7 miRNA family (let-7a, c, d, and g), miR-29a, and miR-143 had significantly decreased levels in serum exosomes on day 23. Interestingly, all of these miRNAs have previously been strongly associated with glucose tolerance and insulin sensitivity (Rottiers and Näär, 2012). It has been reported that the overexpression of let-7 miRNAs results in insulin resistance while global knockdown improved glucose tolerance and insulin sensitivity in liver and muscle of mice (Frost and Olson, 2011). This may suggest that a diet containing non-structural carbohydrates, which leads to increased blood glucose, will trigger the decrease of miRNAs that suppress insulin sensitivity. Insulin sensitivity and glucose tolerance is necessary for maintaining normal glucose metabolism in health horses. Additionally, several other miRNAs involved in adipocyte development were significantly changed in the corn diet. miR-27b, shown to be down regulated during adipogenesis, was decreased in serum exosomes of horses fed the corn diet on day 23. In contrast, miR-17, -20a, and -20b, which are associated with adipocyte development, were significantly increased in serum exosomes (Romao et al., 2011)(Figure 7).

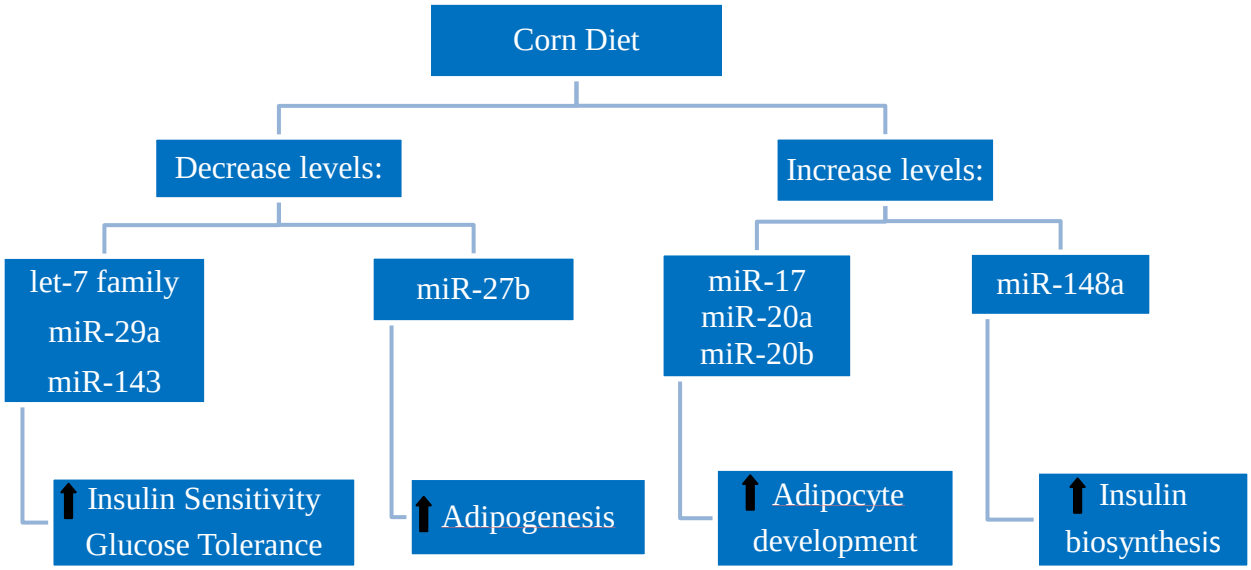


Figure 7: Corn Diet Significantly Different miRNAs Involved in Metabolism.

The horses fed rice bran also showed a significant increase in miR-20b on day 23. Furthermore, rice fed horses demonstrated a decrease in miR-33b, which has been linked to formation of high-density lipoprotein formation and control of fatty acid oxidation. Inhibition of miR-33a and -b expressions increases fatty acid oxidation and insulin signaling in hepatic cell lines (Dávalos et al., 2011). Interestingly, miR-129a-3p was not detectable in horses on day 0 of the feed trial, but was detected in serum exosomes of rice bran fed horses on day 23. This miRNA has been described to be at higher expression levels in normal tissues verses tumors and has been reported to inhibit cell proliferation (Wu et al., 2010)(Figure 8).

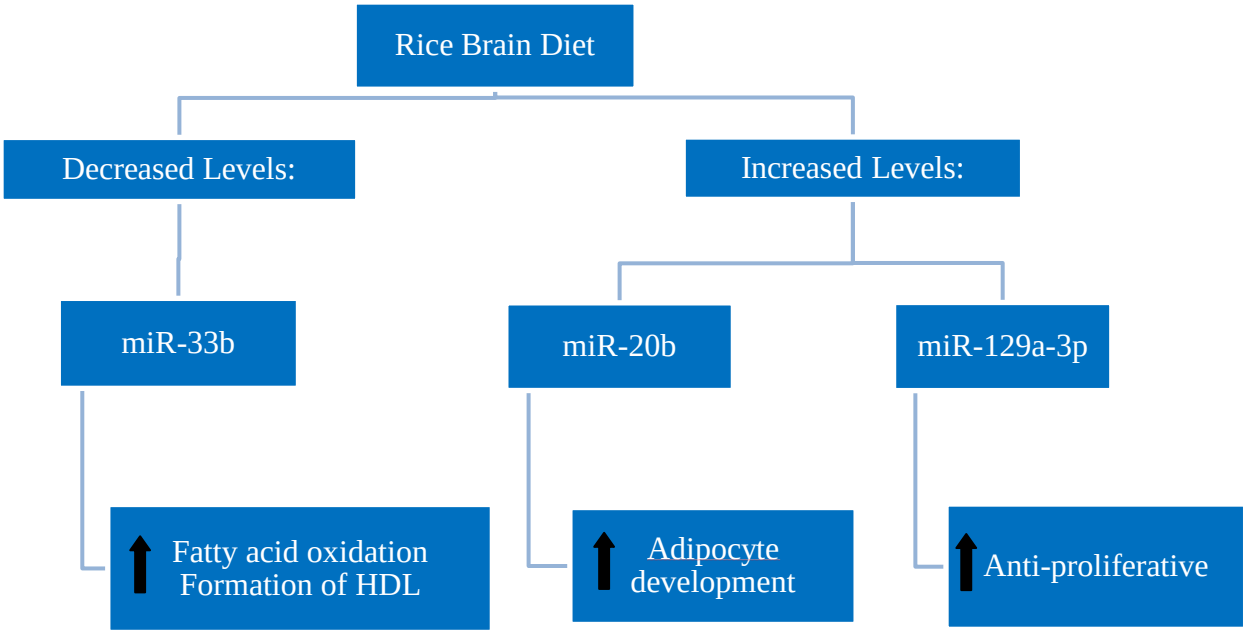


Figure 8: Rice Diet Significantly Different miRNAs Involved in Metabolism.

Several exosomal miRNAs were also differentially expressed in horses fed the alfalfa only diet. This was unexpected because all horses were kept in a dry lot on free choice alfalfa prior to the feed trial. However, this result could be due to a difference in nutrient content between the alfalfa they received in the paddock and the alfalfa they were fed during the trial. Also, when horses are housed in groups, there will be competition for food resulting in horses within the same field eating different amounts on a daily basis. Once the horses were brought into stalls, all horses received the same amount of alfalfa throughout the trial. Although weights were not recorded in this study, the change in miRNA express could also be a result of weight gain and fat disposition. This is suggested by the increased expression of miR-335 in the alfalfa only diet on day 23. The up-regulation of this miRNA has been linked to increased body weight, hepatic triglycerides, and cholesterol in mice (Nakanishi et al., 2009).

These results support the emerging role of diet on miRNA expression and suggests that diet can impact cell homeostasis. This information will allow for the assessment of nutritional status as well as management and prevention of disease through diet. These findings may also contribute to the understanding of metabolic disease in both horses and humans. Further research investigating the mechanisms in which nutrient content influences miRNA expression should be pursued.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

From the first experiment in this study, we can conclude that diet-derived plant miRNAs can be absorbed, packaged into endogenous exosomes, released into the blood and taken up by tissue in the horse. These findings provide intriguing evidence of interspecies communication. Identifying the presences of xenomiRNAs in serum exosomes and tissues is an important step to understanding the mechanisms by which dietary-derived plant miRNAs are taken in and what role they may play in gene regulation of the animal. Secondly, this study is the first to show a modulation of endogenous exosomal miRNAs by diet in the horse. Serum exosomal miRNAs, whether exogenous or endogenous, can serve as non-invasive biomarkers of disease as well as assessment of nutrition status. The aim of this study was to identify the presence of xenomiRNA in serum and to describe the effect of diet on exosomal miRNAs.

These findings further support the hypothesis that diet-derived plant miRNAs are absorbed in the intestinal track and act on endogenous gene expression via translational repression of endogenous mRNAs. We were not able to show a change in plant miRNA levels over time or detect plant specific miRNAs in the serum of horses. However, this could be due to several factors including: the dilution of plant specific miRNA within the diet, length of dietary trial, and dilution in equine circulation. Furthermore, appropriate normalizers need to be indentified and differences in digestion/metabolism between animals models and cell culture needs to be examined.

Examination of exosomal miRNAs in serum at day 23 of different diets indicated several miRNAs that are differentially expressed after dietary supplementation. 37 miRNAs were differentially expressed in horses fed a corn diet that contained high starch and sugar on day 23 when compared to day 0. Many of these miRNAs have been reported to control insulin sensitivity, glucose tolerance, and adipocyte development. This indicates that a diet higher in non-structural carbohydrates will trigger changes in miRNA expression that result in increased glucose uptake and insulin release. It can be suggested that a dysregulation of these miRNAs under influence of a diet high in starch and sugar, similar to corn, could result in glucose intolerance and insulin resistance, common symptoms of Equine Metabolic Syndrome. Further investigation of the role these miRNAs play in metabolic diseases could lead to the discovery of miRNA biomarkers for specific disease states. Additionally, these findings will contribute to the understanding of the mechanisms behind dietary intervention for treatment of metabolic disorders in the horse.

Ultimately, this data strongly supports the concept that diet plays an important role in endogenous miRNA expression and the potential for xenomiRNAs to regulate endogenous mRNA. Further research is needed to confirm the effects of diet-derived miRNAs on functional gene expression and their mechanism of uptake into mammalian tissue. This is of particular importance for understanding the etiology of metabolic diseases, an ever-increasing disorder in both horses and humans.

CHAPTER V: REFERENCES

Admyre, C., S. M. Johansson, K. R. Qazi, J.-J. Filén, R. Lahesmaa, M. Norman, E. P. A. Neve, A. Scheynius, and S. Gabrielsson. 2007. Exosomes with immune modulatory features are present in human breast milk. J. Immunol. 179:1969–1978.

Alemán, L. M., J. Doench, and P. A. Sharp. 2007. Comparison of siRNA-induced off-target RNA and protein effects. RNA 13:385–395.

Alisi, A., L. Da Sacco, G. Bruscalupi, F. Piemonte, N. Panera, R. De Vito, S. Leoni, G. F. Bottazzo, A. Masotti, and V. Nobili. 2011. Mirnome analysis reveals novel molecular determinants in the pathogenesis of diet-induced nonalcoholic fatty liver disease. Lab. Invest. 91:283–293.

Ambros, V. 2004. The functions of animal microRNAs. Nature 431:350–5.

Aukerman, M. J., and H. Sakai. 2003. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15:2730–2741.

Bartel, D. P., R. Lee, and R. Feinbaum. 2004. MicroRNAs : Genomics , Biogenesis , Mechanism, and Function Genomics : The miRNA Genes. Cell 116:281–297.

Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz. 2006. Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell 125:1111–1124.

Binder, H.J. and A. Reuben. 2009. Ch. 45. Medical Physiology, Second Edition. Philadelphia, PA: Saunders Elsevier.

Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, and C. Hivroz. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J. Immunol. 168:3235–3241.

Bologna, N. G., J. L. Mateos, E. G. Bresso, and J. F. Palatnik. 2009. A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159. EMBO J. 28:3646–3656.

Boron, W.F. and E.L. Boulpaep. 2009. Medical Physiology, Second Edition. Philadelphia, PA: Saunders Elsevier.

Brodersen, P., L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y. Y. Yamamoto, L. Sieburth, and O. Voinnet. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320:1185–1190.

Caby, M.-P., D. Lankar, C. Vincendeau-Scherrer, G. Raposo, and C. Bonnerot. 2005. Exosomal-like vesicles are present in human blood plasma. Int. Immunol. 17:879–887.

Cameron, A. 2012. Exosomal microRNA as a Biological Marker of Maternal Recognition of Pregnancy in the Mare. Colorado State University.

Carrington, J. C., and V. Ambros. 2003. Role of microRNAs in plant and animal development. Science 301:336–338.

Chatterjee, S., and H. Grosshans. 2009. Active turnover modulates mature microRNA activity in Caenorhabditis elegans. Nature 461:546–549.

Chen, C.-Z., L. Li, H. F. Lodish, and D. P. Bartel. 2004. MicroRNAs modulate hematopoietic lineage differentiation. Science 303:83–86.

Chen, X. 2005. MicroRNA biogenesis and function in plants. FEBS Lett. 579:5923–5931.

Chendrimada, T. P., K. J. Finn, X. Ji, D. Baillat, R. I. Gregory, S. A. Liebhaber, A. E. Pasquinelli, and R. Shiekhattar. 2007. MicroRNA silencing through RISC recruitment of eIF6. Nature 447:823–828.

Chendrimada, T. P., R. I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura, and R. Shiekhattar. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436:740–744.

Cheng, H., and C. P. Leblond. 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. Am. J. Anat. 141:461–479.

Clayton, A., A. Turkes, S. Dewitt, R. Steadman, M. D. Mason, and M. B. Hallett. 2004. Adhesion and signaling by B cell-derived exosomes: the role of integrins. FASEB J. 18:977–979.

Cullen, B. R. 2004. Transcription and processing of human microRNA precursors. Mol. Cell 16:861–865.

Dávalos, A., L. Goedeke, P. Smibert, C. M. Ramírez, N. P. Warrier, U. Andreo, D. Cirera-Salinas, K. Rayner, U. Suresh, J. C. Pastor-Pareja, E. Esplugues, E. A. Fisher, L. O. F. Penalva, K. J. Moore, Y. Suárez, E. C. Lai, and C. Fernández-Hernando. 2011. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proc. Natl. Acad. Sci. U. S. A. 108:9232–9237.

Davidson, L. A., N. Wang, M. S. Shah, J. R. Lupton, I. Ivanov, and R. S. Chapkin. 2009. n-3 Polyunsaturated fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon. Carcinogenesis 30:2077–2084.

Davis, C. D., & Ross, S. A. 2008. Evidence for dietary regulation of microRNA expression in cancer cells. Nutrition reviews. *66*(8): 477-482.

Dey, N., F. Das, M. M. Mariappan, C. C. Mandal, N. Ghosh-Choudhury, B. S. Kasinath, and G. G. Choudhury. 2011. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. J. Biol. Chem. 286:25586–25603.

Doench, J., and P. Sharp. 2004. Specificity of microRNA target selection in translational repression. Genes Dev. 18:504–511.

Dolganiuc, A., J. Petrasek, K. Kodys, D. Catalano, P. Mandrekar, A. Velayudham, and G. Szabo. 2009. MicroRNA expression profile in lieber-decarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. Alcohol. Clin. Exp. Res. 33:1704–1710.

Drummond, M. J., E. L. Glynn, C. S. Fry, S. Dhanani, E. Volpi, and B. B. Rasmussen. 2009. Essential amino acids increase microRNA-499, -208b, and -23a and downregulate myostatin and myocyte enhancer factor 2C mRNA expression in human skeletal muscle. J. Nutr. 139:2279–2284.

Druz, A., M. Betenbaugh, J. Shiloach. 2012. Glucose depletion activates mmu-miR-466h-5p expression through oxidative stress and inhibition of histone deacetylation. Nucleic Acids Res. 40: 7291-7302.

Duxbury, M. S., S. W. Ashley, and E. E. Whang. 2005. RNA interference: A mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. Biochem. Biophys. Res. Commun. 331:459–463.

Dyer, J., E. Fernandez-Castaño Merediz, K. S. H. Salmon, C. J. Proudman, G. B. Edwards, and S. P. Shirazi-Beechey. 2002. Molecular characterisation of carbohydrate digestion and absorption in equine small intestine. Equine Vet. J. 34:349–358.

Elhassan, M. O., J. Christie, and M. S. Duxbury. 2012. Homo sapiens Systemic RNA Interference-defective-1 Transmembrane Family Member 1 (SIDT1) Protein Mediates Contact-dependent Small RNA Transfer and MicroRNA-21-driven Chemoresistance. J. Biol. Chem. 287:5267–5277.

Esau, C., S. Davis, S. F. Murray, X. X. Yu, S. K. Pandey, M. Pear, L. Watts, S. L. Booten, M. Graham, R. McKay, A. Subramaniam, S. Propp, B. A. Lollo, S. Freier, C. F. Bennett, S. Bhanot, and B. P. Monia. 2006. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 3:87–98.

Eulalio, A., S. Helms, C. Fritzsch, M. Fauser, and E. Izaurralde. 2009. A C-terminal silencing domain in GW182 is essential for miRNA function. RNA 15:1067–1077.

Evans, W.J., A. Botton, H.F. Hintz, and L.D. Van Vleck. 1990. The Horse, Second Edition. New York, NY: W.H. Freeman and Company.

Fabian, M. R., N. Sonenberg, and W. Filipowicz. 2010. Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. 79:351–79.

Feinberg, E. H., and C. P. Hunter. 2003. Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301:1545–1547.

de Fombelle AG, Goachet M, Varloud P, et al. Effects of the diet on prececal digestion of different starches in the horse measured with the mobile bag technique. Proc Eq Nutr Physiol Symp 2003;18:113–5.

Franco-Zorrilla, J. M., A. Valli, M. Todesco, I. Mateos, M. I. Puga, I. Rubio-Somoza, A. Leyva, D. Weigel, J. A. García, and J. Paz-Ares. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39:1033–1037.

Frank, N. 2011. Equine metabolic syndrome. Vet. Clin. North Am. Equine Pract. 27:73–92.

Frost, R. J. A., and E. N. Olson. 2011. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc. Natl. Acad. Sci. 108:21075–21080.

García-Segura, L., M. Pérez-Andrade, and J. Miranda-Ríos. 2013. The emerging role of MicroRNAs in the regulation of gene expression by nutrients. J. Nutrigenet. Nutrigenomics 6:16–31.

Geor, R.J. 2008. Metabolic Predispositions to Laminitis in Horses and Ponies: Obesity, Insulin Resistance and Metabolic Syndromes. J. Equine Vet. Sci. 28:753-759.

Gibbings, D. J., C. Ciaudo, M. Erhardt, and O. Voinnet. 2009. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat. Cell Biol. 11:1143–1149.

Gori, M., M. Arciello, and C. Balsano. 2014. MicroRNAs in nonalcoholic fatty liver disease: Novel biomarkers and prognostic tools during the transition from steatosis to hepatocarcinoma. Biomed Res. Int. 2014.

Gregory, R. I., K.-P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, and R. Shiekhattar. 2004. The Microprocessor complex mediates the genesis of microRNAs. Nature 432:235–240.

Grimson, A., K. K. H. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim, and D. P. Bartel. 2007. MicroRNA Targeting Specificity in Mammals: Determinants beyond Seed Pairing. Mol. Cell 27:91–105.

Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun, and C. C. Mello. 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106:23–34.

Henke, J. I., D. Goergen, J. Zheng, Y. Song, C. G. Schüttler, C. Fehr, C. Jünemann, and M. Niepmann. 2008. microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J. 27:3300–3310.

Hoffman, R. M., R. C. Boston, D. Stefanovski, D. S. Kronfeld, and P. A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. J. Anim. Sci. 81:2333–2342.

Humphreys, D. T., B. J. Westman, D. I. K. Martin, and T. Preiss. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc. Natl. Acad. Sci. U. S. A. 102:16961–16966.

Hutvágner, G., and P. D. Zamore. 2002. A microRNA in a multiple-turnover RNAi enzyme complex. Science 297:2056–2060.

Hwang, H.-W., E. A. Wentzel, and J. T. Mendell. 2007. A hexanucleotide element directs microRNA nuclear import. Science 315:97–100.

Jenkins, D. J. A., C. W. C. Kendall, G. Mckeown-eyssen, R. G. Josse, J. Silverberg, G. L. Booth, E. Vidgen, S. Corrigan, S. Ares, L. S. A. Augustin, T. L. Parker, L. A. Leiter, H. E. Need, and F. O. R. Implementa. 2013. Effect of a Low – Glycemic Index or a High – Cereal Fiber Diet on Type 2 Diabetes. JAMA J. Am. Med. Assoc. 300:2742–2753.

Jiang, M., X. Sang, and Z. Hong. 2012. Beyond nutrients: food-derived microRNAs provide cross-kingdom regulation. Bioessays 34:280–4.

Jiao, Y., W. Song, M. Zhang, and J. Lai. 2011. Identification of novel maize miRNAs by measuring the precision of precursor processing. BMC Plant Biol. 11:141.

Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J. Biol. Chem. 262:9412–9420.

Jordan, S. D., M. Krüger, D. M. Willmes, N. Redemann, F. T. Wunderlich, H. S. Brönneke, C. Merkwirth, H. Kashkar, V. M. Olkkonen, T. Böttger, T. Braun, J. Seibler, and J. C. Brüning. 2011. Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. Nat. Cell Biol. 13:434–446.

Kararli, T. T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm. Drug Dispos. 16:351–380.

Katoh, T., Y. Sakaguchi, K. Miyauchi, T. Suzuki, S.-I. Kashiwabara, T. Baba, and T. Suzuki. 2009. Selective stabilization of mammalian microRNAs by 3’ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. Genes Dev. 23:433–438.

Kawamata, T., H. Seitz, and Y. Tomari. 2009. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nat. Struct. Mol. Biol. 16:953–960.

Keller, S., C. Rupp, A. Stoeck, S. Runz, M. Fogel, S. Lugert, H.-D. Hager, M. S. Abdel-Bakky, P. Gutwein, and P. Altevogt. 2007. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int. 72:1095–1102.

Keller, S., M. P. Sanderson, A. Stoeck, and P. Altevogt. 2006. Exosomes: From biogenesis and secretion to biological function. Immunol. Lett. 107:102–108.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, and R. H. Plasterk. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 15:2654–2659.

Khvorova, A., A. Reynolds, and S. D. Jayasena. 2003. Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209–216.

Kim, J., K. Inoue, J. Ishii, W. B. Vanti, S. V Voronov, E. Murchison, G. Hannon, and A. Abeliovich. 2007. A MicroRNA feedback circuit in midbrain dopamine neurons. Science 317:1220–1224.

Kim, V. N. 2005. MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. 6:376–385.

Kiriakidou, M., G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson, and Z. Mourelatos. 2007. An mRNA m7G Cap Binding-like Motif within Human Ago2 Represses Translation. Cell 129:1141–1151.

Kozuka, C., K. Yabiku, S. Sunagawa, R. Ueda, S. -i. Taira, H. Ohshiro, T. Ikema, K. Yamakawa, M. Higa, H. Tanaka, C. Takayama, M. Matsushita, S. Oyadomari, M. Shimabukuro, and H. Masuzaki. 2012a. Brown Rice and Its Component, -Oryzanol, Attenuate the Preference for High-Fat Diet by Decreasing Hypothalamic Endoplasmic Reticulum Stress in Mice. Diabetes.

Kozuka, C., K. Yabiku, S. Sunagawa, R. Ueda, S. -i. Taira, H. Ohshiro, T. Ikema, K. Yamakawa, M. Higa, H. Tanaka, C. Takayama, M. Matsushita, S. Oyadomari, M. Shimabukuro, and H. Masuzaki. 2012b. Brown Rice and Its Component, -Oryzanol, Attenuate the Preference for High-Fat Diet by Decreasing Hypothalamic Endoplasmic Reticulum Stress in Mice. Diabetes.

Kriegel, C., H. Attarwala, and M. Amiji. 2013. Multi-compartmental oral delivery systems for nucleic acid therapy in the gastrointestinal tract. Adv. Drug Deliv. Rev. 65:891–901.

Krol, J., V. Busskamp, I. Markiewicz, M. B. Stadler, S. Ribi, J. Richter, J. Duebel, S. Bicker, H. J. Fehling, D. Schubeler, T. G. Oertner, G. Schratt, M. Bibel, B. Roska, and W. Filipowicz. 2010. Characterizing Light-Regulated Retinal MicroRNAs Reveals Rapid Turnover as a Common Property of Neuronal MicroRNAs. Cell 141:618–631.

Krol, J., I. Loedige, and W. Filipowicz. 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11:597–610.

Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl. 2001. Identification of novel genes coding for small expressed RNAs. Science 294:853–858.

Lau, N. C., L. P. Lim, E. G. Weinstein, and D. P. Bartel. 2001. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294:858–862.

Lee, R. C., and V. Ambros. 2001. An extensive class of small RNAs in Caenorhabditis elegans. Science 294:862–864.

Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854.

Lee, Y., K. Jeon, J. T. Lee, S. Kim, and V. N. Kim. 2002. MicroRNA maturation: Stepwise processing and subcellular localization. EMBO J. 21:4663–4670.

Lim, L. P., N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, and J. M. Johnson. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769–773.

Liu, J., M. A. Carmell, F. V Rivas, C. G. Marsden, J. M. Thomson, J.-J. Song, S. M. Hammond, L. Joshua-Tor, and G. J. Hannon. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305:1437–1441.

Liu, J., F. V Rivas, J. Wohlschlegel, J. R. Yates, R. Parker, and G. J. Hannon. 2005. A role for the P-body component GW182 in microRNA function. Nat. Cell Biol. 7:1261–1266.

Lu, S., Y. H. Sun, and V. L. Chiang. 2009. Adenylation of plant miRNAs. Nucleic Acids Res. 37:1878–1885.

Lund, E., S. Güttinger, A. Calado, J. E. Dahlberg, and U. Kutay. 2004. Nuclear export of microRNA precursors. Science. 303:95–98.

Mathonnet, G., M. R. Fabian, Y. V Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W. C. Merrick, E. Darzynkiewicz, R. S. Pillai, W. Filipowicz, T. F. Duchaine, and N. Sonenberg. 2007. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science. 317:1764–1767.

McKenzie, E.C., S.J. Valberg, and S.M. Goden. 2003. Effect of Dietary Starch, Fat and Bicarbonate Content on Excercise Responses and Serum Creatine Kinase Activity in Equine Recurrent Exertional Rhabdomyolysis. J. Vet. Intern. Med. 17:693-701.

Mears, R., R. A. Craven, S. Hanrahan, N. Totty, C. Upton, S. L. Young, P. Patel, P. J. Selby, and R. E. Banks. 2004. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics 4:4019–4031.

Meister, G., and T. Tuschl. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349.

Milenkovic, D., C. Deval, E. Gouranton, J. F. Landrier, A. Scalbert, C. Morand, and A. Mazur. 2012. Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: A new mechanism of the action of polyphenols. PLoS One 7.

Mittelbrunn, M., C. Gutiérrez-Vázquez, C. Villarroya-Beltri, S. González, F. Sánchez-Cabo, M. Á. González, A. Bernad, and F. Sánchez-Madrid. 2011. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2:282.

Morel, J.-B., C. Godon, P. Mourrain, C. Béclin, S. Boutet, F. Feuerbach, F. Proux, and H. Vaucheret. 2002. Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell. 14:629–639.

Morelli, A. E., A. T. Larregina, W. J. Shufesky, M. L. G. Sullivan, D. B. Stolz, G. D. Papworth, A. F. Zahorchak, A. J. Logar, Z. Wang, S. C. Watkins, L. D. Falo, and A. W. Thomson. 2004. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood. 104:3257–3266.

Mourelatos, Z., J. Dostie, S. Paushkin, A. Sharma, B. Charroux, L. Abel, J. Rappsilber, M. Mann, and G. Dreyfuss. 2002. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. 16:720–728.

Mu, J., X. Zhuang, Q. Wang, H. Jiang, Z.-B. Deng, B. Wang, L. Zhang, S. Kakar, Y. Jun, D. Miller, and H.-G. Zhang. 2014. Interspecies communication between plant and mouse gut host cells through edible plant derived exosome-like nanoparticles. Mol. Nutr. Food Res.:1–13.

Munch, E. M., R. A. Harris, M. Mohammad, A. L. Benham, S. M. Pejerrey, L. Showalter, M. Hu, C. D. Shope, P. D. Maningat, P. H. Gunaratne, M. Haymond, and K. Aagaard. 2013. Transcriptome Profiling of microRNA by Next-Gen Deep Sequencing Reveals Known and Novel miRNA Species in the Lipid Fraction of Human Breast Milk. PLoS One 8.

Nakanishi, N., Y. Nakagawa, N. Tokushige, N. Aoki, T. Matsuzaka, K. Ishii, N. Yahagi, K. Kobayashi, S. Yatoh, A. Takahashi, H. Suzuki, O. Urayama, N. Yamada, and H. Shimano. 2009. The up-regulation of microRNA-335 is associated with lipid metabolism in liver and white adipose tissue of genetically obese mice. Biochem. Biophys. Res. Commun. 385:492–496.

Naqvi, A. R., M. Sarwat, S. Hasan, and N. Roychodhury. 2012. Biogenesis, functions and fate of plant microRNAs. J. Cell. Physiol. 227:3163–8.

Van Niel, G., I. Porto-Carreiro, S. Simoes, and G. Raposo. 2006. Exosomes: A common pathway for a specialized function. J. Biochem. 140:13–21.

Van Niel, G., G. Raposo, C. Candalh, M. Boussac, R. Hershberg, N. Cerf-Bensussan, and M. Heyman. 2001. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology 121:337–349.

Nottrott, S., M. J. Simard, and J. D. Richter. 2006. Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat. Struct. Mol. Biol. 13:1108–1114.

Ogawa, Y., M. Kanai-Azuma, Y. Akimoto, H. Kawakami, and R. Yanoshita. 2008. Exosome-like vesicles with dipeptidyl peptidase IV in human saliva. Biol. Pharm. Bull. 31:1059–1062.

Olsen, P. H., and V. Ambros. 1999. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 216:671–680.

Orom, U. A., F. C. Nielsen, and A. H. Lund. 2008. MicroRNA-10a Binds the 5'UTR of Ribosomal Protein mRNAs and Enhances Their Translation. Mol. Cell 30:460–471.

Palatnik, J. F., E. Allen, X. Wu, C. Schommer, R. Schwab, J. C. Carrington, and D. Weigel. 2003. Control of leaf morphogenesis by microRNAs. Nature 425:257–263.

Pan, B. T., and R. M. Johnstone. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell 33:967–978.

Pan, B. T., K. Teng, C. Wu, M. Adam, and R. M. Johnstone. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J. Cell Biol. 101:942–948.

Park, M. Y., G. Wu, A. Gonzalez-Sulser, H. Vaucheret, and R. S. Poethig. 2005. Nuclear processing and export of microRNAs in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 102:3691–3696.

Patrick, C., G.-S. Wang, D. E. Lefebvre, J. a Crookshank, B. Sonier, C. Eberhard, M. Mojibian, C. R. Kennedy, S. P. J. Brooks, M. L. Kalmokoff, M. Maglio, R. Troncone, P. Poussier, and F. W. Scott. 2013. Promotion of Autoimmune Diabetes by Cereal Diet in the Presence or Absence of Microbes Associated With Gut Immune Activation, Regulatory Imbalance, and Altered Cathelicidin Antimicrobial Peptide. Diabetes:1–12.

Peters, L., and G. Meister. 2007. Argonaute Proteins: Mediators of RNA Silencing. Mol. Cell. 26:611–623.

Petersen, C. P., M. E. Bordeleau, J. Pelletier, and P. A. Sharp. 2006. Short RNAs repress translation after initiation in mammalian cells. Mol. Cell. 21:533–542.

Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand, and W. Filipowicz. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 309:1573–1576.

Pisitkun, T., R.-F. Shen, and M. A. Knepper. 2004. Identification and proteomic profiling of exosomes in human urine. Proc. Natl. Acad. Sci. U. S. A. 101:13368–13373.

Price, D. R. G., and J. A. Gatehouse. 2008. RNAi-mediated crop protection against insects. Trends Biotechnol. 26:393–400.

Ralston, S. L. 2007. Evidence-based equine nutrition. Vet. Clin. North Am. Equine Pract. 23:365–84.

Ramachandran, V., and X. Chen. 2008. Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. Science 321:1490–1492.

Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V Harding, C. J. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. J. Exp. Med. 183:1161–1172.

Raposo, G., D. Tenza, S. Mecheri, R. Peronet, C. Bonnerot, and C. Desaymard. 1997. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol. Biol. Cell 8:2631–2645.

Reed , S.M., B.M. Warwick, D.C. Sellon. 2009. Equine Internal Medicine. St. Louis, MO : Saunders/Elsevier.

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:901–906.

Reinhart, B. J., E. G. Weinstein, M. W. Rhoades, B. Bartel, and D. P. Bartel. 2002. MicroRNAs in plants. Genes Dev. 16:1616–1626.

Romao, J. M., W. Jin, M. V Dodson, G. J. Hausman, S. S. Moore, and L. L. Guan. 2011. MicroRNA regulation in mammalian adipogenesis. Exp. Biol. Med. (Maywood). 236:997–1004.

Van Rooij, E., L. B. Sutherland, X. Qi, J. A. Richardson, J. Hill, and E. N. Olson. 2007. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 316:575–579.

Rottiers, V., and A. M. Näär. 2012. MicroRNAs in metabolism and metabolic disorders. Nat. Rev. Mol. Cell Biol. 13:239–250.

Seggerson, K., L. Tang, and E. G. Moss. 2002. Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev. Biol. 243:215–225.

Da Silveira, J. C., D. N. R. Veeramachaneni, Q. a Winger, E. M. Carnevale, and G. J. Bouma. 2012. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. Biol. Reprod. 86:71.

Simpson, R. J., J. W. Lim, R. L. Moritz, and S. Mathivanan. 2009. Exosomes: proteomic insights and diagnostic potential. Expert Rev. Proteomics. 6:267–283.

Smith, H.W. 1965. Observations on the Flora of the Alimentary Tract of Animals and Factors Affecting its Composition. J. Pathol. Bacteriol. 89:95–122.

Snow, J. W., A. E. Hale, S. K. Isaacs, A. L. Baggish, and S. Y. Chan. 2013. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biol. 10:1107–16.

Song, J.-J., S. K. Smith, G. J. Hannon, and L. Joshua-Tor. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. Science. 305:1434–1437.

Tabara, H., A. Grishok, and C. C. Mello. 1998a. RNAi in C. elegans: soaking in the genome sequence. Science. 282:430–431.

Tabara, H., A. Grishok, and C. C. Mello. 1998b. RNAi in C. elegans: soaking in the genome sequence. Science. 282:430–431.

Taylor, D. D., and C. Gercel-Taylor. 2008. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol. Oncol. 110:13–21.

Théry, C., S. Amigorena, G. Raposo, and A. Clayton. 2006. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr. Protoc. Cell Biol. Chapter 3:Unit 3.22.

Théry, C., A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena. 1999. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. J. Cell Biol. 147:599–610.

Timmons, L., and A. Fire. 1998. Specific interference by ingested dsRNA. Nature. 395:854.

Tomari, Y., T. Du, and P. D. Zamore. 2007. Sorting of Drosophila Small Silencing RNAs. Cell. 130:299–308.

Triboulet, R., H.-M. Chang, R. J. Lapierre, and R. I. Gregory. 2009. Post-transcriptional control of DGCR8 expression by the Microprocessor. RNA. 15:1005–1011.

Tsang, J., J. Zhu, and A. van Oudenaarden. 2007. MicroRNA-Mediated Feedback and Feedforward Loops Are Recurrent Network Motifs in Mammals. Mol. Cell. 26:753–767.

Valadi, H., K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9:654–659.

Vlassov, A. V., S. Magdaleno, R. Setterquist, and R. Conrad. 2012. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim. Biophys. Acta - Gen. Subj. 1820:940–948.

Wakiyama, M., K. Takimoto, O. Ohara, and S. Yokoyama. 2007. Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev. 21:1857–1862.

Wightman, B., I. Ha, and G. Ruvkun. 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 75:855–862.

Williams, C. A., D. S. Kronfeld, W. B. Staniar, and P. A. Harris. 2001. Plasma glucose and insulin responses of Thoroughbred mares fed a meal high in starch and sugar or fat and fiber. J. Anim. Sci. 79:2196–2201.

Winston, W. M., C. Molodowitch, and C. P. Hunter. 2002. Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science. 295:2456–2459.

Winston, W. M., M. Sutherlin, A. J. Wright, E. H. Feinberg, and C. P. Hunter. 2007a. Caenorhabditis elegans SID-2 is required for environmental RNA interference. Proc. Natl. Acad. Sci. U. S. A. 104:10565–10570.

Winston, W. M., M. Sutherlin, A. J. Wright, E. H. Feinberg, and C. P. Hunter. 2007b. Caenorhabditis elegans SID-2 is required for environmental RNA interference. Proc. Natl. Acad. Sci. U. S. A. 104:10565–10570.

Winter, J., S. Jung, S. Keller, R. I. Gregory, and S. Diederichs. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat. Cell Biol. 11:228–234.

Witwer, K. W., and K. D. Hirschi. 2014. Transfer and functional consequences of dietary microRNAs in vertebrates: Concepts in search of corroboration. BioEssays 36:394–406.

Witwer, K. W., M. A. Mcalexander, S. E. Queen, and R. J. Adams. 2013. Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs Limited evidence for general uptake of dietary plant xenomiRs. 10:1080–1086.

Wolfrum, C., S. Shi, K. N. Jayaprakash, M. Jayaraman, G. Wang, R. K. Pandey, K. G. Rajeev, T. Nakayama, K. Charrise, E. M. Ndungo, T. Zimmermann, V. Koteliansky, M. Manoharan, and M. Stoffel. 2007. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat. Biotechnol. 25:1149–1157.

Wu, J., J. Qian, C. Li, L. Kwok, F. Cheng, P. Liu, C. Perdomo, D. Kotton, C. Vaziri, C. Anderlind, A. Spira, W. V Cardoso, and J. Lü. 2010. miR-129 regulates cell proliferation by downregulating Cdk6 expression. Cell Cycle. 9:1809–1818.

Xue, L. J., J. J. Zhang, and H. W. Xue. 2009. Characterization and expression profiles of miRNAs in rice seeds. Nucleic Acids Res. 37:916–930.

Yoda, M., T. Kawamata, Z. Paroo, X. Ye, S. Iwasaki, Q. Liu, and Y. Tomari. 2010. ATP-dependent human RISC assembly pathways. Nat. Struct. Mol. Biol. 17:17–23.

Yu, B., Z. Yang, J. Li, S. Minakhina, M. Yang, R. W. Padgett, R. Steward, and X. Chen. 2005. Methylation as a crucial step in plant microRNA biogenesis. Science. 307:932–935.

Zhang, L., J. M. Chia, S. Kumari, J. C. Stein, Z. Liu, A. Narechania, C. A. Maher, K. Guill, M. D. McMullen, and D. Ware. 2009. A genome-wide characterization of microRNA genes in maize. PLoS Genet. 5.

Zhang, L., D. Hou, X. Chen, D. Li, L. Zhu, Y. Zhang, J. Li, Z. Bian, X. Liang, X. Cai, Y. Yin, C. Wang, T. Zhang, D. Zhu, D. Zhang, J. Xu, Q. Chen, Y. Ba, J. Liu, Q. Wang, J. Chen, J. Wang, M. Wang, Q. Zhang, J. Zhang, K. Zen, and C.-Y. Zhang. 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res. 22:273–274.

Zhou, M., Q. Wang, J. Sun, X. Li, L. Xu, H. Yang, H. Shi, S. Ning, L. Chen, Y. Li, T. He, and Y. Zheng. 2009. In silico detection and characteristics of novel microRNA genes in the Equus caballus genome using an integrated ab initio and comparative genomic approach. Genomics. 94:125–131.

Zhu, H., S. C. Ng, A. V. Segr, G. Shinoda, S. P. Shah, W. S. Einhorn, A. Takeuchi, J. M. Engreitz, J. P. Hagan, M. G. Kharas, A. Urbach, J. E. Thornton, R. Triboulet, R. I. Gregory, D. Altshuler, and G. Q. Daley. 2011. The Lin28/let-7 axis regulates glucose metabolism. Cell. 147:81–94.

APPENDICES

Appendix Table I: 340 Equine miRNA primer sequences

Mature miRNA ID	Target miRNA Mature Sequence
eca-let-7a	ugagguaguagguuguauaguu
eca-let-7c	ugagguaguagguuguaugguu
eca-let-7d	agagguaguagguugcauaguu
eca-let-7e	ugagguaggagguuguauaguu
eca-let-7f	ugagguaguagauuguauaguu
eca-let-7g	ugagguaguaguuuguacaguu
eca-mir-1	uggaauguaaagaaguauguau
eca-mir-7	uggaagacuagugauuuuguugu
eca-mir-9a	ucuuugguuaucuagcuguauga
eca-mir-10a	uacccuguagauccgaauuugug
eca-mir-10b	uacccuguagaaccgaauuugug
eca-mir-15a	uagcagcacauaaugguuugug
eca-mir-15b	uagcagcacaucaugguuuaca
eca-mir-16	uagcagcacguaaauauuggcg
eca-mir-17	caaagugcuuacagugcagguag
eca-mir-18a	uaaggugcaucuagugcagauag
eca-mir-18b	uaaggugcaucuagugcaguuag
eca-mir-19a	ugugcaaaucuaugcaaaacuga
eca-mir-19b	ugugcaaaucuaugcaaaacuga
eca-mir-20a	uaaagugcuuauagugcagguag
eca-mir-20b	caaagugcucauagugcagguag
eca-mir-21	uagcuuaucagacugauguuga
eca-mir-22	aagcugccaguugaagaacugu
eca-mir-23a	aucacauugccagggauuucc
eca-mir-23b	aucacauugccagggauuacc
eca-mir-24	uggcucaguucagcaggaacag
eca-mir-25	cauugcacuugucucggucuga
eca-mir-26a	uucaaguaauccaggauaggcu
eca-mir-27a	uucacaguggcuaaguuccgc
eca-mir-27b	uucacaguggcuaaguucugc
eca-mir-283p	cacuagauugugagcuccugga
eca-mir-285p	aaggagcucacagucuauugag
eca-mir-29a	uagcaccaucugaaaucgguua

eca-mir-29b	uagcaccauuugaaaucaguguu
eca-mir-29c	uagcaccauuugaaaucgguua
eca-mir-30b	uguaaacauccuacacucagcu
eca-mir-30c	uguaaacauccuacacucucagc
eca-mir-30d	uguaaacaucccgacuggaag
eca-mir-30e	uguaaacauccuugacuggaag
eca-mir-31	aggcaagaugcuggcauagcu
eca-mir-32	uauugcacauuacuaaguugca
eca-mir-33a	gugcauuguaguugcauugca
eca-mir-33b	gugcauugcuguugcauugc
eca-mir-34	uggcagugucuuagcugguugu
eca-mir-92a	uauugcacuugucccggccugu
eca-mir-92b	uauugcacucgucccggccucc
eca-mir-93	caaagugcuguucgugcagguag
eca-mir-95	uucaacgggucuuuauugagca
eca-mir-96	uuuggcacuagcacauuuuugcu
eca-mir-98	ugagguaguaaguuguauuguu
eca-mir-99a	aacccguagauccgaucuugug
eca-mir-99b	cacccguagaaccgaccuugcg
eca-mir-100	aacccguagauccgaacuugug
eca-mir-101	uacaguacugugauaacugaa
eca-mir-103	agcagcauuguacagggcuauga
eca-mir-105	ucaaaugcucagacuccuguggu
eca-mir-106a	caaagugcuuacagugcagguag
eca-mir-106b	uaaagugcugacagugcagau
eca-mir-107b	agcagcauuguacagggcuauca
eca-mir-122	uggagugugacaaugguguuug
eca-mir-124	uaaggcacgcggugaaugcc
eca-mir-125a3p	acaggugagguucuugggagcc
eca-mir-125a5p	ucccugagacccuuuaaccuguga
eca-mir-125b	ucccugagacccuaacuuguga
eca-mir-1263p	ucguaccgugaguaauaaugcg
eca-mir-127	ucggauccgucugagcuuggcu
eca-mir-128	ucacagugaaccggucucuuu
eca-mir-129a3p	aagcccuuaccccaaaaaguau
eca-mir-129a5p	cuuuuugcggucugggcuugc
eca-mir-130a	cagugcaauguuaaaagggcau
eca-mir-130b	cagugcaaugaugaaagggcau
eca-mir-132	uaacagucuacagccauggucg
eca-mir-133a	uuuggucccuucaaccagcug

eca-mir-133b	uuuggucccuucaaccagcua
eca-mir-134	ugugacugguugaccagagggg
eca-mir-135a	uauggcuuuuuauuccuauguga
eca-mir-135b	uauggcuuuucauuccuauguga
eca-mir-136	acuccauuuguuuugaugaugg
eca-mir-137	uuauugcuuaagaauacgcguag
eca-mir-138	agcugguguugugaaucaggccg
eca-mir-1393p	ggagacgcggcccuguuggagu
eca-mir-1395p	ucuacagugcacgugucuccag
eca-mir-1403p	uaccacaggguagaaccacgg
eca-mir-1405p	cagugguuuuacccuaugguag
eca-mir-141	uaacacugucugguaaagaugg
eca-mir-1423p	uguaguguuuccuacuuuaugga
eca-mir-1425p	cauaaaguagaaagcacuacu
eca-mir-143	ugagaugaagcacuguagcuc
eca-mir-144	uacaguauagaugauguacu
eca-mir-145	guccaguuuucccaggaaucccu
eca-mir-146a	ugagaacugaauuccauggguu
eca-mir-146b3p	ugcccuagggacucaguucugg
eca-mir-146b5p	ugagaacugaauuccauaggcu
eca-mir-147b	gugugccgaaaugcuucugcua
eca-mir-148a	ucagugcacuacagaacuuugu
eca-mir-148b3p	ucagugcaucacagaacuuugu
eca-mir-149	ucuggcuccgugucuucacuccc
eca-mir-150	ucucccaacccuuguaccagug
eca-mir-1515p	ucgaggagcucacagucuagu
eca-mir-153	uugcauagucacaaaagugauc
eca-mir-154	uagguuauccguguugccuucg
eca-mir-155	uuaaugcuaaucgugauaggggu
eca-mir-181a	aacauucaacgcugucggugagu
eca-mir-181b	aacauucauugcugucggugggu
eca-mir-182	uuuggcaaugguagaacucacacug
eca-mir-183	uauggcacugguagaauucacu
eca-mir-184	uggacggagaacugauaagggu
eca-mir-186	caaagaauucuccuuuugggcu
eca-mir-187	ucgugucuuguguugcagccgg
eca-mir-1883p	cucccacaugcaggguuugca
eca-mir-1885p	caucccuugcaugguggaggg
eca-mir-190	ugauauguuugauauauuaggu
eca-mir-190b	ugauauguuugauauuggguu

eca-mir-191	caacggaaucccaaaagcagcug
eca-mir-192	cugaccuaugaauugacagcc
eca-mir-193a3p	aacuggccuacaaagucccagu
eca-mir-193a5p	uggguuuugcgggcgagauga
eca-mir-193b	aacuggcccacaaagucccgcu
eca-mir-194	uguaacagcaacuccaugugga
eca-mir-195	uagcagcacagaaauauuggc
eca-mir-196a	uagguaguuucauguuguuggg
eca-mir-196b	uagguaguuuccuguuguuggg
eca-mir-197	uucaccaccuucuccacccagc
eca-mir-199a3p	acaguagucugcacauugguag
eca-mir-199a5p	cccaguguucagacuaccuguuc
eca-mir-199b3p	acaguagucugcacauugguua
eca-mir-199b5p	cccaguguuuagacuaucuguuc
eca-mir-200a	uaacacugucugguaacgaugu
eca-mir-200b	uaauacugccugguaaugauga
eca-mir-200c	uaauacugccggguaaugaugga
eca-mir-204b	uucccuuugucauccuaugccu
eca-mir-205	uccuucauuccaccggagucug
eca-mir-206	uggaauguaaggaagugugugg
eca-mir-208a	auaagacgagcaaaaagcuugu
eca-mir-208b	auaagacgaacaaaagguuugu
eca-mir-211	uucccuuugucauccuuugccu
eca-mir-212	uaacagucuccagucacggcc
eca-mir-214	acagcaggcacagacaggcagu
eca-mir-215	augaccuaugaauugacagac
eca-mir-216a	uaaucucagcuggcaacuguga
eca-mir-216b	aaaucucugcaggcaaauguga
eca-mir-217	uacugcaucaggaacugauugga
eca-mir-218	uugugcuugaucuaaccaugu
eca-mir-2195p	ugauuguccaaacgcaauucu
eca-mir-221	agcuacauugucugcuggguuuc
eca-mir-222	agcuacaucuggcuacugggu
eca-mir-223	ugucaguuugucaaauacccca
eca-mir-224	caagucacuagugguuccguu
eca-mir-296	gaggguuggguggaggcuuucc
eca-mir-299	uaugugggaugguaaaccgcuu
eca-mir-301a	cagugcaauaguauugucaaagc
eca-mir-301b3p	cagugcaaugauauugucaaagc
eca-mir-302a	uaagugcuuccauguuuuaguga

eca-mir-302b	uaagugcuuccauguuuuaguag
eca-mir-302c	uaagugcuuccauguuucagugg
eca-mir-302d	uaagugcuuccauguuuuagugu
eca-mir-3233p	cacauuacacggucgaccucu
eca-mir-3235p	aggugguccguggcgcguucgc
eca-mir-3243p	ccacugccccaggugcugcugg
eca-mir-3245p	cgcaucccccuagggcauuggugu
eca-mir-326	ccucugggcccuuccuccagc
eca-mir-328	cuggcccucucugcccuuccgu
eca-mir-329	aacacaccuaguuaaccucuuu
eca-mir-330	ucucugggccugugucuuaggc
eca-mir-331	gccccugggccuauccuagaa
eca-mir-335	ucaagagcaauaacgaaaaaugu
eca-mir-3373p	cuccuaugagaugccuuuccuc
eca-mir-3375p	gaacggcuucauacaggagcu
eca-mir-3383p	uccagcaucagugauuuuguug
eca-mir-3385p	aacaauauccuggugcugagug
eca-mir-3405p	uuauaaagcaaugagacugauu
eca-mir-3423p	ucucacacagaaaucgcacccgu
eca-mir-3425p	aggggugcuaucugugauugag
eca-mir-3455p	gcugacuccuaguccagugcuc
eca-mir-346	ugucugcccgcaugccugccucu
eca-mir-3613p	uccccaggcgugauucugauuu
eca-mir-3615p	uuaucagaaucuccagggguac
eca-mir-3623p	aacacaccuauucaaggauuca
eca-mir-3625p	aauccuuggaaccuaggugugagu
eca-mir-363	aauugcacgguauccaucugua
eca-mir-365	uaaugccccuaaaaauccuuau
eca-mir-367	aauugcacuuuagcaaugguga
eca-mir-3693p	aauaauacaugguugaucuuu
eca-mir-3695p	agaucgaccgugucauauucgc
eca-mir-370	gccugcugggguggaaccuggu
eca-mir-3713p	aagugccgccauuuuuugagugu
eca-mir-3715p	acucaaacuguggggggcacu
eca-mir-374a	uuauaauacaaccugauaagug
eca-mir-374b	auauaauacaaccugcuaagug
eca-mir-376a	aucauagaggaaaauccacgu
eca-mir-376b	aucauagaggaaaauccaugu
eca-mir-376c	aacauagaggaaauuccacgu
eca-mir-377	aucacacaaaggcaacuuuugu

eca-mir-378	acuggacuuggagucagaagg
eca-mir-379	ugguagacuauggaacguagg
eca-mir-380	uauguaauaugguccacgucuu
eca-mir-381	uauacaagggcaagcucucugu
eca-mir-382	gaaguuguucgugguggauucg
eca-mir-383	agaucagaaggugauuguggcu
eca-mir-384	auuccuagaaauuguucaca
eca-mir-4093p	gaauguugcucggugaaccccu
eca-mir-4095p	agguuacccgagcaacuuugcau
eca-mir-410	aauauaacacagauggccugu
eca-mir-411	uaguagaccguauagcguacg
eca-mir-412	uucaccugguccacuagccg
eca-mir-421	ggccucauuaaaauguuuguug
eca-mir-4233p	agcucggucugaggccccucagu
eca-mir-4235p	ugaggggcagagagcgagacuuu
eca-mir-424	cagcagcaauucauguuuugaa
eca-mir-429	uaauacugucugguaaugccg
eca-mir-431	ugucuugcaggccgucaugcagg
eca-mir-432	ucuuggaguaggucauugggugg
eca-mir-433	aucaugaugggcuccucggugu
eca-mir-448	uugcauauguaggaugucccau
eca-mir-449a	uggcaguguauuguuagcuggu
eca-mir-450a	uuuugcgauguguuccuaauau
eca-mir-450b3p	uugggaacauuuuugcauccaua
eca-mir-450b5p	uuuugcaauauguuccugaaua
eca-mir-451	aaaccguuaccauuacuguguu
eca-mir-454	uagugcaauauugcuuauagggu
eca-mir-4853p	gucauacacggcucuccucucu
eca-mir-4855p	agaggcuggccgugaugaauuc
eca-mir-4863p	cggggcagcucaguacaggau
eca-mir-4865p	uccuguacugagcugccccgag
eca-mir-487a	aaucauacagggacauccaguu
eca-mir-487b	aaucguacagggucauccacuu
eca-mir-488	uugaaaggcuauuucuugguc
eca-mir-489	gugacaucacauauacggcggc
eca-mir-4903p	caaccuggaggacuccaugcug
eca-mir-4905p	ccauggaucuccaggugggu
eca-mir-4913p	cuuaugcaagauucccuucuac
eca-mir-4915p	agugggggaacccuuccaugagg
eca-mir-492	aggagcugcgggacaagauucuu

eca-mir-493b	ugaaggucuuccgugugccagg
eca-mir-494	ugaaacauacacgggaaaccuc
eca-mir-495	aaacaaacauggugcacuucuu
eca-mir-496	ugaguauuacauggccaaucuc
eca-mir-497	cagcagcacacugugguuugu
eca-mir-4993p	aacaucacagcaagucugugcu
eca-mir-4995p	uuaagacuugcagugauguuu
eca-mir-500	uaauccuugcuaccugggugaga
eca-mir-501	auccuucgucccugggugaga
eca-mir-5023p	aaugcaccugggcaaggauuca
eca-mir-5025p	auccuugcuaucugggugcua
eca-mir-503	uagcagcgggaacaguacugcag
eca-mir-504	agacccuggucugcacucuauc
eca-mir-505	cgucaacacuugcugguuuccu
eca-mir-507	auuggcaccucuuagagugaa
eca-mir-5083p	ugauugucaccuuuuggaguaga
eca-mir-5085p	uacuccagagggugucauucaca
eca-mir-5095p	uacugcagacaguggcaauca
eca-mir-514	auugacaccucugugagugga
eca-mir-5323p	ccucccacacccaaggcuugca
eca-mir-5325p	caugccuugaguguaggaccgu
eca-mir-539	ggagaaauuauccuugcugugu
eca-mir-541	uggugggcacagaauccagucu
eca-mir-5423p	ugugacagauugauaacugaaa
eca-mir-5425p	cucggggaucaucaugucacga
eca-mir-543	aaacauucgcggugcacuucuu
eca-mir-544b	auucugcauuuuuaacaaguuc
eca-mir-545	ucaacaaacauuuauugugugc
eca-mir-551a	gcgacccacucuugguuucca
eca-mir-551b	gcgacccauacuugguuucag
eca-mir-568	auguauaaauguauacacac
eca-mir-5825p	uuacaguuguucaaccaguuacu
eca-mir-5823p	uaaccgguugaacaacugaacc
eca-mir-5905p	gagcuuauucauaaaaguacag
eca-mir-5903p	uaauuuuauguauaagcuagu
eca-mir-592	uugugucaauaugcgaugaugu
eca-mir-598	uacgucaucguugucaucguca
eca-mir-6155p	ggggguccccggugcucggauc
eca-mir-6153p	uccgagccugggucucccucuc
eca-mir-628a	augcugacauauuuacuagagg

eca-mir-632	gugccuguuuccuguggga
eca-mir-652	aauggcgccacuaggguugug
eca-mir-653	guguugaaacaaucucugcug
eca-mir-655	auaauacaugguuaaccucuuu
eca-mir-656	aauauuauacagucaaccucu
eca-mir-660	uacccauugcauaucggaguug
eca-mir-664	uauucauuuaucuccuagccuaca
eca-mir-670	gucccugaguguauguggugaa
eca-mir-6715p	aggaagcccuggaggggcuggag
eca-mir-6713p	uccgguucucagggcuccacc
eca-mir-672	ugagguugguguacugugugga
eca-mir-6745p	ggugcucacuuguccuccu
eca-mir-6743p	aggaggccauaguggcaacugu
eca-mir-675	uggcgcggagagggcccacagug
eca-mir-684	aguuuucccuucaauucag
eca-mir-703	aaaaccuucagaaggaaagga
eca-mir-708	aaggagcuuacaaucuagcuggg
eca-mir-711	gggacccagggagagacguaag
eca-mir-758	uuugugaccugguccacuaacc
eca-mir-761	gcagcagggugaaacugacaca
eca-mir-763	ccagcugggaggaaccaguggc
eca-mir-7675p	ugcaccaugguugucugagcaug
eca-mir-7673p	ucugcucauacuccaugguuccu
eca-mir-7695p	ggagaccucuggguucugagcu
eca-mir-7693p	cugggaucucgggggucuugguu
eca-mir-769b	ggaaaccucuggguucugagcu
eca-mir-770	agcaccacgugucugggccaug
eca-mir-802	caguaacaaagauucauccuugu
eca-mir-872	aagguuacuuguuaguucagg
eca-mir-873	gcaggaacuugugagucuccu
eca-mir-874	cugcccuggcccgagggaccga
eca-mir-8765p	uggauuucuuugugaaucacca
eca-mir-8763p	ugguggugguuuacaaaguaauuca
eca-mir-8855p	uccauuacacuacccugccucu
eca-mir-8853p	aggcagcgggguguaguggaua
eca-mir-889	uuaauaucggacaaccauugu
eca-mir-1179	aagcauucuuucauugguugg
eca-mir-1180	uuuccggcucgaguggguguu
eca-mir-1185	agaggauacccuuuguauguu
eca-mir-1193	uaggucacccguuugacuauc

eca-mir-1197	uaggacacauggucuacuucu
eca-mir-1204	ucguggccuggucccacuau
eca-mir-1244	gagugguugguuuguaugagaugguu
eca-mir-1248	uccuucuuguauaaagcacugugcuaaa
eca-mir-1255b	cggauaagcaaagaaaagugguu
eca-mir-1261	guggauuaggcuuuggcuu
eca-mir-1264	caagucuuauuugagcaccuguu
eca-mir-1271	cuuggcaccucguaagcacuca
eca-mir-1282	agugguugguuuguaugagaugguu
eca-mir-1289	uggaguccaggaaucugcauuuu
eca-mir-1291a	uggcccugacugaagaccagcagu
eca-mir-1291b	aggcccugaaucaagaccagcagu
eca-mir-1296	uuagggcccuggcuccaucucc
eca-mir-1298	uucauucggcuguccagaugua
eca-mir-1301	uugcagcugccuggggagugauuuc
eca-mir-13021	uugggacauacuuauacuaaa
eca-mir-1302b2	uugggacauacuuauacuaga
eca-mir-1302d4	uugggacauacuuaugcuaaa
eca-mir-1302e6	uugggauauacuuauacuaaa
eca-mir-1302e7	uugggauauacuuauacuaaa
eca-mir-1302c5	uugcgacauacuuauacuaaa
eca-mir-1461	aucucuacggguaaguguguga
eca-mir-1468	cuccguuugccuguuuugcug
eca-mir-1597	ugaggagcucugcgagcaugua
eca-mir-1839	aagguagauagaacaggucuug
eca-mir-1842	uggcucugugaggucggcuca
eca-mir-1892	auuuggggugggggaugggga
eca-mir-1898	aggucaagguucacaggggauc
eca-mir-1902	agaggugcaguaggcaugacuu
eca-mir-1905a	caccacgagcccuaccacgcgguag
eca-mir-1905b	caccagccccacuacgcgguag
eca-mir-1905c	caccaccagccccaccacgcgguag
eca-mir-1912	uacccagagcgugcagugugaa

Appendix Table II: TaqMan® miRNA Primer Sequences. A.) Mammalian B.) Plant

A.

Mature miRNA ID	**Target Mature miRNA Sequence**
has-mir-16	UAGCAGCACGUAAAUAUUGGCG

B.

Mature miRNA ID	**Target Mature miRNA Sequence**
ath-mir-156a	UGACAGAAGAGAGUGAGCAC
zma-mir-827-5p	UUUGUUGGUGGUCAUUUAACC
osa-mir-1866-3p	UGAAAUUCCUGUAAAAUUCUUG

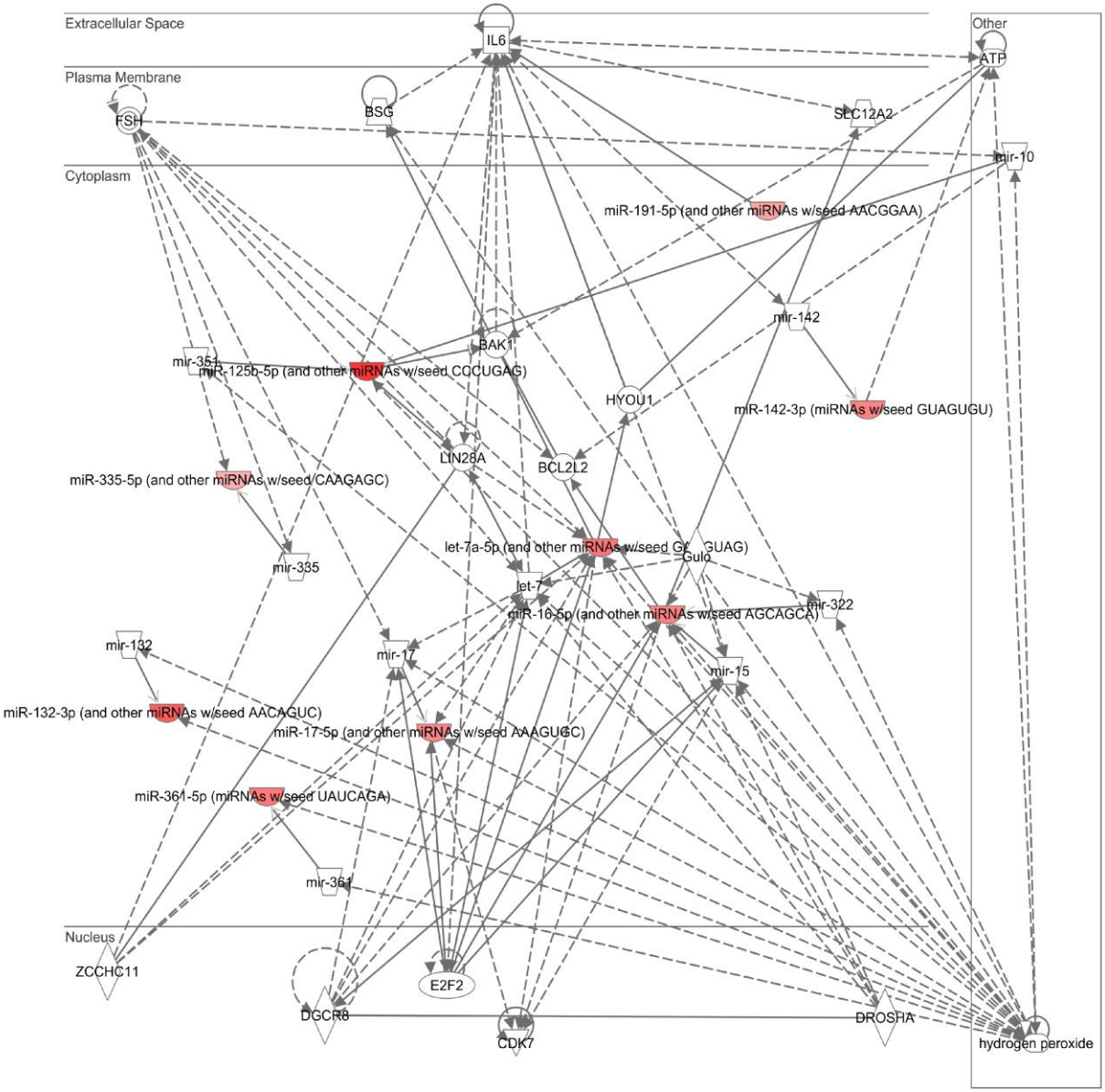
Appendix Table III: Feed Analyses

Formula	Kibbled Corn	Formula	Rice Bran
Ground Corn	80%	Stabilized Rice Bran	94.75%
Wheat Midds	20%	Calcium Carbonate	5.25%
100% DM	Kibbled Corn	100% DM	Rice Bran
Crude Protein	10.545%	Crude Protein	11.939%
Lysine	0.366%	Lysine	0.497%
Methionine	0.215%	Methionine	0.169%
Crude Fat	4.000%	Crude Fat	19.898%
Crude Fiber	4.068%	Crude Fiber	12.436%
ADF	5.182%	ADF	--
NDF	14.864%	NDF	--
Calcium	0.400%	Calcium	2.194%
Phosphorus	0.152%	Phosphorus	1.691%
DE	3.78 Mcal/kg	DE	2.607 Mcal/kg

CSU Mature Horse Pellet	
Crude Protein (min.)	12.00%
Crude Fat (min.)	3.20%
Crude Fiber (max.)	14.50%
Major Ingredients: Grain Products, Forage Products	

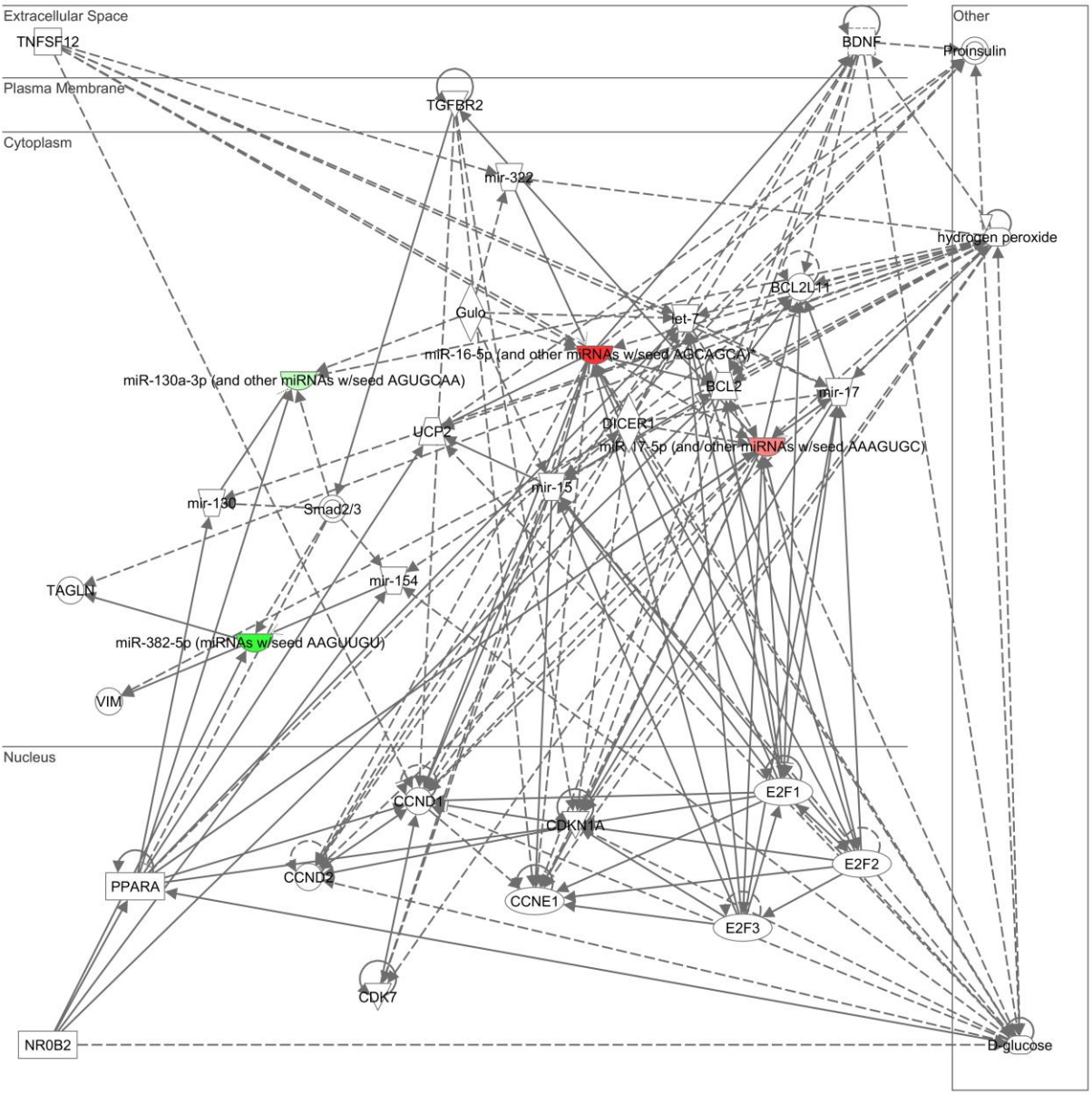
miRNA Acc.	Target Acc.	Expectation (E)	Target Accessibility (UPE)	Alignment	Target Description	Inhibition	Multiplicity
UGACAGAAGAGAGUGAGCAC	NM_033087|ALG2	3.0	15.213	miRNA 20 CACGAGUGAGAGAAGACAGU 1 ::::::::: :: ::::::.: Target 432 GUGUUCACUGUCAUCUGUUA 451		Translation	1
UGACAGAAGAGAGUGAGCAC	NM_003848|SUCLG2	3.0	20.101	miRNA 20 CACGAGUGAGAGAAGACAGU 1 :: :::::. ::.:::::::: Target 210 GUUCUCAUACUUUUCUGUCA 229		Cleavage	1
UGACAGAAGAGAGUGAGCAC	NM_152795|HIF3A	3.0	21.17	miRNA 20 CACGAGUGAGAGAAGACAGU 1 :::::.:::: ::.:::::: Target 3601 CUGCUCGCUCUGUUUUGUCA 3620		Translation	1
UGACAGAAGAGAGUGAGCAC	NM_016946|F11R	3.0	23.039	miRNA 20 CACGAGUGAGAGAAGACAGU 1 : :: :::::::::::::: Target 504 GGGCCCACUCUCUUCUGUCU 523		Cleavage	1

Appendix Figure I: Target Analysis. Predicted mammalian gene targets of ath-miR-156



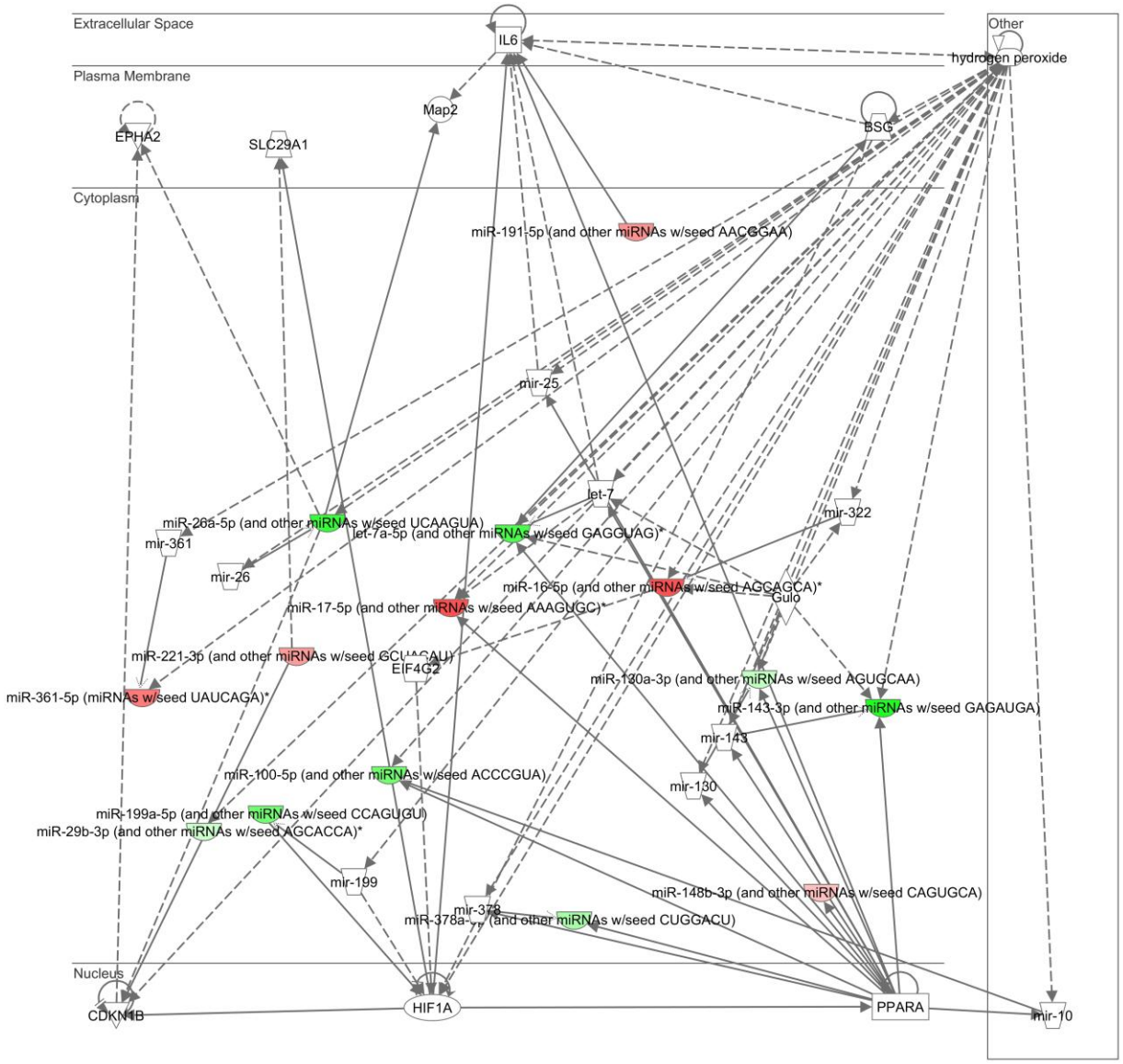
Appendix Figure II.I: Alfalfa Diet Pathway Analysis

Connective Tissue Disorders, Inflammatory Response, Respiratory Disease networks associated with differentially expressed serum exosomal miRNAs at day 23 of feed trial



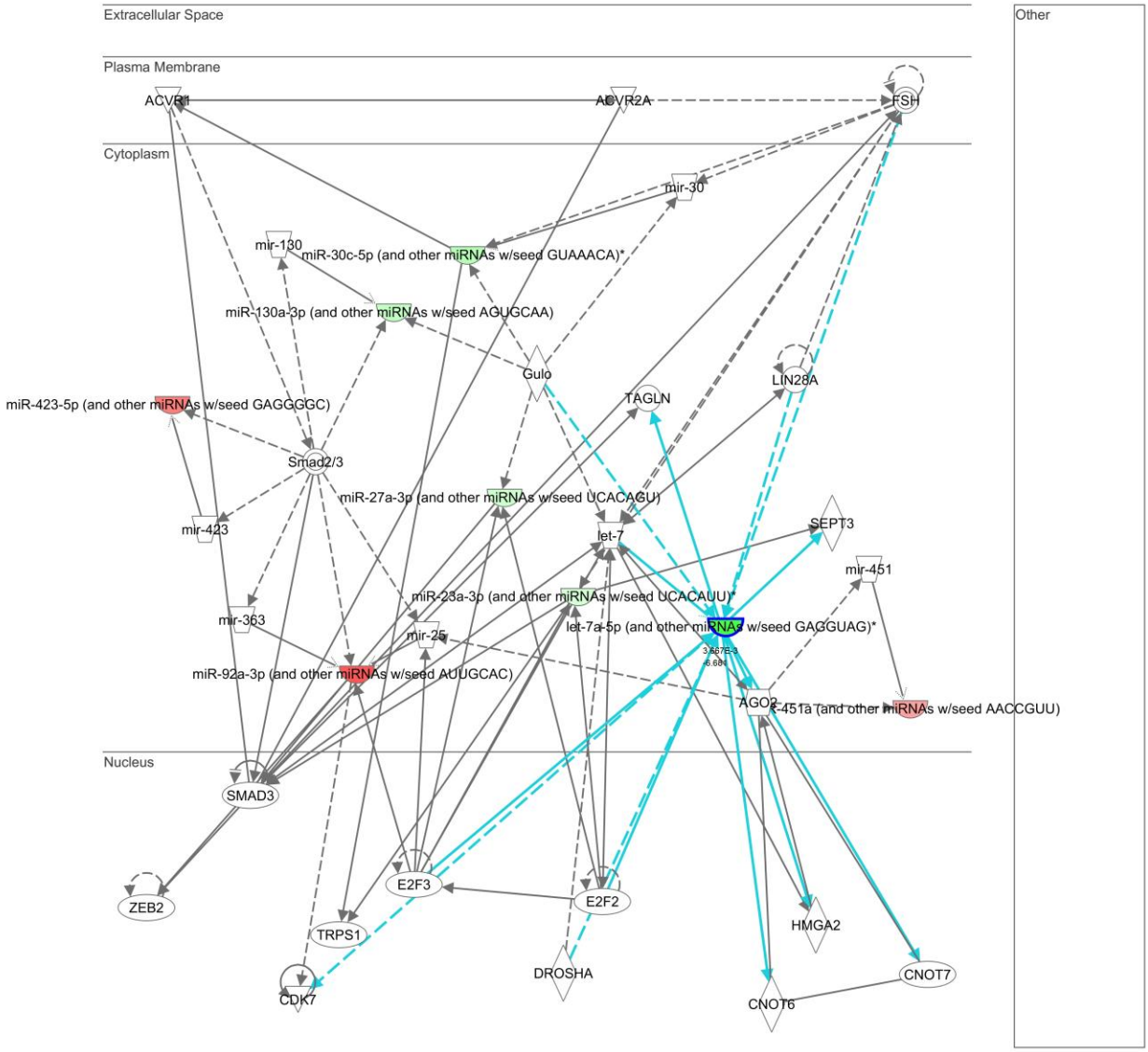
Appendix Figure II.II: Rice Diet Pathway Analysis

Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function networks associated with differentially expressed serum exosomal miRNAs at day 23 of feed trial



Appendix Figure II.III: Corn Diet Pathway Analysis 1

Cancer, Gastrointestinal Disease, Hepatic System Disease networks associated with differentially expressed serum exosomal miRNAs at day 23 of feed trial



Appendix Figure II.IV: Corn Diet Pathway Analysis 2

Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response networks associated with differentially expressed serum exosomal miRNAs at day 23 of feed trial