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Diet-Derived MicroRNAs: Separating the Dream from Reality

Abstract: Background: Both pleiotropic and ubiquitous, microRNAs (miRNAs) exert control over a wide range of cellular functions. They have been detected in virtually every extracellular fluid in the mammalian body, and many circulate substantial anatomical distances in plasma. Thus, secreted miRNAs are valuable not only as diagnostic tools but also may serve as novel biological effectors that can be transmitted between source and recipient tissue. **Design:** This review will discuss the possibility of delivering functional miRNAs from exogenously derived dietary sources. We will examine prior research interrogating the existence and relevance of such a mechanism. **Findings:** Recent findings have reported cross-kingdom transfer of specific plant-derived miRNAs to mammalian tissue following consumption of plant-based foods. These exogenous miRNAs were reported to be active in the recipient organisms, directing changes in gene expression at distant organ sites. In spite of this, subsequent studies have been unable to find evidence of substantial exogenous diet-derived miRNAs in mammalian circulation or tissues, regardless of diet. **Conclusion:** Further examination of diet-derived miRNA uptake is ongoing, but it does not appear that horizontal delivery of miRNAs via normal dietary intake is a generalizable or frequent process to maintain robust expression of these miRNAs in most higher-order animal organisms.

Keywords: microRNA, nutrition, cross-kingdom delivery, diet

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1 Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules that engage in post-transcriptional gene silencing. Found throughout the eukaryotic domain, miRNA sequences have been highly conserved across evolutionary time and are believed to play a crucial role in cellular function. Each miRNA binds a complementary sequence (its "seed sequence") embedded within the 3' untranslated region of its mRNA targets, allowing it to inhibit expression of the encoded protein by either blocking its translation or inducing its degradation [1]. miRNAs are highly pleiotropic, and a single miRNA is often capable of recognizing hundreds of distinct mRNA transcripts, making them potent regulators of a diverse array of cellular pathways. A striking example of this flexibility can be seen in the human genome, which is estimated to encode nearly 1,500 miRNA genes [2]. Put together, these miRNAs are estimated to regulate between 30-60% of all mRNA transcripts produced in the human body [3].

Until very recently, research has focused primarily on the biology of intracellular miRNA molecules. However, extracellular miRNAs have been discovered in a variety of bodily fluids including serum, plasma, urine, saliva, breast milk, and others [4-9]. Also referred to as secreted or released miRNAs, these molecules are remarkably stable and do not degrade in the presence of RNase [10]. They have been found packaged within microvesicles (MVs) [11,12], as well as partnered with a wide variety of lipoproteins and RNA-binding molecules [13-15], affording them a high degree of protection and allowing them to travel great distances within the circulatory system. They may also be taken up by recipient cells where they remain fully capable of suppressing their target genes [16-20]. Given their pleiotropic nature, released miRNAs may thus be at the center of a vast intercellular communication network that is only just now being understood.

Recent reports have indicated that oral consumption of plant-based food products may lead to the cross-kingdom transfer of plant-derived miRNAs into mouse and human tissues [21]. These molecules were found to be active in

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recipient cells, suggesting that miRNA uptake in the gut may mediate the cross-kingdom regulation of gene expression in mammalian species. If these findings can be explained and replicated, they will have broad implications for our understanding of nutrition, agriculture, and human ecology. Potential applications might include therapeutic targeting of nutrition, efficient control of pests in the absence of insecticides, and improved assessment of environmentally derived disease risk. Some have also suggested that diet-derived miRNA may provide an explanation for the efficacy of certain herbal folk remedies [22], and help us to assess the safety of genetically engineered livestock and crops [23]. In spite of this, the presence of meaningful diet-derived miRNA uptake has not been confirmed by any subsequent study, and several groups have found that oral ingestion and gastrointestinal uptake of normal mammalian diets is not a viable means of cross-kingdom miRNA transfer and gene regulation [23,24]. In light of these negative findings, the question of diet-derived miRNA must be examined as critically as possible in order to determine the most solid foundation for future research.

2 MiRNAs Inside and Outside of the Cell

Following transcription and processing within the nucleus, an immature, hairpin-looped miRNA precursor (termed pre-miRNA) is exported into the cytoplasm where it is cleaved by the RNA endonuclease Dicer [1]. The resulting molecule is then incorporated into the RNA-induced silencing complex (RISC), at which point the primary strand of the pre-miRNA duplex sheds its antisense (miRNA*) strand and becomes fully active. Once the RISC has been assembled, the miRNA molecule acts by binding a complementary sequence within the 3' untranslated region of its mRNA targets, a site typically referred to as the "seed sequence." If the degree of complementarity is very high, the mRNA transcript may then be degraded [25]. If not, the miRNA strand remains bound to its target and suppresses protein translation [1].

As previously mentioned, extracellular miRNAs are known to be present in the blood, as well as in a variety of other bodily fluids [4-9]. Extracellular miRNAs are attractive biomarkers as they are highly stable, both in bodily fluids and in formalin-fixed tissues [10], and can be detected without the need for invasive diagnostic procedures. Recent studies have indicated that the miRNA content of plasma is heavily influenced both by the miRNA content of circulating blood cells, and by the number of residual platelets left behind after plasma processing, factors which must be accounted for before circulating miRNA levels can be accurately assessed [26,27]. In spite of these obstacles, however, a variety of cancers [10,28-33], as well as cardiovascular disease [34,35], sepsis [36], and liver disease [37], among others, have been associated with distinct miRNA profiles that may be used to predict the presence of disease with reasonable fidelity.

There exists a wealth of studies detailing the relevance of blood-borne miRNAs in the early detection of injury and disease. miR-141, for example, shows marked differential expression in prostate cancer, and can be used to detect the cancer in individuals with a great degree of accuracy [10]. Other examples include miR-208a [38,39], a marker of cardiac injury and acute myocardial infarction, miR-106b and miR-181b, markers of liver cirrhosis [37], and miR-150, a marker of late-stage sepsis [36]. Similarly, miRNA biomarkers have been discovered in urine for the detection of bladder cancer [28], in sputum for the detection of lung cancer and tuberculosis [5,9], in semen for the detection of non-obstructive azoospermia [7], and in saliva for the detection of esophageal cancer [8]. While a detailed discussion of these studies is beyond the scope of this paper, the sheer number of recent reports in this area is indicative of how prevalent miRNAs are in the extracellular space, and how ubiquitous the mechanisms are for their packaging and secretion.

Importantly, miRNAs remain active after they are secreted, and can continue repressing their mRNA targets as soon as they are absorbed into a recipient cell. It has been shown, for instance, that endothelial cells release miR-126 in the early stages of apoptosis to promote expression of pro-survival chemokines in neighboring cells [40]. Adipocytes, as well, have been shown to make use of miRNAs for intercellular communication. Large adipocytes are known to transfer a variety of miRNAs, including miR-16, miR-222, miR-27a, and miR-146b to small adipocytes in order to stimulate lipid storage [41]. Several *in vivo* studies have confirmed the uptake of secreted miRNAs by recipient tissues in mice, with implications for cellular function in a variety of pathological contexts [16,18-20,42], including tumor metastasis [43] and the formation of atherosclerotic lesions [42]. The mechanisms of miRNA secretion and transport are not fully understood, and are highly complex, highlighting the importance of fine-tuned miRNA signaling between cells.

3 Packaging of Extracellular MicroRNA

While examining the stability of miRNA in human plasma, Mitchell and colleagues isolated the 18- to 24-nucleotide

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RNA fraction from the plasma of a healthy human donor and exposed it to a variety of environmental extremes [10]. These included incubation at room temperature for up to 24 hours, and eight consecutive freeze-thaw cycles. In spite of this treatment, levels of miR-15b, miR-16, and miR-24 remained roughly constant throughout the experiment [10]. Synthetic miRNA, on the other hand, was found to degrade rapidly when introduced into human plasma. The same has held true for naked exogenous miRNA, as well as endogenous miRNA that has been isolated and purified, indicating that these substances lack the protections afforded to miRNA that is secreted from the cell [16].

Initially believed to be cellular debris [44], MVs are now believed to play a crucial role in intercellular communication by shuttling protein, RNA, and surface ligands through the blood and other intercellular fluid spaces [45-51]. miRNAs are detectable in blood-borne MVs isolated from human subjects [11,12], and these are thought to be one of the main mechanisms by which miRNAs are transferred between cells. In order to demonstrate this capacity, Yuan and colleagues incubated mouse embryonic stem cell microvesicles with gamma-irradiated mouse embryonic fibroblasts, comparing the relative abundance of several miRNA transcripts before and after the incubation [52]. The majority of their chosen miRNAs were seen to increase in the fibroblast cells, beginning 1 hour after incubation. Another study examined MVs derived from the peripheral blood of healthy human subjects, reporting 33 known miRNAs to be differentially expressed in these vesicles relative to their expression in peripheral blood mononuclear cells [53]. Many of these were found to have known functions in the cellular differentiation of blood cells and in the immune system, indicating that they may have been secreted into circulation for such purposes.

The means by which miRNAs are selected for extracellular transport is not known. One theory suggests that the vesicle contents are random, and therefore a direct reflection of the miRNAs present in the cytosol of the parent cell [50]. An alternative theory suggests that the contents are actively selected according to the needs of the cell, as well as in response to environmental stimulation. Several studies have reported extracellular vesicles with miRNA profiles that differ substantially from those of their parent cell types, lending support to this second notion [20,54,55]. A 2010 study, for instance, indicated that blood cells exposed to immunogenic lipopolysaccaride secreted MVs with a greater relative number of immune-related miRNAs such as miR-146a, miR-181a, and miR-150 [20]. Additionally, MVs released from tumor cells have been found to contain miRNAs that are virtually absent from their parent cell, suggesting that some miRNAs are transcribed for the explicit purpose of exportation [56,57].

Several forms of MVs exist in the extracellular space (Figure 1). These include both microparticles, large vesicles with nonspecific cargo, formed by the outward protrusion of the plasma membrane, and exosomes, smaller vesicles with actively selected cargo, formed when a multivesicular body fuses with the plasma membrane [58]. Both of these are likely capable of transporting miRNA molecules. Highly expressed miRNAs may be packaged by chance into microparticles, while miRNAs that are scarcer may be selected for exosomal transport only when there is a specific need.

While vesicles play an important role in intercellular communication, it is believed that they account for only a small minority of miRNA transport in circulation. When the number of circulating miRNAs found within secreted MVs is compared with the total number of miRNAs that are detectable in mammalian plasma, only 1-5% of circulating miRNAs are vesicle-bound [19,55,59]. Secreted miRNAs have also been found in association with lipoproteins, such as the high- and low-density lipoproteins (HDL and LDL, respectively) that enable the transport of lipids and fat-soluble vitamins through the bloodstream [13,14]. Interestingly, one of the miRNAs most frequently found in association with HDL particles is miR-223, a miRNA that is highly enriched in blood-borne cells such as monocytes and macrophages [53]. This raises the intriguing possibility that lipoproteins are able to pick up miRNAs off-loaded from cells other than those responsible for the biogenesis of the lipoprotein itself [58]. The functional significance of lipoprotein-bound miRNA, however, remains uncertain, and recent work has shown miRNA binding by HDL to be largely nonspecific. Furthermore, delivery of HDL-bound miRNAs to recipient tissues *in vitro* appears to be inefficient and unreliable [60], suggesting that this is not a robust method of miRNA transport in all contexts. Additional carriers include the argonaute proteins (Ago1 and Ago2), more commonly known as the catalytic components of the RISC, which have been detected in circulation with bound miRNAs both in the presence and the absence of any lipid-based carriers [15].

Whether or not a particular miRNA will be transported within a vesicle or by some other cofactor seems to depend largely on the miRNA in question. Some, such as let-7a, are found exclusively within vesicle carriers, while others, such as miR-16 and miR-92a, are typically transported by other means [59]. The complexity of the extracellular miRNA transport system serves to emphasize the important role that these compounds play in the web of long-range intercellular

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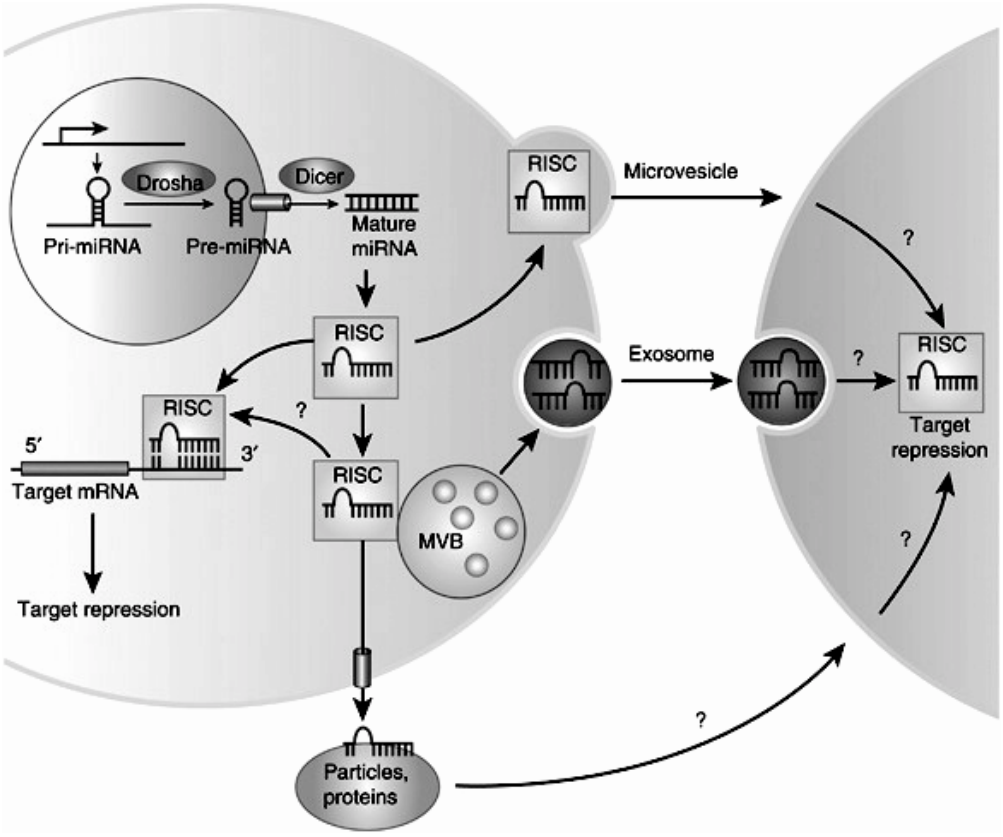

Figure 1: (Adapted with permission from Bitzer *et al*. (2012) Microparticles and microRNAs of endothelial progenitor cells ameliorate acute kidney injury. *Kidney International* 82: 375-377.) Following nuclear transcription and processing, miRNAs may be secreted from the cell by one of two mechanisms. Vesicular miRNAs are carried by: (i) microparticles (MPs) produced by outward budding of the plasma membrane, and (ii) exosomes, produced when a multivesicular body (MVB) fuses with the plasma membrane. Nonvesicular miRNAs are bound by a variety of lipoproteins and other RNA-binding proteins, such as the high- and low-density lipoproteins, and the Argonaute (AGO) protein.

communication. It also highlights the unfavorable conditions facing exogenous miRNAs that manage to enter into mammalian intercellular fluid spaces. Exogenous miRNAs entering the mammalian body via oral ingestion, direct injection, or any other means, require their own intact packaging in order to survive the harsh environment of the circulatory system and other such spaces. Failing this, they must utilize the native packaging system, either through recognition by circulating lipoproteins and RNA-binding factors or through rapid uptake by the surrounding cellular population.

4 Exogenous RNA: Nematodes, Pest-Control, and the Host-Virus Interplay

Given the conserved nature of miRNA throughout the metazoan kingdom, and in some cases throughout the majority of the eukaryotic domain, regulation by exogenous miRNA transmitted between species is plausible. In fact, ingestion of exogenous dsRNA and miRNA is known to be biologically effective in a wide range of lower eukaryotic species such as worms and insects [61-63], where alternative nutrient uptake mechanisms are thought to facilitate the transfer of these molecules [64,65].

It has been known for some time now that bacteria are capable of transmitting small dsRNAs to lower metazoan life forms such as nematodes [61-63]. In 1998, it was discovered that microinjection of a double-stranded RNA (dsRNA) fragment into the nematode *Caenorhabditis elegans* (*C. elegans*) reduced the expression of the corresponding complementary gene through the process of RNAi [66]. A subsequent experiment by Timmons and Fire in the same year involved feeding *C. elegans* specimens with *E. coli* that had been engineered to express the appropriate dsRNA segment [63]. *C. elegans* specimens that had consumed bacteria expressing dsRNA complementary to the *unc-22* and *fem-1* genes presented

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with phenotypes that were virtually identical to specimens with loss-of-function mutations in these same genes, indicating successful transfer of dsRNA through the gut [63]. Similar studies were conducted in the nematode *T. colubriformis* [63] and in a variety of planarian species, [62] and have involved both ingestion of genetically modified bacteria [61-63] as well as soaking in concentrated dsRNA solutions [100]. Studies of this nature contributed to the functional characterization of miRNA in 2000 [67], but they demonstrate another important principle as well: uptake of small RNA from environmental sources, including diet, is feasible in simpler metazoan organisms.

Transmission of dsRNA through the digestive tract has gained attention in more recent years as an alternative to traditional pesticides for the protection of agricultural crops. The cotton bollworm (*Helicoverpa armigera*), a common cotton pest, relies on a cytochrome P450 gene, CYP6AE14, to tolerate the otherwise toxic accumulation of the cotton metabolite gossypol [68]. In 2007, Mao and colleagues demonstrated that larvae fed plants expressing small hairpin RNA (shRNA) complementary to the *CYP6AE14* gene showed decreased expression of this transcript. In the presence of gossypol, their growth was also markedly slowed [68]. Similarly, miRNAs of probable plant origin (MIR166 and MIR168) were found in small RNA libraries sequenced from aphids harvested 48 hours after feeding on melon plants, though no notable target regulation was seen, and it was not known if these transcripts had been transferred into the tissues of the insects or if they were derived from undigested melon remaining in the gut [69].

Further examples of cross-kingdom regulation by exogenous RNA exist in host-virus systems. The cells of infected plants and insects are known to process viral dsRNA into small interfering RNA (siRNA) for the purpose of suppressing viral genes [70]. Some mammals are also capable of this type of defense, and may encode miRNAs to target viral genomes [70]. The primate miRNA, miR-32, for instance, has been shown to inhibit the accumulation of primate foamy virus type 1 (PFV-1) in human cells. Viruses can mount their own defense against these measures, as evidenced by the PFV-1 protein Tas, which inhibits miR-32 [71].

5 Diet-Derived MicroRNA in Mammals

The transfer of functional exogenous RNA is less well characterized in mammals, but it has been demonstrated in cases of bacterial and viral infection [72], and theorized in special circumstances, such as the potential transfer of miRNAs from mother to child during breast-feeding [73]. In 2006, Xiang and colleagues engineered a bacterial plasmid expressing shRNA complementary to the catenin beta-1 gene (CTNNB1). *E. coli* containing this plasmid were incubated with human colon cancer cells. The plasmid was also engineered to contain the *Inv* locus, coding for Invasin, which permitted the noninvasive strain of *E. coli* to infiltrate B1-integrin-positive mammalian cells [74]. The plasmid also contained the *HlyA* locus, coding for Listeriolysin O, which permitted the escape of genetic material from the *E. coli* entry vesicles [75,76]. Following incubation, CTNNB1 was silenced at the mRNA and protein levels in the colon cells, with no additional silencing of unrelated genes, such as KRAS [72]. Similar results were obtained *in vivo*, as nude mice xenografted with human colon cancer cells showed silencing of CTNNB1 in tumor tissue following systemic oral administration of the engineered *E. coli*. Given similar treatment, wild-type mice showed silencing of CTNNB1 in their intestinal epithelium as well, indicating that the effect may be generalizable [72].

While this study was conducted with the aid of a specially engineered strain of *E. coli*, dsRNA and miRNA uptake through the mammalian digestive system has been theorized in other contexts as well. Several immune-related miRNAs have been detected in both human [73,77], and bovine [78] breast milk. These include miR-181a and miR-181b, known as regulators of B cell differentiation and CD4+ T cell selection, as well as the miR-17/92 cluster, known to regulate B cell, T cell, and monocyte development [73]. As with secreted miRNAs in other bodily fluids, the miRNAs in breast milk are largely immune to the effects of RNase, and can withstand other sorts of rough handling, including freeze-thaw cycles and low pH [73]. Notably, in one study, such miRNAs remained intact following exposure to highly acidic conditions (pH 1) for up to an hour at a time, suggesting that they would indeed be able to survive the harsh environment of the human intestine. These results are consistent with the known benefits of breast-feeding in human infants, compared with formula-based diets. Formula-fed infants are more prone to digestive problems, and more likely to contract gastrointestinal and respiratory infections when compared to breast-fed age-mates [73]. Further support can be derived from a related study which showed that exosomes isolated from human breast milk were able to provoke an expansion of the regulatory T cell population in human blood [79]. Such a link, however, remains speculative, as direct transfer of miRNAs through breast milk has yet to be observed, and many of the known immunological advantages to breast feeding are derived

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from other factors as well, such as the transfer of maternal antibodies from mother to child [80].

In 2011, Zhang and colleagues published an intriguing set of results suggesting that diet-derived miRNAs regularly pass through the mammalian gut and into circulation [21]. Their study focused on the plant-derived miRNA MIR168a, which has been shown to suppress low-density lipoprotein receptor adapter protein 1 (LDLRAP1), a liver-enriched protein that regulates the removal of LDL from circulation [81]. Their team discovered relatively high levels of MIR156, MIR166a, and MIR168a in the serum of a cohort of healthy Chinese men and women. Exposing these miRNAs to sodium periodate, an oxidizing agent, confirmed that they were of plant origin, as plant-derived RNA strands are 2'-O-methylated on their terminal nucleotides, rendering them immune to oxidation in this way [82]. As oxidized terminal nucleotides cannot be ligated to the cloning adaptor, most mammalian miRNAs failed to be sequenced after this treatment.

Zhang and colleagues fed mice with a diet of either standard chow, containing low levels of MIR156a, MIR166a, and MIR168a, or fresh rice, containing substantially higher levels of these transcripts [21]. They found higher levels of MIR168a in the serum and in the stomach and small intestine of the rice-fed mice, compared with the chow-fed group. In the serum of mice gavage-fed with double-stranded, single-stranded, and precursor miR-168a, only the single-stranded form was detected by qRT-PCR [21]. Finally, mice fed with a diet of fresh rice for seven days showed substantial elevation of MIR168a levels in the liver and concordantly low levels of LDLRAP1 [21].

Lending further support to the presence of diet-derived miRNAs in mammalian circulation, a study by Wang and colleagues detected the presence of a wide variety of exogenous miRNAs in human blood. Examining the RNA profile of human serum, Wang and colleagues determined that less than 60% of the processed reads mapped to known human transcripts, with roughly 42% of them mapping to known human miRNAs when up to two mismatches were allowed [83]. Of the remaining reads, several mapped to known RNA transcripts, miRNA included, from bacteria, fungi, and other species, with very few mismatches overall. They also found RNA transcripts of probable bacterial, fungal, and plant origin in human lung tissue, bovine milk, and murine plasma [83]. Intriguingly, many of the most common reads mapped to species commonly associated with agriculture. The most common fungal mapping, for example, belonged to *Metarhizium anisoplia*, a common soil fungus [83]. Several reads mapped to *Saccharomyces cerevisae*, otherwise known as baking yeast. Finally, reads mapping to common cereal grains, such as corn and rice, were also present, including the plant-derived MIR168a. Most interestingly, the number of reads mapping to corn was 66 times higher than the number of those mapping to rice in the serum of subjects consuming a Western diet, while the serum of a Chinese individual showed the opposite ratio, with 55 times the number of reads mapping to rice as mapped to corn. Reads mapping to miRNAs and other genetic material from soybean, tomato, and grape species were also seen [83]. Importantly, however, all of these reads were seen at relatively low frequencies, suggesting that few or none of the plant-derived miRNAs found in the blood were present in sufficient quantities to suppress their gene targets in the canonical fashion.

Both of these studies raise the question of whether miRNAs are transferred through the lining of the gastrointestinal tract. In *C. elegans*, the widely expressed systemic RNAi deficient-1 protein (SID-1) is thought to allow for passive diffusion of dsRNA through the cell membrane [64]. The more recently discovered SID-2 protein is localized to the intestine and is thought to mediate endocytosis of dsRNA from the lumen [65]. The mammalian SID-1 homolog is known to enhance siRNA uptake by cells *in vitro* [64,84] and has been hypothesized to be a good candidate for regulating miRNA uptake from the intestine. An exact mechanism in mammals, however, remains undescribed.

6 Diet-Derived MiRNA: A Question of Applicability

In spite of these intriguing results, several follow-up studies since then have raised doubts about the generalizability of these findings (Figure 2). In 2013, Snow and colleagues selected three highly conserved plant-derived RNAs (MIR156A, MIR159A, and MIR169A) known to be highly expressed in a number of fruits that are common to the human diet in Western countries, such as apples and bananas [23]. They also selected one highly conserved miRNA found in animal tissues (miR-21), that can be found at high levels in raw and cooked meat products. They examined the expression of these miRNAs in the plasma of 10 healthy human subjects 24 hours after a breakfast containing fruits. They also examined the plasma of mice that had been fed a soy-enriched chow diet with high levels of the three plant-derived miRNAs, and miR-21$^{-/-}$ mice that had been fed a lard-enriched chow diet with high levels of miR-21 [23]. Though all four exogenous miRNAs were detectable in some subjects, they were present at concentrations of less than 1 copy per

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FOR	AGAINST
Detection of fungal, bacterial, and plant derived miRNA in mammalian serum/plasma is consistently reported [21, 23, 24, 77, 85]	Exogenous miRNA levels in mammalian serum/plasma are inconsistent and typically low [23, 24, 85]
Tissue-level target suppression by diet derived miRNA observed in at least one instance [21]	Mechanism of miRNA transfer through the gut is likely inefficient [23]
Oral uptake of exogenous dsRNA and miRNA is characterized in nematodes and insects [58-60, 63, 64]	Common food sources contain insufficient miRNA copy numbers to support canonical miRNA functions [23]
Ubiquitous diet-derived miRNA are more easily detected in recipient serum/plasma than miRNA that are less well-expressed in food sources [21, 23]	Species (e.g. honey bees) that successfully take up orally administered dsRNA fail to take up miRNA from food sources [23, 96]

Figure 2: Evidence for and against the functional impact of diet-derived miRNAs. **For:** Exogenous miRNAs have been consistently detected in the serum and plasma of mammals [21,23,24,77,85], with accompanying tissue-level effects in one case [21]. Detection of miRNAs in plasma of recipient mammals may be dose-dependent [21,23]. Oral uptake of small RNA molecules occurs more readily in lower metazoan organisms [58-60,63,64]. **Against:** Reported levels of exogenous miRNAs in mammalian serum and plasma have been inconsistent [23,24,85] and often too low to support canonical miRNAs functions, suggesting an inefficient mechanism of transfer through the gut [23]. Common food sources do not typically contain high enough levels of miRNAs to support these functions, even assuming perfect transfer [23]. Experimental attempts to replicate oral uptake of miRNAs in mammals have been largely unsuccessful, even in species previously known to take up orally delivered dsRNAs as well as in a direct replication of the experimental conditions under which MIR168a was first reported to be taken up by rice-fed mice [23,86,87].

cell, making it highly unlikely that any canonical miRNA activity could occur. Much like the copy numbers seen by Wang and colleagues [83], these concentrations are at odds with the very high copy numbers seen for MIR168a in the work by Zhang and colleagues, which reached a reported average of 850 copies per cell [21].

These results aligned with an independent study by Witwer and colleagues, which examined the plasma miRNA profile of two male pigtailed macaques following ingestion of a plant miRNA-enriched fruit and soy protein shake. Plasma samples were taken at 1, 4, and 12 hours post-ingestion, as compared with the 6 hours post-ingestion used by Zhang and colleagues [24]. Of their chosen plant-derived miRNAs, they were only able to detect one (MIR160) within the plasma, and even this was present only at very low levels both before and after ingestion of the soy protein shake [24]. Additionally, prior to the publication of the mechanistic findings of Snow *et al.* and Witwer *et al.*, examination of publicly available sequencing data by a third group could not confirm the high levels of plant-derived miRNAs found by Zhang and colleagues in the serum of mouse and human individuals prior to any form of specialized diet [85]. Indeed, MIR168 was the only plant-derived miRNA consistently detected in a majority of 63 high-throughput sequencing studies representing a number of species, ranging from humans to insects. Even more bafflingly, though MIR168 is a monocot-specific molecule, this miRNA was found in

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animals known to feed almost exclusively on dicot species, raising concerns about the possibility of contamination of samples, especially when measuring extremely low levels of miRNA [86].

Most recently, a Miragen-led study by Dickinson and colleagues attempted to directly replicate the experimental conditions of the original MIR168a study, as performed by Zhang and colleagues. They examined MIR168a and LDLRAP1 levels in the liver and serum of mice fed with one of three diets, a synthetic chow, a nutritionally balanced chow containing 40.8% rice, and a rice-based chow containing 75% rice [87]. They found fewer than ten exact matches to known rice miRNAs in liver and serum samples derived from five of the eight rice-fed mice. More concerningly, they found similar levels of MIR168a in liver and serum samples derived from mice fed with synthetic chow, which contains no unprocessed plant matter. As in the study by Witwer and colleagues, these results raise the possibility that sequencing errors and sample contamination may contribute to the detection of these very low levels of plant-derived miRNA in mammalian circulation [87].

Importantly, the Miragen study also addressed the effects of 2'-O-methylation on ligation activity in the construction of their small RNA libraries. Plant-derived miRNA species are typically 2'-O-methylated at their 3'-terminal nucleotides, and this modification has been found to result in their underrepresentation in miRNA quantification experiments [88]. However, this study showed little or no sequencing bias against small RNA with this modification, as both synthetic and plant-derived 2'-O-methylated RNA were easily detected when artificially spiked into mouse plasma prior to sequencing [87]. Finally, Dickinson and colleagues performed a ligation-independent quantification of small RNA levels using qPCR, again finding notable MIR168a levels in both rice-containing chows, but none in the liver or plasma of animals fed with any of the three experimental diets. Interestingly, their team did observe an increase in LDL levels 3 and 7 days after initiation of a rice-based (75% rice) diet, consistent with the findings of Zhang and colleagues. These effects, however, were not manifest in animals fed with the nutritionally balanced (40.8% rice) diet, suggesting that the relative absence of fat, cholesterol, and protein in the rice-heavy diet, and not the presence of rice-derived miRNA, is responsible for these changes. This notion is further supported by LDLRAP1 protein levels, which remained constant in liver samples taken 1, 3, and 7 days after feeding, regardless of diet [87].

These studies draw attention to several important gaps in our understanding of exogenous miRNA uptake in mammals. Importantly, it should be emphasized that the detectability of exogenous miRNAs in the bloodstream of recipient mammals appears to be a reproducible finding among numerous independent studies. However, the high levels of diet-derived miRNA uptake observed in the original MIR168a study, and the concordant system-level effects, have since been unreplicable at the bench [87]. A counterpoint reply by Zhang and colleagues to the recent Miragen study contains several technical criticisms regarding the bias inherent in ligation-dependent sequencing methods, as well as the need for standard-curve based qPCR assays for the accurate quantification of low copy number targets [89]. These criticisms, however, fail to acknowledge the breadth of evidence against the robust uptake of exogenous miRNA through the mammalian gut. Ligation-independent methods have been attempted by several of the studies discussed above [23,87], including both standard-curve qPCR and digital PCR [23], for the quantification of absolute copy numbers of exogenous miRNA in circulation. More importantly, methodological differences such as these do not explain the large discrepancy between the dramatic results reported by Zhang and colleagues and those reported by numerous follow-up studies with similar experimental design [23,24,87].

In light of these uniformly negative findings, it is important to examine whether physiologically relevant concentrations of miRNA could be achieved at all, given the limited number of copies present for even the most highly expressed miRNAs in animal- or plant-derived dietary items. To provide a concrete example, it has been estimated that at least 100 copies of a given miRNA are necessary for canonical intracellular target gene repression [90]. The plant-derived MIR156a is relatively abundant in cantaloupe, yet in order to account for enough copies of this molecule for delivery to every cell in the human body, Snow and colleagues reported that a person would need to ingest roughly 1,670 kilograms of cantaloupe, without accounting for any inefficiencies of transfer through the intestinal wall [23]. Clearly, this is an unrealistically large amount of food to incorporate into any reasonable human diet. A similar observation was made by Petrick and colleagues in a recent report on the safety of biotechnically-modified crops. Using the highest recorded levels of total RNA per gram of plant tissue, as well as the highest reported estimates of daily soy and maize consumption, they calculated that dietary exposure to plant-derived small RNA would reach, at most, 170 mg per kilogram of body mass per day, far below the concentration required for meaningful biological effects [91]. It remains possible that food items artificially

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concentrated with a particular miRNA could overcome the limitations of dietary dose in order to realize higher levels of exogenous miRNA delivery. However, it appears unlikely that a high percentage of miRNA molecules contained in a given meal could, in general, make their way into the circulation and recipient tissue.

7 Conclusions and Future Directions

Conclusive validation of diet-derived miRNA uptake and function in mammalian organisms would radically change our understanding of nutrition and ecology. However, the notion that miRNAs from these sources could be shown to act in a systematic fashion in mammalian cells remains in doubt. The inconsistency of miRNA delivery as demonstrated in recent reports suggests great limitations to the effectiveness of oral miRNA uptake, and further studies will be required to determine if and how exogenous miRNAs are taken up from the digestive tract in a biologically relevant context (Figure 2).

With this in mind, several other alternatives exist for the problem of transferring therapeutic miRNAs into circulation (Figure 3). It may be possible, for instance, to load food sources with a sufficient quantity of a given miRNA to allow for canonical functions. Alternatively, there may exist certain tissue niches within the body that act as repositories for circulating miRNAs, thereby raising their concentrations to more biologically relevant levels. Uptake may also be improved by specialized packaging, as has been theorized for immune-modulatory miRNAs found in mammalian breast milk [73,77,78]. Even without increasing the efficiency of miRNA intake, the modest number of exogenous miRNAs that are already taken up into circulation may still engage in non-canonical functions, such as cell-surface receptor interactions, theoretically requiring a lower copy number of miRNAs to act upon recipient cells. Finally, it may be possible to bypass the gut altogether. Exogenous miRNAs could be transferred directly into the blood, through blood transfusions and solid organ transplantations, or into the mucosal membranes, through more modern procedures such as fecal transplantation (currently used for the treatment of *C. difficile* infection [92]). Nanoparticles may also be used as packaging for miRNA molecules to promote more efficient uptake and delivery [93].

On the whole, cross-kingdom regulation by diet-derived miRNA is a fascinating concept with a wide range of putative applications. The reality, however, may not live

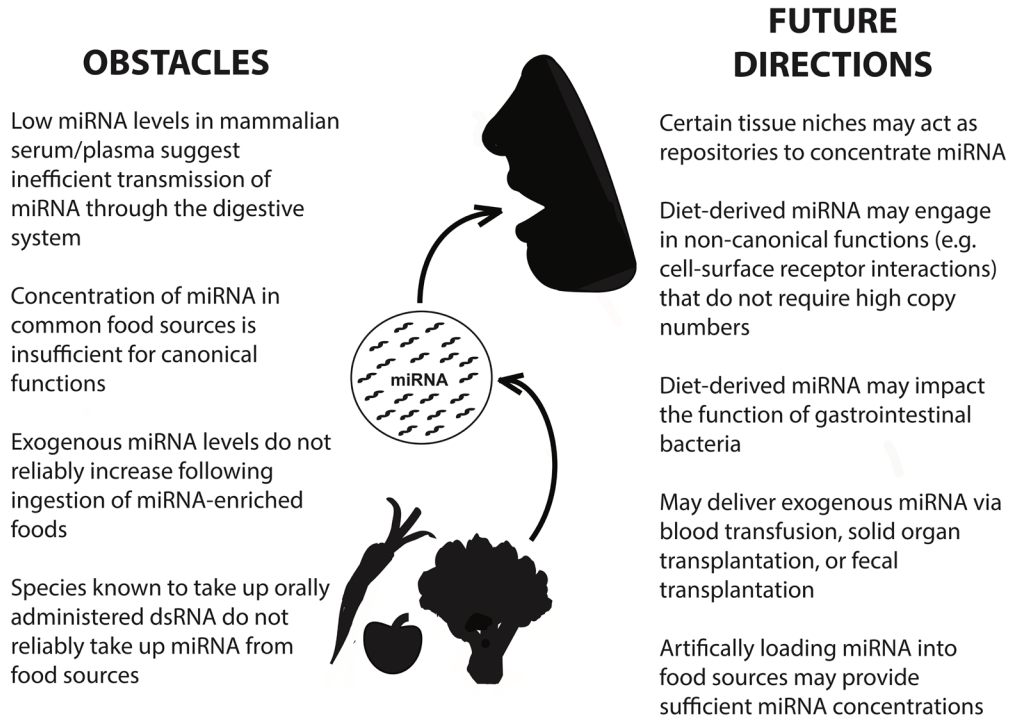

Figure 3: Theoretical possibilities for diet-derived miRNA: Barriers to miRNA uptake through the gut. A number of major obstacles exist that prevent significant delivery of diet-derived miRNAs through oral ingestion. Avenues for future research exist, but the current weight of evidence makes meaningful diet-derived delivery of miRNAs unlikely.

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up to such high expectations. Recent data indicate that, despite its relevance in primitive organisms, this is not an effective or ubiquitous mechanism for generalized miRNA uptake in mammalian species. Thus, although alternative mechanisms may exist for diet-derived miRNA function, it is likely that the most promising avenues for exogenous miRNA research lie elsewhere.

Abbreviations:

AGO - argonaute protein
dsRNA - double-stranded RNA
LDLRAP1 - low-density lipoprotein receptor adapter protein 1
miRNA - microRNA
MV - microvesicle
RISC - RNA-induced silencing complex
siRNA - small interfering RNA

Funding: This work was supported by the National Institutes of Health Grant number HL096834, the McArthur-Radovsky, Lerner, Harris, and Watkins Funds, Gilead Research Scholars Fund, and the Pulmonary Hypertension Association (S.Y.C.).

Acknowledgments: We thank J. Snow for critical reading of the manuscript.

Disclosures: None

Received: August 29, 2013; Accepted: December 30, 2013.

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