REVIEW

Dietary microRNA—A Novel Functional Component of Food

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ABSTRACT

MicroRNAs are a class of small RNAs that play essential roles in various biological processes by silencing genes. Evidence emerging in recent years suggests that microRNAs in food can be absorbed into the circulatory system and organs of humans and other animals, where they regulate gene expression and biological processes. These food-derived dietary microRNAs may serve as a novel functional component of food, a role that has been neglected to date. However, a significant amount of evidence challenges this new concept. The absorption, stability, and physiological effects of dietary microRNA in recipients, especially in mammals, are currently under heavy debate. In this review, we summarize our current understanding of the unique characteristics of dietary microRNAs and concerns about both the mechanistic and methodological basis for studying the biological significance of dietary microRNAs. Such efforts will benefit continuing investigations and offer new perspectives for the interpretation of the roles of dietary microRNA with respect to the health and disease of humans and animals. *Adv Nutr* 2019;10:711–721.

Keywords: microRNA, functional food component; exosome; gastrointestinal tract; plant; mammal; cross-kingdom regulation

Introduction

MicroRNAs are a class of small noncoding RNAs that play essential roles in gene regulation and numerous biological processes (1–3). The results of recent studies suggest that microRNAs have regulatory roles at both the intracellular and intercellular levels, and they can even function in an interspecies manner (4, 5). Recently, it was reported that microRNAs can mediate plant–plant interactions, with evidence showing that *Cuscuta campestris* can accumulate 22-nucleotide microRNAs in haustoria and transport these microRNAs to their host plants to silence host genes and facilitate *C. campestris* growth during parasitism (6). Another study observed a plant–fungal microRNA interaction in the *Arabidopsis-Botrytis cinerea* pathosystem, with evidence suggesting that exosome-like extracellular vesicles secreted from *Arabidopsis* cells can transport small RNAs into the fungal pathogen *B. cinerea* which suppress its pathogenicity by silencing fungal virulence genes (7). Most recently, Teng et al. (8) reported that plant-derived exosome-like nanoparticles (ELNs) from ginger carrying microRNAs such as mdo-miR7267-3p are preferentially taken up by *Lactobacillus rhamnosus*. By mdo-miR7267-3p–mediated targeting of the *L. rhamnosus* monooxygenase *ycnE*, ginger ELN-RNAs can eventually induce IL-22 production and ameliorate mouse colitis, because IL-22 is linked to barrier function improvement. These extracellular microRNA communications have greatly expanded our understanding of microRNAs, and several years ago we detected the presence of plant microRNAs in human and animal sera and tissues (9). Further study showed that these plant microRNAs were absorbed from food, and 1 microRNA with relatively high concentrations in serum, miR168a, directly targeted LDL receptor adaptor protein 1 (*LDLRAP1*) in liver cells and decreased the clearance of LDL from the blood. Subsequently, additional

Supported by National Natural Science Foundation of China grants 31802068 (to LZ), 31802156 (to TC), 31790411 (to LZ), and 31472163 (to Y-LZ); National Key Research and Development Programme grant 2016YFD0500503 (to Y-LZ); Innovation Team Project in Universities of Guangdong Province grant 2017KCXTD002 (to LZ); and National Natural Science Foundation of Guangdong Province grant 2018A030310145 (to LZ).
Author disclosures: LZ, TC, YY, C-YZ, and Y-LZ, no conflicts of interest.
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Abbreviations used: Ago2, Argonaute-2; ELN, exosome-like nanoparticle; GI, gastrointestinal; LDLRAP1, LDL receptor adaptor protein 1; MV, microvesicle; NGS, next-generation sequencing; 3′ UTR, 3′ untranslated region.

evidence suggested that microRNAs from food can be absorbed and delivered into cells, where these food-derived exogenous microRNAs can exert a regulatory function on gene expression and biological processes (10–18). This food-derived exogenous microRNA is not limited to plant microRNAs, because roles for mammalian microRNAs have also been reported. A study on miR-144/451 null mice showed that the consumption of miR-451 by these mice can increase the concentration of miR-451 in the circulating blood and improve antioxidant capacity of red blood cells by targeting the 14-3-3ζ/forkhead box O3 (Foxo3) pathway (19). Dietary microRNA may have various biological implications. For example, the results of our studies showed that dietary plant miR162a can directly target mammalian target of rapamycin (*mTOR*) to regulate honeybee larval differentiation and affect co-evolution (20). Moreover, studies have shown that milk, a common food of mammals, contains both endogenous mammalian microRNAs (9, 21) and food-derived plant microRNAs (22) and that these microRNAs can function as a nutritional component of milk that affects the offspring (23) Increasing data have indicated that these dietary microRNAs could be considered to be a novel functional component of food.

Despite being an interesting new concept, there is evidence that challenges the idea that dietary microRNAs can be absorbed and exert function in recipient organisms, especially mammals. For example, Dickinson et al. (24) were unable to reproduce the effect of miR168a in regulating *LDLRAP1* expression in mouse liver and LDL concentrations. Witwer (25) suggested that dietary plant microRNAs such as MIR2910 and MIR2911 were originated from either sequencing contamination [e.g., human ribosomal RNA (rRNA)] or other artifacts in plants based on reanalysis of public data. Moreover, Mico et al. (26) were unsuccessful in detecting plant microRNAs in plant-based processed products, such as beer and extra virgin olive oil. Huang et al. (27) reported that most corn microRNAs degraded during the digestion process and only minimal amounts were recovered after oral consumption (<0.3% in the stomach, 0.1% in the intestine and feces, and 0.01% in the colon and cecum), and no increases in corn microRNAs were detected in whole blood and tissue samples of the recipient mice.

In this review, we summarize current knowledge on dietary microRNAs based on multiple independent studies as well as our understanding of the unique characteristics of dietary microRNAs with respect to their stability, absorption, delivery, and mechanisms of gene expression regulation. We also address the significant amount of evidence that challenges this new concept. There are both mechanistic and methodological concerns regarding the study of the biological significance of dietary microRNAs. Hopefully, such efforts will benefit continuing investigations and offer new perspectives for the interpretation of the role of dietary microRNA with respect to the health and disease of humans and animals.

Current State of Knowledge

Dietary microRNA—a novel functional component of food

MicroRNAs have been detected extensively in various dietary sources, including plants and animals (28, 29). Studies have shown that both plant and animal microRNAs can be absorbed and function within recipient organisms. Plant dietary microRNAs can be detected and measured in humans and animals using next-generation sequencing (NGS) and qRT-PCR. In our first study, ~30 plant microRNAs were detected in a healthy Chinese population using NGS based on the identification of sequences that showed no match within the entire human genome and perfectly matched plant microRNAs commonly present in food. Because of this sequence difference, we were able to measure the expression level of plant microRNAs in animal consumers using qRT-PCR. Compared with endogenous microRNAs, plant microRNAs are present at relatively lower levels that fall within a similar concentration range but exhibit a high degree of variation (30). To date, we have evaluated a large number of serum samples from healthy male and female individuals, and our results show that the abundance of plant microRNAs in certain individuals is comparable with that of endogenous microRNA, whereas the same plant microRNA is undetectable in samples from other individuals.

Food-derived animal microRNAs are more difficult to detect and measure than those of plants owing to the high sequence conservation among mammalian microRNAs, which obscures the differences between dietary and endogenous microRNAs. Evidence that the endogenous synthesis of microRNAs does not compensate for a dietary microRNA deficiency strongly indicates that the uptake of animal microRNAs from food occurs (12, 31), yet distinguishing dietary animal microRNAs from endogenous microRNAs to accurately assess the concentrations of food-derived animal microRNAs remains difficult. Plant microRNAs bear 2'-O-methylated 3′ ends and are resistant to periodate (32). In our study, the results of oxidized deep sequencing and semi-quantitative RT-PCR analyses showed that exogenous plant microRNAs remained modified in animal serum and tissues. Such modification of dietary animal microRNAs was undetectable because animal microRNAs are not modified in this manner. However, synthetic microRNAs remained methylated at their 3′ ends after absorption (33), suggesting that some modifications, such as methylation, could serve as a distinguishing marker because they could remain preserved.

Function

Although their abundance is relatively low in recipient organisms, dietary microRNAs remain functional. Given the conservation of mammalian microRNAs, a bioinformatic analysis identified tens of thousands of putative target genes for milk-associated microRNAs, a typical source of dietary animal microRNAs, and suggested that milk-associated microRNAs play important roles in consumers, including

in metabolic syndromes, immune function, and cancers (11). Dietary plant microRNAs can also have a functional impact on consumer organisms in a cross-kingdom manner (34), and experimental results have shown that dietary microRNAs affect multiple processes in animal consumers, such as metabolism, immune responses, and cancer, for which some in vivo functional studies are discussed below.

Metabolism

Compared with mice fed an unpurified diet, mice fed rice exhibited significantly higher serum LDL-cholesterol concentrations (≥30%) (9). This effect was caused by the inhibition of *LDLRAP1* in liver cells by miR168a from rice. Importantly, this LDL increase was blocked upon administration of an anti-miR168a antisense oligonucleotide.

Immune response

Small plant RNAs reduced the onset and severity of experimental autoimmune encephalomyelitis by limiting dendritic cell migration (33). This immunomodulatory effect is associated with plant microRNAs binding to toll-like receptor 3 (*TLR3*) and impairing TIR-domain-containing adapter-inducing IFN-β signaling. Among the microRNAs present in milk, many immune-related microRNAs (35–38) have important effects on the infant immune system. Milk miR-155 could regulate the maturation of T cells and B cells as well as the innate immune response, whereas milk-associated miR-181a and miR-181b could regulate B cell differentiation and $CD4^+$ T cell selection (37). Moreover, the milk exosome–derived miR-155 is shown to be involved in the regulation of forkhead box P3 (*FoxP3*) expression, IL-4 signaling, and the control of the binding of IgE to the high-affinity IgE receptor (*FcϵRI*) switch to regulate immune responses in regulatory T cells (39).

Cancer

Oral administration of tumor suppressor microRNAs was observed to reduce the tumor burden in a mouse model of colon cancer (40). It is noteworthy that administration of plant RNA alone could potentially reduce tumor burden compared with the control group ($P = 0.28$), although additional studies using a larger sample size are needed to confirm the therapeutic effects of plant RNA. In the same study, the administration of a combination of plant and tumor suppressor microRNAs further reduced the tumor burden (40). Moreover, the administration of extracellular vesicles from healthy donor sera containing detectable concentrations of miR159 was shown to inhibit breast cancer cell growth, an effect that was partially reversed by the administration of an anti-MIR159 nucleic acid (41). In this study, miR159 was shown to directly target transcription factor 7 (*TCF7*), which encodes a Wnt signaling transcription factor, and decreased myelocytomatosis oncogene protein concentrations (41).

In summary, microRNAs in food can be detected in consumers and function within various biological processes, indicating that dietary microRNA may be a novel functional component of food.

Dietary microRNA stability

In principle, dietary microRNAs undergo processing, absorption by the gastrointestinal (GI) tract, delivery by the circulatory system, and transport into multiple tissues before exerting its functions.

The results of previous studies showed that plant-derived microRNAs remain stable during food processing and cooking. One study reported that the concentrations of 18 maize microRNAs decreased by only one-thirtieth compared with those in fresh maize after puffing treatment (i.e., high-temperature and -pressure conditions with apparent starch dextrinization and protein denaturation) and that these maize microRNAs could be absorbed by porcine serum (42). Plant microRNAs can further survive in the digestive system for >1 h without any significant decrease in their concentrations (43). By contrast, similar treatments with a synthetic cel-lin-4 microRNA showed the instability of the synthesized microRNA under adverse chemical and physical conditions during processing, cooking, and digestion. Our previous results (data not shown) suggested that the gavage-feeding of cel-lin-4 did not elicit a robust response in mouse serum. Taken together, these data indicate that plant microRNAs exhibit a unique bioavailability for absorption. This unique stability may be partially attributed to the modification and packaging of dietary microRNAs. In plants, ELNs could transport microRNAs into consumers (44, 45). These packaging structures, as well as the modification of plant microRNAs, may confer resistance to cooking and digestion and enhance the uptake of dietary microRNA.

In milk, a large fraction of microRNAs appeared to be encapsulated in exosomes (46), and these milk exosomal microRNAs could resist low-pH conditions (pH 2.0), RNase, and freeze-thaw cycles at −20°C (37, 47, 48), whereas Triton X-100-containing regents (47), fermentation (49), and microwave (50) treatments partially altered milk exosomal microRNA concentrations. These results suggest that exosomes have beneficial effects on the stability of milk-associated microRNAs. Notably, unlike plant microRNAs, the milk exosomal microRNAs were not resistant to boiling (35). However, endogenous giant panda milk–associated microRNAs are shown to be more resistant to prolonged incubation at 37°C, low pH values, and even to treatment with high concentrations of exogenous RNase than synthetic worm-specific microRNAs (cel-miR-2 and cel-miR-93) (51).

Absorption of dietary microRNA

Based on our results, dietary microRNAs were observed to display a kinetic absorption curve. For example, when volunteers were fed watermelon juice and mixed fruits, the concentrations of 6 out of 16 microRNAs assayed showed a dynamic physiological pattern in plasma, with absorption rates ranging from 0.04% to 1.31% (52). The concentrations of dietary plant microRNAs peaked within 3–6 h after

intake in serum and tissues, indicating that the GI tract is responsible for absorption. In addition, an independent group showed that dietary microRNAs can survive for 36 h or longer in tissues (53). Specifically, the concentration of miR172 was ∼4.5–0.4% (2–24 h after feeding) in the stomach, ∼2.4–0.2% (2–36 h) in the intestines, ∼1.3–0.2% (2–72 h) in the blood, and ∼0.38–0.04% (2–72 h) in the spleen. Absorbed dietary microRNAs may be metabolized similarly to endogenous microRNAs, and with continuous feeding, the concentration of dietary microRNAs may remain stable. For example, in pigs fed fresh maize, the concentrations of maize microRNAs (zma-miR164a-5p, zma-miR166a-3p, zma-miR167e-5p, zma-miR168a-5p, and zma-miR319a-3p) in serum samples reached peak values between 6 and 12 h after consumption. Subsequently, with animals fed a fresh maize diet ad libitum, concentrations of serum maize microRNAs remained stable during the following 7 d (42). These data suggest that dietary microRNAs are a real physiological phenomenon, although the exact mechanism of their absorption remains unclear and requires further investigation. Notably, dietary microRNAs are quickly incorporated into exosomes, with the results of 1 study suggesting that half of the exogenous lettuce miR156a could be detected in circulating human exosomes 3 h after the individuals have consumed lettuce (54).

The abundance of dietary microRNAs is approximately correlated with dietary intake. Wang et al. (10) reported that the number of reads specific to corn was 66-fold higher on average than those specific to rice in the serum of Western individuals, whereas the number of reads specific to rice was 55 times higher than those specific to corn in the serum of Chinese individuals. These results could be attributed to dietary intake differences between the 2 populations. In addition, plasma from an individual who reported to follow a vegetarian diet was shown to exhibit a relatively high proportion of plant microRNA sequences (55). Similar results were reported for dietary animal microRNAs. After being fed a microRNA-depleted milk diet for 4 wk, the concentration of plasma miR-29b in mice was significantly decreased (by 61%) compared with microRNA-sufficient controls (12). Interestingly, the abundances of exogenous bamboo microRNAs in giant panda milk were closely correlated with their intrinsic expression levels in bamboo leaves (51).

However, independent studies have shown that food uptake is not the sole determinant of dietary microRNA abundance (52). Only a fraction of food microRNAs is present in consumers, indicating that microRNAs in food undergo selective absorption. The specific characteristics of microRNAs are important contributors to the efficacy of food-derived microRNA absorption. In particular, the intrinsic stability conferred by the nucleotide sequence and composition of dietary microRNAs could determine their absorption. In our experience, although MIR2911 has been re-recognized as a ribosomal RNA rather than a microRNA, small RNAs such as MIR2911 that could essentially apply to the same system as microRNA are stable and show significant uptake in consumers owing to their unique sequences and high guanine and cytosine (GC) content. Disruption of only 2 GC nucleotides in the MIR2911 sequence abolishes its stability and absorption. The uptake efficiency of the packaging structures may also be responsible for selective dietary microRNA absorption. However, the exact mechanisms by which nucleotide sequence and composition and packaging structures affect the selective absorption of dietary microRNAs remain unclear and require further investigation.

The exclusive detection of mature, single-stranded dietary microRNAs that remained functional was observed in a previous study (53). One explanation for this result is because the structure of double-stranded RNA is more susceptible to RNase clearance, and the single-stranded form of microRNA provides greater stability than other forms of RNA and DNA in the GI tracts of animals.

Multiple mechanisms, such as endocytosis and transcytosis, may be responsible for dietary microRNA absorption and delivery. However, because specific microRNAs that are abundant in food are detected at low concentrations in consumers, and because of the limited variety of dietary microRNAs detected in consumers, there appears to be a larger role for selective uptake mechanisms, such as receptor-mediated endocytosis or specific microRNA transporters in the intestinal system and peripheral organs. Currently, although systemic RNA interference defective 1 (SID1) has been identified as a membrane transporter for RNA interference (RNAi) in *Caenorhabditis elegans* (56), the transporters used for dietary microRNAs remain unclear. The results of several studies suggest that dietary microRNAs are primarily carried by exosomes or ELNs, and dietary microRNAs, such as milk-associated microRNAs, could be functionally incorporated into breastfeeding infants through exosome-mediated transportation and intestinal endocytosis (36, 57, 58). Thus, the interactions between receptors or transporters and proteins in exosomes or ELNs may offer clues in identifying the membrane receptors and transporters of interest. To date, studies have shown that the disruption of bovine milk exosome surface proteins by proteinase K or cytochalasin D treatment will lead to decreased transport rates in human endothelial cells, suggesting that the surface proteins of exosomes, particularly glycoproteins, are involved in endocytosis carrier-mediated transport (59). Additional studies by the same group showed that β-galactoside, core 1 and core 3 O-linked disaccharide, and N-acetylglucosamine modifications of the surfaces of both bovine milk–associated exosomes and intestinal cells have important roles during the endocytosis process (60).

Notably, the direct absorption and stability of food-derived microRNAs through the human GI tract have not yet been convincingly demonstrated, and even with animal models, several reports have failed to detect exogenous dietary microRNAs (24, 61–63). Thus, the absorption of

dietary microRNAs requires further investigation, especially in mammals.

Delivery of dietary microRNA

After cellular uptake in the GI tract, dietary microRNAs may be packaged and released into the circulation for delivery into tissues similarly to endogenous microRNAs (64).

Although RNAs are subjected to prompt degradation by endogenous RNases and are inherently unstable molecules, there are 2 possible mechanisms for the protection of endogenous microRNAs in the circulation: *1*) protection by the membrane structures of microvesicles (MVs) or exosomes; and *2*) stabilization by association with RNA-binding proteins, such as Argonaute-2 (Ago2). The observation that miR168a was more abundant in MVs than in MV-free serum (9) suggests the ability of intestinal epithelial cells to package dietary microRNAs into MVs for release into the circulation. This packaging of dietary microRNAs into MVs or exosomes would protect them from degradation. Results of immunoprecipitation experiments have shown that dietary microRNAs in the circulation are associated with Ago2 (9), which also protects them from degradation. Interestingly, although MIR2911 does not coimmunoprecipitate with Ago2, it remains resistant to nuclease clearance (18). An additional study showed that the MIR2911 containing complex from cabbage transformed from being proteinase K–sensitive to proteinase K–resistant during digestion or absorption (18), suggesting that it is modified in transit, possibly in intestinal epithelial cells, to enhance the stability of MIR2911 (18). Taken together, these results suggest that the further characterization of dietary microRNA forms in the circulation is crucial to improving our understanding of dietary microRNA absorption and delivery mechanisms.

Similarly to endogenous microRNAs, dietary microRNAs undergo sorting during packaging and delivery, i.e., the ratios of microRNAs taken up by cells are not necessarily the same as those of microRNAs secreted into the extracellular space. Thus, the specific microRNAs delivered to target cells will also differ. For example, in pigs fed fresh maize ad libitum for 7 d, 16 of the 18 assayed maize microRNAs were detected in serum and solid tissues. Among the assayed tissues, the heart, brain, lung, and kidney samples displayed relatively high abundances of microRNAs, whereas pancreas and longissimus dorsi muscle samples displayed low abundances (42). In prostatic hyperplasia rats, plant miR5338 was enriched in the posterior lobe of the prostate after the animals were administered rape bee pollen for 5 wk (65). Furthermore, our group detected miR-130-3p and miR-130-5p in the intestines, liver, and lungs of miR-130 knockout mice 12 h after being intragastrically fed porcine milk–derived exosomes containing miR-130 (Bing Zen, 2019). The underlying mechanisms of dietary microRNA delivery remain unclear and require further investigation to increase our understanding of the biological roles of dietary microRNAs.

Mechanisms of gene silencing by dietary microRNAs

After being released into the circulation, dietary microRNAs can be transported to multiple types of cells and tissues, such as liver, lung, mammary gland, and immune cells, to directly regulate gene expression.

In plants, endogenous microRNAs affect target gene mRNA cleavage and degradation through the complementarity of nucleotide sequences with target mRNA transcripts. In animals, endogenous microRNAs affect translational repression through largely imperfect complementarity. Exogenous plant microRNAs in mammalian cells could regulate target gene expression by partial sequence complementarity and translational repression as well as by mRNA cleavage. Interestingly, miR168a decreased the expression of LDLRAP1 protein in mouse liver cells without affecting the encoding mRNA levels.

In animals, endogenous microRNAs typically regulate gene expression by targeting the 3′ untranslated regions (3′ UTRs) of genes, which can be similarly targeted by exogenous plant microRNAs. For example, an miR156a mimic from broccoli was observed to inhibit epithelial–mesenchymal transition in nasopharyngeal cancer cells by targeting the 3′ UTR of the junctional adhesion molecule A (*JAM-A*) gene (66). We also observed that exogenous animal microRNAs function in the same manner. Our results showed that porcine milk–associated microRNAs promoted cell proliferation in mice (38, 67), likely by targeting the 3′ UTRs of genes (data under review). Exogenous plant microRNAs can also target animal genes outside of their 3′ UTRs, as our previous results showed that miR168a decreased *LDLRAP1* expression by directly targeting exon 4 (9). Computational studies have been conducted to predict putative targets for dietary plant microRNAs (68). Pirrò et al. (68) developed an algorithm to identify targets by searching for functional sequence homologies between plant and mammalian microRNAs and successfully showed that miR168a can regulate the expression of silent information regulator 1 (*SIRT1*) in human cell lines. Food-derived plant microRNAs can also function in recipient cells in a sequence-independent manner (33), with plant microRNAs having been shown to reduce inflammation by binding to the toll-like receptor 3 (TLR3) of dendritic cells. Both methylated and unmethylated plant microRNAs exhibit immunomodulatory effects but with differing efficacies, suggesting that the methylation of plant microRNAs confers a portion of their inflammation-reducing activity.

The results of immunoprecipitation experiments have shown that dietary microRNA is associated with Ago2 in the circulation. This association increases the functionality of dietary microRNAs by increasing the formation of Ago2-associated RNA–induced silencing complexes and regulates target gene expression. As a result, the working concentrations of dietary microRNAs in mammalian cells would be much lower than expected. In addition, we have summarized the major dietary microRNA studies in **Table 1**, and the results will help

TABLE 1 Exogenous miRNA studies of cross-kingdom regulation[1]

miRNA	Source	Target cell or animal	Target gene	Function	N/P	Reference
miR168a	Rice	Humans/mice	*LDLRAP1*	Decreases LDL removal from mouse plasma	P	(9)
miR-451	Human	*Plasmodium falciparum*	*PKA-R*	Inhibits parasite growth	P	(70)
let-7	—	—	*REX1*	—	—	—
miR-BART5	Epstein-Barr virus	Human NPC cells	*PUMA*	Facilitates the establishment of latent infection by promoting host cell survival	P	(72)
miR-K12-6, miR-K12-11	*Kaposi sarcoma herpesvirus*	Lymphatic endothelial cells/blood vessel endothelial cells	*MAF*	Influences the differentiation status of infected cells and contribute to KSHV-induced oncogenesis	P	(71)
mir-BHRF1-3	Epstein-Barr virus	Two BL early-passage cell lines (BL-5 and BL-8)	*CXCL-11*	Cancer immunosurveillance	P	(73)
miR-172	*Brassica. oleracea*	Mice	/	Absorbed	P	(53, 54)
mol-miR168a	*Moringa oleifera*	Human hepatoma cell line G2 (HEPG2) cells	*SIRT1*	Absorbed	P	(68, 69)
hsa-miR-515-5p	Mouse	*Fusobacterium nucleatum*	16S *rRNA*	Promotes bacterial growth	P	(74)
hsa-miR-1226-5p	—	*Escherichia coli*	*yegH*	—	—	—
zma-miR164a-5p	Maize	Pigs	*OTX1, PLAGL2, CSPG4*	Absorbed	P	(42, 43)
miR166a	*Arabidopsis thaliana*	Human breast milk exosomes	/	Absorbed	P	(21)
miR951	*Picea abies*	—	—	—	—	—
miR472a	*Populus trichocarpa*	—	—	—	—	—
miR168	*Brachypodium distachyon Viridiplantae*	—	—	—	—	—
miR168a	Maize	Porcine breast milk exosomes	/	Absorbed	P	—
miR166a and so on	*Arabidopsis thaliana*	—	—	—	—	—
miR156a	Maize	—	—	—	—	—
miR159	*Arabidopsis thaliana*	Mice	*TCF7*	Suppresses the growth of xenograft breast tumors	P	(41, 42)
miR162a	Bee pollen	*Drosophila* phenotypes	*dmTOR*	Delays the development and decreases the body and ovary size in honeybees	P	(20)

(*Continued*)

TABLE 1 (*Continued*)

miRNA	Source	Target cell or animal	Target gene	Function	N/P	Reference
miR166a, miR156a, miR157a, miR172a, miR168a	Tomato etc.	Human breast milk	/	Absorbed	P	(22,23)
miR156a	Cabbage, spinach, and lettuce	Human aortic endothelial cells	*JAM-A*	Suppresses the progression of atherosclerosis	P	(54)
TAS1c-siR483, TAS2-siR453IG, N-siR1, miRNA166	*Arabidopsis thaliana*	Fungal pathogen *Botrytis cinerea*	/	Combats fungal infection	P	(7)
miR12480	*Cuscuta campestris*	*Arabidopsis thaliana*	*SEOR1*	Restricts *C. campestris* growth on *A. thaliana*	P	(6)
miR12497b	*Cuscuta campestris*	*Arabidopsis thaliana*	*TIR1*		—	—
miR168	Corn	Fall army worm and silkworms	Plant microRNAs in animal sRNA data sets can originate in the process of sequencing		N	(71, 75)
miR156a, miR159a, miR169a	Ripened avocado, apple, banana, orange, cantaloupe	Honeybees	Negligible delivery of these molecules was observed in recipient honeybees		N	(69)
miR168a	*Oryza sativa*	Mice	LDL concentrations may be due to the release of endogenous cholesterol stores in response to negligible dietary cholesterol intake in mice fed only rice		N	(76)
miR172, miRs166, miR167, miR168	Soy/fruit substance	Macaques	Their concentrations were quite low and/or amplification was nonspecific in pigtailed macaque plasma		N	(63, 64)
miR156a, miR166a, miR167a, miR168a	/	Humans	Most donors may not have had detectable plant microRNAs in serum		N	(61)
miR2911, miR168	Honeysuckle	Mice	Kidneys clear dietary microRNAs from circulation		N	(14)
miR-375	Murine milk	Mice	Rapid degradation of milk microRNAs in the intestinal fluid		N	(46)
miR156a	Pollen	Honeybees	Not efficiently taken up by the digestive tract or dispersed to distal tissues		N	(77)
miR156a, miR164a, miR167a	Corn	Mice	Corn microRNAs are degraded by digestive process and not uptake into circulation or tissues		N	(27)

[1] BL, Burkitt's lymphomas; CSPG4, chondroitin sulfate proteoglycan 4; CXCL-11, C-X-C motif chemokine ligand 11; dmTOR, *Drosophila melanogaster* target of rapamycin; JAM-A, junctional adhesion molecule A; KSHV, Kaposi sarcoma herpesvirus; LDLRAP1, LDL receptor adaptor protein 1; MAF, avian musculoaponeurotic fibrosarcoma oncogene homolog; miRNA, microRNA; N, negative results; NPC, nasopharyngeal carcinoma; OTX1, orthodenticle homeobox 1; P, positive results; PKA-R, subunit of cAMP-dependent protein kinase; PLAGL2, pleiomorphic adenoma gene-like 2; PUMA, p53 upregulated modulator of apoptosis; REX1, reduced expression 1; SEOR1, Sieve-Element-Occlusion-Related1; rRNA, ribosomal RNA; SIRT1, silent information regulator 1; sRNA, small RNA; TCF7, transcription factor 7; TIR1, F-box/RNI-like superfamily protein; yegH, inner membrane protein.

us understand the cross-kingdom regulation of dietary microRNAs.

Questions remaining to be answered regarding dietary microRNAs and future directions

Independent studies have been conducted to validate the newly discovered roles of dietary microRNAs, particularly the regulatory roles of microRNAs associated with milk exosomes in offspring. However, there is a significant amount of evidence challenging this concept that should not be ignored (24, 73). First, Dickinson et al. (24) attempted to reproduce the observation that rice-derived miR168a could be absorbed by mice and regulate *LDLRAP1* expression and LDL concentrations. They were unable to detect any rice microRNAs, including miR168a, in the plasma or liver samples of mice fed balanced rice feed pellets or rice feed pellets compared with grain-free diet control mice, strongly suggesting the reads detected in the original study were derived from sequencing artifacts or contamination. In addition, they observed a significant increase in plasma LDL concentrations in the high-rice group, with an apparent absence of miR168a uptake. Furthermore, neither chow containing ~40% rice nor osa-miR168a concentrations of 54 fmol/g (much higher than the amount used in the original study) increased plasma LDL concentrations, leading Dickinson's group to propose that the increased LDL concentrations were due to the complementary reactions resulting from nutritional differences, because rice is low in fat and cholesterol. In addition, they did not detect any reduction in LDLRAP1 expression in the livers of mice that had been fed rice (24). In addition to the study by Dickinson et al., the generation of negative results demonstrated the obstacles faced by the dietary microRNA field.

Snow et al. (73) conducted several experiments on honeybees, human athletes, and miR-21 null mice, yet were unable to detect the robust presence of dietary microRNAs. One consideration to note is the origin of dietary microRNAs, which affects the sequence, nucleotide composition, modification, packaging, and protein association of dietary microRNAs. Although all of these factors contribute to the efficacy of microRNA uptake, the exact mechanisms by which they do so remain unclear. Certain microRNAs in food may remain undetectable regardless of the amount of intake. Exactly what type of sequence arrangement or nucleotide composition makes a microRNA bioaccessible? What types of modifications or molecular encapsulation result in the best efficacy of microRNA uptake and functionality? Computational studies will aid in providing theoretical solutions to address issues such as what types of dietary microRNAs are absorbed. Shu et al. (78) conducted such studies and focused on selecting and comparing the structural and sequence features of microRNAs to predict the likelihood of their transportation based on classification.

The so-called "barrier of consumption" mechanisms of dietary microRNA absorption and delivery directly affect their dynamics and require further study to allow for better experimental design for studying the actual effects of dietary microRNAs. Although the results of multiple studies using animal tissues and fluids or feeding experiments showed good agreement that the studied mammals could take up the assayed dietary microRNAs (10, 12, 53, 75, 79), the results of other reports are considered to be artifactual (24, 63, 73, 80, 81). Another aspect to consider is the efficient concentration of dietary microRNAs. The amount of small RNA believed to be required for effective biological regulation has been reported to be 100–10,000 copies per target cell, depending on the amount of target transcript present (82–84). Thus, the mechanisms by which animals generate physiologically relevant concentrations of exogenous microRNAs remained unclear.

More accurate detection methods for dietary microRNAs are also needed. NGS and qRT-PCR are the most commonly used techniques to study dietary microRNAs. However, these methods have been hypothesized to generate inaccurate results because of possible issues such as contamination (75, 81). For example, Tosar et al. (81) analyzed the spectra of plant microRNAs generated from small RNA sequencing studies and observed a strong correlation between different studies. They suggested that cross-contamination between samples from the same organism could be easily overlooked, limiting the accuracy of the results obtained by NGS in the dietary microRNA field. Witwer et al. (63) did not observe a consistent presence of dietary microRNAs (miRNAs 156, 160, 166, 167, 168, and 172 from soy and fruit substance) in the blood of pigtailed macaques by qRT-PCR after gastric gavage of "silk" fruit, and they suggested that nonspecific qRT-PCR amplification is an important factor in limiting its accuracy and application in the dietary microRNA field. Kang et al. (85) reanalyzed 824 sequencing data sets and observed only a 0.001% abundance of exogenous microRNAs in various human tissues and body fluids. Kang et al. (85) also performed an animal feeding study, the results of which showed that no exogenous microRNAs were transferred into animal blood, indicating that exogenous microRNAs may originate from technical artifacts rather than dietary intake. Moreover, the modifications of natural plant microRNAs as well as their low expression levels make sequencing difficult. Notably, appropriate controls are very important in periodate oxidation experiments, and high concentrations of RNAs in the reaction system were observed to result in the incomplete oxidation of unmethylated microRNAs. The improved methodology resulted in the elimination of false positives and optimized plant microRNA detection that worked well in different sample matrices (86). The conservation of sequences among animal microRNAs makes it even more difficult to distinguish dietary animal microRNAs from endogenous microRNAs. In milk, some microRNAs may escape detection, and >245 microRNAs are believed to be present in cow milk (31). Thus, more accurate and sensitive sequencing methods are needed.

By reviewing these negative results, we can better address future research directions for dietary microRNAs and optimize experimental conditions.

Conclusions

Changes in human diet due to increased global development have resulted in new challenges to human health. Dietary microRNAs represent a new area in food science, and based on our studies, these molecules could potentially contribute to a better understanding of the molecular mechanisms of ancient Chinese herbal medicine and provide preventive strategies and safer and more "natural" treatments for various diseases. As whole and functional small RNA molecules, not only consisting of nucleotides, dietary microRNAs have unique characteristics in their absorption, delivery, and functional mechanisms. However, there is currently a substantial lack of mechanistic insight into these aspects of microRNAs resulting from both biological and technical obstacles, and concerted efforts should be made in future investigations to address crucial issues, such as the identification of dietary microRNA transporters and intercellular machinery for exogenous microRNA gene expression regulation. Furthermore, the physiological effects of dietary microRNAs require further elucidation and additional evidence, for which a dietary microRNA deficiency animal model or an appropriate microRNA-deficient diet is needed. Based on the aforementioned currently available information on dietary microRNAs, further studies and efforts are warranted to fully understand the potential impact of dietary microRNAs on health and disease.

Acknowledgments

We thank Dr. Xi Chen for valuable input on the manuscript. The authors' responsibilities were as follows—LZ and TC: drafted the manuscript; YY: helped collect the data and references; C-YZ and Y-LZ: participated in the study design; and all authors: read and approved the final manuscript.

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