REVIEW

Cross-Kingdom Regulation by Plant microRNAs Provides Novel Insight into Gene Regulation

Abdul Fatah A. Samad,[1] Mohd Farizal Kamaroddin,[1] and Muhammad Sajad[2]

[1] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, Skudai, Johor, Malaysia; and [2] Department of Plant Breeding and Genetics, University College of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Punjab, Pakistan

ABSTRACT

microRNAs (miRNAs) are well known as major players in mammalian and plant genetic systems that act by regulating gene expression at the post-transcriptional level. These tiny molecules can regulate target genes (mRNAs) through either cleavage or translational inhibition. Recently, the discovery of plant-derived miRNAs showing cross-kingdom abilities to regulate mammalian gene expression has prompted exciting discussions among researchers. After being acquired orally through the diet, plant miRNAs can survive in the digestive tract, enter the circulatory system, and regulate endogenous mRNAs. Here, we review current knowledge regarding the cross-kingdom mechanisms of plant miRNAs, related controversies, and potential applications of these miRNAs in dietary therapy, which will provide new insights for plant miRNA investigations related to health issues in humans. *Adv Nutr* 2021;12:197–211.

Keywords: microRNA (miRNA), cross-kingdom, plant, post-transcriptional regulation, dietary therapy

Introduction

The emergence of microRNAs (miRNAs) as master regulator in gene regulation has led to interesting discussions. These miRNAs belong to a class of small noncoding RNAs (19–24 nucleotides) derived from secondary structures of transcribed *MIR* genes. The small molecules work by regulating gene expression at the post-transcriptional level in plants, mammals, and viruses (1). According to the public miRNA database, miRBase version 22.1 (http://www.mirbase.org/), a total of 38,589 miRNAs representing hairpin precursor miRNAs (pre-miRNAs) from 271 organisms have been registered (2). These pre-miRNA structures are able to produce 48,860 unique sequences of mature miRNAs (2). Once pre-miRNAs become mature miRNAs, they can target single or multiple target genes. Conversely, a single mRNA target can be targeted by >1 miRNA (3).

The first identified miRNA (lin-4) was discovered in *Caenorhabditis elegans* by Ambros in 1993 through forward genetics (4, 5). Since then, a number of approaches have been utilized for miRNA discovery, including cloning, homology searching and high-throughput sequencing (6, 7). Currently, high-throughput sequencing is the most reliable technique since it can detect miRNAs with low expression levels. miRNAs are well known to be involved in various processes in plants, including growth, development, signal transduction, responses to biotic and abiotic stresses, and regulation of secondary metabolites (1). In addition, miRNAs have been reported to regulate fruit ripening processes in blueberry, tomato, and banana plants (8–10). Recently, cross-kingdom regulation by miRNAs has become a hot topic among researchers. The ability of small RNAs (sRNAs)

This work on miRNA was funded by a UTM Encouragement Research grant (UTMER) (Q.J130000.2654.17J96) awarded to AFAS.
Author disclosures: The authors report no conflicts of interest.
Address correspondence to AFAS (e-mail: abdulfatah@utm.my).
Abbreviations used: ACAT, advanced compartment absorption and transit; AGO, Argonaute; amiRNA, artificial miRNA; BC, breast cancer; BPH, benign prostatic hyperplasia; CAT, compartmental absorption and transit; CKD, chronic kidney disease; COVID-19, coronavirus disease; *CSPG4*, chondroitin sulfate proteoglycan 4; C_T, threshold cycle; CVD, cardiovascular disease; DDL, DAWDLE; DCL1, Dicer-like 1; *DLG2*, discs large MAGUK scaffold protein 2; DNA-pre-miRNA, DNA precursor miRNA; DPI, days after inoculation; dsmiRNA, double-stranded miRNA; dsRNAs, double stranded RNAs; EAE, experimental autoimmune encephalomyelities; ECM, extracellular matrix; EPDENs, edible plant-derived exosome-like nanoparticles; EVs, extracellular vesicles; *Fbn1*, Fibrillin 1; GI, gastrointestinal; *GSK3B*, glycogen synthase kinase 3 beta; Hen 1, Hua enhancer 1; HS, honeysuckle; HST, Hasty; HYL1, Hyponastic leaves 1; IAV, influenza A virus; *ITGA2*, integrin alpha-2; JAK-STAT, janus kinase/signal transducer and activator of transcription; JAM-A, junction adhesion molecule-A; LAMC, laminin subunit gamma; LDL, low-density lipoprotein; *LDLRAP1*, low-density lipoprotein receptor adapter protein 1; MAPK, mitogen-activated protein kinase; miRNAs, microRNAs; mRNAs, messenger RNA; MVs, microvesicles; *NUMB*, NUMB endocytic adaptor protein; *OTX1*, orthodenticle homeobox 1; PAZ, piwi, argonaute and zwille/pinhead; PBPK, physiologically-based pharmacokinetic; *PEG3*, paternally expressed gene 3; *PLAGL2*, PLAG1-like zinc finger 2; Pol II, RNA polymerase II; PPM, Poly(A) polymerase-mediated; pre-miRNAs, precursor miRNAs; pri-miRNAs, primary miRNAs; RACE, rapid amplification of cDNA ends; RISC, RNA-induced silencing complex; RLM, RNA ligase-mediated; RNase, ribonuclease; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SE, C2H2 zinc-finger protein Serrate; SPRY, sprouty RTK signaling antagonist; sRNAs, small RNAs; ssDNA-miRNA, single-stranded DNA miRNA; Th, T-helper; *Tgfb1*, transforming growth factor beta 1; Treg, Regulatory T.

to be transferred from one kingdom to another has been previously shown in lower organisms. A few decades ago, double-stranded RNAs (dsRNAs) were found to be taken up by *C. elegans* and to confer specific interference effects when *C. elegans* were fed a dsRNA-expressing bacteria (11). More recent findings have suggested that *C. elegans* may possess an endocytic pathway for dsRNA uptake by cells (12). However, the transportation mechanism is probably uncommon among invertebrates due to the divergence of digestive tract systems (13).

In 2012, Zhang et al. (14) reported that plant-derived miRNAs can be present in the human serum or plant-feeding mammals and regulate the gene expression of particular targets at the post-transcriptional level (14). A few years later, another study revealed that a honeysuckle (HS) miRNA, miR2911, can be absorbed through the gastrointestinal (GI) tracts of mice infected with influenza A virus (IAV) upon ingestion of an HS decoction and travel via the bloodstream to the lungs where it directly targets IAV (15). These interesting findings have provided initial evidence that plant miRNAs may have the ability to be transmitted from plants to mammals through the GI tract, subsequently regulating their endogenous targets in a cross-kingdom manner and thus affecting the cellular systems of their recipients. The novel ability of plant miRNAs to regulate target genes in different organisms may completely transform our current knowledge and reveal new opportunities for novel therapies through plant-based diets (16).

The main objectives of this review are to analyze current evidence regarding cross-kingdom regulation by plant miRNAs to enhance the understanding of the novel abilities of these regulators as bioactive molecules in plant-based foods. In addition, the possible features of plant miRNAs that are responsible for the efficient transfer of these molecules from plant sources to mammals are discussed. Finally, the future impacts of these new phenomena on dietary therapy are presented.

Biogenesis and Mechanisms of Plant miRNAs

Plant-derived miRNAs are synthesized in the nucleus and mature in the cytoplasm (**Figure 1**). In the nucleus, *MIR* genes are transcribed by RNA polymerase II (Pol II) (17). The first transcription process of *MIR* genes produces primary miRNAs (pri-miRNAs), which consist of thousands of nucleotides together with hairpin structures (18). The pri-miRNA structure is stabilized by the Dawdle (DDL) protein (1, 19). In plants, the essential protein for miRNA processing is Dicer-like 1 (DCL1), which is a type III ribonuclease (RNase) responsible for processing pri-miRNAs by cleaving them to become shorter (19). A pair of proteins, the dsRNA-binding protein Hyponastic leaves 1 (HYL1) and the C2H2 zinc-finger protein Serrate (SE), facilitate DCL1 by improving the efficiency and precision of the DCL1 cleavage mechanism (19). Pri-miRNA processing occurs in the D-body compartment in the nucleus (20). DCL1 performs excision in 2 steps. First, it cleaves the imperfectly folded end of the pri-miRNA to form the hairpin secondary structure of the pre-miRNA. Second, it further slices the pre-miRNA to generate a double-stranded miRNA/miRNA∗ duplex (19).

A notable difference between plant miRNAs and animal miRNAs relates to the methylation process of miRNA/miRNA∗ duplexes, which is carried out by an RNA methyltransferase called Hua enhancer 1 (Hen 1). The methylated miRNA/miRNA∗ duplexes are then exported by the Hasty (HST) protein to the cytoplasm, where the miRNA guide strand is incorporated into the Argonaute (AGO) protein to form an RNA-induced silencing complex (RISC) (21, 22). Meanwhile, the passenger strand (miRNA∗) is removed and degraded (22). AGO proteins mainly consist of PAZ (Piwi, argonaute and zwille/pinhead) and PIWI domains (23). The PIWI domain forms an RNaseH-like fold that catalyzes endonuclease activity (1, 23, 24). The mechanisms of action of plant miRNAs are determined mostly by the degrees of complementary binding between the miRNAs and their target genes. Plant miRNAs tend to bind with their targets with high complementarity, which results in mRNA cleavage, whereas animal miRNAs bind to their targets at seed regions to promote translational inhibition (25). However, in plants, miRNAs also silence their targets through translational inhibition (26, 27).

Cross-Kingdom Regulation by Plant miRNAs Obtained through Dietary Intake

miR168a represents the first evidence of cross-kingdom regulation in plants

Among all organism-derived miRNAs, plant miRNAs are mainly involved in cross-kingdom gene regulation after dietary intake. In addition, only the mature sequences of plant miRNAs have cross-kingdom abilities; other forms of RNA do not have these abilities, such as pre-miRNA, double-stranded miRNA, single-stranded DNA miRNA, and DNA precursor miRNA (14). Due to this phenomenon, the mature sequences of plant miRNAs have the potential to be used in molecular therapy after acquisition through specific diets and food supplements.

The first cross-kingdom study, which was reported in 2012, demonstrated the detection of miR168a in the circulatory systems and organs of humans and mice (14). In this study, a group of healthy Chinese men and women who consumed rice as their main dietary ingredient, ∼30 known plant miRNAs were revealed via Solexa sequencing. Among them, miR156a and miR168a showed dominant levels of expression compared with other miRNAs. In animal studies, miR168a was detected in the livers and serum of 10-week-old mice that were fed fresh rice total RNA (80 μg), synthetic miR168a (300 pmol), or synthetic methylated miR168a (300 pmol) by gavage after feed deprivation overnight. In addition, miR168a was also detected in the serum of 2 other groups of 10-week-old male mice fed a diet of either chow or fresh rice after feed deprivation overnight (14). Further functional studies have shown that the plant miR168a binds to its target, the *LDLRAP1* (LDL receptor adapter protein 1) gene, which leads to decreases in LDLRAP1 levels in

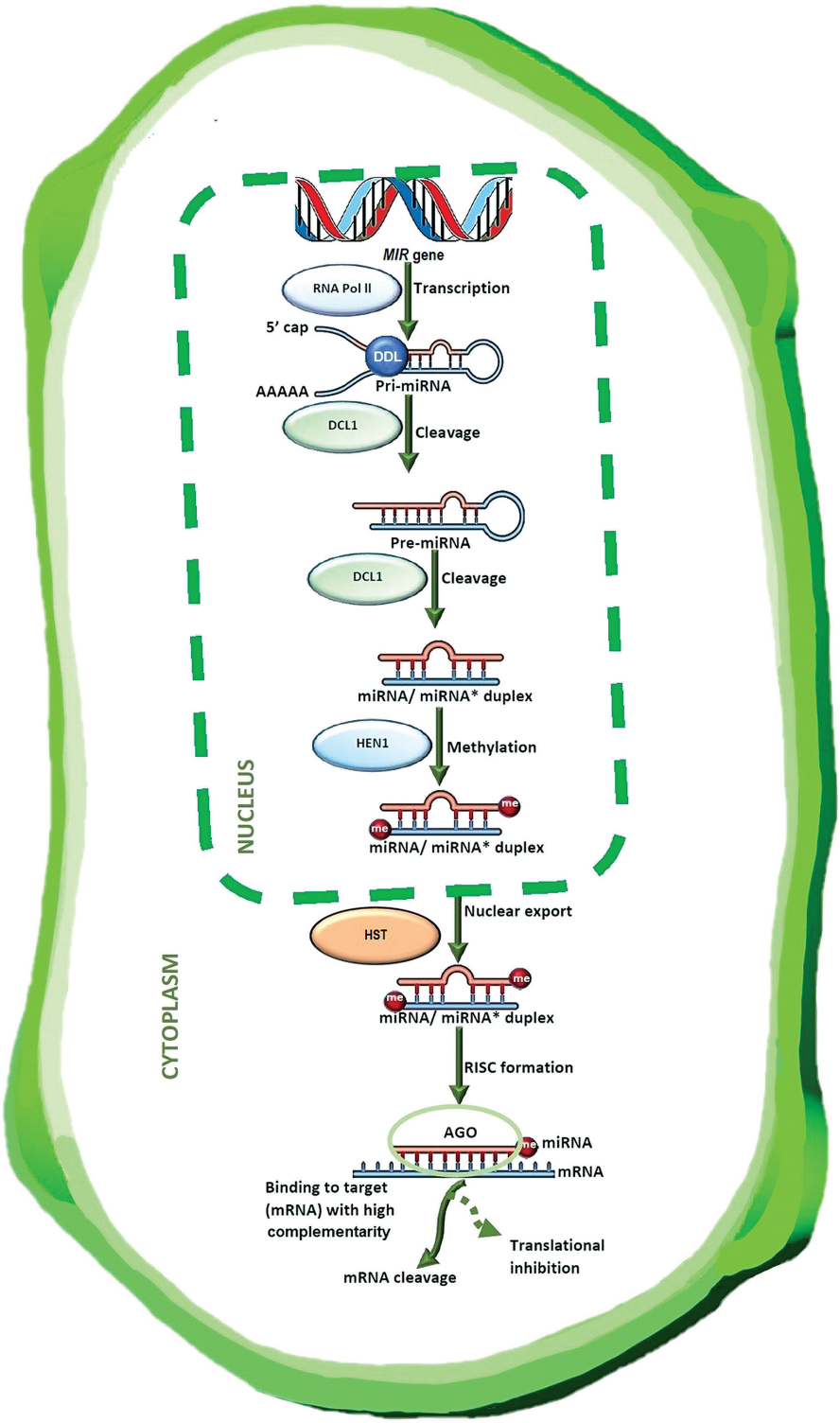

FIGURE 1 miRNA biogenesis pathways in plants. The biogenesis of plant miRNAs begins within the nucleus. The *MIR* gene is transcribed by RNA Pol II to produce pri-miRNAs, which are stabilized by the protein DDL. Pri-miRNAs are cleaved through the action of the endonuclease DCL1 until they become mature miRNA/miRNA∗ duplexes. Subsequently, methylation at the 3′-terminus of the duplex is carried out by Hen 1. Then, HST exports the miRNA/miRNA∗ duplex from the nucleus to the cytoplasm. At the final stage, 1 strand of the miRNA/miRNA∗ duplex is incorporated into AGO as a guide strand to form the RISC where it can perform its function, while the other strand is removed and degraded. Plant miRNAs can regulate gene expression via mRNA cleavage and translational inhibition. However, in plants, the dominant mechanism (solid arrow) occurs by mRNA cleavage, in which the miRNA binds its target with high complementarity, while the alternative mechanism (dashed arrow) occurs by translational inhibition. AGO, Argonaute; DDL, Dawdle; HST, Hasty; Hen 1, Hua enhancer 1; miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RNA Pol II, RNA polymerase II.

liver cells and subsequently decreases the removal of LDL cholesterol (14).

Plant miRNAs remain stable in mammalian systems

Since that exciting breakthrough, more research has been conducted regarding the inter-kingdom transfer of exogenous plant miRNAs. In a study, the most enriched plant miRNA in cabbage, miR172, was identified in the stomachs, intestines, serum, and feces of mice fed cabbage total RNA in quantities ranging from 10 to 50 μg. A maximum of 4.5% of miR172 was reportedly recovered from the stomach of 1 mouse, and the percentages ranged from 0.05% to 4.5% in different organs and tissues. In particular, the concentrations of miR172 were ∼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. This study showed the absorbed dietary miRNAs may be metabolized similarly to endogenous miRNAs, and with continuous feeding, the concentration of dietary miRNAs may remain stable for a certain period of time (28). In a study involving human volunteers, a total of 16 miRNAs (miR156a, miR157a, miR158a, miR159a, miR160a, miR162a, miR163a, miR166a, miR167a, miR168a, miR169a, miR172a, miR390a, miR528, miR824, and miR894) were detected in plasma through qPCR after the volunteers consumed 2.5 L of watermelon juice and 2.5 kg of mixed fruits. However, the C_T (threshold cycle) values of 6 plant miRNAs in human plasma (miR158a, miR159a, miR160a, miR163a, miR169a, and miR824) fell outside the linear ranges of their standard curves, indicating that they were undetectable in plasma and could not be accurately measured, while the remaining 10 miRNAs were expressed at high basal levels (29, 30).

In a study in pigs fed fresh maize, maize-derived miRNAs could be detected in porcine tissues and serum after 7 d of consumption. The detected miRNAs included zma-miR164a-5p, zma-miR166a-3p, zma-miR167e-5p, zma-miR168a-5p, zma-miR319a-3p, and zma-miR408a-3p. The presence of these plant miRNAs was confirmed using oxidization reactions (31). Among these miRNAs, zma-miR164a-5p has 3 potential target genes: *CSPG4* (chondroitin sulfate proteoglycan 4), *OTX1* (orthodenticle homeobox 1), and *PLAGL2* (PLAG1-like zinc finger 2) (31).

Stability and antiviral activity of miR2911 against influenza A viruses (IAV)

In HS, miR2911 was found to be highly stable, and continuous administration of a decoction led to a significant increase in miR2911 in mouse peripheral blood and lungs (15). miR2911 was detected in mice fed by gavage with an HS decoction (10 g HS in 100 mL water) or synthetic miR2911. The HS decoction contained 0.12 nM of miR2911. The synthetic miR2911 was gavage-fed at a dose of 100 pmol/mouse. For both the HS decoction– and synthetic miR2911–fed groups, samples were collected at 0.5, 1, 3, 6, or 12 h for RNA extraction (15). Moreover, synthetic miR2911, RNA extracted from the HS decoction, and the HS decoction itself showed significant antiviral activity against H1N1. Further in vivo study showed miR2911 could inhibit H1N1, H5N1, and H7N9 viral replication in the mouse model, which subsequently prevented viral infection–induced weight loss and reduced the mortality rate (15). This discovery may reveal a novel type of natural product and suggests that miR2911 intake may effectively suppress viral infection.

In a different study, miR2911 from HS was detected in the serum and urine of mice fed a chow diet containing HS after 3.5 d of intake. In another experiment involving gavage-feeding of mice, using the same diet plus HS decoction supplemented with synthetic plant miR168a (400 pmol), both miR2911 and miR168a were detected in the serum of mice by qPCR (32). An additional study also detected abundant miR2911 in mice fed different plant-based diets for 7 d. In that study, mice were administered herb diets, flower diets, and plant chow diets. The herb and flower diets were independently prepared from finely ground tissues of HS, chamomile, sophora, lavender, blue mallow, ginseng, hibiscus, and willow bark plants, which were obtained from various local Chinese herbal medicine and tonic stores. The plant chow diets were prepared by mixing finely ground chow, plant material, and water at a ratio of 2:1:2. The findings showed higher circulating concentrations of miR2911 in mice that were fed diets containing HS (39-fold), chamomile (27-fold), sophora (22-fold), lavender (13-fold), blue mallow (12-fold), and ginseng (5-fold) than in mice that were fed a control chow diet (33).

miR159 as a novel anticancer agent from a plant source

In plants, the miR159 family regulates plant growth, development, and morphology by targeting the transcription factors MYB33 and MYB65. miR159 is a common plant miRNA that can be found in *Arabidopsis thaliana*, *Glycine max*, and broccoli (34). In the context of cross-kingdom gene regulation, a study detected plant miR159 in human serum and breast tumor tissue after dietary consumption. Further computational analysis revealed that plant miR159 directly targeted human *TCF7*, which encodes a Wnt signaling transcription factor. Cell counting assays showed that synthetic miR159 mimicking the duplex of gma-miR159a-3p and gma-miR159a-5p significantly reduced the proliferation of the breast cancer (BC) cell lines MDA-MB-231 and MCFDCIS (34). In xenograft tumor experiments, mice were orally administered 25 mg/kg miR159 or scrambled control oligos for 3 d before their mammary fat pads were implanted with 2×10^5 MDA-MB-231 or MDA-MB-231-TCF7HA BC cells. The group treated with miR159 showed significantly decreased tumor growth and weight on day 16 (34). The ability of plant miR159 to inhibit cancer proliferation was first demonstrated in mammals, suggesting that this particular miRNA may be a potential therapeutic agent for BC.

Plant miRNAs as immunomodulatory agents and their role in improving the antioxidant capacity

A study involving transfection of synthetic miRNAs and native miRNA-enriched miRNAs derived from various plants, such as miR156 and miR168 from strawberry, miR168

from rice, and miR874 from cabbage, revealed that the miRNAs could modify the ability of dendritic cells to respond to inflammatory agents by limiting T-cell proliferation, therefore reducing inflammation (35). In that study, 10 ng/mL of each miRNA was used. Furthermore, intravenous injection of a cocktail of pooled plant sRNAs from cabbage, fern fronds, and apple peel plants (30 μg) into mice every 4 d after inoculation (dpi) from 3 dpi to 22 dpi resulted in a reduction in the onset severity of experimental autoimmune encephalomyelitis (EAE). The plant sRNA cocktail acted by limiting dendritic cell migration and dampening T-helper (Th) 1 and Th17 responses in a Regulatory T (Treg)–independent manner (35). On the other hand, another study demonstrated that uptake of exogenous miR-451 from a chow diet in *miR-144/451*-null mice increased the levels of miR-451. In addition, gavage feeding of 200 μL of wild-type blood also increased the levels of miR-451 in *miR-144/451*-null mice and improved the antioxidant capacity through the Foxo3 pathway. In contrast, when the *miR-144/451*-null mice were fed food lacking miR-451, the antioxidant capacity of the RBCs decreased through the same pathway (36).

Identification of cross-kingdom regulation using a computational approach

In a study involving an in silico approach, plant miRNA sequences were identified in sRNA sequencing data sets from 410 human plasma samples. The data sets were aligned with genomes from plant model organisms, including *A. thaliana*, *Triticum aestivum*, *Oryza sativa*, *Zea mays*, and *Brachypodium distachyon*, using Bowtie and miRDeep2 software (37). Among the miRNAs from plant species, miR2910 from *Populus euphratica*, which was also expressed in *Z. mays*, was found to be more dominant than most of the human miRNAs in the given samples. miR2910 was predicted to target the *SPRY4* (sprouty RTK signalling antagonist 4) gene of the Janus kinase/signal transducer and activator of transcription (JAK-STAT) signaling pathway and transcription regulation genes (37). Another in silico approach was used in a study on the happy tree (*Camptotheca acuminate*), which is recognized as an important medicinal plant with anticancer properties (38). The miRNA from this plant may have the ability to target genes associated with cancer when orally acquired. To assess this possibility, an in silico approach using psRNATarget prediction software was deployed to link the miRNA- and cancer-associated pathways (38). The search predicted that a number of miRNAs in the happy tree, such as cac-miR-4723–3p, miR-5780d, and miR-548d-3p, target genes associated with cancer, such as *DLG2* (discs large MAGUK scaffold protein 2), *NUMB* (NUMB endocytic adaptor protein), and *GSK3B* (glycogen synthase kinase-3B), respectively. Furthermore, a single miRNA, miR-29c-5p, was predicted to target 2 genes associated with cancer: *PEG3* (paternally expressed gene 3) and *ITGA2* (integrin alpha 2) (38). A recent study using a computational approach involved a herb plant, *Ocimum basilicum*, which has considerable therapeutic potential. In *O. basilicum*, oba-miR156, oba-miR531, oba-miR160, oba-miR529b, and oba-miR1118 were predicted to modulate the 87 human target genes associated with the Ras-MAPK (Ras-mitogen-activated protein kinase)signaling cascade, cardiomyopathy, HIV, breast cancer, lung cancer, Alzheimer disease, and several neurological disorders (39).

Plant miR156 as a potential vasoprotective molecule and miR5338 as an alternative treatment for benign prostatic hyperplasia

A recent study stably detected the plant miRNA, miR156a, which is commonly found in dietary green vegetables, such as cabbage, spinach, and lettuce, in 5 healthy human serum samples after the individuals had consumed 500 g of vegetables. The results demonstrated that the miR156a levels peaked 1 or 3 h after lettuce consumption in most people, while the absorption efficiency appeared to vary among individuals (40). Plant miR156a can directly target junction adhesion molecule-A (JAM-A) to reduce inflammatory cytokine–induced monocyte adhesion in human aortic endothelial cells. These findings suggest that plant miRNAs are novel vasoprotective molecules (40). A study has also suggested that plant-derived miR5338 may help ameliorate benign prostatic hyperplasia in rats by inhibiting *Mfn1* gene in the prostate. In that study, the rats in the experimental group were treated with suspensions of rape bee pollen (final concentration: 6.39 g/kg) by gavage (10 mL/kg), whereas the rats in the normal group were given clean water by gavage (10 mL/kg). The levels of the mTtbiR5338 target gene, *Mfn1*, in the posterior lobe of the prostate were found to be significantly lower in the experimental than in the control group (41).

Cross-kingdom dietary miRNA regulation through lactation

In addition to food intake, milk consumption has also been reported to enable the delivery of miRNAs. Unlike plant miRNAs, which are initially hypothesized to be encapsulated in microvesicles (MVs) (14), miRNAs delivered through milk are packaged in exosomes (42). Interestingly, increasing evidence indicates that mammalian breast milk contains both endogenous miRNAs and exogenous plant-derived miRNAs (43–45). In human mature-milk samples collected from 6 mothers who consumed vegetarian or nonvegetarian diets, miR168a was more detectable than other common miRNAs, such as miR156a, miR166a, miR167a, and miR172a. In addition, an endogenous human miRNA, hsa-miR-148a, was detected in all 6 samples (44). The abundant human miRNAs in breast milk participate in regulating various biological processes, such as immune system development, endo- and exocytosis, metabolic processes, cell differentiation, growth, and apoptosis, to ensure proper infant development (44–46). Furthermore, a bioinformatics analysis carried out on human and porcine milk samples revealed an abundance of plant miRNAs; ath-miR166a, pab-miR951, ptc-miR472a, and bdi-miR168 were detected at high levels in human-milk exosomes, while zma-miR168a, zma-miR156a, and ath-miR166a were detected in porcine-milk exosomes (43). **Table 1** shows the current information regarding cross-kingdom regulation by plant miRNAs in mammalian systems.

TABLE 1 Overview of the current status of cross-kingdom regulation of mammalian systems by plant miRNAs[1]

miRNA	Host plant	Target gene/disease	Targeted organism (tissue/organ)	Amount/dose consumed	Detection method	Detection time	Reference(s)
miR168a	Rice	*LDLRAP1*	Human (serum and plasma)	Consumed rice as main diet	Solexa sequencing, oxidation, qPCR, Northern blot, luciferase assay, and AGO2 immunoprecipitation	N/A	(14)
			Mouse (serum, plasma, and liver)	Gavage-fed with total RNA (80 μg), synthetic miR168a (300 pmol), synthetic methylated miR168a (300 pmol), and fresh food, respectively		0.5, 3, or 6 h or 1, 3, or 7 d	
miR2911	Honeysuckle	IAV	Mouse (blood and lung)	10 g of HS in 100 mL	Deep sequencing, qPCR, Northern blot, fluorescent labeled assay, luciferase assay, and AGO2 immunoprecipitation	0.5–12 h	(15)
miR172	Cabbage	Not mentioned	Mouse (stomach, intestine, blood, and spleen)	10–50 μg	RT-PCR, qPCR	2–72 h	(28)
Multiple miRNAs (refer to text)	Watermelon juice and mixed fruit	Not mentioned	Human (plasma)	2.5 L or 2.5 kg	Northern blot, qPCR	0.5–9 h	(30)
zma-miR164a-5p	Fresh maize	*CSPG4*, *OTX1*, and *PLAGL2*	Pig (serum, solid tissue, and intestine)	Not mentioned	Sequencing, oxidation, ex vivo everted gut sac method, qPCR, and dual-luciferase assay	7 d	(31)
zma-miR166a-3p, zma-miR167e-5p, zma-miR168a-5p, zma-miR319a-3p, and zma-miR408a-3p	Fresh maize	Not mentioned	Pig (serum, solid tissue, and intestine)	Not mentioned	Sequencing, oxidation, ex vivo everted gut sac method, and qPCR	7 d	(31)

(*Continued*)

TABLE 1 (*Continued*)

miRNA	Host plant	Target gene/disease	Targeted organism (tissue/organ)	Amount/dose consumed	Detection method	Detection time	Reference(s)
miR2911	Honeysuckle	Not mentioned	Mouse	Gavage-fed with chow diet containing HS decoction	qPCR and droplet digital PCR	3.5 d	(32)
miR168a and miR2911	Honeysuckle	Not mentioned	Mouse	Gavage-fed with chow diet containing HS decoction supplemented with synthetic plant miR168a (400 pmol)	qPCR and droplet digital PCR	3 h	(32)
miR2911	Plant-based diets	Not mentioned	Mouse (serum)	Not mentioned	AGO2 immunoprecipitation, and qPCR	7 d	(33)
miR159	*Arabidopsis thaliana*, *Glycine max*, and broccoli	*TCF7*	Mouse (serum)	Gavage-fed with 25 mg/kg miR159	Solexa sequencing, oxidation, qPCR, in situ hybridization, and luciferase assay	Not mentioned	(34)
miR156 and miR168	Strawberry	Modified the ability of dendritic cells to respond to inflammatory agents	Human (dendritic cell)	10 ng/mL	qPCR, flow cytometry (miR168 only), and fluorescence microscopy (miR168 only)	Not mentioned	(35)
miR168	Rice	Modified the ability of dendritic cells to respond to inflammatory agents	Human (dendritic cell)	10 ng/mL	qPCR	Not mentioned	(35)
miR874	Cabbage	Modified the ability of dendritic cells to respond to inflammatory agents	Human (dendritic cell)	10 ng/mL	qPCR	Not mentioned	(35)

(*Continued*)

TABLE 1 (*Continued*)

miRNA	Host plant	Target gene/disease	Targeted organism (tissue/organ)	Amount/dose consumed	Detection method	Detection time	Reference(s)
Plant sRNA	Cabbage , fern fronds , and apple peel	Reduce the onset and severity of EAE	Mouse	30-μg cocktail of plant sRNA	Not mentioned	Not mentioned	(35)
miR-451	Chow diet enriched with miR-451	Foxo3 pathway	Mouse (blood)	Not mentioned	Two different PCR methods, dual-luciferase assay	Not mentioned	(36)
	Gavage-fed of wild-type blood			200 μL		6 h	
miR2910	*Populus euphratica* and *Zea mays*	*SPRY4*	Human (plasma)	N/A	Computational	N/A	(37)
Cac-miR-4723–3p	*Camptotheca acuminata*	*DLG2*	Human	N/A	Computational	N/A	(38)
miR-5780d		*NUMB*					
miR548d-3p		*GSK3B*					
miR-29c-5p		*PEG3* and *ITGA2*					
Oba-miR156, oba-miR531, oba-miR160, oba-miR529b, and oba-miR1118	*Ocimum basilicum*	87 targets associated with various diseases	Human	N/A	Computational	N/A	(39)
miR156a	Cabbage, spinach, and lettuce	JAM-A	Human (serum)	500 g	qPCR and luciferase assay	Varies among individuals	(40)
miR5338	Rape bee pollen	*Mfn1*	Rats (posterior lobes)	Gavage-fed with 10 mL/kg suspension	Deep sequencing and qPCR	Not mentioned	(41)
miR156a	Breast milk	271 unique targets	Human (milk)	Not mentioned	qPCR	24 h	(44)
miR166a		88 unique targets					
miR167a		15 unique targets					
miR168		4 unique targets					
miR172a		7 unique targets					
miR166, miR167, and miR168	*Glycine max* and *Oryza sativa*	Not mentioned	Survival to pre-treatment (boiling, cooking, storage)	Not mentioned	qPCR	75 min	(47)
miR-34, miR-143, and miR-145	Synthetic 2′-*O*-methylated miRNA	Tumors	Mouse (intestine)	Gavage	qPCR	28 d	(48)

[1] AGO2, Argonaute 2; *CSPG4*, chondroitin sulfate proteoglycan 4; *DLG2*, discs large MAGUK scaffold protein 2; EAE, experimental autoimmune encephalomyelitis; *GSK3B*, glycogen synthase kinase 3B; HS, honeysuckle; IAV, influenza A viruses; *ITGA2*, integrin alpha 2; JAM-A, junction adhesion molecule-A; *LDLRAP1*, LDL receptor adapter protein 1; *Mfn1*, Mitofusin 1; miRNA, microRNA; N/A, not applicable; *NUMB*, NUMB endocytic adaptor protein; *OTX1*, orthodenticle homeobox 1; *PEG3*, paternally expressed gene 3; *PLAGL2*, PLAG1-like zinc finger 2; *SPRY4*, Sprouty RTK signalling antagonist 4; sRNA, small RNA; *TCF7*, transcription factor 7.

Factors that influence the stability and mobilization of plant miRNAs

Previous findings have revealed that food storage, processing, and cooking do not reduce the miRNA content in plants, which might be a crucial factor contributing to the abundance of miRNAs in food products. However, even though plant miRNAs can survive all food-processing steps, they are still prone to degradation in the mammalian circulation system due to endonuclease activity, especially in the spleen and liver (49). To further investigate this topic, a study used a simulated human digestion system, and the results showed significant bioavailability of miR166, miR167, and miR168 (from rice and soybean) after early-stage digestion for 75 min (47). A closer look revealed that one of the biogenesis steps in plants, methylation of miRNA at the 3′ end after the slicing process, might be the first determinant of miRNA stability (Figure 1).

In a plant model system (*A. thaliana*), most miRNAs were found to be 2′-*O*-methylated at their 3′ ends. Northern blotting was carried out to identify the functions of miRNA methylation in wild-type and *hen1* mutants. A decrease in abundance and heterogeneity in size were observed for the unmethylated form of miRNA compared with the methylated form. Then, primer extension studies showed that size heterogeneity existed at the 3′ ends of the miRNAs. Further cloning and Sanger sequencing of miR173 and miR167 in both wild-type and *hen1* mutant plants revealed that the miRNA sequences underwent 3′ truncation and 3′ uridylation, which involves the addition of a short U-rich tail (50). Later comparison studies on sRNAs including miRNAs and siRNAs in *Arabidopsis*, *Drosophila*, and zebrafish showed similar findings: *hen1* mutant individuals were more prone to degradation than wild-type individuals (51). These findings provide fundamental evidence that methylation of plant miRNAs enhances their stability by protecting them from exonuclease and uridylation activities.

The methylated structures at the 3′ ends of plant miRNAs confer the plant miRNAs with greater oxidizing agent resistance than animal miRNAs. Zhang et al. (14) treated both plant and animal miRNAs with sodium periodate (an oxidizing agent), which resulted in modification (oxidation) at the 3′ ends of mammalian miRNAs. The oxidized 3′ ends could not undergo the ligation process during sRNA library preparation and thus failed to be sequenced. However, plant miRNAs that bore the 2′-*O*-methylated moiety were detected in human sera; this moiety rendered them distinguishable from mammalian miRNAs (14). Furthermore, the nucleotide compositions of plant miRNAs may also contribute to their stability. A study on miR2911 showed that the resistance of this miRNA to boiling and RNase treatment might be due to its unique sequence composition and GC content (15).

Plant miRNAs have been suggested to be packaged into MVs or exosomes before being absorbed via intestinal epithelial cells. Despite all the protective mechanisms of plant miRNAs, the MVs or exosomes add further protection by acting as protective barriers to prevent miRNA degradation. In addition, several types of carriers can transport miRNAs, such as apoptotic bodies and edible plant-derived exosome-like nanoparticles (EPDENs) (52, 53). After packaging, miRNAs are released into the circulation and subsequently transported to various kinds of tissues and organs where they regulate the expression of target genes (14). Moreover, plant miRNAs can utilize the AGO2 protein, which is present in exosomes, to form an RISC, thereby facilitating binding to target genes (54). The route taken by plant miRNAs from when they are present in raw materials until they are absorbed into the mammalian system is summarized in **Figure 2**.

Controversy regarding Cross-Kingdom Regulation

Despite the exciting finding of cross-kingdom regulation by plant-derived miRNAs, several groups of researchers have questioned the discoveries due to a lack of reproducibility. The observed plant miRNAs in animal sRNA data sets may have emerged from the sequencing process or from cross-contamination between samples (55, 56). In a study involving controlled insect feeding, plant-derived miRNAs, such as miR168a, were also detected in insects that were not fed monocot plants (56). Furthermore, in a study conducted to reproduce the detection of plant miRNAs in animal systems, mice were divided into particular diet groups: a synthetic chow group, a balanced rice chow group, and a rice chow group. Unfortunately, insignificant levels of plant miRNAs or no plant miRNAs (including miR168a) were detected in the plasma or livers of the mice fed the rice-containing diets (57). Only 192 and 153 reads of miR168 were reportedly detected in the rice-containing chow and rice grain groups, respectively. However, consistent with Zhang et al. (14), animals that ate chow containing high amounts of uncooked rice had significantly increased LDL-cholesterol levels at 3 and 7 d after treatment. The authors suggested that the increases in LDL-cholesterol levels were due to short-term nutritional impacts that may have occurred due to the release of endogenous cholesterol stores in response to negligible dietary cholesterol intake in the mice fed rice only (57). Therefore, the findings of previous cross-kingdom regulation studies may have resulted from contamination by miRNAs instead of from the presence of miRNAs transmitted from other kingdoms.

However, Zhang et al. (14) replied that the scientists questioning their findings may have used a sequencing approach that was biased between plant and animal miRNAs. This possibility became evident because only small quantities of rice miRNAs were detectable in both mouse plasma and livers in that study, while a previous study indicated that rice miRNAs should represent at least 10% of the total reads (58, 59). miR156 and miR168 are the most abundant miRNAs in rice grain, and the total count should have therefore been at least 10,000 reads per million

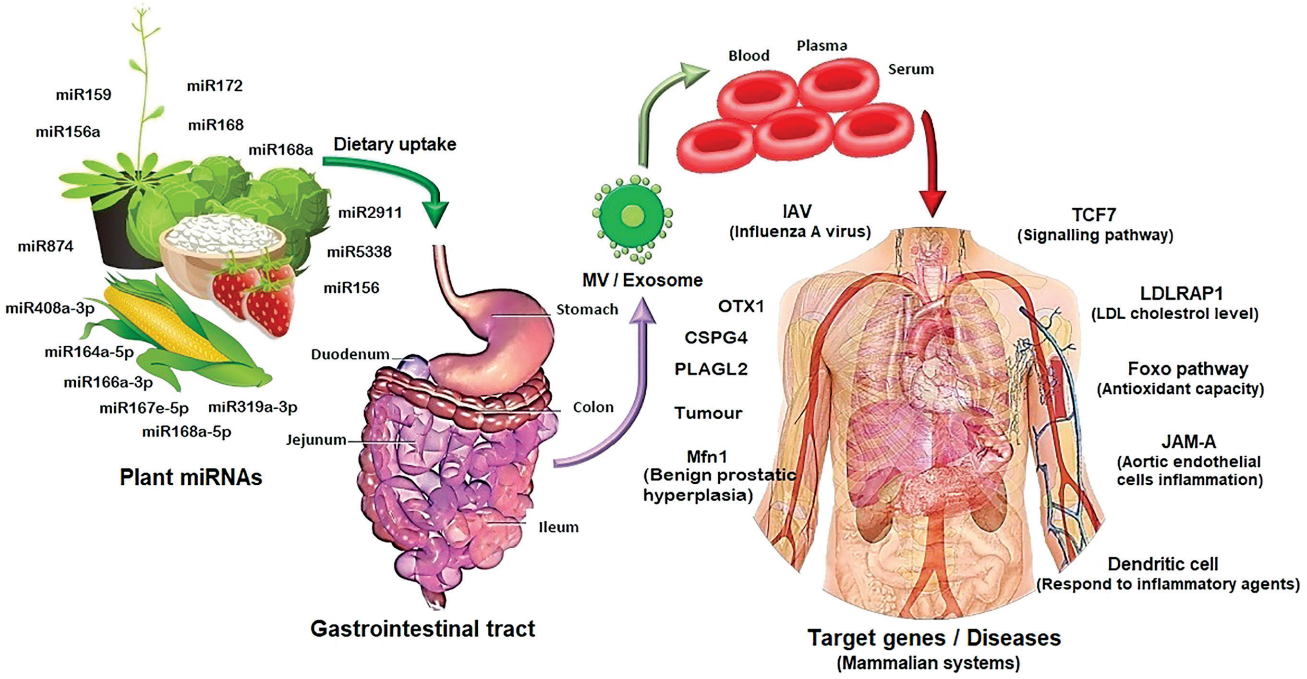

FIGURE 2 An overview of the miRNA journey from raw materials into the human digestive and circulatory systems. miRNAs are acquired through the diet and absorbed in the GI tract before being transported to the circulatory system (blood/plasma/serum) via MV/exosome. In the circulatory system, plant miRNAs are further transported to specific cells/organs to regulate certain diseases or target genes (shown in the figure is the identified target through experimental work). *CSPG4*, chondroitin sulfate proteoglycan 4; GI, gastrointestinal; JAM-A, junction adhesion molecule-A; *LDLRAP1*, LDL receptor adapter protein 1; *Mfn1*, Mitofusin 1; miRNA, microRNA; MV, microvesicle; *OTX1*, orthodenticle homeobox 1; *PLAGL2*, PLAG1-like zinc finger 2; *TCF7*, transcription factor 7.

instead of the 192 and 153 reads for miR168 in the rice-containing chow and rice grain, respectively. The low levels of plant miRNAs indicate that the sequencing platform deployed in that study was less efficient than the platform used in the previous study in measuring plant miRNAs (60). Therefore, only small amounts of exogenous plant miRNAs could be detected in the mouse livers and plasma (60).

Additionally, another group of scientists criticized the findings of the involvement of plant miR156a in human aortic endothelial cells by supporting the opinion of Dickinson et al. (57) that the functional relevance was traced to an experimental design artifact (61). They mentioned that the findings of cross-kingdom miRNAs are most likely due to cross-contamination during library preparation and the use of low-biomass samples combined with some widely used miRNA extraction kits. An additional study carried out by the group detected low levels of plant miRNAs in 800 sRNA-Seq data sets from human tissues and body fluids. Moreover, in well-designed and controlled animal feeding experiments, no cross-kingdom transfer of plant miRNAs was observed (61). On the other hand, the group that carried out the original work responded that they deployed 3 different approaches: *1*) high-throughput sequencing with high numbers of miRNA sequencing reads ($n > 100$ copies after normalization) since high numbers of miRNA reads support the validity of the findings; *2*) qPCR with no-template controls, proper standard curves, and internal standards to ensure accuracy; and *3*) Northern blot analysis. Therefore, the plant miRNAs identified by these 3 different techniques having been derived from contamination or technical artefacts is highly implausible. The issues encountered by the group that could not reproduce the results may have been due to sequencing bias (62). Furthermore, synthetic, fluorophore-labeled miRNAs transfected into bovine-milk exosomes and administered to mice have been shown to exhibit unique distribution profiles and to accumulate in the intestinal mucosa, spleen, liver, heart, or brain (63). Supposedly, no contamination issues can arise during fluorophore-based labeling because the labels can be observed directly using a proper microscopic system. The addressed concerns, resolutions, and remaining issues are summarized in **Table 2**.

Potential Applications and Strategies for Cross-Kingdom miRNA Use in Dietary Therapy

Growing evidence has shown that exogenous plant miRNAs can mobilize and migrate from plants to mammalian cells and regulate certain genes or processes. This unique ability allows these tiny molecules to serve as alternative agents in human therapies. Based on the above discussion, a number of identified plant miRNAs are ideal candidates for dietary therapy approaches, especially those that have already been tested in the laboratory, such as miR2911 from HS, which

TABLE 2 List of issues with and possible responses to cross-kingdom regulation by plant miRNAs[1]

Description of the issue(s)	Resolution/response	Remaining issue(s)
Lack of reproducibility and cross-contamination issues.	These issues may arise due to sequencing bias. Recalibrating the sequencing platform to obtain high copy number ($n > 100$) was suggested.	Some plant miRNAs were detected in mammals; however, whether these miRNAs can regulate mammalian genes/systems remains unclear.
Detection of plant miRNAs in a nonplant diet in a study involving controlled insect feeding.	Further validation using various approaches, such as qPCR, Northern blotting, and fluorophore labelling of miRNAs, confirmed the presence of cross-kingdom transfer and regulation.	Some plant miRNAs were found in mammalian systems and could regulate certain pathways; however, the exact target gene is still unknown.
Detection of a small amount or total absence of plant miRNAs in mice fed a rice-containing diet.		Computationally predicted cross-kingdom regulation may require further experimental validation.
Use of a low-biomass sample with a miRNA extraction kit.		
Low detection of plant miRNAs from human tissues and bodily fluids.		

[1] miRNA, microRNA.

has a surprising capability to suppress IAV, and miR159 from *A. thaliana*, soybean, and broccoli, which has the ability to suppress cancer cell growth (15, 34). The discovery of these miRNAs may lead to the development of nontoxic and inexpensive strategies to treat human diseases that are carried out through dietary changes. The current progress and development of cross-kingdom regulation by plant miRNA are shown in **Figure 3**.

Another application of plant miRNAs would involve the modification of potential endogenous mammalian miRNAs to mimic plant miRNA structures by adding methyl groups at the 3′ ends of the mammalian miRNAs. The methylated forms of mammalian miRNAs have been demonstrated to be more stable than normal miRNAs (48). In a study, tumor suppressor miRNAs (miR-34a, miR-143, and miR-145) were synthesized according to mouse miRNA sequences; a methyl group was added to each miRNA at the 2′ position of the ribose of the 3′ terminal nucleotide to enable the miRNAs to mimic plant miRNAs. Three groups (7 $Apc^{Min/+}$ colon cancer model mice/group) were treated with the miRNAs by gavage for 28 d starting at 5 weeks to determine the treatment effects on the tumor burden. The experimental group received total plant RNA spiked with the 3 tumor suppressor miRNAs, while 2 other groups served as negative controls and received either total plant RNA alone or water. The study showed that the tumor burden was reduced by miRNA administration in the $Apc^{Min/+}$ mouse model (48). This means of modification may reflect an effective oral delivery system for therapeutic miRNAs that show promising results in the treatment of chronic diseases.

In cardiovascular disease, 2 adenovirus-delivered miRNAs, miR-214 and miR-21, have shown positive results in improving left ventricular remodeling and decreasing myocardial apoptosis in a rat model; however, the utilization of viral vectors for delivery of the desired miRNAs to specific target cells may have major drawbacks, such as potential insertional mutagenesis and immune response induction (64). In this case, oral administration of plant miRNAs through diet may become an alternative to deliver the miRNAs. To apply this approach, the plant miRNAs must have the exact target gene as miR-214 and miR-21, so the same result can be produced. However, in this study, no genes are mentioned as miR-214 and miR-21 targets. Thus, target identification work is required. Additionally, if no plant miRNAs are available, modifying available miRNAs by adding a methyl group may be considered.

Similarly, in chronic kidney disease, miR-29 family members have been recognized to act as potential antifibrotic agents by targeting extracellular matrix genes such as *Spry1* (sprouty RTK signaling antagonist 1), *TGFB1*, *Tgfb1* (transforming growth factor beta 1), *Col1a2*, *LAMC1* (laminin subunit gamma 1), *LAMC2* (laminin subunit gamma 2), *FBN1*, *Fbn1* (Fibrillin 1), *Eln* (elastin), and *Itgb1* (integrin subunit beta 1) (65). These miRNAs were delivered to the kidneys using direct renal interstitial infusion, pelvic perfusion, or systemic delivery methods through transfection (65). Identifying plant miRNAs that target the same genes may become another option to achieve the therapeutic effect of miRNAs through the diet. In this case, target validation is not required since it is already validated by previous work (65), unless novel miRNA candidates are identified. Likewise, if no plant miRNAs are available, modifying available miRNAs with a methyl group may be considered. The modified miRNAs (with methylation at their 3′ ends) mimicking natural mammalian miRNAs are expected to deliver therapeutic effects by restoring the levels of miRNAs that are downregulated during certain diseases or by downregulating signaling pathways involved in the diseases (66). A patient would need only to consume a specific diet designed by an expert for the delivery of therapeutic miRNA to target cells.

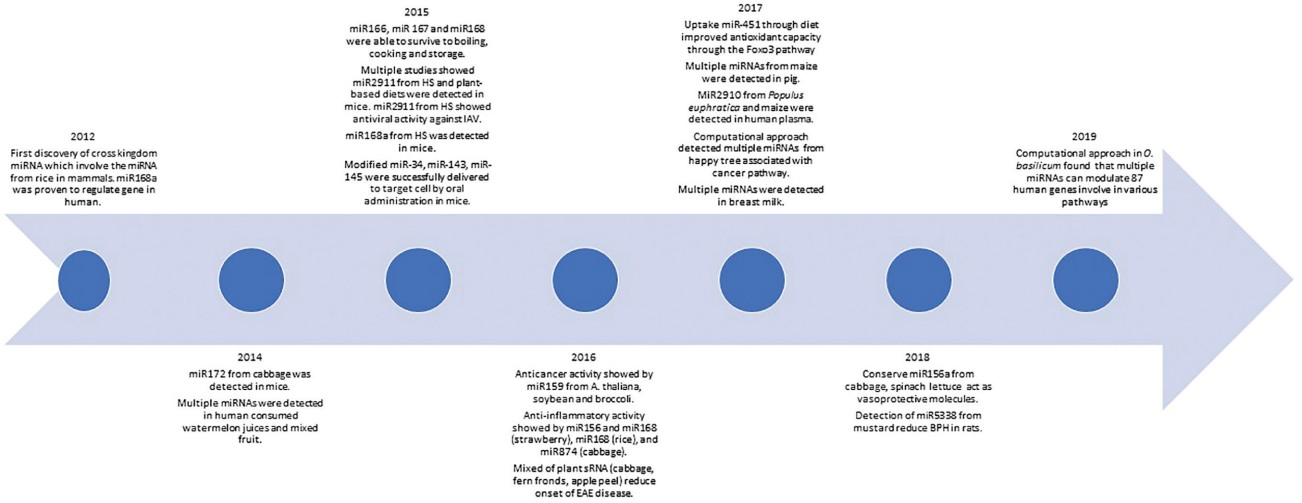

FIGURE 3 The current progress and development of cross-kingdom regulation by plant miRNAs, which started in 2012 and is expected to further expand in the future. BPH, benign prostatic hyperplasia; EAE, experimental autoimmune encephalomyelitis; HS, honeysuckle; IAV, influenza A virus; miRNA, microRNA.

However, before these therapeutic miRNAs can be applied efficiently, some gaps require special attention. The exact transportation system of plant miRNAs must be identified to elucidate how diet-derived miRNAs are transported to target cells. Previously, circulating miRNAs in mammals were found in small membranes called extracellular vesicles (EVs) (67). Based on the aforementioned studies, the EVs involved in dietary miRNA uptake are probably MVs and exosomes. In this matter, the term MVs and exosomes need to be clarified to avoid confusion. Besides, EPDENs also have been shown to carry diet-derived miRNAs to target organisms (42, 53). Additionally, factors that allow plant miRNAs detection in mammalian systems also need to be identified to ensure reproducibility. Currently, the ideas are more focused on plant miRNA stability (due to the methylated structure) and the doses of the plants consumed. A previous study has shown that consumption of a minimum of 500 g and a maximum of 2.5 kg of raw material is sufficient for plant miRNAs to be detected in human plasma (30, 40). These steps are important to ensure that candidate therapeutic miRNAs can be transferred efficiently to target cells.

In addition, another crucial aspect that must be addressed in cross-kingdom regulation is the target gene of the plant miRNAs in the mammalian system. Some miRNAs are known to affect certain metabolic or diseases pathways, but the exact target genes (mRNAs) are still unclear. Additionally, further validation is required for plant miRNAs that have been computationally predicted to be involved in cross-kingdom regulation. Multiple approaches can be deployed to confirm the targets, such as degradome sequencing, RNA ligase-mediated (RLM)–rapid amplification of cDNA ends (RACE), poly(A) polymerase–mediated (PPM)-RACE, and qPCR (68). Last but not least, the absorption rate of the introduced plant miRNAs in the human system must be clarified to ensure that the miRNAs can diffuse to the target cells. Previously, the absorption rate was identified based on the ratio of the total absorption amount to the total uptake amount (30). The total absorption amount is determined by calculating the area under the plasma concentration-time curve (AUC) of each plant miRNA, while the total uptake amount is determined by calculations of the concentrations of plant miRNAs (30). Additionally, the absorption rate also can be identified by considering drug absorption as a model in which 2 fundamental processes accounted for oral absorption: *1*) the dissolution of a molecule in GI fluid and *2*) permeation of a dissolved molecule through the intestinal wall and into the bloodstream (69). The absorption rate can be predicted using models such as the physiologically-based pharmacokinetic (PBPK) model, the mixing tank model, the compartmental absorption and transit (CAT) model, and the advanced compartment absorption and transit (ACAT) model (69).

On the other hand, for plant miRNAs that cannot target a specific gene naturally, bioengineering of selected plants to synthesize specific miRNA sequences is an absolute solution to mimic a particular mammalian miRNA with an established therapeutic effect; this technology is called artificial miRNA (amiRNA) (70). With this technology, an miRNA sequence can be designed to efficiently regulate both single and multiple target genes, which is consistent with findings for most natural plant miRNAs (71, 72). Furthermore, the quantity of raw material that needs to be consumed by a patient can be reduced since the miRNA is already highly expressed in the plant. An issue that may arise with this approach is whether the transgenic plant is safe to consume. Thus, further biosafety work must be carried out. All these suggestions to utilize plant miRNAs as therapeutic tools are summarized in **Table 3**.

TABLE 3 Strategies for the use of dietary or plant miRNAs as therapeutic tools[1]

Description of application	Corresponding study needed to achieve the application
Oral administration of miRNAs to improve heart condition in CVD	Identification of plants with the most similar miRNAs to mammalian miRNAs (miR-214 and miR-21) shown to improve the heart condition; if not applicable, mimicry of the mammal miRNA structures according to plant miRNAs may be considered Identification of EVs used to transport miRNAs to the target cell/tissue Determination of the correct dosage of raw materials for the miRNAs to exert their effect on the heart Identification of the exact target (mRNA) of the candidate miRNAs through computational approach or luciferase assay Confirmation of the targets through degradome sequencing, RLM-RACE, PPM-RACE, and qPCR Determination of miRNA diffusion rate through ratio (total absorption:total uptake) and/or modeling
Oral administration of miRNAs as anti-fibrosis agents in CKD	Identification of plants with the most similar miRNAs to mammalian miRNAs (miR-29 members) that act as antifibrosis agents; if not applicable, mimicry of the miRNA structures according to plant miRNAs may be considered Identification of EVs used to transport miRNAs to the target cell/tissue Determination of the correct dosage of raw materials for the miRNAs to exert their effect on the kidney No target confirmation required since it was previously validated, unless novel candidate miRNAs appear Determination of miRNA diffusion rate through ratio (total absorption:total uptake) and/or modeling
Bioengineering of selected plants to synthesize specific miRNA sequences	Identification of potential plant crops that can be utilized as a biofactory Determination of the correct dosage of raw materials for the miRNAs to exert their effect on the targeted cell/tissue Assessment of biosafety issues regarding the consumption of transgenic products

[1] CKD, chronic kidney disease; CVD, cardiovascular disease; EV, extracellular vesicle; miRNA, microRNA; PPM, poly(a) polymerase-mediated; RACE, rapid amplification of cDNA ends; RLM, RNA ligase-mediated.

Currently, the world is still in a state of alert regarding coronavirus disease (COVID-19), which is caused by a novel type of coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Rapidly growing in cross-kingdom of plant miRNA field has led to a recent finding that showed miR2911 from HS can be absorbed in human serum and significantly inhibit the replication of SARS-CoV-2 when orally administered as a decoction (30 g dry honeysuckle in 200 mL/donor; equal to 10.5 pmol miR2911) (73). In addition, a clinical study showed that COVID-19 patients who received additional miR2911 treatment had a higher negative conversion rate on the 7th day (83.3%) of the treatment than those without miR2911 treatment (26.1%) (73). This finding suggests that plant-derived miRNA has the potential to inhibit viral replication, and that this research area deserves more attention in terms of mechanisms-of-action and to determine if there is therapeutic value to supplements or foods containing these specific miRNAs.

Conclusions and Future Remarks

In conclusion, the discovery of cross-kingdom regulation of gene expression by plant miRNAs suggests a novel approach for dietary therapy that may be of great value in medicine. Extensive studies in this field may lead to the discovery that many diseases can be cured by consuming specific diets containing plant miRNAs. However, many issues must be considered since several researchers have questioned the reproducibility of related findings. Some have questioned whether the copy numbers of exogenous miRNAs are sufficiently high to affect the functions of endogenous target genes. Technical issues may have been the fundamental causes of the reproducibility of the research, such as issues in sRNA library preparation and with sequencing platforms. Moreover, using engineered plant machinery to produce specific therapeutic miRNAs that are subsequently consumed by patients may provide a new perspective for the treatment of many diseases, especially those that develop due to dietary habits. Indeed, cross-kingdom regulation by plant miRNAs and the therapeutic potential of plant miRNAs must be further explored, and any potential risks should be carefully considered.

Acknowledgments

The authors' responsibilities were as follows—AFAS: drafted and revised the main manuscript; MFK: prepared the figures; MS: provided the initial proofreading; and all authors: made substantial contributions and approved the final manuscript.

References

1. Samad AFA, Sajad M, Nazaruddin N, Fauzi IA, Murad AMA, Zainal Z, Ismail I. MicroRNA and transcription factor: key players in plant regulatory network. Front Plant Sci 2017;8(565). doi:10.3389/fpls.2017.00565.
2. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. Nucleic Acids Res 2019;47(D1):D155–62. doi:10.1093/nar/gky1141.
3. Perge P, Nagy Z, Decmann A, Igaz I, Igaz P. Potential relevance of microRNAs in inter-species epigenetic communication, and implications for disease pathogenesis. RNA Biology 2017;14(4):391–401.
4. Lee R, Feinbaum R, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–54.
5. Esquela-Kerscher A. The lin-4 microRNA: the ultimate micromanager. Cell Cycle 2014;13(7):1060–1.
6. Jones-Rhoades M, Bartel D, Bartel B. MicroRNAS and their regulatory roles in plants. Annu Rev Plant Biol 2006;57:19–53.
7. Mehta JP. Sequencing small RNA: introduction and data analysis fundamentals. Methods Mol Biol 2014;1182:93–103.

8. Hou Y, Zhai L, Li X, Xue Y, Wang J, Yang P, Cao C, Li H, Cui Y, Bian S. Comparative analysis of fruit Ripening-Related miRNAs and their targets in blueberry using small RNA and degradome sequencing. Int J Mol Sci 2017;18(12):2767. doi:10.3390/ijms18122767.
9. Gao C, Ju Z, Cao D, Zhai B, Qin G, Zhu H, Fu D, Luo Y, Zhu B. MicroRNA profiling analysis throughout tomato fruit development and ripening reveals potential regulatory role of RIN on microRNAs accumulation. Plant Biotechnol J 2015;13(3):370–82.
10. Bi F, Meng X, Ma C, Yi G. Identification of miRNAs involved in fruit ripening in Cavendish bananas by deep sequencing. BMC Genomics 2015;16. doi:10.1186/s12864-015-1995-1.
11. Timmons L, Fire A. Specific interference by ingested dsRNA. Nature 1998;395(6705):854. doi:10.1038/27579.
12. McEwan DL, Weisman AS, Hunter CP. Uptake of extracellular double-stranded RNA by SID-2. Mol Cell 2012;47(5):746–54.
13. Karasov WH, Martinez del Rio C, Caviedes-Vidal E. Ecological physiology of diet and digestive systems. Annu Rev Physiol 2011;73:69–93.
14. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, Li J, Bian Z, Liang X, Cai X, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res 2012;22(1):107–26.
15. Zhou Z, Li X, Liu J, Dong L, Chen Q, Liu J, Kong H, Zhang Q, Qi X, Hou D, et al. Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. Cell Res 2015;25(1):39–49.
16. Xie W, Weng A, Melzig MF. MicroRNAs as new bioactive components in medicinal plants. Planta Med 2016;82(13):1153–62.
17. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004;23(20):4051–60.
18. Bartel D. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
19. Bologna NG, Schapire AL, Palatnik JF. Processing of plant microRNA precursors. Briefings Funct Genomics 2013;12(1):37–45.
20. Fang Y, Spector DL. Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants. Curr Biol 2007;17(9):818–23.
21. Wahid F., Shehzad A., Khan T., Kim YY, MicroRNAs: synthesis, mechanism, function, and recent clinical trials., Biochim Biophys Acta, 2010, 1803(11):1231–43. doi: 10.1016/j.bbamcr.2010.06.013.
22. Yu Y, Jia T, Chen X. The "how" and "where" of plant microRNAs. New Phytol 2017;216(4):1002–17.
23. Miyoshi T, Ito K, Murakami R, Uchiumi T. Structural basis for the recognition of guide RNA and target DNA heteroduplex by Argonaute. Nat Commun 2016;7:11846. doi:10.1038/ncomms11846.
24. Wilson RC, Doudna JA. Molecular mechanisms of RNA interference. Annu Rev Biophys 2013;42:217–39.
25. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 2011;12:99. doi:10.1038/nrg2936.
26. Beauclair L, Yu A, Bouche N. MicroRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J 2010;62(3):454–62.
27. Li S, Liu L, Zhuang X, Yu Y, Liu X, Cui X, Ji L, Pan Z, Cao X, Mo B, et al. MicroRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. Cell 2013;153(3):562–74.
28. Liang G, Zhu Y, Sun B, Shao Y, Jing A, Wang J, Xiao Z. Assessing the survival of exogenous plant microRNA in mice. Food Sci Nutr 2014;2(4):380–8.
29. Snow JW, Hale AE, Isaacs SK, Baggish AL, Chan SY. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biology 2013;10(7):1107–16.
30. Liang H, Zhang S, Fu Z, Wang Y, Wang N, Liu Y, Zhao C, Wu J, Hu Y, Zhang J, et al. Effective detection and quantification of dietetically absorbed plant microRNAs in human plasma. J Nutr Biochem 2015;26(5):505–12.
31. Luo Y, Wang P, Wang X, Wang Y, Mu Z, Li Q, Fu Y, Xiao J, Li G, Ma Y, et al. Detection of dietetically absorbed maize-derived microRNAs in pigs. Sci Rep 2017;7(1):645. doi:10.1038/s41598-017-00488-y.
32. Yang J, Farmer LM, Agyekum AAA, Hirschi KD. Detection of dietary plant-based small RNAs in animals. Cell Res 2015;25(4):517–20.
33. Yang J, Farmer LM, Agyekum AAA, Elbaz-Younes I, Hirschi KD. Detection of an abundant plant-based small RNA in healthy consumers. PLoS One 2015;10(9):e0137516–e. doi:10.1371/journal.pone.0137516.
34. Chin AR, Fong MY, Somlo G, Wu J, Swiderski P, Wu X, Wang SE. Cross-kingdom inhibition of breast cancer growth by plant miR159. Cell Res 2016;26(2):217–28.
35. Cavalieri D, Rizzetto L, Tocci N, Rivero D, Asquini E, Si-Ammour A, Bonechi E, Ballerini C, Viola R. Plant microRNAs as novel immunomodulatory agents. Sci Rep 2016;6:25761. doi:10.1038/srep25761.
36. Wang W, Hang C, Zhang Y, Chen M, Meng X, Cao Q, Song N, Itkow J, Shen F, Yu D. Dietary miR-451 protects erythroid cells from oxidative stress via increasing the activity of Foxo3 pathway. Oncotarget 2017;8(63):107109–24.
37. Liu YC, Chen WL, Kung WH, Huang HD. Plant miRNAs found in human circulating system provide evidences of cross kingdom RNAi. BMC Genomics 2017;18(Suppl 2):112. doi:10.1186/s12864-017-3502-3.
38. Kumar D, Kumar S, Ayachit G, Bhairappanavar SB, Ansari A, Sharma P, Soni S, Das J. Cross-kingdom regulation of putative miRNAs derived from happy tree in cancer pathway: a systems biology approach. Int J Mol Sci 2017;18(6):1191. doi:10.3390/ijms18061191.
39. Patel M, Mangukia N, Jha N, Gadhavi H, Shah K, Patel S, Mankad A, Pandya H, Rawal R. Computational identification of miRNA and their cross kingdom targets from expressed sequence tags of Ocimum basilicum. Mol Biol Rep 2019;46(3):2979–95.
40. Hou D, He F, Ma L, Cao M, Zhou Z, Wei Z, Xue Y, Sang X, Chong H, Tian C, et al. The potential atheroprotective role of plant MIR156a as a repressor of monocyte recruitment on inflamed human endothelial cells. J Nutr Biochem 2018;57:197–205.
41. Chen X, Wu R-Z, Zhu Y-Q, Ren Z-M, Tong Y-L, Yang F, Dai G-H. Study on the inhibition of Mfn1 by plant-derived miR5338 mediating the treatment of BPH with rape bee pollen. BMC Complement Altern Med 2018;18(1):38. doi:10.1186/s12906-018-2107-y.
42. Title AC, Denzler R, Stoffel M. Uptake and function studies of maternal milk-derived microRNAs. J Biol Chem 2015;290(39):23680–91.
43. Lukasik A, Zielenkiewicz P. In silico identification of plant miRNAs in mammalian breast milk exosomes—a small step forward? PLoS One 2014;9(6):e99963. doi:10.1371/journal.pone.0099963.
44. Lukasik A, Brzozowska I, Zielenkiewicz U, Zielenkiewicz P. Detection of plant miRNAs abundance in human breast milk. Int J Mol Sci 2017;19(1):37. doi:10.3390/ijms19010037.
45. Gu Y, Li M, Wang T, Liang Y, Zhong Z, Wang X, Zhou Q, Chen L, Lang Q, He Z, et al. Lactation-related microRNA expression profiles of porcine breast milk exosomes. PLoS One 2012;7(8):e43691. doi:10.1371/journal.pone.0043691.
46. Alsaweed M, Hartmann PE, Geddes DT, Kakulas F. MicroRNAs in breastmilk and the lactating breast: potential immunoprotectors and developmental regulators for the infant and the mother. Int J Environ Res Public Health 2015;12(11):13981–4020.
47. Philip A, Ferro VA, Tate RJ. Determination of the potential bioavailability of plant microRNAs using a simulated human digestion process. Mol Nutr Food Res 2015;59(10):1962–72.
48. Mlotshwa S, Pruss GJ, MacArthur JL, Endres MW, Davis C, Hofseth LJ, Peña MM, Vance V. A novel chemopreventive strategy based on therapeutic microRNAs produced in plants. Cell Res 2015;25(4):521–4.
49. Sidova M, Tomankova S, Abaffy P, Kubista M, Sindelka R. Effects of post-mortem and physical degradation on RNA integrity and quality. Biomol Detect Quantif 2015;5:3–9.
50. Li J, Yang Z, Yu B, Liu J, Chen X. Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in Arabidopsis. Curr Biol 2005;15(16):1501–7.

51. Zhao Y, Yu Y, Zhai J, Ramachandran V, Dinh TT, Meyers BC, Mo B, Chen X. The Arabidopsis nucleotidyl transferase HESO1 uridylates unmethylated small RNAs to trigger their degradation. Curr Biol 2012;22(8):689–94.
52. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev 2012;64(3):676–705.
53. Mu J, Zhuang X, Wang Q, Jiang H, Deng ZB, Wang B, Zhang L, Kakar S, Jun Y, Miller D, et al. Interspecies communication between plant and mouse gut host cells through edible plant derived exosome-like nanoparticles. Mol Nutr Food Res 2014;58(7):1561–73.
54. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol 2009;11(9):1143–9.
55. Tosar JP, Rovira C, Naya H, Cayota A. Mining of public sequencing databases supports a non-dietary origin for putative foreign miRNAs: underestimated effects of contamination in NGS. RNA 2014;20(6):754–7.
56. Zhang Y, Wiggins BE, Lawrence C, Petrick J, Ivashuta S, Heck G. Analysis of plant-derived miRNAs in animal small RNA datasets. BMC Genomics 2012;13:381. doi:10.1186/1471-2164-13-381.
57. Dickinson B, Zhang Y, Petrick JS, Heck G, Ivashuta S, Marshall WS. Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat Biotechnol 2013;31(11):965–7.
58. Jeong DH, Park S, Zhai J, Gurazada SG, De Paoli E, Meyers BC, Green PJ. Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. Plant Cell 2011;23(12):4185–207.
59. Yi R, Zhu Z, Hu J, Qian Q, Dai J, Ding Y. Identification and expression analysis of microRNAs at the grain filling stage in rice (Oryza sativa L.) via deep sequencing. PLoS One 2013;8(3):e57863. doi:10.1371/journal.pone.0057863.
60. Chen X, Zen K, Zhang CY. Reply to Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat Biotechnol 2013;31(11):967–9.
61. Fromm B, Kang W, Rovira C, Cayota A, Witwer K, Friedländer MR, Tosar JP. Plant microRNAs in human sera are likely contaminants. J Nutr Biochem 2019;65:139–40.
62. Hou D, Zhou Z, Chen X, Jiang X, Zen K, Zhang C-Y. Reply to Fromm et al. J Nutr Biochem 2019;65:140–1.
63. Manca S, Upadhyaya B, Mutai E, Desaulniers AT, Cederberg RA, White BR, Zempleni J. Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. Sci Rep 2018;8(1):11321. doi:10.1038/s41598-018-29780-1.
64. Anguela XM, High KA. Entering the modern era of gene therapy. Annu Rev Med 2019;70(1):273–88.
65. Lv W, Fan F, Wang Y, Gonzalez-Fernandez E, Wang C, Yang L, Booz GW, Roman RJ. Therapeutic potential of microRNAs for the treatment of renal fibrosis and CKD. Physiol Genomics 2018;50(1):20–34.
66. Kreth S, Hubner M, Hinske LC. MicroRNAs as clinical biomarkers and therapeutic tools in perioperative medicine. Anesth Analg 2018;126(2):670–81.
67. Bayraktar R, Van Roosbroeck K, Calin GA. Cell-to-cell communication: microRNAs as hormones. Mol Oncol 2017;11(12):1673–86.
68. Wang C, Fang J. RLM-RACE, PPM-RACE, and qRT-PCR: an integrated strategy to accurately validate miRNA target genes. Methods Mol Biol 2015;1296:175–86.
69. Lin L, Wong H. Predicting oral drug absorption: mini review on physiologically-based pharmacokinetic models. Pharmaceutics 2017;9(4):41. doi:10.3390/pharmaceutics9040041.
70. Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D. Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 2006;18(5):1121–33.
71. Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ. Elucidation of the small RNA component of the transcriptome. Science 2005;309(5740):1567–9.
72. Zhang N, Zhang D, Chen SL, Gong B-Q, Guo Y, Xu L, Zhang X-N, Li J-F. Engineering artificial microRNAs for multiplex gene silencing and simplified transgenic screen. Plant Physiol 2018;178(3):989–1001.
73. Zhou L-K, Zhou Z, Jiang X-M, Zheng Y, Chen Y, Fu Z, Xiao G, Zhang C-Y, Zhang L-K, Yi Y. Absorbed plant MIR2911 in honeysuckle decoction inhibits SARS-CoV-2 replication and accelerates the negative conversion of infected patients. Cell Discovery 2020;6(1):54.