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Review

Plant-derived xenomiRs and cancer: Cross-kingdom gene regulation

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ABSTRACT

Exosomal microRNAs (miRNAs) critically regulate several major intracellular and metabolic activities, including cancer evolution. Currently, increasing evidence indicates that exosome harbor and transport these miRNAs from donor cells to neighboring and distantly related recipient cells, often in a cross-species manner. Several studies have reported that plant-based miRNAs can be absorbed into the serum of humans, where they hinder the expression of human disease-related genes. Moreover, few recent studies have demonstrated the role of these xenomiRs in cancer development and progression. However, the cross-kingdom gene regulation hypothesis remains highly debatable, and many follow up studies fail to reproduce the same. There are reports that show no effect of plant-derived miRNAs on mammalian cancers. The foremost cause of this controversy remains the lack of reproducibility of the results. Here, we reassess the latest developments in the field of cross-kingdom transference of miRNAs, emphasizing on the role of the diet-based xenomiRs on cancer progression.

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1. Cancer: A multifaceted disease

Cancer is a heterogeneous group of diseases and has become a prominent reason of death worldwide (Fitzmaurice et al., 2017). The global cancer burden amounts to 14.1 million worldwide, while ~ 9.6 million deaths occurred in 2018 due to cancer (WHO 2018). The number of expected cancer cases may reach 23.6

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million by 2030 (Si et al., 2019). Cancer is a complex pathological condition characterized by altered gene expression, aberrant genetic mutations and genomic instability. Moreover, the tumor microenvironment (TME), comprising of cellular and non-cellular components of the tumoral niche, plays a crucial role in cancer development as well as metastasis (Wang et al., 2017a; Sun et al., 2018). Accumulating evidence suggests that TME greatly influences the metabolic rewiring of cancer cells via extracellular vesicles (EVs) (Sullivan et al., 2017).

EVs are spherical, bilayered, heterogeneous cell derived membranous organelles (from normal/cancerous cells) that package cellular material; thereby influencing neighboring as well as remote cells with its payload (Colombo et al., 2014). The components of the cargo varies highly and depends on the kind of cell it originated from. Cancer cells are known to secrete many-fold vesicles than normal dividing cells (Akers et al., 2013; Mao et al., 2018). Studies suggests that their content plays a critical function as the crosstalk between constituent cells of the TME through the transport of specific cargo molecules, such as growth factors, chemokines, and microRNAs (miRNAs) (Kahlert et al., 2013; Benmoussa et al., 2017). EVs secreted from cancer cells can remodel the microenvironment and promote cancer progression, metastasis, and drug resistance (Xie et al., 2019; Chiarugi and Cirri, 2016; Tkach and Thery, 2016).

Recent evidences have shown that EVs transport miRNAs that act as messengers between tumoral and stromal cells (Donnarumma et al., 2017). This interaction can affect the TME, influencing the extracellular matrix (ECM), epithelial-mesenchymal transition, angiogenesis, and reprogramming of immune function (Ingenito et al., 2019). Altered miRNA expression in TME favors the acquisition of cancer hallmarks, leading to the development and progression of tumors. (Soon and Kiaris 2013; Rupaimoole et al., 2016; Kunitny et al., 2016; Fanini and Fabbri 2017; Curtale, 2018; Bandini et al., 2019).

miRNAs can circulate in the blood and may be absorbed by cells and tissues at distant sites (Hasegawa et al., 2017). Plant-based exosomal miRNA (which protects RNase) was shown to be delivered to humans in a cross-kingdom fashion (Zhang et al., 2012a), where they can mediate cancer metastasis (Zhou et al., 2014a,b, c). Further studies showed that plant exomiRs may regulate the expression of cancer-related genes and proteins (Pastrello et al., 2016; Chin et al., 2016). In this article, we have summarized and reviewed cross-kingdom gene regulation by plant-derived exomiRs with special emphasis on cancer progression.

2. Extracellular vesicles – Connecting kingdoms

Currently, EVs are known to play a pivotal function in diverse cell-to-cell signaling processes. Multivesicular bodies (MVBs), later revealed to be exosomes, were documented in the 1950s, first in algae (Sager and Palade, 1957) and later in mammalian cells (Sotelo et al., 1959). Simultaneously, outer membrane vesicles (OMVs) have been discovered in bacteria (De, 1959; Chatterjee et al., 1959). Soon after, MVBs were found in higher plants (Jensen, 1965) as well as in fungi (Takeo et al., 1973). At that time, the researchers could not realize the significance of this discovery. Two decades later, exosomes were found to function as garbage-dumping devices. It was assumed that their function was to simply “defenestrate” remnants instead of its degradation (Harding et al., 1983; Pan et al., 1985; Johnstone et al., 1987; Thery, 2011). This notion of waste disposal remained until 1996 when Raposo et al. (1996) discovered that EVs may influence antigen presentation in vivo. Since then, tremendous research on EVs has suggested their importance in cellular communication.

Presently, three different types of EVs are widely accepted according to the consensus nomenclature (Han et al 2019) (Table 1): apoptotic bodies, microvesicles, and exosomes (Fuhrmann et al., 2017; Mathieu et al., 2019). Apoptotic bodies (ApoBDs), the largest EVs (with a diameter of 1–5 µm), are discharged as membrane-surrounded fragments during apoptosis. ApoBDs can carry protein, lipid, DNA, RNA as well as cell organelles (van Niel et al., 2018). Microvesicles (ectosomes) are larger in size (0.1–1.0 µm) than exosomes, originating from outward budding and fission of the plasma membrane (Théry, 2011; Rutter and Innes, 2016; Zemleni 2017; Raposo and Stoorvogel, 2013). Oncosomes are 100–400 nm, specialized vehicles transporting abnormal proteins that play crucial role in vascular growth, cancer evolution and metastasis (Minciacchi et al., 2015; Jaiswal and Sedger 2019). Exosomes, the smallest (30–100 nm) among EVs, are membranous vesicles secreted by a plethora of cell types, including cancer cells; and are released extracellularly via fusion of MVBs with the cell membrane (Hessvik et al., 2013). These secreted EVs has been detected in virtually every kind of body fluids; such as blood (serum and plasma) (Caby et al., 2005; McDonald et al., 2013), seminal fluid (Hoog and Lotvall, 2015), urine (Pisitkun et al., 2004), saliva (Machida et al., 2015; Han et al., 2018), ascites (Keller et al., 2011), breast milk (Lasser et al., 2011; Wang, 2017), cerebrospinal fluid (Bachy et al., 2008), and amniotic fluid (Keller et al., 2011; Ebert and Rai, 2019).

Table 1

Major subtypes of EVs and their representative features (Modified from Han et al 2019, Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

EV subtype	Exosomes	Microvesicles (MVs)/ ectosomes/microparticles	Apoptotic bodies (ABs)
Subcellular origin	Multivesicular bodies (MVBs)/ endosomes	Plasma membrane	Apoptotic blebs
Regular diameter	30–120 nm	100–1000 nm	0.8–5.0 µm
Sedimentation force	100,000–120,000 × g	10,000 g	2000 g
Types of generation	Constitutive	Regulated	Regulated
Filtration	20–200 nm	>200 nm	>1000 nm
Intracellular storage	Yes	No	No
Biogenetic mechanisms	Rab proteins (i.e. Rab7, Rab11, Rab27A/B, Rab35), NSMase, ESCRTs, syndecan, syntenin, ATG12, tetraspanins	ASMase, flippase, flippase and scramblase (TMEM16F), ARF6	Annexin V, Caspase 3
Organelles	No	No	Yes
Content	Proteins, cholesterol, ceramide, noncoding RNA, mRNA, miRNA, and cytosol	Proteins, phosphatidylserine, cholesterol, mRNA, miRNA, and cytosol	Proteins, phosphatidylserine, DNA, rRNA, and cytosol
Marker proteins	CD 9, CD63 and CD61, tetraspanins, HSP70, HSP90, Alix, Rab5a/b	TyA and C1a, ARF6 and VCAMP3, β1 integrins, selectins, CD40, MMP, lineage markers, and ezrin	Calreticulin, TSP and C3b, and histones
Impact on the immune system	Immunostimulators	Immunosuppressors	Immunosuppressors

Exosomes packed with donor tumor cell-derived biologically-active oncogenic proteins and nucleic acids can affect the cellular functions of nearby and remote normal recipient cells (Whiteside 2016). Accumulating evidence reveals that tumor cell-derived exosomes have become a central candidate for promoting tumor cell proliferation, invasion, angiogenesis, distant metastasis and remodeling of the TME through transmitting onco-miRNAs (Rahbarghazi et al., 2019). These exosome-derived miRNAs can regulate intercellular signaling pathways (Tan et al., 2020); inducing functional alterations that impact on tumor progression and metastasis (Whiteside 2016). It has been demonstrated that tumor cell-released exosomal miRNAs such as miR-9, miR-23a, miR-92a, miR-103, miR-105, miR-126, miR-132, miR-135b, miR-210, miR-221 and cytokines (e.g., interleukins: IL-6 and IL-8, TNF- α , transforming growth factor β , FGF2, and VEGF) are proangiogenic factors to promote neovascularization and metastasis (Umezue et al., 2014; Zhou et al., 2014a,b,c; Martinelli 2017; Mentkowski et al., 2018; Ma et al., 2018; Fortunato et al., 2019; Wortzel et al., 2019). It has also been suggested that exosomal miRNAs play a key role in surpassing endothelial cells, cells in the stroma and blood-brain barrier, important in the development of brain metastases (Tominaga et al., 2015; Zhou et al., 2014a,b,c). Considering these findings, *exo*-miRNAs might play a key function during the transformation of normal cells into malignant cells.

In comparison to intercellular communication displayed by low molecular weight compounds (e.g., hormones), EVs transport and deliver their payload in a highly concentrated form, unaltered by diffusion or dilution (Wolf and Casadevall, 2014). Exchange of information can be done either by intake of EV by receptor-mediated internalization, endocytosis, docking, and fusion to target cells or interaction through cell surface receptor (van Niel et al., 2018; Mallocci et al., 2019). Since EVs ferry nucleic acids, they participate in post-transcriptional gene regulation in the target cell.

3. Small RNAs – Key regulators in inter-kingdom communication

In 2007, researchers discovered that EVs can transport messenger RNA (mRNA) and small non-coding RNA (sRNA), allowing cells to trade genetic message; thus, renewing the understanding of cell-cell interaction (Valadi et al., 2007; Simons and Rapasso, 2009; Vlassov et al., 2012; Otsuka et al., 2018). sRNAs (~22 nucleotides single-stranded RNA), including small interfering RNAs (siRNAs) and microRNAs (miRNAs), are crucial managers of several intracellular activities. The sRNA binds to its complementary mRNA, which results in post-transcriptional gene-silencing (Bartel, 2004). These sRNAs can influence several biological processes, including cell growth, differentiation, metabolism, and apoptosis (Bartel, 2004). Thus, sRNAs may act as key regulatory elements in EV-mediated (inter-kingdom) interactions (Record, 2013).

Two most critical parameters of bioavailability of sRNA are its stability and absorption. The vesicular envelope of EVs protect miRNAs from degradation by extracellular RNases enabling them to be more stable than free miRNAs and to be efficiently integrated by specific recipient cells (Kosaka et al., 2010a,b; Zhang et al., 2010; Koga et al., 2011; Keller et al., 2011; Buck et al., 2014; Zempleni et al., 2015; Vautrot et al 2019). This can be evident from the fact that processes such as ultrasonication, pasteurization and homogenization that disrupt the vesicular integrity of the exosomes, greatly reduces detectable levels of sRNA (Zempleni et al., 2017; Baier et al., 2014; Howard et al., 2015).

4. miRNA – A small RNA with diverse functions

miRNAs are endogenous, highly conserved, non-coding single stranded RNAs consisting of ~ 20–25 nucleotides that suppress

translation or stimulate target mRNA degradation (Bartel, 2004). miRNA was initially unearthed in *Caenorhabditis elegans* (*C. elegans*) by Ambros and coworkers who named it as lin-4 (Lee et al., 1993). In 2000, another miRNA (termed let-7) was detected in *C. elegans* (Reinhart et al., 2000). Subsequently, miRNAs have been isolated from almost all eukaryotes. Some miRNAs are highly conserved and highlight their evolutionarily functional relevance across species in post-transcriptional gene silencing. (Lagos-Quintana et al., 2001; Lau et al., 2001). Today, the expression of approximately 30–60% of protein-coding mRNAs is estimated to be controlled by miRNAs (Berezikov et al., 2005; Boffelli et al., 2003; Lewis et al., 2005; John et al., 2004).

4.1. miRNA biogenesis

The biogenesis of miRNAs is complex (Breving and Esquela-Kerscher, 2012). miRNAs are transcribed in the nucleus by RNA polymerase II (although Pol III transcription has also been observed) to generate a precursor, primary (pri)-miRNA, which are often 5' capped and 3' polyadenylated. A pri-miRNA transcript can encode multiple miRNA genes, and each gene is processed into a ~70-nucleotide pre-miRNA hairpin precursor by the RNase III enzyme Drosha and its cofactor, DiGeorge syndrome critical region 8 (DGCR8). An independent subclass of pre-miRNAs, termed “mirtrons,” do not rely on Drosha processing and are generated from mRNA transcripts as by-products of exon splicing and intron disbranching phenomena (Chan and Slack, 2007).

Pre-miRNAs are subsequently exported out of the nucleus by RAN-GTP and Exportin. Once in the cytoplasm, pre-miRNAs are processed further by the RNase III enzyme, Dicer, and its cofactor, TAR RNA-binding protein (TRBP) to generate a ~ 22-nucleotide double-stranded RNA duplex. One strand of the duplex attaches with a large multiprotein miRNA-associated RNA-induced silencing complex (miRISC). Argonaute (AGO) is the key catalytic component of this complex responsible for miRNA strand selection and mediating miRNA-based alterations of gene expression (Peng and Croce, 2016). The associated miRNA escorts the AGO/miRISC complex to the targeted site via miRNA binding to the complementary sequences within the mRNA transcript, and ultimately results in target mRNA degradation and/or blocked protein translation (Fig. 1).

Human miRNAs binds to the 3' untranslated regions (3' UTR) of the target mRNA with imperfect complementarity; although miRNA binding within the 5' UTR and coding regions of the mRNA target can also modulate gene expression. Perfect base pairing of a highly conserved “seed sequence” (nucleotides 2–8) in the miRNA is important for proper miRNA targeting (Doench and Sharp, 2004). A few notable exceptions exist where miRNAs activate target gene expression via epigenetic regulation of the enhancer regions in the nucleus or post-transcriptionally to induce protein translation (Valinezhad et al., 2014; Xiao et al., 2017). Evidence show that a single miRNA can recognize up to 100 well-defined targets (Brennecke et al 2005), and therefore can regulate multiple non-overlapping biological pathways simultaneously. From a therapeutic standpoint, single miRNA therapy could be a powerful tool to treat cancer in patients that harbor an accumulation of genetic alterations and can be effective without identifying the key mutations that lead to tumor formation.

MiRNA gene silencing machinery is highly conserved in most classes of eukaryotes; however substantial differences have been identified between the plant and animal counterparts at the level of the biogenesis, maturation, and mode of action (Achkar et al., 2016). In plants, a ribonuclease III enzyme, Dicer-like 1 (DCL1), plays major role in processing miRNA (Fukudome et al., 2017). This mechanism of miRNA processing can be understood by dividing this process in two distinct steps. In the first step, DCL1 along with

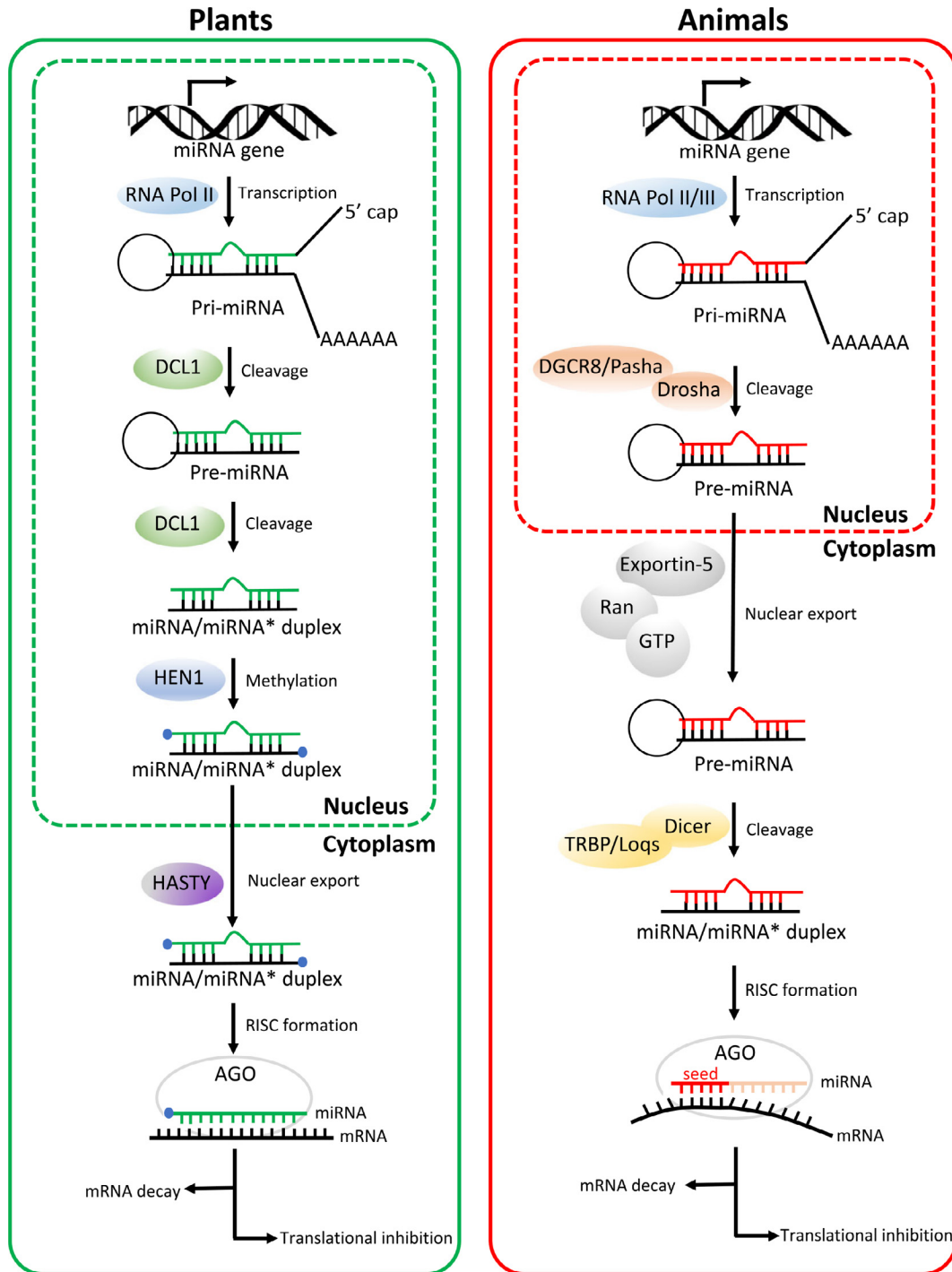


Fig. 1. Comparison of miRNA biogenesis and activity pathways in plants and animals. In both plants and animals, the biogenesis of miRNAs initiates within the nucleus. In plants, miRNA/miRNA* duplexes are cleaved from pri-miRNAs through the action of DCL1 endonuclease in two steps. DCL1 firstly cuts off the imperfectly folded ends of pri-miRNAs to generate pre-miRNAs with stem-loop hairpin secondary structures. The resulting pre-miRNAs are further excised by DCL1 to mature miRNA/miRNA* duplexes. Then the 3'-terminal of duplexes is methylated by HEN1. By contrast, in animals, pre-miRNAs are produced in the nucleus by the action of the Drosha enzyme, together with its DGCR8 protein (in mammals) or Pasha protein (in flies). Duplexes of miRNA/miRNA* are further processed after being exported from the nucleus to the cytoplasm, where pre-miRNAs are cleaved by Dicer and TRBP (in mammals) or Loqs (in flies). In plants, HASTY is responsible for the transport of miRNA/miRNA* duplexes from the nucleus to the cytoplasm, whereas in animals' pre-miRNAs are recognized and then exported by Exportin-5 in a Ran-GTP-dependent manner. During RISC loading, one strand of the small RNA duplexes is selected as the guide strand (green in plants or red in animals) and incorporated into AGO to form a functional RISC, whereas the other strand is removed and degraded. In plants, miRNAs have near-perfect complementarity to their target mRNAs. By contrast, animal miRNAs often have targets with imperfect complementarity and the major determinant for the binding of animal miRNAs to their target mRNAs is a 6–8 nucleotide domain at the 5' extremity or seed sequence. The arrows indicate the direction of the subsequent activity pathways. Both plant and animal miRNAs can regulate gene expression via mRNA decay and translational inhibition (Adapted from Li et al., 2018, an Open access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

its accessory proteins, SERRATE (SE) and HYPONASTIC LEAVES1 (HYL1), forms a 'dicing complex' inside the nucleus (Fang and Spector 2007), which catalyzes the conversion of pri-miRNA to

pre-miRNA by cleaving the imperfectly-folded end of the pri-miRNA to create a stem-loop hairpin secondary structure of pre-miRNA (Dong et al 2008). In the second step, pre-miRNA is

expurgated by DCL1 generating a nascent miRNA/miRNA* duplex (Wang et al., 2019). This second step also takes place in the nucleus at the nuclear processing centre termed as dicing bodies (D-bodies) (Fang and Spector 2007; Song et al., 2007). In the animal counterparts, maturation of pri-miRNA into a mature miRNA involves two different proteins namely 'Drosha' and 'Dicer' which sequentially carry out the process in two different compartments of the cell i.e. nucleus and cytoplasm (O'Brien et al., 2018).

Unlike animals, nascent miRNA/miRNA* duplex generated in the nucleus of plant cells are universally methylated at their 3' ends before being exported to the cytoplasm. Both the strands of nascent miRNA/miRNA* have 3' nucleotide overhangs, phosphate group at 5' ends, and two hydroxyl groups at the 3' ends (2'-OH and 3'-OH). The 2'-hydroxyl group at 3' end of nascent miRNA/miRNA* is methylated by HUA Enhancer (HEN1) which has been identified as a small RNA methyltransferase (Singh et al., 2018). This step prevents uridylation and degradation of the methylated miRNA/miRNA* (Li et al., 2005). Export of methylated miRNA/miRNA* duplex from nucleus to cytoplasm is performed by 'Hasty' (HST) protein which is a homologue of animal Exportin 5 (EXPO 5) (Wahid et al., 2010). Inside cytoplasm, guide strand of matured duplex is loaded onto Argonaute (AGO) protein and the complex so formed targets specific genes through base-pairing, in-turn mediating gene-silencing (Wang and Xu, 2015).

4.2. miRNA and cancer

The association between miRNA and cancer was demonstrated by Dr. Croce and colleagues while trying to recognize the tumor suppressor genes in the chromosomal region 13q14 in B-cell chronic lymphocytic leukemia (BLL) cells (Calin et al., 2002). They found that this region, often deleted or downregulated in BLL, codes for two miRNAs, namely miR-15a and miR-16-1. Further studies revealed the role of miR-15 and miR-16-1 as tumor-suppressor genes that repress Bcl-2, an anti-apoptotic protein known to overexpress in malignant non-dividing B cells, and several solid tumors (Cimmino et al., 2005; Calin et al., 2008). In later years, miRNA profiling and deep sequencing shed some direct evidence on relationship between miRNA expression and cancer dysregulation, providing more focus on its usage in tumor classification, diagnosis, and prognosis.

miRNAs extensively impact the progression of many human cancers (Calin and Croce, 2006a, 2006b; Berindan-Neagoe et al., 2014) acting both as tumor suppressors (Fang et al., 2012; Hatziaepostolou et al., 2011) and promoters (Mi et al., 2017; Zhu et al., 2018). Abnormal upregulation or downregulation of miRNAs has been shown to play a major role in tumor proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, and metastasis.

5. Cross-species sRNA transfer

Experimentations have unveiled that sRNAs are not confined to the cells, which produce them but exhibit inter-species/inter-kingdom communication within an organism or between hosts and pathogens, such as parasites (Choy et al., 2008; Sarkies and Miska, 2014; Colombo et al., 2014; Islam et al., 2017; Knip et al., 2014). Several studies have demonstrated inter-species translocation of sRNA molecules (Xia et al., 2008; Hansen et al., 2010; Knip et al., 2014; Philip et al., 2015). This makes the cross-species transfer of sRNAs a novel form of intra-species and inter-species cross-talk (Knip et al., 2014).

miRNAs have often been reported to be transferred across species, regulating key processes (Table 2). Several reports identify the unique phenomenon of transfer of miRNA from parasites into their vector and host species. Garcia-Silva et al., (2014) demonstrated the channelization of tRNA derived small RNAs (tsRNAs) along with distinctive proteins, to their mammalian host cells packaged as vesicular structures resembling Golgi complex in parasitic protozoan *Trypanosoma cruzi*. Another study shows miRNAs produced in *Schistosoma mansoni*, a blood fluke, to exist very stably in the tissues and body fluids of their host like mice and humans and being used as a bio-marker for the disease (Hoy et al., 2014). According to Buck et al., (2014), *Heligmosomoides polygyrus*, a gastrointestinal parasitic nematode, can manipulate the innate immunity of their hosts by secretion of exosomal vesicles containing miRNAs and parasite derived AGO proteins, thereby elucidating a novel mechanism of RNA transfer between species.

Alternatively, it has been seen that miRNAs may be transferred from host cells into the parasites as a means of building resistance against the pathogen. The anomalous production of two miRNAs (miR-451 and let7i) in erythrocytes having alleles for sickle-cell

Table 2
Exogenous miRNA studies of cross-kingdom regulation (Adapted with permission from Zhang et al 2019).

miRNA	Source	Target cell or animal	Target gene	Function	N/P	Reference
miR-451	Human	Plasmodium falciparum	PKA-R	Inhibits parasite growth	P	Choy et al., 2008
miR-BART5	Epstein-Barr virus	Human NPC cells	PUMA	Facilitates the establishment of latent infection by promoting host cell survival	P	Xia et al., 2008
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	Hansen et al., 2010
miR-BHRF 1–3	Epstein-Barr virus	Two BL early-passage cell lines (BL-5 and BL-8)	CXCL-11	Cancer immunosurveillance	P	Snow et al., 2013
miR-172	Brassica oleracea	Mice	—	Absorbed	P	Liang et al 2014, Hou et al 2018)
mol-miR168a	Moringa oleifera	Human hepatoma cell line G2 (HEPG2) cells	SIRT1	Absorbed	P	(Pirò et al., 2016, Lamonte et al., 2012
miR166a	Arabidopsis thaliana	Human breast milk exosomes	—	Absorbed	P	Lukasik & Zielenkiewicz 2014
miR159	Arabidopsis thaliana	Mice	TCF7	Suppresses the growth of xenograft breast tumors	P	Chin et al., 2016, Luo et al., 2017
miR162a	Bee pollen	Drosophila	dmTOR	Delays the development and decreases the body and ovary size in bees	P	Zhu et al., 2017
zma-miR164a-5p	Maize	Pigs	OTX1, PLAGL2, CSPG4	Absorbed		Luo et al., 2017, Philip et al., 2015

disease, led to impairment of translational machinery inside *Plasmodium falciparum*, thereby disturbing the intra-erythrocytic life-cycle of the parasite and providing resistance against the disease (LaMonte et al., 2012). This non-canonical RNAi mechanism act most likely by forming chimeric fusions miRNA transfer from host to its parasite and may act as a mechanism of selection and regulation of parasitic population. The gut-microbiota consisting of various species of bacteria has also been shown to be controlled by the host. This is done through host miRNA mediated regulation of bacterial genes resulting in their growth perturbations (Liu et al., 2016). Altogether, these reports suggest that cross-species/cross-kingdom transfer of miRNA is not uncommon.

6. Plants and bidirectional cross-kingdom sRNA transport

Plants also exhibit cross-kingdom transfer of double-stranded siRNAs (Pirò et al., 2016; Hou et al., 2018). This strategy known as plant-mediated RNA interference (PM-RNAi), is used by plants to suppress the growth of harmful insects. A study by Baum et al. (2005) found out that the ingestion of plant dsRNAs, through feeding or topical application, can lead to larval stunting and morbidity in several coleopteran insects. They suggested that the RNAi pathway can be exploited to control insect pests via *in-planta* expression of a particular dsRNA. Similarly, plants like cotton have been shown to export plant-specific miRNAs into their fungal pathogens to induce cross-kingdom gene silencing and confer disease resistance. This study conducted by Zhang et al. (2016), showed that two miRNAs (miR166 and miR159) produced in cotton plants are exported to the hyphae of *Verticillium dahliae* (pathogenic fungus causing wilt disease in crops). These miRNAs specifically target two *V. dahliae* genes viz. a Ca²⁺-dependent cysteine protease (Clp-1) and an isotrichodermin C-15 hydroxylase (HiC-15) that are essential for fungal virulence.

A revealing study by Weiberg et al (2013) exemplifies cross-kingdom RNAi as a virulence mechanism of *Botrytis cinerea*, a fungal pathogen responsible for gray mould disease in many plant species. sRNAs of this pathogen (Bc-sRNAs) vandalize host's immune response by selectively paralysing the host RNAi machinery and building infection. Compared to the sRNA transfer from pathogen to host plants, the functional movement of sRNAs from parasitic plants to host plants has rarely been reported. However, recent cases of miRNA movements, where double-stranded small RNA molecules (~22 nucleotide long) move into host plants from their parasitic-plant counterparts like *Cuscuta campestris*, have revealed robust roles of miRNA species in host-parasite interaction and regulation through siRNA mediated target gene silencing (Shahid et al., 2018).

A highly debatable and unresolved issue is whether a cross-kingdom transmission of miRNA from plants to humans can occur. Growing experimental evidence has suggested that the regular usage of plant-based food substances is beneficial against metabolic disease and cancer (Willett, 2002; Liu, 2004). Since daily food intake of humans is considerable, it is intriguing to speculate that exogenous plant-based miRNA (termed xenomiRs) can affect human health and disease pathology (Motti and Meccariello, 2018). Foods, including vegetables, fruits and whole grains, have been shown to contain miRNAs that can survive digestion, translocate into the human blood, and exhibit vital role in regulating gene expression in a cross-kingdom fashion (Witwer 2012; Chin et al., 2016; Zhang et al., 2012a; Zhou et al., 2015) (Fig. 2). This natural uptake of diet-derived xenomiRs is reported to be biologically active in vast variety of lower eukaryotes or invertebrates (Li et al., 2018). Recent studies has proven that xenomiRs can be effectively packaged into exosomal-like nanoparticles that provides resistance against harsh conditions (RNase and extreme pH) and

can be relocated into adjacent/distant cells where they regulate gene function (Luo et al., 2017). However, the actual underlying mechanism still needs to be elucidated.

Several reports have suggested the prospect of cross-kingdom gene regulation by plant-derived miRNAs (Table 3). A breakthrough (yet controversial) study by Zhang et al. (2012a) presented high levels of plant-based miRNAs in human sera. They demonstrated that the miR-168a enriched in rice (*Oryza sativa*), may be absorbed through intestines, where it inhibits the expression of its target low-density lipoprotein receptor 1 (LDLRAP1), in liver (Zhang et al., 2012a). Researchers proposed that ingested plant-based miRNAs can be selectively loaded into microvesicles (exosomes), subsequently being absorbed by the gastrointestinal epithelial cells, which repackage them into EVs and unfetter them in blood. The study was challenged by Dickinson et al (2013) that showed no evidence of intake of ingested plant miRNAs by mice nor any reduction in LDLRAP1 levels in liver. To address this, Chen et al., (2013) proposed that the alteration in results could be due to the nutritional disparities in the diet constituents. They also argued that the sequencing strategy employed by Dickinson et al (2013) may not be able to accurately measure the plant miRNAs.

In another study, Liang et al. (2014) administered cruciferous miRNAs extracted from *Brassica oleracea* (a member of the cabbage family) in food or oral gavage to mice (Liang et al., 2014). *B. oleracea* miR-172 was found to be resistant to the gastrointestinal tract degradation, and this miRNA was recovered in the blood, stomach, intestines, spleen, and feces of treated animals up to 72 h after feeding. This study is particularly intriguing from the perspective of nutritional therapeutics because epidemiological data support the cancer-protective role of cruciferous vegetables such as broccoli (Higdon et al., 2007). Additional studies indicate that miRNAs from *Zea mays* (corn), *Solanum lycopersicum* (tomato), *Vitis vinifera* (grapes), and *Glycine max* (soybean) were found to be present in the blood of animals when they were fed on diets containing these plants (Wang et al., 2012; Yang et al., 2015a, 2015b).

Similarly, Zhou et al., (2015) showed that a plant-based miRNA from honeysuckle inhibits the replication of influenza A. In a similar study, significantly higher levels of a plant specific miRNA were found to be abundantly present in the serum of mice consuming diets rich in vegetables (Yang et al., 2016). They further studied the abnormal abundance of miR2911 and its distinctive origin. The sRNA was found to be closely associated with the mammalian miRISC (Yang et al., 2017). In a recent study, Luo et al., (2017) demonstrated that the dietary maize miRNAs were able to cross the pig gastrointestinal tract and can be detected in porcine tissues and serum. Therefore, dietary intake of exogenous plant miRNAs may be more widespread than initially appreciated and can contribute to recipient gene expression and human health.

On the other hand, there have been many contrasting views regarding presence of foreign miRNAs in mammalian specimens that were absorbed through dietary sources (Table 4), especially plants (Tosar et al., 2014; Snow et al., 2013; Witwer et al., 2013; Dickinson et al., 2013; Chen et al., 2013). Although several previous studies have reported the detection of miRNAs from exogenous organisms in human serum (Wang et al., 2012) and breast milk (Lukasik and Zielenkiewicz, 2014); however, other set of reports have not been able to identify foreign miRNAs after exposure to various food types (Snow et al., 2013; Witwer et al., 2013). There are arguments regarding this contrasting set of opinions, largely based of methodological aspects of the later studies, where a selective set of miRNAs were focused for the study rather than sequencing and screening a complete dataset of all known plant miRNAs. Another group of studies done by a relatively smaller number of researchers reveal another aspect of plant miRNAs in mammals, where miRNAs have been shown to have potential functions in

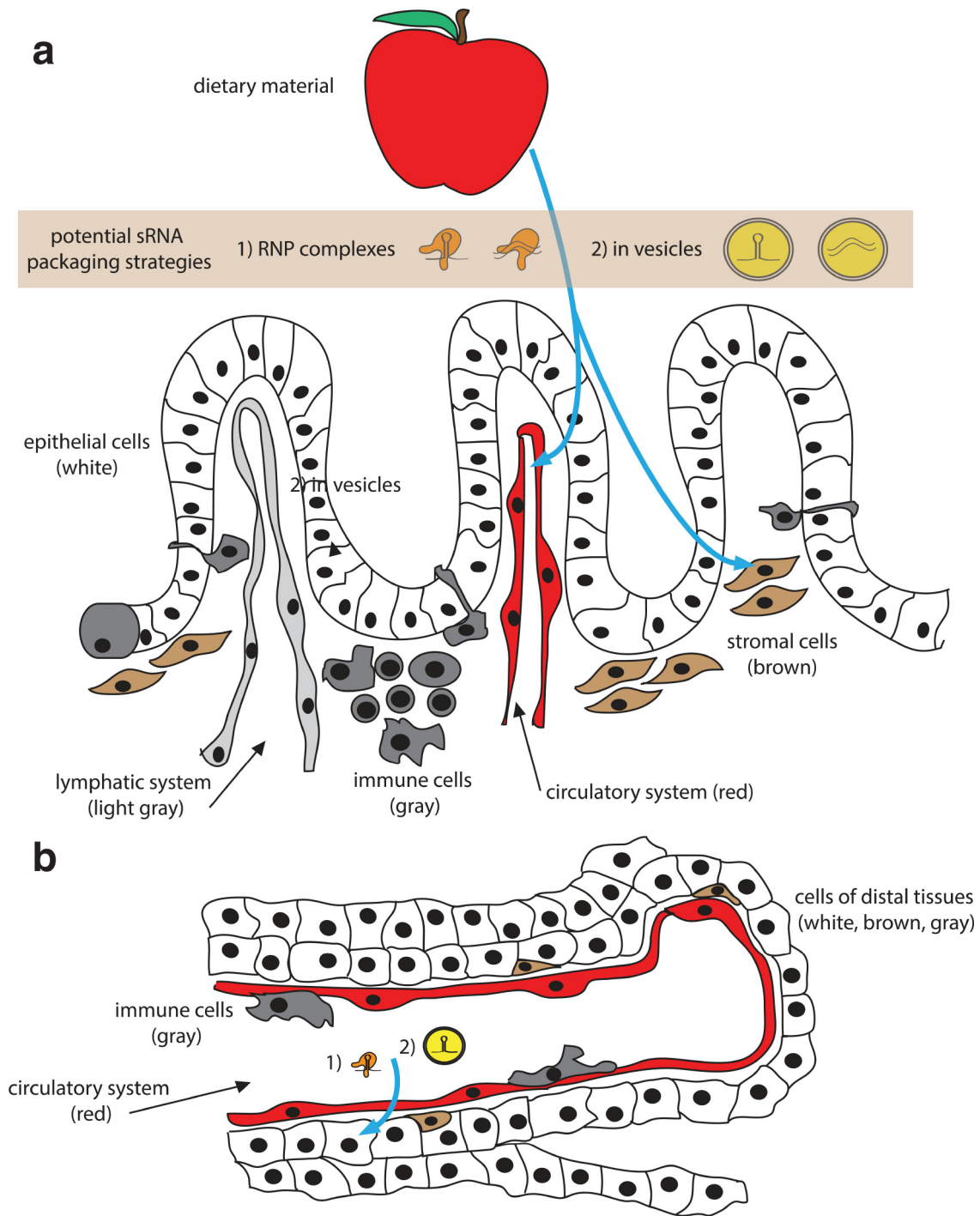


Fig. 2. Model for the uptake of dietary sRNA from the digestive tract. To carry RNAi regulatory activity on gene expression in an ingesting organism, (a) sRNAs from the diet (potentially packaged in (1) ribonucleoprotein (RNP) complexes or (2) in vesicles) should cross the epithelial cell (white) barrier via transcellular or paracellular mechanisms or conveyance by immune cells (gray). They should then be absorbed by proximal cells, such as stromal cells (brown) or must gain access to the circulatory (red) or lymphatic system (light gray) for systemic dissemination. (b) Subsequently, after exiting from the circulatory system (red), uptake of sRNAs is ensued by the cells of various tissues and organs (gray, brown, and yellow). None of these putative steps is understood at the level of the molecular mechanism (Adapted and modified from Chan and Snow, 2017, Open Access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

regulating mammalian cells, however several aspects in this area need further exploration (Zhou et al., 2015; Liang et al., 2014; 2015; Yang et al., 2015b).

The debate has, since then, continued in the form of follow-up studies that have provided evidences either for or against the notion concerning stability and biological function of plant miRNAs consumed by mammals through various dietary sources. A study by Dickinson et al (2013) forms a major cornerstone of this

extended discussion, where miR168a derived from rice, exhibited no cross-kingdom modulation in the levels of a LDLRAP1 found in mouse liver, as discussed earlier. Another study on mice showed negligible evidences for the presence of miRNAs in the blood plasma after ingestion of plant-based diet (Snow et al., 2013). Further, some reports on macaque species have concluded that there has been no detection of dietary miRNAs in blood samples when investigated using droplet-digital PCR (Witwer et al., 2013).

Table 3

Supporting evidence of cross-kingdom communication by diet/plant-derived miRNAs (Adapted with permission from Li et al., 2018, an Open access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

Year	miRNAs involved	Contents	Source origin	miRNA levels	Potential mechanism	Function	Detection methods	Conclusion	Reference
2012	miR168a	Plant miRNAs were present in human and animal sera and organs. miR168a regulated mouse	Rice	fM Level	Associated with AGO2 complex and packaged in MVs	LDLRAP1 expression and consequently decreased LDL removal from mouse plasma.	HTS, RT-qPCR, Bioinformatics, NB, WB, AGO2 immunoprecipitation	Exogenous plant miRNAs in food could regulate the expression of target genes in mammals.	Zhang et al., 2012a
2014	miR172	miR172 from cabbage (<i>Brassica oleracea</i>) was detected in blood, spleen, liver and kidney of mice after feeding with plant extract.	Cabbage	Stomach contained about 4.5–0.4% (2–24 h after feeding), intestines 2.4–0.2% (2–36 h), blood 1.3–0.2% (2–72 h) and spleen 0.38–0.04% (2–72 h) of the miR172 orally administered.	sRNA could survive for >36 h in blood and fecal samples	No phenotypic changes were found in all the mice fed with the foreign RNA.	RT-qPCR, Electrophoresis	Exogenous plant miRNAs could survive in the murine GI tract, enter peripheral blood and continue to access other organs.	Liang et al., 2014
2015	18 plant miRNAs (miR156a, miR157a, miR158a, etc.)	Plant miRNAs were detectable in human plasma of volunteers after drinking juice.	Watermelon juice and mixed fruits	fM Level	Largely encapsulated in MVs	Not mentioned	RT-qPCR, NB	Plant miRNAs in human plasma could be efficiently detected and reliably compared by RT-qPCR. Provided a SOP for measuring plant miRNAs in human and animal plasma.	Liang et al.; 2015
2015	miR166, miR167, miR168	Even after an extensive pretreatment, plant-derived miRNA delivered by typical dietary ingestion remained bioavailable for uptake during early digestion.	Soybean and rice	In vitro methods	Not mentioned	Not mentioned	RT-qPCR	Storage, processing and cooking did not abolish plant miRNAs in food.	Philip et al., 2015
2015	miR2911	miR2911 was highly stable in honeysuckle decoction, and continuous drinking or gavage feeding of honeysuckle decoction significantly elevated miR2911 levels in mouse blood and lung.	Honeysuckle	fM Level	A unique sequence and high GC content, MVs mediated pathway	miR2911 could directly target multiple viral genes and suppress viral infections.	HTS, RT-qPCR, NB, Fluorescent labeled tracing assay, Luciferase reporter assay, Ago2 Immunoprecipitation	Provided evidence of physiological function of exogenous plant miRNAs in human and animals.	Zhou et al., 2015
2015	miR2911, miR168a	Using a chow diet containing	Honeysuckle	fM Level	Consumers of particular diets and/or with increased	Altered or damaged guts lining could	RT-qPCR, droplet digital PCR	Dietary sRNAs could survive circulation and	Yang et al., 2015a

(continued on next page)

Table 3 (continued)

Year	miRNAs involved	Contents	Source origin	miRNA levels	Potential mechanism	Function	Detection methods	Conclusion	Reference
2015	miR2911	honeysuckle plant-based sRNAs could be detected in sera and urine of mice miR2911 was detectable in sera and urine of the honeysuckle decoction consuming mice.	Dried herbs or flowers	fM Level	Intestinal permeability Circulating miR2911 was not bound by AGO2, but due to high GC content.	enhance dietary miRNA uptake Not mentioned	RT-qPCR, AGO2 immunoprecipitation	are excreted in urine The uptake of miR2911 might be a more commonplace phenomenon that could occur when eating a variety of plant-based foods.	Yang et al., 2015b
2016	miR2911	Plant-based miR2911 was measured 7 days after feeding in animals	Plants	fM Level	Circulating miR2911 was not associated with exosomes, but possibly with a protein	Not mentioned	RT-qPCR	Mice consuming diets rich in vegetables displayed enhanced serum levels of plant specific miR2911.	Yang et al., 2016
2017	miR2911	Plant-derived miR2911 was detectable in sera of mice fed with various vegetables.	Cabbage	Arabidopsis	miR2911 was detectable while other plant-based miRNAs failed to detect. Increased levels of miR2911 correlated with the degradation of plant foods and rRNAs.	Not mentioned	RT-qPCR, Dual-luciferase reporter assay, Bioinformatics	Provided insights into the atypical bioavailability of miR2911 and offered engineering strategies for plant-based sRNA therapeutics.	Yang et al., 2017
2015	miR34a, miR143, miR145	Orally administered tumor suppressor miRNAs reduced tumor burden in ApcMin/+ mice and were detectable in intestinal tissue	Synthesized methylated miRNAs	Intestinal miR34a was at a detectable level; detection of miR143 and miR145 in mouse intestines were failed	Not mentioned	Reduced tumor burden in the well-established ApcMin/+ mouse model of colon cancer.	RT-qPCR, Dissecting microscope	Tumor suppressor miRNAs designed to mimic sRNAs produced in plants were taken up by the digestive tract of ApcMin/+ mice upon ingestion.	Mlotshwa et al., 2015
2016	miR159	Plant miR159 could be detected in human sera and tumor tissues and was associated with breast cancer progression.	Synthesized methylated miRNAs	fM Level	Predominantly present in MVs	The miR159 in human serum was capable of inhibiting cell proliferation.	RT-qPCR, HTS, Dual-luciferase reporter assay, WB, In situ hybridization, Immunohistochemistry,	The feasibility of using synthetic forms of plant miRNAs as dietary supplements in the treatment of human cancers, including those outside of the GI track.	Chin et al., 2016
2016	FvmiR168	Strawberry fruit FvmiR168 affected properties of dendritic cells and their ability to respond to inflammatory stimuli	Strawberry fruit	biologically relevant amount	The immunomodulatory effect of plant miRNA was not sequence or plant specific	Plant-based miRNAs modified dendritic cells ability to respond to inflammatory agents by limiting T cell proliferation	RT-qPCR, Flow cytometry, Fluorescence microscopy	A potential for therapeutic use of plant miRNAs in the prevention of chronic inflammation related diseases	Cavalieri et al., 2016

Table 3 (continued)

Year	miRNAs involved	Contents	Source origin	miRNA levels	Potential mechanism	Function	Detection methods	Conclusion	Reference
2017	miR451 miR144	Ingestion of wild type blood increased the levels of miR451 and miR144 in peripheral blood of miR144/451-null mice	Wild type mice blood	At very low level but biologically relevant amount	Exosomes	Exogenous miR451 existing in miR144/451 knockout mice enhanced antioxidant activity in vivo via increasing the activity of Foxo3 pathway	Two different RT-qPCR, Dual-luciferase reporter assay, WB, FACS	miRNAs in foods or dietary supplements could affect the functions of the consumer	Wang et al., 2017b

MVs microvesicles, HTS high-throughput sequencing; RT-qPCR quantitative real time polymerase chain reaction, NB Northern blot, WB Western blot, ELISA enzyme-linked immunosorbent assay, FACS Fluorescent

Huang et al., (2018) recovered less than 1% of corn miRNAs in the gastrointestinal tract of animals fed with plant-based diet.

Arguments putting up questions against such studies generally root from the notion concerning construction and sequencing of plant sRNA libraries. It has been identified that as compared to animal miRNA libraries, plant miRNA libraries are not constructed with the same precision due to presence of 2'-O-methylated 3' end modification (Yu et al., 2005); which has a negative influence on the efficiency of adapter ligation, thereby underestimating the quantification of plant-based miRNAs (Munafa & Robb, 2010). Studies showing contrasting results due to methodological dissimilarities have strengthened the ground for such debates. For example, Zhang et al (2012a) utilized oxidized deep sequencing to retrieve miRNAs of plant origin in human and mice blood samples, whereas Dickinson et al., (2013) could not detect any such small RNAs in mice samples, demonstrating a bias for sequencing. An additional dispute involved in the debate for existence of cross-kingdom miRNAs in human fluids is that nucleic acids are common contaminants in experiments involving genomic library preparations and sequencing, thereby showing false positive results (Tosar et al., 2014). Hence, when extremely low quantities of miRNAs are detected, the first assumption is whether any contamination or background noise exists in the apparatus. Although, these experimental issues can be resolved through cross-validation of results using multiple assay platforms including Chip-seq, RT-PCR and deep sequencing techniques.

7. Plant-derived xenomiRs affect cancer progression

Exosomal miRNAs delivered through the diet may mediate cellular effects in the human body (Fig. 2), including development of cancer. Wolf et al., (2015) showed that bovine exosomes are transported into human colon carcinoma Caco-2 cells in culture, resulting in elevated miR-29b and miR-200c levels. Interestingly, both miRNAs are shown to be incriminated in human cancers (Kwon et al., 2018; Kumar et al., 2015). These studies may open a new arena of treatment of human diseases (including cancer) exploiting plant-based miRNAs.

Although the source and scope of plant miRNAs detected in mammalian specimens remain debatable, Mlotshwa et al., (2015) described that mammalian miRNAs that were 2'-O-methylated like plant miRNAs (Yu et al 2005), could reduce mouse intestinal tumor burden when administered orally for longer durations. They also unraveled that further reduction in the tumor load is possible if

tumor suppressor miRNAs are administered together with the plant miRNAs in the similar subjects (Mlotshwa et al., 2015). This study, however, concluded that a larger sample size may be required to confirm the therapeutic role of plant-based xenomiRs ingested by mammals. Moreover, it is still a subject of investigation, whether these cross-kingdom miRNAs have any beneficiary therapeutic role in treating cancers outside of the GI tract.

Recently, an inverse association between the abundance of plant miRNA and breast cancer incidence and progression was reported (Chin et al., 2016). The authors detected the presence of a plant originating miRNA, miR159 in the sera of human EVs that exhibited resistance to sodium periodate oxidation, which may be due to the 2'-O-methylation. They also found that the concentration of orally ingested miRNA was inversely related to the chances and progression of the cancer. Through administration of synthetic mimic of miR159 in animal model, a transcription factor encoded by TCF7 gene was identified as the target gene for RNAi mediated silencing. This study concluded that miR159 can significantly reduce the growth of breast tumors in mammals, becoming the first report showing the role of a plant-based miRNA in mammalian cancer. More recently, a plant-based mir167e-5p was found to be inhibiting the proliferation of Caco-2 cancer cell lines dose- and time-dependently in an epigenetic manner by targeting β -catenin pathway (Li et al., 2019). However, another study showed no such effect of plant derived miRNAs on mammalian cancers (Xiao et al., 2020). Using deep sequencing analysis, the researchers identified ~750 miRNA from sprouts of *B. rapa sylvestris* (commonly called broccoletti) and predicted their human targets using bioinformatics techniques. Two new miRNAs were identified from this database. It was observed that the overexpression of selected miRNAs and their administration, did not affect any major metabolic activity of pancreatic cancer cells. This study creates scientific evidence against the perception of regulation of human genes from plant-derived miRNAs present in food (Xiao et al., 2020).

8. Conclusion

The role of mobile miRNAs in cross-kingdom RNAi is intriguing; yet, it remains skeptical how these sRNAs 'traverse' long distances surviving diverse cellular boundaries between plants and their target entities. Notably, this cross-kingdom miRNA transfer may not be due to chance, rather it may occur through a selective transport of functional miRNAs. miRNAs present in diets are now being detected in the sera of the consumer where they function to

Table 4

Contradicting evidence of cross-kingdom communication by diet/plant-derived miRNAs (Adapted with permission from Li et al., 2018, an Open access article distributed under the terms of the Creative Commons Attribution 4.0 International License).

Year	Contents	miRNAs involved	Source origin	miRNA levels	Refuting points	Detection methods	Conclusion	Reference
2013	Little or no plant miRNAs or miR168a were detected in blood or liver of mice fed with rice-containing diets	miR168a	Rice	Unmeasurable	The observed changes in LDL levels might be due to the release of endogenous cholesterol stores in response to negligible dietary cholesterol intake in mice fed with only rice	HTS, RT-qPCR, ELISA	Dietary exposure to miR168a did not affect plasma LDL levels. Plasma LDL changes reported by Zhang resulted from nutritional imbalances between test and control groups rather than an RNAi mediated effect of consuming miR168a in rice	Dickinson et al., 2013
2013	Plant miRNAs were not detectable in the plasma from healthy human subjects after intake of a western diet containing fruits. Negligible expression of miR21 in plasma or organ tissue in miR21 knockout mice after oral diets replete with endogenous miR21. Negligible expression of miR156a, miR159a and miR169a in plasma or organs in mice after diets replete with these miRNAs. Negligible expression of plant-derived miRNAs in recipient honeybee tissues.	miR156a miR159a miR169a miR21 miR156a miR159a miR169a miR156a miR159a miR169a	Plant material Animal lard diet replete With miR21 Vegetariandiets replete with these miRNAs Plant derived miRNA	Undetectable Undetectable in plasma; less than one copy per cell in the liver, lungs, kidneys and stomach miR156a: far less than one copy of miRNA per cell in liver, lungs, kidneys and stomach; miR159a and miR169a: undetectable in either plasma and/or organs. Only miR156a but not miR159a or miR169a, was detected in abdominal tissue derived from nurses and foragers, but again at exceptionally low levels.	Low measurable uptake	RT-qPCR	Horizontal delivery of miRNAs via oral ingestion of a typical diet was neither a frequent nor a prevalent event across multiple recipient animal organisms	Snow et al., 2013
2012	Predominant monocot miR168 sequence was present as a result of contamination from a non-plant source	miR168a	Plant	Not available	Contamination	HTS, NB	The observed plant miRNAs in animal sRNA datasets could originate in the process of sequencing, and accumulation of plant miRNAs via dietary exposure was not universal in animals.	Zhang et al., 2012b
2014	Cross-contamination during library preparation was a source of exogenous RNAs	miR168a miR156a miR167a	Plant	Not available	Contamination	HTS	Variable amounts of plant miRNAs were found in publicly available sRNA-seq data sets of human tissues.	Tosar et al., 2014
2014	Failed to observe a postprandial increase in the brassica-specific miR824 or miR167a in broccoli sprouts feeding study	miR167a miR824	Broccoli sprouts	Below detection limit	Low measurable uptake	RT-qPCR	Skeptical of the bioavailability and biologic activity of plant-borne miRNAs	Baier et al., 2014

Table 4 (continued)

Year	Contents	miRNAs involved	Source origin	miRNA levels	Refuting points	Detection methods	Conclusion	Reference
2013	Nonhuman primates failed to uptake dietary plant miRNAs	miR156 miR160 miR166 miR167 miR168 miR172	Fruit	Not available	The concentrations were too low to be specific and reliable.	RT-qPCR, Droplet digital PCR	The level of miRNAs was too low to be true and/or amplification was non-specific	Witwer et al., 2013
2018	Corn miRNA was extensively degraded in the GI tract and that the uptake into circulation and tissues was minimal	miR156a miR164a miR167a	Corn	No corn miRNAs could be detected in whole blood, fecal or liver of animals	Significant degradation of corn miRNAs occurred during digestion	—	No evidence of increased levels of corn miRNAs in whole blood or tissues after supplementation of corn miRNAs in the diet was observed in a mouse model.	Huang et al., 2018

MVs microvesicles, HTS high-throughput sequencing, NB Northern blot, WB Western blot, ELISA enzyme-linked immunosorbent assay, LDL low-density lipoprotein, sRNA small RNA

regulate various biological processes including cancer. Recent evidence suggests that plant xenomiRs may hinder the expression of human disease-related genes (Zhou et al., 2015; Chin et al., 2016; Sharma et al., 2016; Tian et al., 2016; Choi et al., 2017). However, our understanding of the cross-kingdom gene regulation remains inconclusive (Mar-Aguilar et al., 2020), and it is too early to make a final stand in favor or against of the notion that the plant-based xenomiRs regulate gene expression in humans.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further Reading

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