Gene regulation by dietary microRNAs[1]

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Abstract

MicroRNAs (miRNAs) silence genes through destabilizing mRNA or preventing translation of mRNA, thereby playing an essential role in gene silencing. Traditionally, miRNAs have been considered endogenous regulators of genes, i.e., miRNAs synthesized by an organism regulate the genes in that organism. Recently, that dogma has been challenged in studies suggesting that food-borne miRNAs are bioavailable and affect gene expression in mice and humans. While the evidence in support of this theory may be considered weak for miRNAs that originate in plants, there is compelling evidence to suggest that humans use bovine miRNAs in cow's milk and avian miRNAs in chicken eggs for gene regulation. Importantly, evidence also suggests that mice fed a miRNA-depleted diet cannot compensate for dietary depletion by increased endogenous synthesis. Bioinformatics predictions implicate bovine miRNAs in the regulation of genes that play roles in human health and development. Current challenges in this area of research include that some miRNAs are unable to establish a cause-and-effect between miRNA depletion and disease in miRNA knockout mice, and sequence similarities and identities for bovine and human miRNAs render it difficult to distinguish between exogenous and endogenous miRNAs. Based on what is currently known about dietary miRNAs, the body of evidence appears to be sufficient to consider milk miRNA bioactive compounds in foods, and to increase research activities in this field.

Keywords

diet; gene regulation; eggs; miRNA; milk

Plain language summary

MicroRNAs are short stretches of RNA that play essential roles in gene regulation. Traditionally, it was believed that microRNAs synthesized by a certain species regulate genes in that species. This review discusses evidence that microRNAs in food from animal species affect the expression of human genes and the implications of this observation for human nutrition and health.

Gene regulation by endogenous miRNAs

MicroRNAs (miRNAs, miRs) are small noncoding RNAs encoded by their own genes, introns, or exons of long nonprotein-coding transcripts (Rodriguez et al. 2004). miRNAs are approximately 22 nucleotides long (Chen and Rajewsky 2007), hybridize with complementary sequences in the 3′-untranslated regions (UTRs) in mRNA (Chen and Rajewsky 2007), and silence genes through destabilizing mRNA or preventing translation of mRNA (Fig. 1) (Jing et al. 2005; Djuranovic et al. 2012). In contrast, miRNA-binding sites in open reading frames and 5′-UTRs contribute only marginally to gene silencing (Grimson et al. 2007; Baek et al. 2008). The sequence complementarity in the "seed region" (nucleotides 2–8) in miRNAs is of particular importance for binding to target transcripts. The miRNA-dependent degradation of mRNA takes place in the RNA-induced silencing complex (RISC) through miRNA binding to Argonaute proteins; in the process of mRNA degradation, miRNAs are also degraded (Rana 2007). Gene regulation by miRNAs is a complex network of events. miRNAs can bind to multiple and even thousands of gene targets, and genes can be regulated by multiple miRNAs (Bartel 2004; Grun et al. 2005; Cannell et al. 2008; Seitz 2009). It has been estimated that more than 30% of genes are regulated by miRNAs in humans (Lewis 2005). Traditionally, miRNAs have been considered endogenous regulators of genes, i.e., miRNAs synthesized by a given host regulate the expression of genes in that host.

Gene regulation by exogenous dietary miRNAs

The paradigm that miRNAs originate exclusively through endogenous synthesis was recently challenged by a report that humans and mice may absorb miR-168a from rice and that miR-168a from rice alters the expression of the low-density lipoprotein receptor adaptor protein 1 mRNA in humans and mice (Zhang et al. 2012). However, the fascinating theory that dietary miRNAs from the plant kingdom affect gene expression in mammals is highly controversial and has largely been dismissed by the scientific community (Wang et al. 2012; Chen et al. 2013; Dickinson et al. 2013; Snow 2013), including our studies (Baier et al. 2014).

In contrast to the controversial observations regarding miRNAs from plants, mammalian miRNAs are likely transferred among species by dietary means. We have provided unambiguous evidence that (*i*) humans absorb biologically meaningful amounts of miRNAs from nutritionally relevant doses of cow's milk, (*ii*) bovine miRNAs enter peripheral blood mononuclear cells and presumably other peripheral tissues, and (*iii*) physiological concentrations of milk miRNAs affect human gene expression in vivo and in cell cultures (Fig. 2A–C) (Baier et al. 2014). Consistent with these observations, miRNAs in sow's milk

are absorbed by newborn piglets (Gu et al. 2012). Importantly, when mice were fed a milk-miRNA-depleted diet for 4 weeks, plasma concentrations of miR-29b and miR-200c decreased by 61%, suggesting that endogenous synthesis of miRNAs does not compensate for dietary miRNA deficiency (Fig. 2D) (Baier et al. 2014). The above discoveries were largely modeled based on miR-29b and miR-200c (Baier et al. 2014). However, one can assume with a reasonable level of confidence that humans also absorb milk miRNAs other than miR-29b and miR-200c, based on the following rationale. A large fraction of miRNAs in milk is contained in exosomes (Kosaka 2010; Zhou 2012), and the entire miRNA cargo will be delivered to cells that take up dietary miRNAs. Glycoproteins on the surface of both exosomes and target cells play crucial roles in exosome uptake by cells (Escrevente 2011).

While the above observations support the theory that bovine miRNAs have biological activity in humans, the milk studies did not answer the following questions with 100% certainty. (*i*) Are postprandial increases in plasma miRNAs due to absorption from dietary sources or does milk elicit an increase in the expression of genes coding for miRNAs? (*ii*) Do miRNAs from foods of animal origin other than milk also have biological activity in humans? Studies are emerging to suggest that humans absorb miRNAs from nutritionally relevant amounts of hard-boiled chicken eggs; egg-borne miRNAs are delivered to peripheral human tissues, and chicken miRNAs affect gene expression in peripheral blood mononuclear cells (Zempleni et al. 2015). Importantly, the postprandial increase in plasma miRNAs included avian miRNAs that humans cannot synthesize, consistent with a dietary origin of miRNAs (Zempleni et al. 2015).

Following cellular uptake, miRNAs undergo sorting, i.e., the ratios of miRNAs taken up or synthesized by cells are not necessarily the same as the ratios of miRNAs secreted into the extracellular space. The secretion of miRNAs appears to be regulated by the abundance of miRNA binding sites in a population of cells and is also impacted by the activation status of cells (Squadrito 2014). miRNA sorting may lead to scenarios under which a given dietary miRNA is absorbed by intestinal cells, and possibly delivered to the liver, but will not appear in the peripheral circulation and tissues, falsely suggesting that the miRNA was not absorbed. In classical pharmacokinetics, this scenario is referred to as first-pass elimination (Pond and Tozer 1984).

miRNA binding in plasma and tissue is different from that in milk. In milk, a large fraction of miRNAs is contained in exosomes, providing protection against degradation (Hata et al. 2010; Kosaka et al. 2010; Zhou et al. 2012). In contrast, in plasma and tissues, miRNAs are associated with 2 major fractions: one encapsulated in microvesicles (Ohshima et al. 2010; Huang et al. 2013) and one associated with the Argonaute2 protein (Arroyo et al. 2011; Turchinovich et al. 2011), both secreted by mammalian cells and conferring protection against premature degradation.

Snow et al. (2013) did not observe a transfer of dietary miR-21 in miR-21 knockout mice. However, the dietary supply of miR-21 in the studies by Snow et al. (2013) was about 100 times less than the lowest dose (0.25 L) used in the above-mentioned milk feeding study (Baier et al. 2014). If the amount of milk in human feeding studies were lowered by a factor of 100 to 2.5 mL, one also would not anticipate observing a rise in miR-29b plasma levels

(Fig. 2A). Clearly, the provocative concept of exogenous food-borne miRNAs eliciting biological effects in humans warrants confirmation by other laboratories before far-reaching conclusions can be drawn.

Are miRNAs degraded during the processing and storage of milk? When synthetic miRNAs are added to bovine milk, they are rapidly degraded; in contrast, endogenous milk miRNAs are resistant to treatment with acid and RNase (Kosaka et al. 2010; Izumi et al. 2012), presumably because of encapsulation and protein binding as described above. Stability tests suggest that approximately 50% of the miRNAs present in raw milk are degraded during homogenization and sterilization with an additional minor loss of less than 10% during cold storage and cooking; for most dairy products the miRNA content is less than that in processed milk (Howard et al. 2015).

Dietary compounds affecting the expression of miRNAs

Nutrients and bioactive food compounds may affect the expression of genes coding for miRNAs. For example, evidence has been provided that dietary polyphenols alter the expression of miRNAs 291b-5p, 296-5p, 30c-1*, 467b*, and 374* in apolipoprotein-E-deficient mice (Milenkovic et al. 2012). This paper will focus on dietary miRNAs, whereas the nutritional regulation of miRNA expression will be disregarded. For a recent review on the dietary regulation of miRNA expression, see Ross and Davis (2011).

miRNAs in human health

The Human miRNA Disease Database links 572 miRNA genes with 378 diseases in humans (Lu et al. 2008). Because of the large number of entries in the database, the reader is directed to browsing the database for his or her miRNA or disease of interest (Systems Biology and Biomedical Informatics Lab 2014), as opposed to us providing a lengthy description in this paper. Note that researchers may submit entries to the database's curators for review. Using the TargetScan algorithm (Lewis et al. 2005), we identified 11 119 unique human gene targets (unpublished information) for 175 out of the 245 miRNAs in cow's milk (Chen et al. 2010; Izumi et al. 2012). Based on this large number of target genes it is reasonable to propose that milk miRNAs are important regulators in a variety of metabolic pathways. Our own studies suggest that some miRNAs have escaped detection in previous studies, and cow's milk contains more than 245 miRNAs. miRNAs have been implicated in all aspects of health and disease. In the following sections, we highlight potential roles of milk miRNAs in 5 areas that pose important health threats to humans.

Example 1: Bone health

miR-29b is an important regulator of bone mineralization in humans, mediated by a miR-29b-dependent increase in osteoblast differentiation (Li et al. 2009) and decrease in osteoclast differentiation and function (Rossi et al. 2013). The National Osteoporosis Foundation estimates that 44 million women and men in the US aged 50 years and older suffer from osteoporosis and low bone mass (National Osteoprosis Foundation 2011), representing 55% of the people in that age group. These problems may be compounded by low levels of miRNAs in infant formulas compared with breast milk. The concentrations of

miRNAs decrease by 95% in the processing of cow's milk to infant formulas (Chen et al. 2010), and no miR-29b and miR-200c are detectable in hydrolyzed, hypoallergenic formulas and soy-based formulas (Izumi et al. 2012; unpublished data). Consistent with the role of miR-29b in promoting bone mineralization (Li et al. 2009; Rossi et al. 2013), the bone mineral content is 10% higher in breast-fed infants compared with infants fed cow's milk formula, which is 10% higher than in infants fed a soy-based formula at age 3 months (Andres et al. 2013). For about 3.7 million infants, formulas are the primary source of nutrition in the US (McDowell et al. 2008; Cox 2011).

Example 2: Myeloma

miR-29b sensitizes multiple myeloma cells to bortezomib-induced apoptosis (Amodio et al. 2012). In the US, during 2014, there will be an estimated 24 050 new cases and 11 090 deaths (National Cancer Institute 2014*c*). Among African-Americans, myeloma is one of the top 10 leading causes of cancer death. In addition, miRNAs are considered biomarkers for cancer (Pritchard et al. 2012; Schultz et al. 2014).

Example 3: Immune function, arthritis, and inflammatory bowel disease

Cow's milk contains miRNAs implicated in immune function (miR-15b, miR-27b, miR-34a, miR-106b, miR-130a, miR-155, miR-200c, and miR-223) and Crohn's disease (miR-21 and miR-223) in quantities sufficiently high to alter human immune function (Arnold et al. 2012; Izumi et al. 2012). Evidence has been provided that miRNAs in breast milk modulate the immune system in infants (Gu et al. 2012). Both innate and adaptive immunity can be impacted by miRNAs, as reviewed by Lindsay (2008). Specifically, miR-155 is essential for the normal development and function of B cells, T cells, and dendritic cells (Rodriguez et al. 2007), and expression of miR-223 dampens the activation of neutrophils (Johnnidis et al. 2008). Theoretically, having higher dietary intake of miR-223 (among other immune-related miRNAs) may help limit the severity of Crohn's disease. Note that miRNAs bind to Toll-like receptors, which has major implications for tumor biology and immune function (Fabbri 2012; Fabbri et al. 2012).

Example 4: Metabolic syndrome

Pathway analysis (Ogata et al. 1999; Biocarta 2014; National Cancer Institute 2014*b*; Reactome 2014) of human transcripts targeted by bovine miRNAs revealed a significant enrichment of pathways in the metabolism of glucose, cholesterol, and triglycerides, which are pathways implicated in metabolic syndrome. The definition of metabolic syndrome differs somewhat among agencies, but there is general consensus that metabolic syndrome is diagnosed if 3 out of the following 5 conditions co-occur: central obesity, high blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density cholesterol levels (Alberti et al. 2009). miRNAs have been implicated in the pathogenesis of diseases linked to the metabolic syndrome. For example, (*i*) 27 miRNAs are differentially expressed in patients with essential hypertension (Li et al. 2011), (*ii*) more than 30 miRNAs, including miR-29b, have been implicated in beta cell biology and insulin resistance, as well as diabetes and its complications (Fernandez-Valverde et al. 2011), (*iii*) miR-30c decreases hyperlipidemia and atherosclerosis by decreasing lipid synthesis and lipoprotein secretion in

mice (Soh et al. 2013), and (*iv*) low plasma levels of miRs 20b, 21, 24, 29b, 15a, 126, 191, 197, 223, 320, and 486 impair peripheral angiogenic signaling in patients with type-2 diabetes (Zampetaki et al. 2010).

Example 5: Reproduction

miRNAs are essential in reproduction, based on the following observations. Extracellular vesicles (EVs) in human semen carry a distinctive repertoire of small noncoding RNAs with potential regulatory functions (Vojtech et al. 2014). Placenta-derived EVs continuously increase in maternal circulation over the first trimester of pregnancy (Sarker et al. 2014), and uterine luminal fluid EVs deliver miRNAs to the embryo, which is important for embryonic development and establishment and maintenance of pregnancy (Burns et al. 2014). miRNAs also play crucial roles in postnatal growth, including organs and tissues such as heart, aorta, cartilage (Kalsotra et al. 2010; Ott et al. 2011; Bakhshandeh et al. 2012), and bone (see above).

For many miRNAs, the link with diseases is based on correlations rather than unambiguous cause-and-effect relationships. Theoretically, one could establish cause-and-effect relationships between miRNAs and disease by using knockout mice. However, creating knockout mice can pose a problem for some miRNAs. For example, miR-29b knockout is lethal because of a severely compromised immune system (Smith et al. 2012). Normally, one would tackle this problem by creating a conditional knockout model. In the case of miRNAs, this may not be an efficient approach because they may be encoded by multiple copies. For example, murine miR-29b is encoded by 2 distinct loci, i.e., chr6: 31063023–31063093 [−] for miR-29a/b1 and chr1: 195037040–195037120 [+] for miR-29b2/c. Knocking out the gene coding for miR-29b-1 also knocks out miR-29a and severely compromises the immune system (Smith et al. 2012); knocking out the gene coding for miR-29c also knocks out miR-29b-2, but leaves miR-29b-1 unaltered. This is not a problem specific to miR-29b but also applies to 11 other murine miRNAs including miR-467a (6 loci) and miR-669a (9 loci). Some miRNAs are encoded as clusters of 2–19 miRNA hairpins in close proximity (Davis and Hata 2009). While some miRNAs can arise from independent transcripts, e.g., miR-433 and miR-127 (Song and Wang 2008), a single miRNA locus may also give rise to 2 miRNAs with distinct sequences because of bidirectional transcription, as observed in *Drosophila melanogaster* (Stark et al. 2008; Tyler et al. 2008). In addition, selective deletion of only the miRNA sequences juxtaposed between exons, introns, and 3′-UTRs in genes without disrupting gene function is practically impossible in many cases. Moreover, knocking out miRNAs that are encoded in multiple genomic loci would require generating many independent knockout models and subsequent breeding to maintain multiple alleles in one mouse; any such protocols require a significant amount of time and resources. Creating miRNA knockout mice is a priority in the pursuit of establishing cause-and-effect between (dietary) miRNAs and disease and for assessing the phenotypes of dietary miRNA depletion. The use of CRISP/Cas9-based protocols may be a viable approach to address the above difficulties in some cases (Wang et al. 2014).

Implication of dietary miRNAs for using miRNAs as biomarkers

miRNAs in body fluids are considered viable candidates for disease biomarkers, partially because of their extraordinary stability in body fluids compared with proteins and mRNAs (Turchinovich et al. 2011). In particular, cancer researchers have developed an interest in studying miRNAs as potential biomarkers for cancer risk (Schultz et al. 2014; Schwarzenbach et al. 2014). However, the observation that dietary miRNA intake might affect their concentrations in body fluids and tissues suggests that dietary intake needs to be considered a potential confounder when using miRNAs to assess disease risk. Concerns have also been raised about the effects of blood cell count and hemolysis on the concentrations of circulating miRNA (Pritchard et al. 2012).

Features that discriminate exogenous dietary miRNAs and endogenous miRNAs

Mammalian miRNAs and their mRNA targets are highly conserved (Friedman et al. 2009; Warnefors et al. 2014). For example, 245 miRNAs have been identified in cow's milk (Chen et al. 2010; Izumi et al. 2012). A preliminary analysis of these 245 bovine miRNAs using the TargetScan database (Lewis et al. 2005) identified 11 119 putative targets for 175 of these bovine miRNAs, including miR-29b and miR-200c, in the human transcriptome (unpublished observation). This prediction is consistent with the notion that miRNAs typically target more than 1 gene. Seventy-nine and 59 transcripts are experimentally validated targets for bovine miR-29b and miR-200c, respectively, in human bone matrix cells, as per MirTarBase (Fig. 3) (Hsu et al. 2014). Note the crosstalk between the 2 networks.

While the sequence similarities between bovine and human miRNAs increase the probability that miRNAs in milk affect human gene expression, it also creates a problem when seeking to distinguish between dietary and endogenous miRNAs. For example, 155 out of the 245 cow milk miRNAs have identical nucleotide sequences with human miRNAs. In contrast, only 11 out of the 245 cow milk miRNAs have nucleotide sequences that are distinguishable from their human orthologs by quantitative real-time PCR or next generation sequencing; these miRNAs include miR-16-5p, miR-146a, miR-320b, miR-345, miR-452, miR-502b, miR-503, miR-542-3p, miR-660, and miR-363 (Faculty of Life Sciences 2014).

Other features that may determine miRNA activity include the conservation of the seed:target match, the composition of the seed:target flanking region, target site accessibility, and free-energy-based determinants (Wen et al. 2011; Squadrito et al. 2014), as well as the sequence-based features including nucleotide composition and palindromic properties. In addition, it is reasonable to propose that the following factors contribute to regulating the biological activity of (dietary) miRNAs: encapsulation in exosomes and microvesicles (Ohshima et al. 2010; Huang et al. 2013), binding to Argonaute2 (Arroyo et al. 2011; Turchinovich et al. 2011) and high density lipoproteins (Vickers et al. 2011), and proteins on the surface of exosomes and cells (Escrevente et al. 2011). These factors may differ between miRNAs obtained from exogenous and endogenous sources.

Do dietary miRNAs qualify as bioactive food compounds or essential nutrients?

The National Cancer Institute defines bioactive compounds as "*a type of chemical found in small amounts in plants and certain foods […]. Bioactive compounds have actions in the body that may promote good health. They are being studied in the prevention of […] diseases*" (National Cancer Institute 2014*a*). Based on this definition and the above evidence that humans absorb milk miRNAs that regulate genes implicated in human health, milk miRNAs qualify for consideration as bioactive food compounds. It remains to be determined whether miRNAs from dietary sources other than milk and eggs fulfill similar functions. As of today, there is insufficient evidence to rank dietary miRNAs among essential nutrients. The National Research Council defines an essential nutrient as "*a nutrient required for normal human body function that either cannot be synthesized by the body […] in amounts adequate for good health, and thus must be obtained from a dietary source* (National Research Council 1998)". Additional research is warranted to assess the potential role of dietary miRNAs as a bioactive or essential food compound.

Acknowledgments

Abbreviations

EV   extracellular vesicle

miR   miRNA, microRNA

UTR   untranslated region

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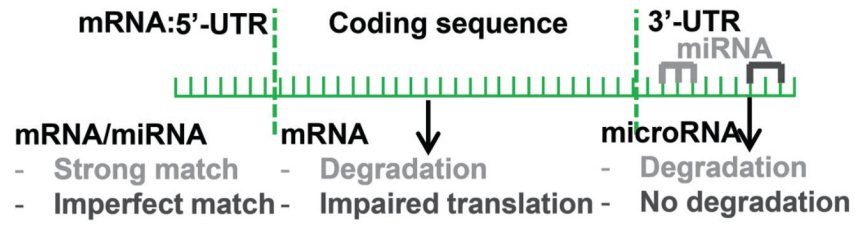
Fig. 1.
MicroRNAs (miRNAs) bind to complementary sequences in the 3′-untranslated region (3′-UTR) in mRNA, triggering mRNA degradation or inhibiting mRNA translation; miRNAs are degraded in the process.
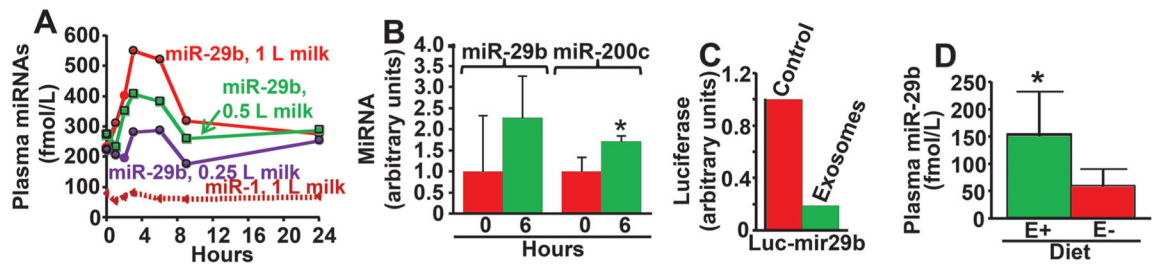
Fig. 2.
(A) Plasma time curves of miR-29b and miR-1 (negative control) in healthy adults following consumption of 0.25 to 1 L milk ($n = 5$). (B) Abundance of miR-29b and miR-200c in peripheral blood mononuclear cells before (0 h) and after (6 h) consuming 1 L of milk ($n = 5$). (C) Treatment of human cells with 200 fmol/L miR-29b in milk exosomes decreases reporter gene activity ($n = 2$). (D) Plasma concentrations of miR-29b in mice fed an miRNA-normal (E+) vs. miRNA-depleted (E−) diet for 4 weeks ($n = 5$); *, $p < 0.05$. Reprinted with permission from Baier et al. (2014).
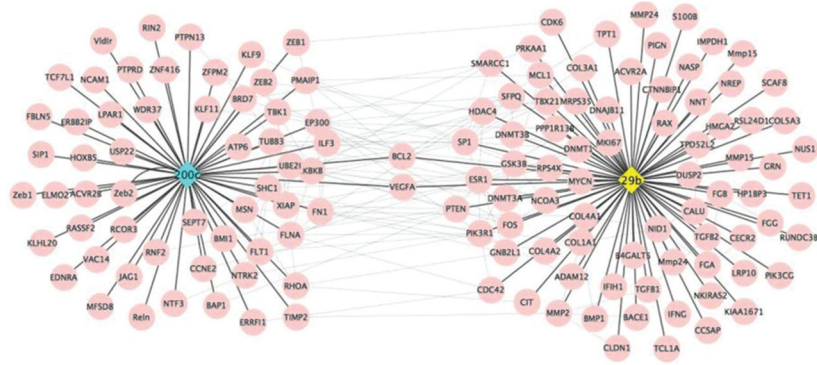
Fig. 3.
Gene network predictions for milk-borne miR-200c (left) and miR-29b (right) in human bone matrix cells.