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Stability and Biological Activity of Dietary MicroRNAs

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Stability and Biological Activity of Dietary MicroRNAs

by

Katherine Howard

A THESIS

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Stability and Biological Activity of Dietary MicroRNAs

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MicroRNAs play important roles in gene regulation by binding to complimentary sites at the 3' untranslated region of target mRNA molecules. Binding results in inhibition or degradation of target mRNA. Many bovine and chicken microRNA are homologous with human counterparts enabling gene regulation. A recent study in our lab provided undisputable evidence that endogenous milk microRNAs are bioavailable in humans; resulting in regulation of human gene expression. Based on these findings, we wanted to explore the possibility that other exogenous food borne microRNAs are able to be absorbed through the diet. My thesis surrounds two aims: 1) assessing the stability of milk borne microRNAs and 2) analyzing the bioavailability of egg borne microRNAs and effects on gene expression following an egg meal.

Aim 1: We assessed the effects of milk processing, storage, somatic cell content and handling by consumers on the degradation of miRNAs in milk. Pasteurization and homogenization caused a 63% loss of miR-200c, whereas a 67% loss observed for miR-29b was statistically significant only in skim milk. Effects of cold storage and somatic cell content were quantitatively minor.

Aim 2: Humans absorbed a significant amount of egg borne microRNAs following an egg meal. Increased plasma levels of miR-181b from baseline showed to be dose dependent. Plasma concentrations of miR-181b peaked 9 hours after egg consumption to 150% above baseline levels. The abundance of miR-181a was also increased in erythrocytes 9 hours after egg consumption to levels 154% higher than baseline values. Expression of BCL2 and BCL2A1, experimentally validated targets of miR-181a/b, was 56% and 19% lower, respectively, in human lymphocytes nine hours after egg consumption.

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Chapter 1

Literature Review

Background on MicroRNAs

MicroRNAs (MiRNAs) are a class of small non-coding RNAs about 22 nucleotides long that play important roles in gene expression through base-pairing with complementary sites at the 3' untranslated region in target mRNA molecules [1] .This binding inhibits translation or can cause degradation of target mRNA. The target seed sequence which is made of nucleotides 2-7 in microRNA is deemed necessary for binding to target transcripts [2].

Over 8000 predicted plant, animal and virus microRNAs are included in the miRNA sequence database. There are 2588 known mature human microRNAs [3] for humans alone suggesting that miRNAs might make up 3% of the human genome and regulate over 60% of protein-coding human genes [4]. MiRNAs can regulate multiple genes and in turn each mRNA can be regulated by several different miRNAs [5].

Mechanism of microRNA formation and function

Most primary microRNA are transcribed by RNA polymerase II. The primary microRNA (pri-miRNA) hairpins are encoded in both protein coding and non-coding transcription units. Often times, multiple pri-miRNA hairpins are encoded by a single transcript. Biogenesis of the mature microRNA begins with cleavage by an RNase III enzyme Drosha which recognizes the 10 bp stem of the pri-miRNA This cleavage results in pre-microRNA, an approximately 55-70 nucleotide hairpin product [6, 7]. Exportin 5 recognizes the overhangs of the pre-microRNA, enabling transport from the nucleus to the cytoplasm. In the cytoplasm Dicer RNase III enzyme cleaves the pre-microRNA hairpin into an approximately 22nt double stranded product. One strand is preferentially chosen and is incorporated into an Ago protein complex to form the mature product. The mature miRNA is guided to its target mRNA with partial complimentary binding sites on the 3' UTR causing repression or degradation of the mRNA [7].
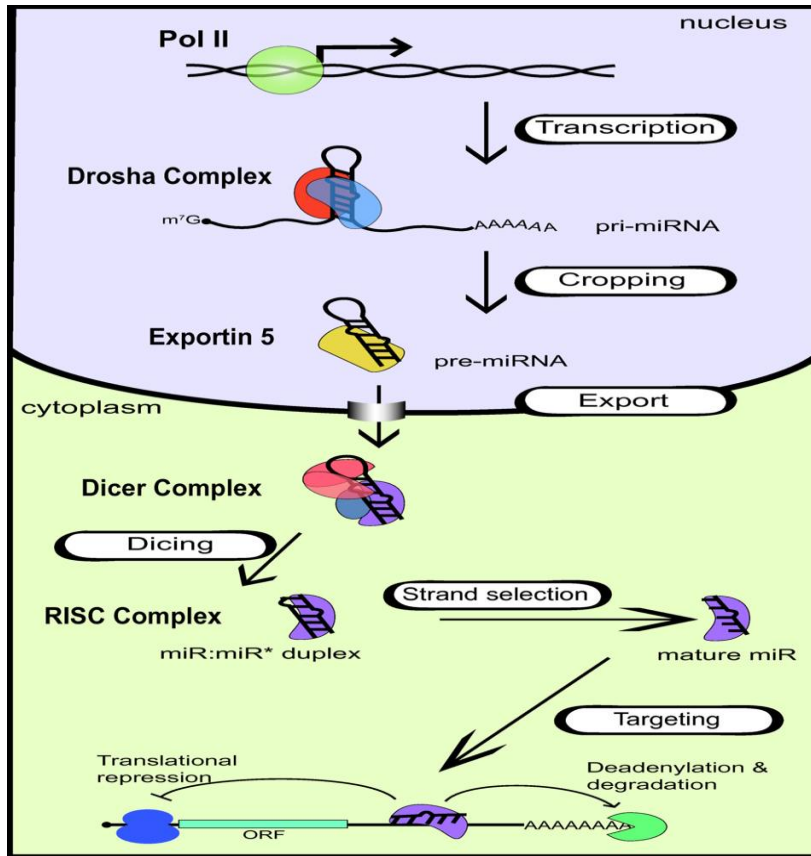
Figure 1.1: MicroRNA Biogenesis: Biogenesis of miRNAs and assembly into RISC complex (Adapted from Davis, 2009)

MicroRNA Encapsulation and Transport via Exosomes

MicroRNAs are encapsulated inside of cell derived vesicles, microvesicles and exosomes, along with mRNAs and proteins. Exosomes are products derived from the endocytic recycling pathway and released into the extracellular space by the fusion of multivesicular bodies with the plasma membrane. Exosomes may impact cells via binding to the cell surface, fusing with the plasma membrane and/ or internalization by the recipient cell. Various endocytic pathways have shown to play a role in exosome uptake although there is dispute as to which method is most prominent. Exosome uptake was found to occur by clathrin- mediated endocytosis in dendritic cells and phagocytosis in phagocytic cells. In phagocytic cells exosomes are more effectively internalized via phagocytosis versus non phagocytic cells [8, 9].

Exosomes and their contents, including mRNA and microRNA, can be transferred between different cell types and species allowing for intercellular communication. These vesicles are able to protect their contents from degradation during transportation through the extracellular environment. Vesicle contents are able to regulate gene expression and alterations in miRNAs levels have shown to be especially important during development and stress response [10, 11]. There is evidence for the importance of extracellular vesicles in maintaining homeostasis. EV dysregulation has been linked to disease and promotion of tumor development [11].

Milk Consumption

Milk is commonly consumed in the United States despite its decline from 236 pounds in 1982 to 195 pounds in 2012. In spite of the decline in milk consumption, total dairy consumption increased by 11% during the same time period [12]. In most states in the United States it is required that milk sold for human consumption is first pasteurized. Pasteurization of milk began in the mid-nineteenth century as a public health precaution to destroy heat resistant non-spore forming bacteria present in raw milk. The most common pasteurization process is known as the high temperature, short- time where milk is heated to 71.7 degrees Celsius and held at room temperature for 15 seconds. Alternatively, milk can be heated to and held at 63.5 degrees for 30 min which is referred to as the standard holder method [13].

Role of MicroRNA-29b and MicroRNA-200c

Potentially, milk derived miR-29b can improve bone health as previous research has shown miR-29b contributes to the prevention of bone deterioration seen with osteoporosis. MiR-29b has been shown to directly downregulate inhibitors of osteoblast differentiation, HDAC4, TGFβ3, ACVR2A, CTNNBIP1, and DUSP2 proteins by binding to target sequences in their mRNAs [14]. Many of the targets of miR-29b are inhibitors of osteoblast differentiation by affecting the functional activity of Runx2, a transcription factor that encourages differentiation of osteoblasts and bone formation. MiR-29b also targets collagens, suppressing their synthesis and preventing degradation of bone matrix [14]. Optimal bone formation is dependent on the number and activity of osteoblasts and osteoclasts. Increasing the number and functionality of osteoblasts while decreasing the action of osteoclasts enhances bone formation; ultimately preventing bone deterioration [15]. MiR-29b has also been found in dendritic cells of the intestines and to carry a gut specific role. Compared to controls, mice deficient in miR-29b showed a more severe case of microbial dependent colitis [16]. Members of the MiR-200 family have been shown to reduce the activity of ZEB1, a known promoter of tumorigenesis. Overexpression of these microRNAs downregulated metastasis by positively regulating E-cadherin expression via ZEB1 targeting, a transcriptional repressor of E-cadherin [17].

MicroRNA-29b Related Health Implications

Osteoporosis is a disorder of the skeleton characterized by compromised bone health leading to a greater risk of fractures [18]. It is estimated to affect 200 million people worldwide; 44 million people in the United States. The prevalence of osteoporosis is continuing to increase among the elderly population putting them at risk for fragility fractures which can lead to morbidity and even mortality. Each year in the United States 1.5 million fractures are attributed to osteoporosis and cost the nation an estimated $17 billion in medical costs. It also results in personal burdens such as pain and disability which can lead to a loss of work and a decreased quality of life [19]. A risk factor for osteoporosis is low peak bone mass which is determined early in life. Ultimately, bone mineral density is determined by the opposition of forces of osteoblasts and osteoclasts [15, 18].

It is commonly thought that calcium and vitamin D are the main nutritional contributors to bone health. While the results of some studies regarding the relationship between calcium intake and bone health showed modest positive benefits on bone mineralization, other studies showed no relationship between calcium and bone health or positive effects were confounded by other variables [20]. Dairy sources of calcium specifically have been reasoned to contribute to a greater bone density although other research has suggested that increased bone health may not be due to calcium [21].

Milk MicroRNAs and Bioavailability

In bovine milk 245 microRNAs have been identified [22, 23]. MiR-29b and MiR200c were detected to be among the most abundant miRNA in bovine and breast milk through microarray and quantitative PCR analysis [23]. The bovine nucleotide sequences of miR-29b and miR200c are each identical to the nucleotide sequence of miR-29b and miR200c in humans [3].

A large amount of milk microRNAs are encapsulated in exosomes allowing them to be resistant to degradation and absorbed into the body. This discovery lead it to be hypothesized that mothers are able to transfer miRNA to their infants through breast milk, which may play an important role in the infant's immune system [23, 24]. In contrast to synthesized miRNAs, endogenous breast milk miRNAs were found to be stable during acidic conditions, multiple freeze thaw cycles, and RNase treatment suggesting their ability to be transferred and survive in the infant gastrointestinal tract, potentially regulating immune function [22, 25]. Cow's milk contains multiple microRNAs involved in immunity, miR-15b, miR-27b, miR-34a, miR-106b, miR-130a, miR-155, and miR-223, many of which are similar to those found in breast milk [22, 23]. Importantly, a recent study in our lab has shown that milk- borne microRNA's are bioavailable in humans, milk microRNAs from nutritionally relevant doses of cow's milk effect human gene expression and endogenous microRNA synthesis does not compensate for dietary deficiency [26]. It has been proposed that MIR 168a, a miRNA abundant in rice, is present in the sera of human subjects [27], although these claims are controversial partially due to the fact that the plasma concentrations found from rice microRNAs are

low. A recent study in our lab contradicted these claims and was unable to detect a postprandial rise in plasma concentration of the brassica-specific *osa*-miR-167a *osa*-miR-824 after consuming a broccoli (*Brassica oleracea var. italica*) sprouts meal. This may be partially due to the much high concentration of microRNAs in milk compared to broccoli. Postprandial levels of microRNAs detected in humans following milk consumption were significantly higher (200-300 times) than those detected from *osa*-miR-167a. This finding is consistent with the theory that in contrast to plant microRNAs, a large amount of milk microRNAs are encapsulated in exosomes allowing them to be resistant to degradation and absorbed into the body [26].

Dietary Contribution of Eggs

Few foods are as nutrient dense as eggs, which meet the criteria for a functional food; a food which provides added physiological benefit beyond that of meeting nutritional needs. Although eggs have received a bad reputation in the American diet due to concerns of a positive correlation between egg consumption and cholesterol [28]. Contrary to popular belief, frequent egg consumption has been found to be inversely correlated with cholesterol levels [29, 30]. Eggs provide many essential nutrients including riboflavin, folate, Iron, Phosphorous, Selenium, Zinc and vitamins A, B, E, and K. They are one of the few dietary contributors of Vitamin K and D. Eggs are also rich in choline, a nutrient important in brain development and memory and carotenoids, known for their role in disease prevention [28]. When compared to egg consumers, nonconsumers were more likely to not meet the RDA for Vitamins A, E and B12 [29]. Additionally, atherosclerotic burden was found to be inversely correlated with consumption of one or more eggs per week. [31] Furthermore, egg consumption was not found to be associated with risk of coronary heart disease or stroke [30].

Endogenous Chicken MicroRNAs and Gene Regulation

MicroRNA 181a and 181b were analyzed due to their high number of sequence reads in the embryonic small RNA library [32, 33]. Among the most abundant miRNAs in eggs, miR-181a and miR-181b have well defined physiological roles. These miRNA have been shown to inhibit inflammation and atherosclerosis. Additionally, expression of miRNA-181b in plasma levels is reduced in individuals with atherosclerosis [34]. MiR-181a plays a role in B cell differentiation as well as T cell maturation. [35]

The sequence of miRNA-181b is homologous between chickens and humans and has been shown to reduce NF-κB nuclear translocation resulting in an inhibition of inflammatory gene expression, NF-κB activation and atherosclerosis in mice targeting importin-α3, a protein used for NF-κB translocation to the nucleus [36]. This translocation inhibition results in reduced inflammatory gene expression and downstream reduction of BCL2 family proteins. [37-39].

BCL2 and BCL2A1, known targets of NF-κB, are regulators of cell death presenting anti-apoptotic functions [37, 40]. BCL2A1 has commonly been shown to be overexpressed in many cancer cells including leukemia and lymphoma, exhibiting anti-apoptotic properties by sequestering pro-apoptotic BCL2 family member proteins [38, 41]. MiR-181b levels have also been found to be inversely correlated with progressive lymphocytic leukemia and overexpression of BCL2 in contrast to patients presenting a stable form of the disease where levels remained constant [42]. Patients presenting with sepsis were shown to have deceased levels of miR-181b compared to control patients [36].

MicroRNA related Human Health Implications

MicroRNAs regulate over 30% of mRNAs [4] and have been implicated in various areas of human health including stem cell plasticity, tumor pathogenesis, neurodegeneration, the nervous system, and immune response. [10] Alterations in miRNA expression are contributors to the pathogenesis of many human malignancies. In various tumor tissues and cells many microRNAs are found to be differentially expressed and as in the case of carcinogenesis can act as oncogenes or tumor suppressors. Processes including cell differentiation, growth and death, which microRNAs play a role in, are often dysregulated in cancer; suggesting microRNAs have a significant impact in carcinogenesis [10, 43, 44].

Dysregulation and transfer of microRNAs have been shown to have many implications related to human health and disease. Atherosclerotic HDL-miRNA delivery has shown to target genes involved in lipid metabolism, inflammation and atherosclerosis where specifically, miR-223 has been found to be upregulated [45]. Dysregulation of twenty-seven different microRNAs have been associated with hypertension. A strong link between hcmv-miR-UL112 upregulation in the pathogenesis of hypertension has been found [46]. MiR-1, miR-133a and miR-208a play important roles in the development and function of the heart and have found to be increased in plasma with acute myocardial infarction [47]. Diabetes has been associated by dysregulation of many different microRNAs involved in beta cell biology, especially the downregulation of miR-375, leading to insulin resistance, diabetes and further complications [48]. Fifteen microRNAs are upregulated with inflammatory bowel diseases, two of which are also found to be

upregulated in other autoimmune disorders suggesting an involvement in the

inflammatory response [49]

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CHAPTER 2

Loss of miRNAs during Processing and Storage of Cows (Bos taurus) Milk

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Running head: Loss of miRNAs during processing and storage of milk

ABSTRACT

MicroRNAs (miRs, miRNAs) play central roles in gene regulation. Previously, we reported that miRNAs from pasteurized, store-bought bovine milk have biological activity in humans. Here we assessed the effects of milk processing, storage, somatic cell content, and handling by consumers on the degradation of miRNAs in milk; we also quantified miRNAs in dairy products. Pasteurization and homogenization caused a 63% loss of miR-200c, whereas a 67% loss observed for miR-29b was statistically significant only in skim milk. Effects of cold storage and somatic cell content were quantitatively minor (<2% loss). Heating in the microwave caused a 40% loss of miR-29b but no loss of miR-200c. Milk fat content had no effect on miRNA stability during storage and microwave heating. The concentrations of miRNAs in dairy products were considerably lower than in store-bought milk. We conclude that processing of milk by dairies and handling by consumers causes a significant loss of miRNAs.

INTRODUCTION

MicroRNAs (miRs, miRNAs) are small non-coding RNAs that play essential roles in the regulation of genes at the posttranscriptional level in plants and animals.[1] Mature miRNAs are about 22 nucleotides long and bind to complementary sequences in the 3'-untranslated region of mRNAs. Perfect or near perfect base pairing of the miRNAs and its target mRNAs typically results in mRNAs degradation, whereas less perfect base pairing typically results in inhibition of mRNAs translation.[2-3] 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. Recently, our laboratory refuted this paradigm. We provided strong evidence that 1) humans absorb biologically meaningful amounts of miRNAs from nutritionally relevant doses of cow's milk, 2) physiological concentrations of milk miRNAs affect human gene expression *in vivo* and in cell cultures, and 3) endogenous synthesis of miRNAs does not compensate for dietary miRNA deficiency in mice.[4] Our discoveries were largely modeled on miR-29b and miR-200c, but likely hold true for all miRNAs encapsulated in milk exosomes.[5-6] To the best of our knowledge, our previous paper is the first to provide unambiguous evidence that miRNAs can be transferred between distinct species through dietary means. In contrast, previous claims that miRNAs from plants affect human gene expression[7-8] are highly controversial and were met with skepticism by the scientific community.[4, 9-12] Based on the above observations, milk miRNAs are a novel class of bioactive food compounds as defined by the National Cancer Institute in the United States.[13] The discovery that milk miRNAs are bioactive food compounds has broader implications as miRNAs play essential roles in gene regulation,[2-3] cell communication,[14-15] and human health.[16-23]

This study focused on determining the effect of milk processing, storage, somatic cell content, and handling by consumers on two miRNAs, miR-29b and miR-200c levels based on the following rationale. In bovine milk, miR-29b and miR-200c are among the most abundant miRNAs.[20] MiR-29b is an important regulator of bone mineralization in humans, as it increases osteoblast differentiation[16] and decreases osteoclast differentiation and function.[17] MiR-200c decreases cancer risk by targeting the transcription factor ZEB1, which induces E-cadherin expression, thereby limiting epithelial-to-mesenchymal transition, a key event in metastasis.[24-25] Also, the nucleotide sequences of miR-29b and miR-200c in bovine milk are identical to those of their human orthologs.[26] Our rationale for including the somatic cell count in our analysis was to assess whether an increase in milk cells, as seen in mastitis, might be a confounder in the analysis of milk miRNAs.

In Western societies, the majority of milk is processed prior to consumption. In fact, the production and sale of raw milk dairy products is illegal in many states in the United States and pasteurization is required.[27] Moreover, while the per capita consumption of milk has declined from 236 pounds in 1982 to 195 pounds in 2012, total dairy consumption increased by 11% during the same time period.[28] Therefore, we considered it worthwhile to assess the effects of processing on the miRNA content in both milk and dairy products.

Little is known about the effects of processing and storage on milk miRNAs levels. In two studies, synthetic miRNAs were added to bovine milk and their stability after exposure to harsh treatments such as acid and RNase was assessed and compared to the stability of endogenous miRNAs in milk.[6, 20] Synthetic miRNAs were rapidly degraded, whereas endogenous miRNAs were resistant to treatment. However, the harsh treatments applied in these studies are not representative of the treatments applied in commercial dairy production. In this study, we

assessed the effects of pasteurization, fat content, cold storage, heating as well as processing into dairy products on content of milk miRNAs.

MATERIALS AND METHODS

Chemicals. Guanidinium thiocyanate and ethanol were purchased for use in the NucleoSpin miRNA plasma RNA extraction kit (Macherey-Nagel Inc., Bethlehem, PA). TRIzol was purchased from Life Technologies (Grand Island, NY).

Milk and dairy products. Raw, whole, 2%, and skim cow's (*Bos taurus*) milk was obtained from The Pennsylvania State University Creamery (University Park, PA) from separate collections in three consecutive weeks in May 2014. All milk was procured from the Penn State Animal Science Department's Holsteins breed herd. The milk for this study was processed from using raw milk and cream routinely supplied to the Penn State Berkey Creamery, University Park, PA and stored under intermittent agitation in a 22,712.5 liter raw milk silo at 2.2°C (Feldmeier Equipment, E-015-05; Little Falls, NY). The milk contained 3.25% milk fat and 8.9% milk solids non-fat (near-infrared method, CEM, Turbo Smart5, Model 907990; Matthews, NC).

For the preparation of the product, milk (3.25% Milk Fat; 12.15% Milk Solids Non Fat) was pasteurized at 75.55°C with a 28-s holding time (APV Paraflow, Serial number 20053003000302; Goldsboro, NC). It was homogenized at 145 Bar and 60°C (APV Gaulin Homogenizer, Serial Number 20052410702; Lake Mills, WI). The product was standardized using a Westfalia Separator, type MSE 55-01-177; Oelde, Germany. A details and process diagram for preparation of whole cow's milk is presented in figure 1.

After cooling the product was transferred to a 7200 liter refrigerated storage tank at 2.2°C

(Feldmeier Equipment, E-015-05; Little Falls, NY). The product was bottled on filling machine

(Federal Manufacturing, Serial Number 1/12.4GL843; Milwaukee, WI) and stored in a

conventional cold-milk warehouse at 3.0°C.

On our initial collection dates, milk of all fat levels were stored at 4°C for up to 15 days, and aliquots

were taken and frozen at -80°C every other day. In a separate experiment bovine cells were removed

from raw milk by centrifugation (500 g, 10 min, 4°C) to determine whether somatic cells are a

meaningful confounder when analyzing the concentrations of miRNAs in milk from healthy

cows. Samples were frozen at -80°C and shipped on dry ice to Lincoln, NE, for miRNA analysis.

Samples from all fat levels of milk on day 15 were heated in the microwave for 15 seconds and

analyzed after cooling off to room temperature. Dairy products other than milk were purchased

from grocery stores in Lincoln, NE. All samples were produced and analyzed as biological

repeats in triplicate.

MiRNA analysis. Milk samples were spiked with a synthetic internal standard (twenty-five

attomoles) prior to extraction of miRNAs using miSPIKE Synthetic RNA (IDT Technologies).[4]

Dairy products (100 mg) other than milk were extracted using TRIzol prior to addition of the

synthetic internal standard. MiR-29b and miR-200c were quantified using quantitative real-time

PCR as described previously.[4]

Statistics. Analysis by Bartlett's Test Homogeneity suggested that variances were

homogeneous.[29] The paired t-test was used for pairwise comparisons. One-way analysis of

variance (ANOVA) and Fisher's protected least significant differences were used when

comparing more than two groups. Repeated measures ANOVA was used for assessing the effects

of storage time on miRNA concentration. StatView 5.0.1 (SAS Institute; Cary, NC) was used for

conducting statistical analyses. Means ± SD are reported. Differences were considered statistically significant if $P \leq 0.05$.

RESULTS

Pasteurization and homogenization of raw milk resulted in a 63±28% decrease of miR-200c in whole milk; effects were similar for 2% fat milk and skim milk (**Fig. 2A**). The effect was less pronounced for miR-29b for which a significant decrease (67±18%) was observed only in skim milk (**Fig. 2B**). Cold storage of milk did not affect the concentration of mir-29b and miR-200c in whole milk, 2% milk and skim milk up to 15 days; 2% fat milk is shown as a representative example in **Fig. 3.** Somatic cells are not meaningful confounds regarding the analysis of miRNAs in milk from healthy cows. When somatic cells were removed from raw milk by centrifugation and analyzed for miRNA content, the cellular miRNAs were found to contribute less than 2% of the total miRNAs present in raw milk before centrifugation: 1.1±0.9% for miR-29b and 0.14±0.08% for miR-200c.

Processing in the household has the potential to cause a considerable loss of some miRNAs in milk. For example, the concentration of miR-29b decreased by 40±28% when processed milk was heated in the microwave and cooled to room temperature compared to milk before heating (**Fig. 4**). In contrast, when milk was heated in the microwave the concentration of miR-200c was not statistically different compared with unheated controls.

DISCUSSION

In a recent paper we reported the importance of milk miRNAs for gene regulation in humans.[4] That report has major implications for the roles of milk and possibly other dairy

products in human health. Cow's milk contains meaningful quantities of 245 miRNAs,[20, 30] and 71.4% of these miRNAs are predicted to target about 11,000 human transcripts (unpublished observations). In addition to the roles of miR-29b and miR-200c in bone health and cancer prevention,[16-17, 24-25] respectively, miRNAs have been implicated in various aspects of human health and disease including hypertension, insulin resistance and diabetes, hyperlipidemia and atherosclerosis, reproduction, immune function and Crohn's disease.[18, 20-21, 31-33]

We propose that milk has a meaningful effect on human health, mediated by miRNA-dependent gene regulation. The potential importance of dietary milk miRNA intake is supported by data suggesting that 1) Americans consume large quantities of milk and dairy products,[28] 2) a large proportion of milk miRNAs is encapsulated in extracellular vesicles, thereby providing protection against degradation [5-6] and a pathway for cellular uptake by endocytosis,[34-35], and 3) milk miRNAs are resistant against degradation during storage (this study).

The concentrations of miRNAs varied considerably among the dairy products tested (**Table 1**), but were generally lower than the concentrations in pasteurized whole milk (compare **Fig. 2**). Fresco Queso Dip was a notable exception and contained higher concentrations of miRNAs than those observed in milk.

Our previous studies of milk miRNAs in humans and mice were conducted using 1% fat milk from the grocery store.[4] Based on this study, the content of miRNAs is about two times higher in unprocessed milk compared with pasteurized, store-bought milk. Note that we have no intent recommending the consumption of raw cow's milk by humans, because of food safety concerns associated with raw milk. We observed that a loss of milk miRNAs occurred only during pasteurization, homogenization, and processing to dairy products. This observation is consistent with previous studies of milk miRNAs. For example, endogenous miRNAs were not

degraded when milk was exposed to harsh treatments such as low pH or treatment with RNase.[6, 20] It is reasonable to propose that encapsulation of miRNAs in extracellular vesicles[5] prevents miRNA degradation, based on the following lines of evidence. 1) When synthetic miRNAs are added to milk and subjected to low pH or RNase treatment, the miRNAs are rapidly degraded.[6, 20] 2) When exosome membranes in milk were disrupted by sonication for preparing miRNA-depleted mouse diets in previous studies, miR-29b was rapidly degraded to concentrations below detection limit.[4] Presumably, degradation was due to milk RNases gaining access to miRNAs released from exosomes. 3) When milk was fermented to produce yoghurt, miRNA concentrations decreased to levels much lower than in milk (this study). We speculate that the decrease was due to the lysis of exosomes during fermentation and the large amounts of RNases produced by microbes. 4) When milk was homogenized, miRNA concentrations decreased by on average 50% (this study). We speculate that the decrease was caused by a disruption of exosome membranes by shear forces applied during homogenization. Collectively, our studies suggest that milk, and perhaps dairy products, have the potential to contribute to the miRNA body pool in humans.

Some uncertainties remain to be addressed in future studies. For example, this study was modeled based on miR-29b and miR-200c, however, there is a possibility that distinct miRNAs may be differentially metabolized.[8, 36] Another layer of uncertainty is the possible effects of feeding regimens, season, and breed on the miRNA content in milk. Moreover, while this study suggests that somatic cells in milk from healthy cows do not contribute meaningful amounts to the total miRNA content in milk, it is possible that the increased somatic cell count in milk from cows suffering from mastitis[37] may cause an artificial increase in milk miRNA concentrations. Our previous studies suggest that plasma miRNA concentrations decrease by 61% in mice fed a

milk miRNA-depleted diet for four weeks. This observation is consistent with milk miRNAs contributing meaningful quantities to the miRNA body pool, but does not necessarily establish the essentiality of dietary miRNA intake. Clearly, this is an uncertainty that will need to be addressed in future studies. Finally, it is conceivable that miRNAs from foods other than milk also contribute toward the total body pool of miRNAs.

ABBREVIATIONS USED

miR, microRNA; miRNA, microRNA

ACKNOWLEDGMENT

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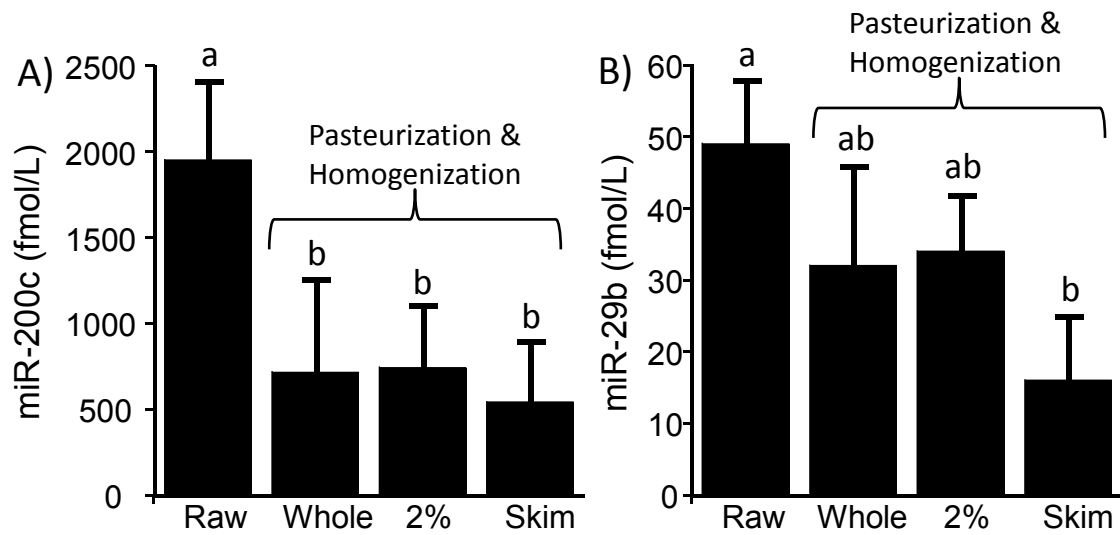
Figure 2.1. Loss of miR-200c (A) and miR-29b (B) during milk pasteurization and homogenization of milk with different fat content. [a,b]Significantly different (n=3 biological replicates, $P<0.05$).
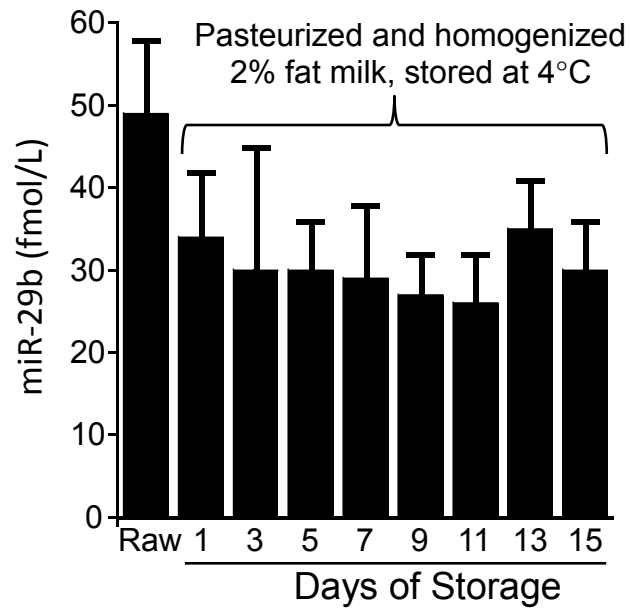
Figure 2.2: Storage at 4°C did not affect the concentrations of miR-29b in pasteurized and

homogenized 2% fat milk (n=3 biological replicates, *P*>0.05).
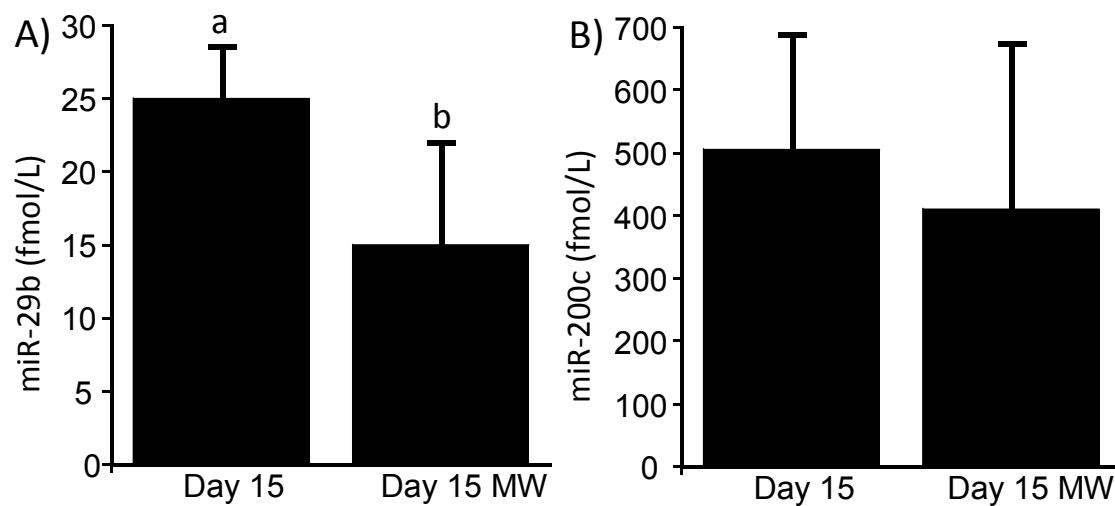
Figure 2.3: Loss of miR-29b (A) and miR-200c (B) during heating of whole milk in the microwave after 15 days of storage at 4°C. Abbreviation: MW, microwaved. [a,b]Significantly different (n=3 biological replicates, *P*<0.05).

Table 2.1. Concentration of miRNAs-29b and -200c in dairy products

Product	MiRNA	
	miR-29b	miR-200c
	fmol/kg	
Best Choice Yogurt	0.9±0.10	37.6±2.8
Fresco Queso Dip	36.1±5.5	1029.8±478.6
Greek Yogurt	14.2±3.9	462.3±126.9
Half and Half	3.0±0.17	513.3±159.2
Heavy Whip Cream	2.6±1.3	342.0±132.9
Parmesan Cheese	4.9±1.9	232.0±64.5
Upstate Farm Yogurt	2.4±1.0	216.9±93.8

Data are means±SD, n=3.

Chapter 3

MicroRNAs in chicken eggs are bioavailable in healthy adults and down-regulate BCL2 and BCL2A1 gene expression in peripheral blood mononuclear cells

MicroRNAs in chicken eggs are bioavailable in healthy adults and down-regulate BCL2 and BCL2A1 gene expression in peripheral blood mononuclear cells

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Running Title: MicroRNAs in chicken eggs are bioavailable

[3]Author disclosures: K. Howard, S. R. Baier, and J. Zempleni, no conflicts of interest.

[4]Abbreviations used: AUC, area under the curve; C_{max}, maximal plasma concentration; miR, microRNA; PBMC, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; RBC, red blood cell; t_{max}, time of peak concentration.

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<u>Abstract</u>

Previous research has demonstrated the bioavailability of milk-borne microRNAs following milk consumption by healthy adults. An important question left unanswered at the conclusion of that study was if this phenomenon is unique to milk or if other foods could also be a source of exogenous microRNA. To explore this possibility, we tested the hypothesis that chicken egg microRNAs are bioavailable following consumption of hard boiled eggs by healthy adult humans. Plasma concentrations of miR-181b peaked 9 hours after egg consumption to 150% above baseline levels. The abundance of miR-181a was also increased in erythrocytes 9 hours after egg consumption to levels 154% higher than baseline values. Importantly, a chicken microRNA not synthesized by humans, gga-miR-1451-5p, was not detectable in plasma samples before egg consumption but was detected at hour nine following egg consumption. Expression of BCL2 and BCL2A1, experimentally validated targets of miR-181a/b, was 56% and 19% lower, respectively, in human lymphocytes nine hours after egg consumption. We conclude that chicken egg microRNAs are bioavailable and have the potential to influence endogenous gene expression in humans following consumption of physiologically relevant doses of eggs.

<u>Introduction</u>

MicroRNAs (MiRNAs) are a class of small non-coding RNAs about 22 nucleotides long that play important roles in gene expression through base-pairing with complementary sites at the 3' untranslated region in target mRNA molecules (1). This binding inhibits translation or can cause degradation of target mRNA. The target seed sequence which is made of nucleotides 2-7 in microRNA is of particular importance for binding to target transcripts (2). Most miRNA are transcribed in the nucleus like protein coding genes and are processed in both the nucleus and cytoplasm into the active, mature form. Traditionally, the miRNA made within one cell are thought to regulate genes in the same cell or perhaps other cells and tissues within the same organism if transported via extracellular vesicles (3). This paradigm was recently refuted by a recent publication from our lab that has provided strong evidence that humans absorb milk miRNAs which in turn have an effect on gene expression (4). Based on these novel and impactful findings, we wanted to explore the possibility that other foods could provide exogenous miRNA through the diet. To explore that aim, this study analyzed chicken (*gallus gallus*) eggs as a potential dietary source of microRNAs. Eggs are commonly consumed in the United States and a good source of nutrients including folate, iron, phosphorous, selenium, zinc and vitamins B, E, A and K (5). This study analyzed the expression of chicken specific microRNAs as well as those shared between humans and chicken (6, 7). We analyzed miRNA-181a and miR-181b due to their high number of sequence reads in the embryonic small RNA library as well as their role in human health. (7) The sequence of miRNA-181b is homologous between chickens and humans and has been shown to reduce NF-κB nuclear translocation resulting in an inhibition of inflammatory gene expression, NF-κB activation and atherosclerosis in mice by targeting importin-α3, a protein used for NF-κB translocation to the nucleus. (8) By

inhibiting NF-κB translocation, expression of inflammatory genes is reduced (9-11). In addition to indirect targeting through regulation NF-κB signaling, miR-181a/b also directly target the 3' UTR of BCL2 mRNA. (12, 13).

Materials and Methods

Human Feeding Study. Eggs were obtained from a local grocery store and hard boiled the night before consumption. Seven healthy adults consumed three different egg doses in a randomized crossover design with a minimum of a one week wash out period between doses. Subjects were instructed not to consume eggs 24 hours before the egg meal as well as during the period of blood sample collection. Twenty milliliters of blood was collected in EDTA tubes before egg consumption (Hour 0) and at intervals 3, 4.5, 9, 12, and 24 hr after egg consumption. Plasma, PBMC and RBC were collected using gradient centrifugation as described previously (14) and frozen at -80°C until analysis. Urine was collected throughout the 24 hour length of the study. This protocol was approved by the University of Nebraska-Lincoln Institutional Review Board and all participants provided signed informed consent forms prior to participation.

RNA Isolation and Reverse Transcription. Total RNA was isolated from 300uL plasma using the NucleoSpin miRNA Plasma kit with a final elution volume of 30uL (Machery-Nagel Inc., Bethlehem, PA). For PBMC and RBCs, the Zymo Quick RNA mini-prep kit was used with a final elution volume of 30uL (Zymo Research, Irvine, CA). For all microRNA studies, 4 uL of RNA was utilized in the miScript RT II Kit (Qiagen, Valencia, CA). For reverse transcription of RNA in PBMC samples, 100 ng was used with the miScript RT II Kit using the HiFlex Buffer. Plasma samples were spiked with a synthetic internal standard (twenty-five attomoles) prior to extraction of RNA using miSPIKE Synthetic RNA (IDT Technologies).

Gene Expression. MicroRNAs were quantified using quantitative real-time PCR as described previously. (15) PBMCs were assessed to analyze the effects of egg borne miRNA on human gene expression. B-cell lymphoma 2 (BCL2) and BCL2 related protein A1 (BCL2A1) were analyzed using samples from 0 h and 9 h post egg consumption. Expression of mRNA was normalized to GAPDH expression and calculated using the $\Delta\Delta$Ct method. (16)

Pharmacokinetics analysis. The linear trapezoidal rule was used to analyze areas under the curve for plasma microRNA to determine the apparent bioavailability of microRNA. (17)

Statistics. For all data sets, homogeneity of variances was tested by using the Bartlett's Test. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA) was used for calculation of AUC. Pharmacokinetics data were analyzed using repeated measures analysis of variance (ANOVA) and posthoc comparisons were tested with Fisher's PLSD (18). PBMC gene expression data was analyzed using the paired t-test, whereas plasma and RBC miRNA were analyzed using the Wilcoxon-Signed Rank Test. StatView 5.0.1 was used for statistical analyses (SAS Institute; Cary, NC). Differences were considered statistically significant if $p < 0.05$.

Results

Bioavailability of egg-borne microRNA. A significant amount of microRNA was absorbed by humans following egg meals (Table 1, Fig. 1). Increases in the plasma miR-181b concentration were dose-dependent as the consumption of four eggs provided the greatest response in C_{max} and AUC, followed by the three egg and two egg doses (Table 1). Plasma concentration of miR-181b

peaked 9 h following consumption of the 4 egg meal with plasma concentrations returning to baseline values by 24 h after egg consumption. The three and two egg doses saw progressively earlier t_{max} values and return to baseline plasma miR-181b concentrations.

Effects of egg microRNA on PBMC and RBC microRNA concentration. Feeding of four eggs did not result in changes to human PBMC miRNA concentrations (data not shown). In RBCs, miR-181a was 154±41 % higher 9 h following consumption of four eggs (Fig. 2, P = 0.05) while miR-181b concentrations were not significantly higher.

Target gene expression in response to egg microRNA. Analysis of PBMC at hour 9 post egg consumption showed BCL2 and BCL2A1 mRNA to be downregulated. The expression of BCL2 was 56±18% lower (Fig. 3, P = 0.05) 9 h following the four egg meal compared to baseline and the expression of BCL2A1 was19±3 % lower (Fig. 3, P = 0.02)

Discussion

Few foods are as nutrient dense as eggs. Eggs are abundant in fat soluble vitamins, caratenoids, known for their role in disease prevention and are one of the few dietary contributors of vitamin K and vitamin D. Eggs are also rich in choline, a nutrient important in brain development and memory (5). When compared to egg consumers, non-consumers were more likely to not meet the RDA for Vitamins A, E and B12 (19). Additionally, eggs are considered to be a functional food; meaning they provide added physiological benefit beyond that of meeting nutritional needs (5). In spite of these positive benefits of consuming eggs, they have received a bad reputation among the general public due to concerns of a positive correlation between egg consumption and cholesterol (5). Contrary to popular belief, frequent egg consumption has been found to be

inversely correlated with cholesterol levels (19, 20). While this observation has been made by different researchers, there is yet to be a widely accepted mechanism by which egg consumption may protect against increased cholesterol levels.

In a recent study we found humans absorb meaningful quantities of bovine microRNAs which have an effect on gene expression potentially having human health implications. (4) In the present study we have observed similar effects regarding egg miRNAs following consumption of hard boiled eggs by healthy adults. This paper holds importance for eggs as well as other animal food products on potential implications in human health. Many egg borne microRNA are homologous with their human counterparts resulting in predictable gene regulation. Among the most abundant miRNAs in eggs, miR-181a and miR-181b have well defined physiological roles. These miRNA have been shown to inhibit inflammation and atherosclerosis and expression of miRNA-181b plasma levels are reduced in individuals with atherosclerosis (21). Additionally, atherosclerotic burden was found to be inversely correlated with consumption of one or more eggs per week (22). Protection from atherosclerosis may be provided, at least in part, by the additional miR-181b provided by egg consumption. Furthermore, egg consumption was not found to be associated with risk of coronary heart disease or stroke (20). Additionally, patients presenting with sepsis have been shown to have deceased levels of miR-181b compared to control patients (8).

BCL2 and BCL2A1, known targets of NF-κB, are regulators of cell death presenting anti-apoptotic functions (9, 23). BCL2A1 has commonly been shown to be overexpressed in many cancer cells including leukemia and lymphoma, exhibiting anti-apoptotic properties by sequestering pro-apoptotic BCL2 family member proteins (10, 24, 25). MiR-181b levels have also been found to be inversely correlated with progressive lymphocytic leukemia and

overexpression of BCL2 in contrast to patients presenting a stable form of the disease where miR-181b levels remained constant (26).

In summary, this research clearly demonstrates the bioavailability of chicken egg miRNAs after consumption of hard boiled eggs by healthy adults. Importantly, the exogenous miRNA also affect endogenous gene expression in PBMC and presumably other body tissues. Epidemiological evidence regarding egg consumption supports the known role of miR-181a/b with regard to protection from many vascular problems including atherosclerosis, inflammation, and sepsis. While we are still in the early stages of establishing the importance of dietary miRNAs, this research demonstrates that milk is not the only food able to supply exogenous miRNA. While our group and others have doubts about the bioavailability of plant miRNAs, results from this study support the idea that animal-based food products are dietary sources of miRNA.

**TABLE 3.1** Pharmacokinetics analysis of plasma time curves of microRNAs after egg meals in healthy adults.

| Variable | miR-181b | | |
| | Dose | | |
	2 eggs	3 eggs	4 eggs
Baseline (fmol/L)[1]	27 ± 15	22 ± 15	27 ± 17
C_{max} (fmol/L)	47 ± 27^a	67 ± 59^{ab}	77 ± 60^b
t_{max} (h)	4.5 ± 1.2	7.5 ± 2.9	8.4 ± 2.2
AUC (fmol/L*h)[2]	62 ± 51^a	133 ± 109^a	266 ± 279^b

Values are means $\pm$ SD, n=7

[1]Plasma concentration at time 0 hours.

[2]For hours 0 through 24.

[a,b]Within a variable for the same miRNA, means without a common letter differ ($P < 0.05$, n =7).
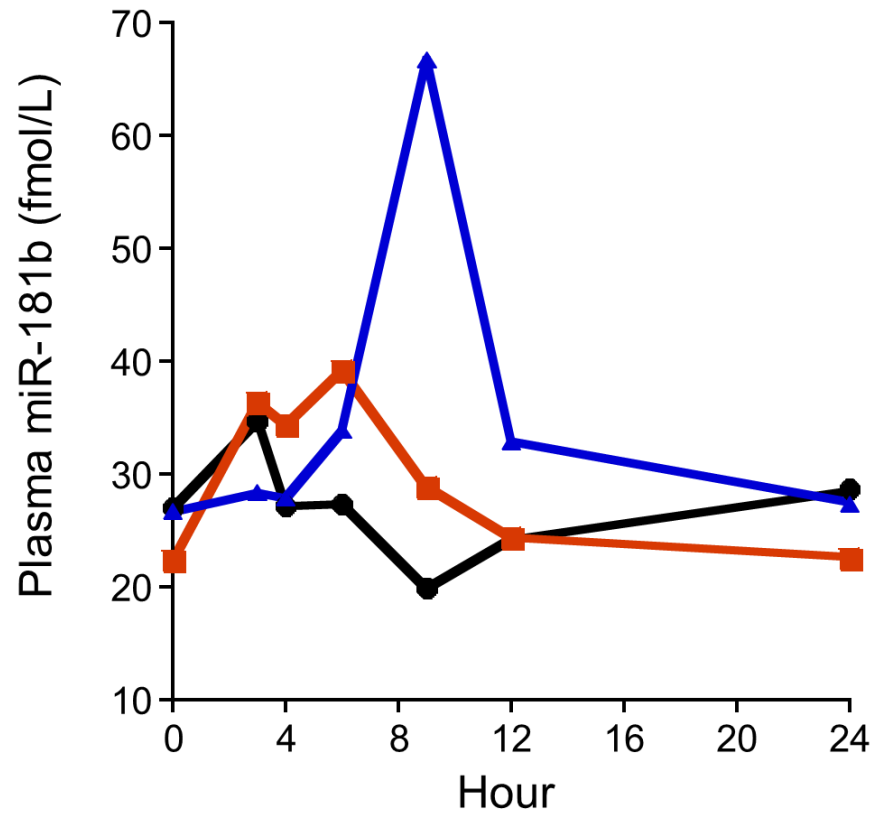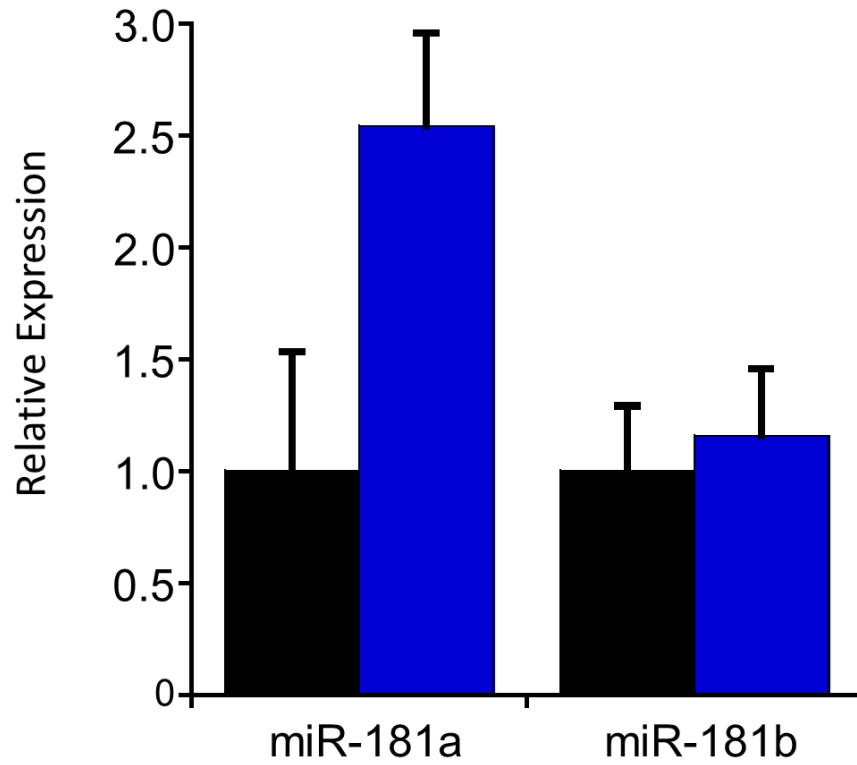
FIGURE 3.1 Plasma time curves of miR-181b following an egg meal in healthy adults.

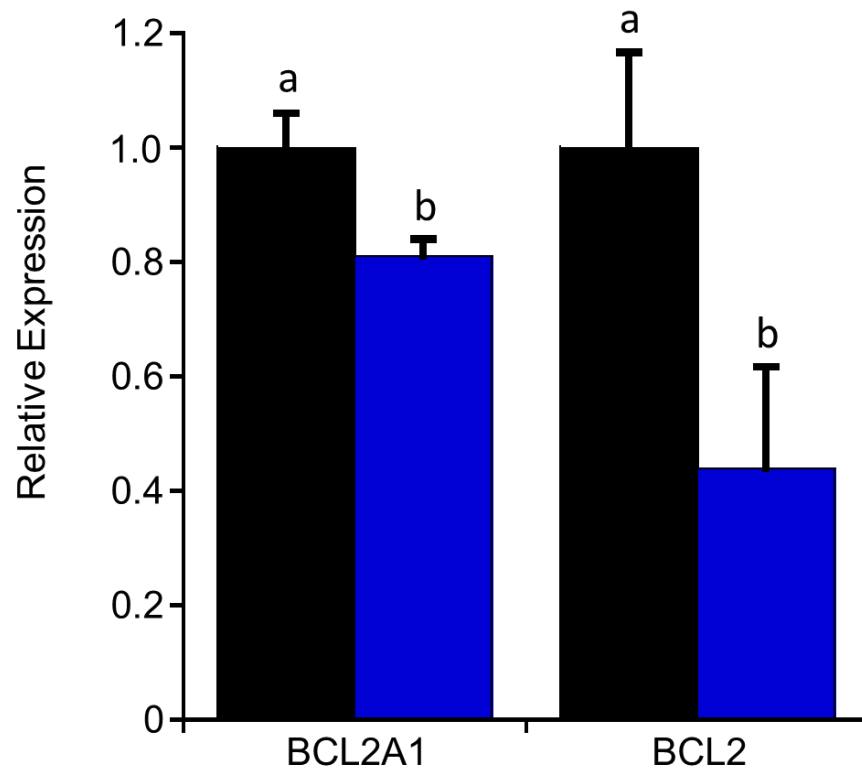Different doses are represented by colored lines: black (2 eggs), red (3 eggs), and blue (4 eggs).

Values are reported as means, n =7. Standard deviations have been omitted for clarity (compare

Table 1).

FIGURE 3.2 Effects of egg microRNAs on the abundance of miRNA in human erythrocytes.

Values are mean ± SD, n = 7. Labeled means without a common letter differ, $P < 0.05$.

FIGURE 3.3 Effects of egg microRNAs on *BCL2* and *BCL2A1* gene expression in PBMC from healthy adults. Data are mean ± SD, n = 7. *Different from hour 0, P < 0.05. *BCL2*, (B-cell lymphoma 2; *BCL2A1*, BCL2-related protein A1.

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Chapter 4

Future Studies

Traditional thought that microRNAs synthesized by a species regulate gene expression in that host has been refuted in our lab. Unambiguous evidence supporting the uptake of dietary microRNAs and subsequent regulation of gene expression warrants future studies addressing the potential effects this bioavailability of exogenous microRNAs has on overall gene expression as well as implications this may have on human health. MicroRNA resistance to degradation during storage and fat adjustment could potentially be ideal findings for milk manufacturers and consumers. Due to the high content of miR-29b, known for its role in bone formation, milk consumption can potentially lead to improved bone health based off of the rationale that miR-29b contributes to the prevention of bone deterioration seen with osteoporosis. Likewise, the increased plasma concentration of miR-181b and downstream gene regulation seen with egg consumption has the potential effect to reduce vascular inflammation. Although, it is necessary to address what other effects upregulation of these microRNAs may have.

A current study in our lab is analyzing phenotypic effects potentially due to microRNA content in the diet. Mice are being fed either an Exo- plus diet where microRNAs are present or Exo- minus diet in which microRNAs are depleted through sonification. Plasma and different tissues will be analyzed at different stages in life as well as success of breeding and offspring.

Cow's milk and breast milk have many similar microRNAs which are degraded in the process of making infant formulas. This degradation surfaces a potential concern for future health, in particular bone health in infants fed formula. An ongoing study in our lab involves the recruitment of mother/infant pairs and collection of monthly infant urine and breast milk or infant formula samples for the first 6 months of life. The microRNA content of the urine and milk will be analyzed along with urinary markers for bone health. We hypothesize that the differing microRNA contents will have an effect on infant bone health. Through assessing the

different levels of microRNAs in breast milk, infant formulas and urine this study will hopefully give insight on ways to prevent age related bone deterioration and promote infant nutrition.

The processed milk used for analysis in this study was both pasteurized and homogenized. Ideas for future studies involve differentiating between the two processes, pasteurization and homogenization, to determine what percent of microRNA loss is due to each. It would also be worthwhile to examine the effect of heating milk at a higher temperature for an extended period of time (i.e. boiling) on miRNA concentration in milk.

MicroRNAs have future potential to serve as biomarkers for various diseases. Dysregulation of microRNA expression has been shown to serve as an indicator for various carcinogenesis and tumorigenesis stages. (Chapter 1, Ref. 44) Concentration of miR-181b was found to be decreased with progressive stages of lymphocytic leukemia, potentially enabling miR-181b to serve as a biomarker and therapeutic agent for patients with progressive lymphocytic leukemia. (Chapter 1, Ref. 42) Addressing parameters to develop known biomarkers would allow microRNAs to serve in the development of disease detection and treatment.

Appendix

qRT-PCR primers used to quantify gene expression

microRNA	Forward Primer Sequence (5-3')	Reverse Primer Sequence (3-5')
miSpike	CTCAGGATGGCGGAGCGGTCT	
miR-29b	GTAGCACCATTTGAATCAGTGTT	
miR-200c	TAATACTGCCGGGTAATGATGGA	
gga-miR-181a	AACAUUCAACGCUGUCGGUGAGU	
gga-miR-181b	AACAUUCAUUGCUGUCGGUGGG	
U6	CGCAAGGATGACACGCAAATT	
GAPDH	TCCACTGGCGTCTTCACC	GGCAGAGATGATGACCCTTT
BCL2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
BCL2A1	TACAGGCTGGCTCAGGACTAT	TTTTGTAGCACTCTGGACGTTT