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MICRORNAs ARE ABSORBED IN BIOLOGICALLY MEANINGFUL AMOUNTS FROM NUTRITIONALLY RELEVANT DOSES OF COW'S MILK AND CHICKEN EGGS AND AFFECT GENE EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS, CELL CULTURES, AND MOUSE LIVERS

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EGGS AND AFFECT GENE EXPRESSION IN PERIPHERAL BLOOD
MONONUCLEAR CELLS, CELL CULTURES, AND MOUSE LIVERS

by

Scott Baier

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Under the Supervision of Professor Janos Zemleni

Lincoln, Nebraska

April, 2015

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Scott Baier, Ph.D.

University of Nebraska, 2015

Advisor: Janos Zempleni

Throughout the twenty-first century, evidence has been continually increasing to show the importance of epigenetic regulation in health. While the term “epigenetics” can be applied to many different processes, the focus of this dissertation will be on microRNAs and chromatin structure. Ultimately, both of these forms of epigenetic regulation can be used to fine tune gene expression based on environmental cues. The first three chapters of the dissertation focus on microRNA bioavailability, stability, and function from two commonly consumed food products: cow's milk and chicken eggs. This important work has been the first of its kind to demonstrate the bioavailability and function of dietary, animal-based microRNAs. In order to test our hypothesis that dietary microRNA are indeed bioavailable, many experimental protocols have been used. Human feeding studies are the prominent tool used, but important discoveries have been made in cell culture and in a mouse feeding study that add to the significance of this work. The final chapter will focus on a well-known histone modifier: sulforaphane. There have been many positive roles attributed to this compound, but the work presented here describes some potentially negative consequences of high sulforaphane intake. Overall, the

conclusion of this work is dietary microRNA are bioavailable and can regulate endogenous gene expression. For the scientific field to determine the contribution of dietary microRNAs to overall health, much more work will need to be performed but the evidence provided in this dissertation indicates it is likely dietary microRNAs play a key role in overall epigenetic regulation. Ongoing studies in our laboratory to further this work and answer some of the current questions are also briefly described.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Janos Zempleni for providing me an opportunity to obtain my doctoral training in his laboratory. Dr. Zempleni not only taught me valuable laboratory skills for use in conducting experiments, but I learned even more from him in how to think like a scientist and critically review and plan scientific work. I also want to extend appreciation to my committee members for their valuable contribution to my dissertation work: Dr. Bin Yu, Dr. Regis Moreau, and Dr. Vicki Schlegel.

In addition to the important role Dr. Zempleni played in my training, many others were crucial for my growth as a researcher. Without a doubt, the most of important of those is a former postdoctoral scientist from our lab, Samudra Wijeratne. When I initially came to work in the lab, Samudra helped teach me many procedures and was always willing to troubleshoot experiments along the way. Other individuals that have helped me in different ways and are in need of recognition are Jing Xue, Mengna Xia, Dandan Liu, Wei Kay Eng, David Giraud, Daniel Câmara Teixeira, Elizabeth Cordonier, Tovah Wolf, Katherine Howard, Rio Jati Kasumi, Dr. Jennifer Wood, and Fang Xie.

While the focus is often on the research done in the lab, there is equally important work done to make sure everything else can run smoothly. Many people are responsible for making this happen, probably more than I even know, but some members of the Nutrition and Health Science and CEHS staff have been especially important and I would like to thank Donna Hahn, Lori Rausch, Jolene Walker, Carrie Brownyard, Connie Wieser, Lori Beals, Ann Grasmick, and Sarah Gibson. I also would like to thank our Department Chair, Dr. Timothy Carr, for his advice and guidance throughout my program.

Finally, saving the most important for last, I want to thank my wife, Ashley, for her unending support and love. I owe my success to her and my parents, without whom this work would not have been possible.

Scott Baier

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INTRODUCTION

While one of just many layers of determining overall gene activity, epigenetic control is an important factor for determining the phenotype at the cellular and organism level.

Literally meaning “over, outside, or around” the genome, epigenetics can refer to many biological events that influence gene expression. One type of epigenetic regulation that is constantly receiving more interest is non-coding RNA. Non-coding RNA can be broken down into many subsets including, but not limited to, long non-coding RNA (lncRNA), piwi interacting RNA (piRNA), and microRNAs (miRNAs). MiRNA, consisting of non-coding RNAs 18-23 nucleotides in length, have received special attention for the role they play in regulating gene expression (1). In spite of not coding for a protein, miRNA have been identified as having key roles in many cellular functions, including developmental timing, cell proliferation and death, hematopoiesis, and nervous system patterning (2).

Canonical miRNA function is accomplished by depressing protein expression of a target gene through binding to that target gene’s mRNA in the 3’ untranslated region (UTR) (3). The seed sequence, made of nucleotides 2-7 in a mature microRNA, is of particular importance for binding to target transcripts (4). The mechanism by which this occurs can be through impairing protein translation or by marking the target gene mRNA for degradation (Fig. 1) (5, 6). While the seed sequence typically needs to be a complete match to the target mRNA for any gene regulation to occur, the similarity, or lack thereof, of the remaining miRNA sequence to its target will determine whether translational inhibition or mRNA degradation are the predominant form of activity.

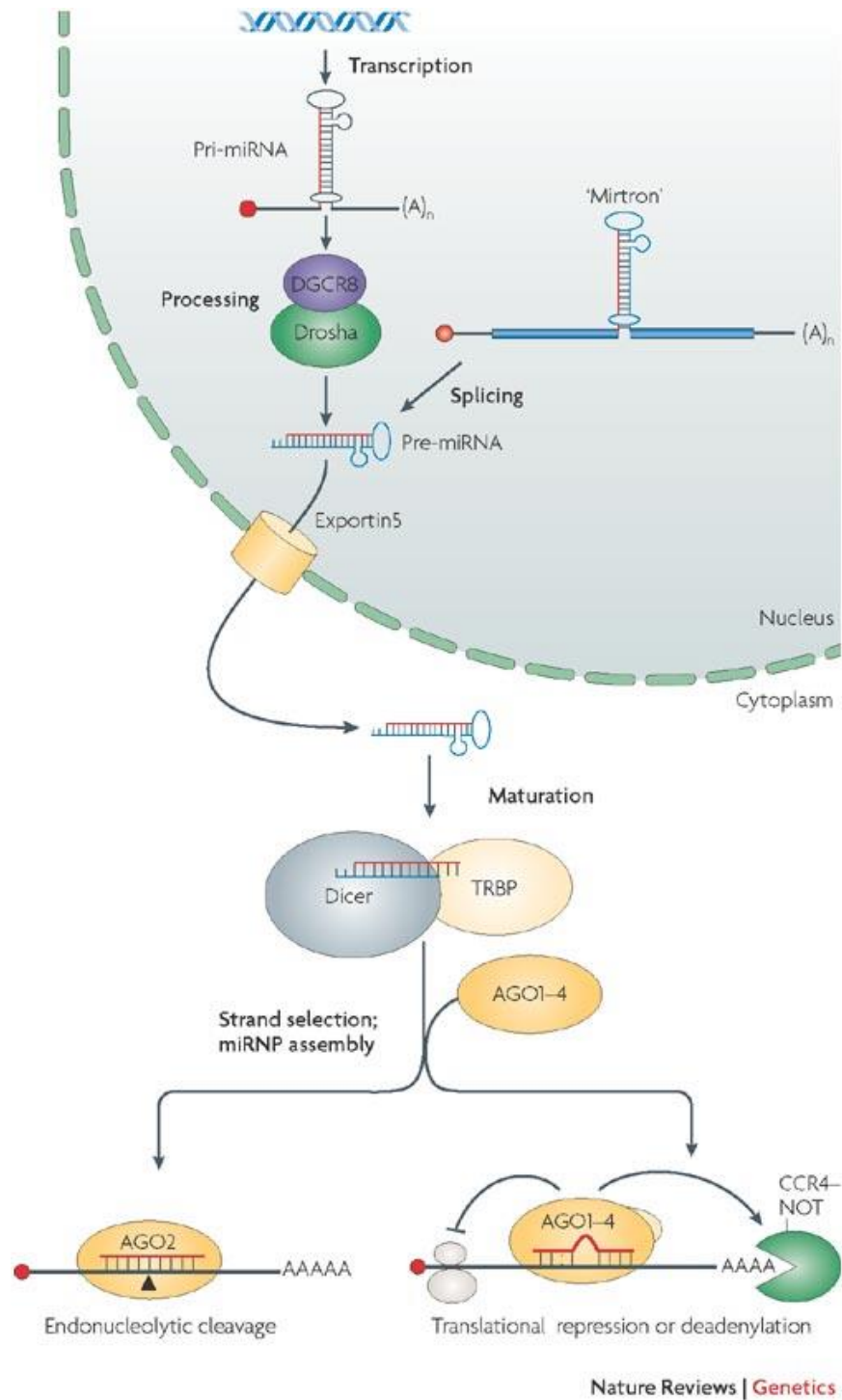


Figure 1. Biogenesis and function of microRNA. Mature microRNA result from processing in the nucleus and cytoplasm and once loaded into protein complexes can inhibit mRNA stability or protein translation. (Adapted from Filipowicz, 2008)

Traditionally, miRNA synthesized in one cell were viewed as specifically regulating genes within the same cell. Over time, this view has expanded to include transfer of miRNA from one cell type to another in the same and even distal tissues in the body, typically through the use of extracellular vesicles (7, 8).

Furthermore, there is precedent for the transfer cross-kingdom transfer of miRNA through the diet. This research suggested that miRNA-168a from rice is detectable in human and animal sera, and osa-miR-168a decreases the expression of low-density lipoprotein receptor adapter protein 1 mRNA, thereby inhibiting LDL receptor expression in mouse liver (9). While the results from this initial study were very interesting, the attempts by other researchers to replicate the data have been, in most cases, contradictory. In short, the bioavailability of plant-borne microRNAs in humans is controversial (10-13).

While there are many uncertainties regarding plant-borne miRNA bioavailability, there is reason to suspect animal-borne miRNA are more likely to survive digestive conditions and cross the intestinal barrier. Along with other nucleic acids and compounds, miRNA are known to be protected from the surrounding environment by extracellular vesicles (Fig. 2) (14, 15). One type of these vesicles, exosomes, have surface proteins that might be important for efficient cellular uptake (16, 17). Plants may have similar structures, but the identity and amino acid sequence of the surface proteins could be different, which in turn would likely negatively affect the uptake of plant exosomes by human intestinal cells.

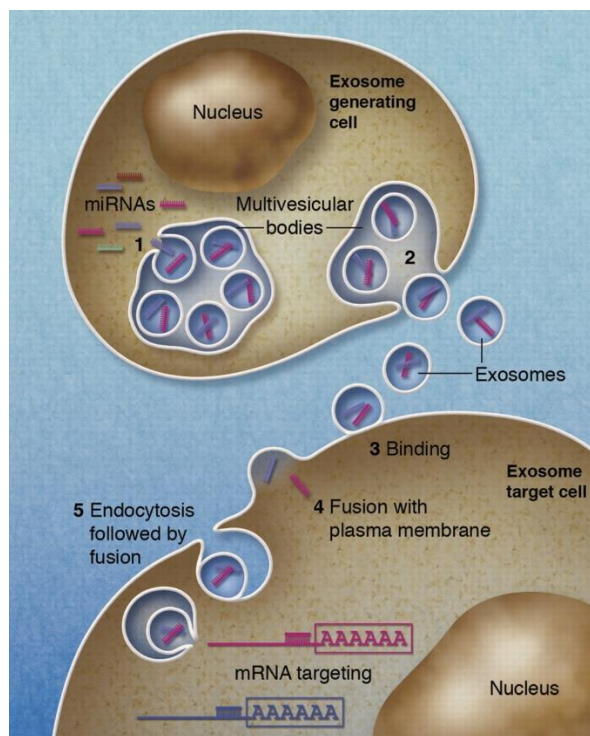


Figure 2. Exosome secretion, stability, and uptake. Initially formed from multivesicular bodies, exosomes are secreted into the extracellular space where the exosomal phospholipid bilayer protects the contents from external conditions until the exosome is taken up by a recipient cell via endocytosis and the contents are unpackaged. (Adapted from Stoorvogel, 2012)

Histone modifications are another subset of epigenetic changes that can lead to greater or lesser gene expression based on the placement and type of modification made to the histone. One type of well-known modification is histone acetylation, which takes place at lysine residues and typically is associated with greater transcriptional activation (18). Equally important in overall gene regulation, the cell has histone deacetylase (HDAC) proteins to remove the acetyl groups from histones when the gene activation they induce is no longer needed.

Epigenetic regulation by dietary compounds may explain some of the presumed health benefits of foods not explained by traditional nutrition. One widely studied food compound, found exclusively in cruciferous vegetables, is sulforaphane (SFN). SFN is purported to have many positive characteristics, including the ability to activate tumor suppressor genes and reduce inflammation (19). There are different hypotheses to explain the cellular responses seen with the administration of SFN, but one that has been experimentally validated is the ability of SFN to act as a histone deacetylase (HDAC) inhibitor (19, 20). Once taken up by a cell, sulforaphane is metabolized into a number of different compounds. While they all confer differing levels of biological activity, SFN-Cysteine seems to have the best HDAC inhibitory activity (20). Experimental and computer modeling results indicate SFN-Cysteine probably functions as a competitive inhibitor (Fig. 3).

By lowering the amount of HDAC activity in cells, there can be greater acetylation in the promoter of genes responsive to HDACs. With regard to observed studies on SFN, suppression of HDAC activity can lead to greater activation of tumor suppressor genes, such as p21. Through the additional protection conferred by having greater p21 expression, SFN acts as a chemoprotective compound. Importantly, the activity of SFN in this manner affects hundreds of genes, as at least 2% of genes are responsive to changing activity of HDACs (21). Additionally, many of those genes are microRNA genes which may have second degree effects on many other downstream targets.

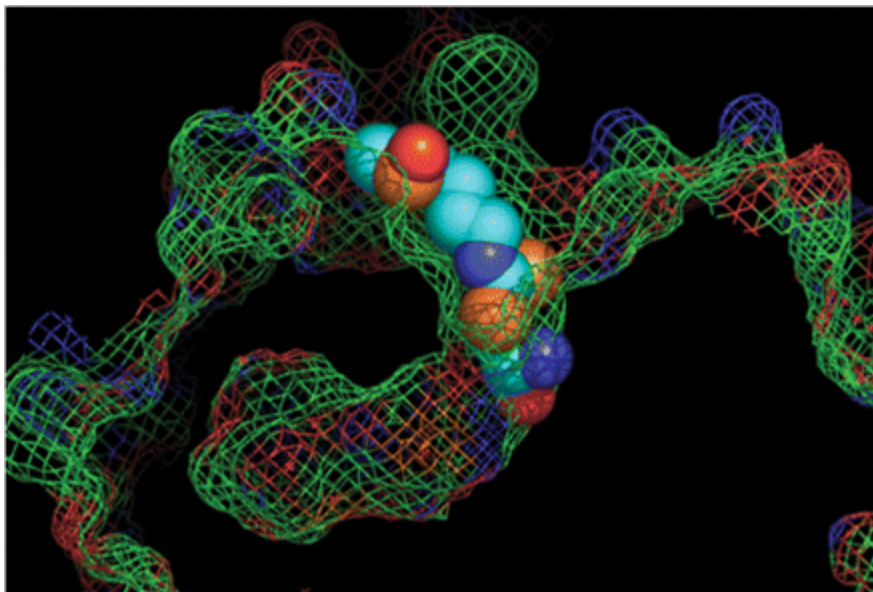


Figure 3. Modeling of a sulforaphane/HDAC interaction. One of the metabolites of sulforaphane, sulforaphane-cysteine fits into the HDAC active site, providing a mechanism by which sulforaphane inhibits HDAC activity. (Adapted from Dashwood, 2005)

One set of genomic loci known to be responsive to hyperacetylation of histones are long-terminal repeats (LTRs) (22). While these loci are typically heavily repressed, they can become active with increased acetylation and when they are de-repressed, it leads to higher risk of cancer and chromosomal abnormalities (23-29). LTRs can exist in a solitary form, meaning it is simply the promoter elements of the retrotransposon at a given genomic loci or they can exist as an in-tact LTR, meaning there are endogenous retrovirus sequences present along with the promoter sequences. While most LTRs exist in the solitary form, at least 60 in-tact, transcriptionally active LTRs have been identified

(30). By modification of global acetylation levels, retrotransposons may become active potentially resulting in translocation from one genomic loci to another (Fig. 4).

Depending on the insertion site of the retrotransposon, the result can be benign or severe, potentially resulting in tumor suppressor inactivation, oncogene activation, or disruption of other essential genes (31-33).

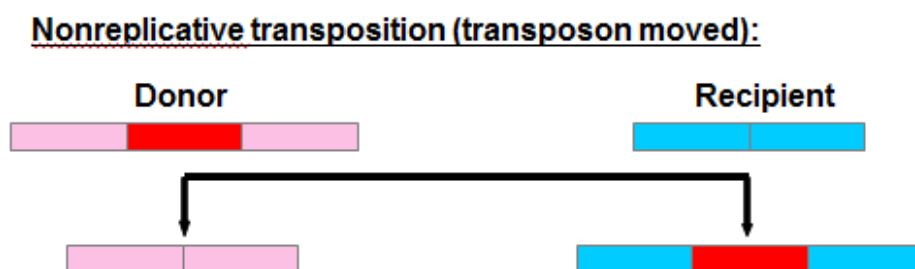


Figure 4. Retrotransposon translocates between different genomic loci. The de-repression of LTRs activates retro-transcription from its promoter, allowing its associated retrotransposon to be transferred from the donor loci to recipient loci in the genome.

(Adapted from Lewin, 2004)

Conclusion

Epigenetic regulation of genes may occur through many different mechanisms.

Transcriptional activity can be increased or decreased by modifying histone positioning based on environmental cues. Even more interestingly, microRNA present in the foods we eat may directly be regulating expression of endogenous genes. The following chapters will explore these ideas further and describe in detail the (i) the bioavailability of milk-derived microRNA as demonstrated in cell culture studies, human feeding studies,

and mouse feeding experiments, (ii) the bioavailability of chicken egg-derived microRNA and the biological activity of those exogenous microRNA, (iii) the stability of milk microRNA throughout processing and conversion of milk into other food products, and (iv) activation of LTRs following consumption of broccoli sprouts by human subjects.

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AUTHOR CONTRIBUTIONS

While a majority of the work presented in this dissertation is my own, I feel it is important to acknowledge where and in what way I contributed to each project. Chapters I and IV consist almost exclusively of my work in performing all experiments, statistical calculations and manuscript preparation. Chapter II is a joint project with Katherine Howard. While we both worked on sample collection, she performed more of that work and sample analysis. Both of us played a role in data analysis and writing for that work. Finally, the contribution I made to Chapter III was in training, supervision, statistical analysis, and helping with manuscript preparation. Each chapter contains a list of all authors who contributed to the content presented below. I thank them all for their efforts.

Scott Baier

April, 2015

CHAPTER I

MicroRNAs are Absorbed in Biologically Meaningful Amounts from Nutritionally Relevant Doses of Cow's Milk and Affect Gene Expression in Peripheral Blood Mononuclear Cells, HEK-293 Kidney Cell Cultures, and Mouse Livers¹⁻³

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Last names are underlined

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³ Supplemental tables 1 and 2 and supplemental figures 1 and 2 are available as Online Supporting Material with the online posting of this paper at <http://jn.nutrition.org>.

⁶Abbreviations used: C_{max}, maximal plasma concentration; *COL1A1*, collagen, type I, alpha 1; *KLF8*, Kruppel-like factor 8; miR, microRNA; PBMC, peripheral blood mononuclear cells; qRT-PCR, quantitative real-time polymerase chain reaction; *RUNX2*, runt-related transcription factor 2; t_{max}, time of peak concentration; *ZEB1*, zinc finger E-box binding homeobox 1.

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Authors' last names: Baier, Nguyen, Xie, Wood, Zempleni

1 **Abstract**

2 *Background* MicroRNAs regulate genes in animals and plants, and can be synthesized
3 endogenously. In milk, microRNAs are encapsulated in exosomes, thereby conferring
4 protection against degradation and facilitating uptake by endocytosis. The majority of
5 bovine microRNAs have nucleotide sequences complimentary to human gene transcripts,
6 suggesting that microRNAs in milk might regulate human genes.

7 *Objective* We tested the hypotheses that humans absorb biologically meaningful
8 microRNAs from nutritionally relevant doses of milk, milk-borne microRNAs regulate
9 human gene expression, and mammals cannot compensate for dietary microRNAs
10 depletion by endogenous microRNA synthesis.

11 *Methods* Healthy adults (3 male, 2 female, ages 26-49 years) consumed 0.25, 0.5, and 1.0
12 liter milk in a randomized cross-over design. Gene expression studies and milk
13 microRNA depletion studies were conducted in human cell cultures and mice,
14 respectively. For comparison, feeding studies with plant microRNAs from broccoli were
15 conducted in humans.

16 *Results* Postprandial concentration time curves suggest that meaningful amounts of
17 microRNA-29b (miR-29b) and microRNA-200c (miR-200c) were absorbed; plasma
18 concentrations of miR-1 did not change (negative control). The expression of *RUNX2*, a
19 known target of miR-29b, increased by 31% in blood mononuclear cells following milk
20 consumption compared with baseline. When milk exosomes were added to cell culture
21 media, mimicking postprandial concentrations of miR-29b and miR200c, reporter gene
22 activities significantly decreased by 44% and 17%, respectively, compared with vehicle
23 controls in HEK-293 human kidney cells. When C57BL/6J mice were fed a milk

24 microRNA-depleted diet for four weeks, plasma miR-29b levels were significantly
25 decreased by 61% compared with microRNA-sufficient controls, i.e., endogenous
26 synthesis did not compensate for dietary depletion. Broccoli sprout feeding studies were
27 conducted as a control and elicited no detectable increase in brassica-specific microRNA.

28 *Conclusion* We conclude that microRNAs in milk are bioactive food compounds that
29 regulate human genes.

30

31 Key words: bioavailability; bone health; dietary miRNA; milk; miR-29b

32 **Introduction**

33 MicroRNAs are small non-coding RNA (about 22 nucleotides in mature microRNA) that
34 may silence genes via destabilizing complementary mRNA sequences or preventing
35 translation of mRNA (1, 2). The nucleotide sequence in the seed region in microRNA
36 (nucleotides 2-7) is of particular importance for binding to target (3); imperfect pairing of
37 sequences in the seed region in microRNA to mRNA impairs gene downregulation at the
38 protein or RNA level (4). MicroRNAs are encoded by their own genes, introns, or exons
39 of long nonprotein-coding transcripts (5). Traditionally, microRNAs are considered
40 endogenous regulators of genes, i.e., microRNAs synthesized by human cells regulate the
41 expression of genes in that host. We challenged this paradigm and tested the hypothesis
42 that humans absorb microRNAs from milk in meaningful quantities and milk-borne
43 microRNAs elicit biologically meaningful changes in human gene expression.

44 There is precedent for the bioavailability of dietary microRNAs in humans. A recent
45 report suggests that microRNA-168a from rice (*Oryza sativa*, *osa*-miR-168a) is
46 detectable in human and animal sera, and *osa*-miR-168a decreases the expression of low-
47 density lipoprotein receptor adapter protein 1 mRNA, thereby inhibiting LDL receptor
48 expression in mouse liver (6). Note that the serum concentration of *osa*-miR-168a is only
49 about 3 fmol/L in humans and that the bioavailability of plant-borne microRNAs in
50 humans is controversial (6-10).

51 This study focused on milk as source of dietary microRNAs, based on the rationale
52 that a large fraction of microRNAs in milk is contained in exosomes, conferring
53 protection against degradation (11, 12), Americans consume 195 pounds of milk annually
54 (13), and cow's milk contains large quantities of 245 microRNA (14, 15). This study of

55 milk-borne microRNAs was modeled primarily on miR-29b based on the rationale that
56 the nucleotide sequence of bovine miR-29b is identical to that of human miR-29b (16),
57 and miR-29b increases bone mineralization in humans through promoting a gain in
58 osteoblast differentiation (17) and a loss in osteoclast differentiation and function (18). In
59 select experiments, miR-200c was included because its concentration is eight times that
60 of miR-29b in cow's milk (15). The nucleotide sequence of bovine miR-200c is identical
61 to that of human miR-200c (16), and miR-200c decreases cancer risk by targeting the
62 transcription factor zinc finger E-box binding homeobox 1 (*ZEB1*⁶); miR-
63 200c—dependent loss of *ZEB1* induces E-cadherin expression, thereby limiting
64 epithelial-to-mesenchymal transition — a key event in metastasis (19, 20).

65 This study was guided by the following aims. First, we determined whether humans
66 absorb quantitatively meaningful amounts of microRNA from nutritionally relevant doses
67 of milk, and characterized the bioavailability of milk microRNAs using pharmacokinetics
68 protocols. Second, we assessed the effects of physiologically relevant microRNA
69 concentrations on the expression of endogenous genes in human peripheral blood
70 mononuclear cells (PBMC)⁶ and reporter genes in human cell cultures. Third, we
71 determined whether mammals compensate for dietary microRNA deficiency by an
72 increased synthesis of endogenous microRNAs in a mouse microRNA depletion study.
73 Fourth, we conducted a broccoli feeding study to determine whether plant-borne
74 microRNAs are bioavailable in humans.

75

76 **Methods and Materials**

77 *Human feeding study.* Five apparently healthy adults (3 male, 2 female) participated in a
78 milk feeding study using three doses of milk in a randomized cross-over design with a
79 washout period of at least a one week between doses. Exclusion criteria included
80 pregnancy, smoking, milk allergies, and self-reported health problems. Preliminary
81 studies suggested that plasma miRNA concentrations remain higher than baseline
82 concentrations until 9 hours after milk consumption; subjects were instructed not to
83 consume milk and dairy products for twelve hours before the milk meal and during the
84 period in which blood samples were collected. Doses of milk were normalized by total
85 body water of subjects, which is the suspected volume of distribution for microRNA.
86 Estimation of total body water was calculated as previously described (21).
87 Normalization of doses by body water resulted in doses of 0.218 ± 0.018 , 0.436 ± 0.037 ,
88 and, 0.872 ± 0.073 L representing the equivalent of 0.25 L, 0.5 L, and 1.0 L in a 26 year-
89 old reference male (75 kg weight, 1.83 m height). Twenty milliliters of blood were
90 collected before milk consumption (baseline, time 0 h) and at timed intervals (1, 2, 3, 6,
91 9, 24 h) after the milk meal. PBMC and plasma were collected using gradient
92 centrifugation as described previously (22) and frozen at -80°C until analysis. This
93 protocol was approved by the University of Nebraska-Lincoln Institutional Review Board
94 and all participants provided signed informed consent forms prior to participation.
95
96 *Quantitative real-time PCR (qRT-PCR).* The sequences of mature bovine miR-29b (*bta*-
97 miR-29), miR-200c (*bta*-miR-200c), and miR-1 (*bta*-miR-1; control) are identical to their
98 human and murine orthologs and, therefore, qRT-PCR amplification targeted the mature
99 human miR-29b (*hsa*-miR-29b-3p), miR-200c (*hsa*-miR-200c-3p), and miR-1 (*hsa*-miR-

1-3p) that originate in the 3' p arm of pre-miRNA (23). RNA was isolated from human and murine (see below) plasma using the NucleoSpin miRNA Plasma kit (Machery-Nagel, Bethlehem, PA). Reverse transcription was performed using the miScript II RT Kit. qRT-PCR was performed using miScript SYBR Green (Qiagen, Valencia, CA) and the universal reverse primer included in the kit primers specific for individual microRNA (**Supplemental Table 1**). MicroRNA-1 is not detectable in milk (data not shown) and served as negative control. Five attomoles of synthetic miRNA, miSPIKE (IDT DNA, Coralville, IA), were added to each sample after denaturation of plasma with lysis buffer and served as external standard and for calibration of qRT-PCR.

Pharmacokinetics analysis. Areas under the curves for plasma miRNA were calculated using the linear trapezoidal rule to assess the apparent bioavailability of microRNA (24). Plasma concentrations of miR-29b and miR-200c returned to baseline levels nine hours after consumption of the two lowest doses of milk. For the calculation of AUC, baseline values were subtracted from postprandial concentrations and the plasma concentrations from the first nine hours after milk meals were used. The maximal plasma concentration (C_{\max}) and the time of peak concentration (t_{\max}) were obtained by visual inspection of the plasma time curves measured for miR-29b and miR-200c. C_{\max} of an orally administered compound is a marker of bioavailability and rate of absorption, whereas t_{\max} is a marker of the approximate absorption site (25).

Gene expression. Effects of milk-borne microRNAs on the expression of human genes were assessed in cell cultures and PBMC from the human milk feeding studies. For

123 studies in cell cultures, HEK-293 human embryonic kidney cells (American Type Culture
124 Collection, Manassas, VA) were cultured in Minimum Essential Medium containing 10%
125 exosome-depleted fetal bovine serum, 0.1% sodium pyruvate, 100,000 U/L penicillin,
126 and 100 mg/L streptomycin for 10 days. Bovine serum was depleted of exosomes by
127 ultracentrifugation at $130,000 \times g$ for 4 hours, leading to the removal of 97% and 81% of
128 miR-29b and miR-200c, respectively. Cells were transfected with microRNA reporter
129 genes as described previously (26). Forty-eight hours after transfection, exosome-
130 depleted media was replaced with exosome-sufficient media at final concentrations of
131 600 fmol/L miR-29b or 1000 fmol/L miR-200c. Exosome-sufficient media were prepared
132 using exosomes collected from cow's milk as previously described (27). The
133 concentrations of miR-29b and miR-200c in exosomes were quantified using qRT-PCR,
134 and exosomes were added back to culture media to produce the desired concentrations of
135 microRNA. Reporter genes for miR-29b and miR-200c were created by inserting the 3'-
136 untranslated regions (3'-UTR) from genes *COL1A1* (three miR-29b binding sites) and
137 *ZEB1* (two miR-200c binding sites), respectively, downstream of the luciferase reading
138 frame, driven by a cytomegalovirus promoter, thereby creating plasmids LUC-mir29b
139 and LUC-mir200c (**Supplemental Fig. 1**). LUC-mir200c was obtained from Dr. Thomas
140 Brabletz (University of Freiburg, Germany) and is denoted *ZEB1* 3'UTR-Luc in the
141 original publication (20). LUC-mir29b was created by digesting LUC-mir200c with *MluI*
142 and *HindIII* to remove the *ZEB1* sequence. The 3'-untranslated regions of *COL1A1* was
143 amplified by PCR (**Supplemental Table 1**) using IMR-90 fibroblast DNA as template
144 and ligated into the reporter plasmid using *MluI* and *HindIII*. Cells were co-transfected
145 with plasmid RSV- β -galactosidase and luciferase plasmids to assess transfection

146 efficiency (28). Reporter gene activities were normalized by transfection efficiency. Our
147 rationale for choosing HEK-293 cells was that these cells can be transfected with near
148 100% efficiency and express *COL1A1* in meaningful quantities (29, 30).

149 For studies in human PBMC, a preliminary time-response screen was conducted to
150 determine the time point when the effects of milk on the expression of Runt-related
151 transcription factor 2 (*RUNX2*) and , zinc finger E-box binding homeobox 1 (*ZEB1*)
152 mRNA were maximal. *RUNX2* is a downstream target of miR-29b-dependent signaling
153 pathways; note that miR-29b is a positive regulator of the expression of *RUNX2* (17). Our
154 preliminary assessment suggested that changes in mRNA abundance were maximal 6 h
155 after milk consumption (**Supplemental Fig. 2**, positive and negative changes for *RUNX2*
156 and *ZEB1* mRNA, respectively). Therefore, subsequent assays of *RUNX2* and *ZEB1*
157 mRNA were conducted using samples from time point 6 h after the milk meal, using
158 qRT-PCR (Online Table 1) and the $\Delta\Delta C_t$ method (31); GAPDH was used to normalize
159 for amplification efficiency (32). Abundance of miRNA was measured as described for
160 plasma samples except that normalization was performed using U6 rather than miSPIKE.

161

162

163 *Mouse feeding study.* A microRNA depletion study was conducted in mice to determine
164 whether endogenous microRNA synthesis compensates for dietary deficiency. Ten
165 female C57BL/6J mice (Jackson Labs, stock 000664), age 3 weeks, were randomly
166 divided between a milk microRNA-depleted treatment group (denoted ExoMinus) and a
167 milk microRNA-sufficient control group (ExoPlus). Diets were based on the AIN-93G
168 formula with the following modifications and were fed for four weeks (33). In the

169 ExoMinus diet, exosome-depleted fat-free cow's milk was substituted for casein and
170 cornstarch in the AIN-93G diet to provide 10% of total calories (**Supplemental Table 2**);
171 the remainder of casein in the AIN-93G diet was replaced with soy protein (Bob's Red
172 Mill, Milwaukee, OR) to eliminate any milk-borne microRNAs. Milk was depleted of
173 exosomes by ultrasonication for 60 min (VWR Aquasonic 250T) and incubation at 37°C
174 for 60 min prior to lyophilization. Ten percent of calories represent a consumption of
175 approximately 0.5 liter of fat-free milk by an adult, based on 352 kcal/L milk and an
176 energy intake of 1760 kcal (34). The ExoPlus control diet was prepared using exosome-
177 containing milk powder, but was otherwise identical to the ExoMinus diet. Components
178 were blended and dried at 40°C overnight. No miR-29b was detectable in the ExoMinus
179 diet whereas the ExoPlus diet contained about 83 fmol/g miR-29b, assessed by qRT-
180 PCR. All mouse protocols used in this study were approved by the University of
181 Nebraska-Lincoln Institutional Animal Care and Use Committee. After four weeks of
182 feeding microRNA-defined diets, mice were euthanized by carbon dioxide and blood was
183 collected in EDTA collection tubes. Livers were excised, rinsed with cold saline, and
184 flash frozen in liquid nitrogen. Ten milligrams of liver were homogenized using lysis
185 buffer in the NucleoSpin miRNA Kit (Machery-Nagel, Bethlehem, PA) and mRNA was
186 purified following the manufacturer's instructions. Reverse transcription was performed
187 with the High Capacity RNA-to-cDNA Kit (Life Technologies, Grand Island, NY) for
188 subsequent analysis of Kruppel-like factor 8 (KLF8) expression using qRT-PCR
189 (Supplemental Table 1).
190

191 *Broccoli-borne microRNAs in humans.* The bioavailability of plant-borne microRNAs is
192 controversial (6-10). We used plasma samples archived from a previous broccoli sprout
193 feeding study in humans to determine whether broccoli-borne microRNAs are detectable
194 in prandial plasma samples (35). In this previous study, eight healthy adults were fed 34,
195 68, and 102 g of broccoli sprouts and timed samples were collected at $t = 0, 2, 4, 8,$ and
196 24 hours. Here, we analyzed samples collected at $t = 0$ and 4 h from the highest dose of
197 broccoli sprouts for miR-824 and miR-167a in four randomly selected subjects. Total
198 RNA was isolated from 75 mg of broccoli sprouts using Trizol. Samples were analyzed
199 for miR-824 and miR-167a using qRT-PCR (Supplemental Table 1). As of today, there is
200 no evidence that humans synthesize miR-824 and miR-167a (36), i.e., the brassica-
201 specific miR-824 and plant-specific miR-167a are good markers of broccoli-borne
202 microRNAs in human blood.

203

204 *Statistics.* Homogeneity of variances was tested using Bartlett's Test. Variances were
205 heterogeneous for microRNA in mouse plasma, i.e., murine plasma data were log
206 transformed prior to subsequent statistical analysis. AUC were calculated using
207 GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Pharmacokinetics data were
208 analyzed using repeated measures analysis of variance (ANOVA) and Fisher's PLSD for
209 posthoc comparisons (37). PBMC gene expression data was analyzed using the paired t-
210 test, whereas plasma miRNA concentrations and liver gene expression in mice were
211 analyzed using the Wilcoxon-Signed Rank Test. StatView 5.0.1 was used for statistical
212 analyses (SAS Institute; Cary, NC). Differences were considered statistically significant
213 if $p < 0.05$.

214

215 **Results**

216

217 *Bioavailability of milk-borne microRNAs in humans.* Cow's milk (1% fat) contained
 218 148±42 pmol/L miR-29b and 680±151 pmol/L miR-200c. Humans absorbed considerable
 219 amounts of miR-29b from cow's milk (**Fig. 1A**). In contrast, the plasma concentration of
 220 miR-1 did not change after the consumption of 1 liter milk (negative control). Plasma
 221 miR-29b concentrations returned to baseline concentrations nine hours after the milk
 222 meals for the 0.25-liter and 0.5-liter doses and 24 hours after the 1-liter dose. The AUC
 223 for miR-29b exhibited a linear dose-response relationship regarding the amount of milk
 224 consumed (**Table 1**). Likewise C_{\max} increased linearly with the amount of milk
 225 consumed, if C_{\max} was corrected for baseline concentrations of miR-29b. T_{\max} was 3.4 to
 226 4.2 hours after the milk meal. MiR-200c is another microRNA present in cow's milk.
 227 Humans also absorbed considerable amounts of miR-200c from cow's milk (**Fig. 1B**).
 228 While the postprandial AUC for miR-200c was significantly higher for the 0.5 L and 1.0
 229 L doses of milk compared to the 0.25 L dose, the AUC did not exhibit a linear dose-
 230 response relationship; the AUC were not significantly different between the 0.5 L dose
 231 and the 1.0 L dose (Table 1).

232

233 *Effects of milk microRNAs on the concentrations of microRNAs in human PBMC.* Milk
 234 feeding elicited an increase in the concentrations of microRNA in human PBMC that was
 235 statistically significant only for miR-200c while trending towards significance for miR-
 236 29b ($P = 0.09$) (**Fig. 2**).

237

238 *Effects of milk microRNAs on human gene expression.* MicroRNA-containing milk
 239 exosomes affected the activity of reporter gene plasmids. When HEK-293 cells were
 240 cultured in media supplemented with milk exosomes providing 600 fmol/L miR-29b for 4
 241 h, the activity of the miR-29b—dependent reporter gene LUC-mir29b decreased by
 242 $44 \pm 13\%$ ($P = 0.04$) compared with cells cultured in exosome-depleted culture media
 243 (**Fig. 3A**). Likewise, when HEK-293 cells were cultured in media supplemented with
 244 milk exosomes providing 1000 fmol/L miR-200c for 4 h, the activity of the miR-
 245 200c—dependent reporter gene LUC-mir200c decreased by $17 \pm 3\%$ ($P = 0.02$) compared
 246 with cells cultured in exosome-depleted culture media.

247 Milk feeding caused changes of microRNA target gene expression in human
 248 PBMC. The expression of *RUNX2* was $31 \pm 13\%$ higher 6 h after the milk meal compared
 249 with time 0 h (Fig. 3B, $P = 0.04$), consistent with the role of miR-29b as positive
 250 regulator of *RUNX2* (17). The difference in the expression of *ZEB1* was not significantly
 251 lower 6 h after the milk meal compared with time 0 h (Fig. 3B).

252

253 *MicroRNA depletion studies in mice.* Endogenous microRNA transcription does not
 254 compensate for dietary deficiency in C57BL/6 mice. When mice were fed miRNA-
 255 defined diet for four weeks, the miR-29b plasma concentration decreased by 61% from
 256 152 ± 79 fmol/L in the microRNA-sufficient ExoPlus group to 60 ± 27 fmol/L in the
 257 microRNA-depleted ExoMinus group ($P = 0.04$). The expression of KLF8 mRNA was
 258 $64 \pm 32\%$ lower in microRNA-depleted mice compared with microRNA-sufficient mice (P
 259 $= 0.04$).

260

261 *Bioavailability of broccoli microRNAs in humans.* Broccoli sprouts contained 53 ± 16
262 pmol/kg of miR-167a and 42 ± 10 pmol/kg miR-824. No change was observed in miR-
263 167a plasma concentration in healthy adults following a broccoli sprout meal (13 ± 4
264 fmol/L at $t=0$ h vs. 15 ± 7 fmol/L at $t=4$ h; $P = 0.85$; $n=4$). The concentration of miR-824
265 was below detection limit (<1 fmol/L) at baseline or 4 h after broccoli sprout feeding in
266 all of the four subjects randomly chosen from our archived plasma samples (35).

267

268 **Discussion**

269 Some non-coding RNA, including microRNAs, play essential roles in gene regulation in
270 plant and animal kingdoms. To the best of our knowledge, this is the first study to report
271 that humans absorb quantitatively meaningful amounts of microRNA from nutritionally
272 relevant amounts of cow's milk. Our study provides unambiguous evidence that the
273 amounts of microRNAs absorbed from milk are sufficient to alter human gene
274 expression, i.e., microRNAs from one mammalian species can affect gene networks in
275 another species. Our observation that endogenous microRNA synthesis cannot
276 compensate for dietary deficiency is of particular interest, because it implies that a
277 regular dietary microRNA intake may be important to prevent aberrant gene regulation.

278 Our observations are important from the perspective of maintaining human health,
279 based on the following rationale. First, 245 microRNAs have been identified in cow's
280 milk (14). A preliminary analysis by sequence alignment suggests that the majority of the
281 nucleotide sequences in bovine milk match the sequences of their human orthologs. We
282 are in the process of developing algorithms for predicting human gene targets for bovine

283 microRNAs, and initial assessments suggest that the number of target genes will exceed
284 11,000. Second, there is unambiguous evidence linking microRNAs with human health.
285 For example, miR-29b promotes bone health through altering osteoblast and osteoclast
286 differentiation (17, 18), miR-200c decreases cancer risk by targeting the transcription
287 factor *ZEB1* (19, 20), and miR-15b, miR-21, miR-27b, miR-34a, miR-106b, miR-130a,
288 miR-155, miR-200c and miR-223 have been implicated in immune function and Crohn's
289 disease (15, 38). Third, supplementation and depletion of milk altered microRNA
290 concentrations and gene expression in human PBMC and mouse liver. For example, the
291 concentration of miR-200c was greater six hours after a milk meal compared with time
292 zero in human PBMC. Likewise, the expression of KLF8 mRNA was greater in livers
293 from milk microRNA-sufficient mice compared with milk microRNA-depleted mice.
294 Fourth, milk is an important staple in many Western diets. For example, Americans
295 consume large quantities of milk, despite a steady decline from about 237 pounds in 1987
296 to 195 pounds in 2012 (13, 39). Note that a large fraction of microRNAs in milk is
297 contained in exosomes, providing protection against degradation (11, 12). Fifth, mice can
298 be depleted of microRNAs by feeding a microRNA-depleted diet.

299 A recent report suggests that miR-168a from rice is detectable in human and
300 animal sera, and *osa*-miR-168a decreases the expression of LDL receptor adapter protein
301 1 in mouse liver (6). The bioavailability of plant-borne microRNAs in humans is
302 controversial (6-10). Note that this research focuses on milk-borne microRNAs for which
303 postprandial plasma levels are 200-300 times higher than those reported for *osa*-miR-
304 168a in humans (6), depending on the species of milk microRNA. We are skeptical of the
305 bioavailability and biological activity of plant-borne microRNAs and conducted a

306 preliminary screen of microRNAs following a meal providing large amounts of broccoli
307 sprouts. In our broccoli sprouts feeding study, we did not observe a postprandial increase
308 in the *Brassica*-specific miR-824 or miR-167a. We speculate that the absence of effect in
309 broccoli feeding studies might be due to one or some of the following factors. First,
310 exosomes carry numerous surface proteins that might be important for cellular uptake
311 (40, 41). The identity and amino acid sequence of surface proteins implicated in the
312 cellular uptake of exosomes differs between plants and mammals, which might adversely
313 affect the uptake of plant exosomes by human intestinal cells. Second, it is possible that
314 the methylation of the 3'-terminal ribose in plant microRNAs by the methyl transferase
315 HEN1 (42) impairs the intestinal transport of microRNAs. Third, the concentrations of
316 microRNAs in broccoli sprouts are moderately below the concentrations present in milk.
317 It is also unclear whether plant-based microRNAs are encapsulated in vesicles that
318 provide protection against enzymatic and non-enzymatic degradation during food
319 processing. Fourth, it is possible that the number of human genes with sequences
320 complimentary to plant microRNAs might be less than those in mammalian microRNAs.
321 We acknowledge that our broccoli feeding study is associated with some uncertainties,
322 e.g., random selection of two microRNAs and one postprandial time point.
323 Notwithstanding these uncertainties, the analysis of samples from the broccoli feeding
324 study provides a valuable negative control for our studies in milk.

325 There are some uncertainties associated with our studies of milk microRNAs.
326 First, this study assessed the apparent bioavailability of microRNAs in milk, without
327 taking into account metabolism and degradation in intestinal cells and liver. Ongoing
328 studies of microRNA transport mechanisms suggest that a substantial fraction of some

329 microRNA is degraded in intestinal cells. Therefore, our bioavailability data probably
330 underestimate the true extent of microRNA absorption in humans, as only a fraction of
331 the absorbed microRNAs will appear in the peripheral circulation. Second our
332 pharmacokinetics analysis suggests that postprandial plasma microRNA concentrations
333 peak about three to four hours after milk consumption. The t_{\max} values for microRNAs
334 are slightly later than t_{\max} observed for the vitamin riboflavin ($t_{\max} \sim 2$ h) (24), which is
335 absorbed in the duodenum (43). Considering that milk meals delay gastric emptying (44),
336 we speculate that milk-borne-microRNAs are absorbed primarily in the upper intestine.
337 Third, this study focused on miR-29b, miR-200c, and miR-1 (negative control) and did
338 not formally study other microRNAs in milk. We speculate that milk exosomes enter
339 human intestinal by a mechanism that is shared by all exosomal microRNAs, and that any
340 discrimination among microRNAs would only occur after intestinal uptake.

341 Ongoing and planned activities in our laboratory include the characterization of
342 intestinal microRNA transport mechanisms, including metabolism and basolateral
343 secretion, and the characterization of milk microRNA-dependent gene networks in
344 humans using computational biology approaches.

345

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347 Each author contributed to the development of this work. SB and JZ designed research
348 and wrote the paper. SB conducted research, analyzed data, and conducted statistical and
349 pharmacokinetics analyses. CN, FX, and JW designed and conducted research. JZ had
350 primary responsibility for final content. All authors read and approved the final
351 manuscript.

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TABLE 1 Pharmacokinetics analysis of plasma time curves of microRNAs after milk meals in healthy adults.

Variable	miR-29b			miR-200c			miR-1
	Dose			Dose			Dose
	0.25 L	0.5 L	1.0 L	0.25 L	0.5 L	1.0L	1.0 L
Baseline (fmol/L) ¹	224 ± 43	274 ± 37	232 ± 51	375 ± 81	468 ± 103	458 ± 37	80 ± 21
C _{max} (fmol/L)	372 ± 40 ^a	484 ± 82 ^{ab}	624 ± 83 ^b	632 ± 104 ^a	819 ± 123 ^{ab}	924 ± 121 ^b	91 ± 18
t _{max} (h)	4.2 ± 1.5	3.4 ± 0.7	3.6 ± 0.6	2.6 ± 0.9	3.8 ± 1.0	3.6 ± 0.6	
AUC (fmol/L*h) ²	327 ± 249 ^a	672 ± 231 ^a	1900 ± 275 ^b	646 ± 187 ^a	1800 ± 566 ^b	2260 ± 555 ^b	

Values are means ± SEMs, n=5

¹Plasma concentration at time 0 hours.

²For hours 0 through 9.

^{a,b}Within a variable for the same miRNA, means without a common letter differ ($P < 0.05$, n =5).

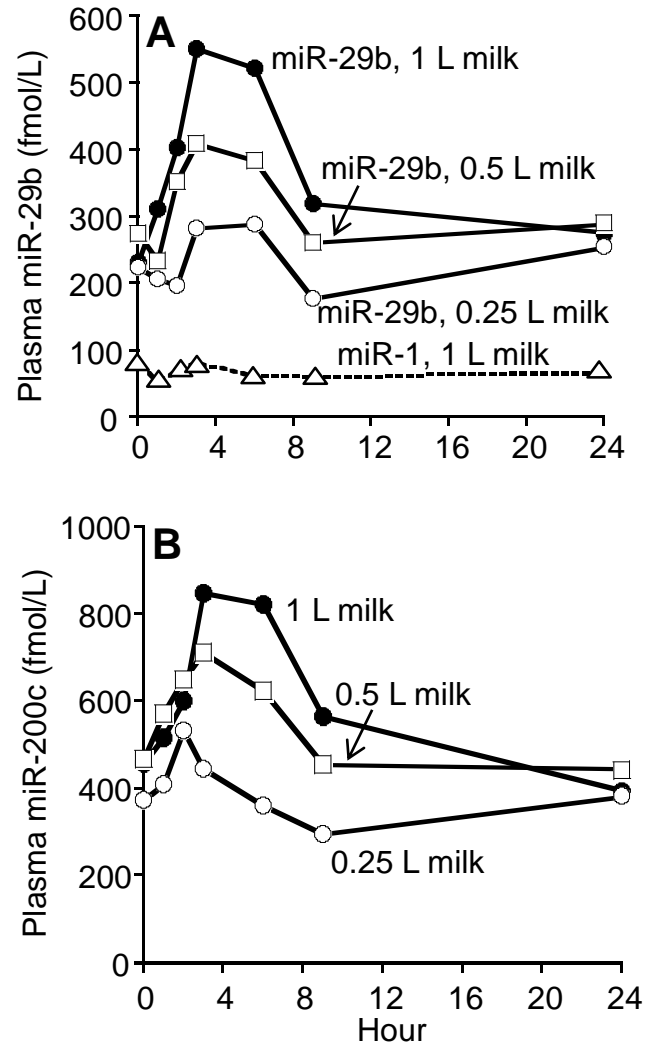


FIGURE 1 Plasma time curves of miR-29b (panel A), miR-1 (negative control, panel A), and miR-200c (panel B) following a milk meal in healthy adults. Values are reported as means, $n = 5$. Standard deviations have been omitted for clarity (compare Table 1).

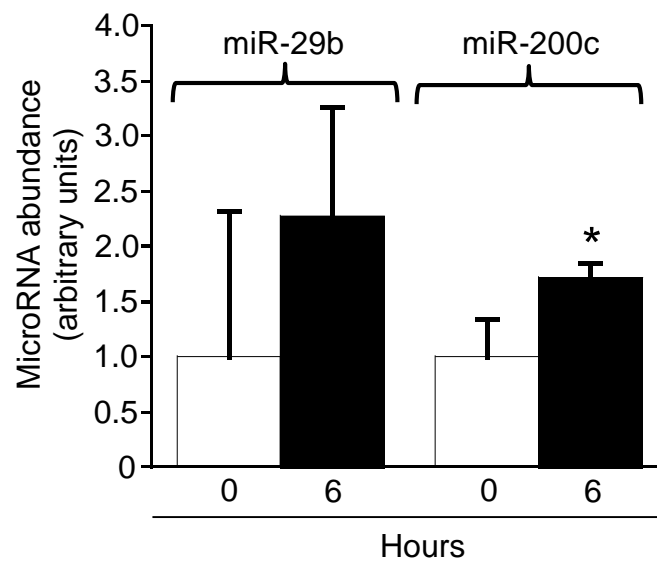


FIGURE 2 Effects of milk microRNAs on the abundance of miRNA in human peripheral blood mononuclear cells. Values are mean \pm SEM, $n = 5$. * Statistically different samples indicated by asterisk, $P < 0.05$.

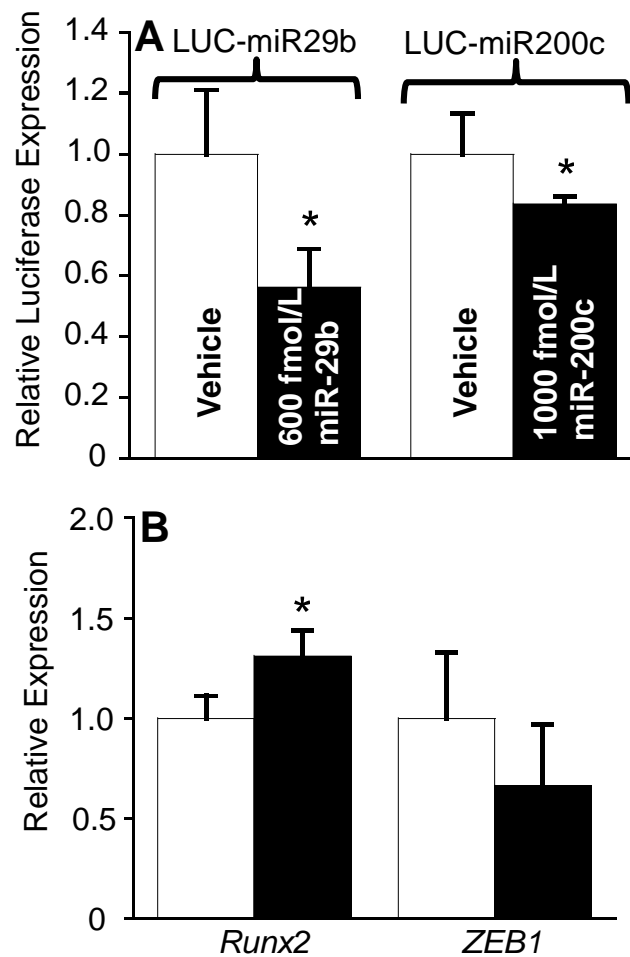


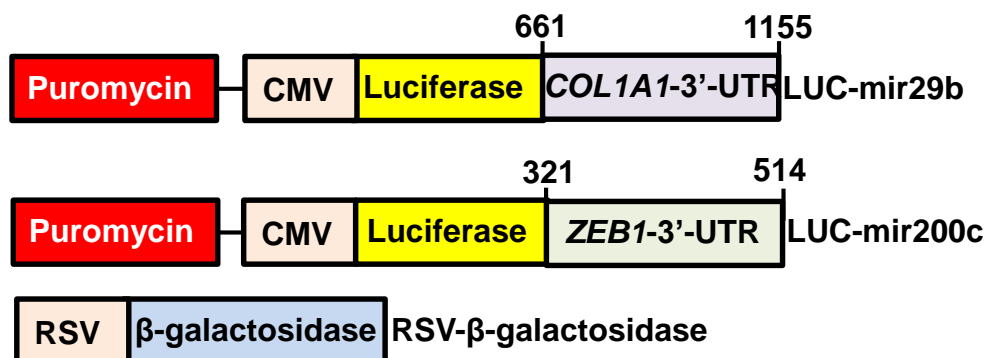
FIGURE 3 Effects of milk microRNAs on gene expression in humans. (A) Activities of microRNA reporter genes in HEK-293 cells. (B) Expression of endogenous *RUNX2* and *ZEB1* genes in PBMC from healthy adults. Data are mean \pm SEM, $n = 4$ (LUC-miR29b), $n = 6$ (LUC-miR200c), $n = 5$ (PBMC). *Different from vehicle, $P < 0.05$. *RUNX2*, Runt-related transcription factor 2; *ZEB1*, zinc finger E-box binding homeobox 1.

SUPPLEMENTAL TABLE 1 Primers used for the quantification of microRNAs in human and murine plasma, mRNA abundance in human PBMC and murine livers, and creating the human COL1A1 reporter plasmid.

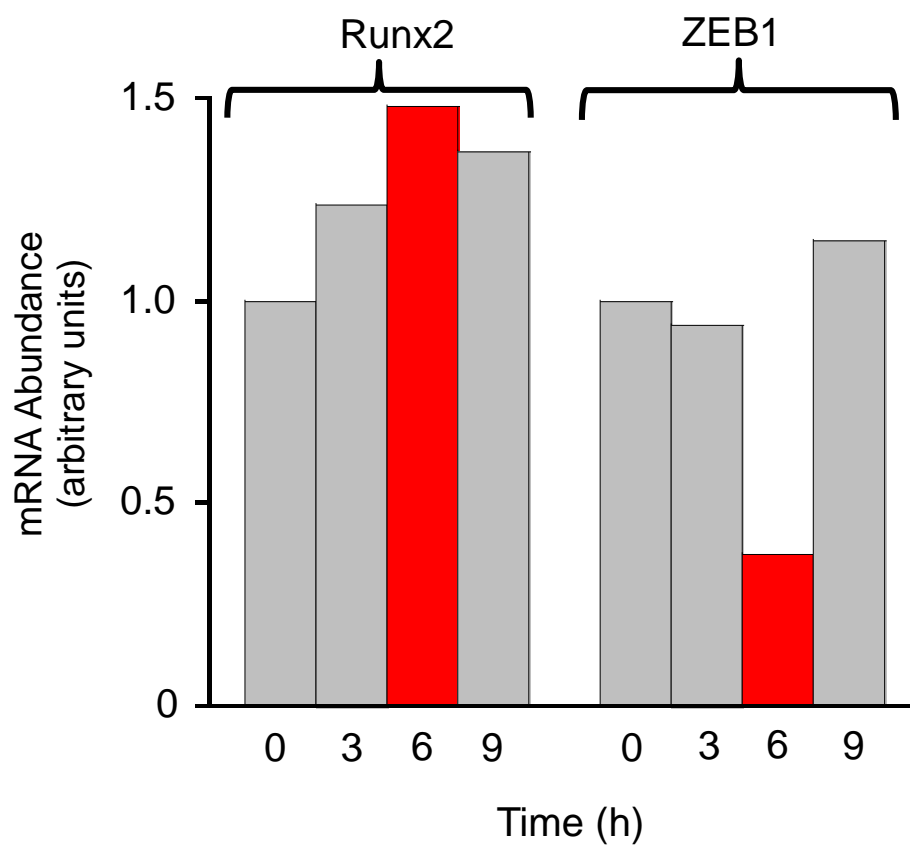
Amplicon	Forward Primer (5'-3')	Reverse Primer (5'-3')
miSPIKE	CTCAGGATGGCGGAGCGGTCT	
U6	CGCAAGGATGACACGCAAATT	
miR-29b	GTAGCACCATTGTAATCAGTGTT	
miR-200c	TAATACTGCCGGGTAATGATGGA	
miR-1	TGGAATGTAAAGAAGTATGTAT	
miR-167a	TGAAGCTGCCAGCATGATCTA	
miR-824	TAGACCATTGTTGAGAAGGGA	
GAPDH	TCCACTGGCGTCTTCACC	GGCAGAGATGATGACCCTTT
ZEB1 ¹	TTCAAACCCATAGTGGTTGCT	TGGGAGATACCAAACCAACTG
RUNX2	CGCCCCTCCCTGAACTCT	TGCCTGCCTGGGATCTGTA
KLF8 (ZNF74)	CAAGCCATTATGGTGCCTAC	ATAGAGCCCGGAGTGAGAAC
COL1A1 3'-UTR	CGTGCGCATTCGGAGGAGAGTC	CGAAGCTTGAGGCCTGAGAAGCC

¹Abbreviations used: *COL1A1*, collagen, type I, alpha 1; *KLF8*, Kruppel-like factor 8; *RUNX2*, runt-related transcription factor 2; *ZEB1*, zinc finger E-box binding homeobox 1.

SUPPLEMENTAL FIGURE 1 Schematic of microRNA reporter plasmids and the β -galactosidase control plasmid. Numerals denote the position of nucleotides in the 3'-UTR in collagen, type I, alpha 1 (*COL1A1*, GenBank NM_000088) and zinc finger E-box binding homeobox 1 (*ZEB1*, GenBank NM_001128128).



SUPPLEMENTAL FIGURE 2 Time courses of runt-related transcription factor 2 (*RUNX2*) and zinc finger E-box binding homeobox 1 (*ZEB1*) mRNA in peripheral blood mononuclear cells from an adult male.



SUPPLEMENTAL TABLE 2 Composition of microRNA-depleted (ExoMinus) and microRNA-sufficient (ExoPlus) diets.

ExoMinus	ExoPlus	g/kg
Cornstarch	Cornstarch	330
Soy Protein	Soy Protein	163
Dextrinized cornstarch	Dextrinized cornstarch	132
Milk Powder (without exosomes) containing ~5% lactose	Milk Powder (with exosomes) containing ~5% lactose	105
Sucrose	Sucrose	100
Soybean oil (no additives)	Soybean oil (no additives)	70
Fiber	Fiber	50
Mineral mix (AIN-93G-MX) ¹	Mineral mix (AIN-93G-MX)	35
Vitamin mix (AIN-93G-VX)	Vitamin mix (AIN-93-VX)	10
L-Cystine	L-Cystine	3
Choline bitartrate (41.1% choline)	Choline bitartrate (41.1% choline)	2.5
Tert-butylhydroquinone	Tert-butylhydroquinone	0.014

¹Abbreviation used: AIN-93G, American Institute of Nutrition growth diet; MX, mineral mixture; VX, vitamin mixture.

CHAPTER II

**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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Tables: 1

Running Title: MicroRNAs in chicken eggs are bioavailable

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Center, the University of Nebraska Agricultural Research Division (Hatch Act), and
USDA multistate group W3002

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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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Abstract

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. 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.

Introduction

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 that 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- κ B nuclear translocation resulting in an inhibition of inflammatory gene expression, NF- κ B

activation and atherosclerosis in mice by targeting importin- α 3, a protein used for NF- κ B translocation to the nucleus. (8) By inhibiting NF- κ B translocation, expression of inflammatory genes is reduced (9-11). In addition to indirect targeting through regulation NF- κ B 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 300 uL plasma using the NucleoSpin miRNA Plasma kit with a final elution volume of 30 uL (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 $\Delta\Delta C_t$ 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 was 19 ± 3 % lower (Fig. 3, $P = 0.02$)

Discussion

Few foods are as nutrient dense as eggs. Eggs are abundant in fat soluble vitamins, carotenoids, 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 determined 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 observed similar effects regarding egg miRNAs following consumption of hard boiled eggs by healthy adults. This paper shows the importance of 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 associated with risk of coronary heart disease or stroke (20). Additionally, patients presenting with sepsis have decreased levels of miR-181b compared to control patients (8).

BCL2 and BCL2A1, known targets of NF- κ B, are regulators of cell death exhibiting both pro-apoptotic and 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 shown 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 hypothesis that animal-based food products are dietary sources of miRNA.

TABLE 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 ± SD, n=7

¹Plasma concentration at time 0 hours.

²For hours 0 through 24.

^{a,b}Within a variable for the same miRNA, means without a common letter differ ($P < 0.05$, n =7).

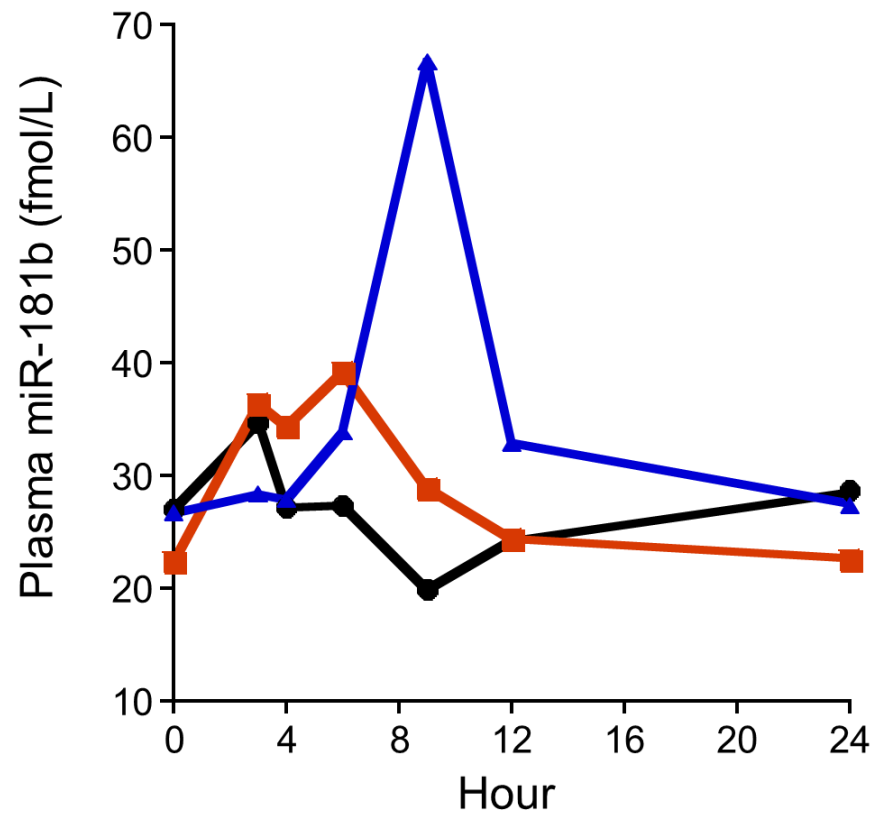


FIGURE 1 Plasma time curves of miR-181b following an egg meal in healthy adults. Different doses are represented by colored lines: black (2 eggs), red (3 eggs), and blue (4 eggs). Values are reported as means, $n = 7$. Standard deviations have been omitted for clarity (compare Table 1).

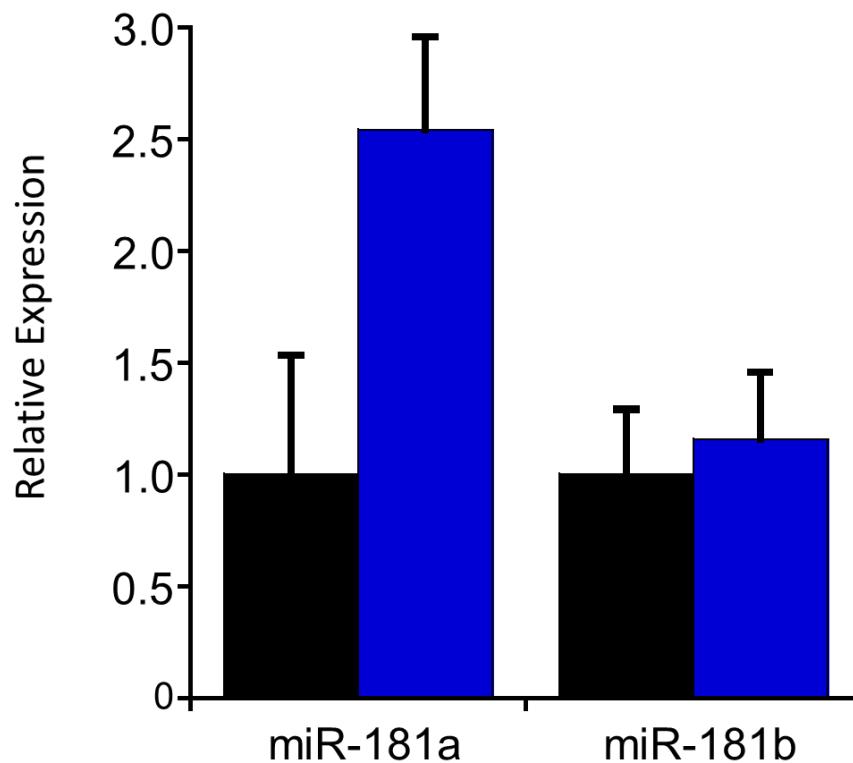


FIGURE 2 Effects of egg microRNAs on the abundance of miRNA in human erythrocytes. Black bars represent baseline (Hour 0) values and blue bars represent miRNA abundance following egg consumption (Hour 9). Values are mean \pm SD, $n = 7$. Labeled means without a common letter differ, $P < 0.05$.

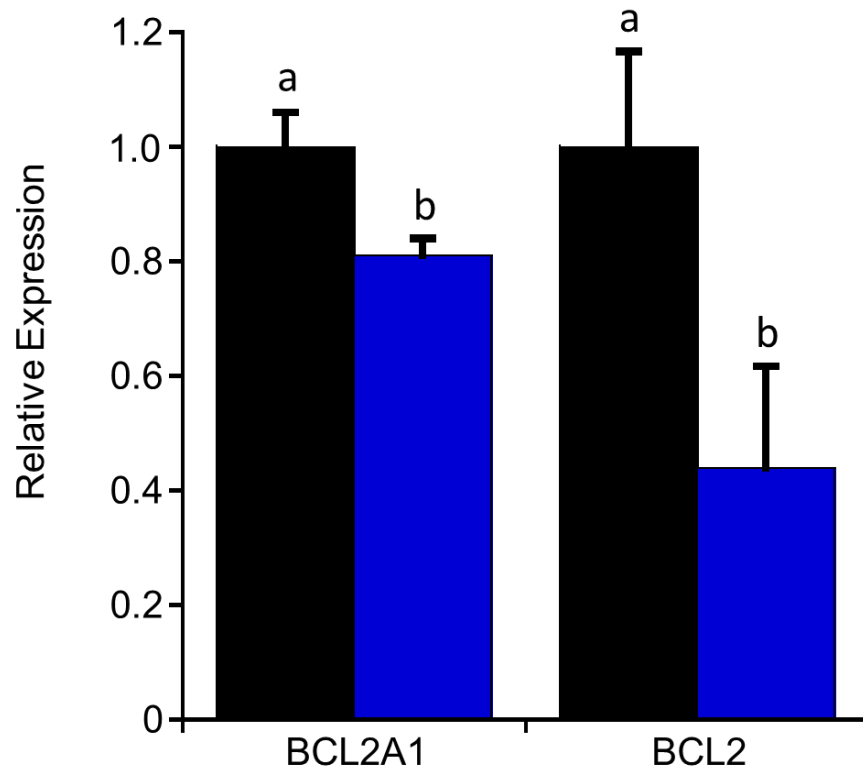


FIGURE 3 Effects of egg microRNAs on *BCL2* and *BCL2A1* gene expression in PBMC from healthy adults. Data are mean \pm SD, $n = 7$ (Hour 0, black bars; Hour 9, blue bars). Labeled means without a common letter differ, $P < 0.05$. *BCL2*, (B-cell lymphoma 2; *BCL2A1*, BCL2-related protein A1.

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CHAPTER III

Loss of MiRNAs During Processing and Storage of Cow's (*Bos taurus*) Milk

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

1 **ABSTRACT**

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

14

15 Key words: heating, MiRNAs, milk, processing, storage

16

17 INTRODUCTION

18 MicroRNAs (miRs, miRNAs) are small non-coding RNAs that play essential roles in the
19 regulation of genes at the posttranscriptional level in plants and animals.¹ Mature
20 miRNAs are about 22 nucleotides long and bind to complementary sequences in the 3'-
21 untranslated region of mRNAs. Perfect or near perfect base pairing of the miRNAs and
22 its target mRNAs typically results in mRNAs degradation, whereas less perfect base
23 pairing typically results in inhibition of mRNAs translation.²⁻³ Traditionally, miRNAs
24 have been considered endogenous regulators of genes, i.e., miRNAs synthesized by a
25 given host regulate the expression of genes in that host. Recently, our laboratory refuted
26 this paradigm. We provided strong evidence that 1) humans absorb biologically
27 meaningful amounts of miRNAs from nutritionally relevant doses of cow's milk, 2)
28 physiological concentrations of milk miRNAs affect human gene expression *in vivo* and
29 in cell cultures, and 3) endogenous synthesis of miRNAs does not compensate for dietary
30 miRNA deficiency in mice.⁴ Our discoveries were largely modeled on miR-29b and miR-
31 200c, but likely hold true for all miRNAs encapsulated in milk exosomes.⁵⁻⁶ To the best
32 of our knowledge, our previous paper is the first to provide unambiguous evidence that
33 miRNAs can be transferred between distinct species through dietary means. In contrast,
34 previous claims that miRNAs from plants affect human gene expression⁷⁻⁸ are highly
35 controversial and were met with skepticism by the scientific community.^{4, 9-12} Based on
36 the above observations, milk miRNAs are a novel class of bioactive food compounds as
37 defined by the National Cancer Institute in the United States.¹³ The discovery that milk
38 miRNAs are bioactive food compounds has broader implications as miRNAs play
39 essential roles in gene regulation,²⁻³ cell communication,¹⁴⁻¹⁵ and human health.¹⁶⁻²³

40 This study focused on determining the effect of milk processing, storage, somatic
41 cell content, and handling by consumers on two miRNAs, miR-29b and miR-200c levels
42 based on the following rationale. Our rationale for including the somatic cell count in our
43 analysis was to assess whether an increase in milk cells, as seen in mastitis, might be a
44 confounder in the analysis of milk miRNAs. In bovine milk, miR-29b and miR-200c are
45 among the most abundant miRNAs.²⁰ MiR-29b is an important regulator of bone
46 mineralization in humans, as it increases osteoblast differentiation¹⁶ and decreases
47 osteoclast differentiation and function.¹⁷ MiR-200c decreases cancer risk by targeting the
48 transcription factor ZEB1, which induces E-cadherin expression, thereby limiting
49 epithelial-to-mesenchymal transition, a key event in metastasis.²⁴⁻²⁵ Also, the nucleotide
50 sequences of miR-29b and miR-200c in bovine milk are identical to those of their human
51 orthologs.²⁶

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

59 Little is known about the effects of processing and storage on milk miRNAs levels.
60 In two studies, synthetic miRNAs were added to bovine milk and their stability after
61 exposure to harsh treatments such as acid and RNase was assessed and compared to the
62 stability of endogenous miRNAs in milk.^{6, 20} Synthetic miRNAs were rapidly degraded,

63 whereas endogenous miRNAs were resistant to treatment. However, the harsh treatments
64 applied in these studies are not representative of the treatments applied in commercial
65 dairy production. In this study, we assessed the effects of pasteurization, fat content, cold
66 storage, heating as well as processing into dairy products on content of milk miRNAs.

67

68 **MATERIALS AND METHODS**

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

72 **Milk and dairy products.** Raw, whole, 2%, and skim cow's (*Bos taurus*) milk was
73 obtained from The Pennsylvania State University Creamery (University Park, PA) from
74 separate collections in three consecutive weeks in May 2014. All milk was procured
75 from the Penn State Animal Science Department's Holsteins breed herd. Milk was
76 pasteurized, homogenized, and bottled in the creamery facility within one day of receipt
77 into bulk tanks. On our initial collection dates, milk of all fat levels were stored at 4°C
78 for up to 15 days, and aliquots were taken and frozen at -80°C every other day. In a
79 separate experiment bovine cells were removed from raw milk by centrifugation (500 g,
80 10 min, 4°C) to determine whether somatic cells are a meaningful confounder when
81 analyzing the concentrations of miRNAs in milk from healthy cows. Samples were
82 frozen at -80°C and shipped on dry ice to Lincoln, NE, for miRNA analysis. Samples
83 from all fat levels of milk on day 15 were heated in the microwave for 15 seconds and
84 analyzed after cooling off to room temperature. Dairy products other than milk were

85 purchased from grocery stores in Lincoln, NE. All samples were produced and analyzed
86 as biological repeats in triplicate.

87 **MiRNA analysis.** Milk samples were spiked with a synthetic internal standard (twenty-
88 five attomoles) prior to extraction of miRNAs using miSPIKE Synthetic RNA (IDT
89 Technologies).⁴ Dairy products (100 mg) other than milk were extracted using TRIzol
90 prior to addition of the synthetic internal standard. MiR-29b and miR-200c were
91 quantified using quantitative real-time PCR as described previously.⁴

92 **Statistics.** Analysis by Bartlett's Test Homogeneity suggested that variances were
93 homogeneous.²⁹ The paired t-test was used for pairwise comparisons. One-way analysis
94 of variance (ANOVA) and Fisher's protected least significant differences were used
95 when comparing more than two groups. Repeated measures ANOVA was used for
96 assessing the effects of storage time on miRNA concentration. StatView 5.0.1 (SAS
97 Institute; Cary, NC) was used for conducting statistical analyses. Means \pm SD are
98 reported. Differences were considered statistically significant if $P \leq 0.05$.

100 **RESULTS**

101 Pasteurization and homogenization of raw milk resulted in a $63 \pm 28\%$ decrease of
102 miR-200c in whole milk; effects were similar for 2% fat milk and skim milk (**Fig. 1A**).
103 The effect was less pronounced for miR-29b for which a significant decrease ($67 \pm 18\%$)
104 was observed only in skim milk (**Fig. 1B**). Cold storage of milk did not affect the
105 concentration of miR-29b and miR-200c in whole milk, 2% milk and skim milk up to 15
106 days; 2% fat milk is shown as a representative example in **Fig. 2**. Somatic cells are not
107 meaningful confounds regarding the analysis of miRNAs in milk from healthy cows.

108 When somatic cells were removed from raw milk by centrifugation and analyzed for
109 miRNA content, the cellular miRNAs were found to contribute less than 2% of the total
110 miRNAs present in raw milk before centrifugation: $1.1 \pm 0.9\%$ for miR-29b and
111 $0.14 \pm 0.08\%$ for miR-200c.

112 Processing in the household has the potential to cause a considerable loss of some
113 miRNAs in milk. For example, the concentration of miR-29b decreased by $40 \pm 28\%$ when
114 processed milk was heated in the microwave and cooled to room temperature compared
115 to milk before heating (**Fig. 3**). In contrast, when milk was heated in the microwave the
116 concentration of miR-200c was not statistically different compared with unheated
117 controls. The concentrations of miRNAs varied considerably among the dairy products
118 tested (**Table 1**), but were generally lower than the concentrations in pasteurized whole
119 milk (compare **Fig. 1**). Fresco Queso Dip was a notable exception and contained higher
120 concentrations of miRNAs than those observed in milk.

121

122 **DISCUSSION**

123 In a recent paper we reported the importance of milk miRNAs for gene regulation in
124 humans.⁴ That report has major implications for the roles of milk and possibly other dairy
125 products in human health. Cow's milk contains meaningful quantities of 245 miRNAs,^{20,}
126 ³⁰ and 71.4% of these miRNAs are predicted to target about 11,000 human transcripts
127 (unpublished observations). In addition to the roles of miR-29b and miR-200c in bone
128 health and cancer prevention,^{16-17, 24-25} respectively, miRNAs have been implicated in
129 various aspects of human health and disease including hypertension, insulin resistance

130 and diabetes, hyperlipidemia and atherosclerosis, reproduction, immune function and
131 Crohn's disease.^{18, 20-21, 31-33}

132 We propose that milk has a meaningful effect on human health, mediated by
133 miRNA-dependent gene regulation. The potential importance of dietary milk miRNA
134 intake is supported by data suggesting that 1) Americans consume large quantities of
135 milk and dairy products,²⁸ 2) a large proportion of milk miRNAs is encapsulated in
136 extracellular vesicles, thereby providing protection against degradation⁵⁻⁶ and a pathway
137 for cellular uptake by endocytosis,³⁴⁻³⁵, and 3) milk miRNAs are resistant against
138 degradation during storage (this study).

139 Our previous studies of milk miRNAs in humans and mice were conducted using 1%
140 fat milk from the grocery store.⁴ Based on this study, the content of miRNAs is about two
141 times higher in unprocessed milk compared with pasteurized, store-bought milk. Note
142 that we have no intent recommending the consumption of raw cow's milk by humans,
143 because of food safety concerns associated with raw milk. We observed that a loss of
144 milk miRNAs occurred only during pasteurization, homogenization, and processing to
145 dairy products. This observation is consistent with previous studies of milk miRNAs. For
146 example, endogenous miRNAs were not degraded when milk was exposed to harsh
147 treatments such as low pH or treatment with RNase.[Kosaka, 2010 #8963; Izumi, 2012
148 #8911] It is reasonable to propose that encapsulation of miRNAs in extracellular vesicles
149 [Zhou, 2012, #8913] prevents miRNA degradation, based on the following lines of
150 evidence. 1) When synthetic miRNAs are added to milk and subjected to low pH or
151 RNase treatment, the miRNAs are rapidly degraded. [Kosaka, 2010 #8963; Izumi, 2012
152 #8911] 2) When exosome membranes in milk were disrupted by sonication for preparing

153 miRNA-depleted mouse diets in previous studies, miR-29b was rapidly degraded to
154 concentrations below detection limit.[Baier, 2014 #10339] Presumably, degradation was
155 due to milk RNases gaining access to miRNAs released from exosomes. 3) When milk
156 was fermented to produce yoghurt, miRNA concentrations decreased to levels much
157 lower than in milk (this study). We speculate that the decrease was due to the lysis of
158 exosomes during fermentation and the large amounts of RNases produced by microbes.
159 4) When milk was homogenized, miRNA concentrations decreased by on average 50%
160 (this study). We speculate that the decrease was caused by a disruption of exosome
161 membranes by shear forces applied during homogenization. Collectively, our studies
162 suggest that milk, and perhaps dairy products, have the potential to contribute to the
163 miRNA body pool in humans.

164 Some uncertainties remain to be addressed in future studies. For example, this study
165 was modeled based on miR-29b and miR-200c, however, there is a possibility that
166 distinct miRNAs may be differentially metabolized.^{8, 36} Another layer of uncertainty is
167 the possible effects of feeding regimens, season, and breed on the miRNA content in
168 milk. Moreover, while this study suggests that somatic cells in milk from healthy cows do
169 not contribute meaningful amounts to the total miRNA content in milk, it is possible that
170 the increased somatic cell count in milk from cows suffering from mastitis³⁷ may cause
171 an artificial increase in milk miRNA concentrations. Our previous studies suggest that
172 plasma miRNA concentrations decrease by 61% in mice fed a milk miRNA-depleted diet
173 for four weeks. This observation is consistent with milk miRNAs contributing
174 meaningful quantities to the miRNA body pool, but does not necessarily establish the
175 essentiality of dietary miRNA intake. Clearly, this is an uncertainty that will need to be

176 addressed in future studies. Finally, it is conceivable that miRNAs from foods other than
177 milk also contribute toward the total body pool of miRNAs.

178

179 **ABBREVIATIONS USED**

180 miR, microRNA; miRNA, microRNA

181

182 **ACKNOWLEDGMENT**

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184 collection and processing.

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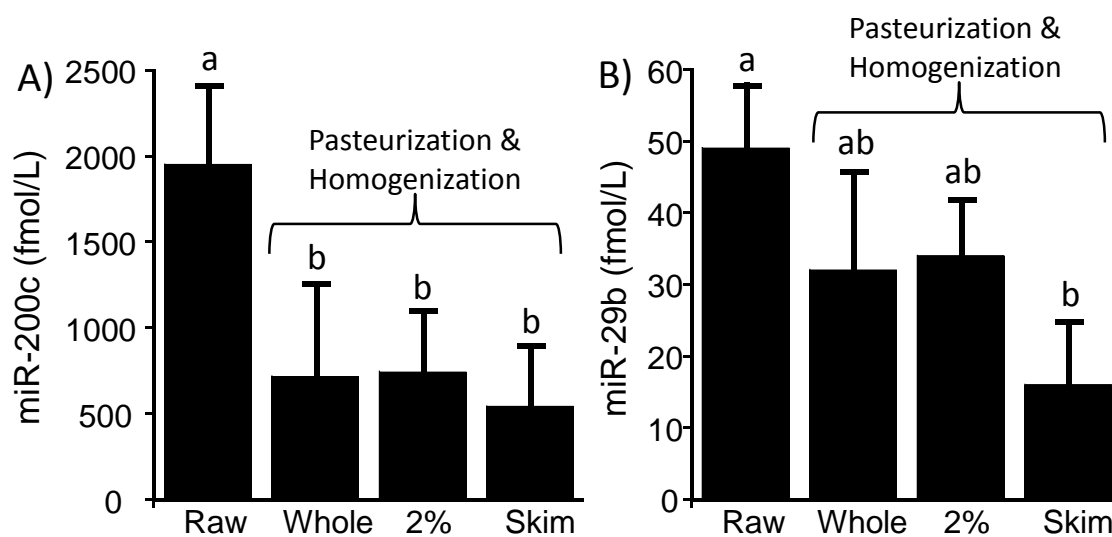


Figure 1. Loss of miR-200c (A) and miR-29b (B) during milk pasteurization and homogenization of milk with different fat content. ^{a,b}Significantly different (n=3 biological replicates, $P<0.05$).

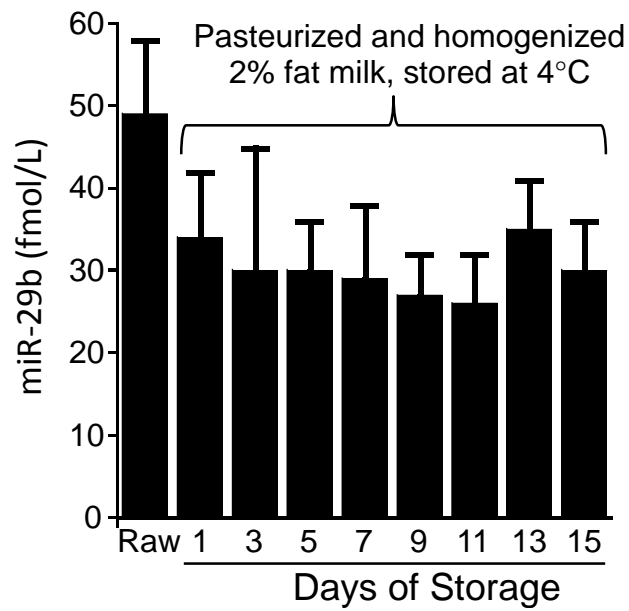


Figure 2. Storage at 4°C did not affect the concentrations of miR-29b in pasteurized and homogenized 2% fat milk (n=3 biological replicates, $P>0.05$).

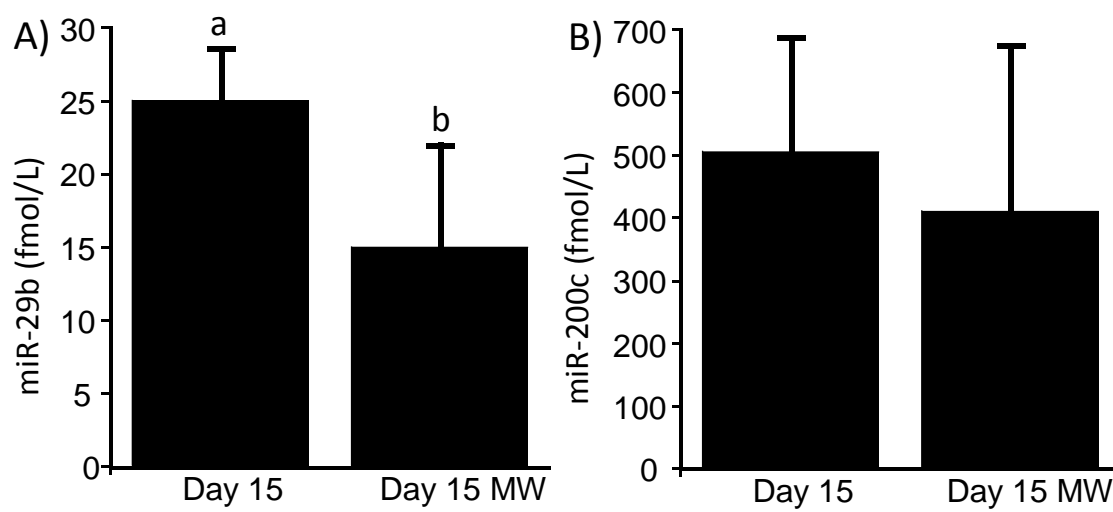


Figure 3. Loss of miR-29b (A) and miR-200c (B) during heating of whole milk in the microwave after 15 days of storage at 4°C. Abbreviation: MW, microwaved.

^{a,b}Significantly different (n=3 biological replicates, $P<0.05$).

Table 1. Concentration of miRNAs-29b and -200c in dairy products

Product	MiRNA	
	miR-29b	miR-200c
	<i>fmol/kg</i>	
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 IV

Off target effects of sulforaphane include the de-repression of long-terminal repeats through histone acetylation events*

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Running Title: Off target effects of sulforaphane

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Key words: Histone deacetylases; long terminal repeats; off-target effects; sulforaphane

Abstract

Sulforaphane is a naturally occurring isothiocyanate in cruciferous vegetables. Sulforaphane inhibits histone deacetylases, leading to the transcriptional activation of genes including tumor suppressor genes. The compound has attracted considerable attention in the chemoprevention of prostate cancer. Here we tested the hypothesis that sulforaphane is not specific for tumor suppressor genes but also activates loci such as long terminal repeats (LTRs), which might impair genome stability. Studies were conducted using chemically pure sulforaphane in primary human IMR-90 fibroblasts and in broccoli sprout feeding studies in healthy adults. Sulforaphane and sprouts caused an increase in LTR transcriptional activity in cell cultures and adults, respectively. For example, the area under the curve for LTR mRNA increased by more than 10 times in adults consuming broccoli sprouts compared with controls. The effect of sulforaphane was dose dependent in the human feeding studies. This increase in transcript levels was associated with an increase in histone H3 K9 acetylation marks in LTR 15 in peripheral blood mononuclear cells from subjects consuming sprouts. Collectively, this study suggests that sulforaphane has off-target effects that warrant further investigation when recommending high levels of sulforaphane intake, despite its promising activities in chemoprevention.

1. Introduction

Cruciferous vegetables such as broccoli and cauliflower contain a large number of glucosinolates including glucoerucin, glucoiberin, and glucoraphanin (1). When unheated vegetables are processed by chopping or chewing, the enzyme myrosinase is released from myrosin grains in myrosin cells and glucosinolates are released from adjacent S cells (2-4). Myrosinase catalyzes the hydrolytic removal of the glucose moiety in glucoraphanin, followed by non-enzymatic release of a hydrogen sulfate moiety and spontaneous rearrangement of the unstable intermediate to form the aliphatic isothiocyanate sulforaphane (SFN) (1). SFN has attracted considerable attention due to its putative role in cancer prevention (5).

Various, not mutually exclusive, mechanisms have been proposed to explain the chemopreventive activities of SFN. One theory is that SFN enhances drug-mediated cytotoxicity against cancer cells including cancer stem cells (6-8). The significance of these observations is not limited to the chemotherapy of cancer but extends to the prevention of cancer through enhancing cellular sensitivity to cell death signals in tumor initiation. SFN-dependent inhibition of anti-apoptotic NF- κ B signaling pathways appear to play a major role in the elimination of abnormal cells (9, 10). A second theory is that SFN-dependent inhibition of histone deacetylases (HDACs) causes an increase in the expression of tumor suppressor genes such as p21 and Bax, leading to cell cycle arrest and apoptosis (11, 12). Evidence suggests that SFN inhibits class I and class II HDACs (13). The locus (gene) specificity of SFN is uncertain, despite a general consensus that chemoprevention needs to pursue gene-specific gene expression through the modulation of epigenetic marks in distinct genomic loci (14). Gene-specific epigenetic editing can,

theoretically, be achieved by fusing enzymes or inhibitors to gene-specific DNA binding domains.

In this paper we tested the hypothesis that the inhibition of HDACs by SFN does not only de-repress tumor suppressor genes, but also has undesirable off-target effects, mediated by de-repression of genes other than tumor suppressor genes. Studies were conducted in both cell cultures and healthy adults to take advantage of the small inter-individual variation in cell cultures and to capture effects of biotransformation in human studies. As model loci for detecting off-target effects we used long-terminal repeats (LTRs), based on the following rationale.

LTRs make up about 8% of the human genome and at least 51 LTRs are transcriptionally competent (15). Repetitive elements such as LTRs pose a burden to genome stability, as their mobilization facilitates recombination between non-homologous loci, leading to chromosomal deletions and translocations (16, 17). Mobilization of LTR transposons is associated with 10% of all spontaneous mutations in mice (18). The transcriptional activity of LTRs is controlled by histone acetylation and other epigenetic marks; inhibition of HDACs leads to an increase in LTR transcription (19). De-repression of LTRs may impair genome stability through insertional mutagenesis, recombination events that cause translocations and other rearrangements, de-regulation of genes in the host genome mediated by LTR promoter activity, and antisense effects if transcription extends into exon sequence downstream of the transposon (20).

2. Methods and Materials

2.1. Cell cultures

Primary human IMR-90 fibroblasts were obtained from American Type Culture Collection (Manassas, VA). We chose primary cells over cell lines to avoid possible artifacts caused by the process of immortalization in cell lines. Fibroblasts (from passages 32-36) were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 0.1% sodium pyruvate, 100,000 U/L penicillin, 100 mg/L streptomycin, and 0.1% non-essential amino acids (final concentrations). R,S-SFN was obtained from LKT Laboratories (St. Paul, MN) and was dissolved in DMSO to prepare a stock solution containing 40 mmol/l SFN. Aliquots were frozen at -20°C until use. SFN concentrations in cell culture media were adjusted to a final concentration of 2.0 µmol/l; controls were treated with solvent. Samples were collected at timed intervals.

2.2. Human feeding study

Eight apparently healthy adults (4 male, 4 female) participated in a broccoli sprout feeding study. Exclusion criteria included pregnancy, smoking, self-reported health problems, and use of SFN supplements. Participants in this study included 6 Caucasians (2 Hispanics) and 2 Asians ages 19-30 years. Subjects were instructed not to consume cruciferous vegetables in the 48 hours leading up to the study and during the eight-hour period in which blood samples were collected. Each subject consumed three doses (34 g, 68 g, 102 g) of BroccoSprouts® broccoli sprouts from a local supermarket

(HyVee, Lincoln, NE). Sprouts were consumed with a bagel and cream cheese (11). Each treatment was separated by a two week washout period and the order of doses was randomized. Thirty milliliters of blood were collected before sprout consumption (baseline, time 0 h) and at timed intervals (2, 4, and 8 h) after consumption. Peripheral blood mononuclear cells (PBMC) and plasma were purified using Histopaque and gradient centrifugation as described previously (21); aliquots were frozen at -80°C until analysis.

2.3. Quantitation of SFN

SFN was extracted and quantitated by HPLC as previously described (22). Briefly, 1 g of BroccoSprouts® was combined with 20 ml of water acidified with 0.1 M hydrochloric acid and homogenized for 10-15 seconds with a Tissue-Tearor model 985370 hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK). Extracts were transferred to a 45°C water bath for 2 hours and then cooled to room temperature. The solution was extracted twice with 20 ml dichloromethane and the solvent phases were collected and combined. The solvent was dried through sodium sulfate and then loaded onto a Supelclean™ LC-Florisil® SPE column (Supelco). The column was washed with 3 ml ethyl acetate and SFN was collected with 3 ml methanol. Samples were passed through a 0.2 µm filter and stored at -20°C until analysis. Determination of SFN concentration was performed by injection on a Waters (Milford, MA) 600S HPLC system equipped with a VyDac C18 (Grace, Deerfield, IL) column and a Waters 2996 Photodiode Array detector. Separation was achieved with an isocratic flow rate of 1.0 ml/min using water and acetonitrile (30:70 v/v). A sample volume of 20 µl was injected.

2.4. *Quantitative Real-Time PCR (qRT-PCR)*

The abundance of p21 mRNA and LTR mRNA (transcript R/U5) was quantified by qRT-PCR as previously described using the cycle threshold method; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize for PCR efficiency (23, 24). PCR primer sequences for p21 were 5'-AGGCGGTTATGAAATTCACC-3' (forward) and 5'-CCCTTCAAAGTGCCATCTG-3' (reverse). LTR primer sequences were the same as previously reported (25). Note that the values for LTR mRNA represent the grand total of all transcriptionally active LTRs due to near-identical sequences in these repeats (25). Areas under the curves (AUCs) for LTR mRNA were calculated using the linear trapezoidal rule (26).

2.5. *Chromatin Immunoprecipitation (ChIP) assays*

ChIP assays were performed as previously described (23). Antibody against K9-acetylated histone H3 (H3K9ac; ab10812) was purchased from Abcam (Cambridge, MA). Data were normalized for nucleosomal occupancy using an antibody to the C-terminus in histone H3. The enrichment of H3K9ac marks in LTR15 {nomenclature as per (15)} were quantified by qRT-PCR, using GAPDH as a control. PCR primer sequences were the same as in our previous studies (25). In case of ChIP assays, as opposed to mRNA quantification, individual LTRs can be distinguished by having one PCR primer anneal with sequences in the host genome adjacent to the LTR of interest.

2.6. Statistics

Homogeneity of variances was tested using Bartlett's Test. If variances were heterogeneous, data was log transformed before analysis. Data from IMR90 cell cultures were analyzed by using the Wilcoxon Signed-Rank Test. Data from PBMC experiments were analyzed using one-way analysis of variance (ANOVA) and Fisher's LSD for posthoc comparisons (27) for gene expression data and Wilcoxon Signed-Rank Test for ChIP data. Differences were considered statistically significant if $p < 0.05$. StatView 5.0.1 was used for conducting statistical analyses (SAS Institute; Cary, NC).

3. Results

3.1. LTR transcript levels increase after SFN treatment in IMR-90 fibroblast cultures

SFN de-repressed LTRs in IMR-90 fibroblasts (Fig. 1). A significant increase in LTR mRNA was detectable at $t = 4$ h, and peak values were achieved at $t = 6$ h compared with vehicle controls. LTR expression levels returned to baseline levels after 16 h of SFN treatment. The expression of the p21 tumor suppressor also increased in response to SFN treatment (Fig. 2), consistent with previous reports in BPH-1, LnCaP, and PC-3 prostate epithelial cells (28), but this increase in p21 mRNA was not statistically significant.

3.2. Human consumption of broccoli sprouts increases LTR expression and histone acetylation

The expression of LTRs increased dose-dependently in response to consumption of broccoli sprouts, as judged by the AUCs for LTR mRNA in PBMC (Fig. 3). The AUCs for LTR transcripts in subjects consuming 34 g, 68 g, and 102 g sprouts were 1.29 ± 2.38 , 5.54 ± 5.26 , and 13.06 ± 23.68 arbitrary units, respectively, compared with baseline. The increase was significantly greater for the 68 g and 102 g doses compared with the 34 g dose ($p < 0.05$, $N = 8$). The AUCs for p21 mRNA in subjects consuming 34 g, 68 g, and 102 g sprouts were 0.80 ± 1.43 , 0.73 ± 0.90 , 2.46 ± 3.00 arbitrary units, respectively, compared with baseline; however the differences were not statistically significant ($p > 0.05$, $N = 8$). Note that the level of SFN in Broccosprouts® was 18 mg/g fresh weight.

The de-repression of LTRs was associated with an increased abundance of H3K9ac marks in LTR15 at $t = 4$ h compared with $t = 0$ h (Fig. 4A). The magnitude of the effect depended on the amount of broccoli sprouts that was consumed; meaningful increases in H3K9ac enrichment at $t = 4$ h were observed when sprout doses exceeded 34 g (Fig. 4B).

4. Discussion

To the best of our knowledge, this is the first report suggesting possible off target effects of SFN in the de-repression of LTRs in both primary cells and human feeding

studies. The increase in LTR mRNA abundance was not only statistically significant but also appears to be of biological importance, considering that the AUC for LTR mRNA increased by more than 10 times for the highest dose of broccoli sprouts. The doses tested in cell cultures and feeding studies are nutritionally relevant (29). Our observations were corroborated by an increase in the abundance of H3K9ac marks in LTRs, consistent with their de-repression. Note that SFN also increased the expression of the tumor suppressor gene p21, albeit to a smaller extent than previously reported (30). The comparably small increase in p21 expression in response to SFN might be due to us using nutritionally meaningful levels, whereas other studies typically concentrations of at least 15 $\mu\text{mol/l}$ SFN (9, 31-33).

Our observations are important for human health, considering the roles of LTRs in impairing genome stability (see Introduction) and the widespread availability of SFN-containing foods and supplements. The sprouts used in this study, Broccosprouts[®], are widely available in the United States; the lowest dose that was tested, 34 g, is about one serving according to product labeling. One serving of broccoli sprouts may contain levels of SFN sufficient to de-repress LTRs. SFN-containing supplements are also available to consumers over the counter. To the best of our knowledge, there are no data available for the consumption of SFN supplements in the United States, but supplement use in general is fairly common. For example, approximately 50% of all Americans and 64-81%, of cancer survivors take nutritional supplements (34). Of the many SFN supplements on the market, wide ranges of doses are available. One product, BroccoMax[®] is a supplement labeled as having 30 mg sulforaphane glucosinolate per serving, providing a dose of SFN similar to the dose tested in this study. One could assume with a reasonable level of

confidence that consuming such doses of SFN on a regular basis could elicit effects exceeding those seen in this study where single doses were administered. Note that we do not dispute the potential benefits of SFN in the chemoprevention of cancer (6-8, 11, 12). However, we propose that SFN needs to be considered in the context of gene-specific editing (14).

There are some uncertainties that need to be addressed in future studies. First, the effects of SFN on the de-repression of LTRs (and p21) are transient, and it remains to be determined whether a de-repression that last a few hours is sufficient to elicit biologically meaningful effects. Second, while the de-repression of LTRs was quantitatively important, it remains to be seen whether the observed increase in LTR expression is sufficient to impair genome stability.

Collectively, this study suggests that sulforaphane has off-target effects that warrant further investigation when recommending high levels of sulforaphane intake, despite its promising activities in chemoprevention.

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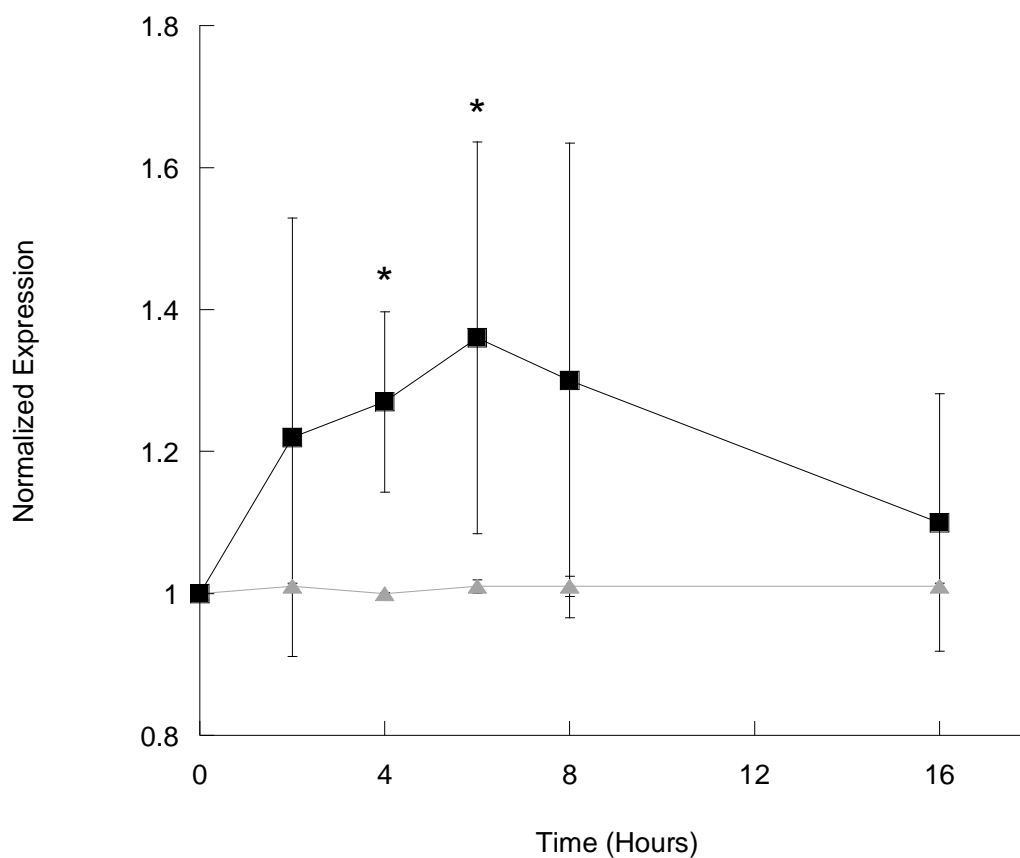
Figure 1

Fig. 1. SFN increases LTR transcription in IMR-90 fibroblast cultures. Fibroblasts were treated with 2.0 $\mu\text{mol/l}$ SFN (squares) or solvent (triangles). *Significantly different compared with vehicle control at the same collection time ($p < 0.05$ by Wilcoxon Signed-Rank test, $N = 4-7$).

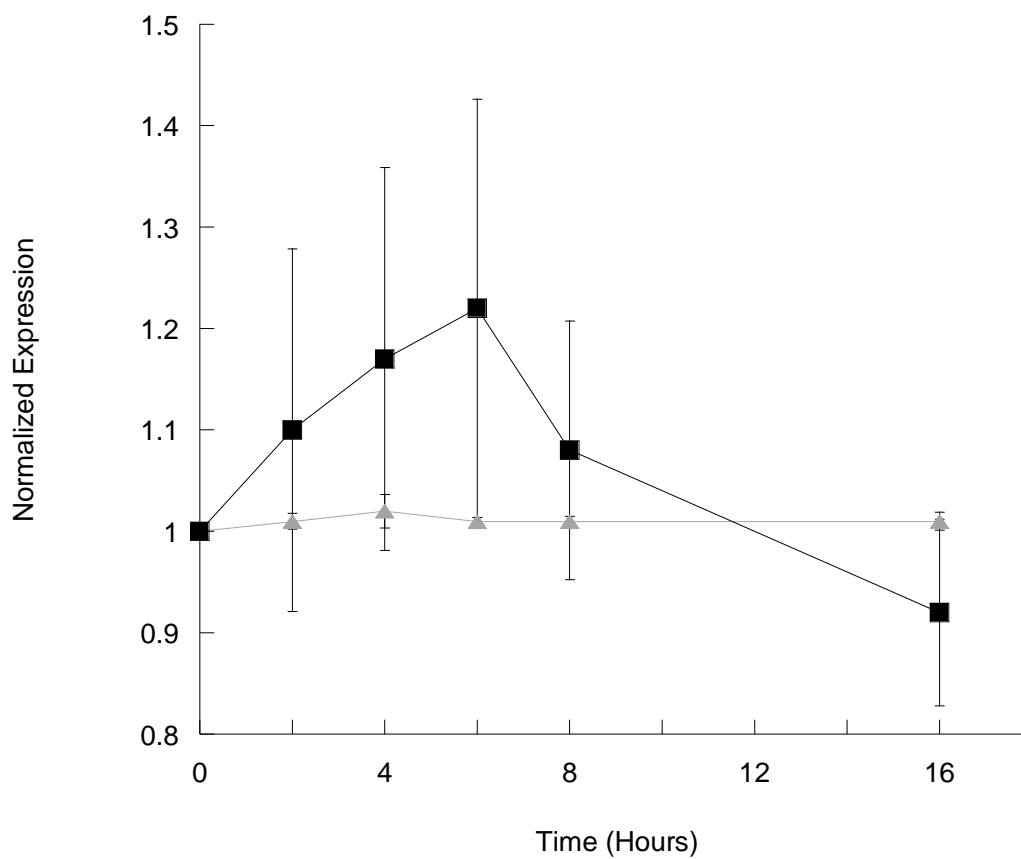
Figure 2

Fig. 2. SFN had no significant effect on p21 transcription in IMR-90 fibroblast cultures. Fibroblasts were treated with 2.0 $\mu\text{mol/l}$ SFN (squares) or solvent (triangles).
*Significantly different compared with vehicle control at the same collection time ($p < 0.05$ by Wilcoxon Signed-Rank test, $N = 4-7$).

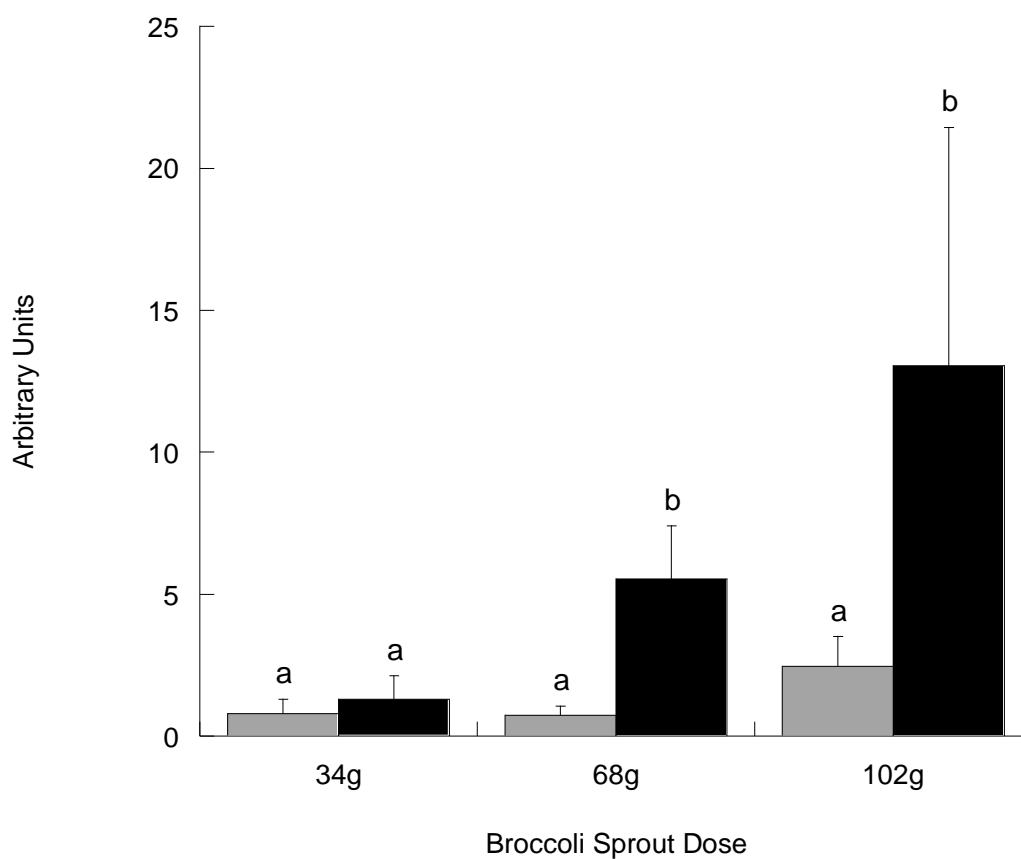
Figure 3

Fig. 3. AUC for LTR (black bars) and p21 (gray bars) expression increases in a dose-dependent manner following three broccoli sprout doses in healthy adults. Statistical significance determined by ANOVA with Fisher's LSD used for posthoc comparisons. ^{a,b}Columns not sharing the same letters are significantly different for the same treatment group (SFN vs. control; $p < 0.05$, $N=8$).

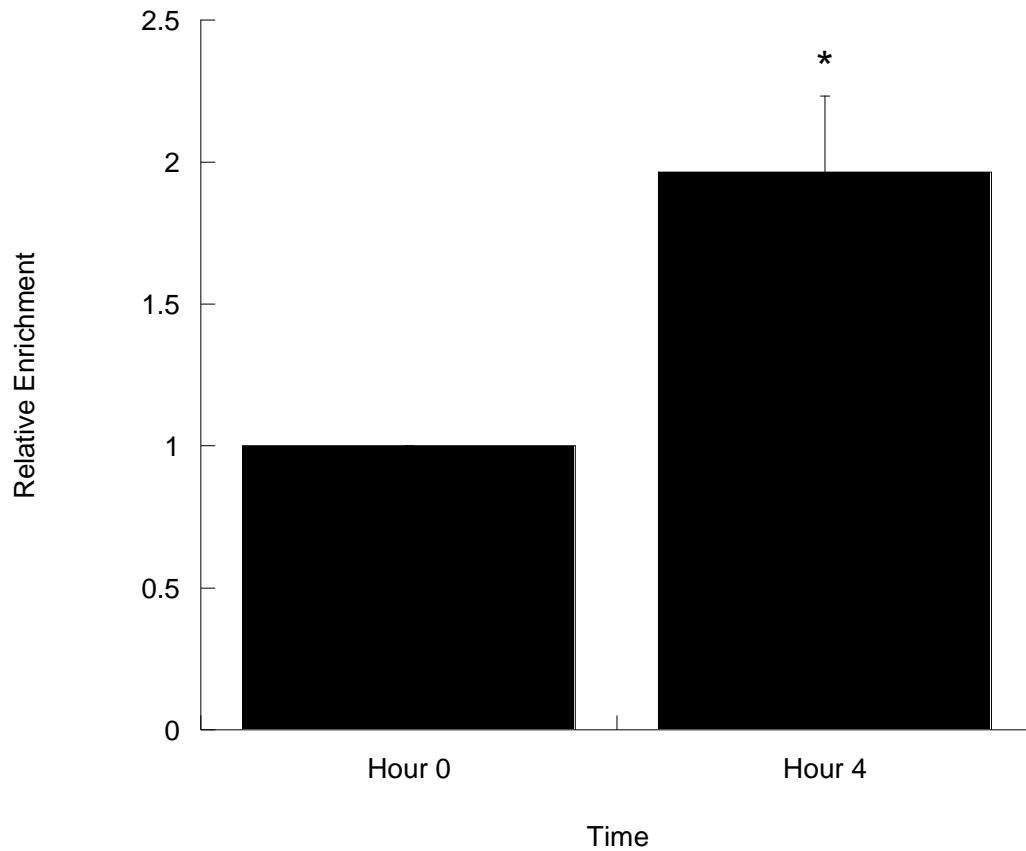
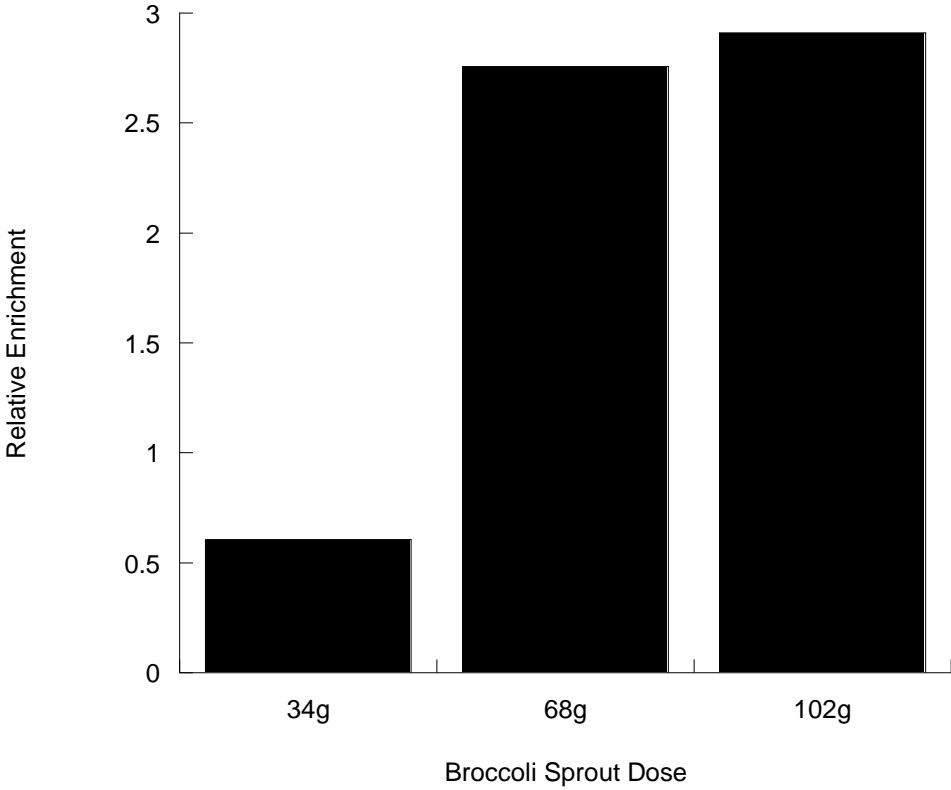
Figure 4

Fig 4. (A) Broccoli sprout consumption increases enrichment of H3K9ac mark in LTR15 in PBMCs from human subjects consuming 102 g broccoli sprouts. *Significantly different than controls as per Wilcoxon Signed-Rank test ($p < 0.05$, $N=8$). (B) Dose-response curve for the H3K9ac mark in LTR15 in PBMCs from a representative subject.

Figure 4B



OUTLOOK

The concept of dietary microRNAs is still relatively new in the nutrition field and while the potential importance of bioavailable nucleic acids cannot be overlooked, much more work needs to be done before we fully understand the function and impact of these molecules. Initial reports indicating the bioavailability of plant-based microRNAs have largely been refuted, but the results presented here in favor of milk and egg-derived microRNA availability show there is great potential for dietary microRNA-based gene regulation that may result in phenotypic changes on the organism level. To investigate dietary microRNA further, certain projects are underway and planned that will greatly increase our knowledge in this area.

1. Sonicated milk/eggs feeding study

Sonication of milk was used in making the ExoMinus diet in the mouse feeding study presented in Chapter I. While not initially planned as part of the human feeding study, we now have started another study with feeding subjects 1.0 L of sonicated milk. Since most miRNA do not remain in the milk following sonication, the expected response in regard to plasma miRNA concentrations and resulting PBMC gene expression changes should not occur, or at least to a much lesser extent. This study will provide important evidence showing the changes in miRNA abundance and gene expression in tissues are truly due to exogenously supplied miRNA. Similar studies will also be performed for the egg feeding study presented in Chapter II.

2. *Mouse phenotyping study*

In Chapter I, a short term mouse feeding study was presented where mice were fed diets named ExoPlus, meaning it was sufficient in exosomal/microRNA content or ExoMinus, where the microRNA were depleted by sonication. While this study provided interesting results showing mice are not able to make up for decreased dietary microRNA by upregulating endogenous genes, it was likely too short of a study and too few mice to determine phenotypic effects of long-term consumption of milk microRNAs. Currently, a larger study is underway where mice are being fed the same two diets with tissue collections occurring at different time points throughout life. In addition, breeding success and the viability and success of offspring will be measured. Also, unlike the pilot study, both male and female mice will be included to examine any potential gender specific effects.

3. *Comparison of milk and dairy consumers versus non-consumers*

One of the outcomes of the pilot study in mice from Chapter I was the observation that some liver metabolites were present in different levels when comparing mice on the ExoPlus versus ExoMinus diets. In particular, metabolites in the purine metabolism pathway, including xanthine and uric acid, were more abundant in the livers of mice on the ExoMinus diet. This observation has also been made in human plasma samples when comparing two consumers of milk to one non-consumer. While in the early stages, the encouraging results from the mouse study and limited analysis of human samples has let

us to begin recruitment of more individuals for the human sample analysis. In addition to plasma samples, 24 hour urine collections will be performed to allow for analysis of urine metabolites.

4. Fortification of infant formulas with milk-derived exosomes

One of the observations made in our lab is breast milk has significantly greater amounts of miRNA than infant formulas. This is probably due, in part, to the large effect processing has on the abundance of miRNA in milk (see Chapter III). Additionally, plant based formulas contain none of the same miRNA as naturally found in breast milk. If possible, the addition of milk exosomes into formula powder preparations could improve the overall nutritional profile of infant formulas and make up for the differences in the health outcomes of infants on formula-based diets compared to breast-fed counterparts. This study will be performed by feeding human adults two different 1.0 L drinks. One will be a soy infant formula mixed according to label recommendations. The other will be the same formula with supplemented cow's milk exosomes to obtain a similar miRNA profile as seen in cow's milk. Theoretically there will not be a change to plasma miRNA following consumption of soy formula alone, but the supplemented formula should result in plasma miRNA increases similar to that of the study performed in Chapter II.

5. Determination of bone health in infants based on feeding regimen

Infants fed breast milk have greater BMD for the first few months of life compared to formula fed infants. We hypothesize the miRNA in breast milk may be partially responsible for this difference (see the effects of miR-29b in Chapter I) and are in the process of collecting samples from infants on different diets. In this study, mothers are providing milk samples, both breast milk and formula milk, and collecting infant urine once monthly until 6 months of age. The urine and milk miRNA content will be analyzed as well as the presence of different markers of bone health in the urine. We expect that the miRNA content of the infant's diet impacts markers of bone formation and breakdown. This study has enrolled a few infant/mother pairs and active recruitment is currently underway.

6. Use of milk-derived exosomes as drug delivery vehicles

Cow's milk exosomes are widely available and cheap to obtain when compared to exosomes produced in cell culture chambers. Additionally, since many cell cultures are from cancer cell lines, there are many concerns about the exosomes derived from those cells having the potential to transfer the cancer phenotype to recipient cells. Since we have observed encouraging results from the studies present in this dissertation and from the work of other students, pilot studies were performed regarding drug loading of exosomes. By using electroporation we have been able to transfect exosomes with drug and plasmid DNA and subsequently measure uptake by recipient cells treated with the

transfected exosomes. This project is in the early stages, but once conditions are optimized it may open the door for use of bovine milk exosomes as an inexpensive way to package drugs that cannot currently be consumed orally because of degradation in the gastrointestinal tract.