Endogenous and exogenous extracellular microRNA transfer

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Abstract

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MicroRNAs are small non-coding RNA molecules that post-transcriptionally regulate gene expression by repressing specific target mRNAs. Although most miRNAs regulate gene expression intracellularly, extracellular miRNAs have been observed in many body fluids. As a result many researchers have focused on understanding the origin and function of extracellular miRNAs. Many studies have reported that extracellular RNAs are transferred between cells and can exert regulatory function, however the biological contexts and mechanisms of miRNA transfer are not well understood. We set out to investigate miRNA transfer in the context of the bone marrow microenvironment and our initial results indicated that there was transfer of miRNAs from monocytic to stromal cells. After further investigation, we determined that these results were artifacts of unbound fluorophore uptake and contamination of cell populations due to inaccurate sorting during flow cytometry-based sorting. These findings indicate that investigators carrying out miRNA transfer studies should exercise caution when relying on fluorescence to track miRNAs, as well as when using flow cytometry-based sorting to separate recipient and donor cells to determine miRNA transfer after co-culture.

Until recently, miRNA transfer studies have focused on endogenous transfer within an organism, but a report suggested exogenous miRNAs are acquired from dietary consumption. This was the first report to indicate that there may be cross-kingdom regulation from dietary-derived miRNAs and sparked intense specialist and lay interest. Research groups conducted follow-up studies to verify these results, however most of these reports have conflicting results to the initial study. None of these studies have been definitive due to limitations in the study design or not being done in humans. We designed the first controlled study in humans, where individuals were fed a specific plant-based meal and collected serial blood draws to determine whether dietary-derived miRNAs in the human bloodstream are acquired orally. Through this study we determined that plant miRNAs, ath-miR-156a and hvu-miR-168a, are not detected at any timepoint in the majority of participant serum samples. The maximum serum concentration of plant miRNA observed in any participant is low with only 22,071 copies per uL of serum. However, even in participants with detectable levels above the limit of quantification, absolute concentrations are low and miRNA levels did not replicate in independent analyses. We concluded that dietary plant miRNA absorption is unlikely to occur in healthy individuals. At best plant miRNA absorption into the human bloodstream is highly inefficient and absolute concentrations detected in this study are so low that they are unlikely to regulate endogenous gene expression.

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Chapter 1

INTRODUCTION

MicroRNA biogenesis and function

MicroRNAs (miRNAs) are short non-coding RNAs between 19 and 24 nucleotides in length that post-transcriptionally regulate gene expression by binding to and repressing specific target mRNAs. MiRNAs were first discovered in *C. elegans* in the 1990s [1]. Since this discovery, miRNAs have been reported in a variety of organisms. MiRNA sequences and processing machinery are highly conserved across species indicating an importance of miRNA function. Primary miRNA transcripts (pri-miRNAs) are often several hundred base pairs long and are transcribed in the same way as protein coding genes [2]. Pri-miRNAs are processed in the nucleus to yield a precursor miRNA (pre-miRNA) about 70 nt in length [3]. The pre-miRNA is exported into the cytoplasm and sequentially processed by the Dicer enzyme complex into a mature double stranded miRNA of 19-24 nt in length [4-6]. The miRNA duplex is then unwound and the guide strand is loaded into the RISC complex. The key components of RISC are Argonaute proteins, and Argonaute 2 (AGO2) plays a critical role in miRNA-mediated mRNA silencing [7, 8].

The passenger strand is usually degraded, but in some cases, there is evidence that both of the strands of the miRNA duplex are loaded into the RISC complex and are functional [9]. Mature miRNAs act as a guide strand within the RISC complex for complementary mRNA sequences, typically within the 3' untranslated region of target mRNAs [10, 11]. The mRNA sequence is then inactivated either by cleavage or translational repression, depending on the degree of complementarity between the miRNA and the

mRNA [10, 11]. A single miRNA can affect multiple target mRNAs within a cell and thus miRNAs are believed to serve as “hubs” in gene regulatory networks.

Extracellular circulating miRNAs

Although the majority of miRNAs are found intracellularly, extracellular miRNAs are also detected in many body fluids. Many groups independently identified the presence of cell-free mature miRNAs in blood plasma and serum [12-15]. Extracellular miRNAs are detected in various body fluids including urine, saliva, and semen [16-19]. Surprisingly, extracellular miRNAs are highly stable despite the presence of ribonucleases in body fluids including serum [15, 20]. This suggests that miRNAs are packaged in some way to protect from degradation. Cells in culture have been shown to export miRNAs in exosomes [21] and miRNAs are also detected in peripheral blood microvesicles [22]. miRNAs are not just protected by microvesicles. The majority of extracellular miRNAs are microvesicle-free and associated with RNA-binding proteins in plasma, serum and cell culture media [23, 24]. Some miRNAs are also found in purified fractions of high-density lipoprotein (HDL) from human plasma [25].

The expression of some miRNAs is specific to tissues or biological stages and some miRNAs are deregulated in association with cancer development and progression, making miRNAs good candidates as biomarkers. For example, serum levels of miR-141 have been used to discriminate advanced prostate cancer patients from healthy individuals [15], and the ratio of miR-126 and miR-182 in urine samples can be used to detect bladder cancer [16]. It has also been established that human blood cells are a major contributor to circulating miRNAs, as perturbation of the blood cells can alter plasma miRNA biomarker

levels [26]. Therefore, some endogenous miRNA levels are altered in blood serum and plasma when hemolysis is present in the samples.

Despite increasing reports of circulating miRNAs in body fluids, the function of these circulating extracellular miRNAs is not well understood. Amongst the questions that remain unanswered are the origin of these miRNAs, how and why these miRNAs are released into the extracellular environment, and whether these miRNAs are functional and able to interact with mRNA targets to regulate gene expression.

Endogenous miRNA transfer

One key question is whether stable extracellular miRNAs in body fluids could facilitate and mediate cell-cell communication between distant cell types. Small RNA communication has been reported in different species. Double stranded RNA fed or injected directly into the gut in *C. elegans* can mediate RNA interference in distant tissues [27]. In many insect species, cells can take up extracellular dsRNA that mediate RNA interference in the recipient cells [28]. Plant cells can also transport small RNAs through plasma membrane-lined cytoplasmic channels to other distant cell types [29]. Studies have suggested that some miRNAs can be transferred between cells to mediate non-cell-autonomous regulation of mRNA, though the native biological contexts and mechanisms of this phenomenon, especially in mammals are not well understood.

In mammals, a few studies have shown selective miRNA export into the extracellular environment by various cell lines [30-32]. These studies suggest that miRNAs are sorted prior to export and transfer of miRNAs could be directional. However, some studies also suggested that miRNAs have different decay kinetics that could account for differential expression in the extracellular environment when compared to levels within cells [33, 34].

It is not known whether miRNAs are selectively secreted and released by cells or if miRNAs are a byproduct of cellular apoptosis.

Many groups have reported that extracellular miRNAs, within apoptotic bodies, microvesicles, and exosomes (30-100 nm vesicles), can be transferred to recipient cells and mediate gene expression changes [21, 35-37]. More specifically, one study observed unidirectional transfer of miRNA loaded exosomes from T cells to antigen presenting cells and showed the exosomes attached to the cell membrane of the recipient cells [37]. Apoptotic bodies from endothelial cells were also shown to deliver miR-126 to recipient vascular cells and mediate production of CXCL12 [38]. In another example of miRNA transfer, HDL particles delivered miRNAs to recipient cells and mediated direct targeting of mRNA [25, 39]. These are just a few examples of extracellular miRNA transfer, through exosomes, apoptotic bodies, and HDL particles, which may regulate mRNA transcripts between distant cell types.

Although there is potential for miRNA transfer between distant cell types, various studies have shown that some miRNA transfer is dependent on close proximity of cells to one another. Adjacent cells can form gap junctions made of connexin proteins, which allow diffusion of molecules less than 1000 Da [40]. Small single stranded RNAs tagged with a fluorophore can traverse gap junctions and are detected in the recipient cell, but larger single stranded or double stranded RNAs are not able to transfer through gap junctions [41, 42]. Glioma cells have also been shown to transfer miRNAs in a gap junction dependent manner and exert regulatory function in recipient glioma cells as determined by a luciferase reporter system [43]. Another compelling example of cell-cell contact dependent miRNA transfer has been demonstrated in the context of B-cells to T-cell interactions (i.e.,

an “immune synapse”) [44]. In this study, fluorescently-tagged small RNA duplexes and endogenous miRNA miR-127, were transferred from B-cells to T-cells through cell-cell contact via mechanisms yet to be determined [44]. These studies represent reports of miRNA transfer dependent on close cell-cell interactions, however the mechanisms of transfer are not well understood.

Challenges in studying miRNA cell-cell transfer

There are many reports of non-cell autonomous transfer of endogenous miRNAs, however these reports do not address potential challenges that studying these mechanisms may pose. We set out to investigate native mechanisms of endogenous contact-dependent miRNA transfer, but instead identified artifacts associated with relying on fluorescence and flow cytometry-based methods to study cell-cell transfer of miRNAs. In Chapter two, we describe our work to understand miRNA cell-cell transfer in the context of the bone marrow microenvironment (BM). The bone marrow microenvironment represents an interesting context in which stromal cells interact and form cell-cell contacts with monocytes resulting in changes in gene expression patterns in both cell types [45, 46]. We hypothesized that miRNAs could mediate the gene expression changes through transfer of miRNAs from monocytes to stromal cells and/or vise versa.

We utilized THP-1 monocytic cells and HS-27a stromal cells as a model for monocytic and stromal interactions within the BM. We used two methods commonly used to study miRNA transfer: 1) a fluorescently-tagged miRNA to allow tracking of the miRNA and 2) flow cytometry to separate cell types after co-culture, followed by qRT-PCR to detect changes in miRNA levels in the donor and recipient cells. Initially, we obtained data that suggested transfer and/or uptake of the fluorescently-tagged miRNA into recipient cells.

However after further investigation, we discovered that the fluorophore did not correlate with miRNA levels within the cells. This indicated that using a fluorescently-tagged miRNA is not an accurate method for tracking miRNA transfer within a cell. We then co-cultured THP-1 and HS-27a cells to determine if miRNA transfer could occur. Surprisingly, our initial experiments suggested transfer between THP-1 and HS-27a cells, but after further investigation the observed 'transfer' was an artifact due to cell contamination of improperly sorted cells through flow cytometry. These findings are especially pertinent because of the wide use of flow cytometry for miRNA-mediated cell-cell communication studies.

Exogenous miRNA transfer

Most studies in the field focus on endogenous miRNA transfer as a mode of communication between cells within an organism, but there are recent reports of potential exogenous miRNA transfer from plant sources into the human bloodstream. A recent study reported that miRNAs from exogenous sources, such as from a plant-based diet, are transferred into the human bloodstream [47]. In this study, plant miRNAs, miR-156a and miR-168a, were detected in human serum samples from men and women through high-throughput sequencing [47]. This group then used a mouse study to characterize the uptake of plant miRNA, miR-168a, and determined regulation of the endogenous transcript, LDLRAP1, by miR-168a in liver tissue [47]. This was the first report of potential cross-kingdom regulation and sparked large interest in the field because it challenged many paradigms, suggesting that exogenous miRNAs can be acquired orally, accumulate in tissues, and exert regulation of endogenous transcripts.

After this initial report, various independent groups have conducted additional studies to determine whether dietary-derived miRNAs are detected in the bloodstream, but these studies have all suffered from significant limitations. Results from two different groups, where controlled feeding studies were conducted with either mice or pigtailed macaques, suggested limited or no detection of plant miRNAs and miRNA levels detected were attributed to artifacts or contamination [48, 49]. Plant miRNAs were also not detected in plasma from healthy athletes after ingestion of fruit [50]. Analysis of small RNA (sRNA) datasets of tissues from a variety of species determined that plant miRNAs in sRNA datasets are an artifact of sequencing [51]. A separate group analyzed deep-sequencing libraries for diet-derived miRNAs and variable amounts of plant miRNAs were detected, however this report suggested that diet-derived miRNAs were due to contamination during library preparation [52].

Although there is increasing evidence that plant miRNAs are not as abundant in human blood as the initial study indicated [47], there was one study that identified a fraction of circulating RNA in human plasma is from exogenous species, including bacteria and fungi [53]. Plant miRNA transfer into the human bloodstream and tissues as a potential mode of cross-kingdom regulation continues to be of interest in the field. Additional controlled human studies are necessary to overcome the limitations in collection and analysis of blood samples. The limitations from previous studies include: blood drawn at random or after 12 hours of fasting with no diet information, lack of negative controls, minimal blood draw time points and potentially not enough plant-based food consumed.

Overcoming limitations of previous plant miRNA studies

As described above, further studies are merited to overcome limitations in previous studies of exogenous plant miRNA absorption into the bloodstream. In Chapter 3, we describe our work to determine whether plant-derived miRNAs are detected in human blood and are acquired orally. We designed the first controlled study in humans, thus far, to include multiple sequential blood draws after consumption of a plant-based meal. Blood samples were collected at baseline to establish background levels of plant miRNAs in each participant. After consumption of the plant-based meal, blood was drawn hourly for 8 hours or every half-hour for 4 hours and at 24 and 48 hours. Serum samples were analyzed for plant miRNAs, ath-miR-156a and hvu-miR-168a, via qRT-PCR. We determined that plant miRNAs, ath-miR-156a and hvu-miR-168a, are not detected above the limit of quantification at any timepoint in the majority of participant serum samples. However, even in participants with detectable levels above the limit of quantification, absolute concentrations are low and in a range associated with experimental noise. We determined that plant miRNA absorption is unlikely to occur and at concentrations detected, plant miRNA absorption is highly inefficient and unlikely to regulate endogenous gene expression.

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Chapter 2

Limitations of fluorescently-tagged miRNA tracking and flow cytometry-based analysis for miRNA transfer studies

Abstract

Recent studies have suggested that miRNAs can be transferred between cells to mediate non-cell-autonomous regulation of mRNA, though the native biological contexts and mechanisms of this phenomenon are not well-studied. We set out to investigate miRNA communication in the context of the bone marrow microenvironment, between monocytic and stromal cells. We studied potential transfer of miRNAs between THP-1 monocytic cells and HS-27a stromal cells using two different methods: 1) a fluorescently-tagged miRNA to allow tracking of the miRNA and 2) flow cytometry to separate cell types after co-culture of distinct cell populations, followed by qRT-PCR to detect changes in miRNA levels in the donor and recipient cells. Both of these studies initially suggested that transfer of miRNA from THP-1 cells to HS-27a cells had occurred. After further investigation, we determined that these results were artifacts of Cy3 fluorescent uptake and contamination of cell populations due to improper sorting during flow cytometry. These findings indicate that investigators carrying out miRNA transfer studies should exercise caution when relying on fluorescence to track miRNAs and flow cytometry to separate recipient and donor cells to determine miRNA transfer after co-culture.

Introduction

Since the discovery of extracellular miRNAs in many body fluids [1-8], there have been extensive studies to better understand the origin and function of extracellular miRNAs. MiRNA transfer that mediates changes in recipient cells has been observed in many species including *C. elegans*, insects, and plants [9, 10]. In mammals, studies have focused on extracellular export and transfer of miRNAs to distinct cell types.

Many studies have investigated the potential for miRNA transfer to recipient cells via exosomes (30-100 nm vesicles) and microvesicles as a means of mediating changes in gene expression in distant cells [11-13]. Glioblastoma microvesicles transport functional RNA and proteins that are taken up by normal cells, such as brain microvascular endothelial cells [14]. Other groups have determined that miRNAs are released through a ceramide-dependent secretory machinery, indicating that export may occur via exosomes and that these secreted miRNAs can be transferred and functional in recipient cells [15, 16]. MiRNA loaded exosomes from T cells have been shown to unidirectionally transfer miRNA to antigen presenting cells and also shown to attach to the cell membrane of the recipient cells [17]. Although there are many reports of miRNA export and transfer by exosomes, HDL particles can also contain miRNAs that can be delivered to recipient cells and mediate direct targeting of mRNA [18, 19]. These reports indicate that miRNA transfer via exosomes and HDL particles could facilitate miRNA-mediated communication between distant cell types.

There have also been reports of miRNA transfer that is dependent on cells being in direct or close contact with one another. One way molecules can be transferred from cells in close contacts is through gap junctions. Gap junctions are channels made of connexin

proteins between adjacent cells and allow diffusion of molecules less than about 1000 Da [20]. It was determined that small single stranded RNAs conjugated to a fluorophore can traverse gap junctions and can be visualized in the recipient cell [21, 22]. Glioma cells transformed to express cel-miR-67 and co-cultured with cells expressing a luciferase reporter with a target for cel-miR-67, resulted in gap junction dependent suppression of luciferase expression [23]. Another compelling example of cell-cell contact dependent miRNA transfer, has been demonstrated in the context of B-cell to T-cell interactions (i.e., an "immune synapse") [24]. In this study, fluorescently-tagged small RNA duplexes, as well as endogenous miR-127, were transferred from B-cell to T-cells through cell-cell contact via mechanisms yet to be determined [24]. These studies suggest that miRNAs can be transferred in a contact dependent manner and regulate mRNAs in the recipient cell.

We investigated miRNA cell-cell communication in a novel context within the bone marrow microenvironment (BM). The BM consists of distinct stromal cells that interact and form cell-cell contacts with monocytes [25, 26]. Two isolated stromal cell types, HS-5 and HS-27a, have diverse functional and gene expression patterns [26]. Most interestingly, these two stromal cell types have been shown to form cell-cell contacts with monocytes resulting in changes in gene expression in both cell types [25]. We hypothesized that miRNAs may mediate some of these gene expression changes as a result of transfer from one cell type to the other. We used two commonly used methods to study the potential transfer of miRNAs between the HS-27a stromal cell line and the THP-1 monocytic cell line. The two methods were: 1) tracking of a fluorescently-tagged miRNA to study localization during and/or after transfer, and 2) co-culture of different cell types followed by separation of the cell types after co-culture to determine whether miRNA transfer occurred.

Although initial results suggested miRNA transfer, we determined that fluorescently-tagged miRNAs are not reliable to detect presence of the miRNA within cells and FACS is not able to fully separate donor and recipient cells.

Methods

Cell culture

THP-1 cells were purchased from ATCC. THP1 cells were grown in RPMI (Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals) and 1X β-mercaptoethanol. GFP-HS-27a cells were acquired from the Torok-Storb Lab at FHCRC. GFP-HS-27a cells were grown in RPMI (Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals) and 1X L-glutamine (Invitrogen).

Transfection

THP-1 cells were transfected by nucleofection (Lonza Nucleofector 4D) with 100nM Cy3-miR-302d, 5' Cy3 ACUUUAACAUGGAGGCACUUGC, (IDT) using the SG Cell Line 4D-Nucleofector X Kit (Lonza) and following manufacturer instructions for the 100 uL Single Nucleocuvette.

Stable expression of mCherry in THP-1 cells

THP1 cells were transduced with p-RSC-mCherry vector (obtained from Torok-Storb Lab) for stable expression of mCherry. Flow cytometry was used to select a purified population of THP-1 cells with stable expression of mCherry. These cells were referred to as mCherry-THP1 cells and used for co-culture experiments.

Co-culture and FACS

GFP-HS-27a cells were seeded on 10cm dishes for 4 hours. Transfected THP-1 cells, mCherry-THP1 cells, or Cy3-miR-302d alone were subsequently added to the seeded cells.

Cells were co-cultured for 15 hrs. Cells were trypsinized using 0.25% trypsin (Invitrogen) and centrifuged at 1000 xg for 5 min to pellet cells. Cells were resuspended in 500 uL of serum free media and strained using a 35um cell strainer cap tube (Falcon) to eliminate cell clumps. A BD FACS Aria II was used to analyse and sort populations (GFP+, mCherry+, and GFP+mCherry+).

HPLC Separation Conditions

Samples were separated using an Agilent 1260 Infinity Bio-inert Quaternary LC system. Solvent A contained 400 mM HFIP in water pH 7.0 and Solvent B contained 400 mM HFIP in 50% methanol and 50% water, the pH of the mobile phases was adjusted with triethylamine. Samples were injected onto a Waters XBridge OST C18 Column (130Å, 2.5 µm, 4.6 mm 50 mm) at a flow rate of 0.5 µL/min for 10 min using Solvent A. The elution gradient included: 0% Solvent B to 15% Solvent B from 0 to 5 min, isocratic 15% Solvent B for 8 min, 15% Solvent B to 55% Solvent B for 17 min, and 55% Solvent B to 90% Solvent B for 5 min. The chromatogram was monitored at 254.4. nm and fractions were collected.

Amnis ImageStreamX Analysis

Cells were co-cultured and prepared the same as for standard FACS sorting. Raymond Kong ran the co-culture sample on the ImageStreamX at Amnis. For analysis, 50uL of the co-culture sample was injected into the ImageStreamX. The flow for the ImageStreamX was set in order for images of each cell to be captured at 20X magnification as they were analysed by the flow cytometer. After the sample was run on the ImageStreamX, all images were analysed for cell size and fluorescence.

RNA and DNA isolation

RNA was isolated from cells using miRNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. RNA was eluted from the column in 30uL RNase free water. DNA was isolated from cells using Blood & Cell Culture DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was resuspended in 200 uL of RNase free water.

miRNA and DNA Analysis in Co-Culture Samples

miRNA profiling was performed for 376 human miRNAs using miRCURY LNA Universal RT microRNA PCR Human panel I V2(Exiqon) using a standard protocol and internal quality controls. The amplification was performed on a Viia7 qPCR machine (Applied Biosystems).

qRT-PCR was performed using Taqman assays (Applied Biosystems) for miR-302d, and miR-142-3p, and the control RNU-48 assay. For each sample a 5uL reverse transcription reaction with 1.67uL input of total RNA was performed. Each RT reaction was diluted with 28.9 uL of water. A 5 uL QPCR reaction with 2.25 uL of the diluted RT sample was added to each well of 384-well plates, and amplification was performed on a Viia7 qPCR machine (Applied Biosystems). Copies of miRNAs present in qRT-PCR reactions were calculated using a standard curve consisting of dilutions of synthetic oligos and normalized to RNU-48 levels.

We designed Taqman assays for eGFP DNA(Fam Probe: CACTACCAGCAGAACAC, Forward Primer: GCATCAAGGTGAACTTCAAGA, Reverse Primer: ACTGGGTGCTCAGGTAGTGG) and mCherry DNA (Fam Probe: TCGCCCTCGCCCTCG, Forward Primer: CAAGGGCGAGGAGGATAAC, Reverse Primer: GAACTGAGGGGACAGGATGT) (Applied Biosystems) for qRT-PCR. A 5 uL QPCR reaction with 2.25 uL of the diluted RT sample was added to each well of 384-well plates, and amplification was performed on a

Viia7 qPCR machine (Applied Biosystems). DNA cell equivalents were calculated by using a standard curve made from serial dilutions of DNA isolated from a known number of mCherry-THP1 cells and GFP-HS-27a cells prior to co-culture.

Results

Fluorescently-tagged miRNA tracking within a cell

Cy3 fluorescence is detected in GFP-HS-27a cells after co-culture

THP-1 and HS-27a cells were utilized as a model for monocytic and bone marrow stromal interactions, as monocytes and stromal cells have been previously shown to affect each other's mRNA expression [25]. We chose to use miR-302d for miRNA tracking during transfer, because endogenous levels of miR-302d are very low in both GFP-HS-27a and THP-1 cells (Figure 2.1). The 5' Cy3-tagged miR-302d (Cy3-miR-302d) oligo was used to visualize transfer from THP-1 cells to GFP-expressing HS-27a cells (GFP-HS-27a). To do so, THP-1 cells were transfected with Cy3-miR-302d and co-cultured with GFP-HS-27a cells (Figure 2.1A). After co-culture cells were analysed for Cy3 fluorescent signal using fluorescence-activated cell sorting (FACS) and distinct cell populations were collected. As expected, we observed significant Cy3 fluorescence in Cy3-miR-302d transfected THP-1 cells, indicating high transfection efficiency (Figure 2.1C).

After co-culture of GFP-HS-27a cells with transfected THP-1 cells, we observed a subpopulation, about 50% of GFP-expressing cells, that were double positive for both GFP and Cy3. The double positive population, suggested potential transfer of Cy3-miR-302d from THP-1 cells to GFP-HS-27a cells (Figure 2.2E, upper right quadrant). As a control for direct uptake (non-transfection mediated) of the Cy3 fluorescent signal, Cy3-miR-302d was added directly to the media of GFP-HS-27a cells. Although, we did not anticipate any uptake

of the Cy3-miR-302d oligo into cells, we did observe a significant Cy3 signal, about 80%, to GFP-HS-27a cells incubated with the oligo (Figure 2.2F, upper right quadrant). As described above, we observed some transfer of the Cy3 fluorescent signal to GFP-HS-27a cells after co-culture with transfected THP-1 cells, but the direct uptake of the Cy3 fluorescent signal to GFP-HS-27a was more robust. This indicated that the Cy3-miR-302d could be directly taken up by cells and not necessarily transferred from one cell type to the other. However, at this point we were not certain whether the presence of Cy3 fluorescence in these cells corresponded to the miR-302d within the cell populations.

Cy3 fluorescent signal does not correlate with miR-302d levels

We measured levels of miR-302d in FACS isolated cell populations to determine whether the presence of a Cy3 fluorescent signal corresponded to transfer of miR-302d. If Cy3 fluorescence was a marker for miR-302d within the cells, we expected the levels of miR-302d in cells to correlate with the presence of Cy3 fluorescence. As expected, THP-1 cells transfected with Cy3-miR-302d showed a 76-fold increase in miR-302d levels when compared to untransfected THP-1 cells (Figure 2.1G). Despite about 80% GFP+Cy3+ cells in GFP-HS-27a cells incubated with Cy3-miR-302d, we did not detect miR-302d levels above endogenous levels in the GFP+Cy3+ population (Figure 2.1G). Consequently, we conclude that the uptake of the Cy3 fluorescent signal by GFP-HS-27a cells does not correlate with the presence of miR-302d within the cell.

Next, we tested whether there was transfer of Cy3-miR-302d from transfected THP-1 cells to GFP-HS-27a cells. We observed a significant population of GFP+Cy3+ cells after co-culture of transfected THP-1 and GFP-HS-27a cells, however miR-302d was not detected above endogenous levels in GFP+Cy3+ cells via qRT-PCR (Figure 2.1G). Again this data

supports the conclusion that there is not a correlation between Cy3 fluorescence and miR-302d within a cell. These data suggests that 5' Cy3-tagged miRNA is not adequate for small RNA tracking within a cell, as there is no correlation between levels of Cy3 and miR-302d within a cell.

Cy3 fluorophore is taken up by cells more rapidly in media supplemented with FBS

Despite obvious uptake of the Cy3 fluorophore, we did not see a concomitant increase in miRNA levels in these cells. Consequently, we wanted to determine the possible mechanism for this observation. To do so, we HPLC purified the Cy3-miR302d oligo to determine whether any free Cy3 was present in the reagent, which would suggest that cells could be absorbing free Cy3 fluorophore. We used free Cy3 fluorophore and a miR-302d synthetic oligo as controls for HPLC (Figure 2.2A,B). The Cy3-miR-302d oligo was HPLC-analyzed and we determined there was no free Cy3 present in the sample, however there was minimal unlabelled miR-302d (Figure 2.2C). Since there was no free Cy3 in the Cy3-miR-302d oligo, we hypothesized that there is disassociation and/or degradation of the miRNA oligo from the Cy3 flurophore, which would allow free Cy3 to be taken up by the cells.

A recent report indicated that the use of a fluorescent tag, specifically Cy3, is not a reliable marker for small RNA transfection in the presence of serum [27]. This study indicated that in the presence of serum a Cy3-tagged miRNA is degraded resulting in release and uptake of the Cy3 fluorophore into the cells [27]. To determine if this occurred during our experiments, we cultured GFP-HS-27a cells in media supplemented with fetal bovine serum (FBS) and in serum-free conditions, and then added Cy3-miR-302d oligo to

the media. We visualized cells at 20X magnification to determine whether the uptake of Cy3 was dependent upon presence of serum in the media.

We observed uptake of the Cy3 fluorescent signal in GFP-HS-27a cells, supplemented with FBS, 3 hours after addition of the Cy3-miR-302d (Figure 2.3A-D). Under serum free conditions we did not observe Cy3 fluorescent signal after 3 hours (Figure 2.3E-H). However, after GFP-HS-27a cells were cultured for 24 hours under serum-free conditions, we observed Cy3 fluorescent signal in the cells (Figure 2.3M-P). The levels of Cy3 were comparable to GFP-HS-27a cells cultured with FBS conditions at 24 hours (Figure 2.2I-L). Our observations suggest that the miRNA is disassociated and/or degraded from the Cy3 fluorophore more readily in the presence of serum-supplemented media resulting in uptake of Cy3. This supports the conclusions by Han J *et al.*, where the miRNA was degraded prior to Cy3 uptake [27]. Through these experiments we determined that there is direct uptake of Cy3, especially in serum-supplemented media, therefore 5' Cy3-tagged miRNAs cannot be used to visualize the presence of miRNAs within a cell.

Co-culture of different cell types expressing different fluorescent proteins

Distinct cell populations were present after co-culture

Knowing that we could no longer rely on a Cy3-tagged miRNA to determine the presence of the miRNA within the cells, we decided to co-culture GFP-HS-27a and THP1 cells to study potential miRNA transfer. After co-culture cells were separated and analysed for changes in miRNA levels. We needed to be able to reliably distinguish cell types; therefore we stably expressed mCherry in THP-1 cells (mCherry-THP1). This allowed for mCherry-THP1 and GFP-HS-27a cells to be distinguished and collected using FACS. mCherry-THP1 cells were co-cultured with GFP-HS-27a cells, with a 2 donor (mCherry-

THP1) to 1 recipient (GFP-HS-27a) ratio, cells were then analysed and sorted using FACS 15 hours after co-culture (Figure 2.4A).

After co-culture, 38% of the population was found to be positive for only GFP. These cells were collected and used to determine if there was miRNA transfer from mCherry-THP1 cells (Figure 2.4B). In order to identify miRNAs that could have potentially been transferred, we profiled the miRNA levels in donor and recipient cells prior to and after co-culture. We looked for miRNAs that increased or decreased in the recipient or donor, respectively. Using miRNA profiling we identified that endogenous miRNAs, miR-142-3p and miR-223, were elevated in GFP-HS-27a cells after co-culture when compared to prior to co-culture (Figure 2.4C). We verified the increase in levels of miR-142-3p in GFP-HS-27a cells after co-culture via qRT-PCR. We determined that GFP-HS-27a cells after co-culture had 8-fold more miR-142-3p present than prior to co-culture and miR-142-3p was highly expressed in mCherry-THP1 cells prior to co-culture (Figure 2.4D). This suggested that miR-142-3p might be transferred from mCherry-THP1 cells to GFP-HS-27a cells.

We expected to observe only two cell populations, mCherry+ and GFP+, after co-culture, which would indicate separation of the two cell populations, mCherry-THP1 and GFP-HS-27a. Surprisingly, we also observed that about 1% of the population was double positive for both mCherry and GFP (Figure 2.4B). We were able to reproducibly detect this 1% population of mCherry+GFP+ cells after co-culture. The qRT-PCR analysis indicated the mCherry+GFP+ population had 80-fold more miR-142-3p levels than is found in GFP-HS-27a cells prior to co-culture (Figure 2.4D). The most likely explanation for the finding of a double positive population of cells would be contamination during the FACS process. Therefore, we took additional preventative measures to avoid contamination from

mCherry-THP1 cells to GFP-HS-27a cells by sorting for GFP+ cells first and flushing lines prior to sorting for mCherry+ cells. These findings indicated that there was potentially two populations present after co-culture that may have received transferred miRNAs. The GFP+ only population, which may represented cells with only miRNA transfer, and the mCherry+GFP+ cells, that could represent transfer of miRNAs in addition to mCherry mRNA or protein.

Amnis ImageStreamX allowed visualization of cell populations

We wanted to further characterize the mCherry+GFP+ population and hypothesized that mCherry+GFP+ cells could result either indicate from cell fusion and/or transfer of mCherry mRNA or protein in addition to miRNAs. In order to better elucidate the mechanism of transfer we needed to determine whether this population was one cell with two nuclei, indicating potential cell fusion, or one cell that is both mCherry+ and GFP+, indicating protein or mRNA transfer. We utilized an Amnis ImageStreamX flow cytometer, which captured images of each cell as the flow cytometer analysed them. This platform allowed visualization and analysis of each cell population after co-culture. A subset of cell images was evaluated for the average area of a cell to establish limits that are used to analyse all images and determine area of each cell. This analysis allowed for separation of each cell image into populations of debris, single cells, and doublet cells (Figure 2.5A). This analysis revealed the existence of a doublet cell population that indicated two cells were being analysed by the flow cytometer at the same time, with either two GFP+ cells, two mCherry+ cells, or one mCherry+ and one GFP+ cell (Figure 2.5B,C). We also observed a single cell population, which were mostly either GFP+ or mCherry+ and only a small population that were both mCherry+ and GFP+ (Figure 2.5D). The few single cells with

mCherry+GFP+ cells had punctate mCherry+ fluorescence, however this population of cells accounted for less than 5% of double positive cells (Figure 2.5F). This data indicated that the initial miRNA transfer that we observed in mCherry+GFP+ cells was not due to transfer of miRNAs but was rather an artifact of cells sticking together and being sorted improperly during FACS.

Repeat flow cytometry revealed impurities in sorted cell populations

In order to determine whether cells were improperly sorted, resulting in contamination of the purified populations, we co-cultured mCherry-THP1 and GFP-HS-27a cells, FACS sorted for distinct populations and then repeated the FACS analysis. After the first round of FACS sorting, mCherry+, GFP+ and mCherry+GFP+ cells were collected, and a fraction was saved for qRT-PCR analysis while the rest of GFP+ and mCherry+GFP+ cells were reanalysed via FACS. In the initial FACS analysis, we observed that 25% of the population consisted of GFP+ only cells, while about 1% of the population of consisted of mCherry+GFP+ cells (Figure 2.6A). Reanalysis of the mCherry+GFP+ population revealed that about 28% and 39% of the cells were mCherry+ only cells and GFP+ only cells, respectively (Figure 2.6B). This indicated that the mCherry+GFP+ population we initially thought was a single cell population was actually composed of cells that were incorrectly sorted due to cells sticking together during FACS analysis. Most surprisingly, when the GFP+ population was reanalysed, we observed a 1% contamination with mCherry+ cells (Figure 2.6C). This indicated that there was contamination of mCherry+ cells to a population believed to be mCherry negative. Given this finding, we needed to determine whether this level of contamination by mCherry would be sufficient to account for the miRNA transfer we initially observed.

We designed Taqman qRT-PCR primers specific for GFP and mCherry DNA to allow us to confirm that there was contamination from the different cell types in the isolated cell populations. Results of the DNA qRT-PCR indicated that the isolated populations after co-culture contained contamination, likely due to inefficient FACS sorting. There was a 1% DNA contamination of GFP DNA in mCherry+ only cells and 1% contamination of mCherry DNA in GFP+ only cells (Figure 2.7A). When contaminated cells were re-sorted, the contamination of GFP+ cells by mCherry DNA was eliminated (Figure 2.7A). We then analysed the sorted populations (mCherry+, GFP+, mCherry+GFP+, and re-sorted GFP+) for miR-142-3p. After re-sorting the GFP+ cells, the levels of miR-142-3p returned to background levels (Figure 2.7B). These findings confirmed that the initial data suggesting transfer of miR-142-3p to GFP+ population was in fact due to contamination by mCherry-THP1 cells, due to improper sorting of mCherry-THP1 cells. Given these findings and the limitations of the available techniques to experimentally address miRNA transfer, we were unable to evaluate true transfer of miRNAs between THP-1 and HS-27a cells. Any transfer that might have occurred would likely have been masked by level of contamination we observed, making it unlikely that we would be able to detect it.

Discussion

We investigated cell-cell transfer of miRNA within the bone marrow microenvironment using two methods commonly used in the field. The first method we used was tracking a fluorescently tagged-miRNA during transfer to determine presence of miRNA within a cell. In our initial experiments, we observed robust Cy3 fluorescent signal transferred from Cy3-miR-302d transfected THP-1 cells to GFP-HS-27a cells after co-culture. We believed this was transfer of the miRNA, but upon further investigation we

found that the miRNA levels did not correlate with the fluorescent signal. This indicated that using a 5' Cy3-tagged miRNA is an inadequate way to visualize and track miRNA cell-cell transfer over time.

The second method we used was co-culturing two different cell types that express different fluorescent proteins and isolating cells after co-culture to determine whether miRNA was transferred between cells. We co-cultured mCherry-THP1 and GFP-HS-27a cells and FACS sorted for cell populations after co-culture. Using this method, we initially detected a transfer of miR-142-3p to isolated GFP-HS-27a cells after co-culture. Most surprisingly, we observed about 1% of the population to be both GFP+ and mCherry+ after co-culture and detected transfer of miR-142-3p to this population. As we were attempting to characterize this phenomenon, we determined that there was about a 1% contamination in isolated single fluorescent populations. We also determined that the 1% mCherry+GFP+ population after co-culture was due to inadequate sorting of cells. The cellular contamination from FACS accounted for the initial observed transfer of miR-142-3p to both of these cell populations. Through these experiments, we discovered that FACS sorting could lead to at least a 1% cross-contamination of individual cell types.

These experiments highlight the potential challenges that could be encountered when studying miRNA cell-cell transfer. We determined that relying on fluorescent-tagged miRNAs is not a reliable method for visualizing miRNA transfer within a cell, especially in serum-supplemented media. We also determined that fluorescence-based methods to separate cell types using FACS are insufficient and cannot be solely relied on to distinguish recipient and donor cells. It is important to be aware of these potential artifacts related to

fluorescently-tagged miRNAs and FACS, as both of these approaches are currently commonly used in miRNA cell-cell transfer studies.

We would like to acknowledge the contributions to this work by:

Majlinda Kulloli, Sharon Pitteri, Aravind Ramakrishnan, Michele Murphy, Beverly Torok-Storb

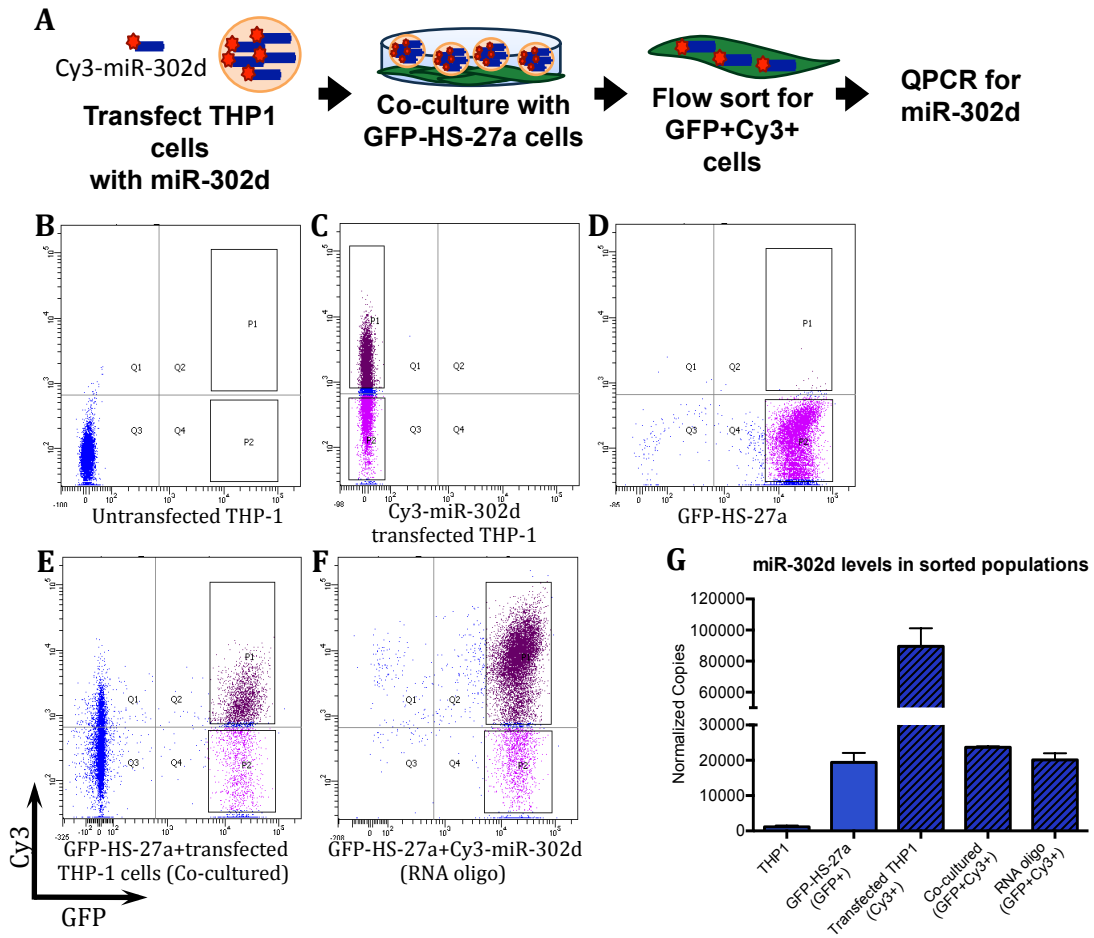

Figure 2.1: Cy3 fluorescent signal does not correlate with miR-302d A) Co-culture schematic. THP-1 cells are transfected with Cy3-tagged miR-302d and co-cultured with GFP-HS-27a cells. Cells are then flow sorted for GFP+Cy3+ cells and analyzed for miR-302d via qRT-PCR. FACS analysis for B) Untransfected THP-1 cells, C) Cy3-miR-302d transfected THP-1 cells, D) GFP-HS-27a cells alone, E) Cy3-miR-302d transfected THP-1 cells co-cultured with GFP-HS-27a cells (Co-cultured), F) Cy3-miR-302d co-cultured with GFP-HS-27a cells (RNA oligo). G) MiR-302d levels were analyzed in sorted samples using qRT-PCR. THP-1 and GFP-HS-27a cells express low levels endogenously of miR-302d. After transfection with Cy3-miR-302d, THP-1 cells have 76 times more miR-302d when compared to untransfected THP-1 cells. Co-cultured GFP+Cy3+ cells and RNA oligo GFP+Cy3+ cells do not have an increase in miR-302d above background levels after co-culture.

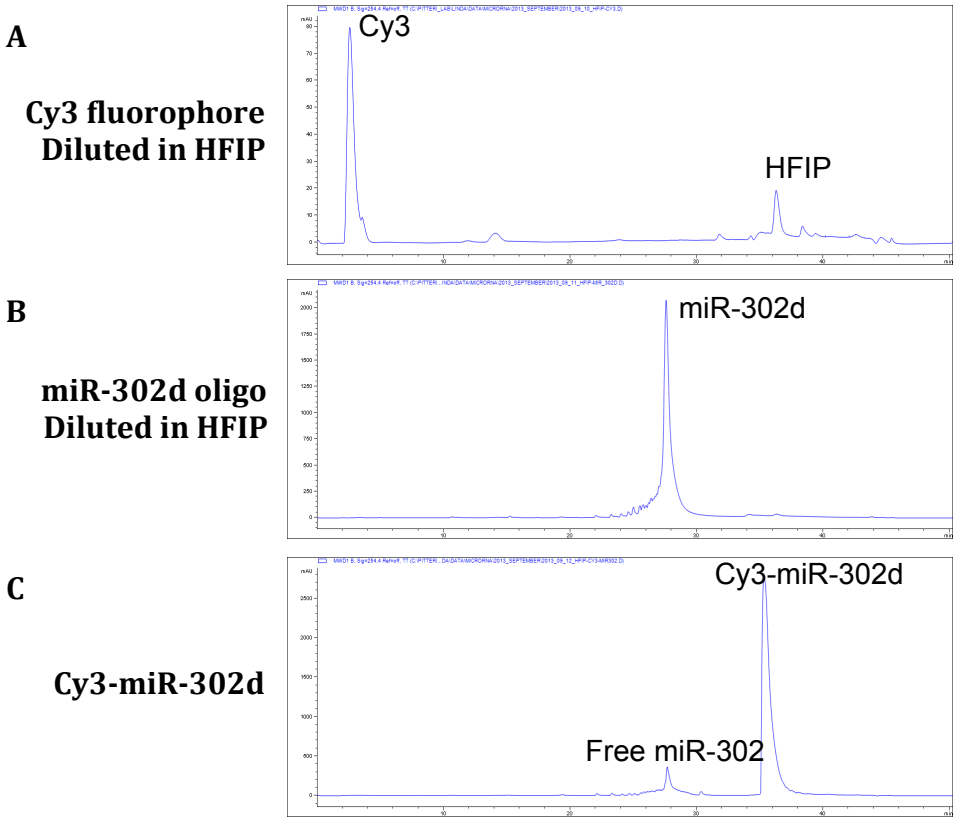

Figure 2.2: HPLC purification of the Cy3-tagged miR-302d oligo reveals no free Cy3. A) Control Cy3-fluorophore alone has one peak for the fluorophore. B) Control miR-302d reveals the peak for miR-302d alone. C) HPLC for Cy3-miR-302d reveals no free Cy3. The majority of the miR-302d oligo is linked to Cy3.

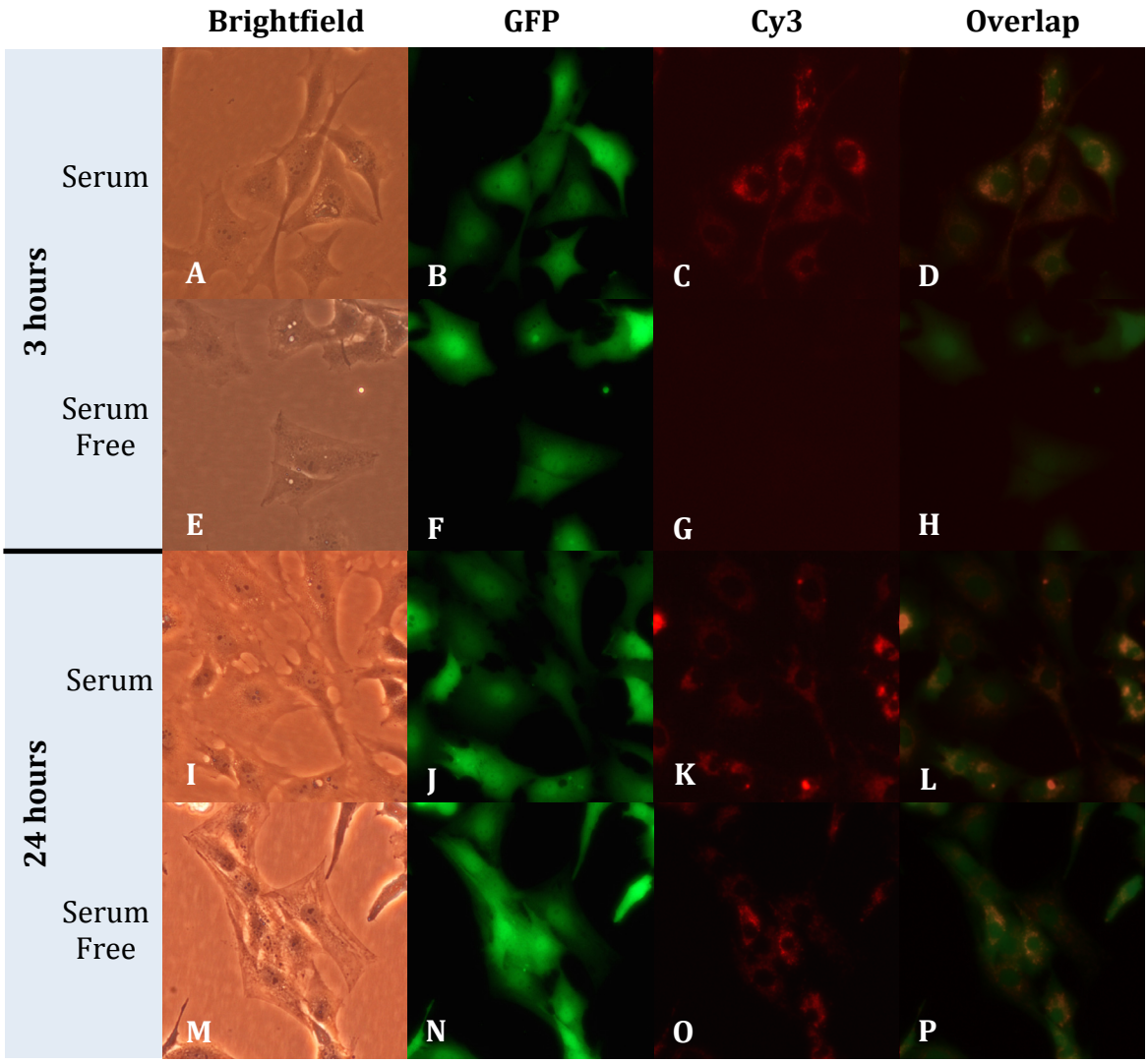

Figure 2.3: Cy3 is taken up by GFP-HS-27a cells more rapidly in the presence of serum supplemented media. GFP-HS-27a cells co-cultured with Cy3-miR-302d oligo A-D) Serum supplemented media for 3 hours, E-H) Serum free supplemented media 3 hours I-L) Serum supplemented media for 24 hours, M-P) Serum free supplemented media for 24 hours. Cells were visualized at 20X magnification.

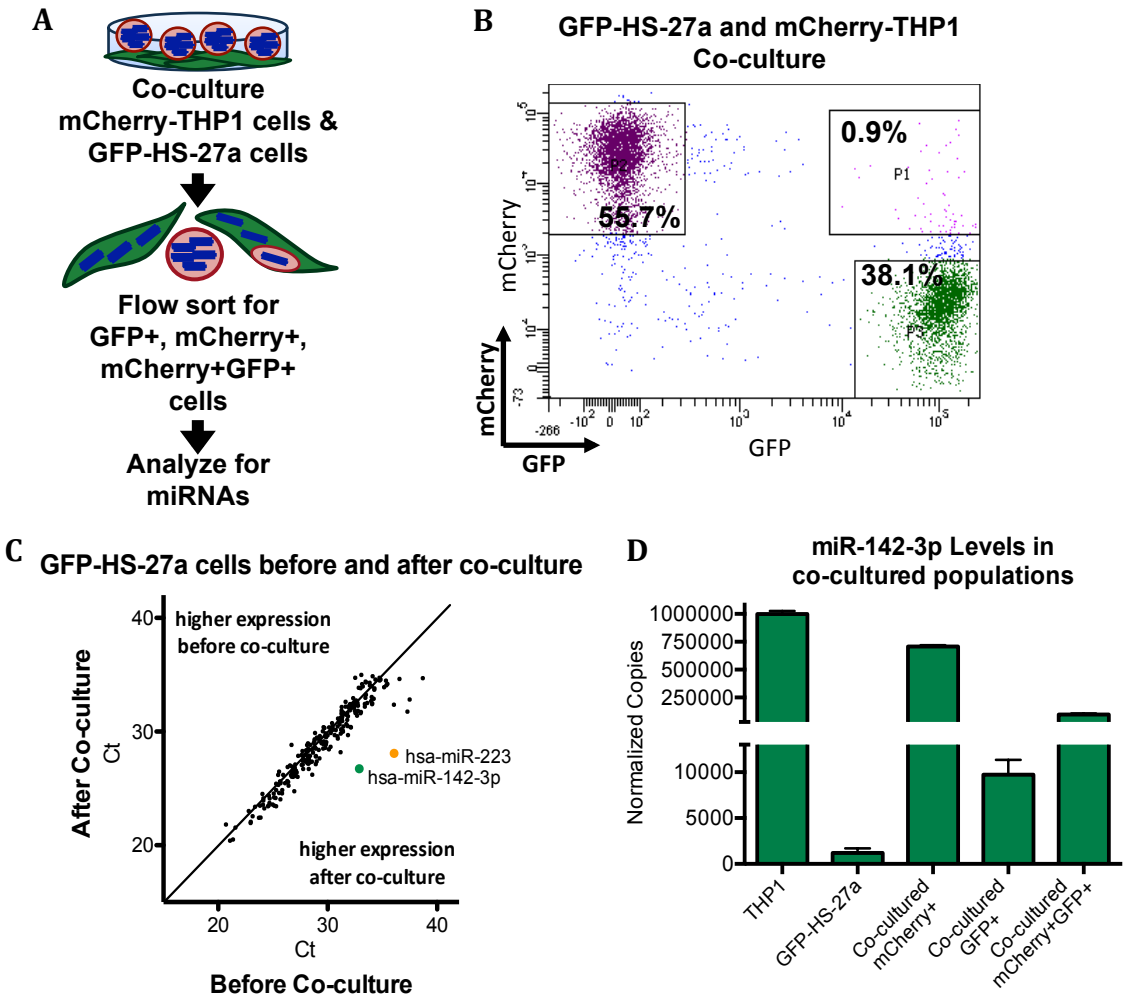

Figure 2.4: Distinct cell populations are present after co-culture and transfer of miR-142-3p is detected in isolated populations A) Co-culture schematic. mCherry expressing THP-1 cells (mCherry-THP1) were co-cultured with GFP-HS-27a cells. Cells were separated by flow cytometry for GFP+, mCherry+ and mCherry+GFP+ and analyzed for miRNAs using miRNA profiling and qRT-PCR. B) Flow cytometry analysis revealed about 38% of the population was GFP+ only cells. Surprisingly there was about 1% of the population that was mCherry+GFP+ cells. C) miRNA profiling indicated that there was miRNA transfer from mCherry-THP1 cells to GFP-HS-27a cells (GFP+ only cells). miR-142-3p and miR-223 were two miRNAs with highest change in GFP-HS-27a cells after co-culture. D) The transfer of miR-142-3p was verified using qRT-PCR. miR-142-3p was about 8 times more abundant in GFP+ only cells than endogenous levels. mCherry+GFP+ cells after co-culture have 80 times more miR-142-3p than endogenous levels in GFP-HS-27a.

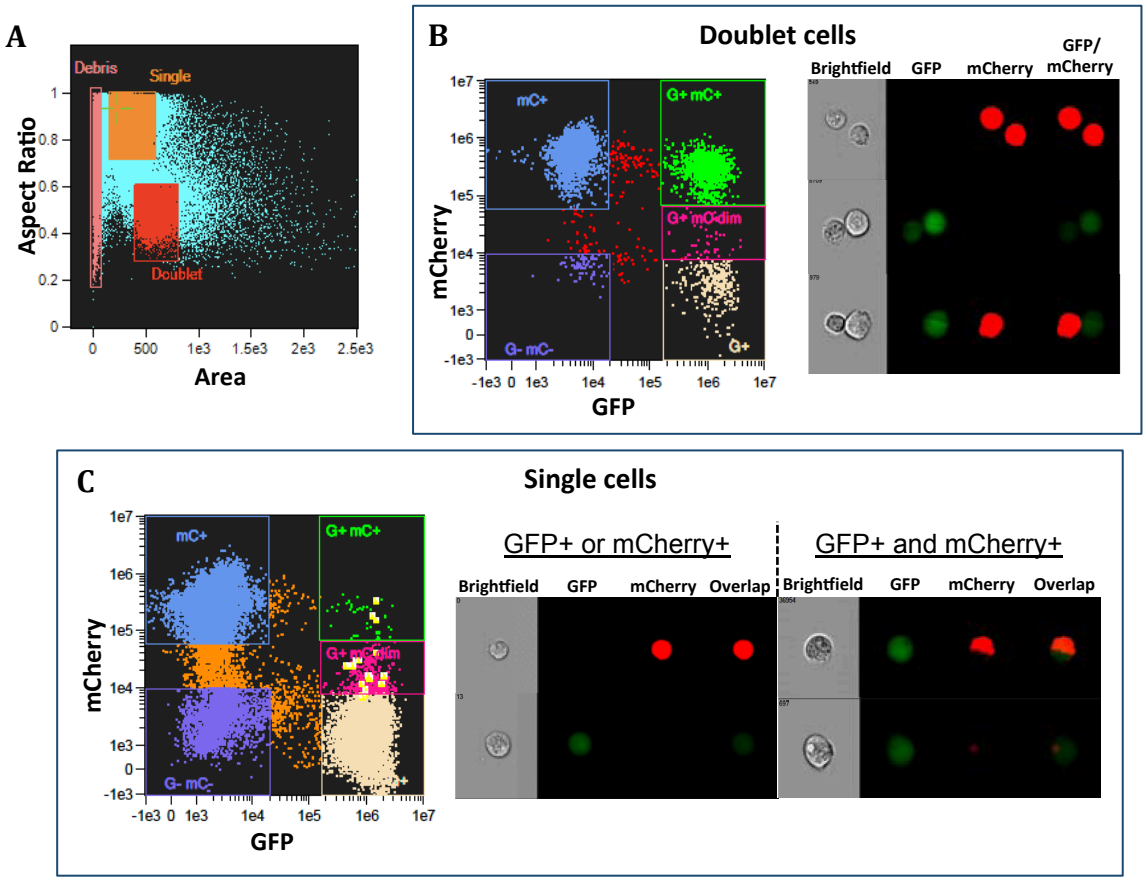

Figure 2.5: Amnis ImageStreamX revealed that isolated populations are contaminated by improper sorting. ImageStream X allows for visualization of each cell during flow cytometry analysis. A) The area of particles are measured and through algorithms can discriminate between debris, single, and doublet cells. B) Analysis of doublet populations revealed that cells are being co-analyzed by the flow cytometer. These cells are a mix of GFP+ only, mCherry+ only and mCherry+GFP+. C) Analysis of the single cell population revealed that most of the population is either GFP+ only or mCherry+ only. A small population of cells are mCherry+GFP+, however these particles are not single cells.

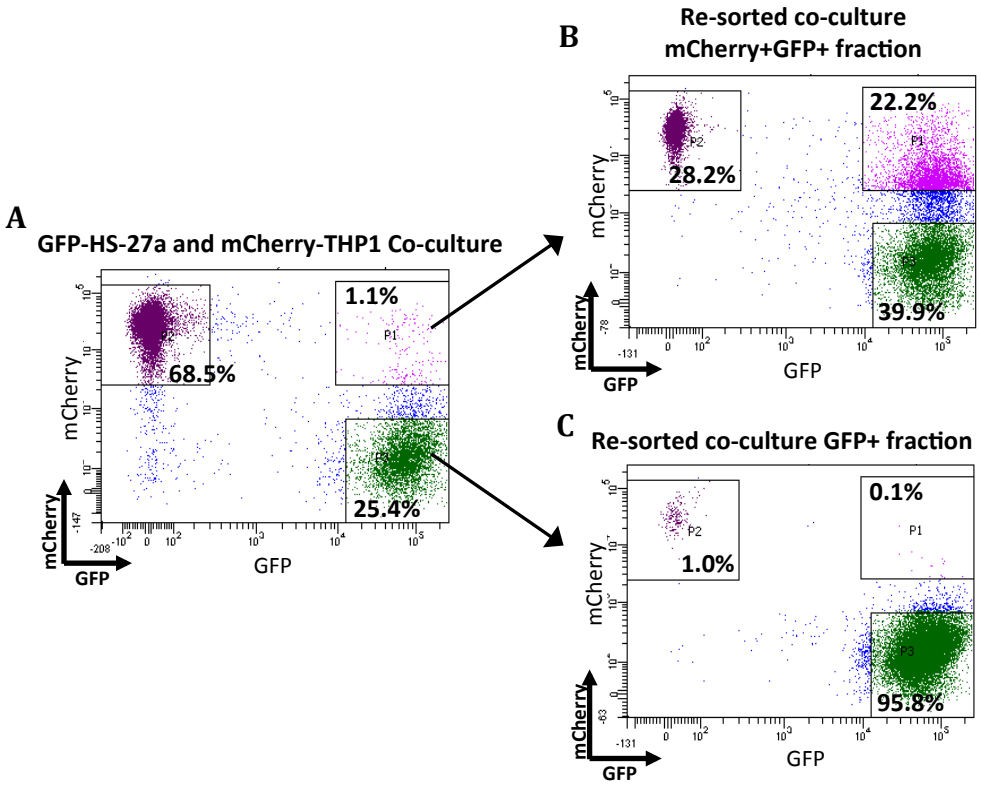

Figure 2.6: Repeat FACS revealed contamination in sorted cell populations. A) mCherry-THP1 and GFP-HS-27a cells were co-cultured and sorted for GFP+ and mCherry+GFP+ cell populations. A fraction of these populations was collected for analysis and the rest was re-sorted to determine if there was incorrect sorting. B) The resorted mCherry+GFP+ cells showed incorrect sorting with about 28% of cells being only mCherry+ and about 40% being only mCherry+. Indicating that a majority what was believed to be an mCherry+GFP+ cell population was actually flow cytometry contamination. C) Most surprisingly, the GFP+ population contained a 1% contamination of mCherry+ only cells. This indicates that there is contamination of the GFP-HS-27a population by mCherry-THP1 cells.

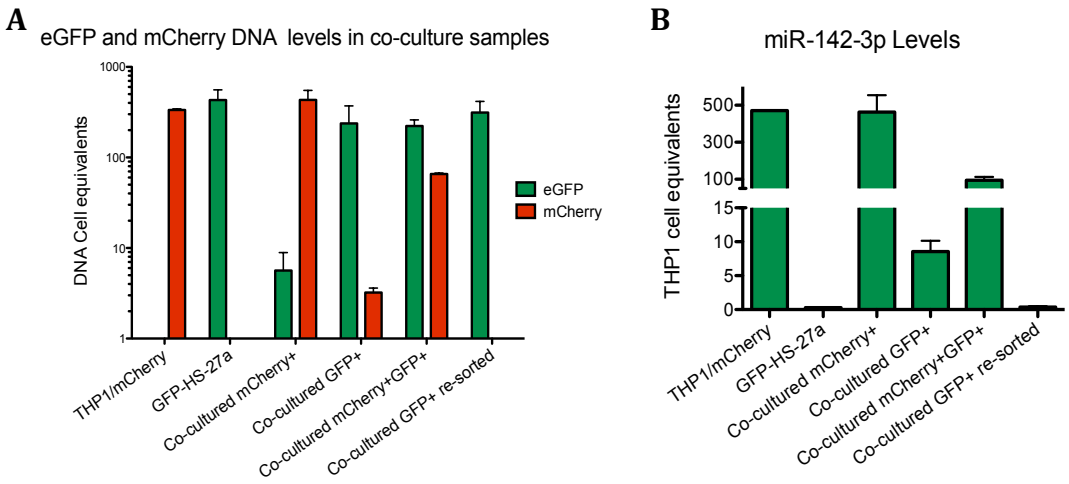

Figure 2.7: DNA and miRNA qRT-PCR revealed initial transfer to be an artifact of FACS sorting.

A) eGFP and mCherry DNA levels in sorted populations. There is about 1% contamination of mCherry DNA in sorted GFP+ population and 1% contamination of GFP DNA in sorted mCherry+ population. The contamination of mCherry DNA disappeared in re-sorted GFP+ cells. B) miR-142-3p levels in sorted populations. The initial transfer present in co-cultured GFP+ cells, disappears after the re-sorting of GFP+ cells. Indicating that the miRNA transfer was due to contamination with mCherry+ cells.

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Chapter 3

Investigating plant miRNA transfer into the human bloodstream

Abstract

Endogenous miRNAs are present in a highly stable, extracellular form in the bloodstream, where they are postulated to be functional. Whereas most studies of circulating miRNAs focus on endogenously-derived miRNAs, a recent study reported that intact rice miRNAs could be detected in human blood serum, raising the possibility that bioactive miRNAs from the diet can be acquired orally. Although this report stimulated much discussion, the hypothesis that dietary miRNAs are acquired orally has not yet been tested under controlled conditions in humans. In order to test this hypothesis, we designed the first controlled study in which we fed individuals a specific plant-based meal and collected serial blood specimens hourly for 8 hours or every half-hour for 4 hours. We analyzed serial blood specimens for the presence of plant-derived miRNAs from the consumed meals. Through this study we determined that plant miRNAs, ath-miR-156a and hvu-miR-168a, are not detected above the limit of quantification at any timepoint in the majority of participant serum samples. The maximum serum concentration of plant miRNA observed in any participant was low with only 22,071 copies per uL of serum. However, even in participants with detectable levels above the limit of quantification, the absolute concentrations are low and miRNA levels did not replicate in independent analyses. Our results indicate that plant miRNA absorption is unlikely to occur in healthy individuals. At best plant miRNA absorption into the human bloodstream is highly inefficient and absolute

concentrations detected in this study are so low that they are unlikely to regulate endogenous gene expression.

Introduction

The majority of miRNAs are found intracellularly, however a significant number of extracellular miRNAs have been observed. Surprisingly, extracellular miRNAs are highly stable, suggesting that miRNAs are protected from degradation in some way. It is now known that miRNAs can be protected from degradation by packaging in exosomes, lipid vesicles, and/or in complexes with RNA-binding proteins [1-5]. Despite evidence of the presence of extracellular miRNAs, the function of these circulating miRNAs remains poorly understood. There are have been many studies conducted by various groups to better understand the function and transfer mechanisms of extracellular miRNAs.

Most studies have focused on endogenous circulating miRNAs, but recently a study discovered a novel phenomenon in which exogenous plant-derived miRNAs where detected in human serum [6]. This study was the first report of potential cross kingdom regulation, and suggested that ingested miRNAs are transferred to the blood, can accumulate in tissues, and exert regulation of endogenous transcripts [6]. Since this initial report there have been follow up studies to investigate whether exogenous miRNAs could be transferred to into the blood. Analysis of small RNA (sRNA) datasets of tissues from mammals, chicken, and insects determined that there are low levels of plant miRNAs detected in sRNA datasets, but these plant miRNA sequences are an artifact due to contamination of sequencing reactions [7]. Plasma from healthy athletes collected after ingestion of fruit was analyzed for the presence of plant miRNAs (miR-156a, miR-159a and

miR169a), but did not carry detectable levels of these molecules [8]. Human plasma was analyzed for miRNA biomarkers using next generation sequencing and it was observed that a fraction of circulating RNA originates from exogenous species including bacteria and fungi [9]. In another study, blood was obtained from pigtailed macaques pre- and post-feeding, but plant miRNAs were not detected via qRT-PCR or droplet digital PCR [10]. One group conducted a controlled mouse feeding study where mice were fed either a rice-based diet or purified chow devoid of plant material and the results suggested that the plant miRNA sequencing reads obtained could be attributed to artifacts or contamination [11]. In the most recent study, deep-sequencing libraries were analyzed for diet-derived miRNAs and variable amounts of plant miRNAs were detected, however this group suggested that diet-derived miRNAs were due to contamination during library preparation [12]. Thus, there are conflicting data that plant miRNAs could be acquired from the diet.

Follow up studies have provided increasing evidence that plant miRNAs may not be as abundant in the human bloodstream as the initial study indicated [6], but these studies all had significant limitations. These limitations include: blood drawn at random or after 12 hours of fasting, lack of negative controls or baseline blood draws, insufficient blood draw time points and insufficient or unknown plant-based diet consumption. We designed a controlled feeding study that would allow us to formally test the hypothesis that plant-derived miRNAs are acquired orally and characterize the efficiency and dynamics into the bloodstream. The information acquired in this study provides a foundation for directing future studies of potential cross-kingdom gene regulation through dietary miRNA uptake.

Methods

Subjects

Healthy non-smoking men and women, ages 21-60 with a BMI between 18.5 and 39.9 kg/m^2, were recruited from the greater Seattle area by distributing flyers at the Fred Hutchinson Cancer Research Center (FHCRC), University of Washington, Seattle Pacific University, other community college campuses in Seattle, and Seattle at large, by placing ads in local newspapers (college, weeklies and community based), and by posting on social media like Craigslist. Potential participants filled out a screening questionnaire, and were excluded if they reported any of the following: medical history of gastrointestinal, hepatic, or renal disorders; history of kidney stones; pregnancy or lactation; antibiotic use within the past 3 months; current use of prescription medication (including oral contraceptives); alcohol intake of more than two drinks per day (two drinks being equivalent to 720 mL beer, 240mL wine, 90mL spirits); inability to consume study foods; inability to schedule the nine hour clinic appointment during the work week. As part of the study participants completed a 2-day food record, and a health and demographic survey. The Institutional Review Board at the Fred Hutchinson Cancer Research Center approved the study and informed consent was obtained from all participants prior to the start of the study.

Feeding study design

The feeding study was conducted between June 2013 and February 2014. We recruited and enrolled 15 participants, 8 males and 7 females. Participants were not allowed to eat any of the study foods for 24-hours, therefore each participant could serve as their own control. The first blood draw was after a 10-hour overnight fast in all participants. Seven participants consumed the study meal and had hourly blood draws for

4 hours, followed by a second study meal and hourly blood draws for 4 hours, for a total of 9 blood draws. In the other eight individuals, participants consumed the study meal and had blood draws every half hour for 4 hours, for a total of 9 blood draws, then consumed the second study meal. All participants returned for a 24 and 48-hour blood draw at fasting (Figure 3.2).

Study Diets

Participants consumed two study meals consisting of solid food and smoothie components. The total amount of food served to each participant was 700 grams per meal (Table 3.1). Participants were expected to eat at least 75% or 525 grams. Participants were allowed to eat additional snacks during the nine-hour clinic visit, after breakfast was served. The allowed snacks included coffee, milk, cheese, potato chips, Nilla Wafers, and Mini Wheats. Participants were sent home with one or more frozen macaroni and cheese dinners and snacks. All food during the clinic visit day was supplied by the FHCRC Prevention Center.

Biological Sample Collection

Participants had an IV catheter inserted with a drip of normal saline started for the clinic visit day. No heparin was used to prevent clotting, as heparin was previously shown to interfere with qRT-PCR reactions [13, 14]. Blood for plasma and serum, 4 mL and 7 mL respectively, was collected from participants at each time point. The volume of each draw was 12 mL including a 1 mL discard of blood (which contains saline from the IV line). For the 24-hour and 48-hour time point blood draws, blood was drawn directly for plasma and serum, 4 mL and 7 mL, respectively.

Plasma and Serum Processing

All blood samples were processed promptly after each draw, aliquoted at 0.25mL per vial and stored at -80°C immediately after processing. Plasma was collected using a 4mL lavender top K2EDTA plasma tube (BD Vacutainer Tube #367862) and centrifuged at 3400xg for 10 minutes at room temperature with high brake. The top three-fourths of liquid volume was transferred to a new pre-labeled 15mL conical tube and centrifuged at 1940xg for 10 minutes at room temperature with no brake. The top three-fourths of liquid volume was transferred to a new pre-labeled 15mL conical tube and aliquoted in 0.25mL aliquots in pre-labeled vials. The remaining pellet was also transferred into a pre-labeled vial. Serum was collected using a 7mL SST tube. Serum was allowed to clot at room temperature for 45 minutes, or until clot was solid and firm, and centrifuged at 1300xg for 15 minutes at room temperature. Serum separated from clotted RBCs was immediately transferred into a new pre-labeled 15 mL tube and aliquoted in 0.25mL aliquots into pre-labeled vials.

RNA was isolated from plasma and serum samples with miRCURY RNA isolation biofluids kit (Exiqon) according to manufacturer's instructions using 200uL per sample. Spike-ins for RNA isolation controls (cel-miR-39, cel-miR-54, cel-miR-238 and MS2 RNA) were added to each sample prior to isolation. RNA was eluted in 30 uL RNase free water.

Food Aliquot Processing

We collected 100mg aliquot of each of the study foods, broccoli, brussel sprouts, spinach, rice, tofu and smoothie in quadruplicate for each participant in Safe-Lock tubes (Eppendorf) and stored immediately at -80°C. RNA was isolated from food aliquots using miRVana miRNA isolation kit (Life Technologies) and Plant RNA isolation aid (Life

Technologies). First we added, 100uL of Plant RNA isolation aid and 100uL Lysis/Binding Buffer to each sample tube. We disrupted the tissue for 3 minutes at 25 Hz using a TissueLyser, then added 900uL Lysis/Binding Buffer. The sample was then homogenized for 1.5 minutes at 25 Hz using a TissueLyser. The lysate was clarified by centrifugation at 13,000 x g for 5 minutes and transferred to a new pre-labeled 2 mL tube and RNA isolation controls (cel-miR-39, cel-miR-54, cel-miR-238) were added. We continued isolation according to miRVana miRNA isolation manufacturer protocol at step E1. RNA was eluted with 100uL of preheated nuclease-free water, according to manufacturer instructions.

Plant miRNA qRT-PCR Analysis

qRT-PCR was performed using Taqman assays (Applied Biosystems) for ath-miR-156a, hvu-miR-168a, bol-miR-824, and hsa-miR-16 and control assay cel-miR-39. For each sample a 5 uL RT reaction was completed with 1.67 uL input of total RNA. Each RT reaction was diluted with 28.9uL of water. A 5 uL QPCR reaction with 2.25 uL of the diluted RT sample was added to each well of the 384-well plates, and amplification was performed on a Viia7 qPCR machine (Applied Biosystems). Copies in qRT-PCR reaction were calculated and normalized to cel-miR-39. Normalized copies were converted to normalized copies per uL of serum or normalized copies per gram of tissue.

Blinded Analysis of Select Samples

Selected participant samples were blinded prior to RNA isolation. RNA was isolated from 200uL using methods described in Plasma and Serum Processing, above, and RNA was divided for analysis via qRT-PCR and ddPCR. Samples were analyzed via qRT-PCR using methods described in Plant miRNA qRT-PCR Analysis, above. Normalized copies per uL serum were then compared to initial qRT-PCR analysis.

Samples were analyzed via ddPCR by the Witwer lab at Johns Hopkins. ddPCR was performed using Taqman Assays (Applied Biosystems) for ath-miR-156a and hvu-miR-168a. For each sample a 10 uL RT reaction was completed with 2 uL input of total RNA. A 20 uL ddPCR reaction with 1 uL cDNA from the RT reaction using ddPCR Supermix (Bio-Rad). Samples were analyzed using QX100 Droplet Digital PCR System (Bio-Rad). Concentrations were determined manually to correct for positive and negative droplet reads.

Results

Plant miRNAs are present in a subset of human serum samples

In order to determine if plant miRNAs could be detected in human serum and are associated with diet consumption, we first analyzed serum samples from a previous feeding study conducted at FHCRC by the Lampe lab. In this study each participant consumed two different diets for two weeks each, one devoid of fruit and vegetables (base diet) and one including fruits and vegetables (F&V diet) [15]. We obtained three serum samples, a baseline, base diet, and F&V diet, each from six individuals and analyzed for plant miRNAs via qRT-PCR. The miRNAs we analyzed for were ath-miR-156a and bol-miR-824. Ath-miR-156a is found in many plants and also the most abundant miRNA in sequencing data from the first plant miRNA transfer paper [6]. The plant miRNA, bol-miR-824, is a miRNA specific to *Brassica* vegetables that were a large component of the F&V diet. The *Brassica* specific miRNA, bol-miR-824, was not detected in any of the serum samples (data not shown). From a total of 18 serum samples, ath-miR-156a was detected in very low abundance in 15 or about 80% of samples (Figure 3.1a). If plant-derived miRNAs were acquired orally we expected an increase in ath-miR-156a after consumption of the

F&V diet when compared to the base diet. Surprisingly, there was not an increase in ath-miR156a levels in the serum after consumption of the F&V diet when compared to the levels of ath-miR-156a after consumption of the base diet (Figure 3.1b). For this study blood was drawn after a 12-hour overnight fast. Since kinetics of absorption are not known, the timing of the blood draw may not have been ideal for the detection of plant miRNAs in the blood.

A larger sample set was subsequently analyzed for ath-miR-156a to determine how frequently plant miRNAs are detected in human serum. However, we did not have any dietary information and samples were also drawn after 12-hour fast, which indicated that these samples might not be ideal for detecting plant miRNAs. The sample set analyzed consisted of a total of 160 female serum samples, 85% were non-ovarian cancer controls and 15% were ovarian cancer patients. Even though the blood was drawn at random, ie. not timed to consumption of a meal, we observed low levels of ath-miR-156a in 58 of 160 samples, about 36% (Figure 3.1C). These data suggested that ath-miR-156a may be detected at a low abundance in a subset is of individuals, however the levels of ath-miR-156a detected are within a range associated with experimental noise.

Plant miRNAs are highly abundant in study foods

Given our initial findings that plant miRNA, ath-miR-156a, is detected in a subset of individuals, we decided to conduct a controlled human study in which blood is collected before and serially after feeding a defined meal. In this controlled study, participants underwent a 24-hour washout period, where they did not consume any of the study foods. On the study day, we collected a baseline blood draw after a 10-hour overnight fast, to establish baseline levels for each participant. After the first draw, participants consumed

the study meal for breakfast, followed by hourly blood draws for 4 hours, then consumed the study meal for lunch and continued with hourly blood draws for an additional 4 hours (Figure 3.2A). Participants returned for 24-hour and 48-hour blood draws after a 10-hour overnight fast. The study meal consisted of two components, a solid food portion, broccoli, brussel sprouts, spinach, rice and tofu, and a smoothie made of apple, orange and kale (Table 3.1). We were most interested in plant miRNAs, ath-miR-156a and hvu-miR-168a, that were the most abundant plant miRNAs in the Zhang L *et al.* study and are expressed in many plants, and bol-miR-824, a *Brassica* specific miRNA.

Aliquots of study foods, broccoli, brussel sprouts, spinach, rice, tofu and smoothie, were collected to quantify the amount of ath-miR-156a, hvu-miR-168a, and bol-miR-824a each participant ingested. Broccoli had the highest expression of in ath-miR-156a, hvu-miR-168a, and bol-miR-824 with $4.5x10^{11}$ copies per gram of tissue, $2.2x10^{10}$ copies per gram of tissue, and $1.5x10^{12}$ copies per gram of tissue, respectively (Figure 3.3A,B,C). The average total amount of ath-miR-156a, hvu-miR-168a, and bol-miR-824 ingested by each participant was approximately $8.3x10^{13}$ copies, $4.6x10^{12}$ copies and $1.8x10^{14}$ copies, respectively, at each meal (Figure 3.3D). Each participant consumed twice this amount during the study day.

bol-miR-824 was not detected in participant samples

A total of 7 individuals were recruited, consented, and participated in the 8-hour study with hourly blood draws. There was no reliable detection of the *Brassica* miRNA bol-miR-824 in any of the participant serum samples (data not shown). It is not clear why this miRNA was not detected in any participant blood samples, despite bol-miR-824 being more abundant, $1.8x10^{14}$ total copies, in the study meal than either ath-miR-156a and hvu-miR-

168a, $8.3x10^{13}$ and $4.6x10^{12}$ total copies, respectively (Figure 3.3D). One hypothesis is that there is less absorption from *Brassica* vegetables, than there is among other foods consumed in the study. We also determined that the bol-miR-824 assay was less sensitive than ath-miR-156a and hvu-miR-168a, based on a control standard curve for each assay. Based on the synthetic standard curve for bol-miR-824, we estimated that if any bol-miR-824 present in participant serum samples it was less than 300 copies per uL of serum.

ath-miR-156a and hvu-miR-168a were detected above levels of quantification in a subset of study participants

Participant serum and plasma samples from all timepoints were analyzed for ath-miR-156a and hvu-miR-168a via qRT-PCR to determine levels of plant miRNAs in participants. For each participant we used synthetic standard curves for ath-miR-156a, hvu-miR-168a, and bol-miR-824 to determine the limit of quantification (LOQ) for each assay. The LOQ represents the point where the assay no longer responds linearly to dilution of the synthetic oligo and varies between participants due to day-to-day qRT-PCR variability. Ath-miR-156a and hvu-miR-168a was not reliably detected in any of the participant plasma samples (data not shown). In 4 out of the 7 participants that completed the 8-hour study, ath-miR-156a was not detected in the serum in any of the timepoints (Figure 3.4). Ath-miR-156a was detected above the LOQ in 3 of the participants that completed the 8-hour study, but absolute concentrations were still low with the highest concentration of only 3,900 copies per uL of serum in participant 6 (Figure 3.5).

We also measured levels of hvu-miR-168a in the serum of the same 7 participants, and did not detect hvu-miR-168a concentrations above the LOQ in any of the timepoints in 4 out of the 7 participants (Figure 3.6). Levels above the LOQ for hvu-miR-168a were

observed in 3 out of 7 participants, but were much lower than ath-miR-156a with only 80 and 540 copies per uL of serum (Figure 3.7). These data suggested that plant miRNAs, specifically ath-miR-156a and hvu-miR-168a, are not detected in any timepoint in most participants and when detected plant miRNA levels are low.

In an attempt to better characterize absorption of ath-miR-156a, the study was modified to obtain blood every 30 minutes for 4 hours after the first meal instead of hourly blood draws for 8 hours (Figure 3.2). A total of 8 participants were recruited, consented and enrolled in the modified 4-hour study. We measured both ath-miR-156a and hvu-miR-168a in participant plasma and serum samples. Again, we were not able to reliably detect ath-miR-156a and hvu-miR168a in any participant plasma samples (data not shown).

Ath-miR-156a was not detected in 3 out of the 8 participants at any timepoint indicating no presence of plant miRNA in the serum (Figure 3.8). We detected low concentrations of ath-miR-156a 5 out of 8 participants (Figure 3.9). Participant 12 and 13 had the highest concentration of ath-miR-156a observed with 2,810 and 22,071 copies per uL serum, respectively (Figure 3.9C,D). The majority of 4-hour study participants, did not have detectable levels of hvu-miR168a at any timepoint (Figure 3.10). In 2 out of 8 participants, hvu-miR-168a concentrations were above the LOQ, but there was no peak elevation after feeding and large variability between replicate RT reactions indicating no uptake after consumption of a study meal (Figure 3.11). In most of the participants, we do not detect plant miRNAs, ath-miR-156a and hvu-miR168a, at any timepoint. We detected ath-miR-156a and hvu-miR-168a above the LOQ in a few participants, however absolute concentrations of these miRNAs were low ranging from 45 to 22,071 copies per uL of serum.

Blinded analysis of plant miRNAs does not directly correlate with initial analysis

In order to determine reproducibility of ath-miR-156a levels in participant serum samples, we blinded serum aliquots corresponding to a subset of the participants and performed an independent RNA isolation on the blinded and subsequent analysis of ath-miR-156a using both qRT-PCR and ddPCR (Figure 3.12A). We did not observe concordance between initial qRT-PCR results and the blinded qRT-PCR results (Figure 3.12B-D). For example, in participant 6, initially we observed a peak concentration of ath-miR-156a 2 hours after the first meal, but in the blinded qRT-PCR and ddPCR analysis we did not observe an increase at the same timepoint (Figure 3.12B). In participant 7, the initial qRT-PCR analysis revealed a peak concentration of ath-miR-156a 6 hours after the first meal (Figure 3.12C). In the blinded qRT-PCR and ddPCR analysis for participant 7, we observed a peak concentration at 7 hours after the first meal instead of 6 hours (Figure 3.12C). In the initial analysis for participant 13, we observed a peak concentration of ath-miR-156a at 2.5 hours after the first meal with 22,071 copies per uL serum. The blinded qRT-PCR for participant 13 also had a peak concentration at 2.5 hours, however the concentration of ath-miR-156a was much lower with 93 copies per uL serum (Figure 3.12D). These data indicate that there is a lack of concordance in the concentrations of ath-miR-156a between separate RNA isolations and using a different PCR-based method to analyze levels of ath-miR-156a. These findings suggest that the miRNA concentrations are very low and consequently subject to experimental noise.

Discussion

We designed and carried out the first controlled feeding study in humans with a defined plant-based meal and multiple blood draw timepoints to determine whether plant

miRNAs in the bloodstream are acquired orally. We used qRT-PCR and ddPCR to measure levels of plant miRNAs in human plasma and serum samples. We were not able to detect ath-miR-156a and hvu-miR-168a in any plasma samples (data not shown). Using qRT-PCR we determined that ath-miR-156a concentrations did not exceed the LOQ at any timepoint in 8 out of 15 participants. In 10 out 15 participants, hvu-miR-168a concentrations did not exceed the LOQ at any time point. Even for participants with values above the limit of quantification, the absolute concentrations are low and miRNA levels did not replicate in independent analyses. The maximum serum concentration of plant miRNA, ath-miR-156a, observed in any participant was still low with only 22,071 copies per uL of serum.

We determined that plant miRNAs were highly abundant in the study meal with a total of 8.26×10^{13} copies of ath-miR-156a per meal. Participants had to consume 820 grams of plant-based food to consume this amount of ath-miR-156a, which is much higher than any average person would consume during a day. The percentage of ath-miR-156a consumed that is detected in human serum ranges from 0.00015% to 0.073% (Figure 3.13). This indicates that uptake of plant miRNA is highly inefficient with the highest case of absorption into the blood representing less than 0.1% of ath-miR-156a consumed. However, we cannot rule out that absorbed miRNAs may be degraded and/or cleared from the human bloodstream.

To put the observed serum concentrations of dietary plant miRNA into context with respect to function in target tissues, we compared serum ath-miR-156a concentrations to miRNA concentrations typically necessary in cell culture transfection experiments. We estimated that for a moderate effect in a typical transfection in cell culture, a minimum of 6×10^{10} miRNA copies per uL media for a 96-well transfection of 5,000 cells is required, as

shown in Arroyo *et al.* [16] (Figure 3.14). In contrast, in the circulation at best we detect 1.21×10^4 ath-miR-156a copies per uL of whole blood for 2.1×10^{11} liver cells (liver was chosen as an example target organ as reported in the original study [6]), which are not likely to have an effect (Figure 3.14). If we assume 100% transfection efficiency and even distribution in cell culture transfection there are 1.2×10^9 total copies per cell required for a moderate effect in a target mRNA, however at best case for plant miRNAs we calculate only 0.29 total copies per cell on average (Figure 3.14). These calculations indicate that serum concentrations of ath-miR-156a detected are unlikely to regulate endogenous gene expression.

This is the first investigation in humans that studied exogenous miRNA absorption at early timepoints and high time resolution (i.e hourly and half-hourly intervals) after consumption of a plant-based meal. We conclude that plant miRNA absorption is not likely to occur from dietary intake in most healthy individuals. In some participants, we detected low levels of plant miRNAs, but the absolute concentrations are in a range associated with experimental noise, as determined by the variability in replicating initial qRT-PCR results. We also determined that if this absorption does occur, at concentrations detected in this study, plant miRNA absorption is highly inefficient and unlikely to regulate endogenous gene expression. However, we only measured three plant miRNAs, did not examine absorption into tissues and collected samples from a limited number of individuals. Therefore, we cannot rule out that absorption may occur in local or target tissues, there may be individuals with higher miRNA absorption and other plant miRNAs or classes of RNA may be more readily absorbed. The results from this study provide a foundation to direct future studies of exogenous miRNA transfer into the human bloodstream.

We would like to acknowledge the contributions to this work by:

Ingrid Ruf, James Yan, Yvonne Schwarz, Melissa Alexander, Kenneth Witwer, Johanna Lampe

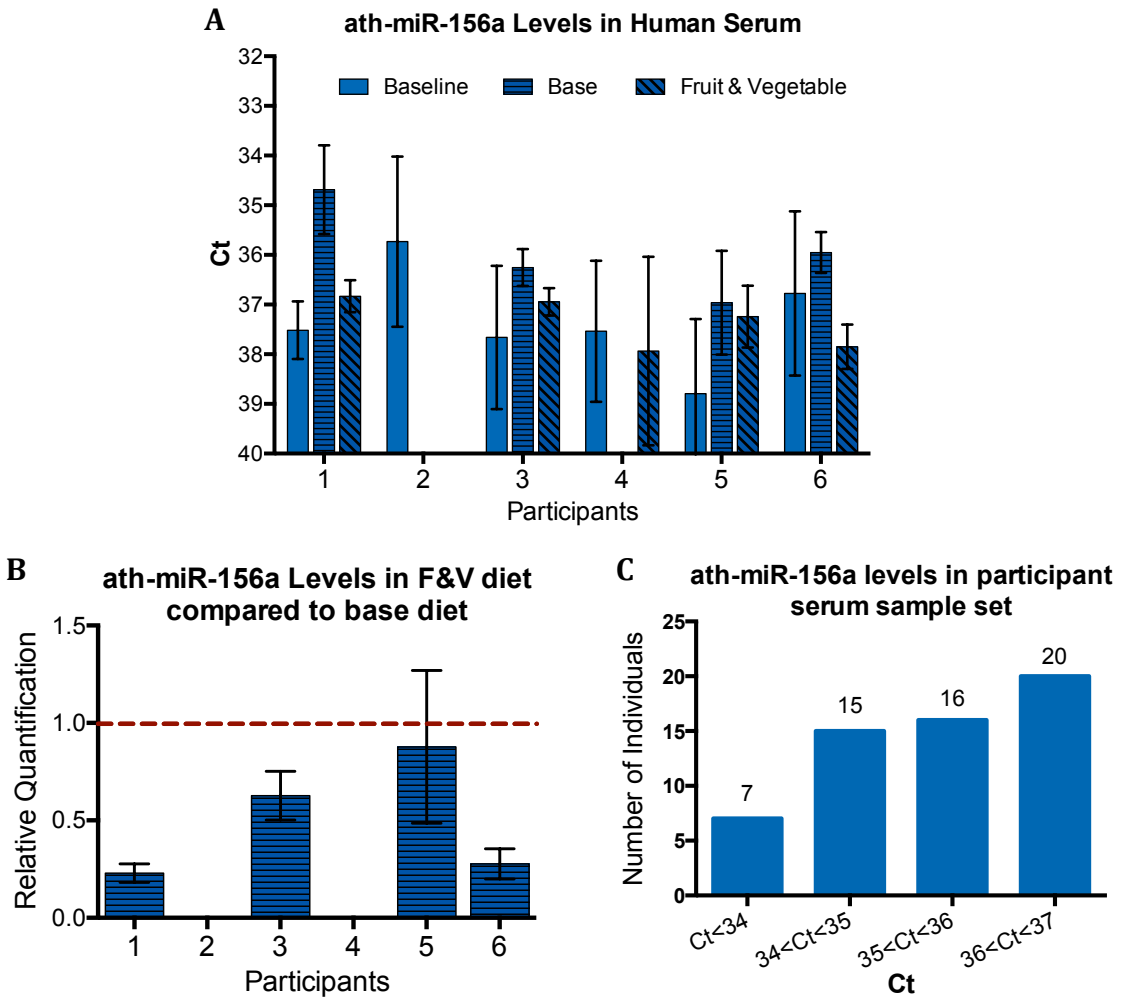


Figure 3.1: Plant miRNA, ath-miR-156a, is detected in a subset of human serum samples. A) qRT-PCR was used to assay levels of ath-miR-156a in human serum samples from 6 participants. Three serum samples were analyzed per participant, a baseline draw, after consumption with a basic diet and after consumption with a fruit & vegetable diet. Ath-miR-156a was detected in low levels in 15 out of 18 samples samples. B) Comparison of ath-miR-156a levels in participant samples from fruit & vegetable diet to basic diet. Indicates that there were no plant-derived miRNAs acquired orally in these samples. C) In a serum sample set from 160 individuals, ath-miR-156a was detected in 58 samples.

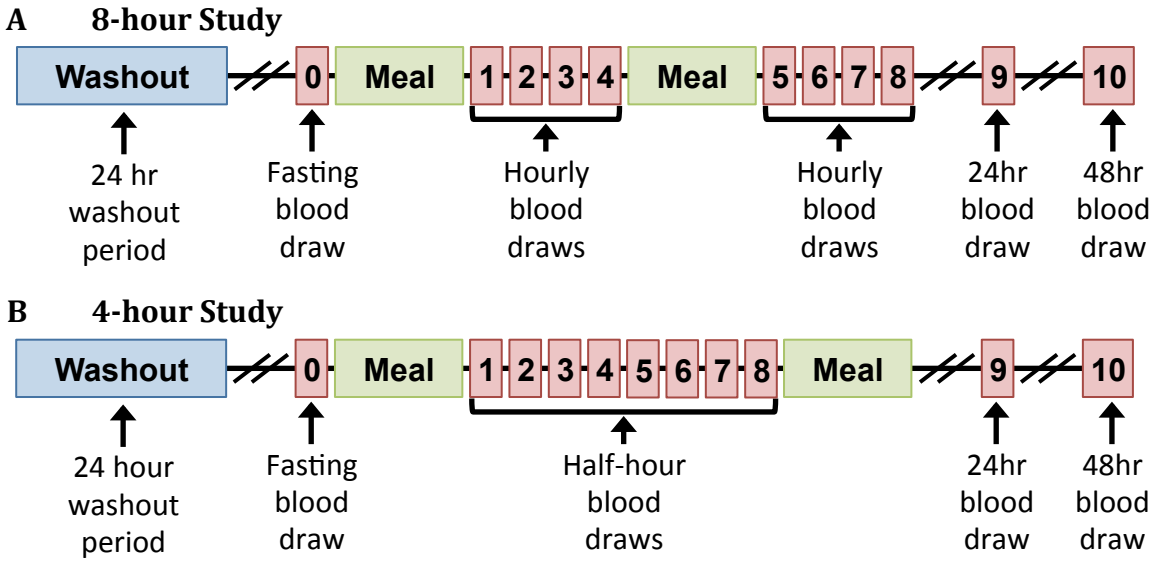


Figure 3.2: Schematic of controlled human study design A) Schematic of hourly blood draws for 8 hours after consuming a study meal. B) Schematic of blood draws every half-hour for 4 hours after study meal.

Solid food		Smoothie	
Food	Raw Weight(g)	Food	Raw Weight(g)
Broccoli	80	Orange	80
Brussel Sprouts	100	Apple	80
Spinach	110	Kale	40
Rice	80	Simple Syrup	20
Tofu	130	Water/Ice	100
TOTAL	**500**	**TOTAL**	**320**

Table 3.1: Study meal components and amount consumed by each participant
The study meal consisted of two components, solid food and a smoothie. Participants were served a total of 820 grams of a plant-based meal and each participant consumed at least 75% or 615 grams. Most participants consumed more than 90% of the meal.

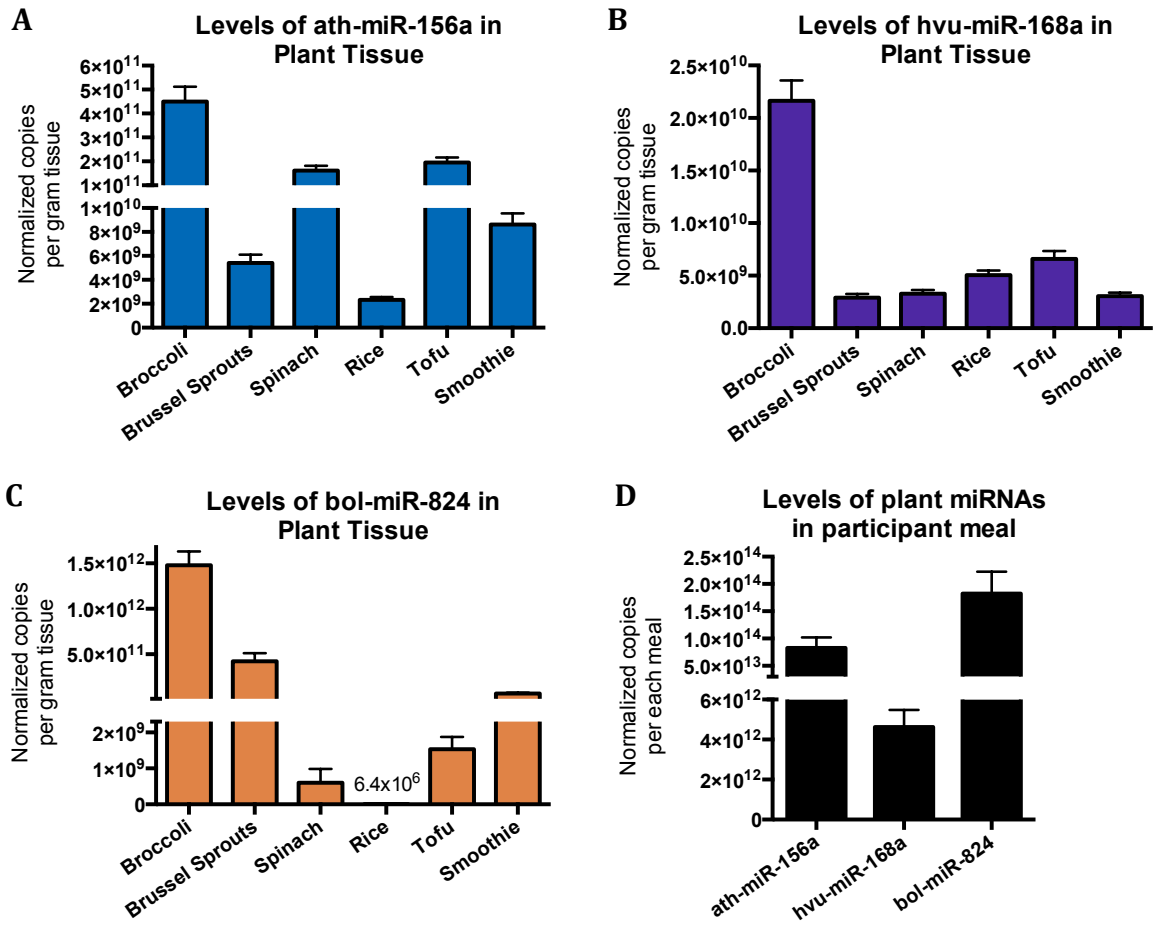


Figure 3.3: Plant miRNAs, ath-miR-156a, hvu-miR-168a and bol-miR-824, are highly abundant in study foods Aliquots of each food (broccoli, brussel sprouts, spinach, rice, tofu and smoothie) were collected and analyzed via qRT-PCR for A) ath-miR-156a, B) hvu-miR-168a, and C) bol-miR-824. D) Total ath-miR-156a, hvu-miR-168a, and bol-miR-824 levels in each participant meal.

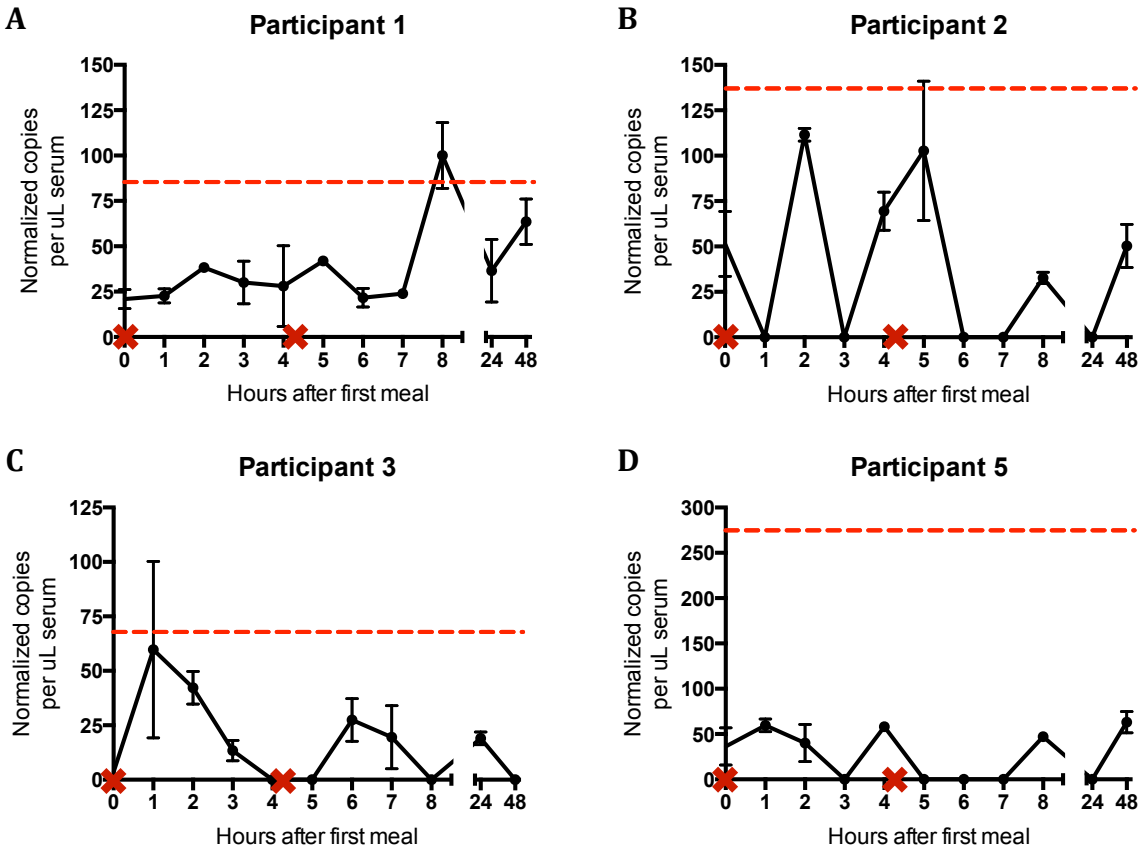


Figure 3.4: Ath-miR-156a is not detected above the limit of quantification in a subset of participants in the 8-hour study. The limit of quantification (- - -) is shown for each participant. Ath-miR-156a is detected below the limit of quantification at all timepoints in serum samples of A) Participant 1, B) Participant 2, C) Participant 3, and D) Participant 5. ✖ = meal served

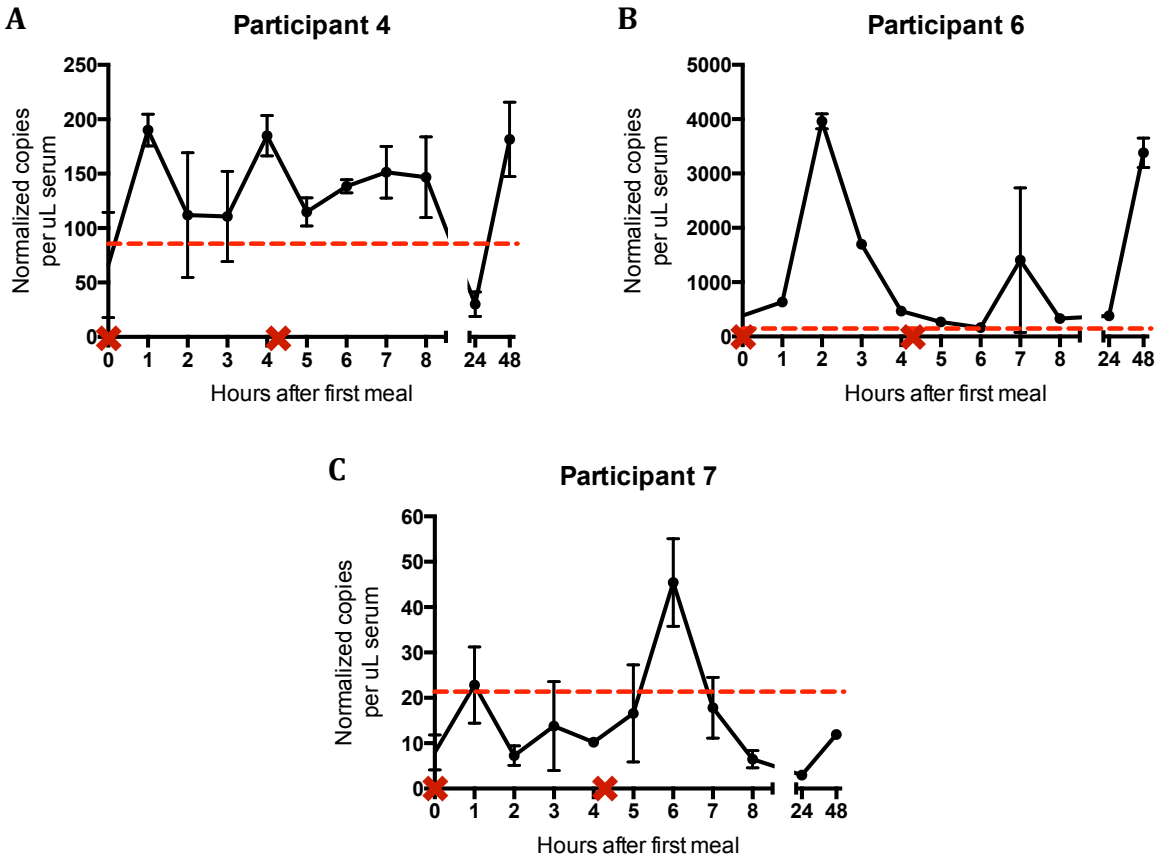


Figure 3.5: Ath-miR-156a is detected above the limit of quantification in a subset of participants in the 8-hour study. The limit of quantification (- - -) is shown for each participant. Ath-miR-156a is detected above the limit of quantification in A) Participant 4 at various timepoints, B) Participant 6 with a peak at 2 hours after the first meal, and C) Participant 7 with a peak at 6 hours after the first meal. ✖ = meal served

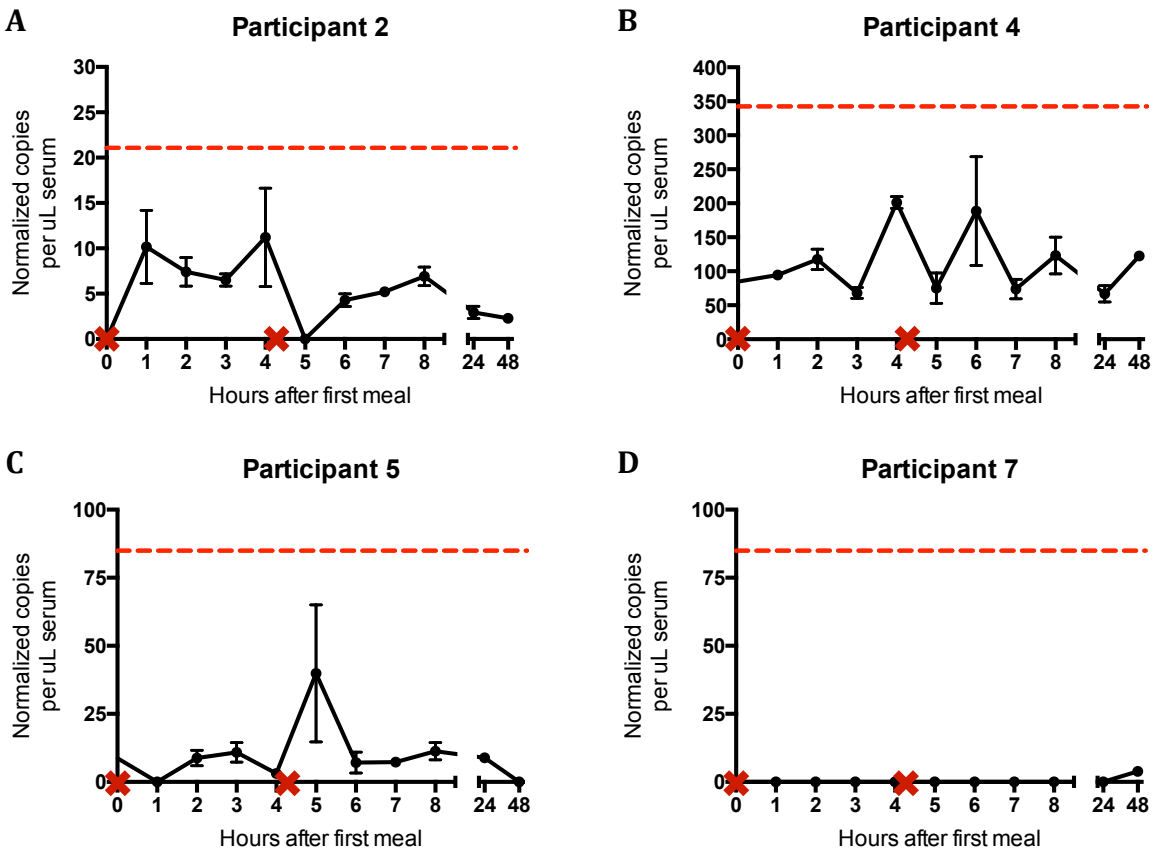


Figure 3.6: Hvu-miR-168a is not detected above the limit of quantification in a subset of participants in the 8-hour study. The limit of quantification (- -) is shown for each participant. Hvu-miR-168a is detected below the limit of quantification at all timepoints in A) Participant 2, B) Participant 4, C) Participant 5, and D) Participant 7. ✖ = meal served

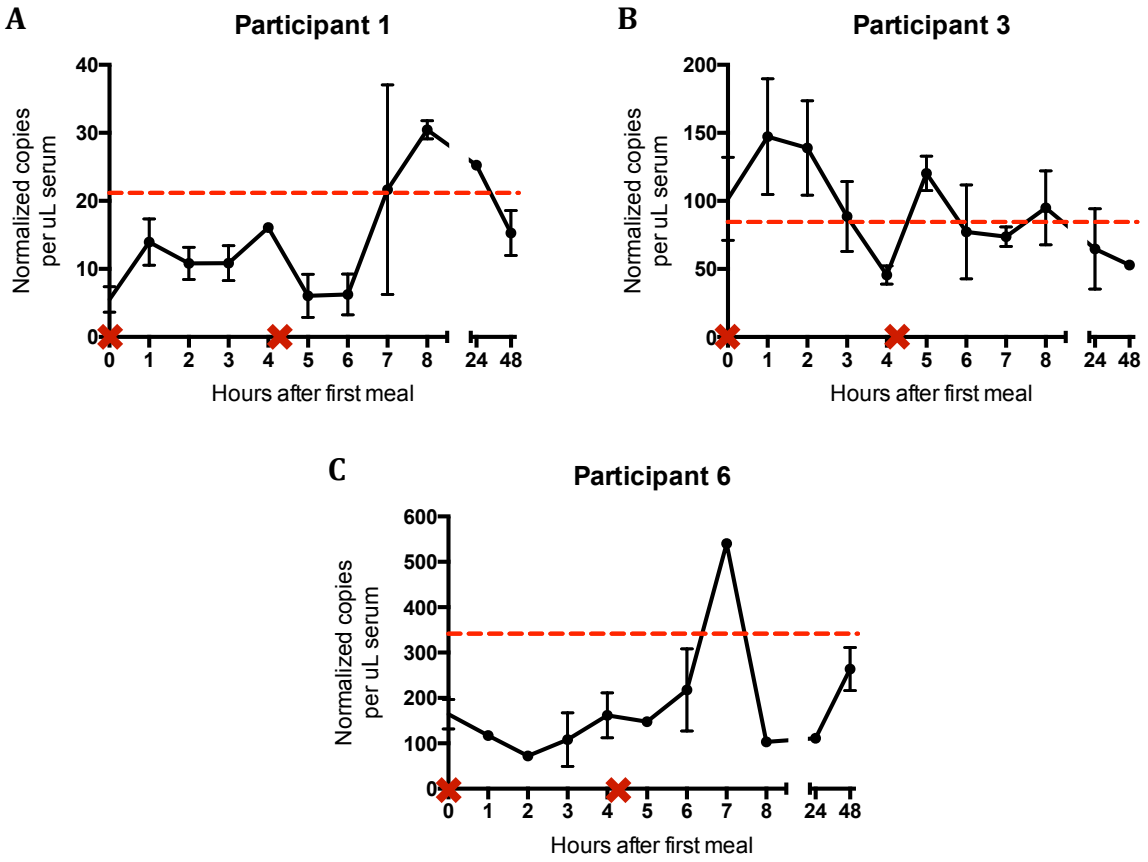


Figure 3.7: Hvu-miR-168a is detected above the limit of quantification in a subset of participants in the 8-hour study. The limit of quantification (- - -) is shown for each participant. Hvu-miR-168a is detected above the limit of quantification in A) Participant 1 with a peak 8 hours after the first meal, B) Participant 3 at various timepoints, and C) Participant 6 with a peak at 7 hours after the first meal. ✖ = meal served

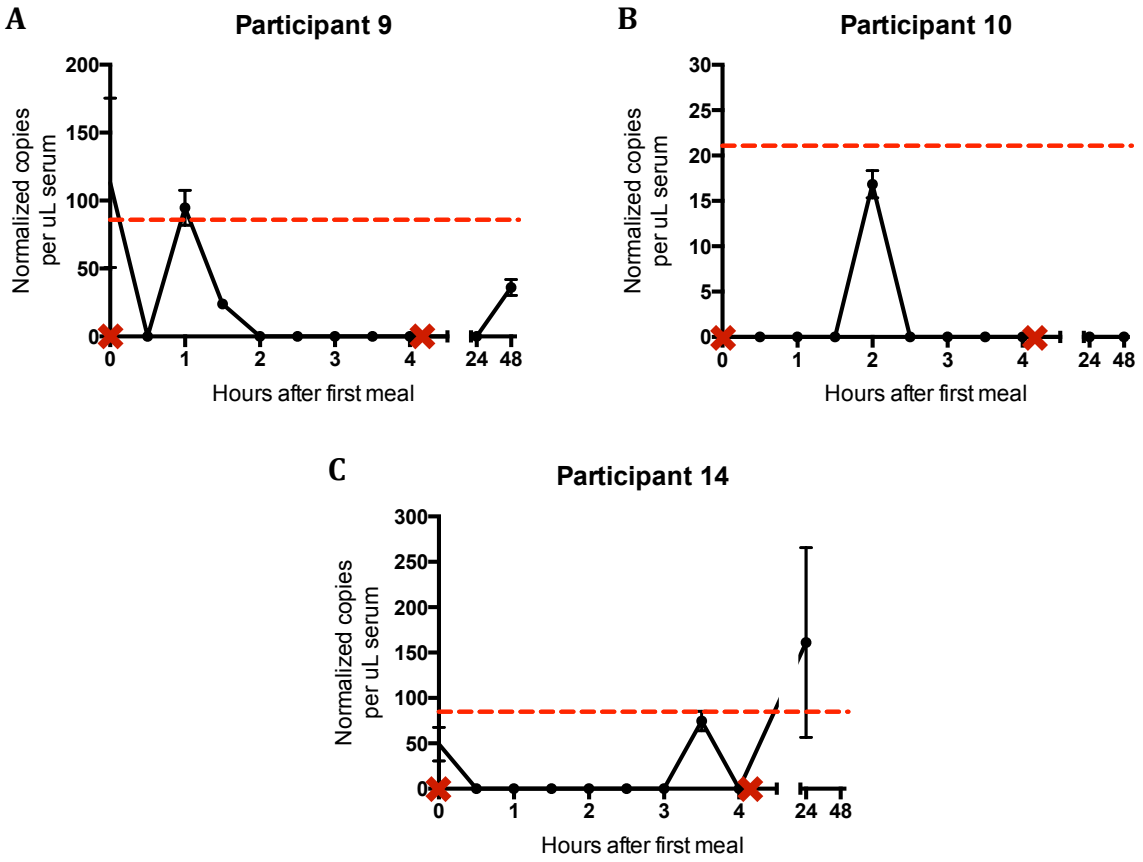


Figure 3.8: Ath-miR-156a is not detected above the limit of quantification in a subset of participants in the 4-hour study. The limit of quantification (- - -) is shown for each participant. Ath-miR-156a is detected below the limit of quantification at all timepoints in A) Participant 9, B) Participant 10, and C) Participant 14. ✖ = meal served

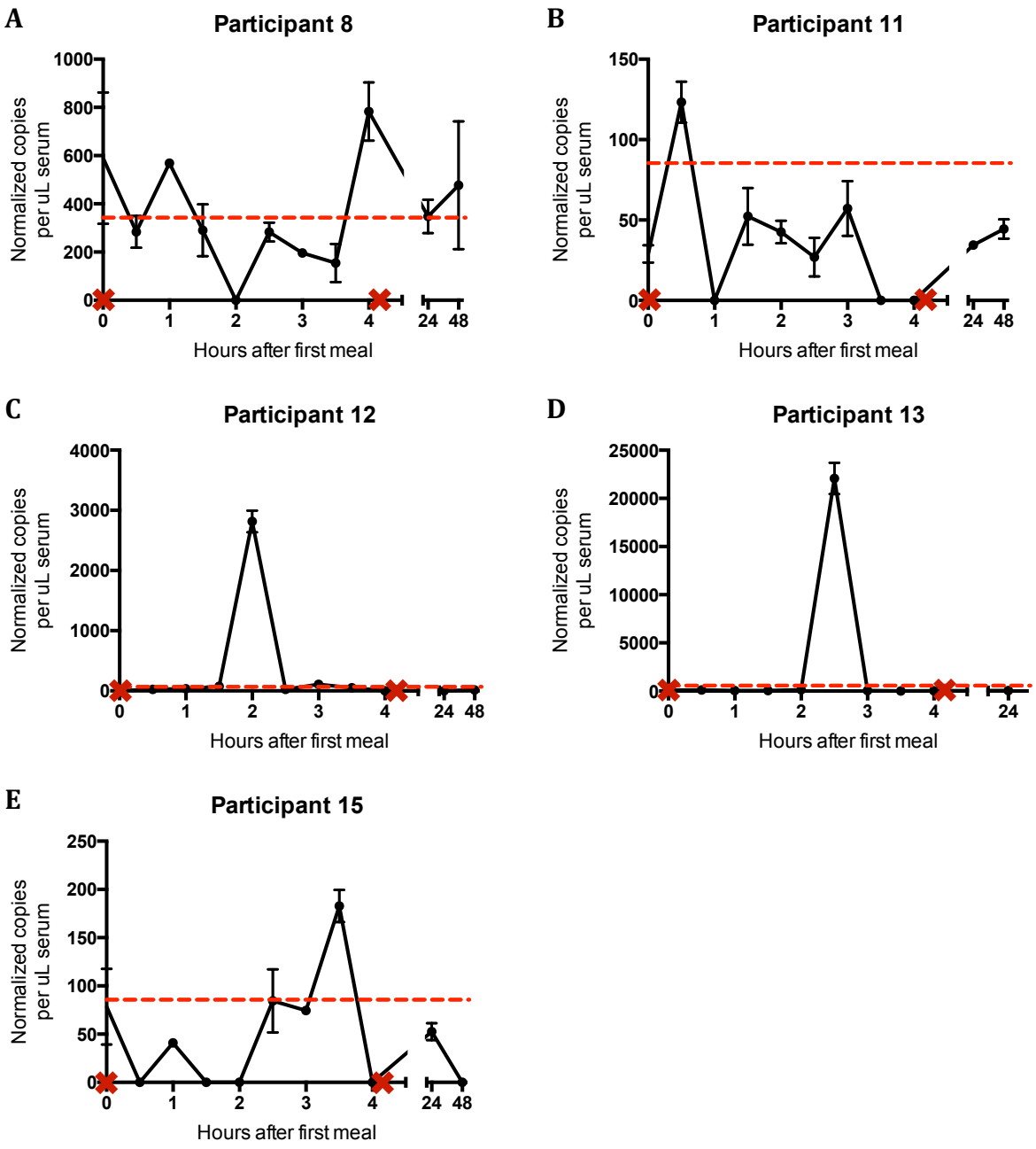


Figure 3.9: Ath-miR-156a is detected above the limit of quantification in a subset of participants in the 4-hour study. The limit of quantification (- - -) is shown for each participant. Ath-miR-156a is detected above the limit of quantification in A) Participant 8 at various timepoints, B) Participant 11 with a peak at 0.5 hours after the first meal, C) Participant 12 with a peak at 2 hours after the first meal, D) Participant 13 with a peak 2.5 hours after the first meal, and E) Participant 15 with a peak 3.5 hours after the first meal. ✖ = meal served

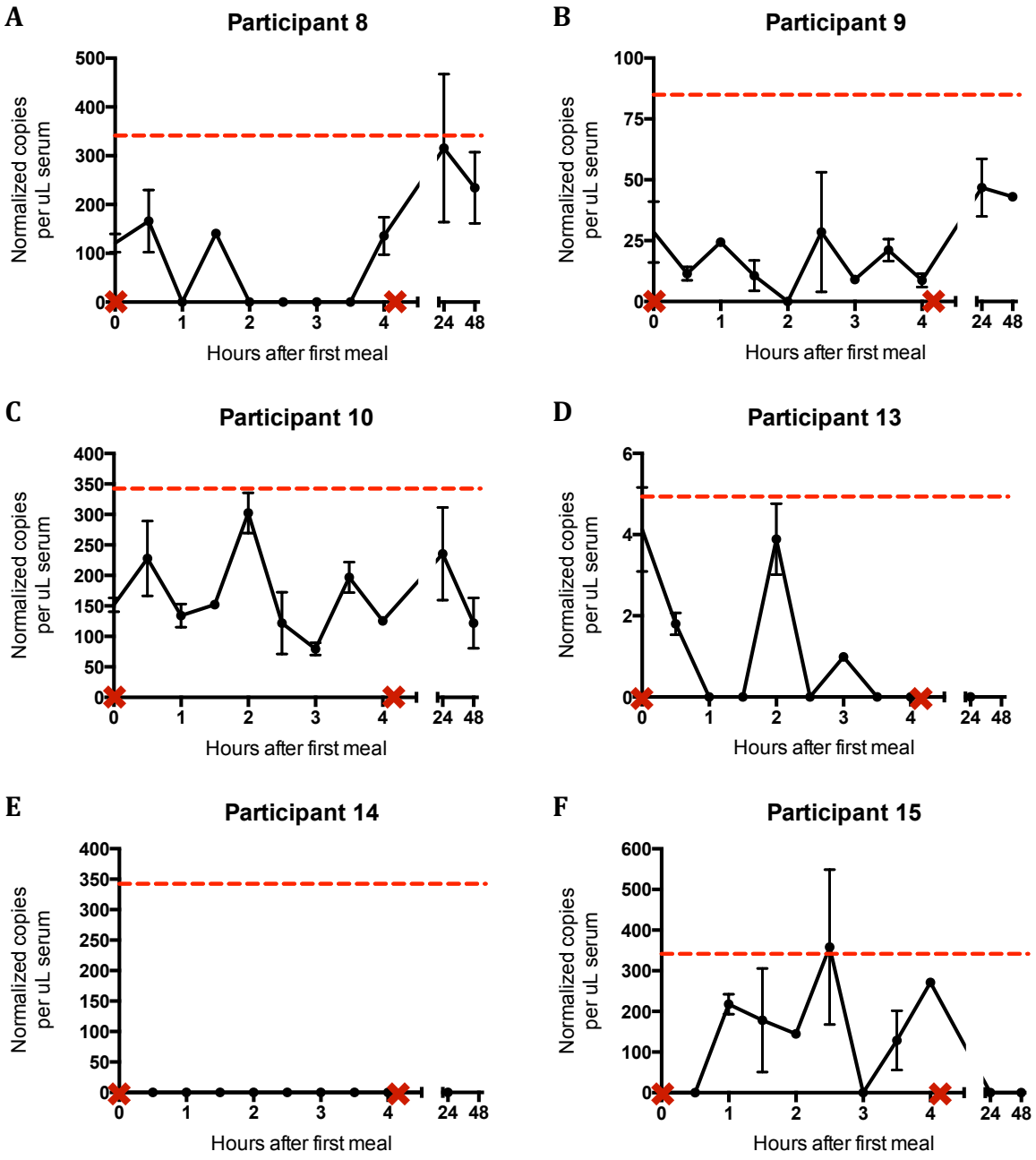


Figure 3.10: Hvu-miR-168a is not detected above the limit of quantification in a subset of participants in the 4-hour study. The limit of quantification (▬) is shown for each participant. Hvu-miR-168a is detected below the limit of quantification at all timepoints in A) Participant 8, B) Participant 9, C) Participant 10, D) Participant 13, E) Participant 14, and F) Participant 15. ✖ = meal served

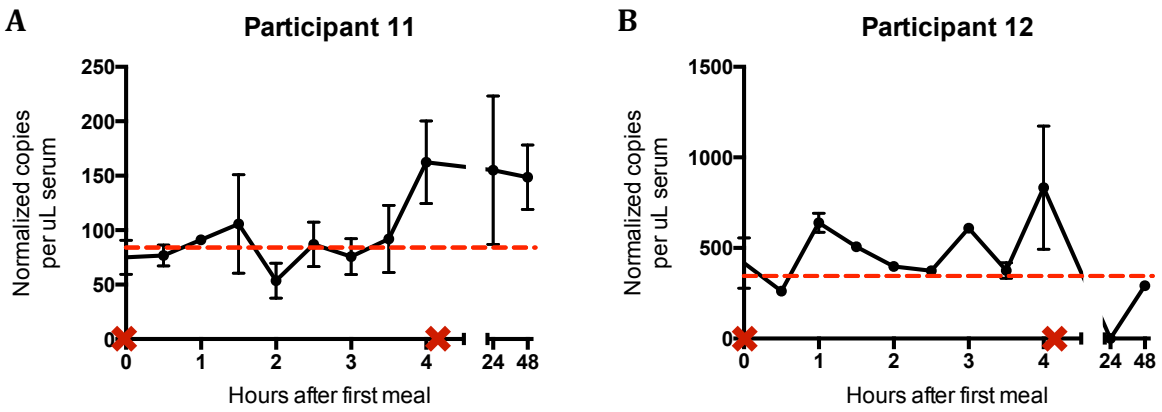


Figure 3.11: Hvu-miR-168a is detected above the limit of quantification in a subset of participants in the 4-hour study. The limit of quantification (- - -) is shown for each participant. Hvu-miR-168a is detected above the limit of quantification in A) Participant 11 at various timepoints, and B) Participant 12 at various timepoints. ✖ = meal served

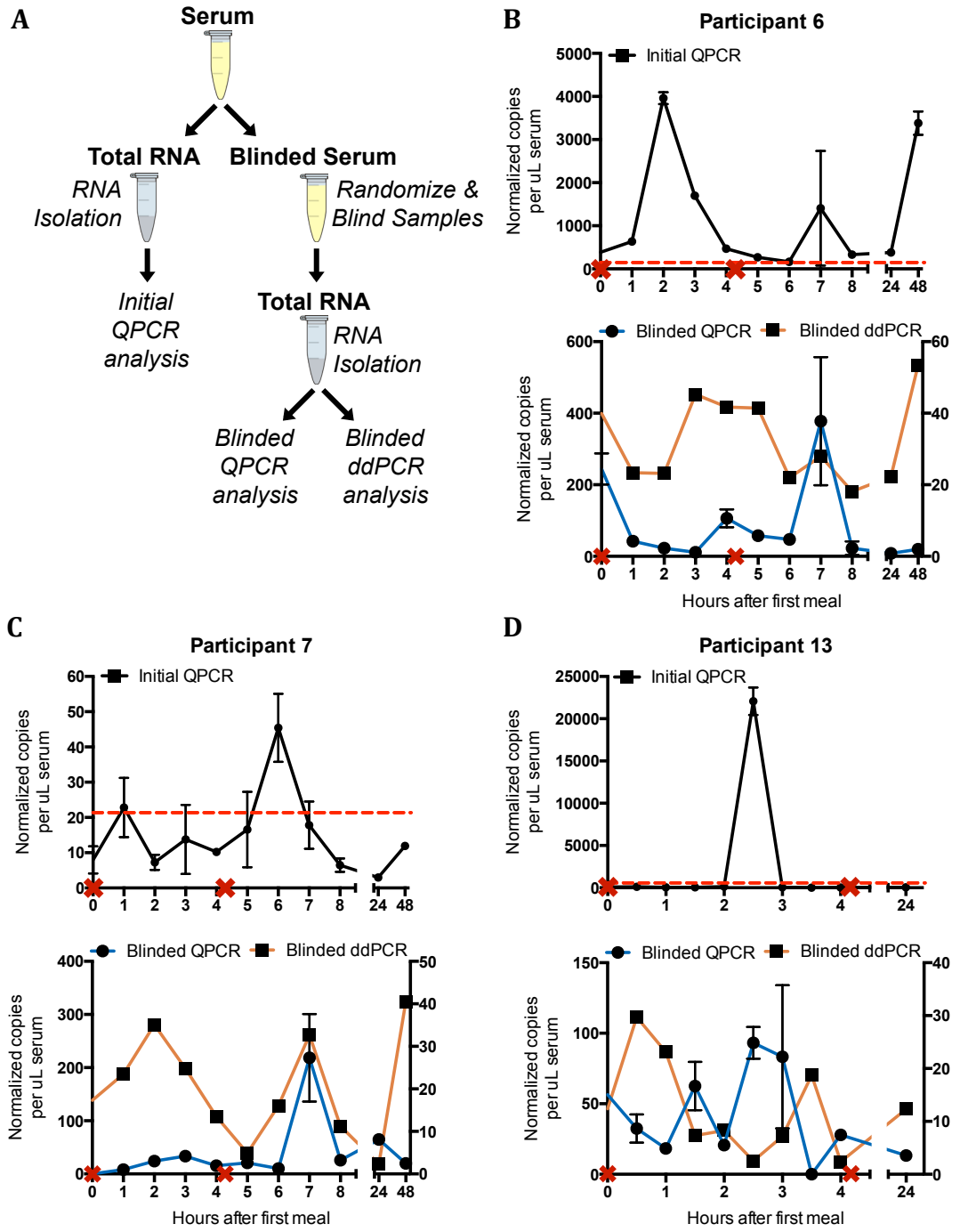


Figure 3.12: Blinded qRT-PCR and ddPCR analysis of select serum samples does not directly correlate with initial analysis. A) Schematic for analysis of RNA samples from two different serum aliquots. The limit of quantification (- - -) is shown for each participant. Participant 6, 7, and 13 have ath-miR-156a detected above the limit of quantification in the initial qRT-PCR. Ath-miR-156a comparison between initial qRT-PCR (top panel) , and blinded qRT-PCR analysis: left y-axis and blinded ddPCR analysis: right y-axis (bottom panel) in B) Participant 6, C) Participant 7, and D) Participant 13.

ath-miR-156a levels in study meal

8.26×10^{13} total copies **in** 830 grams of plant-based food

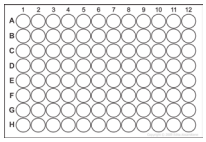
ath-miR-156a levels detected in participant serum at peak elevation

	ath-miR-156a expression at peak elevation	Total normalized copies in serum	% of ath-miR-156a consumed
lowest	45 copies/uL serum	1.24×10^{8}	0.00015%
average	4,206 copies/uL serum	1.16×10^{10}	0.014%
highest	22,071 copies/uL serum	6.07×10^{10}	0.073%

Figure 3.13: Uptake of plant miRNAs is highly inefficient. Comparison of ath-miR-156a concentrations consumed and observed in serum at peak elevation above limit of quantification in participants. Percentage of ath-miR-156a consumed that is detected in human serum ranges from 0.00015% to 0.073%.

Typical transfection in cell culture

96-well plate
5x10^3 cells/ well

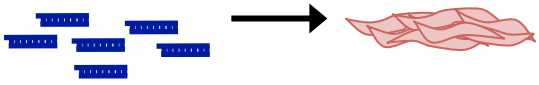


Typical transfection:
10pmol miRNA in
100uL media
(Arroyo *et al.* NAR 2014)

Copies per uL media

A moderate effect of 2-fold decrease or less in target mRNA requires at least:

6x10^{10} miRNA
copies/uL media



5x10^3 cells

Total copies per cell

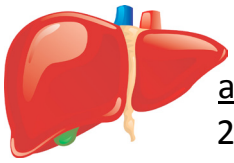
If we assume 100% transfection efficiency and even distribution

1.2x10^9 total copies per cell

are required for a moderate effect of 2-fold decrease or less in target mRNA.

Human blood and liver

Liver
2.1x10^{11} cells



Highest level of
ath-miR-156a detected:
22,071 copies/uL serum

Copies per uL whole blood

1.21x10^4 miRNA
copies/uL whole blood

2.1x10^{11} cells

Compared to transfection conditions there are fewer miRNA copies per uL and many more cells

Total copies per cell

If we assume even distribution there is less than 1 miRNA copy per cell

0.29 total copies per cell

Some cells may acquire multiple copies and others none

Figure 3.14: Serum concentrations of ath-miR-156a are unlikely to regulate endogenous gene expression. Left panel: Concentration of miRNA necessary for a moderate effect in a typical transfection in cell culture. Right Panel: Comparison of plant miRNA concentration in human blood and liver to transfection conditions.

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Chapter 4

CONCLUSIONS

Challenges in understanding origin and function of endogenous extracellular miRNA

Since the discovery of extracellular miRNAs in human plasma and serum in 2008 [1-4], researchers have focused on understanding release, protection and function of these miRNAs. The field has made progress in understanding some aspects of circulating miRNAs and their potential implications to regulate genes through endogenous cell-to-cell transfer. However, the overall biological contexts and mechanisms of miRNA transfer are not completely understood and potential limitations in understanding miRNA transfer have not been reported in detail. We attempted to characterize endogenous miRNA transfer by studying transfer within the context of the bone marrow microenvironment (BM). The BM is composed of distinct stromal and monocytic cells that interact and form cell-cell contacts and alter each other's gene expression [5, 6]. Our initial experiments suggested transfer of miRNAs between THP-1 monocytic cells and HS-27a stromal cells. After further experiments to characterize the mechanism of transfer, we discovered that our initial results were due to artifacts of relying on fluorescence-based methods.

This indicated that methods that rely on fluorescent-based reporters to separate cell types and/or track small RNAs for miRNA transfer might be inadequate. One report, determined that a fluorescent tag was not a reliable marker for small RNA transfection in the presence of serum [7]. Our results using a Cy3-tagged miRNA to detect the presence of a miRNA are consistent with these conclusions. We determined that culture of a 5' Cy3-tagged miRNA in the media of cells resulted in robust uptake of Cy3 fluorescence into cells.

The uptake of Cy3 fluorescence occurred more rapidly in the presence of serum, but after 24 hours we detected robust uptake of Cy3 fluorescence in serum and serum-free conditions. We also determined that flow cytometry does not adequately separate cell types, as we observed contamination due to improper sorting of cells in isolated cell types. Other methods utilized for imaging of miRNAs include fluorescent and bioluminescent enzymes in reporter systems to determine miRNA activity [8]. There are also limitations in these methods, especially for *in vivo* studies, such as high background signal, low sensitivity, innate fluorescent uptake, and poor tissue penetration amongst others [8]. It is important to develop methods to study miRNA transfer that combine strengths of different imaging techniques and functional assays.

For miRNA transfer studies, it may necessary to use a combination of current methods to establish miRNA transfer or function, until more adequate methods are developed. In co-culture experiments, recipient and donor cells must be adequately separated prior to analysis of miRNA levels in individual cell types. This could be accomplished using fluorescence and/or cell surface markers, but special care must be taken to determine complete separation of donor and recipient cells, such as repeat flow sorting. MiRNA levels in recipient cells could be analyzed using qRT-PCR, miRNA profiling, or sequencing, however characterizing functional changes within the cell may provide the most reliable data. Reporter systems and/or measurement of endogenous mRNA and protein levels could determine functional changes after transfer of miRNAs. These methods are most effective for detecting transfer of miRNAs and the associated gene regulation, but subtle changes may not be detected due to limitations (i.e. low sensitivity and high background signals). New technologies need to be developed for increased sensitivity to

better analyze the mechanism and function of miRNA transfer, especially in an *in vivo* system.

Advances in exogenous miRNA transfer

Most recently the field has gained interest in exogenous miRNA transfer from the diet, after a report of plant-derived miRNA transfer into the human bloodstream [9]. These findings were controversial because there were implications that plant miRNAs could regulate endogenous human transcripts after transfer into the bloodstream. This initial study [9], sparked interest in the general public and multiple research groups to conduct follow-up studies to verify these results. Most of the follow-up studies that have been conducted did not support the initial reports by Zhang *et al.* [9]. Instead these reports have attributed exogenous miRNAs in sequencing datasets and independent studies to be artifacts and/or contamination that originated in the sequencing process [10-14]. However, studies that have suggested that plant-derived miRNAs in the human bloodstream are artifacts suffered from significant limitations, including blood drawn at random, lack of negative controls, and insufficient blood draw time points.

We designed the first controlled study in humans with serial blood draws prior to and after consumption of a plant-based meal to determine whether plant-derived miRNAs are absorbed into the human bloodstream. This study was designed to overcome the limitations observed in previous studies by including a baseline blood draw for each participant and serial blood draws initiated after consumption of a defined plant-based meal. In this controlled study, we measured plant miRNAs, ath-miR-156a, hvu-miR-168a and bol-miR-824 in both plasma and serum participant samples. We did not detect ath-miR-156a, hvu-miR-168a and bol-miR-824 in any of the participant plasma samples and

bol-miR-824 was not detected in any serum samples. We also observed that the majority of participants did not have concentration of ath-miR-156a and hvu-miR-168a above the limit of quantification in any of the serum samples. In a subset of participant serum sampled we did detect ath-miR-156a and hvu-miR-168a above the limit of quantification, however the absolute concentrations were low and in a range associated with experimental noise. The highest expression that we observed was in one participant 2 hours after consumption of the first meal with 22,071 copies per uL of serum of ath-miR-156a. In order to estimate the degree of experimental variation in our approach, we compared initial qRT-PCR results to a second round of qRT-PCR and ddPCR measurements from a different serum aliquot. We found that there was substantial variation, presumably due to experimental noise associated with attempting to measure miRNAs at low concentration. Our data suggests that dietary plant miRNAs are not absorbed into the human bloodstream in healthy individuals.

We have determined that even if the plant miRNA concentrations detected in some participants are *bona fide*, plant miRNA absorption is highly inefficient and unlikely to be functional with respect to regulating endogenous gene expression. We determined that plant miRNAs were highly abundant in the study meal with a total of 8.26×10^{13} copies of ath-miR-156a per meal. Participants had to consume 820 grams of plant-based food to consume this amount of ath-miR-156a, which is much higher than what an individual would typically consume during a day. The percentage of ath-miR-156a consumed that is detected in human serum ranges from 0.00015% to 0.073%, indicating that uptake of plant miRNA is highly inefficient. We also compared miRNA concentrations necessary for a moderate effect of 2-fold decrease or less in target mRNA repression in cell culture

transfection experiments, to levels of plant miRNAs we observed in human serum. We determined that for a moderate effect in a typical transfection in cell culture, if we assume 100% transfection efficiency and even distribution per cell, there are 1.2×10^9 total copies per cell required for a moderate effect in a target mRNA. At best case for plant miRNAs, if we assume that plant miRNAs from the blood are transferred to the liver, we calculate average only 0.29 total plant miRNA copies per liver cell indicating that serum concentrations detected are unlikely to regulate endogenous gene expression.

This is the first investigation in humans that studied exogenous miRNA absorption at early timepoints and high time resolution (i.e hourly and half-hourly intervals) after consumption of a plant-based meal. We designed this study to overcome limitations in previous studies, listed above, but we also identified some limitations within our study. First, we only measured three plant miRNAs, ath-miR-156a, hvu-miR-168a and bol-miR-824, and only ath-miR-156a and hvu-miR-168a were detected at low levels in some participants. We cannot rule out the possibility that there are other exogenous miRNAs and/or classes of RNAs that are more readily absorbed into the human bloodstream. We also only examined absorption into the bloodstream and did not examine absorption into tissues. We cannot exclude that exogenous miRNAs could be absorbed locally, after ingestion by the stomach lining and/or the intestine, or be taken up by target tissues, such as the liver.

Lastly, we only examined 15 individuals in our study, therefore we cannot exclude that there are individuals in the population with a greater propensity for dietary miRNA absorption. Studies have observed associations between the gut microbiome and nutrient absorption that suggest regulation of nutrient uptake by microbes [15-17]. The gut

microbiome is also very diverse and varies widely among healthy adults [18-21] suggesting that differences in nutrient uptake by individuals could be attributed to the gut microbiome. We speculate that the gut microbiome may play a role in the absorption of dietary-derived RNAs, which could lead to absorption of higher amounts of plant miRNAs from the diet in some individuals. Genetic variations, variations in the lining of the stomach or intestines, and absorption disorders such as celiac disease, could also influence plant miRNA absorption into the human bloodstream and/or tissues.

Although we do have limitations within our controlled feeding study, we have determined that plant miRNA absorption is not a general phenomenon that routinely occurs with high efficiency in most healthy individuals. Our data suggests that dietary plant miRNA absorption at best yields very low absolute serum concentrations of plant miRNA, is in a range associated with experimental noise, and is unlikely to be functional at the levels detected. Our findings corroborate the follow-up reports by multiple groups [10-14], and we speculate that the initial report by Zhang *et al*. [9] may be attributed to sequencing artifacts due to contamination in library preparations.

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