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TRANSPORT OF BOVINE MILK EXOSOMES BY HUMAN COLON CARCINOMA CACO-2 CELLS AND RAT SMALL INTESTINAL IEC-6 CELLS

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We have reported that microRNAs (miRNAs, miRs) in bovine milk regulate human genes. In milk, many miRNAs are encapsulated in exosomes, thereby conferring protection against degradation and a pathway for intestinal transport of miRNAs. We hypothesized that the uptake of bovine exosomes in human intestinal colon carcinoma Caco-2 cells and rat primary small intestinal IEC-6 cells is mediated by endocytosis. Transport studies were carried out using fluorophore-labeled exosomes purified from bovine milk. The transport of bovine exosomes exhibited saturation kinetics at 37°C (K_m= 55.5±48.6 μg/200 μL, V_{max}= 0.083±0.057 ng exosomal protein *x* 81,750 cells^{-1} *x* hr^{-1}) and decreased by 60% if transport was measured at 4°C in Caco-2 cells, consistent with carrier-mediated transport. Inhibitors of vesicle trafficking and carbohydrate competitors caused a 62-85% and 61-83% decrease, respectively, in exosome transport, consistent with cellular transport of bovine exosomes by endocytosis that depends on surface glycoproteins in Caco-2 cells. Similar patterns were observed in IEC-6 cells. When milk exosomes at a concentration of five times the K_m were added to the upper chamber in transwell plates, Caco-2 cells accumulated miR-29b in the lower chamber, whereas reverse transport was minor. We conclude that the uptake of bovine milk

exosomes is mediated by endocytosis and depends on cell and exosome surface glycoproteins in human and rat intestinal cells.

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CHAPTER 1

LITERATURE REVIEW

Extracellular Vesicles

.Extracellular vesicles (EVs) are secreted by the plasma membrane (1) and range 30-1000 nm in size (2). They are released from many cell types, such as epithelial cells (3), tumor cells (4), and neurons (5). Post-transciption modications (6), intercellular communcation (7), and exchange of genetic material in both eukaryote and prokarytoic cell types (8, 9) are just a few of the notable functional capablities of EVs. These membrane inclosed vesicles are spherical in shape (10), and when mounted for negative staining to be viewed by transmission electron microscopy (TEM), they appear cup-shapped (1, 11).

History and Mechanism of Action

EVs are found in many biologial fluids, such as urine (12, 13), blood (14), salvia (15), and breast milk (16). The major catergories of EVs include exosomes, microvesicles, and apopotoic bodies (Table 1). Table 1 summarizes the main differences between them.

In 1967, Peter Wolf visualized EVs with electron microscopy and described the vesicles as platlet dust while doing coagulation research (17). By 1971 one of Wolf's collegues used electron micrographs to visualize EVs from pig plasma which he described as having a "heterogenous granular" apppearance (18) . Using ultracentrfiguation, George et al. in 1982 analyzed the microvesicles populations in the resulting pellets of human sera and plasma with an immunoelectrophoretic assay. They

discovered circulating micropartices originate from elsewhere in the human body, not just blood platelets as initially thought (19). Research progress has discovered a role for EVs in intracellular and extracellular communication (1, 7), immune response (7, 20, 21) and the delivery of exogenous compounds to recepient cells (8, 22, 23). For the purposes of this review, the primary focus will be exosomes.

Table 1. **Characteristics of different eukaryotic types of cell derived extracellular vesicles**

Vesicles:	**Exosome**	**Microvesicles (Ectosomes)**	**Apoptotic bodies**
Diameter (nm)	30-150	100-1,000	50-5000
Density (g/ml)	1.13-1.19	Unknown	Unknown
Formation	Exocytosis of MVBs	Budding of plasma membrane	Release from dying/apoptotic cells
Morphology (TEM)	Cup-shaped	Irregular shape	Heterogeneous
Origin	Endosomes	Plasma membrane	Blebs released from cells undergoing apoptosis
Protein Enrichment	C81, CD63, CD9, LAMP1, Alix, TSG101	MMP2, Annexin V, integrins, selectins, CD40 ligands	Histones, Annexin V

Adapted from (1, 11, 24-33)

Exosomes

The literature is well established that many cell types shed small vesicles into the extracelluar space. Exosomes are a type of extracellular vesicles that are secreted from various cells types by multivesicular bodies (MVBs) fusing with the plasma membrane (25, 32, 33). Endosomal exosome biogensis occurs by the inward budding of the plasma membrane into multivesicular bodies (MVBs). Johnston et al. proposed the term exosome be given to EVs that originate from MVBs (34). According to the International Society for Extracellular Vesicles, the term exosomes is the most popular word used to classify any type of extracellular vesicle (35).

Exosomes range 30-150 nm in sizes and mediate the transfer of microRNA between cells (30, 31, 36). Raposo et al. demonstrated B cell lymphocyte exosomes play a role in extracelluar commication by eliciting specific T cell responses (1). Dedendritic cell-derived exosomes can diminish the establishment of murine tumors, reinforcing their role in immune response (7). Numerous other studies have demonstrated the role of exosomes in cell to cell signaling activities (37, 38).

Composition

Exosomes are enriched in proteins, and as a result have been associated with specific proteins for indication of their presence. Identification of exosomes became more appart when proteins that are involved with MVB biogensis were detected in exosomes (7, 39). Currently, over 4,500 proteins have been identified in exosomes (40). Eight major proteins have been identifited from murine dendritic derived exosomes, with heat shock cognate protein 73 (hsc73) being adundant and shown to accumulate in the endocytic

compartments of a murine spleen-derived cell line (41). According to ExoCarta, CD63 is among the top 25 proteins that are often identified in exosomes (42). Other proteins commonly used to identify exosomes include heat shock 70kDA protein 8 (HSPA8), CD9, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB), CD81 and annexin A2 (ANXA2) (40). Though exosomes are enriched in many proteins, additional research is needed to distinguish between exosomes and microvesicles if trying to obtain pure isolates.

The lipid content of exosomes has not been studied as intensely as protein. Thus far, studies that focus on exosomes secreted from cells contain saturated fatty acids, cholesterol (43, 44), posphytileserine, and sphingomyelin (44, 45).

Transportation of Exosomes

The research suggests that cellular uptake of exosomes is mediated by an endocytosis-dependent process (46-48) including phagocytosis (49), receptor-mediated endocytosis (50), micropinocytosis (51) and macropinocytosis (see Figure 1.1) (52). According to Tan et al., mescnehymal stem cell released exosomes from an endocytosed lipid raft, suggesting their endosomal origin (53).

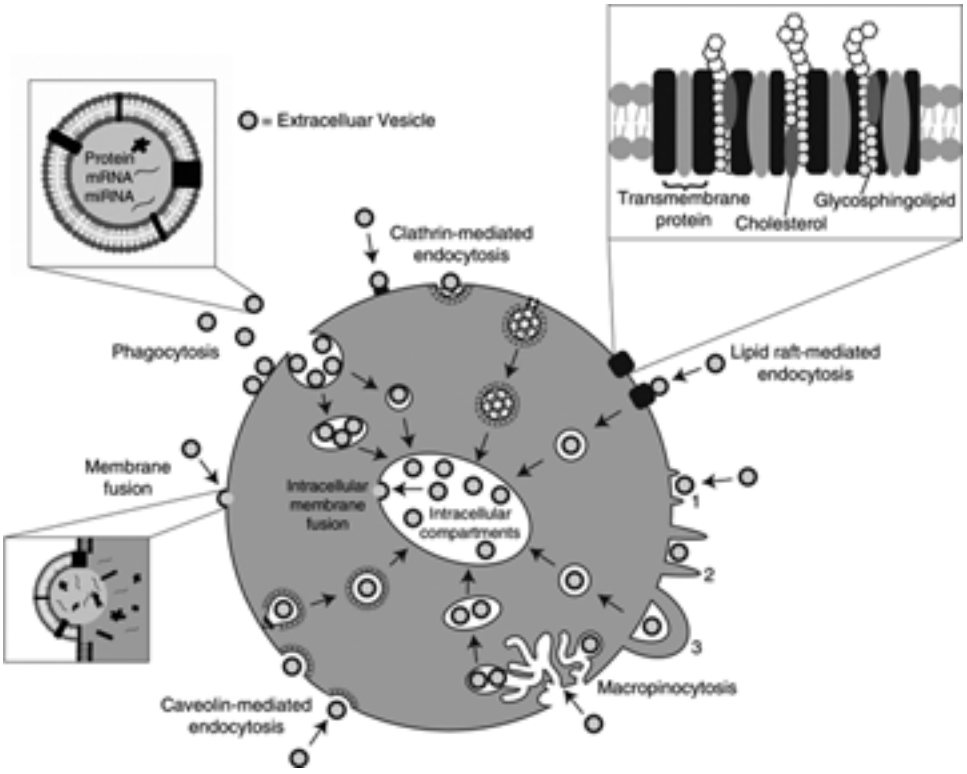

Figure 1.1 Simplified uptake pathways for extracellular vesicles

[Figure source: Review by Mulcahy et al., 2014] (52)

How milk exosomes cross the intestinal muscosa needs further exploration, but one can infer that it might be similar to human EVs. Recently, an independent laboratory suggested that milk exosomes enter the mouse circulation intact by crossing the intestinal mucosa without re-packaging (54). Exosomes secreted by mast cells that contain mRNA and miRNA can be transferred to nearby cells and be operational (8, 55).

Rab proteins are small cytostolic GTPases that are known to control important steps in vesicle formation and trafficking (56). As a result, exosomes of endosomal origin contain proteins that are involved in membrane transport and fusion processes (see Figure 1.2 B) (57). A group of the Rab proteins have been shown to be involved in exosome secretion. For instance, Rab35 is involved in transporting proteolipid protein from a

murine oligodendroglial cell MVBs to exosomes (58). The negative dominant mutant of Rab35 decreased exosomes release in an erythroleukemia cell line (59). Knock down of five Rab proteins: Rab27, Rab2b, Rab5a, Rab9a, and Rab27A, in HeLa cells inhibited exosomes secretion dramatically demonstrating their important role in exosomes secretion (60).

By using inhibitors of transport processes, researchers can gain insight on exosomes methods of entry and secretion from cells. According to Escrevente et al., treatment of ovarian tumor cells with the inhibitor cytochalasin D, resulted in a 36 +/- 13% reduction in cell uptake of isolated labeled SKOV3 cell exosomes (61). A study using a transwell system treating Caco-2 cells with 10 uM of cytochalsin D caused nearly complete depolymerization of actin stress fibers (62). Guanine nucleotide exchange protein (BIG2) is a factor involved in ADP-ribosylation (63, 64). Brefeldin A has been shown to target ADP-ribosylation factor that is responsible for coat proteins associated with clathrin-coated vesicles (65).

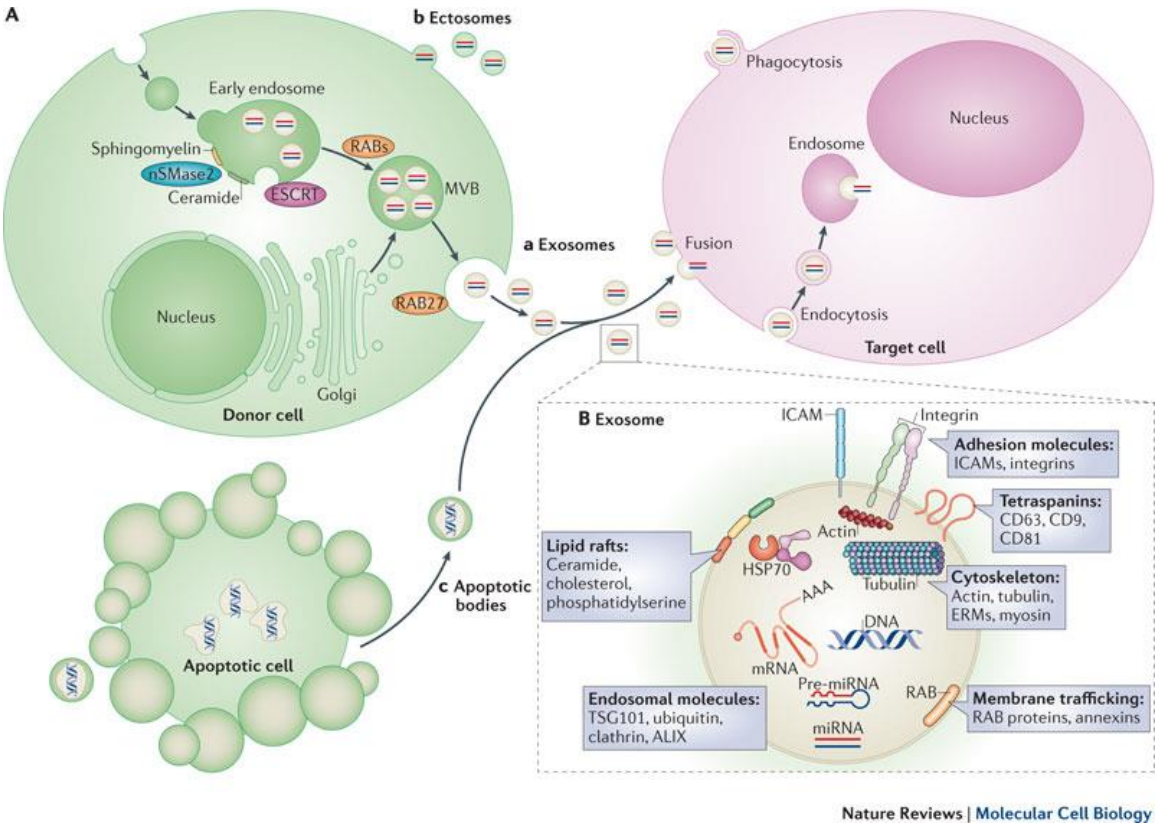

Figure 1.2. Exosomes composition and extracellular vesicles origin of exosomes (a), ectosomes (b), and apoptotic bodies (c).
(B) Exosomes are composed RAB proteins, annexins ,adhesion molecules and tetraspanins, cytoskeletal proteins, and miRNA. Membranes are enriched in raft lipids such a cholesterol, spingolipids, and ceramide. Abbreviations: ALIX- apoptosis-linked gene 2-interacting protein X, ERM- ezrin radixin moesin, HSP70-Heat shock protein 70, TSG101- tumor susceptibility gene 101, ICAM- intercellular cell adhesion molecule. Figure adapted from Mittelbrunn et al., 2012 (57).

Isolation of Extracellular Vesicles

The initial process used to isolate EVs was based on differential centriguation to remove vesicles larger than EVs (1, 7, 34). The final ultracentifugation step typically includes a G-force of at least 100,000 *x g* to pellet the exosomes (66). Centrifuge runs as high as 140,000 g has been used to sediment exosomes as well (67). To ensure that aggregates aren't formed, some researchers use a sucrose cushion, where the aggrages sink though the sucrose, and the lipid containing EVs float on the correct density.

Exosomes float in sucrose gradients between the density of 1.13-1.19 g/mL (26). Though because this method is tedious, it's losing popularity for exosome isolation since more convenient methods are currently being developed. Iodizanol Optiprep™ (Axis-Shield PoCAS) has recently been used with differnetial centrifugation to obtain more pure exosomes (68). Recent reports also suggest improved separation from apopototic bodies and viruses (69). Anti-body coated beads (HansaBioMed) and polymer based precipation kits (exoquick) are other methods that have become available for isolation of extracellular vesicles. An in-depth investigation of all methods of isolation for EVs is needed, as there is currently no gold standard recommendation for pure isolation of EVs.

MicroRNA

MicroRNAs are 19 to 25 nucleotides long (70, 71) noncoding RNAs, that typically bind to the 3' UTR of target mRNAs leading to mRNA break down or translational delay (72, 73). In the nucleus, microRNA is encoded by its own genes and transcribed by RNA polymerase II or RNA polymerase III (73, 74), followed by cleavage of the miRNA transcript by a complex called Drosha (75). The hairpin loop miRNA structure is then transported into the cytoplasm where the premature miRNA is further processed by dicer (76, 77), resulting in double stranded mature miRNA. The mature miRNA typically bind to the 3' UTR of target mRNAs leading to mRNA break down or translational delay (72, 73) (see Figure 1.3) (78).

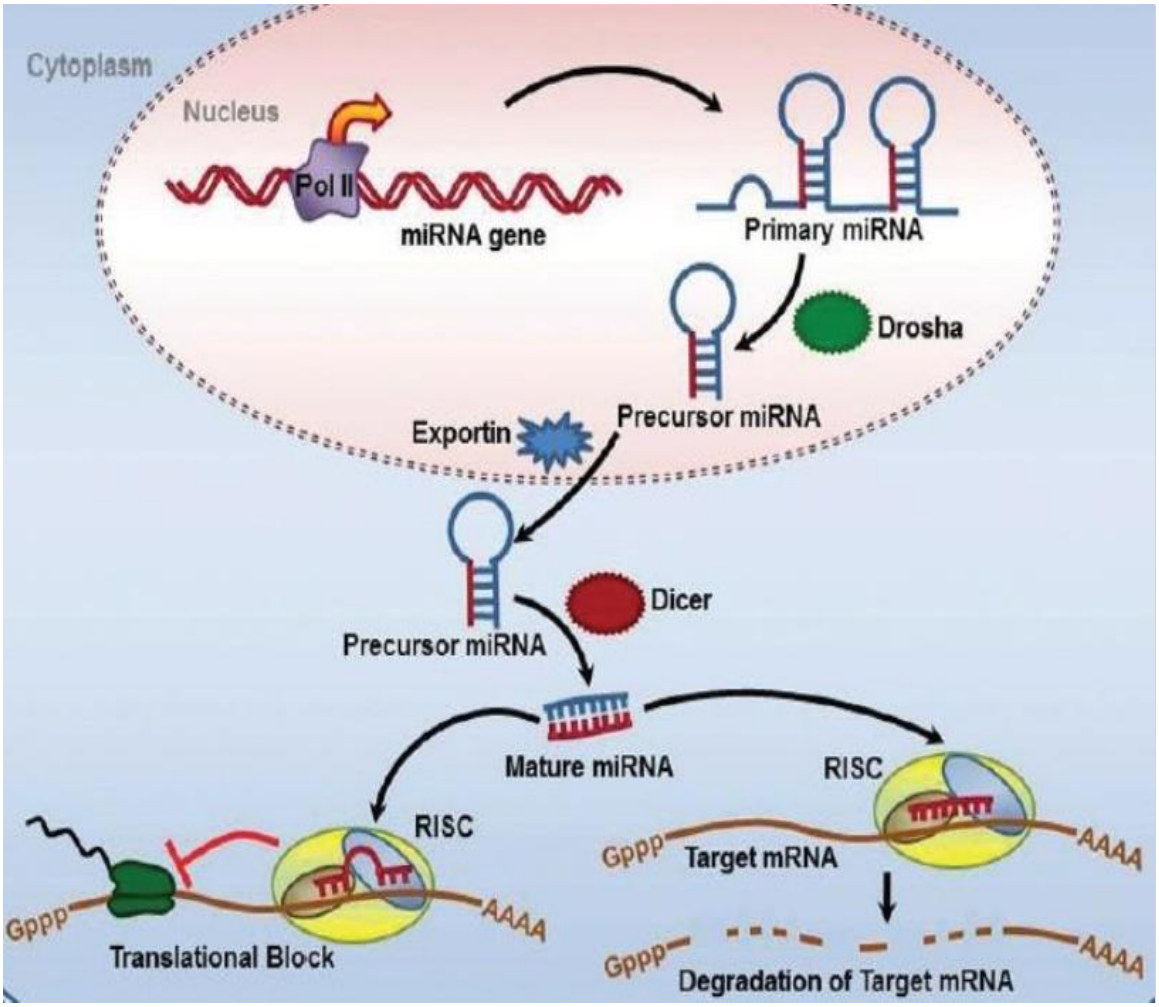

Figure 1.3 The biogenesis of MicroRNA [Figure source: Joshi et al., 2011] (78)

Many mammalian microRNAs are found in introns or exons of long noncoding transcripts (73). Though the majority of human miRNAs are found 8937 intracellularly, they are also present in extracellular fluids such as cell culture supernatants (8, 55), urine (79), saliva (80), and milk (81). Extracellular miRNA in the plasma and serum has been found directly bound to Argonaute2 as part of the RISC complex (RNA induced silencing complex), which makes them resistant to degradation by exonucleases (82). In the plasma, miRNA has been shown to be delivered to cells by with high-density lipoproteins (83).

A pivotal study in 2007 revealed that exosomes from MC/9, BMMC, and HMC-1 cells contained a large amount of RNA, and miRCURY LNA Array detected 121 microRNAs (8). Many miRNAs can be found encapsulated in exosomes in human serum and saliva (84). MicroRNAs have also been detected in exosome originating from cells (55, 85, 86) and from malignant tumors (55).

Bovine MicroRNA

In milk, many miRNAs are encapsulated in extracellular vesicles, such as exosomes, thereby conferring protection against degradation and a pathway for intestinal transport of miRNAs. MiR-29b is moderately abundant in cow's milk, and plays a role in bone health (87). MiR-200c is highly abundant in cow's milk, and plays a role in cancer prevention by causing dependent loss of transcription factor ZEB1 (88). MiR-29b stimulates osteoblast differentiation (89, 90), impairs osteoclast differentiation and as a result, promotes bone health through increasing bone mineral density (91). Recent studies have been conducted isolating exosomes from human and bovine milk that contain miRNA (92-94), as well as plants. Baier et al. was able to detect increased levels of miR-200c and miR-29b after consumption of cow's milk in human plasma (92).

Other Dietary MicroRNA

The first report of plant miRNAs having the potential to modulate human genes was 3 years ago. Zang et al. high in rice, which contains miR-168a, could be detected in the human and animal sera (95). Thus far, other researchers have not been able to replicate their results (92, 96-99). In contrast to mammalian microRNAs, plant miRNAs are methylated at the 3'-terminal ribose by methyl transferase HEN1 (100).

Farmer et al. recently showed that miR-2911 from honeysuckle was detected in mouse sera and urine after consumption (101). Human subjects fed broccoli sprouts had no detectable increase in broccoli borne miR-824 and miR-167a in human blood (92). Continued research in the microRNA research field is needed to confirm if dietary microRNAs can affect human health in physiological relevant concentrations.

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CHAPTER 2

THE TRANSPORT OF BOVINE MILK EXOSOMES BY HUMAN COLON CARCINOMA CACO-2 CELLS AND RAT SMALL INTESTINAL IEC-6 CELLS

Transport of Bovine Exosomes by Human Colon Carcinoma Caco-2 cells and Rat Small Intestinal IEC-6 Cells [1-2]

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Abbreviations used: V_{max}, maximal rate of exosome uptake; Km is the concentration of exosomal protein required to half-saturate the rate of maximum transport under experimental conditions; miR, microRNA; EV, extracellular vesicles; qPCR, quantitative real-time polymerase chain reaction; *RUNX2*, runt-related transcription factor 2; *ZEB1*, zinc finger E-box binding homeobox 1.

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Abstract

We have reported that microRNAs (miRNAs, miRs) in bovine milk regulate human genes. In milk, many miRNAs are encapsulated in exosomes, thereby conferring protection against degradation and a pathway for intestinal transport of miRNAs. We hypothesized that the uptake of bovine exosomes in human intestinal colon carcinoma Caco-2 cells and rat primary small intestinal IEC-6 cells is mediated by endocytosis. Transport studies were carried out using fluorophore-labeled exosomes purified from bovine milk. The transport of bovine exosomes exhibited saturation kinetics at 37°C (K_m= 55.5±48.6 μg/200 μL, V_{max}= 0.083±0.057 ng exosomal protein *x* 81,750 cells^{-1} *x* hr^{-1}) and decreased by 60% if transport was measured at 4°C in Caco-2 cells, consistent with carrier-mediated transport. Inhibitors of vesicle trafficking and carbohydrate competitors caused a 62-85% and 61-83% decrease, respectively, in exosome transport, consistent with cellular transport of bovine exosomes by endocytosis that depends on surface glycoproteins in Caco-2 cells. Similar patterns were observed in IEC-6 cells. When milk exosomes at a concentration of five times the K_m were added to the upper chamber in transwell plates, Caco-2 cells accumulated miR-29b in the lower chamber, whereas reverse transport was minor. We conclude that the uptake of bovine milk exosomes is mediated by endocytosis and depends on cell and exosome surface glycoproteins in human and rat intestinal cells.

Key words: endocytosis; epithelial cell; extracellular vesicles; milk exosomes; bovine milk exosomes; uptake

INTRODUCTION

The literature is well established that many cell types shed small vesicles into the extracellular space. Evidence suggests that exosomes mediate the transfer of microRNA (miRNA) between cells (1, 2). Our lab is the first to show that humans absorb meaningful quantities of mircoRNA-29b (miR-29b) and miR-200c from nutritionally relevant amounts of cow's milk (3). Baier et al. also showed these miRNAs can affect expression in vitro, and that endogenous synthesis does not compensate for dietary miRNA loss in mice (3). MiR-29b is moderately abundant in cow's milk, and plays a role in bone health (4) by repressing runt-related transcription factor 2 (RUNX2) inhibitors (5, 6), and as a result increases bone mineral density (7). MiR-200c is highly abundant in cow's milk, and plays a role in cancer prevention by inhibiting the transcription factor zinc finger E-box binding homebox 1 (ZEB1), thereby limiting epithelial-to-mesenchymal transition (8, 9).

Mature microRNAs (miRNAs) are small noncoding RNAs that typically bind to the 3' UTR of target mRNAs, leading to mRNA break down or translational delay (10, 11). The emerging evidence of human miRNAs modulating human genes has sparked an interest in dietary miRNAs to see if they also play a role in human health. Many microRNAs in milk are protected against degradation (12) by encapsulation in exosomes (13, 14). In 2007, it was discovered that exosomes carry mRNA and miRNA (15). In this study it was demonstrated that a human mast cell line expressed mouse specific proteins after treatment with mouse exosomal mRNA. This important finding indicated that mRNA transported via the exosomes is translated and may hold importance in overall

cell function (15). The aim of our study was to determine mechanisms of milk exosome cellular uptake. This study can help provide further evidence if food miRNAs protected by exosomes can gain entry into human colon carcinoma Caco-2 and rat IEC-6 primary small intestinal cells. Cow's milk contains 245 microRNAs (16) and bioinformatics predictions suggest that 175 of those might interact with 11,199 human transcripts (unpublished data).

In this study we describe studies of human colon carcinoma Caco-2 cells (Caco-2) as a model system for studies of the uptake into human colon cells. We speculate that milk exosomes are absorbed in the small intestine, therefore we also use a rat small intestine epithelial cell (IEC-6) as a model as well. We hypothesize the uptake of bovine exosomes in human intestinal colon carcinoma Caco-2 cells and rat primary small intestinal IEC-6 cells is mediated by endocytosis. Here we will characterize the uptake kinetics of milk exosomes into Caco-2 and IEC-6 cells.

METHODS AND MATERIALS

Cell cultures. Human colon carcinoma Caco-2 cells were purchased from American Type Culture Collection and were used from passage 52-72 for experiments cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1% non-essential amino acids, 10% fetal bovine serum (FBS), 1% L-glutamine, 100,000 U/L penicillin and 100 mg/L streptomycin. In select experiments, Caco-2 cells were cultured for 2 days in exosome-depleted media obtained by ultracentrifugation of FBS at 120,000 *x* g for 6 hours. Cell media was replaced with fresh media every 2 to 3 days. Transport studies were conducted

at 75% confluence in 96-well plates and at 100% confluence in transwell plates. IEC-6 primary rat small intestinal cells were obtained from ATCC at passage 14, and cultured as described for Caco-2 cells except that culture media contained 0.1 unit/mL bovine insulin and that no transwell studies were conducted due to the IEC-6 cells' inability to form a tight monolayer.

Milk exosome isolation and fluorophore conjugation. Skim cow's milk was purchased in a local grocery store. The milk was centrifuged at 13,200 *x* g and 4°C for 30 minutes to remove somatic cells and debris. The supernatant was mixed 1:1 (by volume) with 250 mM EDTA (pH 7.0) on ice for 15 minutes to precipitate milk casein(17). The suspension was ultracentrifuged at 100,000 *x* g and 4°C for 60 minutes (F37L-8x100 rotor; Thermo Scientific, USA) to remove precipitated protein, fat globules, and vesicles larger than exosomes. The clear supernatant was ultracentrifuged at 120,000 *x* g for 90 minutes at 4°C to collect exosomes. The exosome pellet was re-suspended in a small volume of phosphate-buffered saline containing 0.01% sodium azide, filtered twice through a 0.22-µm membrane filter, and stored at 4°C and -20°C if necessary. The exosomes were labeled with the fluorophore, FM 4-64 (Molecular Probes). One microliter of a stock solution of FM 4-64 (.986 mM/L) was added to 1 mL of exosome suspension, incubated for 15 minutes at 37°C and excess FM 4-64 was removed by ultracentrifugation at 120,000 *x* g at 4°C for 90 minutes.

Absence of aggregation and exosome purity was assessed as recommended by the International Society for Extracellular Vesicles (18). Briefly, absence of exosome

aggregation was confirmed using transmission electron microscopy (Hitachi H7500, Japan) in the Microscopy Core Facility in the University of Nebraska-Lincoln (**Fig. 1A**). ImageJ (http://imagej.nih.gov/ij/index.html) was used to analyze the exosome size distribution. Exosome purity and identity was confirmed using whole protein extracts from exosomes and gel electrophoresis (10 μg protein/lane) as described previously (19). Membranes were probed using mouse anti-bovine CD63 (AbD Serotec, UK) as a marker for exosomes, rabbit antiserum to bovine alpha s1-casein as a marker for the animal species of exosome origin, and goat anti-bovine histone H3 (Santa Cruz Biotechnology, USA) as a negative control (all at 1,000-fold dilutions). Bands were visualized using an Odyssey infrared imaging system (Licor, Inc.) and IRDye 800CW-labeled secondary antibodies at a 50,000-fold dilution (**Fig. 1B**).

Transport studies. Caco-2 cells and IEC-6 cells were seeded at a density of 20,000 cells and 7,000 cells per well, respectively, in 96-well plates and allowed to adhere for 48 hours. Transport studies were conducted using FM4-64 labeled exosomes using 3-110 μg exosomal protein/well (Caco-2 cells) or 27-652 μg exosomal protein/well (IEC-6 cells) and incubating cells for periods of time described in Results; blanks were created using solvent. Assays were calibrated by quantifying the fluorescence of a known mass of exosomes labeled with FM 4-64. When indicated, cells or exosomes were treated with 100 μg/mL proteinase K (Caco-2 cells) to remove surface proteins, 10 μg/mL of the endocytosis inhibitor cytochalasin D, 20 μg/mL of brefeldin A to inhibit vesicle trafficking, and 150 mmol/L of the carbohydrate competitors D-glucose or D-galactose 30

minutes before initiation of transport studies and continuing for the duration of transport studies. IEC-6 cells did not survive proteinase K treatment. Therefore, surface proteins were removed by treating IEC-6 cells and exosomes using 0.105 mmol/L for five and 30 minutes, respectively, at room temperature. Exosome uptake was analyzed by measuring the cell fluorescence at 515 nm (excitation) and 640 nm (emission) using a Biotek FLx800 plate reader and Gen5 data analysis software (BioTek, Winooski, VT). Fluorescence readings were corrected for cell autofluoresence by subtracting signals measured in cells incubated with exosome-depleted media. Transport kinetics were modeled using the Michaelis-Menten equation and non-linear regression; modeling was conducted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

In transwell studies, Caco-2 cells were seeded at a density of 9,000 cells per well and 75 μL media in 96-well polycarbonate plates with a pore size of 0.4 μm (EMD Millipore, Billerica, MA), and allowed to grow a differentiated monolayer for 21-24 days (20). Caco-2 cell monolayer integrity was formally confirmed using the Lucifer Yellow (LY) rejection assay according to the manufacturer's instructions at a final concentration of 60 μmol/L (20). LY fluorescence was measured in the transwell apical and basolateral chambers after one hour of incubation at 37°C (480 nm excitation, 530 nm for emission). In parallel experiments, Caco-2 cells were cultured in exosome-depleted media to which milk exosomes were added back to produce either a concentration of 275 μg/100 μL exosomal protein in the upper, apical chamber or the lower, basolateral chamber. Controls were cultured in exosome-depleted media. Aliquots of media were collected from the upper chamber and bottom chamber after two hours of incubation for analysis of

miRNAs. Twenty-five attomoles of internal standard (miSPIKE Synthetic RNA, IDT Technologies) were added to samples prior to miRNA extraction using the nucleospin miRNA plasma kit (Macherey-Nagel). The concentration of miR-29b in transwell chambers was measured by quantitative real-time PCR, using miScript II RT kit, miScript SYBR Green (Qiagen), and miRNA-specific primers (**Table 2**) as described previously (3). Values were corrected for the internal standard to normalize for extraction efficiency.

Statistics. Homogeneity of variances was assessed using the Brown-Forsythe test (21, 22). The data variation for IEC-6 cells was heterogenous, i.e., those data were log transformed prior to statistical analysis. Statistical significance of differences among treatment groups was assessed using one-way ANOVA and Dunnett's test for post hoc comparisons between treatment groups and control. Analyses were performed using GraphPad Prism. Differences were considered significant if $P<0.05$. Results were presented as means ± S.D. and represent independent biological replicates.

RESULTS

Time course. In Caco-2 cells, exosome uptake at 37°C was linear for up to 120 minutes if transport was measured using non-saturating substrate concentrations, i.e., 110 µg exosomal protein/200 µL (**Fig. 2A**): $y=0.001159x + 0.01355$ ($r^2=0.969$). In IEC-6 cells, exosome uptake at 37°C was linear for only up to 60 minutes if transport was measured using non-saturating substrate concentrations, i.e., 55 µg/200 µL (**Fig. 2B**): $y=0.003298x + 0.03304$ ($r^2=0.754$). Subsequent transport studies were conducted using incubation

times of 60 minutes and 30 minutes for Caco-2 cells and IEC-6 cells, respectively.

Transport kinetics. In both Caco-2 and IEC-6 cells, the uptake of bovine exosomes was mediated by saturable transport mechanisms. Transport kinetics were modeled using the Michaelis-Menten equation. In Caco-2 cells, K_m and V_{max} were 55.5±48.6 µg exosomal protein/200 µL medium and 0.083±0.057 ng exosomal protein *x* 81,750 cells^{-1} *x* hr^{-1}, respectively, at 37°C (r^2=0.746; **Fig. 3A**). In IEC-6 cells, K_m and V_{max} were 152.4 ±39.47 µg/200 µL and 0.140±0.0133 ng exosomal protein *x* 36,375 cells^{-1} *x* 30 min^{-1}, respectively, at 37°C (r^2=0.559; **Fig. 3B**). When the incubation temperature was decreased from 37°C to 4°C, the transport rate decreased from 100±56% to 54±13% using a substrate concentration of 55.5 µg exosomal protein/200 µL in Caco-2 cells (*P*<0.05; n=3). Likewise, when the incubation temperature was decreased from 37°C to 4°C, the transport rate decreased from 100±11% to 44±25% using a substrate concentration of 153 µg exosomal protein/200 µL in IEC-6 cells (*P*<0.05; n=3). Subsequent transport studies were conducted using substrate concentrations of 55 µg/200 µL and 153 µg/200 µL in Caco-2 cells and IEC-6 cells, respectively, except for transwell studies.

***Roles of surface glycoproteins and endocytosis*.** The uptake of bovine milk exosomes into human and rat intestinal cells depended on surface proteins on both exosomes and cells. When surface proteins were removed from exosomes or Caco-2 cells were treatment with proteinase K, exosome uptake decreased to 32±25% and 18±16% of

controls (P<0.05, n=3) (**Fig. 4A**). These observations are consistent with previous reports that the cellular uptake of exosomes is initiated by protein/protein interactions, leading to vesicle uptake by endocytosis (23-25). When IEC-6 cells were treated with trypsin, exosome uptake decreased to 82±8% of controls (P<0.05, n=3) (**Fig. 4C**), indicating that proteins containing amino lysine and arginine on the cell surface may be involved in bovine milk exosome uptake. When Caco-2 cells were treated with inhibitors of endocytosis (cytochalasin D), intracellular vesicle trafficking (brefeldin A), or carbohydrate competitors the uptake of exosomes decreased to less than 50% of controls (**Fig. 4B**). Carbohydrate competitors were equally effective in decreasing exosome uptake into Caco-2 cells. The effects of these inhibitors and competitors were similar in IEC-6 cells, although treatments were not statistically significant (P=0.11, n=6) (**Fig. 4D**).

MiRNA sorting in the intestinal mucosa and reverse transport. When Caco-2 cells were provided with exosome-supplemented media (275 μg/100 μL) in the apical chamber and incubated for two hours, the concentrations of miR-29b in the basolateral chamber increased from 0.018±0.03 fmol/L to 0.094±0.16 fmol/L (P<0.05). When exosomes were provided with exosome-supplemented media (275 μg/100 μL) in the basolateral chamber and incubated for two hours, no concentration gradient was detected in the two chambers, suggesting minimal reverse transport under the experimental conditions: 0.067±0.068 miR-29b fmol/L in the basolateral chamber compared to 0.066±0.11 fmol/L miR-29b in the apical chamber. Caco-2 cells formed a tight monolayer, judged by a Lucifer yellow

rejection percentage of 99.7±0.19%.

DISCUSSION

Exosomes are found in many biologial fluids. In the past decade, research has been focused on but not limited to those found in urine (26, 27), salvia (28), blood (29, 30), and breast milk (13, 31). While much has been discovered regarding exosomes, studies on mechanisms of intestinal transport of dietary exosomes are lacking. By understanding the uptake kinetics of bovine milk exosomes, this study provides the foundation to further explore the mechanism of bovine exosomes in regards to human health. Here we provide evidence that bovine milk exosomes are absorbed into human colon carcinoma Caco-2 cells and IEC-6 RAT small intestinal cells.

Evidence continues to emerge surrounding miRNA's and their role in human health (8, 32, 33). The theory of deitary miRNA absorbance into the human intestinal muscosa and inducing biological activity in humans continues to gain evidence (3, 34-36). In contrast to plant microRNAs, many mammalian microRNAs are protected against degradation (12) by encapsulation in microvesicles such as exosomes (13, 14, 37). It has become a controversial topic in the science community if plant miRNAs can in fact effect human gene expression (3, 38-41). In contrary, a recent report suggests that another animal product, eggs in nutritionally relevant amounts, can cause a 100% increase in plasma miRNAs (36).

By using inhibitors of vesicle trafficking and a broad-spectrum proteinase, and by measuring uptake at 4°C and 37°C, we provide evidence that uptake of bovine milk

exosomes is a carrier-mediated process in human intestinal cells and that glycoproteins on the milk exosomes and intestinal cells are involved. High sucrose concentrations have been associated with decreased levels of receptor-mediate endocytosis (42). By increasing the levels of glucose and galactose, it's possible that the hyperosmolar state decreased the uptake of milk exosomes into Caco-2 cells, indicating bovine milk exosomes depend on endocytosis to be taken into the cell. We also think it's possible that glucose and galactose may be acting as carbohydrate competitors with milk exosomes.

There are some uncertainties associated with our studies of bovine milk exosomes and will need to be revisited in future studies. First, FM4-64 might alter the structure of bovine EVs. We are not able to overcome this obstacle at this time. Second, FM4-64 is believed to fluoresce intensely when inserted into the outer leaflet of the surface membrane (43), so it's possible that the bovine exosomes are not fully internalized in the cell. We find this to be an unlikely possibility due to a study from an independent laboratory suggesting that milk exosomes enter the mouse circulation intact by crossing the intestinal mucosa without re-packaging (35). We will carry out future studies to determine if we also can come to this conclusion. Lastly, it is not yet established if surface proteins in human and rat cells can recognize exosomes from other species.

Because low Km is associated with a higher affinity, we can conclude bovine exosomes is higher in human Caco-2 cells than rat IEC-6 cells. One could speculate that the bovine surface glycoproteome is more compatible with human cells than it is with rats. Current studies in our laboratory include characterization of the glycoproteins involved in bovine milk exosome transportation. In contrast, the capacity for transport is

higher in rat IEC-6 cells compared with human Caco-2 cells. One would expect that with more cells, the transport rates would be higher. When using half as many IEC-6 cells, the differences become more dramatic between transport rates. This makes logical sense based on the absorption kinetics of humans absorbing milk microRNA (3). This study out of our laboratory provided the evidence to propose that the upper intestine is the primary site of milk exosome absorption (3), and we validated it with the IEC-6 cell line in this study.

Looking forward, the use of milk exosomes may be a promising vehicle for the oral delivery of drugs. Exosomes can be harvested from cells, but milk proves advantageous due to the large quantities that can be obtained and the practicality when it comes to the cost and availability. Cell cultures are often from cancer or mutant cells which might run the risk of promoting malignant transformation of cells in subjects treated with such vesicles (44).

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T.W., S.R.B, and J.Z. designed the research and wrote the manuscript. T.W. conducted the research, analyzed the data and conducted the statistical analysis. J.Z. had the primary responsibility for the final content. Each author contributed to the development of this work, and all authors have read and approved the final manuscript. The first author would also like to thank Elizabeth Cordonier for advice with laboratory techniques.

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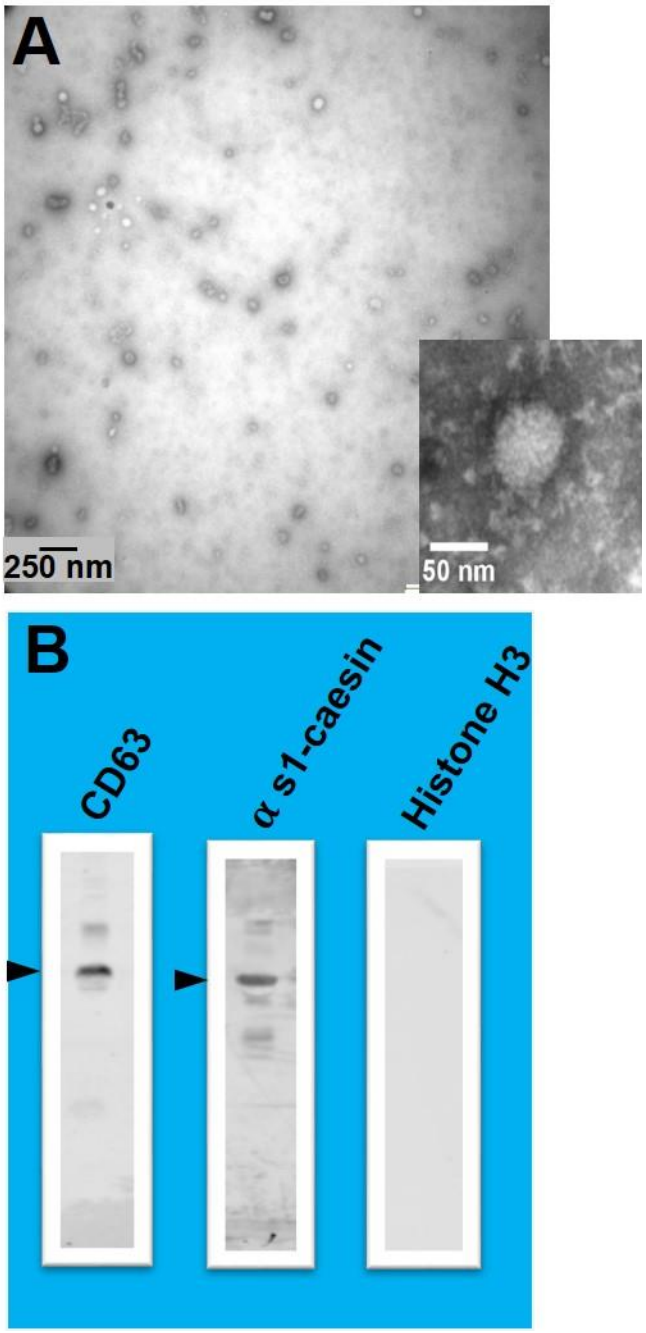


Figure 1
Electron microscopy and western blot.
(*A*) Electron microscopy images: large field image (15,000 x magnification) and single exosomes (60,000X magnification).
(*B*) Western Blot A: anti-C63 (exosome positive control), B: anti-casein (bovine positive control), C: anti-histone H3 (negative control). All samples were run on the same gel but the membrane had to be cut to allow for probing with distinct antibodies.

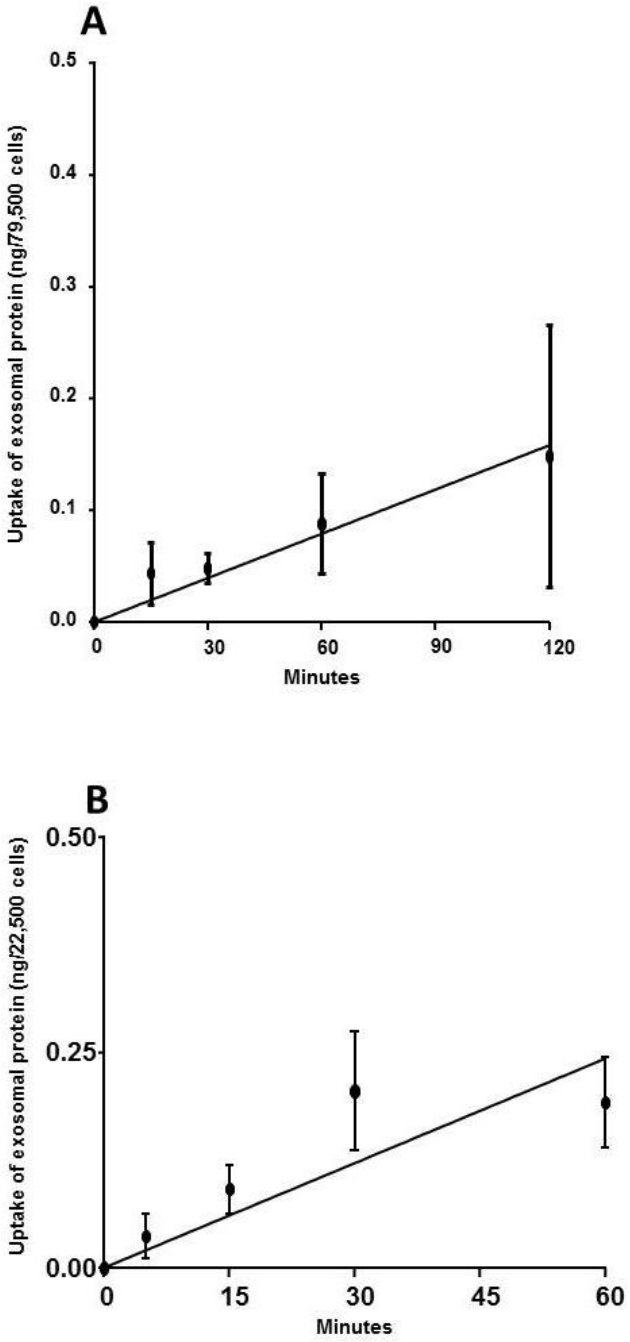


Figure 2

(*A*) Exosome uptake into human colon carcinoma Caco-2 cells as a function of time at a concentration of 110 µg exosome protein/200 µL medium and temperature of 37°C (n=6.)

(*B*) Exosome uptake into rat primary intestinal IEC-6 cells as a function of time at a concentration of 55 µg exosome protein/200 µL medium and temperature of 37°C (n=3). All panels: Means±SD are reported.

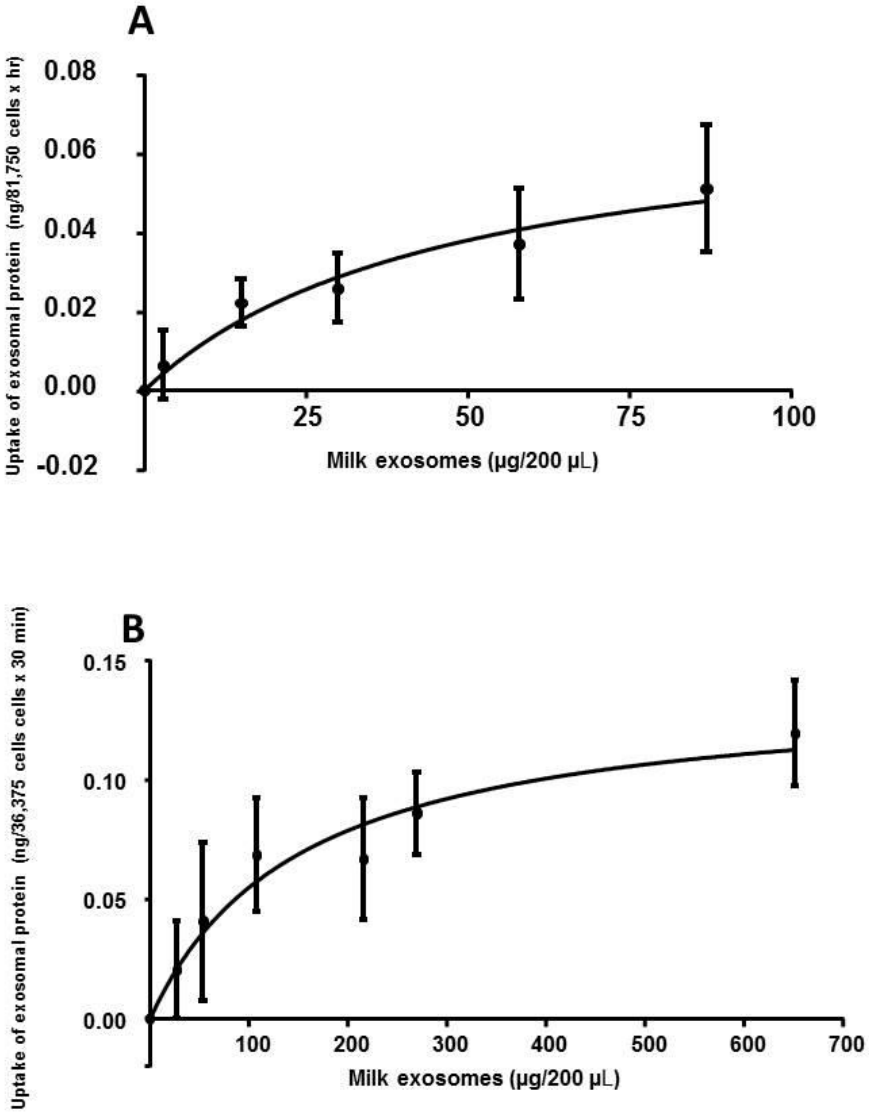
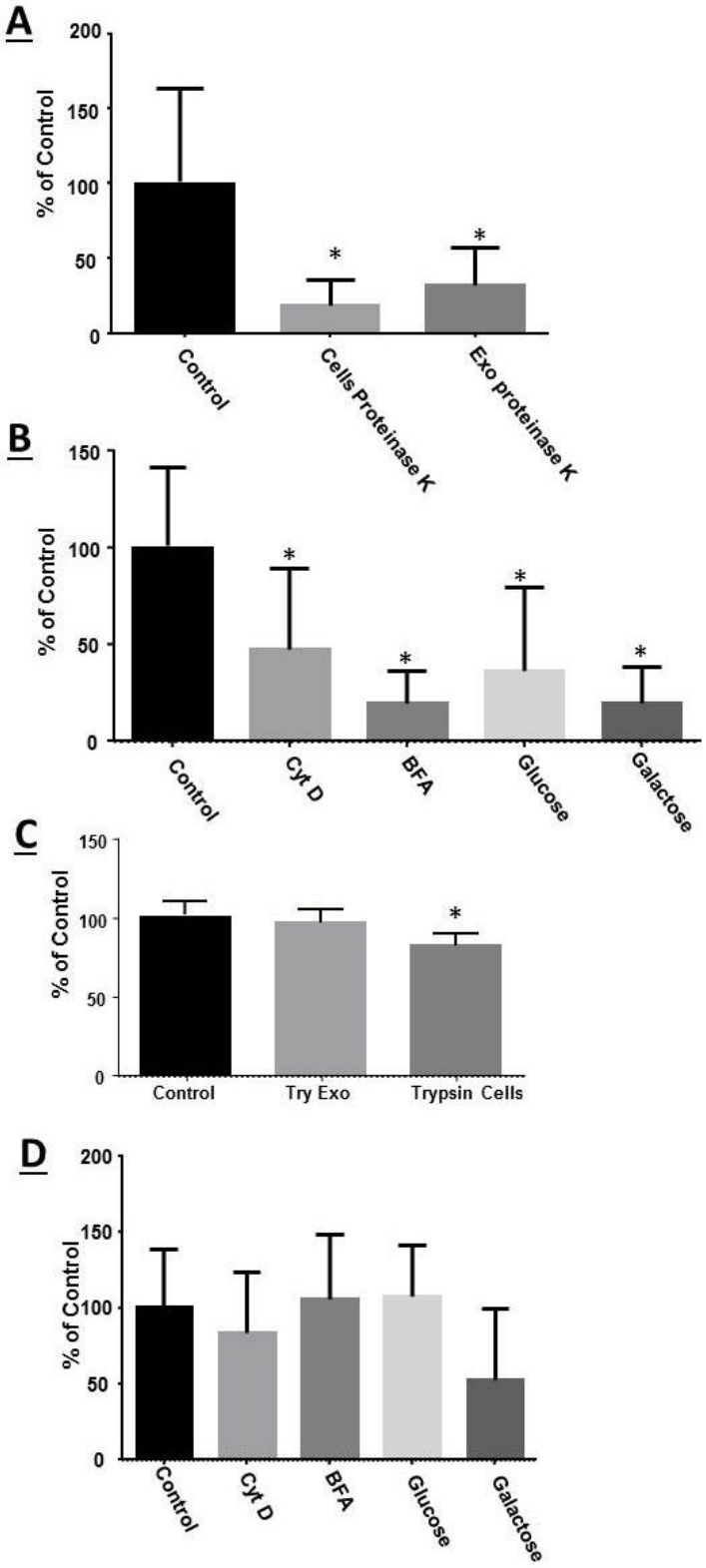


Figure 3
Saturation kinetics of bovine exosome transport in Caco-2 cells.
(*A*) Exosome uptake into human colon carcinoma Caco-2 cells as a function of exosome protein dose in one hour at 37°C (n=6).
(*B*) Exosome uptake into rat primary intestinal IEC-6 cells as a function of exosome protein dose in 30 hour at 37°C (n=3). All panels: Means±SD are reported.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Glucose
Galactose

Figure 4
Effects of inhibitors of endocytosis and vesicle trafficking, and carbohydrate competitors on the uptake of bovine exosomes in human and rat intestinal cells.
(*A*) Treatment of bovine exosomes or Caco-2 cells with proteinase K prior to analysis of transport rates. (n=3).
(*B*) Exosome transport in Caco-2 cells treated with cytochalasin D (Cyt D), brefeldin A (BFA), or in the presence of carbohydrate competitors (n=5).
(*C*) Treatment of bovine exosomes or IEC-6 cells with trypsin prior to analysis of transport rates (n=3).
(*D*) Exosome transport in IEC-6 cells treated with Cyt D, BFA, or in the presence of carbohydrate competitors (n=6).
All panels: Means±SD are reported; *$p<0.05$ vs control.

Table 1
Quantitative PCR Primers Used to Quantify Gene Expression

microRNA	**Forward Primer Sequence 5 – 3'**
miSpike	CTC AGG ATG GCG GAG CGG TCT
miR-29b	GTA GCA CCA TTT GAA TCA GTG TT

CHAPTER 3

CONCLUSIONS

FUTURE STUDIES & CONCLUSION

Moving forward our lab is currently conducting a study analyzing phenotypic effects potentially due to microRNA provided by the diet. Mice are being fed an exosome plus diet, where microRNAs are present, or an exosome minus diet, in which microRNAs are depleted through sonication. Plasma and a number of different tissues will be analyzed at different stages in life. The success of breeding and offspring survival will be analyzed as well. Our lab will also test feeding human adults soy infant formula and the same soy infant formula but supplemented with bovine milk exosomes. We predict the miRNA plasma levels will not change following consumption of soy formula alone, but when supplemented with bovine exosomes, may result in plasma miRNA increases similar to our prior study (1).

Many questions remain unanswered about extracellular vesicles, and much research is still needed, but so far, the future of extracellular vesicles in drug delivery is looking promising (2, 3). A remarkable study recently published created a treatment by loading exosomes with a catalase, a potent antioxidant, for the treatment of Parkinson's Disease in mice (2). A clinical trial currently recruiting participants will access if plant exosomes loaded with curcumin can effectively deliver curcumin to colon cancer and normal colon mucosa tissues as a dietary supplement (4).

Technical aspects of EV research, such as storage, isolation, and other factors are being explored to determine the best technique for harvesting and preserving EVs (5-7). Mammal and food derived EVs currently have no established nomenclature for classification and isolation procedures (8). This study, and many others, is just the

beginning of a path that will perhaps lead to improved insight on drug and nutrient delivery systems.

MicroRNAs have been implicated in many aspects of human health and disease including bone health, inflammatory bowel disease, metabolic syndrome (9-12) cancer (13), and cystic fibrosis (14-16). Though it is important to note that some miRNAs may have adverse effects (17-19). More in-depth studies to determine if dietary microRNAs can survive and pass the human gastrointestinal tract are needed. As the great Frenchmen Brillat-Savarin once said, “Tell me what you eat, and I will tell you what you are.” This may continue to ring true with even deeper meaning than initially thought if dietary microRNAs from other species can indeed impact gene expression in humans.

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