Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages

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ABSTRACT

We reported previously that microRNA (miRNA) are present in whey fractions of human breast milk, bovine milk, and rat milk. Moreover, we also confirmed that so many mRNA species are present in rat milk whey. These RNA were resistant to acidic conditions and to RNase, but were degraded by detergent. Thus, these RNA are likely packaged in membrane vesicles such as exosomes. However, functional extracellular circulating RNA in bodily fluids, such as blood miRNA, are present in various forms. In the current study, we used bovine raw milk and total RNA purified from exosomes (prepared by ultracentrifugation) and ultracentrifuged supernatants, and analyzed them using miRNA and mRNA microarrays to clarify which miRNA and mRNA species are present in exosomes, and which species exist in other forms. Microarray analyses revealed that most mRNA in milk whey were present in exosomes, whereas miRNA in milk whey were present in supernatant as well as exosomes. The RNA in exosomes might exert functional effects because of their stability. Therefore, we also investigated whether bovine milk-derived exosomes could affect human cells using THP-1 cells. Flow cytometry and fluorescent microscopy studies revealed that bovine milk exosomes were incorporated into differentiated THP-1 cells. These results suggest that bovine milk exosomes might have effects in human cells by containing RNA.

Key words: microRNA, mRNA, exosome, milk

INTRODUCTION

Bovine milk and dairy products have a long tradition and are a good source of nutrition to maintain human health (Haug et al., 2007). In addition to nutritional agents, milk also contains a large number of biological components, including cytokines, chemokines, and

hormones (Cross and Gill, 2000; van Hooijdonk et al., 2000). In recent years, studies have revealed that human (Kosaka et al., 2010b; Weber et al., 2010; Zhou et al., 2012; Munch et al., 2013), bovine (Chen et al., 2010; Hata et al., 2010; Izumi et al., 2012, 2013; Sun et al., 2013), pig (Gu et al., 2012), and rat milk (Izumi et al., 2014) also contain microRNA (**miRNA**). Some of these studies reported that mRNA are also contained in whey (Hata et al., 2010; Izumi et al., 2012, 2013, 2014). Moreover, these RNA are stable under harsh conditions, such as low pH, and in the presence of RNase (Hata et al., 2010; Kosaka et al., 2010b; Izumi et al., 2012). These RNA have been detected in commercial dairy products, such as powdered infant formula, that have undergone stringent industrial processes (Chen et al., 2010; Izumi et al., 2012). Based on these studies, it is possible that RNA in milk have functions in the mammalian gastrointestinal tract.

Most body fluids, such as blood, saliva, urine, and amniotic fluid, contain miRNA (Mitchell et al., 2008; Kosaka et al., 2010a; Weber et al., 2010; Argyropoulos et al., 2013; Ogawa et al., 2013). However, these circulating RNA exist in diverse forms, including packaged in exosomes (Simpson et al., 2009; Kosaka et al., 2010a; Lässer et al., 2011), in high-density lipoprotein (Vickers et al., 2011), and as complexes with RNA-binding proteins (Arroyo et al., 2011). For example, our previous study (Izumi et al., 2013) showed that the RNA concentration in bovine milk whey-derived exosomes was lower than was that in whey. Therefore, the aim of the current study was to clarify which miRNA and mRNA species in bovine milk whey are present in exosomes, and assess which species exist in other forms, using microarrays. No comprehensive reports have analyzed the presence of mRNA in bovine milk whey, although mRNA were found in rat milk whey (Izumi et al., 2014), human (Maningat et al., 2009; Lemay et al., 2013) and goat (Brenaut et al., 2012) milk fat globules, and bovine milk somatic cells (Wickramasinghe et al., 2012) using microarrays or next-generation sequencing. Our previous study using rat milk whey (Izumi et al., 2014) detected >10,000 mRNA. Therefore, to our knowledge,

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the current study is the first report to reveal which mRNA are present in bovine milk whey exosomes and in other forms. Previous studies revealed that bovine milk miRNA profiles differ among cows and between colostrum and mature milk (Chen et al., 2010; Izumi et al., 2012). In commercial dairy products, raw milk is collected and mixed from many cows and is usually used. Whey is an important fraction of milk, and is also used in a variety of products (Marshall, 2004). In the current study, we prepared whey fractions from raw milk, which is used for commercial dairy products. In addition, some studies showed that macrophages could take up milk whey-derived exosomes (Lässer et al., 2011; Sun et al., 2013). Therefore, we also investigated whether bovine milk-derived exosomes could be taken up by human cells using human monocytic leukemia THP-1 cells that show macrophage-like functions after differentiation by phorbolmyristate acetate (PMA).

MATERIALS AND METHODS

Milk Whey Sample Preparation

Three bovine raw milk samples were prepared and stored at -80°C until use; these are commonly used for research and development in our laboratory. Whey was prepared as described previously (Izumi et al., 2012, 2013). Briefly, the samples were centrifuged twice ($1,200 \times g$, 4°C , 10 min) to remove fat, cells, and large debris. The defatted supernatant was then centrifuged ($21,500 \times g$, 4°C , 30 min, 1 h) to remove residual fat and casein. The clear supernatant (whey) was then passed through 0.65-, 0.45-, and 0.22- μm filters to remove residual cell debris.

Exosome and Supernatant Preparation

The whey samples (see previous section) were ultracentrifuged ($100,000 \times g$, 4°C , 90 min) using an SR-P70AT rotor (Hitachi Koki, Tokyo, Japan) according to a previous study (Lässer et al., 2011). The resulting supernatant was collected carefully to avoid contaminating the pelleted exosomes. The pelleted exosomes were resuspended in PBS and then ultracentrifuged ($100,000 \times g$, 4°C , 90 min) for washing; this wash step was performed twice. Finally, the exosomes were resuspended in PBS. The protein content of the exosomes was determined using a Micro Bradford protein assay (Bio-Rad, Hercules, CA).

Extraction of Total RNA

Total RNA was extracted from exosomes and supernatant samples, and was purified using an miRNeasy

Serum/Plasma Kit (Qiagen, Hilden, Germany) as described previously (Izumi et al., 2012, 2013, 2014). The quantity and integrity of the RNA were assessed using an RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA), and the miRNA-to-small RNA ratio (default settings: miRNA = 10–40 nucleotides; small RNA = 0–270 nucleotides) was examined on an Agilent 2100 Bioanalyzer (Agilent) using a Small RNA Kit (Agilent).

miRNA Microarray

The RNA samples were derived from an equal mixture of 3 samples. To detect the expression of miRNA in exosomes and supernatant, 40 ng of mixed total RNA was labeled with cyanine-3 and then hybridized to Bovine miRNA Microarray Rel. 17.0 arrays using an miRNA Complete Labeling Reagent and Hyb Kit (Agilent; Wang et al., 2007). The Bovine miRNA Microarray Rel. 17.0 analyzes the expression of 670 bovine miRNA from the Sanger miRBase Rel.17.0 (www.mirbase.org). Signals were detected with an Agilent DNA Microarray Scanner, and the scanned images were analyzed using the Feature Extraction Software (ver. 10.7.3.1; Agilent).

mRNA Microarray

The RNA samples were derived from an equal mixture of 3 samples. To detect the expression of mRNA in exosomes and supernatant, complementary RNA was generated from 100 ng of mixed total RNA and then labeled with cyanine-3 using a Low Input Quick Amp Labeling Kit (Agilent). Labeled cRNA was hybridized to Bovine Oligo DNA Microarray ver. 2.0 ($4 \times 44\text{K}$) using a Gene Expression Hybridization Kit (Agilent). The Bovine Oligo DNA Microarray ver. 2.0 ($4 \times 44\text{K}$) contains 43,713 probes (without control probes). Signals were detected with an Agilent DNA Microarray Scanner, and the scanned images were analyzed using the Feature Extraction Software (ver. 10.7.3.1).

Microarray Data Analysis

Raw data from Feature Extraction Software were exported to GeneSpring GX ver. 11.5.1 (Agilent). Entities were considered present if signal was judged to be present by software. For mRNA microarray data, transcripts with raw signals >20 and that were given an Entrez Gene ID were considered to be present. All microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO), and are accessible through GEO Series accession number GSE61978 (www.ncbi.nlm.nih.gov/geo).

Quantification of miRNA by qPCR

The cDNA was generated using an miScript Reverse Transcription Kit (Qiagen). Briefly, total RNA from exosomes or supernatant (both from 3.2 mL of whey) was polyadenylated using poly(A) polymerase, and cDNA was generated using reverse transcriptase and tagged oligo-dT primers. The cDNA was then diluted in 9 volumes of nuclease-free water and subjected to quantitative (q)PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using an miScript SYBR Green PCR Kit and miScript Primer Assay (Qiagen) according to the manufacturer's instructions. This system was reported that show relatively high accuracy and high sensitivity among several quantification methods (Mestdagh et al., 2014) and was used in our previous studies (Izumi et al., 2012, 2013, 2014). The following real-time PCR protocol was used: initial activation of HotStarTaq DNA Polymerase (from miScript kit; 95°C, 15 min); 40 to 50 cycles of denaturation (94°C, 15 s), annealing (55°C, 30 s), and extension (70°C, 34 s); and melting curve analysis. The miScript Primer Assays (Qiagen) used for the target miRNA are listed in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2014-9076>). The data were analyzed using 7500 Software ver. 2.0.4. (Applied Biosystems) with the fixed cycle threshold (Ct) setting ($\Delta Rn = 0.02$, where ΔRn is the fluorescence of the reporter dye minus the baseline) to assign baseline values and the threshold for Ct determination.

Quantification of mRNA by qPCR

The cDNA to quantify mRNA was generated from total RNA extracted from exosomes or supernatant (both from 3.2 mL of whey) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). The cDNA was diluted in 9 volumes of nuclease-free water, and was then subjected to qPCR on a 7500 Fast Real-Time PCR System (Applied Biosystems) using TaqMan Fast Advanced Master Mix (Applied Biosystems) and TaqMan Probes according to the manufacturer's protocol. The following real-time PCR protocol was used: uracil-N-glycosylase incubation (50°C, 2 min), polymerase activation (95°C, 20 s), 40 to 50 cycles of denaturation (95°C, 3 s), and annealing and extension (60°C, 30 s). The TaqMan Probes used for the target mRNA are listed in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2014-9076>). The data were analyzed using 7500 Software ver. 2.0.4. (Applied Biosystems) with the fixed Ct setting ($\Delta Rn = 0.2$) to assign baseline values and the threshold for Ct determination.

Cell Culture

The THP-1 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (HyClone, Logan, UT), 1% penicillin-streptomycin-glutamine (Gibco, Palo Alto, CA), 1% NEAA (Gibco), and 0.1% β -mercaptoethanol (Gibco; complete RPMI 1640). Cells were incubated at 37°C in humid atmosphere with 5% CO₂. The FBS was ultracentrifuged (100,000 $\times g$, 4°C, 90 min) before use to eliminate bovine serum exosomes.

Exosome Staining

The exosomes were labeled with PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions and a previous study (Lässer et al., 2011), with minor modifications. Briefly, 8 μ L of PKH67 dye was added to 1 mL of Diluent C (from the kit), and was then added to the exosomes and the control sample (PBS). The samples were mixed gently for 4 min, and then 2 mL of 2% FBS (exosome-depleted) Cell Wash (BD Biosciences, San Diego, CA) were added to bind the excess dye. The samples were then transferred to 100-kDa filters (Amicon, Millipore, Billerica, MA). The samples were washed 3 times with 2% FBS (exosome-depleted) Cell Wash, before being transferred to new 100-kDa filters and washed twice with complete RPMI 1640 (see previous sections).

Uptake of Milk-Derived Exosomes by THP-1 Cells

The THP-1 cells were cultured in 12-well plate for flow cytometry and were cultured in 8-well Lab-Tek Chamber Slide (Thermo Fisher Scientific, Waltham, MA) for fluorescence microscopy. The THP-1 cells were differentiated using 50 nM PMA (Sigma) for 60 h or were maintained without PMA, and were washed 3 times with complete RPMI. Ten micrograms of PKH67-labeled exosomes or the same volume of the PKH67-PBS control was added per 1×10^5 cells (differentiated or undifferentiated THP-1 cells), which were then incubated for 2 h at either 37°C or 4°C. The incorporation of exosomes into the macrophages was analyzed using FACSCanto (BD Biosciences) and visualized using a fluorescence microscope BX53 (Olympus, Tokyo, Japan). For flow cytometry, cells were washed twice with PBS, treated with 0.05% trypsin-0.02% EDTA-4Na solution (Gibco), washed twice with 2% FBS (exosome-depleted) Cell Wash, and suspended in 2% FBS (exosome-depleted) Cell Wash. The data were then acquired using FACSCanto. The flow cytometric data were analyzed using the FlowJo software (Tree

Star Inc., Ashland, OR). For fluorescence microscopy, cells were washed twice with PBS, fixed with CellFIX (BD Biosciences) for 15 min, and washed 3 times with PBS. Five microliters of 7-AAD (BD Biosciences) in 200 μ L of PBS was added for 15 min to label nuclei, and the samples were mounted using Vectashield (Vector Laboratories Inc., Burlingame, CA). The fluorescence microscopy data were analyzed using cellSens (Olympus).

Statistical Analyses

The values are expressed as mean $\pm$ SEM. The significance of differences was determined using *t*-test for comparisons between 2 groups, and the Tukey-Kramer honestly significant difference (HSD) test was used for multiple comparisons (JMP software; SAS Institute Inc., Cary, NC). A *P*-value <0.05 was considered significant difference.

RESULTS

Bioanalyzer Analyses

Total RNA was extracted from bovine milk whey-derived exosomes and ultracentrifuged supernatant, and was analyzed using a bioanalyzer. No or very little ribosomal RNA (18S and 28S) was seen, but small RNA (<300 nucleotides) were present (Figure 1). The RNA concentration in the exosome fraction was significantly higher than was that in the supernatant fraction (Figure 2A). The RNA content ratio of exosomes to supernatant was $\sim 7:3$ (Figure 2B). The miRNA-to-small RNA ratio (default setting: miRNA = 10–40 nucleotides; small RNA = 0–270 nucleotides) was significantly higher in the supernatant fraction compared with the exosome fraction (Figure 2C).

Microarray Analyses

The miRNA microarray analyses revealed 79 miRNA in the exosomes, and 91 in the supernatant (Figure 3A). A total of 39 miRNA were common to both fractions (Figure 3A), but their signal intensities differed (Figure 3B). The top 50 highly expressed miRNA in exosomes and supernatant, and their normalized signal intensities, are listed in Table 1. The miRNA that were expressed at high levels differed between exosomes and supernatant (Table 1). The mRNA microarray analyses detected 19,320 mRNA probes in exosomes and 2,634 mRNA in supernatant (Figure 3C). A total of 2,538 mRNA probes (most of the mRNA probes that were detected in supernatant) were common to both exosomes and supernatant (Figure 3C); however, the signal

intensity differed between fractions (Figure 3D). The top 50 highly expressed mRNA probes and their signal intensities in exosomes and supernatant are listed in Tables 2 and 3, respectively. Some mRNA probes were expressed highly in both exosomes and supernatant. However, most of highly expressed mRNA probes were different between exosomes and supernatant (Tables 2 and 3).

qPCR Analyses of miRNA

Twenty miRNA were selected from the top 50 list (Table 3) and were analyzed using qPCR to assess their expression levels in exosomes and supernatants that were isolated from the same volume of whey (Figure 4). Almost all of the selected miRNA (except for miR-133b and miR-1281) were expressed at significantly higher levels in exosomes compared with supernatants.

qPCR Analyses of mRNA

Twenty-three mRNA were selected from those shown in Tables 2 and 3, and were analyzed using qPCR to quantify their expression in exosomes and supernatants that were isolated from the same volume of whey (Figure 5). The selected mRNA were milk- and milk-derived exosome-related mRNA (Figure 5A, B) or mRNA that were reported in other studies to be milk RNA (milk cells and milk fat globule; Figure 5C; Maningat et al., 2009; Brenaut et al., 2012; Wickramasinghe et al., 2012; Lemay et al., 2013). All the selected mRNA were expressed at significantly higher levels in exosomes compared with supernatants (Figure 5).

Cell-Based Experiments

Fluorescence microscopy revealed that bovine raw milk-derived exosomes were taken up by differentiated THP-1 cells (Figure 6A–C). The extent of exosome incorporation into THP-1 cells was analyzed using flow cytometry. Undifferentiated THP-1 cells did not take up exosomes. In contrast, differentiated THP-1 cells (macrophage-like) could uptake exosomes at both 4 and 37°C; however, exosome incorporation was significantly higher at 37 than at 4°C (Figure 6D).

DISCUSSION

Extracellular circulating RNA can be present in forms other than exosomes and microvesicles (Simpson et al., 2009; Kosaka et al., 2010a; Arroyo et al., 2011; Lässer et al., 2011; Vickers et al., 2011). In the current study, data revealed that RNA were also present in ultracentrifuged supernatants of whey fraction (Figure

Figure 1. Analysis of RNA purified from bovine raw milk whey-derived exosomes and ultracentrifuged supernatants using Bioanalyzer (Agilent Technologies, Santa Clara, CA). (A, B) Bovine raw milk whey-derived exosomes; (C, D) ultracentrifuged supernatants from bovine raw milk whey. A and C were analyzed using an RNA 6000 Pico Kit (Agilent); B and D were analyzed using a Small RNA Kit (Agilent). fu = fluorescence unit; nt = nucleotide. Color version available online.

1), although the RNA concentration was significantly lower in supernatants compared with exosomes (Figure 2A). In addition, the RNA content ratio of exosome to supernatant was ~7:3 (Figure 2B); therefore, most RNA was present in exosomes. Ninety-one miRNA were detected in the supernatant; however, 52 miRNA of these were present in only the supernatant. Therefore, the miRNA expression pattern differed between the supernatant and exosomes (Figure 3A, B).

Most (2,538 mRNA transcripts) mRNA detected in the supernatant were also present in exosomes, and the detected number of mRNA only in the supernatant was very few (96 mRNA transcripts), although 2,634 mRNA

transcripts were detected in supernatant (Figure 3C). In contrast, large numbers of mRNA (19,320 mRNA transcripts) were present in exosomes. Therefore, it might be thought that most mRNA detected in milk whey were concentrated in exosomes.

The 50 miRNA expressed at the highest level in exosomes and supernatant according to microarrays are presented in Table 1. Fourteen miRNA (miR-2478, miR-2412, miR-2305, miR-2881, miR-2328*, miR-2888, miR-2304, miR-2391, miR-2892, miR-2887, miR-2316, miR-2374, miR-2291, miR-2284l) in exosomes and 28 miRNA (miR-2305, miR-2412, miR-2888, miR-2316, miR-2328*, miR-2478, miR-2893, miR-2881, miR-2374,

Figure 2. RNA concentrations, RNA concentrate ratio, and microRNA (miRNA)-to-small RNA ratio in bovine raw milk whey. (A) RNA concentrations in bovine raw milk whey-derived exosomes and ultracentrifuged supernatants; (B) ratios of the RNA concentrations in exosomes to those in supernatants from 3 raw milk (RM) whey samples; (C) miRNA-to-small RNA ratio. The values are means $\pm$ SEM ($n = 3$). * $P < 0.05$ (compared with supernatant), ** $P < 0.01$ (compared with exosome).

miR-2391, miR-2348, miR2892, miR-2885, miR-2428, miR-2882, miR-2486, miR-2455, miR-2898, miR-2407, miR-2436-5p, miR-2389, miR-2309, miR-2340, miR-2902, miR-2454, miR-2373*, miR-2899, miR-2300a-5p) in supernatant are bovine-specific. Generally, species-specific miRNA are likely to be evolutionally younger and expressed at lower levels than highly conserved miRNA. The biological roles of these miRNA are unknown; however, some (miR-2478, miR-2412, miR-2305, miR-2881, miR-2328*, miR-2888, miR-2391, miR-2374, miR-2882, miR-2455, miR-2898) are predicted to regulate carbohydrate or lipid metabolism (Romao et al., 2014). Zhou et al. (2012) and Gu et al. (2012) reported the top 10 miRNA expressed in human and in pig milk exosomes, respectively. Seven (miR-148a, miR-30b-5p, let-7f, miR-29a, let-7a, miR-141, and miR-200a) of the top 10 miRNA in human milk exosomes and 5 miRNA (miR-148a, miR-30a-5p, miR-30d, miR-200c, and let-7a) from pig milk exosomes were also present in the 50 miRNA expressed at the highest levels in bovine milk exosomes. Nevertheless, 14 of the top 50 miRNA were bovine-specific. These results suggest that milk exosome miRNA have some common roles among species, whereas other functions are species-specific. The biological functions of most miRNA remain unknown. In the current study, we selected 20 miRNA from the top 50 whose function was relatively well characterized (Table 1) and quantified them using qPCR in exosomes and supernatants that were isolated from the same volume of whey (Figure 4). Data revealed almost all of the miRNA (except for miR-133b and miR-1281) were expressed at significantly higher levels in exosomes than in supernatants. Both miR-133b and miR-1281 were among the 50 species expressed at the highest levels in supernatants, but not in exosomes. Therefore, these data do not contradict the microarray results because we used same amount of RNA from exosomes and supernatants in the microarrays. Two miRNA (miR-148a and let-7a) are common in human and pig milk exosomes (Gu et al., 2012; Zhou et al., 2012). These 2 miRNA were also expressed highly in bovine raw milk exosomes (Table 1, Figure 4). A previous study reported that miR-148a is a negative regulator of the innate immune response and antigen-presenting function of dendritic cells (Liu et al., 2010). It is also upregulated during intestinal cell differentiation (Hino et al., 2008), and targets DNA methyltransferase 3b (Sato et al., 2011). Let-7a inhibits Th17 differentiation by downregulating IL-6 secretion (Zhang et al. 2013). In addition, let-7a is upregulated during intestinal cell differentiation (Papetti and Augenlicht, 2011). It is very interesting that the miRNA that are expressed highly in milk exosomes from several species exhibit common functions, such as regulating immune function and intestinal maturation.

Figure 3. MicroRNA (miRNA) and mRNA microarray results of bovine raw milk whey-derived exosomes and ultracentrifuged supernatants. (A) Venn diagram showing the numbers of miRNA expressed in exosomes and supernatants; (B) heat map of the normalized array data for miRNA detected in exosomes or supernatants; (C) Venn diagram showing the numbers of mRNA probes expressed in exosomes and the supernatants; (D) heat map of the normalized array data for mRNA detected in exosomes or supernatants.

Table 1. Top 50 most highly expressed microRNA in bovine whey exosome or supernatant

Exosome			Supernatant		
Rank	Name	Signal intensity	Rank	Name	Signal intensity
1	miR-2478	19.16	1	miR-1777a	20.02
2	miR-1777b	14.11	2	miR-1777b	19.74
3	miR-1777a	13.44	3	miR-2305	18.93
4	let-7b	12.78	4	miR-1343	17.76
5	miR-1224	12.60	5	miR-2412	17.48
6	miR-2412	12.54	6	miR-2888	17.42
7	miR-2305	11.70	7	miR-2316	16.97
8	let-7a	11.67	8	miR-2328*	16.86
9	miR-200c	11.51	9	miR-1224	16.04
10	miR-141	11.32	10	miR-2478	16.02
11	miR-2881	11.29	11	miR-2893	15.92
12	miR-2328*	9.92	12	miR-2881	15.89
13	let-7c	9.89	13	miR-2374	14.91
14	miR-148a	9.87	14	miR-1584	14.84
15	miR-320	9.81	15	miR-1249	14.47
16	miR-2888	9.74	16	miR-671	14.45
17	let-7f	9.73	17	miR-2391	14.22
18	miR-200b	9.55	18	miR-2348	13.75
19	miR-1584	9.51	19	miR-2892	13.64
20	miR-26a	9.30	20	miR-2885	12.76
21	miR-20a	9.18	21	miR-2428	12.41
22	miR-103	9.15	22	miR-2882	12.39
23	miR-29c	8.83	23	miR-2486	11.57
24	miR-30d	8.80	24	miR-2455	11.36
25	miR-92	8.63	25	miR-2898	11.31
26	miR-2304	8.41	26	miR-1225-3p	11.13
27	miR-375	8.41	27	miR-2407	11.12
28	miR-2391	8.40	28	miR-2436-5p	10.89
29	let-7g	8.39	29	miR-2389	10.85
30	miR-30a-5p	8.24	30	miR-320	10.82
31	miR-1343	8.19	31	miR-141	10.58
32	miR-125b	8.17	32	miR-2476	10.38
33	miR-30b-5p	8.16	33	miR-489	10.24
34	miR-26b	7.99	34	miR-345-3p	10.16
35	miR-2892	7.96	35	miR-2392	10.11
36	miR-24-3p	7.94	36	miR-30d	10.01
37	miR-1249	7.88	37	miR-365-3p	9.99
38	miR-423-5p	7.85	38	miR-2309	9.95
39	miR-664	7.75	39	miR-2340	9.91
40	miR-2887	7.75	40	miR-2902	9.74
41	miR-2284d	7.68	41	miR-2454	9.64
42	miR-2316	7.63	42	miR-2373*	9.61
43	miR-2374	7.61	43	miR-1835	9.56
44	miR-29a	7.61	44	miR-2899	9.45
45	let-7d	7.53	45	miR-30a-5p	9.42
46	miR-200a	7.51	46	miR-425-3p	9.40
47	miR-151*	7.46	47	miR-2300a-5p	9.39
48	miR-30f	7.38	48	miR-133a	9.39
49	miR-2291	7.32	49	miR-1281	9.38
50	miR-2284l	7.24	50	miR-133b	9.31

The 50 mRNA that were expressed at the highest level in exosomes and supernatants according to microarrays are shown in Tables 2 and 3, respectively. Most of mRNA expressed in exosomes are milk protein- and ribosomal protein-related mRNA. We observed similar results with rat milk whey-derived mRNA previously (Izumi et al., 2014). Consistent with this, previous microarray analysis of RNA extracted and purified from rat milk whey revealed that milk protein-derived and

ribosomal mRNA transcripts were expressed highly (Izumi et al., 2014). Few studies of milk mRNA have been conducted. However, Maningat et al. (2009) and Lemay et al. (2013) studied human milk fat-derived mRNA. Both of these studies showed that milk protein- and ribosomal protein-related mRNA transcripts were expressed highly in the milk fat layer (Maningat et al., 2009; Lemay et al., 2013). However, Wickramasinghe et al. (2012) reported that no ribosomal protein-related

Table 2. Top 50 most highly expressed mRNA probes in bovine whey exosome

Rank	Probe name	Gene symbol	Gene name	Signal intensity
1	A_73_P100256	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	18.27
2	A_73_113728	LALBA	α -LA	18.26
3	A_73_P459006	PAEP	Progesterone-associated endometrial protein (also known as β -LG)	18.14
4	A_73_P311621	CSN2	Casein β	18.04
5	A_73_116854	CSN2	Casein β	18.03
6	A_73_P393463	CSN1S1	Casein α -s1	17.95
7	A_73_P084401	RPS28	Ribosomal protein S28	17.95
8	A_73_P100346	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	17.94
9	A_73_P504783	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	17.92
10	A_73_111101	CSN3	Casein kappa	17.91
11	A_73_P287836	CSN1S2	Casein α -S2	17.90
12	A_73_P050936	CSN1S1	Casein α -s1	17.88
13	A_73_P468825	CSN1S1	Casein α -s1	17.88
14	A_73_115729	PAEP	Progesterone-associated endometrial protein (also known as β -LG)	17.84
15	A_73_P232452	CSN2	Casein β	17.79
16	A_73_111985	RPS3	Ribosomal protein S3	17.77
17	A_73_112949	RPLP0	Ribosomal protein, large, P0	17.77
18	A_73_P411496	PAEP	Progesterone-associated endometrial protein (also known as β -LG)	17.76
19	A_73_P038871	LALBA	α -LA	17.76
20	A_73_P107731	CSN3	Casein kappa	17.76
21	A_73_P097536	RPL23	Ribosomal protein L23	17.74
22	A_73_120857	RPL21	Ribosomal protein L21	17.73
23	A_73_107578	H1FX	H1 histone family, member X	17.70
24	A_73_P045606	CSN2	Casein β	17.68
25	A_73_114056	RPS2	Ribosomal protein S2	17.63
26	A_73_P093916	RPS16	Ribosomal protein S16	17.63
27	A_73_115443	RPL18	Ribosomal protein L18	17.63
28	A_73_106136	RPS8	Ribosomal protein S8	17.62
29	A_73_P462036	RPLP1	Ribosomal protein, large, P1	17.59
30	A_73_111582	FABP3	FA binding protein 3, muscle and heart (mammary-derived growth inhibitor)	17.58
31	A_73_109246	RPS10	Ribosomal protein S10	17.58
32	A_73_120860	RPL31	Ribosomal protein L31	17.58
33	A_73_P073166	RPL23A	Ribosomal protein L23a	17.56
34	A_73_P103236	RPS28	Ribosomal protein S28	17.55
35	A_73_100438	CSN1S1	Casein α -s1	17.54
36	A_73_P291436	RPS18	Ribosomal protein S18	17.54
37	A_73_P041941	EEF1A1	Eukaryotic translation elongation factor 1 α 1	17.53
38	A_73_P068826	RPS3A	Ribosomal protein S3A	17.52
39	A_73_P056536	RPLP2	Ribosomal protein, large, P2	17.51
40	A_73_P040046	UBA52	Ubiquitin A-52 residue ribosomal protein fusion product 1	17.50
41	A_73_P057311	RPL31	Ribosomal protein L31	17.49
42	A_73_P044606	PLIN2	Perilipin 2	17.49
43	A_73_P035681	RPS27A	Ribosomal protein S27a	17.48
44	A_73_P046936	RPS16	Ribosomal protein S16	17.46
45	A_73_115118	RPL10	Ribosomal protein L10	17.44
46	A_73_P097551	RPS8	Ribosomal protein S8	17.43
47	A_73_114339	GLYCAM1	Glycosylation-dependent cell adhesion molecule 1	17.43
48	A_73_P041201	TPT1	Tumor protein, translationally-controlled 1	17.43
49	A_73_P097526	RPL10	Ribosomal protein L10	17.42
50	A_73_P266861	CSN1S2	Casein α -S2	17.41

mRNA were among the 7 mRNA species expressed at the highest level in bovine milk somatic cells (cells from 15, 90, and 250 d of lactation), although milk protein-related mRNA were included in the list. These results suggest that some roles of mRNA might be common to milk whey, milk whey-derived exosomes, the milk fat layer, and milk cells, whereas others might differ or the roles of milk mRNA might differ among species.

In the current study, a large number of mRNA transcripts (19,320 mRNA transcripts) were detected

in exosomes. However, selecting specific mRNA transcripts is challenging. Therefore, we selected milk protein-, milk-derived exosome-, and milk fat layer-related mRNA that were expressed at high levels in milk whey, the milk fat layer, or in milk somatic cells in previous studies (Maningat et al., 2009; Izumi et al., 2012, 2014; Wickramasinghe et al., 2012; Lemay et al., 2013). These mRNA transcripts were then analyzed in exosomes and supernatants isolated from same volume of whey using qPCR (Figure 5). Data revealed that all mRNA were

Table 3. Top 50 most highly expressed mRNA probes in bovine whey supernatant

Rank	Probe name	Gene symbol	Gene name	Signal intensity
1	A_73_P504783	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	17.40
2	A_73_P153516	POLR2K	Polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	17.36
3	A_73_P455626	TSPYL4	TSPY-like 4	16.52
4	A_73_P361931	FBXO16	F-box protein 16	16.06
5	A_73_P491198	TMEM173	Transmembrane protein 173	15.65
6	A_73_P215177	TLR2	Toll-like receptor 2	15.46
7	A_73_P093286	C3H1orf87	Uncharacterized protein C1orf87-like	15.36
8	A_73_117796	IL1RL1	Interleukin 1 receptor-like 1	15.30
9	A_73_P420956	PFDN5	Prefoldin subunit 5	15.29
10	A_73_P190192	AGTR1	Angiotensin II receptor, type 1	15.28
11	A_73_P213062	ANKRD39	Ankyrin repeat domain 39	14.90
12	A_73_P180692	ACOT11	Acyl-CoA thioesterase 11	14.83
13	A_73_P486468	TNS4	Tensin 4	14.80
14	A_73_P224702	ANKRD39	Ankyrin repeat domain 39	14.70
15	A_73_121245	LOC511936	Cytochrome P450, family 2, subfamily J	14.70
16	A_73_P051356	MGC152278	Similar to Myeloid-associated differentiation marker	14.66
17	A_73_P048836	ZNF674	Zinc finger protein 674	14.64
18	A_73_P405601	AGTR1	Angiotensin II receptor, type 1	14.61
19	A_73_P240340	HRASLS	HRAS-like suppressor	14.59
20	A_73_P459006	PAEP	Progesterone-associated endometrial protein (also known as β -LG)	14.58
21	A_73_P191827	AGTR1	Angiotensin II receptor, type 1	14.54
22	A_73_103111	GTF2H5	General transcription factor IIH, polypeptide 5	14.48
23	A_73_107578	H1FX	H1 histone family, member X	14.46
24	A_73_P192282	CHRNA1	Cholinergic receptor, nicotinic, α 1 (muscle)	14.46
25	A_73_P476773	SLC5A12	Solute carrier family 5 (sodium/glucose cotransporter), member 12	14.41
26	A_73_P033441	H2B	Histone H2B	14.40
27	A_73_P242615	CHMP1B	Chromatin modifying protein 1B	14.40
28	A_73_P461676	HERC1	Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	14.38
29	A_73_P246691	C3H1orf109	Hypothetical LOC508021	14.34
30	A_73_P374271	SECTM1	Secreted and transmembrane 1	14.31
31	A_73_P195057	NIPSNAP1	Nipsnap homolog 1 (C. elegans)	14.28
32	A_73_P134616	ACPP	Acid phosphatase, prostate	14.26
33	A_73_P132081	ACPP	Acid phosphatase, prostate	14.18
34	A_73_P100256	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	14.06
35	A_73_P187832	ACPP	Acid phosphatase, prostate	14.05
36	A_73_P200172	POLH	Polymerase (DNA directed), eta	14.04
37	A_73_P218867	IFT80	Intraflagellar transport 80 homolog (Chlamydomonas)	14.02
38	A_73_P171747	LOC100139208	Hypothetical protein LOC100139208	14.02
39	A_73_P149206	FGF2	Fibroblast growth factor 2 (basic)	13.96
40	A_73_P288576	CWC25	CWC25 spliceosome-associated protein homolog (S. cerevisiae)	13.91
41	A_73_P211172	MBL2	Mannose-binding lectin (protein C) 2, soluble	13.85
42	A_73_P504858	NEPN	Nephrocan	13.83
43	A_73_P211497	C12H13orf18	Uncharacterized protein C13orf18 homolog	13.83
44	A_73_P465708	ACPP	Acid phosphatase, prostate	13.75
45	A_73_P134026	ACOT11	Acyl-CoA thioesterase 11	13.74
46	A_73_P465498	ACPP	Acid phosphatase, prostate	13.72
47	A_73_113343	LOC517043	Leucine-rich repeat-containing G protein-coupled receptor 6-like	13.71
48	A_73_P187927	ACOT11	Acyl-CoA thioesterase 11	13.69
49	A_73_P465423	PIGM	Phosphatidylinositol glycan anchor biosynthesis, class M	13.63
50	A_73_100232	LOC508118	Zinc finger and BTB domain containing 3	13.63

expressed at significantly higher levels in exosomes than in supernatants. Among the selected mRNA, some transcripts were expressed highly in bovine milk somatic cells and the human milk fat layer in previous reports (Maningat et al., 2009; Wickramasinghe et al., 2012; Lemay et al., 2013), but were not listed among the 50 highly expressed transcripts in exosomes in the current study. These results support the hypothesis that mRNA might be concentrated in exosomes. Unfortunately, in the current study, we did not investigate

the presence of forms of RNA in supernatant in detail or the size range of exosomes. We prepared exosomes by ultracentrifugation (most commonly used method); however, the size of exosomes ranges widely. Thus, it is possible that extensively small or light exosomes remained in supernatant. It might be the reason that small numbers of mRNA detected in supernatant.

Exosomes isolated from bovine milk could be taken up by human macrophages (Figure 6). Consistent with this, Lässer et al. (2011) reported that exosomes from

Figure 4. Quantitative PCR analysis [cycle threshold (Ct) values] of select microRNA (miRNA) in exosomes and supernatants isolated from equal volumes of bovine raw milk whey. Total RNA from exosomes and supernatants isolated from 3.2 mL of raw milk whey was used. The values are means $\pm$ SEM ($n = 3$); * $P < 0.05$ (compared with supernatant), ** $P < 0.01$ (compared with supernatant).

human breast milk could be taken up by human macrophages. Moreover, Sun et al. (2013) reported that bovine milk exosomes could be taken up by murine macrophages, and that the functions of macrophages that incorporated bovine milk exosomes were altered. This suggests that human cells can uptake exosomes

from other species, which is consistent with the current results.

It remains unknown whether milk-derived exosomes exert functions in vivo. However, previous studies, including those from our laboratory, showed that milk whey-derived RNA are resistant to acidic conditions

Figure 5. Quantitative PCR analysis [cycle threshold (Ct) values] of select mRNA in exosomes and supernatants isolated from equal volumes of bovine raw milk whey. Total RNA from exosomes and supernatants from 3.2 mL of raw milk whey was used. (A) Selected transcripts from the 50 species expressed at the highest level in exosomes; (B) milk-derived exosome-related transcripts; (C) selected transcripts identified in previous studies of milk somatic cells and the milk fat layer. The values are the mean $\pm$ SEM ($n = 3$); * $P < 0.05$ (compared with supernatant), ** $P < 0.01$ (compared with supernatant). Actb = β -actin; EEF1 α 1 = eukaryotic translation elongation factor 1 α 1; FAS = fatty acid synthase; FTH1 = ferritin, heavy polypeptide 1; GLYCAM1 = glycosylation-dependent cell adhesion molecule 1; LF = lactoferrin; LPO = lactoperoxidase; MFG-E8 = milk fat globule-EGF factor 8; MUC1 = mucin 1; pIgR = poly Ig receptor; SPP1 = secreted phosphoprotein 1; TPT1 = tumor protein, translationally controlled 1; XDH = xanthine dehydrogenase; CD36 = cluster of differentiation 36, CD63 = cluster of differentiation 63; ApoE = apolipoprotein E, CD74 = cluster of differentiation 74.

and RNase (Hata et al., 2010; Kosaka et al., 2010b; Izumi et al., 2012). Therefore, it is possible that bovine milk-derived exosomes could exert functions in human

body. In recent years, studies demonstrated that food-derived miRNA could be incorporated and exert function in vivo. For example, Zhang et al. (2012) reported that osa-MIR-168a (from rice) could be detected in human and mouse sera, and osa-MIR-168a decreased low-density lipoprotein receptor adapter protein 1 mRNA (the predicted target mRNA of osa-MIR-168a) in the mouse liver. In addition, Ju et al. (2013) reported that grape exosome-like nanoparticles mediate intestinal tissue remodeling to induce intestinal stem cells and protect intestinal tissue from dextran sulfate sodium-induced colitis. Moreover, Lukasik and Zielenkiewicz (2014) reported that some miRNAs from *Arabidopsis thaliana*, *Picea abies*, *Populus trichocarpa*, *Brachypodium distachyon*, and *Zea mays* were present in human and porcine breast milk exosomes by analyzing publicly available data obtained from high-throughput sequencing using bioinformatics. In contrast, studies regarding the bioavailability of plant-borne miRNA in humans are controversial (Dickinson et al., 2013; Snow et al., 2013). Recently, Baier et al. (2014) reported that meaningful amounts of miRNA were detected in plasma isolated from healthy human subjects after the ingestion of nutritionally relevant doses of cow milk. They also revealed that *Brassica*-specific miRNA could not be detected in a broccoli sprout-feeding study. Therefore, it is important to consider why only milk-borne miRNA were absorbed. In the current study, we showed that human macrophages could take up bovine raw milk-derived exosomes. However, it is unknown whether bovine raw milk-derived exosomes are taken up by only macrophages.

In conclusion, the current study demonstrated that most mRNA in bovine raw milk whey were present in exosomes, whereas miRNA were present both in exosomes and in other forms. Most miRNA and mRNA that are present in bovine raw milk whey were expressed at significantly higher levels in exosomes than in other forms. In addition, human macrophages could uptake bovine raw milk whey-derived exosomes. Moreover, a recent study (Baier et al., 2014) suggested that functional RNA in milk exert functions in the human body. However, it is unclear whether bovine raw milk whey-derived exosomes affect the function of macrophages. Moreover, the different size exosomes contain different types of RNA and other exosome-like vesicles (e.g., ectosomes) might be contained in milk-derived exosomes. As such, several aspects of milk-derived RNA remain unclear. Bovine raw milk is used widely in many dairy products and is an important food source. Therefore, additional studies regarding the physiological and biological roles of these functional RNA are needed. The current data describing RNA in bovine raw milk whey-derived exosomes might facilitate future studies.

Figure 6. Uptake of bovine raw milk whey-derived exosomes by macrophages. (A–C) Fluorescence microscopy images; 7-AAD (BD Biosciences, San Diego, CA) was used to label the nuclei of THP-1 cells (red), and PKH67 (Sigma-Aldrich, St. Louis, MO) was used to label the exosomes (green). (A) PBS-PKH67 was added to differentiated THP-1 cells, followed by incubation at 37°C. (B) PKH67-labeled exosomes were added to differentiated THP-1 cells, followed by incubation at 4°C. (C) PKH67-labeled exosomes were added to differentiated THP-1 cells, followed by incubation at 37°C. (D) The uptake of fluorescence-labeled exosomes into THP-1 cells at various conditions was evaluated using a flow cytometer. PBS 37°C: PBS-PKH67 was added to differentiated THP-1 cells and cells were incubated at 37°C. UD Exo 37°C: PKH67-labeled exosomes were added to undifferentiated THP-1 cells, followed by incubation at 37°C. Exo 37°C: PKH67-labeled exosomes were added to differentiated THP-1 cells, followed by incubation at 37°C. Exo 4°C: PKH67-labeled exosomes were added to differentiated THP-1 cells, followed by incubation at 4°C. The values are the mean $\pm$ SEM ($n = 3$). Means without common letter (a–c) differ significantly ($P < 0.05$). Exo = exosome; MFI = mean fluorescence intensity; UD = undifferentiated.

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