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Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs

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ABSTRACT

By a series of centrifugation and ultracentrifugation, we could isolate microvesicles with approximately 100 nm in diameter from bovine milk. We also found that approximately 1700 and 1000 ng of total RNA, in which small RNAs were major components, was contained inside the microvesicles isolated from 6 ml of colostrum and mature milk, respectively, despite high RNase activity in the milk. Polyadenylated gene transcripts for major milk proteins and translation elongation factor-1α (EF-1α) were present in the microvesicles, and integrity of some transcripts was confirmed by real-time PCR targeting 5′- and 3′-ends of mRNA and by in vitro translation analysis. Moreover, a considerable amount of mammary gland and immune-related microRNAs were present in the milk-derived microvesicles. Acidification of milk to mimic gastrointestinal tract did not mostly affected RNA yield and quality. The milk related gene transcripts were detected in cultured cells when incubated with milk-derived microvesicles, suggesting cellular uptake of the microvesicle contents including RNA. Our findings suggest that bovine breast milk contains RNAs capable for being transferred to living cells and involved in the development of calf's gastrointestinal and immune systems.

1. Introduction

Microvesicles including exosomes are secreted from a variety of cells under both normal and pathophysiological conditions and are heterogeneous in size ranging from 30 nm to 1 μm in diameter. Microvesicles have been so far reported to be secreted by many different types of cells including reticulocytes [1], dendritic cells [2], mast cells [3], epithelial cells [4,5], and adipocytes [6]. In addition, microvesicles have been identified in biological fluids such as blood [7], urine [8], and epididymal fluid [9].

Microvesicles are now recognized as important mediators of intercellular communication, though they had previously been believed to be inert cell debris. The mechanisms by which microvesicles might mediate intercellular signaling could involve the activation of receptors on the plasma membrane of the recipient cells. Alternatively, microvesicles might bring out signals to target cells by directly transferring bioactive molecules inside the vesicles. It has been demonstrated that microvesicles can transfer some of their components to other cell types. For instance, oncogenic form of epidermal growth factor receptor, known as EGFRvIII, was shown to be transferred between glioma cells via microvesicles, contributing to a horizontal propagation of oncogenes and their associated transforming phenotype [10]. More interestingly, it has recently reported that exosomal vesicles secreted by mast cells [11], glioblastoma cells [12], and embryonic stem cells [13] contained mRNA and microRNA (miRNA) inside the vesicles, which could be transferred to neighboring cells and be functional in their new location.

Milk is a sole nutritional supply to newborn infants. In addition, many biologically active materials such as hormones and cytokines are known to be present in the milk. Like other biological fluids, we have found that microvesicle-like particles are present in the mouse milk [14]. More recently, exosome-like microvesicles have been identified in the human milk [15]. In the present study, we identified the presence of microvesicles in the bovine milk and further showed the presence of mRNAs and micro(mi)RNAs in the vesicles. Potential physiologic relevance of milk-derived microvesicles was also examined.

2. Materials and methods

2.1. Materials

A monoclonal antibody to bovine MFG-E8 (3F11) was previously described [16]. Rabbit antisera to bovine αs1- and β-caseins were a generous gift from Dr. Hajime Ohtani (Shinsyu University,

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Japan). All other reagents were from Sigma or Wako Pure Chemicals (Osaka, Japan), unless otherwise noted.

2.2. Milk samples

Bovine colostrum and milk samples were individually collected from the 1st/2nd-day lactating and mid-lactating (2–4 months after parturition) healthy Holstein cows, respectively, bred in the dairy farm of Mie Prefecture Livestock Research Institute. Milk samples were frozen immediately after milking and were kept at −80 °C until use.

2.3. Preparation of membrane fractions from milk

Bovine milk samples were centrifuged first at 5000*g* for 30 min at 4 °C to remove milk fat globules (MFGs) as well as mammary gland-derived cells. Defatted samples were then subjected to three successive centrifugations at 4 °C for 1 h each at 12,000*g*, 35,000*g*, and finally at 70,000*g* to remove residual MFGs, casein, and other debris. Then, from the final supernatant (so-called whey or milk serum) the membrane fraction was prepared by ultracentrifugation at 100,000*g* for 1 h by using an SW41T rotor (Beckman Coulter Instruments, Fullerton, CA).

2.4. Sucrose-density gradient ultracentrifugation

For further fractionation of the membrane fraction described above, the 100,000*g* precipitates from 120 ml of milk were suspended in 0.5 ml of PBS and layered on the top of a linear sucrose-density gradient (SDG) (5–40%, w/v) solution (12 ml) prepared with Gradient Mate device (BIOCOMP, Fredericton, Canada) and ultracentrifuged at 200,000*g* for 18 h using an ultracentrifuge (Beckman Coulter Instruments) equipped with an SW41T rotor. Then, 0.9 ml of each gradient fraction was collected from the top of the tube (numbered from 1 to 13). Each of the SDG fractions was diluted four times with PBS and ultracentrifuged again at 100,000*g* for 1 h to precipitate membrane vesicles, and then the precipitated membranes were gently suspended in small volumes of PBS and used for the subsequent analyses. Density of each fraction was determined by weighing an aliquot of the solution.

2.5. Electron microscopy

The SDG fraction 13 as described above was diluted 1:10 with PBS and ultracentrifuged again to recover microvesicles as pellets. Following fixation in 2% glutaraldehyde, microvesicles were negatively stained with uranyl acetate, and observed by a transmittance electron microscope (JEOL JEM2000EX, Tokyo, Japan). For observation of ultrathin section samples, the glutaraldehyde-fixed samples were post-fixed in OsO4, dehydrated with ethanol, and embedded in Epon 812. Ultrathin sections (80 nm in thickness) were contrast-stained with uranyl acetate and observed as above.

2.6. RNA purification

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). When isolated from milk-derived microvesicles, ethachinmate (Nippon Gene, Tokyo, Japan) was used to yield RNA with higher recovery. The RNA was quantified by using e-spect UV–vis spectrometer (Malcom, Tokyo, Japan) and confirmed by using Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.7. Quantitative RT-PCR

Total RNA was first digested with DNase I (Invitrogen), reverse transcribed using a cDNA synthesis kit (Roche, Basel, Switzerland) for mRNA detection, and subjected to quantitative real-time PCR on a BioFlux LineGene (TOYOBO, Osaka, Japan) using a SYBR Green real-time PCR master mix (TOYOBO) according to the manufacturer's instructions. Primer sets for individual genes are listed in Table 1. For amplification of miRNA, DNase I-digested total RNA was reverse transcribed using a Mir-X™ miRNA First Strand Synthesis Kit (Takara Bio Inc., Ohtsu, Japan), and subjected to quantitative real-time PCR as above using a SYBR Advantage qPCR Premix (Takara Bio Inc.) according to the manufacturer's instructions (Table 2). For all SYBR Green assays, standard curves were generated for each primer set to determine their efficiency, and dissociation curves were generated to detect non-specific amplified products and primer-dimers. PCR products were also run on an agarose gel to confirm that a single PCR product was generated.

Table 1
PCR primers for mRNAs.

Target gene	Accession no.	Sequence	Target region	Ampricon (bp)
α-s1-Casein (5′-terminal)	NM_181029	CTCAAGAAGTCCTCAATGAA TCCAGATAACCCAGGTAAC	79–99 313–333	255
α-s1-Casein (3′-terminal)	NM_181029	TTCCACTAGGCACACAATAC GTAGAGCATGGAATTTTCAG	608–627 758–777	169
α-s2-Casein	NM_174528	TTTGCTCCACATTCTGCAAG ATCCCATGGGTTCAAAACAA	149–168 356–375	153
β-Casein (5′-terminal)	NM_181008	AGCCTTTCAAGCAGTGAGGA GATGTTTTGTGGGAGGCTGT	88–107 248–267	180
β-Casein (3′-terminal)	NM_181008	GGATTTCAAAGTGAATGCCC TGATGCAAGGATTGAAAAGTTG	737–756 795–816	80
κ-Casein	NM_174294 229	AGTTGTGACTATCCTGGCAT GCATAATATGGGTATGGCAG	20–40 229–248	229
β-Lactoglobulin	NM_173929	CGATGCCTTGAATGAGAACA TTTGTCGAATTTCTCCAGGG	300–319 443–462	163
MFG-E8	X91895	GTGAGACCACCTGCACCTCA GACAAAAACCCCTGGATCCA	341–360 511–530	190
EF-1α	AB060107	ATTTGTGCCAATTTCTGGCT AGACATCCTGGAGAGGCAAA	567–586 741–760	194
GAPDH	BC102589	GGGTCATCATCTCTGCACCT ATCCACAGTCTTCTGGGTGG	347–366 542–561	215

Table 2
PCR primers for miRNAs.

Target miRNA	Accession no.	Sequence
bta-mir-101	MIMAT0003520	TACAGTACTGTGATAACTGAA
bta-mir-125b	MIMAT0003539	TCCCTGAGACCCTAACTTGTGA
bta-mir-150	MIMAT0003845	TCTCCCAACCCTTGTACCAGTGT
bta-mir-223	MIMAT0009270	TGTCAGTTTGTCAAATACCCCA
bta-mir-24-1	MIMAT0009250	GTGCCTACTGAGCTGATATCAGT
bta-mir-93	MIMAT0003837	CAAAGTGCTGTTCGTGCAGGTA

2.8. In vitro translation

An aliquot (1.4 μg) of total RNA, which was purified from mature milk-derived microvesicles, was translated by using an in vitro translation kit (Wako PURE system, Osaka, Japan) according to the manufacturer's instructions. Translated products were immunoblotted with rabbit antibodies to bovine αs1- and β-casein, and protein bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and Light Capture system (AE-6962; ATTO, Tokyo, Japan).

2.9. Acidification of milk

Mature milk whey fractions were adjusted to pH 2.0 with HCl and kept at 37 °C for 30 min. Following brief centrifugation to remove debris, resultant supernatant was subjected to microvesicle preparation as above.

2.10. Uptake of milk-derived microvesicles by cultured cells

NIH-3T3 cells were maintained in DMEM supplemented with fetal calf serum (FCS). NIH-3T3 (1×10^5 cells) were incubated with 2 ml of medium containing microvesicles derived from 12 ml milk samples for 24 h in 6-well plate (Techno Plastic Products AG, Switzerland). Following washing with PBS twice, cells were detached from the plate with 0.02% (w/v) EDTA·2Na solution. Cells were pelleted by centrifugation and washed with PBS twice. Cells were inoculated into new a new plate and cultured for another 24 h. Cells were lysed with Trizol reagent for RNA preparation and subsequent RT-PCR analyses.

3. Results

3.1. Isolation of microvesicles from bovine milk

According to the previously reported methods for preparation of exosomal microvesicles, bovine colostrum and mature milk were first centrifuged at low speed to remove casein and fat, and the resultant whey fraction was fractionated by a serial centrifugation. Following final ultracentrifugation, equivalent aliquots of resultant pellets were subjected to immunoblotting. MFG-E8, a major component of microvesicles, was clearly detected and its amount was higher in the colostrum-derived pellet (Fig. 1A). Colostrum-derived microvesicle fraction was further fractionated on a sucrose-density gradient by ultracentrifugation. As shown in Fig. 1B, MFG-E8 was detected in the fractions with sucrose densities 1.14–1.20, which have been reported for exosomes [17]. The fraction with 1.20 g/ml sucrose density was observed by a scanning electron microscope following negative staining (Fig. 1C). Microvesicles with approximately 100 nm in diameter were obviously detected (left panel). To clarify microstructure of these vesicular materials, the precipitates of the fraction were embedded, cut into ultrathin sections, and observed by a transmittance electron microscope. Cross-sections of microvesicles with approximately 100 nm in diameter were observed in accordance with the above described negatively stained samples (right panel). Furthermore, bi-layer structures of microvesicles were clearly observed, which looked like phospholipid bi-layer of biological membranes.

RNA contents of the microvesicles prepared from 6 ml of colostrum and mature milk were spectrophotometrically estimated to be 1704.1 ± 70.0 ng and 988.4 ± 81.2 ng, respectively (Fig. 2A). Separation by Bioanalyzer revealed that the microvesicle-derived RNA was heterogeneous in size, but contained no or little 28S and 18S ribosomal RNA (Fig. 2B). Instead, the majority of total RNA in the microvesicles was concentrated below 200 nt. RNase activity in both milk samples was examined, because relatively higher RNase activity has been reported in milk [18]. Typical 28S and 18S ribosomal RNA from NIH-3T3 cells was mostly digested when mixed with 1 μl of both milk samples (Fig. 2C). Thus, it is most likely that almost all the RNA in milk is present inside the microvesicles and is protected from RNase by surrounding membranes.

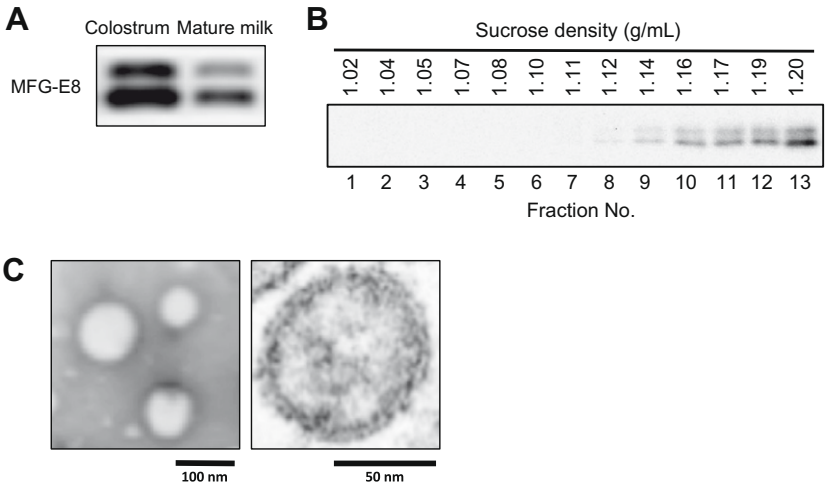

Fig. 1. Isolation of microvesicles from bovine milk. (A) Microvesicles were prepared from bovine colostrum and mature milk and immunoblotted with anti-MFG-E8 antibody as described in Section 2. (B,C) Colostrum-derived microvesicles were sub-fractionated on a sucrose-density gradient by ultracentrifugation and each fraction was immunoblotted with anti-MFG-E8 antibody. The fraction 13 with 1.20 g/ml sucrose density was observed by a scanning electron microscope (left panel) or a transmission electron microscope (right panel).

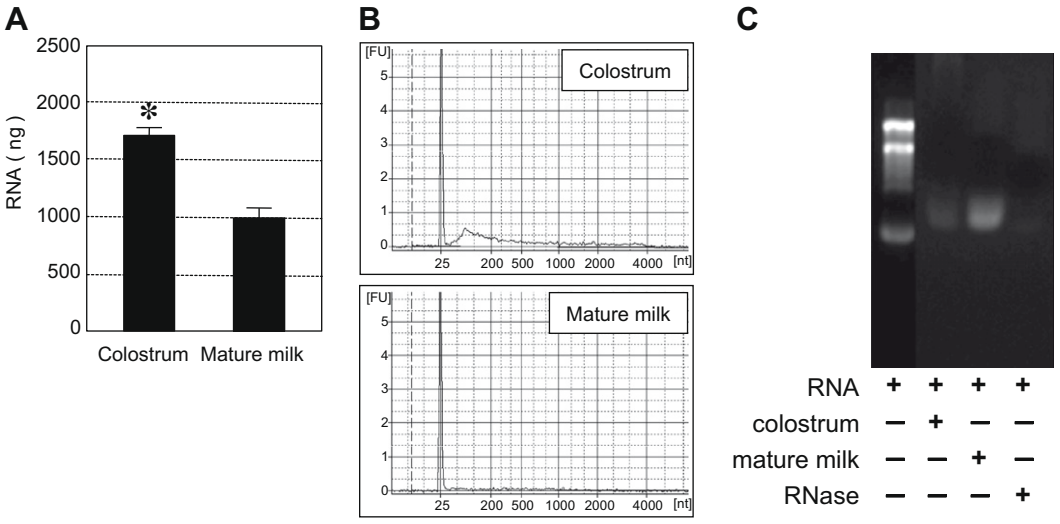

Fig. 2. Milk-derived microvesicles contain RNA. (A and B) RNA was purified from microvesicles isolated from 6 ml of bovine colostrum and mature milk, and spectrophotometrically determined. An aliquot (100 ng) of each RNA sample was analyzed by Bioanalyzer. (C) An aliquot (1 μg) of NIH-3T3-derived total RNA was left untreated or treated with 1 μl of colostrum, mature milk or RNase A solution (100 μg/ml) at 37 °C for 15 min, and then separated on an agarose gel followed by ethidium bromide staining.

3.2. Presence of mRNAs and miRNAs in the milk-derived microvesicles

Milk microvesicle-derived RNA was purified and reverse transcribed with oligo (dT) primer and resultant cDNA pool was amplified with primer sets for mammary gland-specific and dominant genes. As shown in Fig. 3A, all the genes including milk proteins and elongation factor-1α (EF-1α) could be successfully amplified to a varying extent. Without reverse transcription, no amplification was obtained, strongly suggesting the presence of polyadenylated RNA within milk-derived microvesicles and genomic DNA was not contaminated. All the amplified products were confirmed by direct sequencing (data not shown). To confirm the integrity of the mRNAs isolated from milk-derived microvesicles, we compared the 5′-end amplicons with 3′-end ones of individual

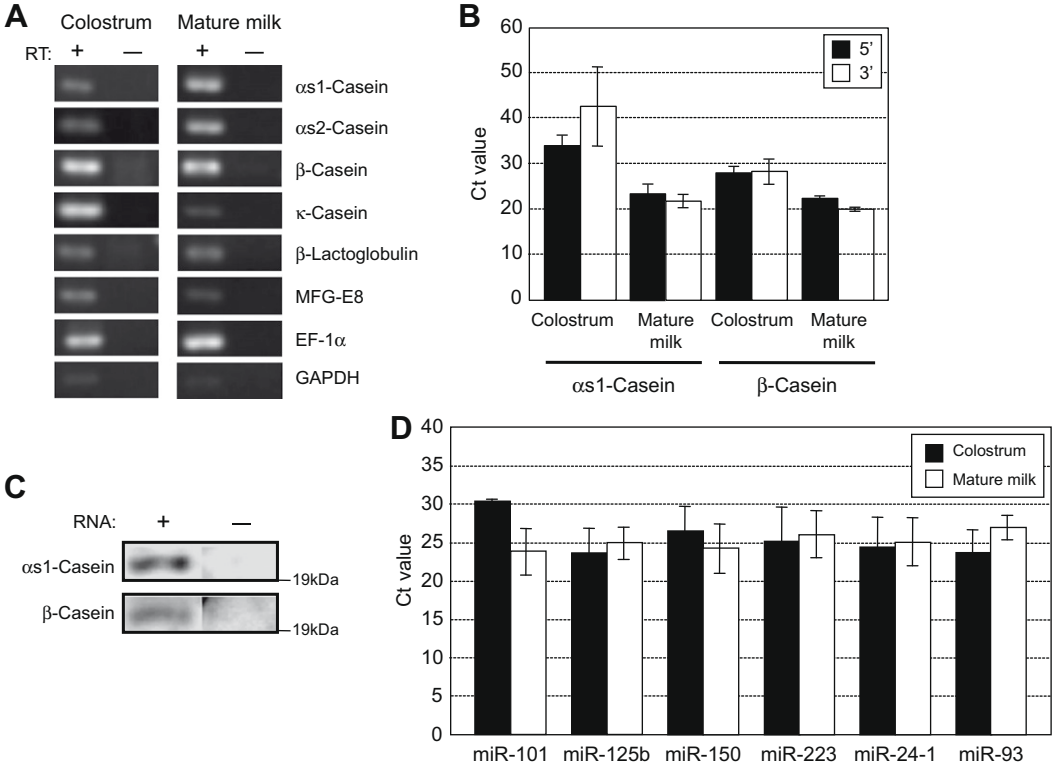

Fig. 3. Presence of RNAs in the milk-derived microvesicles. (A) An aliquot (20 ng) of the colostrum and mature milk microvesicle-derived RNA was reverse transcribed with (+) or without (−) reverse transcriptase followed by PCR amplification with the indicated primer sets. (B) Integrity of αs1-casein, β-casein, and β-lactoglobulin mRNAs was confirmed by real-time PCR as described in Section 2. C_t values for 5′- and 3′-ends for each gene transcript were compared. Results are shown as means ± SEM ($n = 3$). (C) An aliquot (1.4 μg) of total RNA, which was purified from mature milk-derived microvesicles, was translated in vitro as described in Section 2. Translated products were immunoblotted with indicated antibodies. (D) An aliquot (1 ng) of the colostrum and mature milk microvesicle-derived RNA was reverse transcribed followed by real-time PCR amplification for the indicated miRNAs. Results are shown as means ± SEM ($n = 3$).

transcripts by real-time quantitative RT-PCR method; the 5′-end amplicon should be ideally equal in amount to 3′-end one unless RNA is degraded. Real-time quantitative RT-PCR was done for αs1-casein and β-casein mRNAs as a model. As shown in Fig. 3B, the 5′- and 3′-end amplicons for both transcripts were almost the same in the C_t value in both colostrum and mature milk-derived RNA samples. Moreover, both casein transcripts could be translated into approximately 25 kDa proteins under an in vitro system (Fig. 3C), suggesting that mRNAs in the microvesicles were intact and not degraded.

Based on the total RNA profile (Fig. 2B), a significant portion of RNA may be composed of small RNAs, though the analytical method has a limitation for very small RNAs. We hypothesized that milk-derived microvesicles contain miRNAs in addition to mRNAs. We performed real-time quantitative RT-PCR to quantify the abundance of several miRNAs, which have been reported to be expressed in the mammary glands [19]. We also chose immune-related miRNAs [20] with a role of milk for infants in mind. As shown in Fig. 3D, bta-miR-101, bta-miR-125b, bta-miR-150, bta-miR-223, bta-miR-24-1, and bta-miR-93 were comparably detected in both milk-derived microvesicles.

3.3. Potential physiologic relevance of milk-derived microvesicles

To mimic gastrointestinal environment, the effect of acidification of whey on the recovery and quality of microvesicle-derived RNA was examined. Acidification did not affect the recovery of microvesicle-derived RNA (Fig. 4A) and amplification of αs1-casein, β-casein, and β-lactoglobulin was comparable with or without acidification (Fig. 4B). Comparable amplification of some miRNAs was also confirmed (Fig. 4C). RNA transfer capability of milk-derived microvesicles was finally examined. Milk specific gene transcripts were clearly amplified only when NIH-3T3 cells were incubated with milk-derived microvesicles (Fig. 4D). Transfer of bovine miRNAs was not concluded because their sequences between mouse and bovine species were too similar to be distinguished by PCR amplification (data not shown).

4. Discussion

In the present study, we showed the presence of microvesicles in bovine colostrum and mature milk, which contained an exosomal marker protein, MFG-E8 and exhibited lipid bi-layer structure with approximately 100 nm in diameter (Fig. 1C). The presence of an MFG-E8, the morphological properties and the density distribution on the bovine milk microvesicles resembled those of recently reported exosomal vesicles from cultured cells [2–6], letting us hypothesize that milk membrane vesicles also plays some roles in protein and nucleotide transfer for the vesicle-mediated intercellular communication as shown in these previous studies [10–13]. As expected, we found that milk-derived microvesicles contained mRNAs and miRNAs despite extremely high RNase activity in the milk. Colostrum-derived microvesicles contained by about 1.7-fold higher amount of total RNA than mature milk-derived ones (Fig. 2A), which was well consistent with the case of MFG-E8 content in the microvesicles (Fig. 1A). As reported for other microvesicles, milk-derived microvesicles were mostly absent from ribosomal RNA. We tried to identify all the mRNA species present in the microvesicles by using microarray techniques but fluorescence labeling could not be achieved due to unknown reasons. Instead, we showed that mammary gland-specific and dominant gene transcripts were present in the microvesicles by

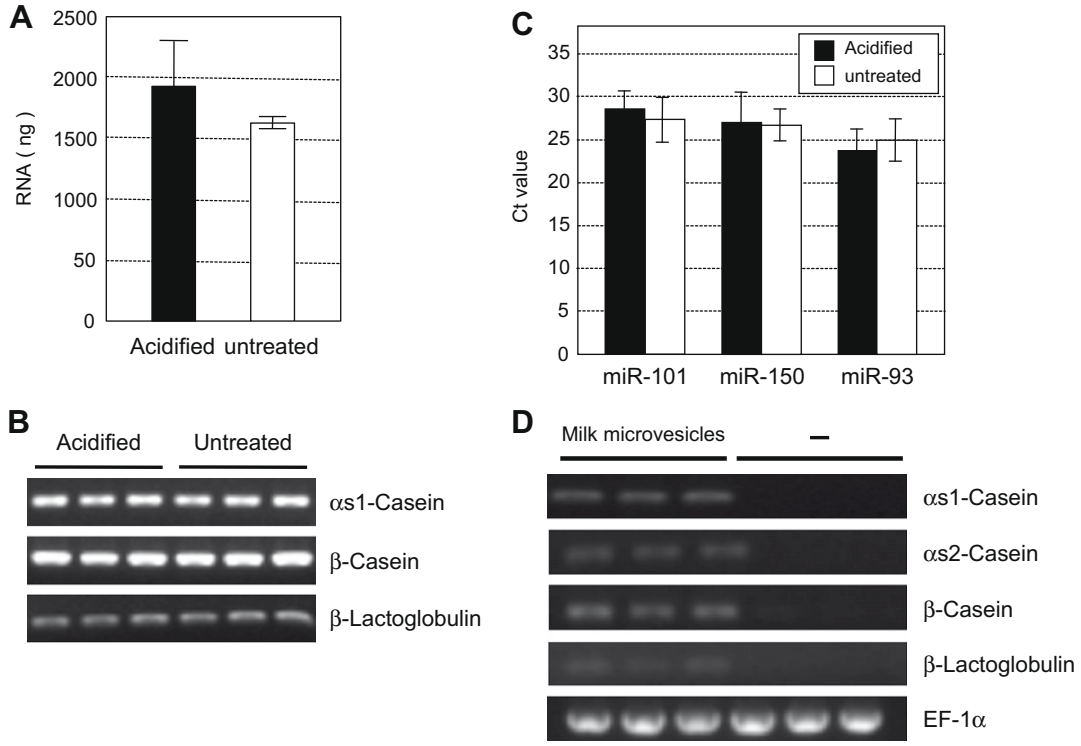

Fig. 4. Potential physiologic relevance of milk-derived microvesicles. (A) Defatted whey fraction of colostrum was left untreated or acidified by HCl to pH 2.0 and incubated for 30 min at 37 °C, and then subjected to microvesicle preparation followed by RNA purification and determination. Results are shown as means ± SEM (n = 3). (B) An aliquot (20 ng) of the above RNA samples was reverse transcribed followed by PCR amplification for the indicated mRNAs. (C) An aliquot (1 ng) of the above RNA samples was reverse transcribed followed by PCR amplification for the indicated miRNAs. (D) NIH-3T3 cells were incubated with or without mature milk-derived microvesicles for 24 h. Cells were detached, washed, inoculated into cell culture dishes with fresh medium, and cultured for another 24 h. Cells were lysed for RNA preparation and subsequent RT-PCR analyses.

RT-PCR (Fig. 3A) and, moreover, casein transcripts were shown to be translated in vitro (Fig. 3C). These results in addition to our previous observation that mouse mammary epithelial COMMA-1D cells secreted MFG-E8-containing microvesicles [5] indicate that milk microvesicles are originated mainly from mammary epithelial cells, which produce nutrients and some other biologically important factors for infants of mammals.

Several miRNAs, which have been reported to be expressed in mammary glands [15] and be related to immune system [20], were also present in the milk-derived microvesicles (Fig. 3D). The miR101 and miR150 have recently been reported to modulate the functional development of the effecter [21] and regulatory [22] T cells, respectively. Moreover, the miR-125b targeting tumor necrosis factor-α is down-regulated in macrophage upon LPS stimulation [23], and the miR-223 has been shown to play some roles in innate immune cells such as granulocytes [24] and neutrophils [25]. Since the acid treatment of milk did not drain the microvesicle miRNAs, it could be possible for the milk miRNAs to reach innate and acquired immune cells in gut-associated lymphoid tissues of sucking calves.

Biological significance of milk protein mRNAs in milk microvesicles remains uncertain at this moment, though the mRNA transfer from the milk microvesicles to cells in vitro was suggested (Fig. 4B). It is worth noticing that an earlier study on mRNA expression of hematopoietic cells demonstrated the expression of casein mRNAs in some cytotoxic T cell lines and mentioned potential roles in the T cell function [26]. Further studies would be required to clarify whether caseins have other biological functions than the protein nutrient for infants. Nevertheless, detection of milk protein mRNAs in the cultured cells indicate the transfer of microvesicle contents such as miRNA into the cells, supporting above described possibility on milk miRNA function.

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