REVIEW

Biological Characteristics and Roles of Noncoding RNAs in Milk-Derived Extracellular Vesicles

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ABSTRACT

Extracellular vesicles (EVs) have diverse roles in the transport of proteins, lipids, and nucleic acids between cells, and they serve as mediators of intercellular communication. Noncoding RNAs (ncRNAs) that are present in EVs, including microRNAs, long noncoding RNAs, and circular RNAs, have been found to participate in complex networks of interactions and regulate a wide variety of genes in animals. Milk is an important source of nutrition for humans and other mammals. Evidence suggests that milk-derived EVs contain abundant ncRNAs, which are stable and can be transported to the offspring and other consumers. Current data suggest a strong link between milk EV ncRNAs and many biological processes, and these ncRNAs have been drawing increasing attention and might play an epigenetic regulatory role in recipients, though further research is still necessary to understand their precise roles. The present review introduces basic information about milk EV ncRNAs, summarizes their expression profiles, biological characteristics, and functions based on current knowledge, and discusses their biological roles, indeterminate issues, and perspectives. Our goal is to provide a deeper understanding of the physiological effects of milk EV ncRNAs on offspring and to provide a reference for future research in this field. *Adv Nutr* 2021;12:1006–1019.

Keywords: extracellular vesicles, exosome, milk, microRNAs, long noncoding RNAs, circular RNAs

Introduction

Breast milk is the perfect nutrition for infants, as a result of millions of years of finetuning to the requirements of growing mammals. Breast milk is rich in proteins, lipids, and carbohydrates, which are the primary source of nutrition for infants (1). Dairy products are also full of nutrients for people of different ages. Immunoglobulins and nonnutritional bioactive factors in milk are generally considered as the main functional substances in organismic regulation. However, in 2007 Valadi et al. (2) reported a new mechanism of gene communication between cells, namely, through transport of exosomal RNAs. In the same year, Admyre et al. (3) found exosomes—small extracellular vesicles (sEVs)—in milk. In 2012, Zhang et al. (4) reported that exogenous plant (rice) microRNAs (miRNAs) in food can regulate the expression of target genes in mammals. Since then, scientific studies on milk-derived exosomes and their RNA cargoes have been drawing much attention.

Exosomes, defined as a subtype of extracellular vesicles (EVs), originate from late endosomes (5, 6). Several proteins, including tetraspanins (CD9, CD63, and CD81), endosomal sorting complex required for transport (ESCRT), tumor susceptibility gene 101 (*TSG101*), ALG-2-interacting protein X (Alix), and heat stress protein 70 (HSP70) are often used as "exosome markers" (6, 7). EVs play an important role as mediators in cell-to-cell communication by transporting cargoes from donor to recipient cells (8). Among EV cargoes, noncoding RNAs (ncRNAs) are of particular interest. They participate in complex interactions with other nucleic acids and proteins, and they often have wide-reaching effects on cell biology and regulate a majority of genes in mammals in

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Abbreviations used: AF, Alexa Fluor; Alix, ALG-2-interacting protein X; BCAA, branched-chain amino acid; *Bach2*, BTB domain and CNC homolog 2; circRNA, circular RNA; *CRNDE*, Colorectal neoplasia differentially expressed; *DANCR*, Differentiation antagonizing non-protein coding RNA; DNMT, DNA methyltransferase; ESCRT, Endosomal sorting complex required for transport; EV, extracellular vesicle; *FAS*, TNF receptor superfamily member 6; *FOXP3*, forkhead box P3; *GAS5*, Growth arrest specific transcript 5; *Hmga2*, High mobility group AT-hook 2; HSP70, Heat stress protein 70; IEC, intestinal epithelial cell; Igf2bp1, insulin-like growth factor 2 binding protein 1; IPEC-J2, Epithelial cells of porcine small intestine; lncRNA, long noncoding RNA; Mdr, multiple drug resistance; miRNA, microRNA; *Mitf*, Melanocyte inducing transcription factor; ncRNA, noncoding RNA; p53/Tp53, protein 53; RNase, ribonuclease; *SERPINE1*, Serine proteinase inhibitor, member 1; sEV, small extracellular vesicle; siRNA, short interfering RNA; *SRA1*, Steroid receptor RNA activator 1; T2DM, type 2 diabetes mellitus; *TSG101*, Tumor susceptibility gene 101; *Wnt1*, Wnt family member 1; *ZFAS1*, Zinc finger NFX1-type antisense RNA 1.

an epigenetic way (9–13). It has been reported that sEVs in breast milk from many mammals, including human (14), cow (15), pig (16), panda (17), sheep (18), rat (19), and wallaby (20), also contain abundant ncRNAs. The particle size of milk exosomes mainly ranges from 100 to 200 nm, and the positive markers, CD9, CD63, CD81, HSP70, TSG101, and Alix, can also be detected (21–23). The exosomal lipid membrane helps to protect milk-derived RNAs against degradation by ribonucleases (RNases) (15), low pH, and digestive enzymes in vitro (21, 24), and thereby protects the important functions of milk EV–encapsulated RNAs in the communication from mother to child. Importantly, unlike other exosomes, milk exosomes can transport their cargoes to the progeny and even to other species (25), leading to mother-child or interspecies communication.

Increasing evidence has clearly indicated that milk exosomal RNAs can be taken up by cells, are permeable to the intestinal barrier, and enter the blood circulation, although this phenomenon has been controversial in past years (26, 27). In vitro experiments have shown that milk exosome–derived RNAs can be absorbed by intestinal and immune cells (24, 28). Bovine milk exosomes and their RNA cargo could enter the blood circulation of the milk consumer and distribute into various tissues in mice after application of labeled bovine milk exosomes (25, 29). Endothelial cells transport milk exosomes by endocytosis, and this is an important step in the delivery of exosomes and their RNA cargo to peripheral tissues (30, 31). Recent articles have strengthened the evidence that bovine milk exosomes and RNAs play an important role in purine metabolism, fecundity, and intestinal immune responses (32, 33).

Based on these studies, it is extremely likely that EV-derived ncRNAs in breast milk, serving as a type of biomolecular software, are important for the epigenetic regulation of genes and developmental processes in newborn infants and have significant regulatory effects. Here, we summarize the composition, biological characteristics, and functions of milk EV ncRNAs based on current knowledge, and discuss the questions and perspectives of milk EV ncRNAs for future research. This review will benefit our understanding and research of milk EV ncRNAs, their physiological functions, and the underlying molecular events.

Current State of Knowledge

Isolation of milk EV RNAs

Although most of the milk RNA is contained inside sEVs such as exosomes, other components of milk also contain RNAs. Milk RNA concentrations differ between different milk fractions, such as milk cells, the lipid fraction, and whey (34). Milk cells that contain RNA include somatic cells, originating from breast epithelial cells, and white blood cells, which are involved in inflammation (35, 36). Milk RNAs are also found in milk lipids (36, 37). Isolation of milk exosomes is a prerequisite for obtaining milk exosomal RNA. Fractional centrifugation combined with supercentrifugation or an exosome extraction reagent is a common and effective method for the separation of milk exosomes from fresh milk (22, 38, 39). However, isolation of EVs from breast milk is influenced by sample collection and storage procedures. It has been reported that the storage of unprocessed breast milk at −80°C or 4°C causes cell death in breast milk, leading to contamination of the breast milk EV population by storage-induced EVs (40). Therefore, for EV isolation, fractional centrifugation steps (to remove fat, deposited cells, and debris) should be performed prior to freezing, to reduce the number of apoptotic bodies contaminating the sample.

Studies have reported that milk exosomal RNA from human (41), cow (15), pig (42), and panda (17), contains very little 18S and 28S ribosomal RNA, but many small RNA molecules. This suggests that the RNAs in milk exosomes are specific, and different from those in eukaryotic cells. To date, there is no established endogenous RNA control in milk exosomal RNA research. To normalize the ncRNA expression data obtained by qPCR, synthetic/exogenetic RNA is usually added as a control (15, 21). The RNA concentration in milk exosomes shows variations across different stages of lactation and among different species. The RNA concentration in colostrum is significantly higher than that in mature milk (15). After normalization to protein concentrations, the total RNA concentration in porcine milk exosomes is 50–100 times higher than that in bovine milk exosomes (Bin Zeng, Ting Chen, Yongliang Zhang, unpublished results). These data suggest that the milk EV RNA concentration is possibly programmed to meet the requirements of different species and different growth stages of mammals.

The Landscape of ncRNAs in Milk EVs

MicroRNAs

The best-studied ncRNA is the miRNA, which is evolutionarily conserved and involved in posttranscriptional regulation of gene expression. Mature miRNAs are ∼22 nucleotides long and hybridize with complementary sequences in the 3′-untranslated regions in mRNAs, thus silencing genes by destabilizing mRNAs or preventing translation (43). Chen et al. (44) have identified miRNAs in raw milk, commercial fluid milk, and powdered milk products. miRNAs have also been found in bovine milk–derived EVs (45). miRNAs have been verified to be present in milk EVs of many mammalian species by deep sequencing or microarray analysis. Interestingly, van Herwijnen et al. (46), combining their own research with published studies, reported that miRNAs abundantly present in milk-derived EVs are conserved among mammals. Here, 12 studies in total, including human, cow, pig, panda, and sheep, are selected to compare the top 10 most abundant miRNAs (**Table 1**). Six miRNAs are identified in high abundance in all 5 examined species, namely, miRNA-148a (miR-148a), let-7a, let-7b, let-7f, miR-30a, and miR-30d (Table 1, which flags these miRNAs as abundant in 5 species' milk EVs). Note that miR-148a always ranks in the top 4. Furthermore, we also identified miRNAs that are abundant in milk EVs from 2 or 3 species. For example, miR-21 and miR-200c are in the top 10 most

TABLE 1 Top 10 most abundant miRNAs detected in milk extracellular vesicles from different species and studies[1]

Top 10 ranked miRNA	Species and lactation period										
	Human (2 mo)	Human (3 mo)	Human (6–8 mo)	Human (3–9 mo)	Cow (Not provided)	Cow (Not provided)	Cow (3 mo)	Pig (0–28 d)	Pig (1–5 d)	Pig 3–4 wk	Panda 15 d	Sheep Mid-lactation
1	miR-148a*	miR-148a*	miR-22	miR-30d*	miR-2478	miR-148a*	miR-30a*	miR-148a*	miR-193a	Let-7a*	miR-148a*	miR-148a*
2	miR-30b	miR-22	miR-30d*	miR-148a*	miR-1777b	Let-7a*	miR-148a*	miR-30a*	miR-423	miR-30a*	Let-7b*	Let-7b*
3	Let-7f*	miR-30d*	miR-181a	miR-200a	miR-1777a	Let-7b*	miR-141	miR-25b	miR-320	miR-191	Let-7a*	Let-7a*
4	miR-146b	Let-7b*	miR-148a*	miR-200c	Let-7b*	miR-21	miR-22	miR-182	miR-181a	miR-21	miR-30a*	miR-21
5	miR-29a	miR-200a	miR-30b	Let-7a*	miR-1224	miR-99a	miR-26a	miR-30d*	miR-30a*	miR-30d*	miR-92a	Let-7c
6	Let-7a*	Let-7a*	miR-141	miR-200b	miR-2412	Let-7f*	miR-186	miR-574	miR-378	Let-7f*	miR-181a	Let-7i
7	miR-141	Let-7f*	miR-92a	miR-21	miR-2305	Let-7c	miR-182	miR-30c	miR-191	Let-7c	Let-7g	miR-26a
8	miR-182	miR-146b	miR-26a	Let-7b*	Let-7a*	miR-200c	miR-181a	miR-200c	Let-7a*	miR-200c	miR-30d*	Let-7f*
9	miR-200a	miR-24	miR-375	Let-7f*	miR-200c	miR-26a	miR-191	miR-191	Let-7f*	Let-7g	Let-7i	miR-125b
10	miR-378	miR-21	miR-30a*	miR-30a*	miR-141	miR-30d*	miR-27b	Let-7a*	Let-7c	miR-320	Let-7f*	miR-143
Reference	(14)	(47)	(24)	(46)	(28)	(48)	(49)	(50)	(16)	(46)	(17)	(18)

[1] d, days; miRNA, microRNA; mo, month; wk, week.
* miRNAs abundant in 5 species' milk extracellular vesicles.

abundant miRNAs in humans, cows, and pigs. miR-141 is in the top 10 most abundant miRNAs in humans and cows. In addition, miR-200a, miR-30b, and miR-146b are only in the top 10 most abundant miRNAs in humans, whereas miR-191 is only in the top 10 in pigs. The high abundance of these miRNAs in milk EVs indicates that they might play a relatively important role in physiological function through milk EVs. As shown in Table 1, miRNA profiles in milk EVs of the same species were different in different studies. The possible causes of this phenomenon are differences in nutrient concentrations, breed, lactation number, lactation period, sample treatments, and sequencing analysis.

Furthermore, many factors, including sample collection at lactation period, disease, and change of nutrition and environment, could also underlie the varied concentrations of milk EV miRNAs. The concentrations of 7 immune-related miRNAs (miR-24, miR-30d, miR-93, miR-106a, miR-181a, miR-200a, and miR-451) in human colostrum EVs are higher than in mature milk EVs (51). The expression patterns of miRNAs in pig (42) or panda (17) milk EVs are distinct across the lactation period. Cai et al. (48) and Sun et al. (49) reported that miR-142-5p and miR-223 in bovine milk EVs are upregulated upon *Staphylococcus aureus* infection. These miRNAs are potential biomarkers for early detection of bacterial infection in mammary glands. Quan et al. (52) partly replaced alfalfa hay with whole cotton seed and soybean hull (nonforage fiber source) in the feed formula of cows and identified 9 differently expressed miRNAs (4 upregulated and 5 downregulated) in milk EVs. Moreover, miR-142, miR-135, and miR-320a in milk exosomes are found to be most responsive to group relocation of cows (53).

However, research is lacking on the profound biological roles of miRNAs that are highly expressed and relatively conserved in mammalian milk EVs, as well as those whose concentrations change under the influence of different factors. Future studies should focus on these aspects.

Long noncoding RNAs and circular RNAs

Long noncoding RNAs (lncRNAs) are >200 nucleotides in length and lack protein-coding capacity. They comprise a heterogeneous class of intergenic transcripts, enhancer RNAs, and sense or antisense transcripts that overlap other genes. lncRNAs have been proposed to carry out transcriptional regulation in cis or trans, organize nuclear domains, and regulate proteins and RNAs (54). Circular RNAs (circRNAs) are classified as a new type of endogenous ncRNAs, and they are different from common linear RNAs. They are characterized by covalently closed loops without 5′ or 3′ polarities (13). Recent studies have demonstrated that circRNAs can adhere to miRNAs by stable complementary binding and serve as miRNA sponges to regulate gene expression (55). lncRNAs and circRNAs have received much attention in recent years, and they have been found to play important functional roles in numerous biological processes across every branch of life, including transcriptional regulation, epigenetic gene regulation, and disease (56, 57). To

date, there have been few reports of lncRNAs and circRNAs in milk EVs. Karlsson et al. (41) used qRT-PCR to analyze 87 lncRNAs, which had been previously reported to be important for developmental processes, in human breast milk EVs. Results revealed the presence of 55 lncRNAs in EVs from ≥1 of the analyzed individual breast milk samples ($n = 30$). Among these, 5 lncRNAs [Colorectal neoplasia differentially expressed (*CRNDE*), Differentiation antagonizing non-protein coding RNA (*DANCR*), Growth arrest specific transcript 5 (*GAS5*), Steroid receptor RNA activator 1 (*SRA1*), and Zinc finger NFX1-type antisense RNA 1 (*ZFAS1*)] were detected in 90–100% of the breast milk samples. Our previous work identified 3475 novel lncRNAs and 6 annotated lncRNAs in bovine milk EVs by RNA sequencing, and lncRNAs showed higher expression levels than mRNAs (21). lncRNAs in bovine milk EVs are also varied across different stages of lactation, as revealed by qRT-PCR analysis. Expression levels of LNC_0,01182 and LNC_0,02303 are higher in colostrum than in mature milk. Conversely, levels of LNC_0,01442 are higher in the mid- and late lactation periods (150 and 270 d) than in colostrum (2 d) and early lactation (30 d). circRNAs have also been found in bovine milk EVs. Wang et al. (58) reported 2059 distinct circRNAs identified in milk EVs by high-throughput RNA sequencing, most of them specifically expressed either in colostrum or in mature milk EVs. In porcine milk EVs, 3205 lncRNAs were identified, but only 61 circRNAs were found (59). Here, we have collated the detailed information (location in the genome) of the top 20 most abundant lncRNAs/circRNAs in bovine and porcine milk EVs (**Table 2**).

In general, lncRNAs and circRNAs are less conserved in different species than miRNAs, and the biological functions of most of them are unknown. Thus, it is more difficult to research lncRNAs and circRNAs in milk EVs. The establishment and gradual improvement of the ncRNA database will be helpful in the exploration and analysis of lncRNAs and circRNAs in milk EVs.

The biogenesis, stability, and uptake of ncRNAs in milk-derived EVs

To date, there is no direct and convincing evidence about the source of RNA in milk EVs. Alsaweed et al. (60) used TaqMan OpenArrays to compare human milk RNAs with those in mammary epithelium cells, maternal peripheral blood mononuclear cells, and plasma, and suggested that milk miRNAs primarily originate from mammary epithelia, whereas the maternal circulation makes a smaller contribution. Based on previous reports, we summarized the top 15 most abundant miRNAs in the mammary gland (lactating period) and in milk EVs from 3 mammals (**Table 3**). Interestingly, the results from different studies show that the top 15 most abundant miRNAs in the bovine or porcine mammary gland share 10 common members with the top 15 most abundant miRNAs in their milk EVs. Also, there are 9 common miRNAs between ovine mammary glands and ovine milk EVs (Table 3, which flags miRNAs abundant in both mammary glands and milk EVs). Le Guillou et al. (61) conducted a comparative analysis of the miRNome between milk and lactating mammary glands. Results showed that of 487 annotated miRNAs in lactating mammary glands, 433 (88.9%) were detected in milk. Sixteen miRNAs are present in the top 30 of both the lactating mammary gland and the milk. These results suggest that endogenous synthesis in the lactating mammary gland is likely to be one source of miRNAs in milk EVs, but much more work is needed to confirm this. Analysis of ncRNAs in milk EVs and mammary glands from the same individual is a useful strategy. ncRNAs in milk can exist in other forms. Immunoprecipitation assays have shown that miRNAs in porcine milk are bound to proteins, including argonaute 2 (Ago2), nucleophosmin 1 (NPM1), and HDLs (Ting Chen, Bin Zheng, Delin Lin, Yongliang Zhang, unpublished results). In addition, milk fat globules, immune cells, and nonimmune milk cells, such as milk epithelial cells and milk stem cells, might all be sources of milk RNAs (51, 62, 63).

Many studies indicate that miRNAs in milk EVs are very stable and resistant to harsh conditions, including low pH, RNase digestion, and freeze-thaw cycles, because the lipid bilayer acts as a protective covering (15, 64, 65). Milk ncRNAs are packaged in EVs, allowing them to avoid degradation in the gastrointestinal tract. Our previous study also demonstrated that lncRNAs in bovine milk EVs are resistant to in vitro digestion with different digestive juices, including saliva, gastric juice, pancreatic juice, and bile (21). However, milk EV ncRNAs could be degraded by the addition of detergent (1% Triton X-100) (15), bacterial fermentation (66), milk processing (67), microwave heating (68), and ultrasound treatment (69). Notably, when milk EVs were heated to 100°C for 10 min, the encapsulated miRNAs and lncRNAs could still be detected by PCR, despite the fact that heating resulted in degradation of the majority of ncRNAs (Bin Zeng, Ting Chen, Yongling Zhang, unpublished results). This result provides a physiological basis for the absorption of milk EV ncRNAs in the intestine. Moreover, the comparison of RNA stability between milk EVs and other EVs (nonfood sources) upon treatment with various digestive juices will likely shed more light on the generality and characteristics of milk EVs.

EV uptake is linked to multiple mechanisms and endocytic pathways, including clathrin-dependent endocytosis and clathrin-independent pathways such as caveolin-mediated uptake, macropinocytosis, phagocytosis, and lipid raft–mediated internalization (70). A recent assay revealed that the membrane fusion of exosomes could be enhanced at pH 6.0 (71). Indeed, it seems likely that a heterogeneous population of EVs can gain entry into a cell via >1 route. Evidence has suggested that the entry of milk miRNAs into recipient cells is mediated by endocytosis and depends on cell and exosome surface glycoproteins (30, 31). It has been experimentally demonstrated that bovine milk EV miRNAs could be taken up by human macrophages (28), and human milk exosomes and their miRNAs could be taken up by human intestinal crypt-like cells (24). Zempleni's team (72)

TABLE 2 Detailed genetic information of top 20 most abundant lncRNAs/circRNAs in bovine and porcine milk EVs[1]

Top 20	lncRNA ID Bovine milk EV (50–100 d)	lncRNA ID Porcine milk EV (3 d)	circRNA ID Bovine milk EV (2 d)	circRNA ID Bovine milk EV (90 d)	circRNA ID Porcine milk EV (3 d)
1	Chr10: 42,863,856/42,864,152	AEMK02000696.1: 79,195/97,543	Chr3: 638,422/739,656	Chr8: 100,351,443/100,353,491	Chr14: 121,316,587/121,320,413
2	Chr26: 17,917,472/17,972,241	Chr7: 10,867,838/10,868,358	Chr6: 87,270,299/87,276,280	Chr6: 87,152,488/87,157,921	Chr15: 77,234,645/77,283,482
3	Chr6: 77,982,100/77,982,100	AEMK02000407.1: 82,569/83,800	Chr5: 41,157,982/41,297,645	Chr6: 87,145,048/87,150,953	Chr1: 119,487,613/119,500,634
4	Chr6: 77,983,357/77,986,216	AEMK02000603.1: 28,188/36,155	Chr6: 109,933,165/110,018,362	Chr26: 46,866,552/46,926,465	Chr9: 7,476,477/7,481,586
5	Chr8: 74,783,032/74,789,504	AEMK02000358.1: 14,710/22,171	Chr16: 58,714,416/58,733,499	Chr6: 87,181,195/87,186,019	Chr10: 29,037,749/29,038,934
6	Chr27: 43,452,486/43,560,243	AEMK02000665.1: 68,696/81,137	Chr14: 65,829,343/65,832,220	Chr3: 680,930/739,656	Chr15: 60,407,129/60,414,442
7	GJ058845.1: 24/1211	Chr4: 101,819,215/101,872,430	Chr3: 51,745,964/51,792,200	Chr4: 79,458,313/79,618,721	Chr1: 216,148,994/216,177,064
8	Chr8: 61,266,610/61,284,332	Chr5: 42,366,339/42,372,651	Chr1: 155,995,947/156,077,037	Chr5: 118,108,657/118,281,103	Chr11: 5,503,064/5,507,316
9	Chr2: 100,162,502/100,165,205	AEMK02000495.1: 152,240/153,686	Chr18: 15,677,553/15,769,596	Chr2: 63,170,683/63,228,633	Chr1: 45,173,523/45,175,843
10	Chr20: 70,185,321/70,185,894	Chr9: 42,551,562/42,617,514	Chr5: 38,456,047/38,485,210	Chr11: 25,674,108/25,745,152	Chr3: 93,174,831/93,179,511
11	Chr6: 19,792,457/19,802,292	AEMK02000328.1: 317,535/358,163	Chr10: 79,243,596/79,335,928	Chr29: 25,303,168/25,338,898	Chr18: 54,393,585/54,404,485
12	Chr26: 17,969,399/17,971,287	Chr14: 109,907,139/109,967,090	Chr14: 73,650,350/73,691,478	Chr13: 19,157,964/19,330,999	Chr10: 69,201,375/69,235,186
13	Chr8: 620,509/6,207,288	AEMK02000301.1: 78,258/97,242	Chr6: 20,604,830/20,700,712	Chr4: 92,833,700/92,950,401	Chr17: 13,008,091/13,031,589
14	GJ058260.1: 2/1519	AEMK02000135.1: 3328/8711	Chr17: 7,077,165/7,125,454	Chr3: 102,324,607/102,362,801	Chr1: 1,654,691/1,663,793
15	Chr8: 61,266,453/61,284,374	AEMK02000205.1: 16,230/24,313	Chr12: 11,730,279/11,824,957	Chr6: 70,427,322/70,515,551	Chr7: 113,369,840/113,371,752
16	Chr6: 19,799,937/19,802,292	Chr5: 61,105,793/61,114,297	Chr14: 4,513,617/4,527,572	Chr16: 58,714,416/58,733,499	Chr8: 18,861,487/18,879,156
17	Chr8: 6,205,093/6,207,288	AEMK02000220.1: 7139/8444	Chr10: 45,489,842/45,610,796	Chr4: 105,645,266/105,685,635	Chr12: 59,319,171/59,331,703
18	GJ058260.1: 2/1519	Chr6: 62,037,122/62,045,112	Chr11: 40,550,350/40,623,558	Chr17: 10,435,130/10,463,829	Chr18: 10,283,698/10,300,806
19	GJ058845.1: 69/1107	AEMK02000220.1: 15,348/18,156	Chr10: 93,250,197/93,310,748	Chr26: 43,911,446/43,954,980	Chr18: 54,260,018/54,270,584
20	Chr6: 17,938,105/17,939,902	Chr4: 46,447,193/46,450,808	Chr9: 95,143,978/95,207,141	Chr13: 7,280,480/7,331,583	Chr1: 263,932,774/263,940,366
Reference	(21)	(59)	(58)	(58)	(59)

[1] Chr, chromosome; circRNA, circular RNA; d, days; EV, extracellular vesicle; ID, identity document; lncRNA, long noncoding RNA.

TABLE 3 Top 15 most abundant miRNAs detected in mammary glands and milk EVs from different studies and species[1]

	Cow		Pig		Sheep	
Top 15 ranked miRNAs	Mammary glands	Milk EVs	Mammary glands	Milk EVs	Mammary glands	Milk EVs
1	Let-7a*	miR-148a*	miR-21*	Let-7a*	miR-143*	miR-148a*
2	Let-7f*	Let-7a*	miR-30a*	miR-30a*	Let-7b*	Let-7b*
3	Let-7b*	Let-7b*	Let-7a*	miR-191*	Let-7a*	Let-7a*
4	Let-7g*	miR-21*	miR-200c*	miR-21*	miR-378	miR-21*
5	miR-26a*	miR-99a	Let-7f*	miR-30d*	miR-148a*	Let-7c*
6	Let-7c*	Let-7f*	miR-191*	Let-7f*	Let-7f*	Let-7i
7	miR-21*	Let-7c*	miR-30d*	Let-7c*	miR-30a*	miR-26a
8	miR-103	miR-200c*	Let-7c*	miR-200c*	Let-7c*	Let-7f*
9	miR-29a	miR-26a*	miR-200b	Let-7g*	miR-146b	miR-125b
10	miR-30a*	miR-30d	miR-30c*	miR-320	miR-21*	miR-143*
11	miR-26b	Let-7g*	Let-7g*	miR-99a	miR-103	miR-30a*
12	miR-200c*	miR-30a*	miR-375	miR-30c*	miR-200c	miR-27a
13	miR-148a*	miR-200a	miR-24	miR-92a	miR-30d	miR-127
14	miR-1	miR-151	miR-26a	miR-425	miR-126	miR-181a
15	Let-7i	miR-423	miR-186	miR-20a	Let-7g*	Let-7g*
Reference	(73)	(48)	(74)	(46)	(75)	(18)

[1] EV, extracellular vesicle; miRNA, microRNA.
*miRNAs abundant in both mammary glands and milk EVs.

has reported that meaningful amounts of miR-29b and miR-200c are absorbed when adults consumed 0.25, 0.5, and 1.0 L of milk in a randomized crossover design. However, Auerbach et al. (27) reported a failure to detect bovine miR-29b and miR-200c in human plasma after milk consumption. Title et al. (26) detected only trace amounts of miR-375 (magnitude below the threshold for target genes) in the plasma of miR-375–knockout mouse pups receiving wild-type milk. Subsequently, Zempleni's team (29) suggested that the integrity of the samples used in the study by Auerbach et al. (27) was compromised and the RNA was degraded. They also suggested that, unlike many other miRNAs, miR-375 in milk is subjected to "first passage elimination" in intestinal mucosa and liver, and therefore its concentrations in the blood circulation and peripheral tissues are low (76). More direct evidence about milk EV ncRNA uptake has been provided by Zempleni's team. They transfected synthetic, fluorophore-labeled miRNAs into bovine milk exosomes and administered these to mice, and found that distinct species of miRNAs demonstrated unique distribution profiles and accumulated in intestinal mucosa, spleen, liver, heart, and brain (25). Further, miRNAs present in milk are able to enter normal and tumor cells (77). Milk miRNAs encapsulated in exosomes have been confirmed to be able to cross the intestinal barrier (65, 78). Our previous study suggested porcine milk exosomal miRNAs could be taken up by intestinal epithelial cells (79, 80). Our recent work demonstrated that the concentrations of 4 miRNAs (miR-2284, miR-2291, miR-7134, and miR-1343) were significantly different in piglet serum after feeding porcine or bovine milk, which is in accordance with their original concentrations in porcine and bovine whey (which contains milk EVs). Interestingly, these milk-derived miRNAs showed differences in piglet serum at different time points (days 0, 3, 6, and 12), which could be relevant to the variation in absorbance of miRNAs after milk feeding (81). Moreover, data from studies in dual-chamber systems have suggested that some miRNAs of milk EVs cross the intestinal mucosa more efficiently than others, and reverse transport from the basolateral to the luminal side is minimal in human Caco-2 colon carcinoma cells (30).

Biological Roles of ncRNAs in Milk EVs

Intestinal health

Milk EVs and their RNA cargoes, after resisting digestion by gastric juices, reach the intestines. Recent studies suggested that milk EVs and their RNA cargoes are beneficial for intestinal health. Intestinal epithelial cells play a crucial role in the regulation of development and health, forming an essential barrier between the exterior and the interior of the body, and are responsible for the first physiological step of transporting nutrients to the body's cells. It has been shown that milk exosomes promote intestinal epithelial cell growth (82) and protect them against intestinal injury under oxidative stress (83, 84), intestinal inflammation (85), and necrotizing enterocolitis (23, 86). Recent research has shown that dietary depletion of milk exosomes and their miRNA cargoes exacerbates cecal inflammation in multiple drug resistance (*Mdr*)1a$^{-/-}$ mice (*Mdr*1a$^{-/-}$ mice spontaneously develop clinical signs of inflammatory bowel disease). Bovine milk exosomal miR-200a-3p plays an important role in alleviating cecal inflammation by downregulating the expression of the proinflammatory chemokine (C-X-C motif) ligand 9 (*CXCL9*) (87). Furthermore, our recent work demonstrated that porcine exosomal miR-4334 and miR-219 reduce LPS-induced intestinal epithelial cell (IEC) inflammation through the NF-κB pathway, porcine milk exosomal miR-338 inhibits

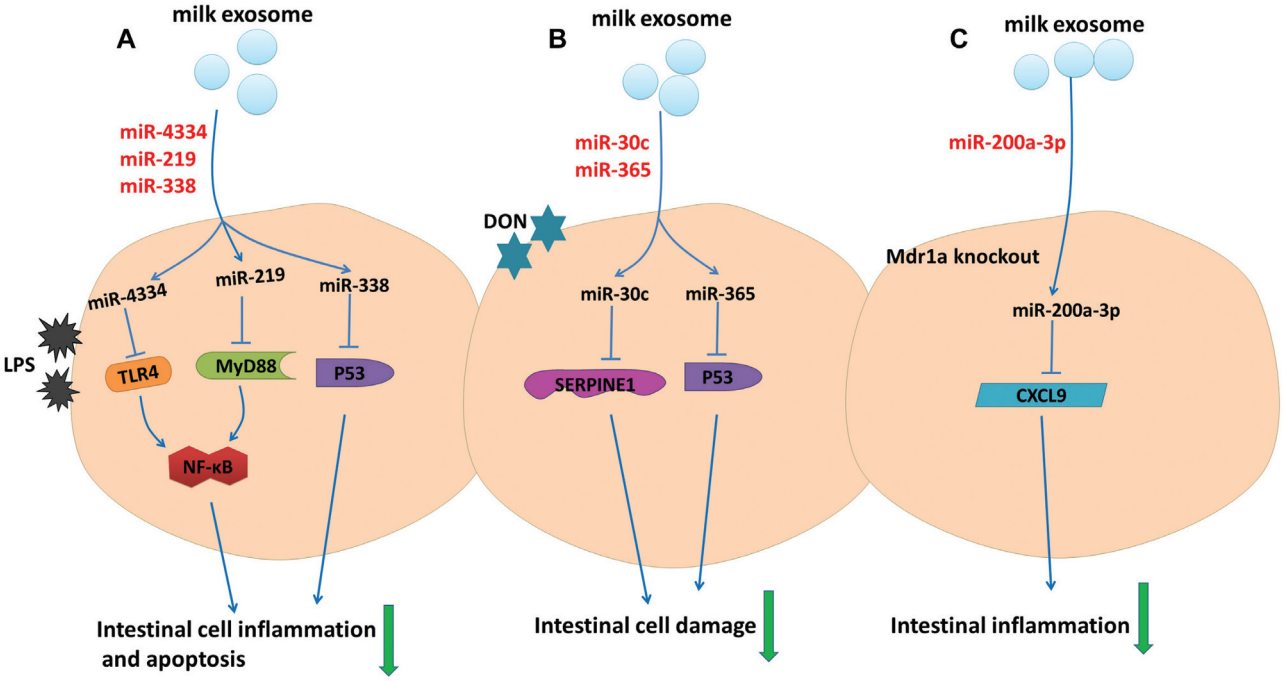

FIGURE 1 Milk exosomal miRNAs inhibit intestinal inflammation or damage caused by different factors. (A) Porcine milk exosomal miRNAs (miR-4334, miR-219, and miR-338) reduced LPS-induced intestinal cell inflammation and apoptosis. (B) Porcine milk exosomal miRNAs (miR-30c and miR-365-5p) attenuated deoxynivalenol (DON)-induced intestinal cell damage. (C) Bovine milk exosomal miR-200a-3p alleviated cecal inflammation in Mdr1a-knockout mice. CXCL9, chemokine (C-X-C motif) ligand 9; Mdr1a, multiple drug resistance 1a; miRNA, microRNA; MyD88, myeloid differentiation factor 88; p53, protein 53; SERPINE1, serine proteinase inhibitor, member 1; TLR4, toll-like receptor 4.

the LPS-induced IEC apoptosis via the p53 pathway (79), and porcine milk exosomal miR-30c and miR-365–5p attenuate deoxynivalenol-induced IEC damage by downregulating Serine proteinase inhibitor type-1 (*SERPINE1*) and protein 53 (*Tp53*) expression (80). These findings show that several milk exosomal miRNAs are involved in the suppression of intestinal inflammation and damage by affecting target gene expression (**Figure 1**).

Many studies have indicated the potential of milk exosomal miRNAs to regulate intestinal health. Our previous study (88) and Gao et al. (22) suggested that both porcine and yak milk exosomes could promote intestinal proliferation by inhibiting protein 53 (*p53*) gene expression, and milk EV miR-2320, miR-181a, miR-1343, miR-128, and miR-769–3p concentrations were significantly increased in epithelial cells of porcine small intestine (IPEC-J2) after treatment with porcine milk-derived exosomes. Bioinformatics analysis suggested that TNF receptor superfamily member 6 (*FAS*) is targeted by miR-2320 and miR-181a and that *SERPINE* is targeted by miR-769-3p and miR-128 in the p53 signaling pathway. Breast milk exosomal miR-125b, miR-30d, and miR-25 can play an important role in attenuating cell death of intestinal epithelial cells (83). A recent study suggested that oral administration of moderate amounts of bovine milk–derived EVs enhances intestinal immunity in mice (89). Interestingly, highly abundant miRNAs in the milk EVs of many species, including miR-148a and let-7 family and miR-30 family miRNAs (Table 1), have been reported to play a critical role in intestinal mucosal immunity regulation (90). For example, Paneth cell differentiation is important for producing defensins and antimicrobial peptides of the intestinal tract. The let-7 miRNA family facilitates Paneth cell differentiation by suppressing expression of their target genes [High mobility group AT-hook 2 (*Hmga2*) or insulin-like growth factor 2 binding protein 1 (*Igf2bp1*)] (91). Plasma cells are essential in IgA production in the intestinal tract. miR-30 family members have been reported to regulate the gene expression of *Blimp-1*, which is a transcription factor that is critical for the transition of germinal center B cells into plasma cells (92). miR-148a has been found to orchestrate the regulatory network to facilitate plasma cell differentiation by regulating Melanocyte inducing transcription factor (*Mitf*) and BTB domain and CNC homolog 2 (*Bach2*) expression (93). miR-375 is detected in the top 10 of human milk exosomes, and it is also thought to function in epithelial goblet cell differentiation (94). Together, these reports suggest the involvement and significance of highly expressed milk EV miRNAs in intestinal health, and provide sufficient hypotheses for future studies (**Figure 2**).

There is little literature about ncRNAs other than miRNAs in milk EVs in relation to intestinal health. Gene

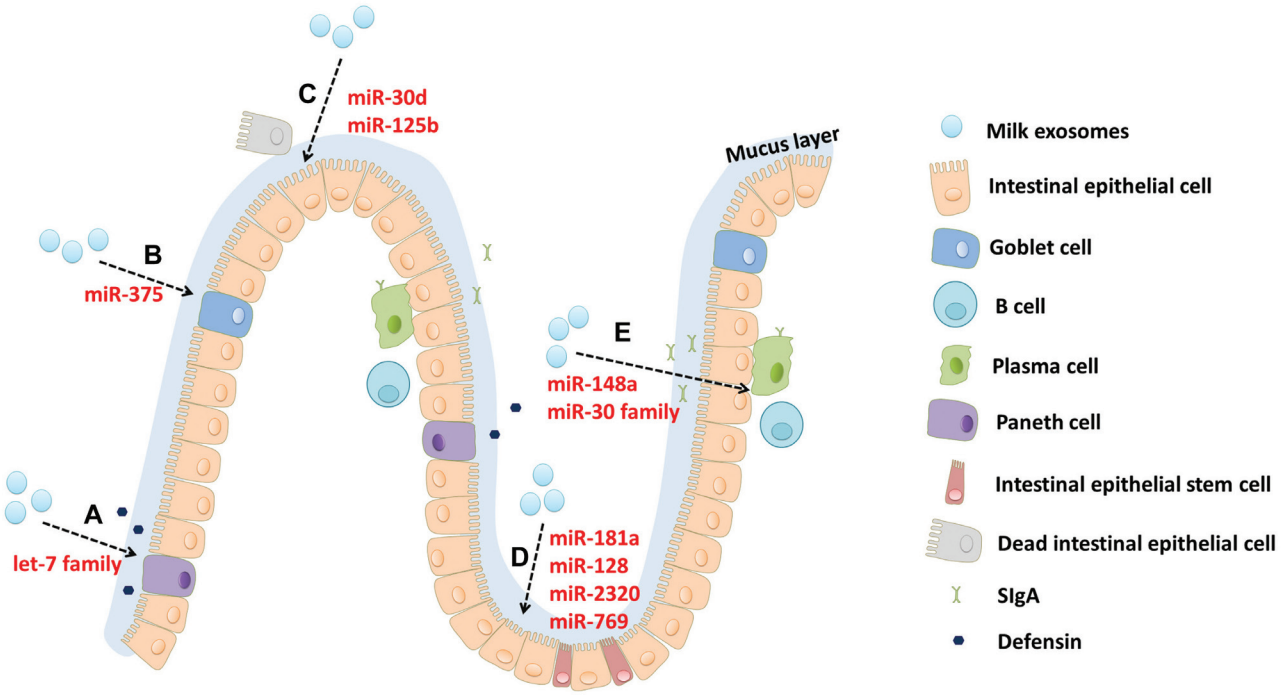

FIGURE 2 Potential regulation of milk exosomal miRNAs on intestinal health. (A) Paneth cell differentiation. (B) Goblet cell differentiation. (C) Attenuation of intestinal epithelial cell death. (D) Promotion of intestinal epithelial cell proliferation. (E) B-cell and plasma cell differentiation. miRNA, microRNA; SIgA, Secretory immunoglobulin A.

Ontology annotation indicated that the predicted target genes of bovine milk EV lncRNAs are enriched in the intestinal immune network for IgA production (21). In addition, gut microbiota and their metabolites play an important role in intestinal health. It has been reported that plant-derived exosome-like nanoparticles are taken up by the gut microbiota and contain miRNAs that alter microbiome composition and host physiology (95). Studies have reported that dietary milk EVs elicit changes in gut microbiota in mice (89, 96), but more research is necessary to determine whether milk EV ncRNAs influence the gut microbiota.

Immune regulation

Breast milk is important in the development of a child's immune system (97). Milk EVs have immune modulatory effects, and their ncRNA cargoes might play a role in this process, but research on the exact mechanism is still insufficient. A potential role for milk-derived EVs in immune modulation was first suggested by Admyre et al. (3), who found that human breast milk–derived EVs could facilitate regulatory T-cell induction. A recent publication showed that milk-derived EVs modulate cyclophosphamide-induced immunotoxicity in rats (98). Kosaka et al. (99) detected high abundances of immune-related miRNAs in the first 6 mo of lactation and proposed breast milk miRNA as a new immunoregulatory agent. Moreover, Sun et al. (51) found that bovine colostrum–derived EVs contain higher concentrations of immune-related miRNAs and display immune modulatory effects. Bovine milk exosomes containing miRNAs have been found to be taken up by human macrophages (28) and peripheral blood mononuclear cells (72). Our previous work suggested 14 of the top 20 miRNAs in porcine milk EVs possibly participate in the regulation of the IgA immune network (16). Quan et al. (18) reported that 14 sheep milk EV miRNAs of the top 20, accounting for 98% of the total expression, are immune related. These reports suggest that miRNAs in milk EVs are potential immune protectors. As mentioned earlier, immune-related miRNAs are also found to be abundantly present in milk EVs of many species. miR-148a directly targets DNA methyltransferase 1 (*DNMT1*), which is associated with the forkhead box P3 (*FOXP3*) locus in $CD4^+$ T cells. *DNMT1* deficiency results in highly efficient *FOXP3* induction following T-cell receptor stimulation (100). miR-148a functions as a critical regulator of B-cell tolerance and autoimmunity. Elevated miR-148a expression impairs B-cell tolerance by promoting the survival of immature B cells after engagement of the B-cell antigen receptor by suppressing the expression of the autoimmune suppressor growth arrest and DNA-damage-inducible 45 alpha (*Gadd45α*) (101). miR-30a directly targets myeloid differentiation factor 88 (*MyD88*) and suppresses Toll-like receptor (*TLR*)/*MyD88* activation and cytokine expression in Human acute monocytic leukemia (THP-1) cells during *Mycobacterium tuberculosis* H37Rv infection (102). Let-7f is involved in the promotion of memory cell generation

(103). Let-7a regulates the survival mechanisms mediated by the inflammatory cytokine IL-6 (104). In addition to miRNAs, the detection of specific lncRNAs in human breast milk EVs indicates that lncRNAs could also be important for programming the neonatal immune system (41). For instance, lnc-*ZFAS1* appears to have an important role in cell cycle control (105), whereas lnc-*GAS5* is essential in apoptosis and normal growth arrest in T cells (106). lnc-*DANCR* has been demonstrated to control the expression of IL-6 and TNFα in blood mononuclear cells (107). lnc-*SRA1* could be important for the infant immune system because it regulates genes in the TNFα signaling pathway (108). In addition, functional enrichment analysis of the target genes of porcine milk EV lncRNAs indicated that these lncRNAs are involved in immune processes, including the regulation of the adaptive immune system, IL-8 production, and IL-6 secretion (59).

Epigenetic regulation

ncRNAs in milk EVs could be a class of key molecules in epigenetic regulation. miRNAs (109), lncRNAs (110), and circular RNAs (111) have been found to affect the epigenetic machinery. It is well known that the particular composition of breast milk gives it a role in epigenetics. Evidence is accumulating that epigenetic signaling of milk promotes the development of the infant's gastrointestinal tract and immune system, and also osteogenesis, myogenesis, adipogenesis, and neurogenesis (63). Milk nutritional epigenetics concerns the effects of nutrients on gene expression (112). In the past decade, ncRNAs have been found to be abundant in milk EVs, which has led to a new understanding of milk epigenetic regulation. Milk EVs provide high amounts of miR-148a, which targets *DNMT*s to potentially affect the whole genomic DNA methylation patterns (63). After incubation of normal and cancer cells with human milk–derived miRNAs, the expression of miR-148a was upregulated and the expression of its target, *DNMT1*, was downregulated (77). Milk EV miR-152, miR-29b, and miR-21 also target *DNMT*s (63). Moreover, milk EV miR-125b, miR-30d, and miR-25 might downregulate *p53*, which physically interacts with and stabilizes *DNMT1* (113). Therefore, milk EV miRNAs can function as potential epigenetic modifiers of the milk recipient.

Metabolic disease

Milk EVs are rich in ncRNAs, some of which are associated with metabolic diseases. Many metabolic diseases, including obesity, type 2 diabetes mellitus (T2DM), osteoporosis, and Parkinson disease, have steadily increased in prevalence since the 1950s, the period of widespread distribution of refrigerated pasteurized cow milk (114). Much evidence has shown that ncRNAs in milk EVs can be absorbed by animals with potential benefits for intestinal health and immune function. But it raises concerns about metabolic diseases caused by ncRNAs in milk EVs. Young mice with long-term ad libitum access to commercial whole cow milk exhibit increased body weight and epididymal fat mass, compared with controls with no access to dairy milk (115). However, whether obesity is promoted by milk EV ncRNAs is not clear. miR-148a, a highly abundant miRNA in milk EVs, suppresses its target gene Wnt family member 1 (*Wnt1*), an endogenous inhibitor of adipogenesis. Ectopic expression of miR-148a accelerates differentiation and partially rescues Wnt1-mediated inhibition of adipogenesis, whereas knockdown of miR-148a inhibits adipogenesis (116, 117). Remarkably, the miR-148a gene has been identified as an obesity risk gene in humans who are exhibiting single nucleotide polymorphisms (118, 119). miR-29b and miR-21, 2 other abundant mammalian milk EV–derived miRNAs (Table 1), are also involved in adipogenesis (120, 121). Notably, miR-29b also plays a role in T2DM. miR-29b mediates increases in branched-chain amino acid (BCAA) concentrations and BCAA-driven mammalian target of rapamycin complex 1 (*mTORC1*) activation in peripheral tissues, which explains why Ribosomal protein S6 kinase- (*S6K1*-)mediated inhibitory phosphorylation of insulin receptor substrate 1, a key checkpoint of insulin signaling, causes insulin resistance (122). Meanwhile, Kelch et al. (123) reported that miR-21 is significantly upregulated in serum and osteoclasts of patients with osteoporosis. miR-21 suppresses the expression of programmed cell death 4 (*PDCD4*) (124), which is important for the differentiation of preosteoclasts to osteoclasts (123). The above data indicate that milk EV ncRNAs can exert regulatory effects on metabolism of the recipient, but much more research is needed to determine the exact role of milk ncRNAs in this process.

Targeted Therapeutic Potential

Because of the therapeutic significance of exosomal miRNAs in an array of diseases, drug development focusing on the release of exosomal miRNA contents has begun (125). Due to their simplicity to obtain, stability in the gastrointestinal tract, and ability to efficiently shuttle small molecules to specific organs or the circulatory system, milk sEVs (exosomes) are an extremely promising therapeutic tool for numerous diseases. Munagala et al. (126) demonstrated that milk exosomes exhibit cross-species tolerance with no adverse immune and inflammatory response, and possess tremendous potential as drug carriers for hydrophilic and lipophilic agents. Shandilya et al. (78) encapsulated scrambled Alexa Fluor (AF)-488 short interfering RNA (siRNA) in milk exosomes using lipofection, and found that milk exosomal siRNAs are resistant to different digestive juices, and could be internalized by Caco-2 cells. The stable delivery of exosomal AF-488 siRNA and its transepithelial transport were confirmed by fluorescence microscopy and fluorescence intensity measurements. Of particular note, a recent study reported that siRNAs against specific genes, including vascular endothelial growth factor (*VEGF*), epidermal growth factor receptor (*EGFR*), protein kinase B (*AKT*), mitogen-activated protein kinase (*MAPK*), and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), can be loaded in milk exosomes by electroporation or chemical transfection, and the expression levels of target genes after knockdown were 2- to

10-times lower in various cancers (127). Meanwhile, natural and unprocessed milk exosome ncRNAs also have potential for therapeutic applications. Metabolism-related miRNAs in breast milk are influenced by premature delivery (128), with miR-148a expression being higher and miR-320a expression being lower in preterm human milk compared with term human milk (129). Alterations in miRNA expression in milk EVs can affect biological function in infants and could serve as a nutritional therapeutic target (129). Our recent work suggested that miR-4334, miR-219, and miR-338 in porcine milk exosomes could have potential for application in therapies for necrotizing enterocolitis (79). However, there are bottlenecks in milk EV ncRNA-based target therapies. The transportation, uptake, and in vivo effector mechanisms of milk EV ncRNAs remain largely obscure. Moreover, milk EVs contain diverse contents and exert different functions. More methods must be developed to obtain milk EVs containing purified ncRNAs.

Questions and Perspectives of Milk EV ncRNAs

Because of its unique nutritional value and unique effects on humans and other mammals, the analysis of breast milk has never ceased. The field of milk EVs and their ncRNA cargoes has witnessed rapid expansion and progress in recent years. Collectively, milk EVs of humans and other mammals contain abundant ncRNAs, which have been proven: *1*) to be stable under harsh conditions, and *2*) to be able to enter the circulatory system through the intestinal barrier and influence target gene expression. Their roles as biological regulators in milk have been supported or predicted by many studies. All of these strongly indicate a potential function of milk EV ncRNAs in the recipient or offspring. However, a wide uncharted territory remains to be explored and verified, and the following issues should be considered in future research.

First, little is known about the expression profiles of the milk EV ncRNAs other than miRNAs. It is unknown whether highly expressed circRNAs and lncRNAs play a similar role in different mammalian milk EVs as miRNAs. From the perspective of milk nutrition evolution in different mammals, the ncRNAs in milk EVs could perform their characteristic nutritional regulatory functions just like lactose and proteins in milk. Therefore, exploration and analysis of ncRNAs in milk EVs is meaningful work. In addition, a quantitative survey of EV ncRNAs present in raw milk from different mammals and their products is equally important. This is an infrastructural project to replenish the milk ncRNA database. More research in this field will be helpful to elucidate the role of breast milk EV ncRNAs in infant or adult recipients.

Second, the exact origin, packaging mechanism, and uptake approach of milk EV ncRNAs remain unclear. For instance, the mammary gland is one of the possible sources of milk EV miRNAs (Table 3), but we still lack solid evidence supporting this hypothesis. Moreover, the following aspects remain unclear: *1*) the mechanisms by which ncRNAs are selectively encapsulated into milk EVs; *2*) the pathways by which they enter intestinal epithelial cells and the circulatory system; and *3*) the mechanisms by which they are distributed to target tissues and their final destination. These issues are important but challenging to elucidate. Moreover, although controversy about the absorption of milk EV ncRNAs into the circulatory system, tissues, or organs is decreasing, attention should be paid to the specific design of experimental protocols, such as animal models, applied dosage, and sampling time, to avoid controversial results.

Finally, the effects of milk containing EVs that are (partly) depleted of ncRNAs on offspring phenotypes and the underlying mechanisms need to be explored to determine the importance of maternal milk EV ncRNAs for babies. However, solid evidence that convincingly demonstrates the biological functions and exact mechanisms of milk EV ncRNAs under physiological or pathological conditions, especially in vivo, is still lacking, although many studies on this topic have been conducted. A progressive approach has been used to explore milk exosomes and RNA cargoes at Zempleni's laboratory. They fed mice with ultrasound-treated cow milk in which most of the exosomes and RNA cargoes were destroyed; this resulted in a series of phenotypic changes in mice (32, 87). Because milk or milk EVs are composed of multiple complex ingredients, ultrasound can also alter or degrade other components, not just EV RNAs. To this end, the construction of an animal model that lacks biogenesis of milk EV RNAs or certain ncRNA(s) in milk EVs is a potential key approach for further elucidating the physiological effects of milk EV ncRNAs. Importantly, it is necessary to distinguish milk EV ncRNA uptake from endogenous synthesis when a physiological feeding study is conducted in animals given milk EVs. Hopefully, a series of pioneering biotechnologies, including gene editing, nucleic acid tracing, isotopic tracing, and RNAscope, will rapidly advance this line of research in the near future.

Conclusions

ncRNAs in milk EVs represent a new research area in food science and milk nutrition. This field has expanded rapidly in recent years. Based on current studies, ncRNAs are abundant in milk EVs and have high stability. Some miRNAs are highly expressed in the milk EVs of different mammals and show a similar expression pattern. lncRNAs and circRNAs are present in milk EVs of humans, cows, and pigs. miRNAs in milk EVs are beneficial for intestinal health. These ncRNAs possibly also play biologically functional roles in immune regulation, epigenetic regulation, metabolic disease, and targeted therapy. Nevertheless, there are still many open questions that urgently require experimental analysis, such as the origin, uptake, and physiological effects of ncRNAs in milk EVs. Research in this field should be encouraged. As more detailed information in this field is being revealed, this will bring new insights into the importance of milk nutrition for human or animal health, and even into disease treatments.

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