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Journal

Advances in Nutrition, 12(5)

ISSN

2161-8313

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Publication Date

2021-09-01

DOI

10.1093/advances/nmab059

Peer reviewed

PERSPECTIVE

Perspective: Milk microRNAs as Important Players in Infant Physiology and Development

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ABSTRACT

Evolutionary selective pressure on lactation has resulted in milk that provides far more than simply essential nutrients, delivering a complex repertoire of agents from hormones to intact cells. Human infants are born with low barrier integrity of their gut, which means that many of the complex biopolymer components of milk enter and circulate in lymph and blood, reaching organs throughout the body. Due to this state of gut maturation, all components of milk are potentially part of the crosstalk between mother and infants. This article highlights the functions of milk's complex biopolymers, more specifically the potential role of microRNAs (miRNAs) contained in extracellular vesicles in human milk. miRNAs are key effectors in the regulation of many biological processes during early-age development, and consequently milk-sourced miRNAs must be considered to provide unique biological assets to the infant during breastfeeding. This article interprets the evidence of the potential action of human milk miRNAs on infant development, taking into account their abundance in milk based on the literature and current knowledge. Human milk miRNAs appear to influence lipid and glucose metabolism, gut maturation, neurogenesis, and immunity. We also show growing evidence that human milk miRNAs are epigenetic modulators that play a pivotal role in the regulation of tissue-specific gene expression throughout life. Furthermore, this article addresses the ongoing debate regarding the potential influence of human milk miRNAs on viral infection as a new research area. This article highlights that these bioactive molecules are now being incorporated into our overall understanding of nutrient needs for healthy infant development, preparing each individual infant to succeed as a healthy and protected adult throughout its life. In essence, miRNAs are a new language in the Rosetta stone of health that is mammalian lactation. *Adv Nutr* 2021;12:1625–1635.

Statement of Significance: This manuscript presents analyses that are new in the field.

Keywords: human milk, exosome, miRNA, infant, health

Introduction

The emergence of lactation as the sole source of nourishment for neonatal mammalian infants has been central to the success of mammals (1), providing a complete system of nourishment. The composition of milk has been a singularly valuable guide for nutrition scientists to identify nutrients, their quantitative requirements, and various mechanisms that ensure their successful absorption (2, 3).

The current paradigm for nourishment is that biopolymers, including proteins, saccharides, polynucleotides, and complex lipids, are denatured by stomach acid, dispersed by bile acids, and attacked by endogenous hydrolytic enzymes. This adult model envisions a milk digestive process that rapidly leads to the release and complete absorption of monomeric amino acids, sugars, nucleotides, and lipids by the intestinal epithelia (4). However, human infants are developmentally naive, produce little gastric acid or bile acids, and express low levels of digestive enzyme activities (5). As a result, the infant is exposed to far more of the components of milk intact. Additionally, the lower barrier integrity of the infant intestine means that many of the complex biopolymer components of milk enter and circulate in lymph and blood, reaching targets throughout the infant and playing a role in establishing immunity in newborns (6). The chemical and biological examination of milk must now consider the functions of those intact and semi-intact biopolymers and the ensembles of molecules from the mammary gland in the infant.

The most innovative opportunity for milk research is to discover the targets on which milk acts to protect infants, support development, and prevent diseases proactively (7). With the goal of identifying mechanisms of "function for

prevention," multiple independent biopolymers have been selected for efficacy as ingested components towards the targets that guide the success of the mother-infant dyad.

Neonates and infants are particularly challenging as models of biological functions because all of their complex systems are actively proceeding through their development processes. Many systems (e.g., cardiovascular, respiratory, gastrointestinal) undergo significant changes at birth, and many others (such as neural systems) have not yet completed their development.

Human milk (HM) contains a complex combination of lipids, proteins, carbohydrates, and minerals that are essential for infant growth, development, and immune system (8, 9). Identifying and annotating the complex repertoire of milk components is part of the daunting scientific task. Evidence has shown the importance of milk constituents for infant development. For example, milk lipids illuminate the complexity of milk's diverse roles in infant nutrition. Their presence and abundance are the keys to diverse levels of metabolic regulation, from subcellular compartments to whole-body energy control and signaling (10). Intensive research over the last decade has highlighted oligosaccharides as having a very important role in infant development, in particular, acting on the gut microbiota (GM) (11). More recently, scientists have focused on nucleic acids present in milk. Life scientists have now recognized that cells transcribe far more RNAs than simply those encoding structural proteins.

Of special interest are microRNAs (miRNAs), which are now considered key regulators of numerous biological processes (12). They are actively secreted out of cells that synthesize them, including in milk. The discovery of miRNAs in milk in significant amounts has led the scientific milk community to focus their attention on the potential role of milk in the health of infants (13–15). In this article, we provide evidence in support of the multieffector strategy of milk and the use of miRNAs in milk as a conceptual window into the targets of those tactics. After a brief description of miRNA biogenesis and evidences of food-source miRNAs in humans, this article focuses on small noncoding RNAs (miRNAs) in human breast milk and their potential effects on infant development, finishing with the concept of their role in counteracting viral infections.

Supported by NIH grant (no: 1R01DK124193-01).
Author disclosures: The authors report no conflicts of interest.
Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/advances/.
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Abbreviations used: *AGPAT6*, 1-acylglycerol-3-phosphate *O*-acyltransferase 6; DNMT, DNA methyltransferase; EMT, epithelial-mesenchymal transition; EV, extracellular vesicle; GM, gut microbiota; HM, human milk; miRNA, microRNA; nt, nucleotide; QKI, Quaking; UTR, untranslated region.

MicroRNA Biogenesis

miRNAs are small noncoding RNAs [~22 nucleotides (nts)] transcribed by RNA polymerase II as part of "pri-miRNAs" (16, 17). Each pri-miRNA forms a hairpin that is a substrate for the Drosha-DGCR8 complex (18). Drosha has 2 RNase III domains that each cut the pri-miRNA hairpin, liberating a ~60-nt stem-loop called a "pre-miRNA" (19). This pre-miRNA is exported to the cytoplasm via exportin 5 and RAN(RAS-related Nuclear protein)-GTP) (20–22), where Dicer, an endonuclease with 2 RNase III domains, cuts both strands near the loop to generate the miRNA duplex. Then, the miRNA duplex is loaded into an Argonaute protein with chaperone proteins (HSC70/HSP90) to expulse the second strand of the duplex (named miRNA∗) to form the mature silencing complex (23). Next, the miRNA targets mRNAs by base pairing to direct their posttranscriptional repression. miRNAs control various biological processes and are thought to regulate ≥60% of genes at the posttranslational level (12, 24). miRNAs have been described as participating in the crosstalk between cells in the same organ (25). However, their isolation from extracellular vesicles (EVs; so-called exosomes) in bodily fluids (as serum) emphasizes their function in the crosstalk between organs (26, 27).

Milk miRNAs as Food-Sourced miRNAs

There is increasing evidence of the presence of plant food–sourced miRNAs in human plasma (28). As an example, *miR-168a*, an abundant miRNA in rice, was detected in the sera and tissues of the Chinese population. This exogenous plant miRNA could target the human LDL receptor adapter protein 1 (LDLRAP1) mRNA, inhibiting its expression in liver (29). Similarly, a negative correlation between plant *miR-159* and breast cancer incidence and progression was shown in a Western population (30). Such studies suggest cross-kingdom action of dietary intake miRNAs and demonstrate that miRNAs acquired orally through food intake influence human gene expression after migration through the plasma and delivery to specific organs (31, 32). The functional influence of ingested miRNAs on organisms consuming them has already emerged as a new signaling system affecting the physiology of consumers through modulation of host gene expression profiles.

The detection of miRNAs in milk has served as a starting point for studies investigating the role of miRNAs, their transfer to and from milk and potential impact on consumers, and of course, better characterization of miRNAs in milk. Thus, miRNAs were detected in different compartments of milk, such as milk fat globules and exosomes (33–35). A comparison between different fractions of milk showed that the milk lipid fractions contained higher concentrations of miRNA compared with skim milk (33). Milk fat globules have proven to be a complex window to the miRNA repertoire of the mammary gland, with discrepancies (35, 36) due, at least in part, to the secretion mechanisms of milk fat globules (37, 38). Discouragingly, few studies are dedicated to the survival or to the effects of milk fat globule miRNA on infants.

Conversely, the potential biological effects of milk EVs containing miRNAs have been demonstrated both in vitro (39) and in vivo (40), suggesting a role of dietary miRNAs. The stability of miRNAs, including those in milk under various harsh conditions, has led investigators to suggest that their packaging in milk EVs has the net effect of protecting them (41), thus allowing their survival in the gut intestinal tract of offspring (29). This is an important step in the delivery of dietary EVs and their cargo, as reported in human vascular endothelial cells with bovine milk (42). However, few studies have reported the lack of transfer of miRNAs from milk to mouse tissues (43, 44). The disagreement between the 2 competing hypotheses could be due to actions modulated by their packaging within "transporting vehicles," which play an important role in their transfer and action in the consumer (33, 45). Exosomes containing miRNAs protect them against degradation and facilitate their uptake by endocytosis in bovine milk and HM (46, 47). In vitro studies have revealed the survivability and complexity of HM exosome miRNAs upon simulated gastric/pancreatic digestion (48), demonstrating the same ability as preterm milk exosomes (49), suggesting their functional and nutritive role (50). This is confirmed by the in vivo detection in different tissues (such as intestinal mucosa, spleen, liver, heart, and brain) of fluorophore-labeled synthetic miRNAs administered in milk exosomes to mice and pigs (51). Because miRNAs regulate numerous biological processes, their involvement in infant development is indubitable, and crossing through the intestinal barrier is one mechanism of this action.

The Potential Effects of Breast Milk EV miRNAs on Newborn Development

The abundance of miRNAs in milk and their persistence in milk across millennia of selective pressure argue that they play important roles in the postnatal development of mammalian neonates. Here, we performed a functional annotation of miRNAs present in human breast milk exosomes with recent advances in the understanding of their function related to infant development. Their potential action is predicted from the influence of miRNAs on metabolism, gut, neurogenesis, immunity, and epigenetics.

Strategy

We used CAB Abstract (via Ovid at UC Davis Library) and PubMed to search for articles referring to the effect of miRNA on development, avoiding those related to diseases. We focused on 5 main functions that have an important impact during the first weeks of development in a newborn just after birth when the intestine wall is thin and still under development. The thin intestine wall could more easily allow miRNA–target interactions. The keywords that were used for the search were miRNA, immunity, lipid, glycerol, glucose, gut, intestine, epithelium, neurogenesis, and neuron. We focused on metabolism (lipids and glucose), gut maturation, neurogenesis, and immunity. We searched miRNAs regulating these functions. Then, we took into account only miRNAs detected in HM exosomes (48). The abundance was also taken into account on the basis of the total of read counts calculated from Liao et al. (48) with the categories defined as grade A > 250.00, 250.00 ≥ B > 150.00, 150.00 ≥ C > 50.00, and 50.00 ≥ D > 0 sum of the total normalized read counts. We identified 32, 86,170, and 302 miRNAs in grades A, B, C, and D, respectively (**Supplemental Table 1**). Then, we created a list of the miRNAs that have a potential role in the selected functions based on the rank of abundance (**Table 1**).

The ranking allowed evaluation of the bioavailability of each miRNA. In this Perspective article, we chose to discuss only the high- and medium-ranking miRNAs. We assume that very low concentrations of miRNA in milk are less captured by the intestinal cells and thus have less effect on the infant. However, it should be noted that considering that exosomes protect their cargoes against the intestinal environment (low pH, enzymatic activities, etc.), we cannot exclude that miRNAs in very small quantities might also have effects.

Potential of human milk miRNAs in infant lipid metabolism

An increasing number of publications report the effects of miRNAs on metabolism regulation. Such miRNAs underlying metabolic regulation were detected in HM (Table 1; **Figure 1**). Indeed, we identified highly expressed miRNAs in HM that were shown to target genes involved in lipid metabolism, such as *miR-182-5p*, *miR-148a-3p*, and *miR-22-3p*, which were among the top 25 most abundant miRNAs in HM exosomes (48). They regulate the expression of the *AGPAT6* gene (coding for 1-acylglycerol-3-phosphate *O*-acyltransferase 6), having a direct effect on the synthesis of triacylglycerol and long-chain acyl-CoA fatty acids in cells (52). Lipid metabolism could also be regulated by *miR-26a,* which regulates glucose and lipid metabolism in the liver of mice fed a high-fat diet (53), and *miR-30a-5p* has been reported to influence fatty acid synthesis by regulating the expression of *THEM4 (Thioesterase Superfamily Member 4)*, a member of the thioesterase superfamily (54). *let-7f-5p* (rank A for abundance) is a member of the large *let-7* family. This family is highly conserved across species in sequence and function (55). *let-7f-5p* (rank A) was also identified as potentially targeting mRNA coding for AGPAT6, which is an enzyme involved in the synthesis of triacylglycerol (52), and mRNAs coding for stearoyl-coA desaturase and fatty acid desaturase 2, two enzymes catalyzing the biosynthesis of PUFAs, which play pivotal roles in many biological functions. In addition, the members of this family, *let-7f*, are proposed to regulate stem cells by promoting differentiation during development (56). In addition to these highly abundant miRNAs (rank A) in HM, *miR-33* detected with a lower abundance (rank C) has also been reported to influence lipid metabolism. *miR-33∗* (*miR-33a* and *miR-33b*) is located within sterol regulatory element-binding protein (SREBP) genes and is well known as a key transcription factor regulating lipogenic gene expression. *mir-33∗* regulates host gene expression and therefore lipid metabolism (57, 58).

TABLE 1 Categorization of potential influence of milk microRNAs (miRNAs) on health from bibliographic analyses crossed with human milk miRNA detection[1]

Function	miRNAs	Rank	Observations	Reference[2]
Lipid metabolism	*let-7f-5p, miR-148a-3p, miR-182–5p, miR-22–3p*	A	AGPAT6 is regulated by some of the most highly expressed human milk cell miRNAs, and has a direct effect on the synthesis of triacylglycerol and long chain acyl-CoA	Alsaweed et al. 2016 (45)
	let-7f-5p	A	Modulates FADS2 and involved in oleate biosynthesis	Alsaweed et al. 2016 (45)
	miR-33	C	*miR-33a* and *miR33b* are intronic miRNAs located within the SREBP genes; regulate lipid metabolism in concert with their host genes	Goedeke et al. 2013 (57)
	miR-26a	A	Regulates insulin sensitivity and metabolism of lipids	Fu et al. 2015 (53)
Glucose metabolism	*miR-30a-5p*	A	Controls THEM4, which is essential for the phosphorylation and synthesis of fatty acids	Alsaweed et al. 2016 (45)
	miR-143	B	Induced transgenic overexpression of *miR-143* impairs insulin-stimulated AKT activation and glucose homeostasis	Jordan et al. 2011 (59)
	miR-33	C	Cooperates with SREBP in regulating glucose metabolism by targeting PCK1 and G6PC, key regulatory enzymes of hepatic gluconeogenesis	Ramirez et al. 2013 (60)
	miR-26a	A	Regulates insulin sensitivity and metabolism of glucose	Fu et al. 2015 (53)
	miR-181b	A	Improves glucose homeostasis and insulin sensitivity by regulating endothelial function in white adipose tissue	Sun et al. 2016 (61)
Gut maturation	*miR-375, miR-200c*	A	Both modulate epithelial function, which can influence exosomal endocytosis and thus uptake of the miRNAs	Alsaweed et al. 2016 (45)
	miR-200b	B	Inhibits tight junction disruption of intestinal epithelial cells in vitro	Shen et al. 2017 (62)
	miR-21	A	Regulates intestinal epithelial tight junction permeability	Yang et al. 2013 (63)
	miR-99b	A	Inhibits the gene expression of *MFG-E8*, known to maintain intestinal homeostasis by enhancing enterocyte migration	Wang et al. 2016 (64)
	miR-200 family	A–D	Critical gatekeepers of the epithelial state linked to epithelial-mesenchymal transition	Pillman et al. 2018 (65)
	miR-30 family	A–D	Control proliferation and differentiation of intestinal epithelial cells	Peck et al. 2016 (66)
Neurogenesis	*let-7* family	A–D	Neural differentiation of EC cells was accompanied by an increase in *let-7* precursor processing activity	Wulczyn et al. 2007 (67)
	miR-574	B	Promotes neurogenesis, but reduces the neural progenitor pool	Zhang et al. 2014 (68)
	miR-15b	C	Inhibits cortical neural progenitor cell proliferation and promotes cell-cycle exit and neuronal differentiation	Lv et al. 2014 (69)
	miR-210	C	*miR-210* inhibition significantly increased neuronal survival of inflammation but reduced proliferation	Voloboueva et al. 2017 (70)
	miR-29b	C	Plays a pivotal role in fetal neurogenesis by regulating VDAC1	Roshan et al. 2014 (71)
Immunity	*miR-223*	C	Activates proliferation of granulocytes	Johnnidis et al. 2008 (72)
	miR-146b-5p	A	Targets signaling proteins of innate immune responses	Taganov et al. 2006 (73)
	miR-181a	A	Regulates inflammation responses in monocytes and macrophages in part by downregulating IL-1α	Xie et al. 2013 (74)
	miR-150	C	Blocks B-cell development	Zhou et al. 2007 (75)
	miR-182–5p	A	Promotes T-cell–mediated immune responses	Stittrich et al. 2010 (76)
	miR-17, miR-92	C, A	Regulate monocyte development as well as B- and T-cell differentiation and maturation	Mendell 2008 (77)
	miR-29a-3p	A	Suppresses immune responses to intracellular pathogens by targeting IFN-γ	Ma et al. 2011 (78)
	miR-155	C	Regulates T- and B-cell maturation and the innate immune response	Vigorito et al. 2013 (79)

[1] Exosome human milk miRNAs from Liao et al. (48). Abundances of miRNAs in milk are ranked with A > 250.00, 250.00 ≥ B > 150.00, 150.00 ≥ C > 50.00, and 50.00 ≥ D > 0 sum of the total normalized read counts. AGPAT6, 1-acylglycerol-3-phosphate *O*-acyltransferase 6; AKT, Protein kinase; EC, embryocarcinoma; FADS2, fatty acid desaturase 2; G6PC, Glucose-6-phosphatase Catalitic subunit; MFG-E8, milk fat globule EGF and factor V/VIII domain containing; PCK1, phosphoenolpyruvate carboxykinase 1; SREBP, sterol regulatory element-binding protein; THEM4, thioesterase superfamily member 4; VDAC1, Voltage-dependent anion channel 1.

[2] The list of articles reported here is not exhaustive.

FIGURE 1 Potential effects of human milk exosomal microRNAs (miRNAs) on infant development. The font size and color of each miRNA corresponds to the abundance classification. The classification was based on the total count reads reported by Liao et al. (48) with A > 250.00, 250.00 ≥ B > 150.00, 150.00 ≥ C > 50.00, and 50.00 ≥ D > 0 sum of the total normalized read counts (Supplemental Table 1). miRNAs playing a role in ≥2 functions are in bold. miR, microRNA.

Potential of human milk miRNAs in infant glucose metabolism

In addition to their action on lipid metabolism, *miR-33* and *miR-26a* are also depicted as influencing glucose metabolism. *miR-33b* is reported to inhibit the expression of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, 2 key enzymes of hepatic gluconeogenesis leading to the regulation of glucose production (60). The involvement of hepatic *miR-26a* in glucose metabolism, lipid metabolism, and insulin signaling through the regulation of critical metabolic genes suggests *miR-26a* is a promising novel target for the treatment of obesity-associated metabolic syndrome (53). *miR-33a* and *miR-143,* both present in HM EVs, have been associated with glucose homeostasis and energy metabolism in a model of Per-Arnt-Sim kinase (PASK)–deficient mice fed a high-fat diet (80). The action of *miR-143* on glucose homeostasis has also been identified in mice overexpressing this miRNA (59). Additionally, adipose glucose homeostasis and insulin sensitivity are reported to be regulated by *miR-181b*, which is abundant in HM EVs (61). All these data suggest a potentially important role of HM miRNAs in metabolism influencing the development of infants and therefore their immediate health as well as their future.

Potential of human milk miRNAs in the infant gut

An expected potential role of breast milk miRNAs is in gut maturation and function. Our analysis identified ≥4 miRNAs or families of miRNAs with high abundance and potentially influencing gut function and structure (Table 1; Figure 1). This influence is particularly important due to the permeability of the gut during the first days of life. Intestinal epithelial cells play a fundamental role in the selective absorption of nutrients. They are also a major source of immunoregulatory cytokines and a critical part of the physiological epithelial barrier (81). As mentioned above, studies have demonstrated the interaction of milk EVs and intestinal epithelial cells (14, 47), suggesting their regulation by HM miRNAs. Thus, *miR-99b*, which is very abundant (rank A) in milk, is known to enhance intestinal MFG-E8 (also known as milk fat globule membrane protein) and restore enterocyte migration (64). The *miR-200* family, which is also abundant in HM EVs, is known to influence epithelial-mesenchymal transition (EMT), which has a key role in the structure of epithelia. The members of this family are critical gatekeepers of the epithelial state, restraining the expression of promesenchymal genes that drive EMT (65). *miR-200c* was shown to regulate several important signaling pathways, such as transforming growth factor β, PI3K/Akt (Phosphoinositide 3 Kinase/protein kinase), Notch, and NF-κB signaling (82). *miR-200/375* are also reported to control epithelial plasticity–associated alternative splicing by repressing the RNA-binding protein Quaking (QKI) known to directly bind to and regulate alternative splicing targets (65). The *miR-200*/*miR-375*/QKI axis exerts pleiotropic effects, such as increasing cell migration and invasion. Another member of this family, *miR-200b*, inhibits tight junction disruption of intestinal epithelial cells in vitro (62). Similarly, intestinal epithelial barrier function is modified by *miR-21* (63), which is also abundant in breast milk EVs. Gastrointestinal tract

function is influenced by the microbiota. The GM plays an important role in host metabolism and therefore regulates a large number of biological processes. The GM interacts with its host, working together to maintain symbiosis (83). miRNAs have been shown to act in the intercommunication between the host and GM (83, 84). This intercommunication includes an influence of the GM on host miRNA expression but also an influence of host miRNAs on the GM (83). miRNAs can enter bacteria and thus regulate their growth (84). Because the GM is shaped by many factors, including host diet (85), we can hypothesize that dietary miRNAs could influence the GM. However, these potential effects are still unknown. In particular, the role of milk miRNAs in human neonates and their effects on GM are unidentified.

Role in neurogenesis

To date, few highly abundant miRNAs present in breast milk EVs are known to influence neurogenesis (Table 1; Figure 1), and in vivo studies have been performed in rodents. The most abundant miRNAs belong to the *let-7* family. An increase in *let-7* precursor processing activity is associated with neural differentiation of cells, suggesting the role of *let-7* in early developmental regulation of embryonic stem cell differentiation and neurogenesis reported in mice in vivo and in vitro (67). *miR-29b* is also detected in HM EVs and is reported to influence neurogenesis. Its knockdown in vivo in mice results in neural cell death (71). *miR-574-5p,* moderately abundant in HM EVs, is also an actor in neurogenesis. The overexpression and downregulation of this miRNA promotes and inhibits neurogenesis, respectively, in rat mesenchymal stem cells (68). Three other miRNAs (*miR-15b*, *-132*, and *-210*), detected in HM but in lower abundance, were described as influencing neurogenesis. As a *let-7* family member, *miR-15b* promotes neuronal differentiation and inhibits neural progenitor proliferation in mice (69). *miR-132* has been shown to exert effects within the central nervous system by improving memory (86). Conversely, *miR-210* inhibition was reported to increase neuronal survival in vitro (70). These few data report the potential role of some miRNAs on neurogenesis but also underline the need to increase our knowledge on the link between miRNAs and neurogenesis and open up new avenues of investigation.

Influence on immune function development

Milk is well known to transfer immunity-related substances from mother to infants. For instance, HM contains large quantities of immunological components but also several nonspecific factors, such as lysozyme, lactoferrin, and oligosaccharides, which have antimicrobial properties (87–89). In addition, miRNAs could participate in this transfer (Table 1; Figure 1). HM is rich in immune-related miRNAs (innate and acquired), including *miR-223*, *-146b-5p*, *-181a*, *-150*, *-155*, *-92a*, and *-17* (41, 90). Among them, *miR-223*, *-146*, *-181a*, and *-155* have been detected in higher abundance in colostrum than in mature HM (91). Such abundance is in line with the role of transfer of immunity to neonates. Thus, *miR-181a* is reported to target the 3′-untranslated region (UTR) of *IL1a* mRNA, regulating inflammatory responses in monocytes and macrophages in vitro (74), whereas *miR-223* regulates granulocyte function and fine-tunes the inflammatory response in mice (72). Both miRNAs act on human T cell and granulocyte cell populations as selective targets (92), suggesting that miRNAs could affect newborn immune homeostasis at early stages of life. The innate immune response is also regulated by *miR-146b*, which is predicted, in vitro, to base-pair the 3′-UTRs of the TNF receptor-associated factor 6 and IL-1α receptor-associated kinase 1 mRNAs encoding 2 key adapter molecules downstream of Toll-like receptors and cytokine signaling (73). *miR-155* regulates T-helper-cell differentiation and participates in the development of immune cells such as B and T lymphocytes and macrophages by regulating their function and activation (93, 79). *miR-150* is also reported in mice to be an effector controlling B-cell differentiation (94). These studies demonstrate a potential role of HM miRNAs in the establishment of the immune system in infants.

In addition to the potential effects of EV milk miRNAs on neonatal immune function, recent evidence demonstrates the influence of EVs during viral infections. During viral infection, host EVs can package the virus, thus giving them potential protection from the host's immune system and providing secreted entry into host cells. In this way, EVs can contribute to the spread of the virus, as suggested for COVID-19 virus infection in a review (95). However, EVs must be considered to influence various aspects of the infection process, because EVs carry miRNAs, which could affect the interactions between viruses and hosts. A burgeoning body of data suggests a complex 2-way relation between miRNAs and viruses (95). Host miRNAs can act on virus. Host miRNAs can affect RNA virus replication and pathogenesis through direct binding to the RNA virus genome or through virus-mediated changes in the host transcriptome (96). An increasing number of examples have described the influence of host miRNAs during viral infection, leading to the identification of novel mechanisms to block RNA virus replication. As described above, food-source miRNAs can act in consumer cells, and we can hypothesize that they also influence the spread of a viral infection by acting on virus replication (96). To explore this possibility, we compared the 23 miRNAs (Supplemental Table 1) already known to affect virus replication by directly binding numerous RNA virus genomes (96) regardless of the type of virus, with the miRNAs detected in EV milk (48). We observed that among those 23 miRNAs, 16 were present in EV milk (**Figure 2**). We identified *miR-29a*, *miR-21*, and *miR-181*, which can inhibit the replication of different viruses and are abundantly present in milk EVs (rank A or B). For example, *miR-29a*, *miR-21*, *miR-181*, *miR-23*, *miR-28*, and *let-7c* were reported to bind HIV, porcine reproductive and respiratory syndrome virus, infectious bursal disease virus, enterovirus 71, human T-cell leukemia virus 1, and H1N1 influenza virus, respectively. *miR-23*, *miR-28*, and *let-7c* or *miR-150*, *miR-223*, *miR-378*, *miR-505*, and *miR-296* were also identified to act on virus replication and detected in

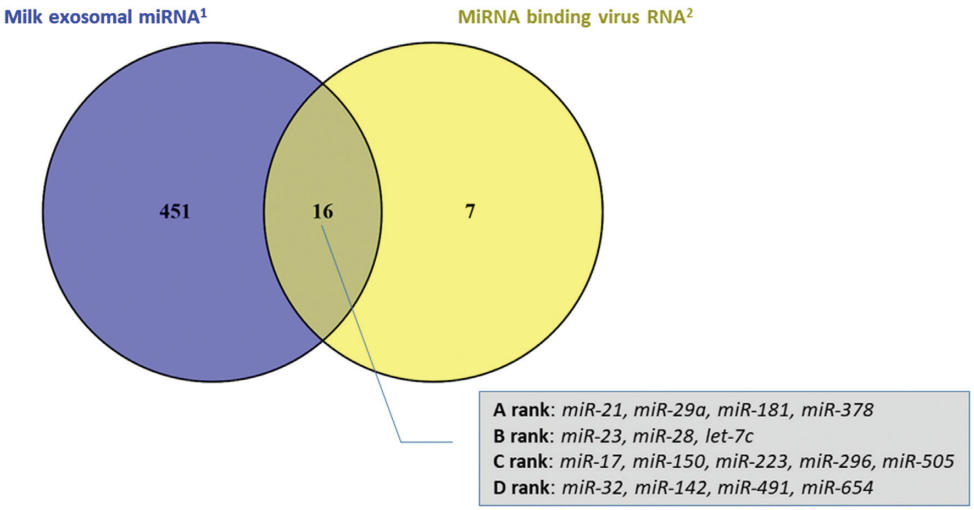

FIGURE 2 Comparison between milk exosomal microRNAs (miRNAs) taking into account their abundance [rank A (high) to D (low) abundance with A > 250.00, 250.00 ≥ B > 150.00, 150.00 ≥ C > 50.00, and 50.00 ≥ D > 0 sum of the total normalized read counts] and the list of miRNAs targeting viral RNA. Additional knowledge on the effects of host miRNAs on virus replication is indicated in the panel bottom right. [1]From Liao et al. (48); [2]from Trobaugh and Klimstra (96). miR, microRNA.

rank A, B, or C abundance as milk exosomal miRNAs. The last 4 miRNAs (*miR-142*, *miR-32*, *miR-491*, *miR-654*) were weakly abundant. In addition, other publications reported a role of host miRNAs in viral replication, because *mir-30a* (97), *miR-16-5p* (98), and *miR-103a* (99) were highly expressed (rank A for abundance in HM exosomes), and *miR-203* (100) had lower expression (rank C). In contrast, *miR-340-5p*, belonging to the rank B class in milk, has been described to enhance influenza A virus replication. The diverse roles of miRNAs at the host–virus interface must be deeply explored as well as the mechanisms of action. The effectiveness of the role of food-source miRNAs on viral replication is one of the exciting challenges of the field, particularly in the COVID-19 pandemic context. We used the same strategy to compare the milk exosomal miRNA (from reference 48) with the miRNA computationally predicted to target SARS-CoV-2 RNA (101). We identified 10 miRNAs (**Figure 3**A). However, their abundances reported by Liao et al. (48) are low. Further analyses of their abundance during different lactation statuses showed that 9 of them were less abundant during late lactation than during early or midlactation (Figure 3B).

In conclusion, based on all these observations, we can suggest that EV miRNAs from milk should be predicted to target viral RNA, including SARS-CoV-2. The determination of the conceivable action of milk miRNAs on viral replication raises the question of their possible role in protecting against viral infection. Such a role should be investigated to be considered in parallel with more conventional therapeutic strategies.

Predicted role of HM in epigenetic regulation

In addition to the diverse roles of miRNAs in neonatal development, long-term effects must be considered such as their actions on epigenetic processes. Epigenetic processes are shown to play a pivotal role in regulating tissue-specific gene expression and can induce long-term changes, which persist throughout the life course (102). There is accumulating evidence that milk is a major epigenetic modulator of gene expression in infants and therefore in adults. miRNAs were demonstrated to act on epigenetics via the expression regulation of DNA methyltransferases (DNMTs) involved in DNA methylation, which is crucial for gene expression and hallmarks of human diseases. For example, the *miR-29* family targets DNMT family members, because *miR-29* reverts aberrant methylation via complementarities to the 3′-UTRs of *DNMT3A* and *DNMT3B* mRNA, encoding 2 key de novo methyltransferases (103). Similarly, *miR-148a-3p* represses human *DNMT3B* gene expression (104). These 2 miRNAs reduced DNA methylation in humans. Thus, the presence of *miR-29b* and *miR-148a-3p* in HM exosomes (41) raises the question of the role of HM miRNAs in the epigenome of infants.

Conclusion

This study highlighted the predicted key role of HM miRNAs in infant development: they participate in the continuum between mother and infant. We related the predicted effects on the infant to the abundance of miRNA in milk, presented here as a ranking. This point is important to strengthen their potential influence. All these data underscore the complexity of regulation by miRNAs. Their abundance in milk must be taken into account to assess their effects on infants. There was compelling evidence showing the importance of milk as a diverse, complex, and highly functional matrix of support systems selected through evolution for newborn development. More recently, the presence of miRNAs in breast milk allowed us to answer the following question:

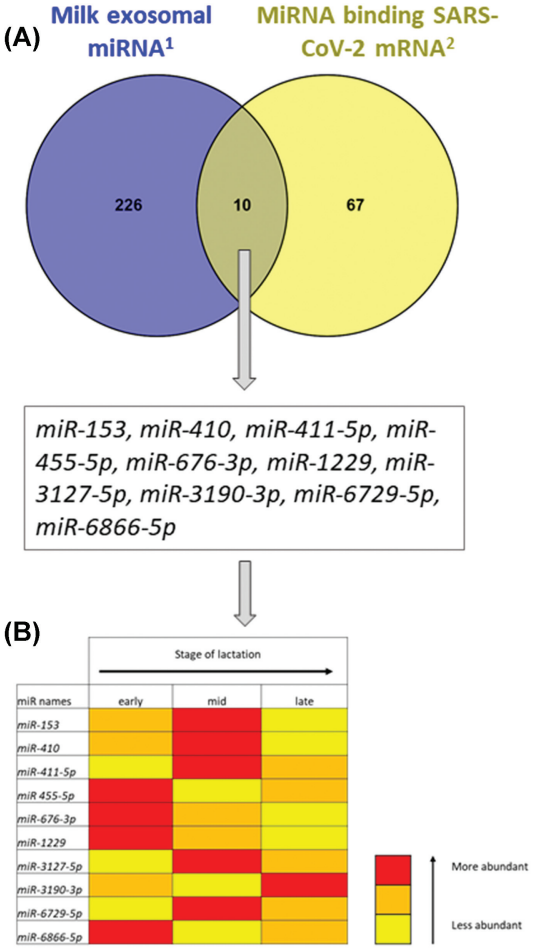

FIGURE 3 Potential relation between milk exosomal microRNAs (miRNAs) and SARS-CoV-2 virus. (A) Comparison between milk exosomal miRNAs and the list of miRNAs targeting SARS-CoV-2 RNA. (B) Abundance of common miRNAs in undigested exosomal miRNAs according to lactation status. Yellow, orange, and red colors indicate the level of abundance, with yellow being less abundant than orange than red. [1]From Liao et al. (48); [2]from Demirci and Adan (101). miR, microRNA.

what targets does milk act upon to improve the health of infants? We report a detailed examination of the effects of several dozen of those miRNAs involved in the control of functions related to infant development. Several miRNAs that are abundant in milk could potentially influence various functions (Figure 1). For example, *miR-21* was reported to be involved in immunity and gut maturation, *miR-29b* could regulate epigenetics and influence neurogenesis, *miR-182-5p* was described to act both on metabolism and immunity, and *miR-148* influenced metabolism and epigenetics. The *let-7* family is represented by several members in milk and is known to be involved in multiple functions (e.g., neurogenesis, immunity, metabolism). Based on this set of evidences, in addition to the greater permeability of the gut during the first days of life, the potential role of miRNAs, especially those abundant in milk, in infant development can reasonably be suggested. These discoveries have to be incorporated into our overall understanding of nutrient needs for healthy infant development, preparing each individual infant to succeed as a healthy and protected adult throughout life. This study builds on the concept of natural selection throughout evolution for a biofluid that transfers bioactive components from mother to infant through milk for successful infant development. In addition, this study strengthens the crucial role of milk in infant development and protection using examples of the importance of multiple milk constituents. This evolutionary perspective on the protection and prevention of disease is envisioned as a complement to the history of therapeutic interventions based on the model of 1 target–1 molecule drug development. In practice, HM is a model combining efficacy and safety in natural selection that takes into account the daunting challenge of this complexity. In the most modern context, milk miRNAs can be considered in the face of viral infection and how to build scientific strategies for prevention. We propose that within the significant ongoing research to fully understand human breast milk and its targeted function, miRNAs provide an attractive path for functional discovery. However, functional redundancy across biopolymers acting upon unique targets in concert should be the goal of future strategies. This massive task of reverse engineering a bioreactor as complex as the mammary gland and a product as dynamic as milk will require new tools, models, and paradigms (105). Nonetheless, accomplishing this task will have immediate benefits on infants and mothers and provide a clear map for guiding improved health for everyone. In essence, miRNAs are a new language in the Rosetta stone of health, mammalian lactation.

Acknowledgments

We thank the NIH for the grant (no. 1R01DK124193-01).

The authors' responsibilities were as follows—MLC: conducted the analyses to build Table 1; JBG, CL: conceptualized the article; CL: wrote the first draft; and all authors: read and approved the final manuscript.

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