Extracellular vesicles in human milk

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Purpose of review
Milk-derived extracellular vesicles (MDEVs) are nanovesicles that carry microRNA (miRNA) DNA, RNA, proteins and lipids. MDEVs have a potential of therapeutic targets, based on their properties and cargo profile. The present review summarizes recent studies on MDEVs, their cargo and potential role in mammalian development.

Recent findings
The detailed characterization of their miRNA cargo leads to the conclusion of their potential importance in the regulation of gene expression, immune function, development and infant growth.
While their miRNAs are important regulatory elements and their profile expression was characterized in various mammalian milk sources, little is known about their effect on infant health and development.
MiRNA activity in breast milk is likely influenced by the overall ecosystem of the early environment, including maternal characteristics, behaviors, and health.

Summary
MDEVs may have an important role in early child development and infant future health. Understanding benefits of MDEVs characteristics have potential role on gut maturation, immune system development and the prevention of metabolic disorders.

Keywords
breastfeeding, exosomes, extracellular vesicles, milk, microRNA

INTRODUCTION

Milk is the main nutritional source for newborn mammals and breastfeeding is recognized as one of the most valuable contributors to infant health. Breastfed infants enjoy significant health benefits in comparison to those fed with infant formulas. Milk comprises a complex and dynamic system that transmits nutritional, hormonal and other molecular signals from mothers to their infants during a sensitive window of postnatal development. In recent years, new components, extracellular vesicles (EVs), have also been identified in milk. EVs are lipid bilayer particles secreted by cells into the extracellular space [1]. The three main subtypes of EVs are *exosomes*, *ectosomes* (microvesicles) and *apoptotic bodies*, which differ in their biogenesis and secretion pathways. Exosomes are generated in the late endosome, ectosomes are formed by budding and subsequent fusion of the plasma membrane and apoptotic bodies are formed during cells apoptosis. EVs carry DNA, RNA, microRNA (miRNA), proteins and lipids, which play important roles in intercellular communication, which modulates and regulates adjacent recipient cells. Exosomes have been found in physiologic fluids such as bronchoalveolar lavage [2], serum [3,4], urine [5] and are most abundant in human milk [6], as well as in bovine, yak, goat and porcine sources [7–11].

MILK-DERIVED EXTRACELLULAR VESICLE CONTENT

Noncoding RNAs

Various types of noncoding RNAs have been identified and isolated from human milk-derived extracellular vesicles (MDEVs), including miRNA, long noncoding RNA (lncRNA) and circular RNA (circRNA). Noncoding RNAs in MDEVs are involved in complex signals and regulation networks.

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KEY POINTS

- Milk-derived extracellular vesicle (MDEVs) contain miRNA, proteins and other noncoding RNA that have regulatory functions.
- MDEVs are therapeutic targets based on their properties and cargo profile.
- MDEVs provide a novel mechanism of biologic communication between nursing mother and infant.
- MDEV content varies in response to the maternal environment, behaviors, nutrition and health.
- MDEVs may have an important role in early child development and future health.

MicroRNAs

Among the most studied cargo molecules in MDEVs are the miRNAs, small noncoding RNAs involved in gene regulation. Each miRNA can bind to hundreds of target messenger RNAs (mRNAs), typically inhibiting their translation into proteins or promoting their degradation, thereby fine-tuning the translation of genome-directed proteins [12]. MiRNAs are important regulatory elements that control a wide range of cellular functions and play an important role in various biologic processes such as proliferation [13–15], cell differentiation [16–18] and inflammatory signal pathways [19–21]. MiRNAs transmitted to the infant's gut are protected from degradation by the MDEVs which encapsulate them. These miRNAs are very well characterized in various sources of mammalian and mainly, human milk [22,23▪]. The most abundant miRNAs in milk are miR-148a-3p, miR-22-3p, miR-200a-3p, miR-146b-5p, miR-30d-5p, let-7a-5p, miR-30a-5p, let-7f-5p, let-7b-5p and miR-21-5p [12,24▪,25,26]. Many of the miRNAs in human milk are implicated in regulating immune function and may be particularly important epigenetic modulators of infant gene expression [26,27]. For example, miR-148, miR-30, miR-181, and let-7a each regulate various immune processes [28]. MiRNAs delivered inside MDEVs can be transferred into the blood circulation, to repress translation of target mRNAs in distant cells [27]. For instance, incubation of transformed and normal cells with human MDEVs resulted in downregulated expression of DNA methyltransferase 1 (DNMT1), a target gene of miR-148a [29,30] and the induction of DNA demethylation, thereby potentially regulating epigenetic modifications of target genes.

Interestingly, the five most abundant miRNAs found in MDEVs, which include miR-148a, are similarly expressed across milk from human, bovine, porcine and ovine sources. These findings indicate an important functional role of these miRNAs in mammalian milk.

Long noncoding RNAs

lncRNAs, defined as transcribed RNA molecules greater than 200 nucleotides in length, have been detected in human MDEVs [31,32▪]. lncRNA interacts with RNA and proteins to regulate gene expression, signal pathways and chromatin function [33]. lncRNAs were identified for the first time in human MDEVs by *Karlsson et al.* [31]. They detected five lncRNAs which are important for developmental processes. The main lncRNAs are colorectal neoplasia differentially expressed (CRNDE), differentiation antagonizing nonprotein coding RNA (DANCR), growth arrest-specific 5 (GAS5), steroid receptor RNA activator 1 (SRA1) and ZNFX1 antisense RNA1 (ZFAS1). The lncRNA NORAD (Noncoding RNA activated at DNA damage) was found to be ubiquitously present in human MDEVs and was significantly downregulated in the MDEVs of mothers who delivered prematurely [32▪].

Circular RNAs

Noncoding circRNAs discovered in recent years are covalently closed, continuous loop RNAs that were found to play important functions in numerous biological processes including transcriptional regulation, epigenetic gene regulation and in diseases [34,35]. CircRNAs were found in human MDEVs [36▪▪] and exert their effects by binding miRNAs. The circRNAs found in human MDEVs such as hsa_circRNA_405708 and 104707, bind to related miRNAs that may be involved in the Vascular Endothelial Growth Factor signaling pathway [36▪▪].

Lipids

The biological functions of lipids in human MDEVs are poorly understood. Lipidomic analysis of MDEVs identified 15 lipid subclasses, including phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE). In particular, PE(18:1/18:1), PC(18:0/18:2), PC(18:1/16:0), PS(18:0/18:1) and PS(18:0/22:6), are enriched in human MDEVs [37▪]. The lipids detected in the human MDEVs were associated with the Extracellular signal-regulated kinase/mitogen-activated protein kinase pathway [37▪]. Moreover, the lipids detected in the MDEVs are related to the central nervous system. For example, choline deficiency may contribute to impaired lean body mass growth and pulmonary and neurocognitive development in infants [38].

Proteins

The protein composition of MDEVs was determined by various proteomics analyses [39,40,41]. The exosome markers CD9 and CD81 tetraspanin proteins were consistently detected by proteomic analysis in human MDEVs [39]. The glycoprotein CD36, one of the abundant proteins detected by the proteomic analysis of the MDEVs, is involved in foreign pathogen recognition, phagocytosis, and pathogen-induced signaling. It is also a receptor for a range of ligands such as TLR4 [42]. Others proteins found in MDEVs are lactotransferrin and heat shock protein 90 (HSp70). Lactotransferrin is an iron-binding glycoprotein, has an antimicrobial function and also stimulates host immunity [43]. Furthermore, MDEVs carry crucial bioactive regulatory proteins such as ransforming Growth Factor (TGF)-β. [44].

Oligosaccharides

The oligosaccharides in MDEVs play critical roles in the development of the infant's immature mucosal immune system. [45]. Fourteen typical encapsulated human milk oligosaccharides (HMOs) were discovered in colostral MDEVs, including 2-fucosyllactose (2-FL), 3-fucosyllactose (3-FL), lacto-N-difucohexaose (LDFH), 6-galactosyllactose (6-GL) and 3-sialyllactose (3-SL) [45]. In contrast, MDEVs from mature maternal milk were found to contain fewer varieties of HMOs. Interestingly, the HMOs in MDEVs were phagocytosed by macrophages and altered the whole transcriptome, allowing macrophages to switch to the alternate activation phenotype.

Milk-derived extracellular vesicles in health and disease

EVs and exosomes in particular, have been found to play a crucial role in different biological processes, including: cell signaling, neuronal function [46], tissue regeneration [47], intercellular communication [48], immune function [49], development and differentiation of stem cells [50], cancer life cycle [51,52] and viral replication [53]. EVs have the ability to transfer their cargo and to be selectively taken up by specific cells, thereby reprogramming the target cell and, possibly, inducing or preventing disease. The MDEVs' cargoes have a therapeutic impact on recipient cells and can provide a new target for treatment and diagnosis.

Uptake of milk-derived extracellular vesicles

The characteristics and regulatory functions of miRNA cargoes within MDEVs have led to extensive investigation of their involvement in human health. MDEVs were found to be taken up by intestinal cells [54] and to survive intestinal proteolytic enzymes [55,56]. Following oral and intravenous administration into mice and pigs, MDEVs and their miRNA cargoes were found to accumulate in the liver, spleen and brain [57,58]. Oral administration of human or bovine MDEVs to mice was shown to accumulate in the intestine [59▪]. Additional *in vitro* studies showed that MDEVs are absorbed into macrophages [57].

The effect of milk-derived extracellular vesicles on target cells

MDEVs can affect gene expression via their regulatory RNA cargo. Cells incubated with human MDEVs change their miRNA expression profile. Incubation of human MDEVs with normal intestinal CRL 1831 cells, K562 leukemic cells and Lim 1215 colon cancer cells, led to increased levels of miR-148a-3p, one the most abundant miRNAs in breast milk, in the cells. This was accompanied by a decrease in the expression of DNA methyltransferase1 (DNMT1), a target of miR-148a-3p [26]. The level of DNMT1 was shown to be regulated by MDEVs at both gene [26] and protein levels, in an miRNA-dependent manner [54]. MDEVs can also change the protein composition of a target cells. For example, transforming growth factors-β1 [59▪] and -β2 [60] (TGF-β1 and β2) are cargo proteins of human MDEVs and TGF-β1 levels were found to be highly present in MDEVs, as compared to other milk fractions [59▪]. In a dextran sulfate sodium (DSS) mouse model of colitis, we found increased levels of TGF-β in the colon following gavage administration of MDEVs, an observation that may have implications for therapeutic options of colitis [59▪].

Milk-derived extracellular vesicles regulation of immune response and inflammation

Human MDEVs inhibit anti-CD-3-induced interleukin-2 (IL-2) and IFN- γ production by peripheral blood mononuclear cells [61]. Furthermore, it was shown that MDEVs enhanced CD69 expression and induced IFN-γ production by NK and also by γδT cells, in the presence of IL-2 and IL-12. Interestingly, MDEVs alone do not activate immune cells but may activate excessively stimulated immune cells under inflammatory conditions [61]. Additionally, human MDEVs can directly inhibit CD4+ T helper cell activation without inducing tolerance [62▪▪].

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC), an acute inflammatory disease, is one of the most severe gastrointestinal

diseases in premature infants and develops in up to 10% of premature infants [63]. Human milk has been proved to reduce the risk of NEC [64,65]. Several in vivo and in vitro studies showed the potential of MDEVs to modulate NEC [37▪,66▪, 67–69,]. Human MDEVs protect intestinal epithelial cells from oxidative stress and enhance epithelial proliferation and migration *in vitro* [12,68,69]. MDEVs decreased inflammation in intestinal organoids exposed to injury [66▪,67] and in animal NEC models, intestinal mucosal injury and inflammation were attenuated by human MDEV treatment [66▪]. MDEVs increased the number of goblet cells and restored impaired NEC-related mucosa production [66▪,70]. Human MDEVs not only reduced inflammation and injury to the intestinal epithelium, but also restored intestinal tight-junction proteins [71▪].

Inflammatory bowel disease

Human MDEVs have a therapeutic and anti-inflammatory effect on colitis, involving several complementary mechanism of action pathways [59▪,72,73]. Human MDEV treatments ameliorate pathologic and clinical scores of the disease and were shown to downregulate major cytokines such as TNFα and IL-6. Moreover, we found that miRNAs highly expressed in milk, such as miRNA-320, 375, and Let-7, are more abundant in the colon of MDEV-treated mice compared with untreated mice and in contrast, the expression of their target genes, mainly DNMT1 and DNMT3, is downregulated. Furthermore, depletion of MDEVs from the diet of mice exacerbated inflammation in a murine model of inflammatory bowel disease. In addition, MDEVs improved several outcomes associated with DSS-induced colitis, i.e., they restored intestinal impermeability, replenished mucin secretion and modulated the gut microbiota [72].

Arthritis

The anti-inflammatory effects of bovine MDEVs have been demonstrated in arthritis mouse models, in which cartilage pathology and inflammation were shown to be decreased following MDEV administration [74▪▪].

Milk-derived extracellular vesicles and cancer

MDEVs promote proliferation of normal intestinal epithelial cells but in contrast, proliferation was not induced in cancer cells lines [54]. Furthermore, epithelial–mesenchymal transition (EMT) was observed following incubation with human MDEVs only in normal, but not in cancer cell lines. The biological effect of MDEVs on cells is miRNA-dependent [54]. MDEVs inhibit the proliferation of MCF7 breast cancer cells *in vitro* and to decrease their migration [75]. In a rat model of breast cancer, administration of camel MDEVs demonstrated an antitumor effect with significantly reduced tumor weight and growth progression by [75].

Milk-derived extracellular vesicle and the microbiome

Consumption of a diet depleted of MDEVs alters certain microbial taxa of the gut microbiome in mice [76]. For example, the relative abundance of Firmicute classes Clostridia (Ruminococcaceae) and Verrucomicrobia classes (Muciniphila) were greater in mice fed without MDEVs, as compared with MDEV-fed mice, tested at 15 weeks of age [77].

Regulation of human milk-derived microRNA

While miRNAs are clearly important regulatory elements, it is important to explore the variation and dynamics of EV miRNA content within milk over time and how they are affected by maternal environments. MiRNA activity in human milk is likely to be influenced by the overall ecosystem of the early environment, including maternal characteristics, behaviors, and health. The expression patterns of MDEV miRNAs showed variation from colostrum to mature milk [78–80]. Thus, the MDEV miRNA cargo content may be programmed to meet requirements of different stages of growth.

The miRNA profile in milk varies also in response to characteristics such as premature delivery [79], hormonal changes and health status of mothers [81]. For example, there are differences in miRNA levels in milk from mothers with type I diabetes as compared to healthy mothers [82]. Moreover, maternal obesity is negatively associated with the content of selected beneficial miRNAs in human milk [83▪]. HIV-1 infection has been shown to perturb the expression of miRNA in the milk of infected *vs* healthy mothers [84]. Additionally, other maternal chronic health conditions may also have important influences on milk miRNA activity.

The mother's diet was shown to affect the MDEV miRNA profile. One study in cows showed that a dietary change from forage fiber (alfalfa hay) to nonforage fiber (whole cottonseed and soybean hull) was associated with 9 differently expressed miRNAs in MDEVs [85]. In a study with nursing rats, an obesogenic dietary pattern, or so-called 'cafeteria diet', led to variations in specific miRNA levels in milk, with higher miR-222 and lower of miR-200 and miR-26 concentrations in animals eating the cafeteria diet, *vs* controls [27]. One study in

mice found that a high-fat diet (HFD) was associated with 25 differentially expressed miRNA in milk [86].

Oxytocin during lactation modulates the expression and secretion of milk-derived miRNAs, miR-148a and miR-320. MiR-148a is upregulated, and miR-320 is downregulated in human colostrum MDEVs of oxytocin-treated mothers [23▪].

The miRNA profile in human milk differs between mothers of preterm infants and mothers of full-term infants [79,87]. MiRNA-320 was more highly expressed in the colostrum of full-term than in preterm human milk. MiRNA-320 was upregulated in cells incubated with MDEVs, leading to a decrease of fatty acid synthase (FASN1) a key enzyme in the metabolism of long-chain fatty acids and a major target of miRNA-320 [88].

MiRNA expression in human MDEVs was shown to be associated with the timing of milk collection after delivery, maternal body mass index, and maternal smoking, but not with maternal parity (nulliparous or multiparous) [89▪▪].

CONCLUSION

In the last two-decades MDEV, exosomes in particular, emerged as promising bioavailable regulatory components, biomarkers, and therapeutic agents. The detailed characterization of their miRNA cargo leads to the conclusion of their potential importance in the regulation of gene expression, immune function, development and infant growth. Furthermore, other cargoes of MDEVs, i.e proteins, lipids and lnc-RNAs, have also been shown to have a regulatory function.

MDEVs are of general interest as therapeutic targets based on their properties and cargo profile. Their therapeutic effect was proven in various animal models of immune-mediated diseases.

MDEVs provide a novel mechanism to better understanding of biologic communication between nursing mother and infant. MDEVs may have an important role in early child development and their future health.

The miRNA profile in MDEVs varies in response to the maternal environment, behaviors, nutrition and health.

The current studies have exiting findings regarding the cargo of MDEVs in particular miRNAs, with beneficial biological functions such as miR-148. It can be speculated although has not been proved that those beneficial miRNAs may contribute to the gut maturation, immune system development and the prevention of metabolic disorders.

Future studies should be focused on determining the effect of MDEVs and their cargo on infant health outcomes. In particular, focus should be placed on proving the transfer of regulatory molecules such as miRNAs, using MDEVs as vehicles, from mother to infant. Furthermore, it will be important to determine the healthy implication of MDEVs addition or their absence in infant nutrition during the lactation period on their long-term health.

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Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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